A Detailed Analysis of Transcriptional Regulators Affecting the *Saccharomyces cerevisiae* Heat-shock Gene *SSA1*

Thesis by

Warren Alden Kibbe

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To my grandparents, who
gave me ideals to strive for,

For my parents, who
worked so hard to raise me,

And for Cate, who
has shown me how to fuse
my ideals and desires into fulfillment

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At a place like Caltech, it is often too easy to become completely immersed in your work and submerge your personal desires in the context of your work. To escape from this abyss of self-inflected servitude, it is sometimes necessary for others with similar interests to your own to call you up and encourage you to take some time away from your work. Thus, it has been a real boon that I have encountered many people that share an interest in gaming during my time at Caltech, and that they have always had a willingness to engage my intellect away from the lab, much to my advisor's chagrin at times, I am sure. Because of these people, I have had the chance to spend many enjoyable hours gaming while at Caltech. Many happy hours have been spent in the company of Winston Wiser, Barry Maurer, Don Montgomery, Mark Lysek, Joel Rushanan, Jeffrey Pugh, Brian Treco, Phil Askenazy, Brooke Anderson, Jane Goldsborough, and multifarious others who came and went.

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Abstract

The yeast *Saccharomyces cerevisiae* contains a family of hsp70 related genes. One member of this family, *SSA1*, encodes a 70kD heat-shock protein which in addition to its heat inducible expression has a significant basal level of expression. The first 500 bp upstream of the *SSA1* start point of transcription was examined by DNAse I protection analysis. The results reveal the presence of at least 14 factor binding sites throughout the upstream promoter region. The function of these binding sites has been examined using a series of 5' promoter deletions fused to the recorder gene lacZ in a centromere-containing yeast shuttle vector. The following sites have been identified in the promoter and their activity in yeast determined individually with a centromere-based recorder plasmid containing a truncated *CYC1*/lacZ fusion: a heat-shock element or HSE which is sufficient to convey heat-shock response on the recorder plasmid; a homology to the SV40 'core' sequence which can repress the GCN4 recognition element (GCRE) and the yAP1 recognition element (ARE), and has been designated a upstream repression element or URE; a 'G'-rich region named G-box which can also convey heat-shock response on the recorder plasmid; and a purine-pyrimidine alternating sequence name GT-box which is an activator of transcription. A series of fusion constructs were made to identify a putative silencer-like element upstream of *SSA1*. This element is position dependent and has been localized to a region containing both an ABF1 binding site and a RAP1 binding site. Five site-specific DNA-binding factors are identified and their purification is presented: the heat-shock transcription factor or HSTF, which recognizes the HSE; the G-box binding factor or GBF; the URE recognition factor or URF; the GT-box binding factor; and the GC-box binding factor or yeast Sp1.

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Introduction

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Introduction

The expression of genetic information in a temporally and spatially regulated fashion is a fundamental problem in all cells. The processes involved in this regulation are known collectively as gene expression. In most organisms, the genetic information (present in the primary sequence of DNA) is transcribed into RNA, translated into protein, and translocated or secreted into appropriate areas of the organism by a series of complex processes. The regulation of gene expression occurs primarily at the level of transcription, and DNA-protein interactions are necessary for transcription and the regulation of transcription.

Currently research is underway in a variety of organisms to identify the components, both *cis* and *trans*-acting, that are involved in the formation of a transcription complex, and the subsequent dissection of these components to understand how they promote transcription. This thesis presents specific examples of several *cis* and *trans*-acting transcription factors present in the yeast *Saccharomyces cerevisiae*. The following introduction will focus on the transcription of protein-encoding genes and will include a discussion of the following topics: 1. The components of the transcription machinery as it is currently understood. 2. The temporal events leading to the proper assembly of a transcription complex on a promoter. 3. The structure of the minimum number of DNA elements necessary to form an active yeast promoter. 4. The conservation of various components of the transcriptional machinery in eukaryotes. 5. The regulation of transcription by site-specific DNA-binding proteins. 6. The structure and function of site-specific DNA-binding proteins.

Components of the transcription apparatus

In this discussion, the term **transcription complex** will be used to describe the components involved in the initiation and elongation of the RNA transcript. The term **transcription apparatus** will be used to describe all of the components required for the active transcription of a gene, including the elements of the promoter, *trans*-acting factors that recognize these elements, the constituents necessary for termination, as well as the components making up the transcription complex. The nomenclature used in this introduction for denoting the components of the transcription complex is as follows: the factor is given an abbreviation to describe the RNA Polymerase for which it is a cofactor, and a letter designating the location in a chromatography scheme. For instance, the TATA-box which is found in many genes transcribed by RNA Polymerase II, is bound by a protein that has been named TFIID, where TFII stands for transcription factor for RNA Polymerase II and D the chromatographic fraction from which it was purified (Mermelstein 1989; Parker 1989; Saltzman and Weinmann 1989).

The main component of the transcription apparatus is RNA Polymerase, which itself is a multiple subunit structure in all but the simplest of organisms (See Figure 1 for a comparison of the yeast and mammalian RNA Polymerase II subunits). Isolated RNA Polymerase has the ability to transcribe RNA, although in a non-specific manner. In eukaryotes, several additional components have been identified that are necessary for specific and accurate transcription *in vitro*, and presumably most, if not all, are also necessary *in vivo*. For the purpose of this discussion, only the components necessary to transcribe genes transcribed by RNA Polymerase II (protein-encoding genes) will be surveyed.

RNA Polymerase II		TFIIA		TFIIB		TFIID		TFIIE		TFIIF		TFIIS	
yeast	mouse	CT	HeLa	yeast	HeLa	yeast	HeLa	yeast	HeLa	yeast	HeLa	yeast	HeLa
220	240	19	43		30	28	30		76		82		38
150	140	13									30		
44	41												
32	29												
27	27												
23	22												
14	19												
12	16												

Figure 1. Comparison of the apparent size of yeast and mammalian transcription factors in kiloDaltons (kD). RNA Polymerase II subunits sizes were determined in denaturing gels and were identified by immunological reactivity in various species (Buhler, et al. 1987). TFIIA was sized by denaturing gels from calf thymus (Samuels and Sharp 1986) and from HeLa cells (Egly, et al. 1984), and by gel filtration (native) from calf thymus (Samuels and Sharp 1986) and HeLa cells (Reinberg and Roeder 1987a), resulting in the larger estimates of 34 kD and 82 kD, respectively. TFIIB has also been isolated from HeLa cells (Reinberg and Roeder 1987a), and TFIID has been found and isolated from yeast (Burakowski, et al. 1988; Eisenmann, et al. 1989; Horikoshi, et al. 1989a), *Drosophila* cells (Parker and Topol 1984) and from HeLa cells (Reinberg, et al. 1987). TFIIE has recently been divided into two components, TFIIE and TFIIF, and TFIIF is apparently still composed of two subunits (Flores, et al. 1989).

In addition to RNA Polymerase II, the following factors are necessary to form a transcription complex: TFIIA, TFIIB, TFIID, TFIIE, and TFIIS (see Figure 1 for a comparison of these factors in various organisms). Other components are involved in the transcriptional apparatus, such as site-specific DNA-binding proteins that regulate the rate of transcription, termination factors, and the DNA sequence of the promoter, gene and termination region. There are several excellent recent reviews of RNA Polymerase II transcription factors and components, each reviewing aspects of transcription from a slightly different viewpoint: see Parker 1989; Mermelstein 1989; Saltzman and Weinmann 1989.

Assembly of the transcription complex

The components necessary for the accurate transcription of a protein-encoding gene *in vivo* have been identified by the fractionation of a crude soluble transcription system capable of specific transcription (Manley, et al. 1980; Matsui, et al. 1980; Weil, et al. 1979). The components, TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIS, and RNA Polymerase II, were highly purified from HeLa cells, and their order of assembly has been shown by sequential addition experiments that examine the kinetics and size of the transcription complex, as well as its resistance to inhibitors of transcription (Burakowski, et al. 1989; Cai and Luse 1987; Flores, et al. 1989; Hawley and Roeder 1985; Matsui, et al. 1980; Nakajima, et al. 1988; Reinberg, et al. 1987; Reinberg and Roeder 1987b; Sawadago and Roeder 1985; Tolunay, et al. 1984; Van Dyke, et al. 1988).

It has been demonstrated that the formation of a preinitiation complex requires the binding of TFIID to DNA containing a TATA-box (Nakajima, et al. 1988). For efficient formation of a preinitiation complex, which has been defined as transcriptionally competent but not containing the initial phosphodiester bond, another factor in addition to TFIID is needed (Davison, et al. 1983; Fire, et al. 1984). One such factor, TFIIA, is not promoter specific and doesn't bind DNA, but does function to promote the formation of a preinitiation complex with TFIID *in vitro* (Reinberg, et al. 1987). TFIIA has been shown to be present in yeast and in mammals (Hahn, et al. 1989a). Three site-specific DNA-binding proteins, USF, ATF and GAL4, have been shown to be capable of functioning in place of TFIIA *in vitro* (Lin, et al. 1988; Sawadago and Roeder 1985; Van Dyke, et al. 1988; Workman and Roeder 1987). USF, ATF or GAL4 can be added to a reconstituted transcription system depleted of TFIIA and will activate transcription when a promoter containing the appro-

priate binding site is used (Carthew, et al. 1985; Hai, et al. 1988; Horikoshi, et al. 1988; Lin, et al. 1988; Miyamoto, et al. 1985; Sawadago and Roeder 1985; Van Dyke, et al. 1988). Although these site-specific transcriptional activators have been shown to work independent of TFIIA to reconstitute transcription *in vitro*, some researchers have claimed that this independence is because the TFIID fractions used were still contaminated with TFIIA, and that even in the presence of site-specific transcription proteins TFIIA is required (Hahn, et al. 1989a). In summary, the addition of TFIID and a cofactor to a promoter is necessary and sufficient for the "commitment" of a template (Van Dyke, et al. 1989) and is the initial, rate-limiting step in the formation of the transcription complex (Reinberg, et al. 1987). This step is shown schematically in Figure 2A.

After the assembly of TFIID and a cofactor (TFIIA, ATF, USF and perhaps others as well), the next step in the assembly of the transcription complex is the recognition of the TFIID/cofactor/DNA complex by TFIIB, TFIIE, TFIIF and RNA Polymerase II (see Figure 2B). Once these components have assembled, TFIIA (if used) may be released and a preinitiation complex involving TFIIB, TFIIE, TFIID and RNA Polymerase is formed (Hawley and Roeder 1985). The physical interactions of TFIID and upstream elements USF (Van Dyke, et al. 1988), or ATF (Horikoshi, et al. 1988) with DNA have been shown to change qualitatively upon the addition of TFIIB, TFIIE and RNA Polymerase II when assayed by DNAse I footprinting. These changes are most noticeable when all of the components are added, although some minor changes occur with single or pairwise addition of the factors (Van Dyke, et al. 1988). This step is shown in Figure 2B.

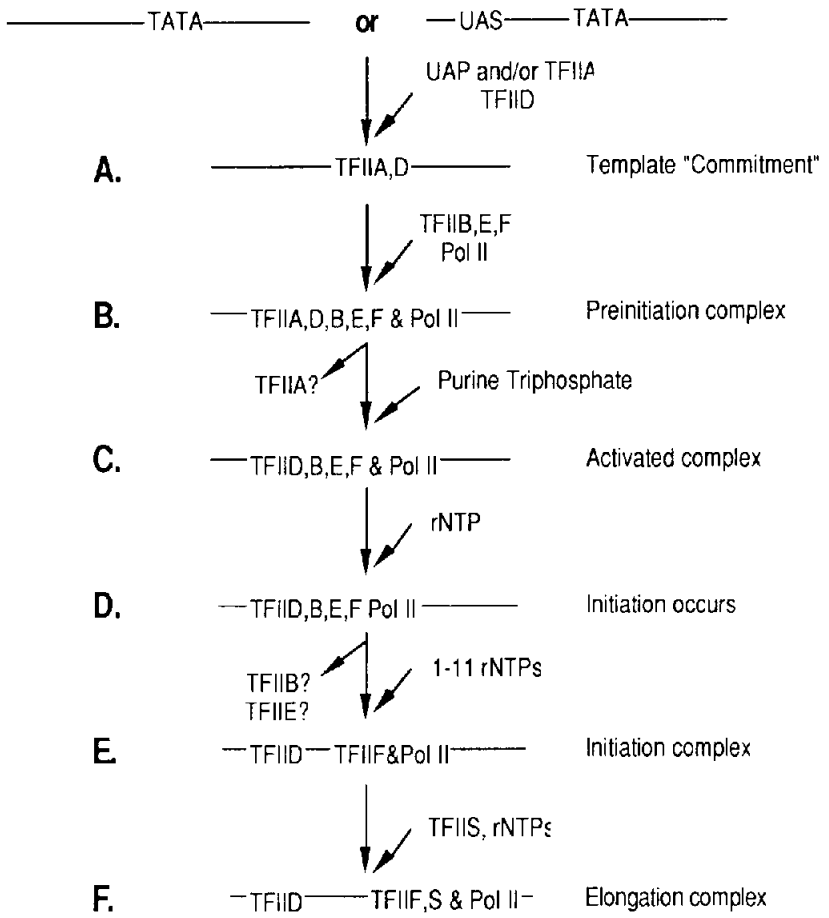

Figure 2. Model showing the sequence of events leading to a transcriptionally competent complex. The TFII (A,B,D,E,F) nomenclature is for Transcription Factor for RNA Polymerase II (chromatographic identification). UAP stands for a upstream activation protein and UAS upstream activation site. The TATA-box is known to be recognized by TFIID, 'B' factor or TATA factor in yeast (Burakowski, et al. 1988), *Drosophila* (Parker and Topol 1984) and HeLa cells (Nakajima, et al. 1988). TFIIA is shown in the various steps, but promoter-specific DNA-binding proteins can also lead to the formation of a preinitiation complex (Hai, et al. 1988).

Energy in the form of purine triphosphates is apparently utilized to alter the conformation of the TFIID, TFIIB, TFIIE, TFIIF and RNA Polymerase II-containing complex (Rappaport and Weinmann 1987) and form an activated complex (shown in Figure 2C). Upon addition of a single ribonucleotide ini-

tiation occurs (Figure 2D) and upon subsequent elongation TFIIB and/or TFIIE is released (Burakowski, et al. 1989, shown in Figure 2E). The release of TFIIA, TFIIB and TFIIE is postulated on the basis of the ability of the transcription complex to reassemble on a newly introduced promoter under limiting factor conditions (Hawley and Roeder 1985), the size of the assembled complexes (Burakowski, et al. 1989; Van Dyke, et al. 1988), and changes in the footprinting region upon purine triphosphate addition (Burakowski, et al. 1989). TFIIE and TFIIF have been shown to associate with RNA Polymerase II, and TFIIF is necessary for elongation (Flores, et al. 1989). After the transcription of the first several nucleotides TFIIS joins the transcription complex (Burakowski, et al. 1989; Cai and Luse 1987; Reinberg and Roeder 1987b), and the transcription complex is fully competent for elongation (see Figure 2F).

Structure of a yeast RNA Polymerase II transcribed promoter

The next aspect of transcription that will be examined is the structural elements located in an active promoter (*cis* elements) and the factors that interact with these elements. For the purpose of this discussion, only elements that are required for the activation of *Saccharomyces cerevisiae* structural genes will be examined. By referring to the generalized form of a yeast promoter in Figure 3, it can be seen that there are 3 distinct elements present in a promoter: the startpoint of transcription (or cap site), the TATA-box (also referred to as the TATA-element or Goldberg-Hogness box) roughly 80 to 100 bases upstream of the startpoint, and one or more upstream activation sites (UASs) located a variable distance upstream of the TATA-box. The UAS is responsible for activating transcription, and the TATA-box directs the transcription complex to the startpoint If all three of these elements are present up-

stream of a gene in the proper spatial orientation, then that gene should be actively transcribed under the appropriate conditions *in vivo*.

Two additional elements which affect the rate of transcription have been found to work at a distance - enhancers (Guarente 1988) and silencers (Mahoney and Broach 1989). Enhancers and silencers for structural genes can act at a distance to *enhance* or to *silence* promoter activity. Both enhancers and silencers can also act either upstream or downstream of a gene and their action is relatively independent of position or orientation. For the purpose of this discussion, enhancers and silencers will be treated as extensions of typical UASs or upstream repression sites (URSs), respectively.

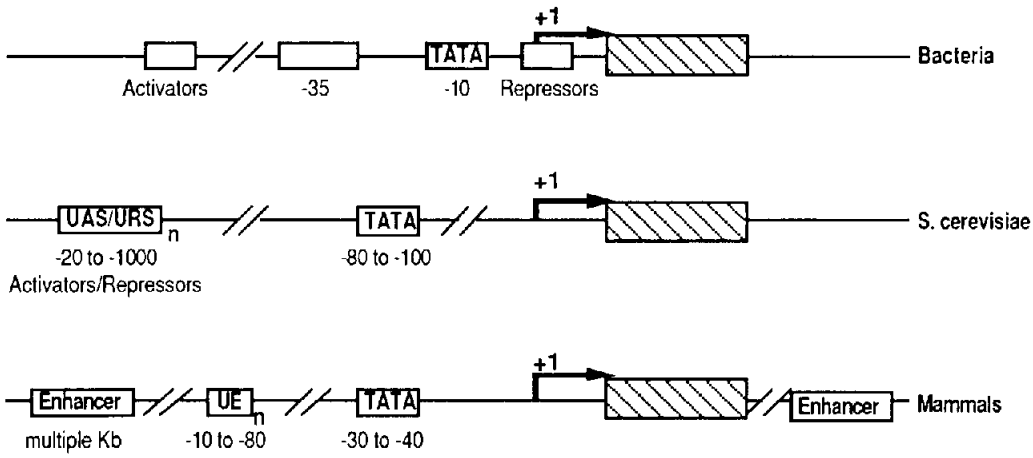

Figure 3. Comparison of features present in bacterial, yeast and mammalian promoters. Examples of genes with promoters containing TATA homologies, and upstream activator elements have been found in all organisms examined so far. Eukaryotic promoters (here represented by idealized fungal and mammalian promoters) have several additional elements, such as upstream repressors and cap sites (at the site of initiation), and are in general less spatially constrained than prokaryotic promoters. Higher eukaryotic promoters, including insect and mammalian promoters, also have enhancer elements which can function at large distances upstream and downstream of the start point of transcription. The acronyms UAS, URS and UE stand for upstream activation site, upstream repression site and upstream element, respectively.

The yeast promoter drawn in Figure 3 is typical (Struhl 1989b), however, there are at least two alternative promoter structures that are functional in yeast. One involves the replacement of the TATA-box with other sequences that function as a "TATA-like element" (Chen and Struhl 1989), while the second functions independent of a either a TATA-box or "TATA-like element" and is composed of repeating 'A' rich regions (Chen, et al. 1987; Struhl 1985a). These three types of promoters are shown diagrammatically in Figure 4. These two alternative promoter structures will be compared and contrasted to the 'classical' or TATA-box containing promoter.

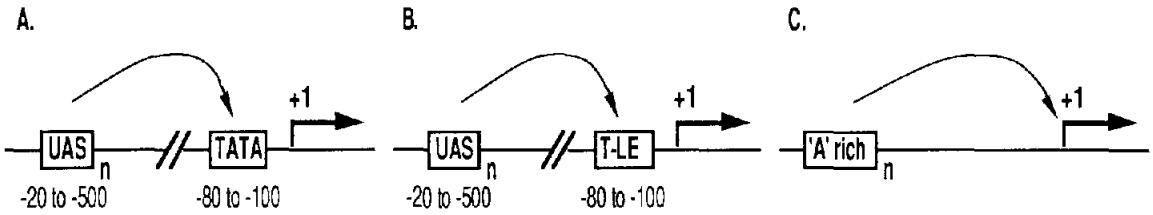

Figure 4. Three types of yeast promoters. 4A depicts the 'classical' or TATA-box containing promoter, 4B depicts a promoter with a "TATA-like element" (T-LE) and 4C shows an 'A' rich region that conveys a low level of activation without a TATA-box or T-LE. The arrows indicate that the activation sequences work through either the TATA-box, T-LE or directly on the startpoint, and are not meant to indicate a molecular mechanism.

In yeast and probably all eukaryotic organisms, the location of the startpoint for RNA Polymerase II transcribed genes is determined by the location of the TATA-box or TATA-like element rather than a specific feature present at the startpoint of transcription (Nakajima, et al. 1988). It is possible that other elements or features present in the primary sequence of the promoter also direct the startpoint, especially in the case of the TATA-box-less promoters, but there is no direct evidence for such a feature other than the preference for the initiation of transcription to occur at the short consensus CA*Pyrimidine* (Heintz and Roeder 1982). This sequence preference is proba-

bly due to some component of the transcription complex, rather than a site-specific DNA-binding factor. Direct evidence that the TATA-box/TFIID complex directs the transcriptional machinery to the startpoint is as follows: In the *S. cerevisiae* gene *CYC1* there are three functional TATA-boxes that drive multiple startpoints which are spread over about 40 bases. By creating a set of mutant promoters, each containing only one of the TATA-boxes, it was shown that each TATA-box directed the activation of a subset of the startpoints of transcription (Hahn, et al. 1985). This indicates that there are spatial constraints placed upon the location of the TATA-box and the subsequent use of start sites.

Apparently the TATA-box/TFIID complex is not the only component of the transcription apparatus that determines the distance from the TATA-box to the startpoint of transcription. This was demonstrated when a cell-free soluble transcription system derived from mammalian cells and depleted of TATA factor (TFIID) was complemented with yeast derived TATA factor (Burakowski, et al. 1988). This heterologous reconstituted transcription system was used to transcribe a synthetic gene containing the Adenovirus 2 major late promoter. In yeast, the startpoint of transcription would be expected to occur from 80 to 100 bases downstream of the TATA-box, whereas in a mammalian system the startpoint would be around 30 bases downstream. The startpoint of transcription in the heterologous system was approximately 30 bp downstream of the TATA-box, indistinguishable from the wild type mammalian startpoint (Burakowski, et al. 1988). Thus, the difference in the distance of the startpoint from the TATA-box between yeast and mammalian promoters is apparently due to differences in some part of the transcription apparatus other than the TATA-box/TFIID complex. Furthermore, this experiment showed that a functional interaction between transcription compo-

nents derived from mammals and from yeast could occur, demonstrating that at least a part of the transcription apparatus is functionally conserved between yeast and mammals. See *Conservation of the transcriptional machinery* for detailed comparison of the elements of the transcriptional machinery that are conserved throughout evolution as well as for other examples of functional conservation.

In the classical TATA-box-containing promoter (See Figure 4a), the UASs apparently direct the transcriptional machinery to the TATA-box which promotes transcription at the correct position (Dynan and Tjian 1985). Certain other *cis* elements, such as the GCN4 recognition element (UAS_{GCN4}), can also function in place of the TATA-box (Chen and Struhl 1989). Apparently the GCN4 protein, when it is in the correct location, can direct the positioning of the startpoint of transcription, just as the TATA factor has been shown to function. A further point of similarity exists between the TATA-box and the "TATA-like element" - the presence of an upstream activation element is required for the efficient promotion of transcription. In other words, when a UAS is functioning as a TATA-box, it cannot also function as an upstream activation element. One experiment that remains to be done is to place a TATA-box upstream of a UAS TATA-like element and see if the TATA-box can activate transcription in this type of promoter. It seems unlikely that the TATA-box will function in this context: if it cannot, than the ability of a UAS to activate transcription is a separate function from its ability to act as a TATA-like element, suggesting that some UASs may lack this TATA-box-like function and may therefore not be able to function in place of the TATA-box.

The third type of active promoter in yeast, which does not activate transcription by the use of a TATA-box or TATA-like element, may operate through a somewhat different mechanism. The best defined (and *perhaps*

only) element of this sort is the 'A' rich element found upstream of several genes (Struhl 1985a; Struhl 1985b; Winter and Varshavsky 1989). Chen and colleagues examined the activity of an 'A' rich element along with the activity of a UAS in a T7 transcribed promoter. They placed either element in a T7 promoter, and transformed this promoter into a yeast strain containing actively expressed bacteriophage T7 polymerase. They found that when a UAS was placed upstream of the T7 promoter, it was unable to promote transcription, however, when the 'A' rich region was placed upstream, it could still function as an activator of transcription (Chen, et al. 1987). This result was interpreted as proof that the 'A' rich region conferred an unusual structure upon the DNA, and that even T7 polymerase could be attracted to this unusual structure. However, recently Winter and Varshavsky have isolated and cloned Datin, a protein that recognizes this region, and shown that at least some of the properties of this 'A' rich region are conveyed by Datin (Winter and Varshavsky 1989). Therefore, it seems likely that the action of the 'A' rich region is through a novel attraction mechanism for the assembly of a transcriptionally competent complex. It is worth noting that it is not known whether the 'A' rich region positions the startpoint, or if another element or component is necessary for this positioning. A representation of this type of promoter is shown in Figure 4C.

Conservation of the transcriptional machinery

The evolutionary divergence of a region of DNA can be shown to occur at a fixed rate (Lewin 1987). However, it has been observed that genes and other regions of DNA that are required for some function diverge slower, and that the rate of divergence is related to the number of constraints placed on the region of DNA, such as the importance of the function of the region or the

number of components that must interact with it (Lewin 1987). For instance, sequence elements and their positions in bacterial, yeast and mammalian promoters are quite similar, demonstrating that the sequence elements necessary to promote transcription are highly conserved, suggesting that a great deal of selective pressure is placed on these elements (idealized promoters are shown in Figure 3). For instance, all three promoters have a similar TATA-box sequence upstream of the startpoint of transcription which is necessary for the spatial alignment of the startpoint. Furthermore, all of these promoters have upstream elements that are necessary to activate transcription.

The level of conservation includes the function and structure of the proteins involved in the transcription complex and in recognizing DNA. The level of conservation of the structures involved in transcription and the ability of the factors to be functionally interchanged among various organisms suggests a high degree of selective pressure to conserve certain functional domains. The apparent molecular weight of many of these factors from various organisms is quite disparate (see Figure 1), suggesting that if these proteins have the same function, there must be domains that are highly conserved with large regions of divergence. The general transcription factors TFIIA and TFIID have been shown to be present in yeast and mammalian cells, and are interchangeable in soluble transcription systems (Burakowski, et al. 1988; Hahn, et al. 1989a), even though the apparent sizes of the mammalian and yeast homologues are quite different (see Figure 1). TATA factor or 'B' factor has also been purified from *Drosophila* cells (Parker and Topol 1984). TFIID or TATA factor has recently been cloned from yeast and shown to be the same as *SPT15*, a yeast gene identified by genetics as a gene involved in sporulation (Eisenmann, et al. 1989; Hahn, et al. 1989b; Horikoshi, et al. 1989a). The DNA-binding domain has some homology with the single-

stranded binding domain of sigma factor, and some limited homology with other sigma factors as well (Eisenmann, et al. 1989).

Other site-specific DNA-binding proteins that are known to be transcription factors are present in yeast and mammals, such as ATF (Jones and Jones 1989), USF (Chodosh, et al. 1989), heat-shock factor (Wiederrecht, et al. 1987), Sp1 (this thesis), AP1 (Moye-Rowley, et al. 1989) and the SV40 'core' recognition protein (this thesis). In particular, heat-shock factor, a site-specific DNA-binding protein has been cloned from yeast, and shown to be capable of recognizing the heat-shock recognition element in a variety of organisms (Wiederrecht, et al. 1987). However, attempts to use this gene to pull out *Drosophila* homologues have thus far failed (G.J.Wiederrecht and J. Nieto, personal communication), suggesting that the sequence homology between the yeast and *Drosophila* genes is quite limited. The yeast HSTF was also demonstrated to activate transcription in a *Drosophila* cell-free soluble transcription system, demonstrating that the HSTF can function in conjunction with the *Drosophila* transcription apparatus.

A comparison of the DNA sequence of the gene encoding the large subunit of RNA Polymerase II from several organisms establishes that the overall DNA and protein homology of this subunit between these organisms is not very great (Greenleaf, et al. 1987; Jokerst, et al. 1989). In addition, unlike TFIID, yeast RNA Polymerase II does not function in a complementation assay using mammalian transcription factors (Horikoshi, et al. 1989b). However, there are localized regions which are highly conserved between yeast, mammals, and E. coli B' (Jokerst, et al. 1989).

These observations strongly suggest that the regions of these proteins involved in protein-protein interactions, protein-DNA interactions or enzymatic or catalytic activities are highly conserved, at least with regard to their

function. It seems likely that proteins with multiple functions have separable domains for each function. Examples of domain modularity have been found in several transcription factors. For example, a domain has been found in AP1 (Moye-Rowley, et al. 1989), Sp1 (Kadonaga, et al. 1987) and in TFIIIA (Ginsberg, et al. 1984) that is associated with activation of transcription and is separable from the DNA-binding domain.

The conservation of small regions of structure and function and the concomitant conservation of DNA sequence coding for those regions has allowed the identification and isolation of families of genes in a single organism, such as the GTP binding superfamily. This conservation can also be used to locate homologous members of a gene in other species, such as specific members of the RAS or heat-shock families. The advent of inexpensive synthesis of oligonucleotides (Pharmacia 1989) and the polymerase chain reactions (PCR) has allowed very small regions of conservation to be used to pull out representative DNAs from genomic and cDNA libraries (Saiki, et al. 1988).

Transcriptional Regulatory mechanisms

So far the components that are a part of a transcriptionally competent complex and the elements necessary to assemble these components in an accurate manner on a promoter have been discussed. However, structural genes are not always actively transcribed, nor are all promoters equal in strength (measured as the rate of transcription from a promoter) under various conditions. In fact, many genes that encode proteins which are members of a particular pathway, such as genes involved in respiration or in metabolism are often regulated at the level of transcription. For instance, the yeast cytochrome-C genes *CYC1* (Guarente, et al. 1984; Guarente and Mason 1983; Lowry, et al. 1983), *CYC7* (Prezant, et al. 1987), the cytochrome-C oxidase

subunit genes *COX4* (Hinnebusch 1988), *COX5a* (Trueblood, et al. 1988), and *COX6* (Trawick, et al. 1989) are transcriptionally regulated by the level of heme present in the cell and carbon source used to grow the cells. Nearly all of the anabolic pathway genes are controlled by the presence or absence of amino-acids (Hinnebusch 1988). This regulatory pathway is referred to as general amino-acid control (Hinnebusch 1988). The components used by the cell to accomplish the regulation of transcription are quite varied, but the basic control of transcription can be broken down into two categories - activation and repression. Even at this level of simplicity, the distinctions between the two categories can blur. For example, some factors that are transcriptional activators in the context of one promoter can be repressors in a different promoter (Diffley and Stillman 1989)! This flexibility of control gives rise to a level of complexity that makes it quite difficult to unravel the function of individual factors. It is past the scope of this discussion to introduce all of the possible mechanisms by which the activation and repression can occur, but a few will be discussed in some detail.

Going back to the basic form of a yeast promoter in Figure 3, we see that the position of the UASs or upstream repression sites (URSs) in yeast are always upstream of the startpoint of transcription. Silencers will be treated as unusual extensions of URSs and enhancers as extensions of UASs. The molecular mechanisms by which UASs or URSs function is not fully understood, however, UASs in conjunction with their binding factors probably interact in concert with TATA factor (TFIID) or with the transcriptional machinery (see Figure 5A). This indicates that the factors interacting with a UAS have at least two domains: the DNA recognition domain and a domain responsible for transcriptional activation. This type of modularity has been demonstrated for a number of transcription factors. A factor *could activate*

transcription by enhancing the binding of the transcriptional machinery, or by catalyzing a conformational change in TFIID or another component of the transcription complex. An interesting characteristic of many UASs and the proteins that bind to them is that the activational effect of multiple UASs is proportional to the number of sites present in the promoter, up to the point of saturation (Harshman, et al. 1988; Park and Craig 1989; Sorger and Pelham 1987). Thus, if a UAS has the ability to convey 5 units of activity on a promoter, the same promoter with 3 of the UASs will have 15 units of activity (assuming non-saturating conditions). This characteristic of a yeast UAS is difficult to explain by a simple one-to-one interaction of the UAS/UAS-binding factor with either the transcription complex or the TATA-box/TFIID complex. One explanation for this additive effect is that the UAS/UAS-binding factor could function by affecting the rate of assembly or initiation of the transcriptional components on the promoter, where each UAS/UAS-binding factor complex could attract or position some component of the transcription complex at a set rate. The total rate of transcription complex assembly or initiation would be the sum of all of the UAS 'attractor' rates. This assumes that the slow step in the assembly of a transcription complex is the assembly or positioning of the transcription complex at the proper startpoint. In this model, saturation of the UAS effect occurs when the assembly or positioning of the transcription complex ceases to be the rate-limiting step.

URSs probably function by changing the conformation or accessibility of either a UAS/UAS-binding factor complex (Figure 5B), the TATA-box/TFIID complex (Figure 5C) or by preventing a conformational change to occur which is necessary for the assembly of a transcriptionally competent complex during the initial formation of a transcription complex (Figure 5D). Repressors that interact specifically with just one UAS probably conform to the model in

Figure 5B and are exemplified by the URF (Chapter 2, this thesis) or URS1 (Sumrada and Cooper 1987). Silencers probably function by interrupting the function of the TATA-box/TFIID complex (similar to the model in 5C) and a 'blocking' activity (this thesis, Chapter 1) must function by somehow blocking the activity of UASs that are further upstream of the blocking region, but not affect UASs downstream of the blocking region (model not shown). This last bit of information indicates that there is a directional component to the action of activation. One possible mechanism to explain this directional blocking of activation is to invoke a mechanism by which a component of the transcriptional machinery is attracted to an activator, the transcriptional machinery is dropped down onto the DNA to either side of the activator, and the transcription component slides either upstream or downstream. Using this mechanism, it becomes quite obvious that the 'blocking' activity would be doing exactly that - preventing the sliding of the transcriptional apparatus past the block. Many different experiments with tethered activators attached to DNA have been tried in order to differentiate between the bending or the sliding mechanism of activation, and so far none of the experiments has been conclusive (Müller, et al. 1989).

In addition to these models for URS mediated repression, there are also two other forms of repression that do not require the presence of a URS. In one, shown in Figure 5E, the repressor binds specifically to an activator without the need to bind DNA. In the other, shown in Figure 5F, the repressor and activator recognize each other, and this interaction prevents the activator from successfully binding to the UAS (Levine and Manley 1989). One other method of regulation would be competition of multiple binding factors for one site, such as is seen between the factors RC2 and HAP1 for the *CYC1*

UAS1 (Pfeifer, et al. 1987), or ABF1 and the GT-box factor for the ABF1 site and GT-box (Chapter 1, this thesis).

The terms upstream activation site (UAS) and upstream repression site (URS) refer to *cis* elements present in the promoter, however, it is the activity of factors that bind to these elements that determine the actual activity of a particular element. As was alluded to in the beginning of this section, sequences (and perhaps factors) that act as repressors in one set of physiological conditions or promoter contexts may in fact be activators under different conditions or contexts. Several examples of elements of these sorts are known, such as RAP1, which functions as a repressor in some promoters, an activator in others, and has been implicated in replication as well (Buchman, et al. 1988). Similarly, ABF1 has been shown to function as a repressor or activator of transcription, as well as to bind to yeast autonomous replication sequences (ARSs) and mediate the replication of ARSs (Dorsman, et al. 1989; Rhode, et al. 1989; Sweder, et al. 1988).

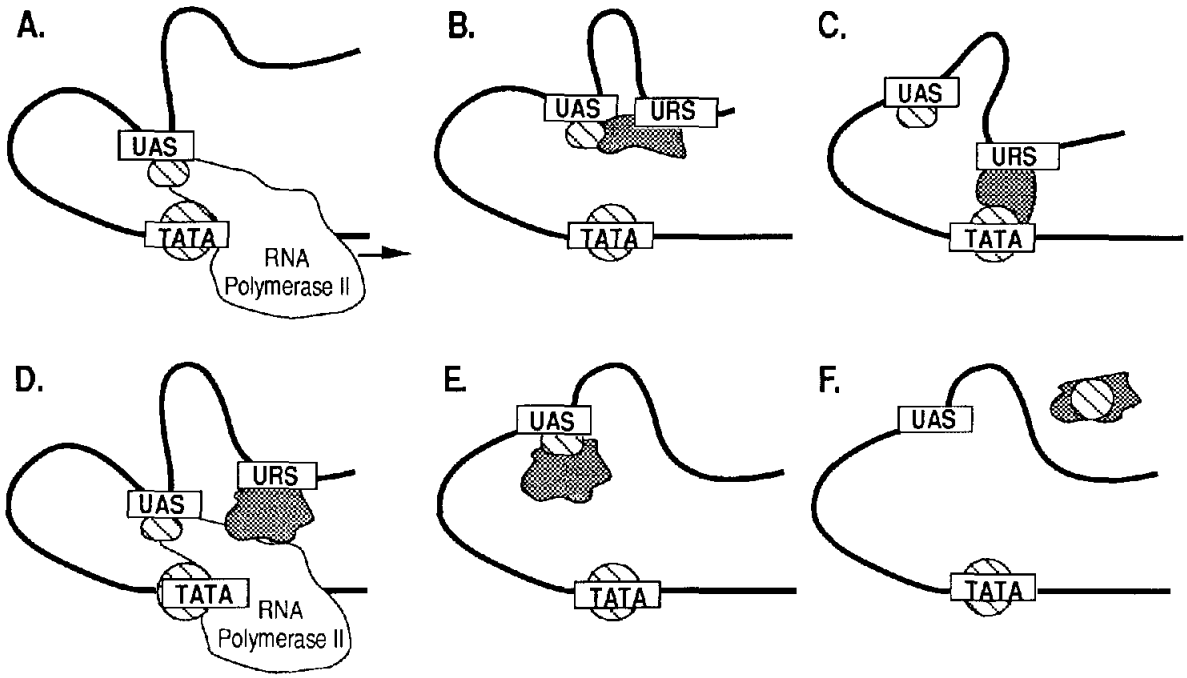

Figure 5. Schematic drawing of a possible mechanisms for transcriptional activation and repression. 5A shows the interaction of an upstream activator protein with its recognition element (UAS) and with RNA Polymerase II. The TATA-box and TFIID are also required for proper assembly of the RNA Polymerase onto the transcription initiation site. In diagrams B through F various modes of transcriptional repression are shown. In 5B, the model predicts that an upstream repressor protein interacts directly and in a one-to-one stoichiometry with an upstream activator protein, and that this interaction changes the conformation or availability of a transcription activation domain so that polymerase does not assemble onto the transcription initiation site. In diagram 5C, the upstream repressor protein functions by interacting directly with TFIID, again inhibiting the assembly of a transcription complex at the transcription initiation site. This second mechanism of inhibition would therefore be dominant over multiple activation proteins. The model of repression in 5D allows polymerase to recognize DNA, but not to fully assemble into a transcriptionally competent complex. This could be accomplished indirectly by a change in the conformation of TFIID or an upstream activation protein, or by a direct interaction of the repressor protein with polymerase or some other component of the transcriptional machinery necessary to convert the polymerase complex into a transcriptionally competent complex. 5E and 5F show two forms of repression where the repressor is not a site-specific DNA-binding protein.

Site-specific DNA-binding factors

DNA-protein interactions are essential for gene expression. In particular, the spatial and temporal control of replication and transcription is mediated by site-specific DNA-binding proteins (Echols 1986). In the model system λ, the interaction of multiple binding sites with two site-specific DNA-binding proteins is sufficient to create a molecular 'switch'. This is exemplified by the interaction of the DNA-binding proteins λ repressor and Cro on the operator sites O_R1, 2 and 3 in the divergent promoter P_R and P_{RM} (Ptashne 1986). Other studies with λ have demonstrated that the differential affinity of the operator sites for λ repressor and cro in combination with the concentration of λ repressor and cro regulates the expression of *cI* (repressor-encoding gene) and *cro* genes. From currently available evidence, the transcription pattern of a gene is sensitive to the affinity, position and context of promoter elements located in the promoter, as well as properties of the proteins interacting with the promoter.

Interactions necessary for binding to DNA

The affinity of a particular DNA site and a DNA-binding factor must be due to the summation of all of the interactions between the DNA site and the factor. These interactions can be viewed as belonging to two types of interactions - non-specific and specific. The non-specific DNA interactions can be due to binding occurring on the phosphate backbone, or with the sugar moieties. The specific interactions are due to interactions in with functional groups that are sequence specific - such as interactions of the binding factor with groups on the bases. These interactions have been generally modeled to be hydrogen bonds with sites available in the major groove of DNA.

DNA-protein interactions, both specific and non-specific, are presumed to derive their specificity from one of 4 types of bonding (Saenger 1984): (1) salt bridges (electrostatic interactions) formed between the phosphate backbone and positively charged amino-acid side chains, (2) hydrogen bonding between phosphate, sugar, or nucleosides moieties and hydrophilic amino-acid side chains in proteins, (3) stacking interactions involving aromatic amino-acid side chains and bases, and (4) hydrophobic interactions associating nucleic acid bases with nonpolar amino-acid side chains.

Protein structural motifs

Many site-specific DNA-binding proteins from a variety of organisms have been cloned and sequenced. Several motifs have been shown to be found in many of these proteins. The three best characterized are the helix-turn-helix, the putative metal binding motif known as the zinc-finger, and the leucine or isoleucine repeat structure known as the leucine-zipper (see review: Struhl, 1989a). Prokaryotic activators and repressors containing the helix-turn-helix motif have been crystallized, and their structure determined by x-ray diffraction. The three motifs (and a variation on the zinc-finger) are shown in Figure 6.

The helix-turn-helix motif

The helix-turn-helix motif is probably the best characterized structural motif associated with the ability of a protein to bind DNA. In the examples of this structure that have been examined, there are two regions necessary for the binding of the protein to DNA: one is the helix-turn-helix domain (also known as the homeodomain) which is sufficient for DNA recognition and the other is a protein dimerization domain. A monomer of a helix-turn-helix containing protein does not bind DNA with as high an affinity as the

multimeric protein, as defined by mutations in the dimerization domain of Cro (a λ site-specific DNA binding protein).

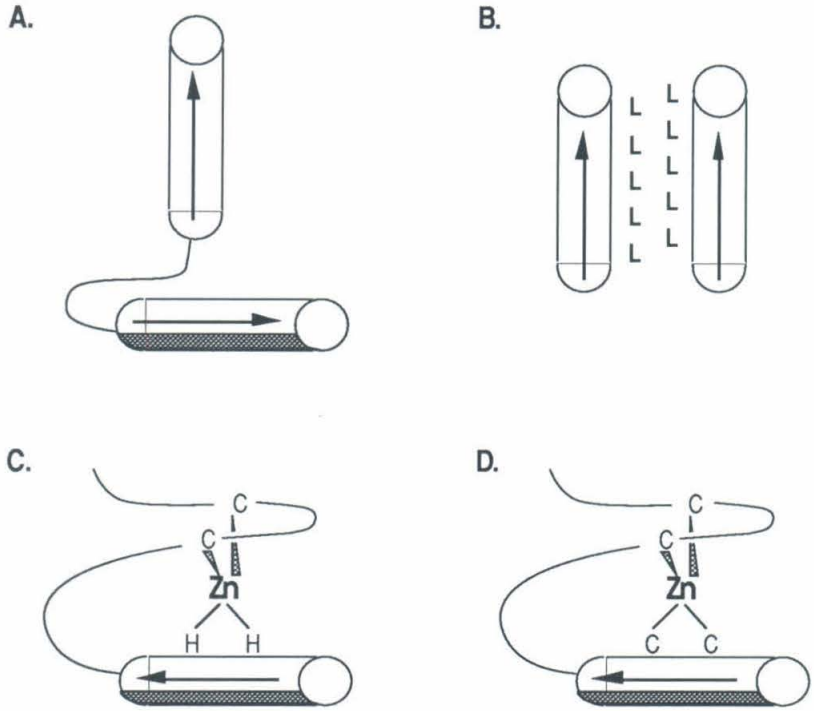

Figure 6. A representation of the 3 structural motifs currently identified in DNA-binding proteins. The cylinders represent α-helices, with the direction of the helix indicated by an arrow. The shaded side of the cylinders represent the side that reputed is involved in DNA interactions. 6A is a schematic of the helix-turn-helix motif, 6B is the leucine-zipper, and 6C and D are representations of the two common zinc-fingers: the cysteine-histidine zinc-finger (C) and the cysteine-cysteine zinc-finger. Figures 6A and 6B are from Struhl 1989a. Figures 6C and D are modified from Berg 1988 and from Gibson, et al. 1988.

The zinc-finger motif

This motif was first identified upon examination of the sequence of TFIIIA, which is a RNA Polymerase III transcription factor necessary for the transcription of 5S RNA genes. TFIIIA is known to bind multiple zinc molecules *in vivo* and *in vitro*, and that the coordination of zinc is necessary for the DNA-binding activity of TFIIIA. A motif, now known as the zinc-finger, was found repeated 9 times in the sequence (Brown, et al. 1985; Miller, et

al. 1985). Several examples of known DNA-binding proteins contain multiple repeats of this motif; *ADR1*, a yeast gene with two repeats involved in glucose regulation (Hartshorne, et al. 1985); *SWI5*, a yeast gene that has 3 repeats and is involved in the regulation of HO expression (the mating type switch in yeast) and binds to a short sequence in the HO promoter (Stillman, et al. 1988); and Sp1 from mammalian cells, also with three repeats (Kadonaga, et al. 1987; Kadonaga, et al. 1988). The zinc-finger has also been found in a number of *Drosophila* gap genes, including *kruppel* (Rosenberg, et al. 1986), and *hunchback* (Tautz, et al. 1987).

In the case of ADR1, SWI5 and TFIIIA, the zinc-finger motif has been shown to be contained in the DNA-binding domain. It has been presumed that the differences in the zinc-finger homologies are responsible for the various DNA sequence specificities associated with each protein. However, in a preliminary experiment in which the yeast PPR1 gene zinc-finger was inserted in place of a GAL4 zinc-finger, the resulting protein still recognized the wild type GAL4 binding site (Struhl 1989a). This suggests that a region outside the zinc-finger motif is responsible for specificity. Recently, three models depicting the tertiary structure of the zinc-finger have been proposed (Berg 1988; Gibson, et al. 1988; Miller, et al. 1985). One of these models (Gibson, et al. 1988) was developed using computer modeling techniques, and incorporates a ß-hairpin structure coupled with an α-helix that recognizes the DNA major groove. Dimethyl sulfate protection studies of these proteins has demonstrated that major groove contacts are important in the recognition of DNA (McKnight and Tjian 1986; Sakonju and Brown 1982). Both models assume that the metal is coordinated between two histidines and two cysteine residues (zinc-finger type I motif) and the second model, (Gibson, et al. 1988), shows a nearly identical structure using the four cysteine-coordinated metal

ion proposed for a second class of zinc-finger motifs (Struhl 1989a). The suggestion is made that for both types of zinc-finger motif containing proteins, the structural rigidity necessary to maintain high specificity is due to the constraints placed on the protein by the bound metal.

The leucine-zipper motif

The leucine-zipper motif has been found in GCN4, yAP1, *jun*, *fos*, *myc* , heat-shock factor and C/EBP proteins. In the *jun* family (in which GCN4 and yAP1 are members), it has been shown that there is a leucine-zipper is closely associated with the DNA-binding domain (Hope and Struhl 1987; Moye-Rowley, et al. 1989). The leucine-zipper has also been found closely associated with the DNA-binding domain in C/EBP (Landschulz, et al. 1988). In *myc* and the heat-shock transcription factor, the DNA-binding domain and the leucine zipper are distinct, indicating that the leucine-zipper itself does not recognize DNA (Sorger and Nelson 1989; Stone, et al. 1987). The motif consists of 4 or 5 leucine or isoleucine residues spaced exactly 7 amino-acids apart, which places these residues on the same face of an α-helix. It has been suggested that this structure is involved in protein-protein contact. Recently, using circular dichroism to measure the amount of α-helix content present in a short peptide, it was shown that a synthetic peptide containing the GCN4 leucine zipper could form a stable dimer that was almost 100% α-helical (O'Shea, et al. 1989) Furthermore, by placing two different peptides in solution, one with a N-terminal cysteine and the other with a C-terminal cysteine, this structure was shown to consist of two **parallel** coils, since only homodimers were formed, demonstrating that the dimerization preferentially occurred to form parallel coils (O'Shea, et al. 1989).

Concluding remarks

It has become apparent that a large number of components interact with the transcription complex to modulate the transcriptional activity of a gene, and that the mechanisms by which these components mediate their effects are quite diverse. Likewise, the assembly, initiation, elongation and termination of the transcription complex requires a number of factors and can be broken down into multiple steps. A great deal of the information that is now known about both the regulation of transcription and the action of the transcriptional machinery can be attributed to the identification and isolation of the factors associated with and comprising the transcription complex.

Because the mechanisms involved in the regulation are so diverse, the identification and isolation of new factors and the examination of their interactions with the transcriptional machinery continues to be a fruitful area of research. The research in this thesis involves the identification and isolation of several of site-specific DNA binding factors and examines their transcriptional activity.

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Chapter 1

Detailed Characterization of the Promoter for the Yeast HSP70 Gene *SSA1*

Warren A. Kibbe and Carl S. Parker

Table of Contents

Detailed Characterization of the Promoter for the Yeast HSP70 Gene *SSA1*

Abstract

The yeast *Saccharomyces cerevisiae* contains a family of hsp70 related genes. The *SSA1* gene encodes a 70kD heat-shock protein, which in addition to its heat inducible expression has a significant basal level of expression. Using DNAse I protection analysis of the promoter, the first 500 bp upstream of the *SSA1* start point of transcription was examined. The results reveal the presence of multiple protein binding sites, throughout the upstream promoter region. The function of these binding sites has been examined using a series of 5' promoter deletions fused to the bacterial gene lacZ encoding the enzyme ß-galactosidase. Some of these binding sites have also been assayed individually to determine their affect on the transcriptional activity of a truncated *CYC1*/lacZ fusion. Finally, a series of fusion constructs were made to identify a putative silencer-like element upstream of *SSA1*. This element is position dependent and has been localized to a region containing both an ABF1 binding site and a RAP1 binding site.

Introduction

It has been clearly demonstrated that the transcription of eukaryotic genes is regulated by sequence-specific DNA binding proteins. Thus, the sequence present in a promoter determines, at least in part, the precise regulation pattern exhibited by a particular gene. Because the yeast *Saccharomyces cerevisiae* is genetically well defined and is particularly amenable to both molecular genetic and biochemical approaches, it has been used as a model eukaryotic organism for studying the interactions of transcriptional elements. Several highly conserved binding elements and the associated binding factors involved in the regulation of RNA polymerase II genes have been studied in great detail in yeast, such as the TATA element, which is found upstream of the start point of transcription in many genes (Buratowski, et al. 1988; Chen and Struhl 1988; Hahn, et al. 1985; Horikoshi, et al. 1989; Nakajima, et al. 1988) and the heat-shock transcription factor recognition element (HSE), and is also found upstream of the start point of transcription (Sorger and Pelham 1987; Wiederrecht, et al. 1987). Some of the binding sites are responsive in a set manner to a particular stimulus, almost independent of other sites present in the promoter, such as the HSE (Park and Craig 1989; Slater and Craig 1987; Sorger and Pelham 1987; Wiederrecht, et al. 1987). Other sites, however, such as the ABF1 binding site or the RAP1 binding site, are context sensitive: they are modulated by other *cis*-elements present in the promoter, presumably through interaction between the factors binding to the cis-elements (Buchman, et al. 1988; Diffley and Stillman 1989; Dorsman, et al. 1989; Halfter, et al. 1989; Kimmerly, et al. 1988; Shore and Nasmyth 1987). Thus, it is necessary to characterize the activity of a particular promoter element not only individually, but also in the presence of other promoter elements.

Because investigating all of the possible permutations necessary to unravel the interactions of multiple trans-acting factors is quite daunting, the standard mechanism for studying the activity of trans-acting factors in yeast has been to define genetically genes that are responsive to a particular type of control, and then examine the promoters of these genes in search of putative *cis*-elements that could convey this response on the promoter. Heat-shock, nitrogen starvation, amino-acid control (called general control of amino-acid biosynthesis) and carbon metabolism are all pathways for which *cis* and *trans*-acting factors have been identified (Hinnebusch 1988; Lindquist and Craig 1988).

Heat-shock proteins (hsps) have been shown to be particularly well conserved across evolutionary boundaries; in particular, at least one representative of the 70 kD hsp is found in plants, animals, and bacteria. The hsp70 multigene family in *Saccharomyces cerevisiae* contains at least eight members (Lindquist and Craig 1988), with different members of the family responding differently to stress. These eight members have been broken down into 4 subfamilies on the basis of homology and complementation, SSA, SSB, SSC, and SSD (for *S*tress *S*eventy subfamily *A-D*) (Lindquist and Craig 1988). The presence of at least one functional gene product from each of the SSA and SSC subfamilies has been shown to be necessary for cell viability under a variety of growth conditions (Werner-Washburne, et al. 1989; Werner-Washburne, et al. 1987).

The regulation of the heat-shock response has been found to occur at the level of transcription, translation, and protein processing/maturation, depending on the organism and the hsp investigated. The RNA levels of individual yeast hsp70 genes including SSA1 respond differently to various physiological conditions (Werner-Washburne, et al. 1989), suggesting that hsp

transcription responds to many different stimuli and that the precise stimuli and temporal control exhibited by an hsp is dependent at least in part on the promoter structure.

In order to identify the *cis* elements and *trans*-acting factors responsible for the various responses of the *SSA1* promoter, we have undertaken the biochemical identification of some of them, notably the heat-shock transcription factor (HSTF) (Wiederrecht, et al. 1988; Wiederrecht, et al. 1987) and the G-box binding factor (GBF, see Chapter 4). Assaying the *SSA1* promoter for binding sites by DNAse I protection, we have found that the promoter contains at least 14 binding sites bound by at least nine different factors. Three of these sites have been previously identified as important for the regulation of the *SSA1*: two heat-shock elements (HSE sites 1 and 2) and a GC-box that is proximal to HSE2 (Park and Craig 1989; Slater and Craig 1987).

General mechanisms of transcriptional regulation are rapidly being elucidated. However, a key question that remains unanswered is how a *cis* element that modulates the transcription of one promoter is "blocked" so that another, nearby promoter does not respond to that element. In many cases the distance between adjacent promoters is far too great for them to utilize each others' regulatory elements; but in other examples, such as divergently transcribed genes sharing a small intergenic control region, another mechanism must be postulated. We have utilized the divergently transcribed promoter *SSA1/FUN15* to look for elements responsible for the "blocking" of transcription signals from one promoter to the other. *FUN15* stands for Function *UN*known gene *15*, and its purpose has not been determined (Craig, et al. 1987). *SSA1* is transcriptionally activated upon heat-shock, whereas *FUN15* is not (Craig, et al. 1987). We have identified a set of sites upstream of the *SSA1* HSE3 that can "block" transcriptional signals positionally. That is,

when these "blocking" elements are placed between known upstream activating sequences (UASs) and the TATA box of a functional gene, the gene no longer responds to the upstream UASs, but will still respond to UASs positioned downstream of the "blocking" sequences. We show the region responsible for conveying this activity, and identify at least two putative factors involved in this activity.

Results

Separation of the various binding activities

Figure 1a shows a summary of the purification scheme for the various DNA-binding factors analyzed in this paper and shows the elution profiles of the factors from several columns. For some of the factors, further characterization of their chromatographic behavior was carried out, and will be mentioned in other sections. The protection experiments described in this paper used the most highly purified fractions of each factor listed in Figure 1, unless otherwise stated in the individual figure legend. All of the fractions used were derived from the *Saccharomyces cerevisiae* strain EJ926. Heparin-Sepharose was the initial column used in all the purification schemes presented in this paper. Affinity chromatography was performed as described in *Materials and methods*.

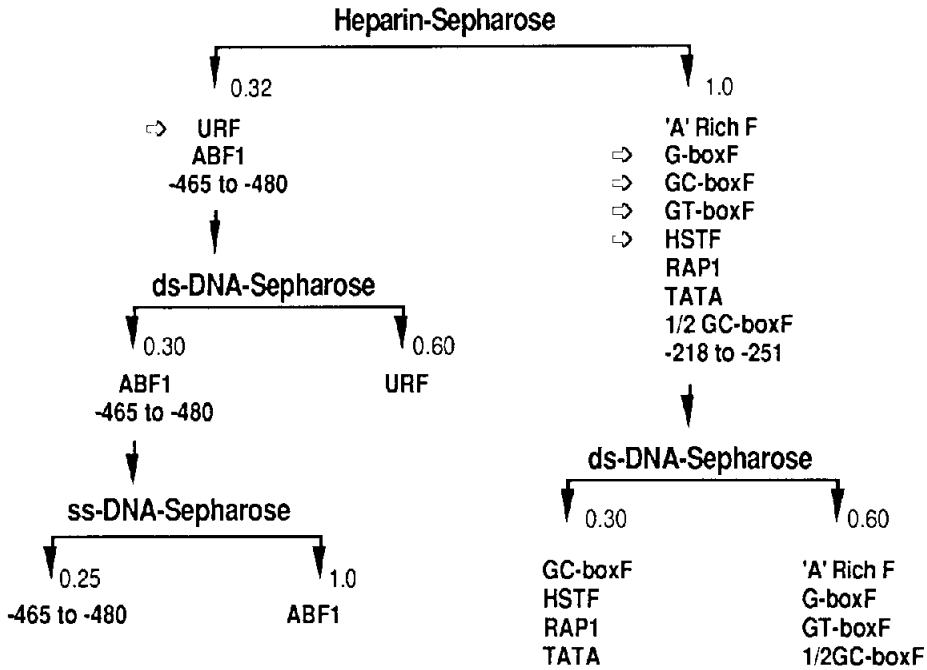


Figure 1A. Summary of the chromatographic behavior of the factors referred to in this paper. Input to the heparin-Sepharose column was a nuclear extract of the *S. cerevisiae* strain EJ926. Vertical lines underneath the column name have the molarity of KCl used to step elute material from the column written to the right of the line. The factors eluting at a particular step are listed underneath the vertical line. The chromatographic behavior of the -218 to -251 DNA-binding factor was not examined on dsDNA-Sepharose. Factors indicated with an arrow were also purified by sequence-specific affinity chromatography (see Figure 1B). See *Materials and methods* for more information.

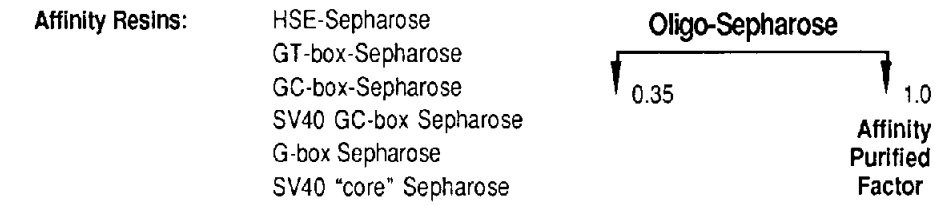


Figure 1B. Affinity purification of five of the binding activities via ligated sequence-specific oligonucleotide-coupled Sepharose. The activities were eluted by high salt off the respective affinity column and were shown to bind only to their own specific DNA sequence on the *SSA1* promoter. The columns were loaded with the chromatographic fractions noted in Figure 1A. The fractions were dialyzed to 0.2 M KCl prior to loading. After loading, the affinity columns were washed at 0.35 M KCl for one hundred column volumes (100 mls) at 10 column volumes per hour. Sequences for each of the specific oligonucleotide columns are given in the *Materials and methods* section.

DNAse I protection analysis

The DNAse I protection pattern obtained using different partially purified yeast protein fractions demonstrated that the *SSA1* promoter has multiple regions of protection. Nine chromatographically distinct factors were identified that interact with the region from -73 to -480 bases, relative to the start point of transcription. Five of these factors were affinity-purified and shown to be separate activities. These five factors bind only to the following regions on the SSA1 promoter: the GT-box factor binds the single GT-box site from -393 to -367; the GC-box factor (ySp1) binds to the GC-box site from -187 to -172 as well as the SV40 GC-box (data not shown); the upstream repression factor (URF) binds to the URE sites (homologous to the SV40 "core" consensus) from -325 to -310 and -101 to -85; the G-box factor (GBF) binds to the G-box sites at -104 to -86 and at -159 to -142; and the heat-shock transcription factor

(HSTF), recognizes the three heat-shock elements or HSEs at -94 to -73, -207 to -181, and -306 to -281.

The other factors that were purified and shown to be independent factors based on their chromatographic behavior recognize the sites labeled the 'A' Rich Region, the RAP1 site, the ABF1 site, and the 1/2GC-box. The 1/2GC-box was named as such because it shares the GCGC motif with the GC-box protection region. The activity binding to the 1/2 GC-box was found to be distinct from the activity protecting GC-box site. This factor does not recognize the SV40 GC-box and was not characterized further. Two additional footprints at -480 to -465 and at -236 to -218 (variably from -251 to -218, may be the same factor) were also not characterized further.

Figures 2 through 5 show footprints on all of the sites listed in Figure 6 except the URF on URE sites 1 and 2, which are in Chapter 2, this thesis. A summary of the sequences protected in DNAse I footprinting experiments is shown in Figure 6a. Figure 6b shows a schematic of the promoter with the protection sites and their boundaries.

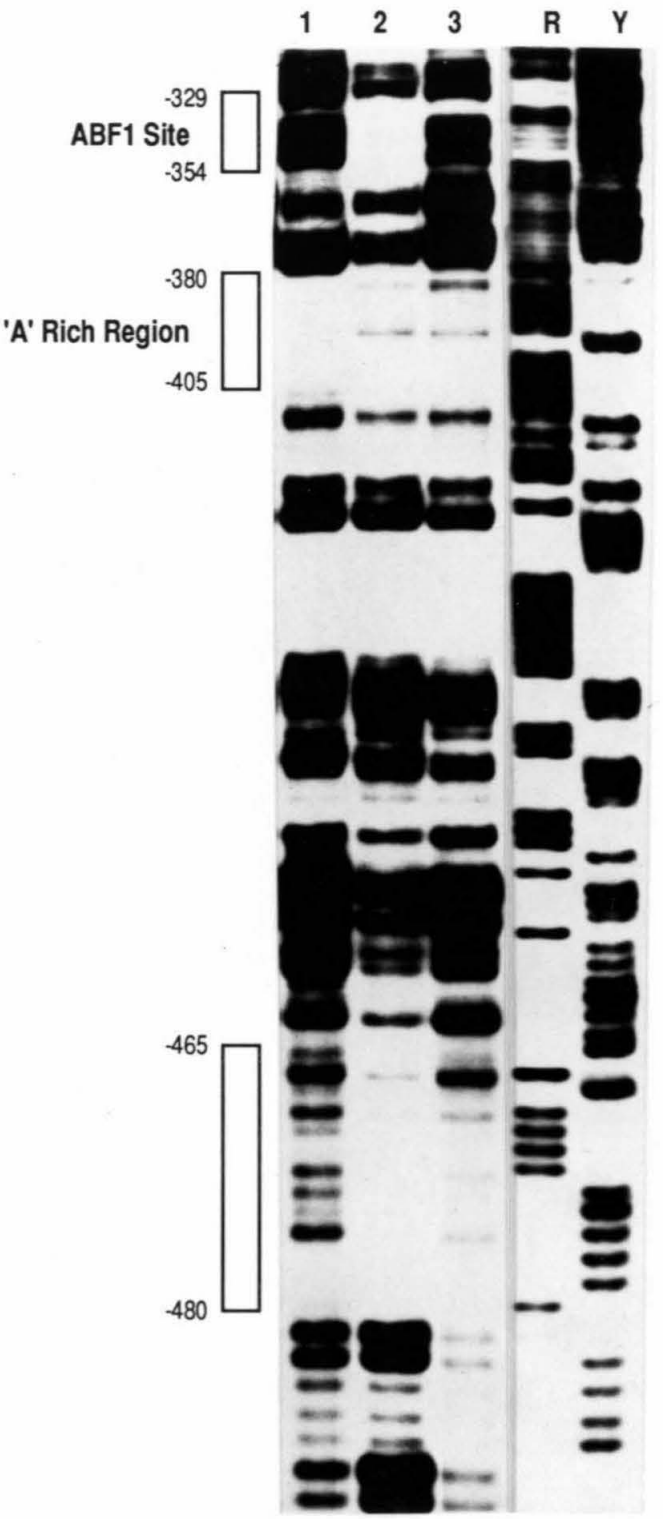


Figure 2.

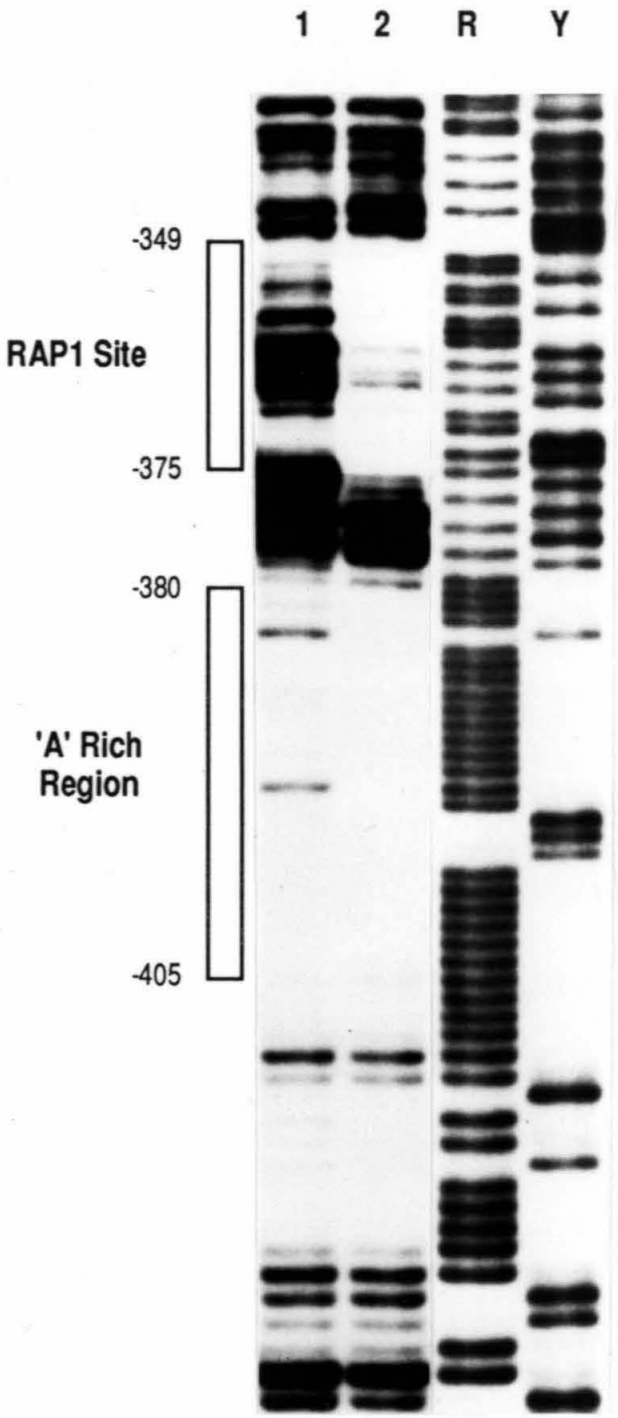


Figure 3.

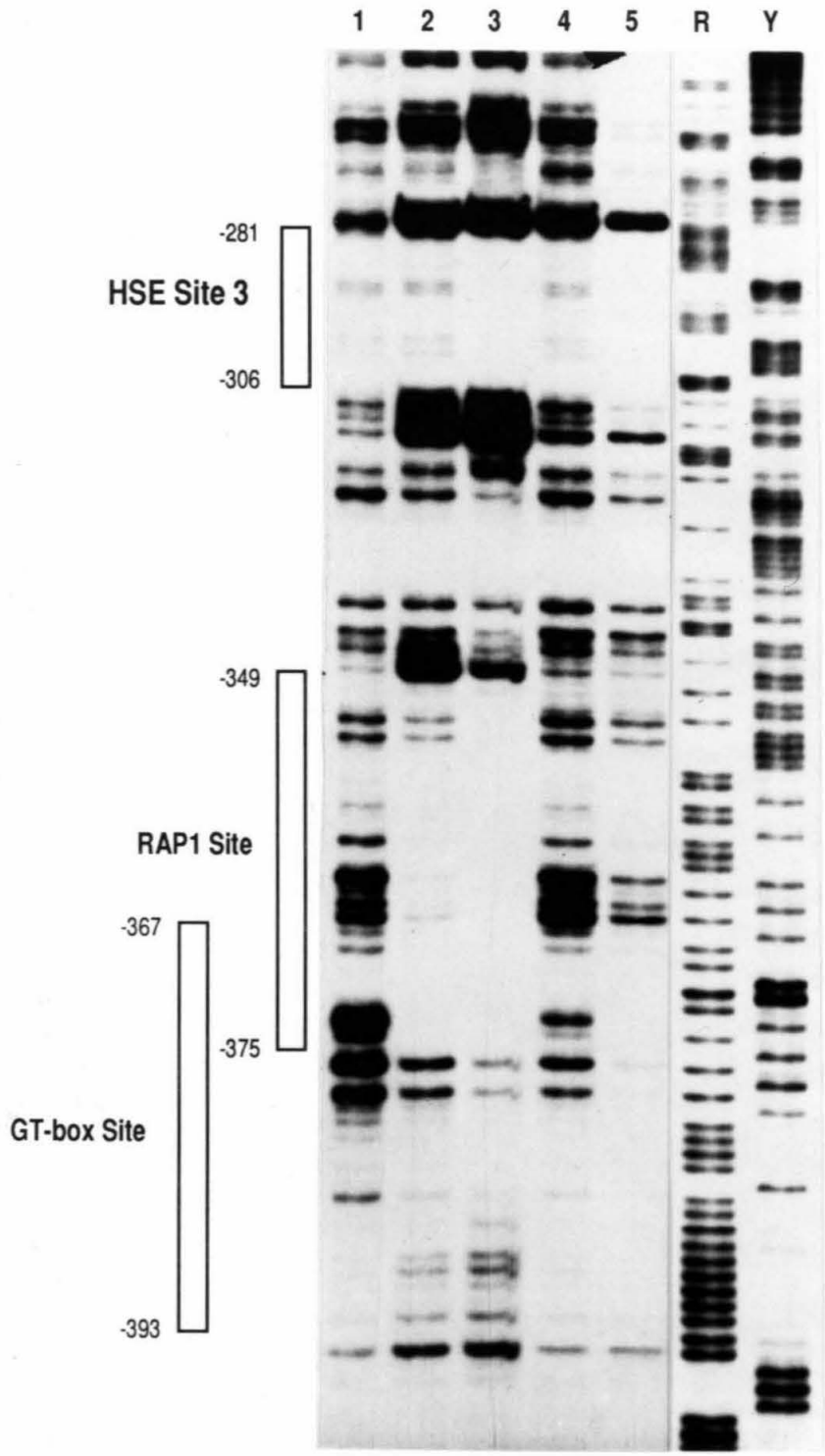

1
2
3
4
5
R
Y
-281
HSE Site 3
-306
-349
RAP1 Site
-367
-375
GT-box Site
-393

Figure 4.

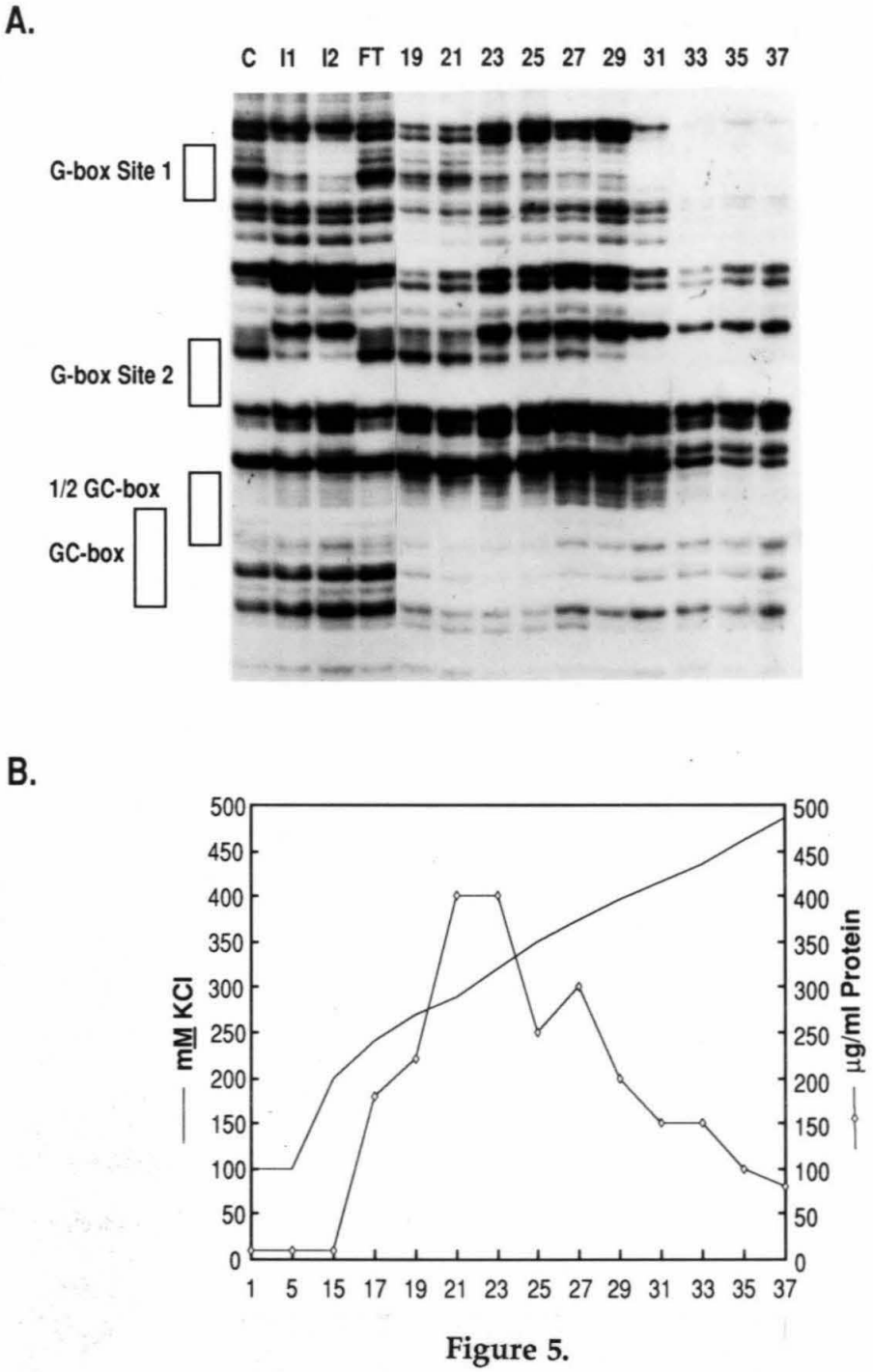

A.
C I1 I2 FT 19 21 23 25 27 29 31 33 35 37
G-box Site 1
G-box Site 2
1/2 GC-box
GC-box
B.
mM KCl
µg/ml Protein
500
450
400
350
300
250
200
150
100
50
0
1 5 15 17 19 21 23 25 27 29 31 33 35 37

Figure 5.

A.

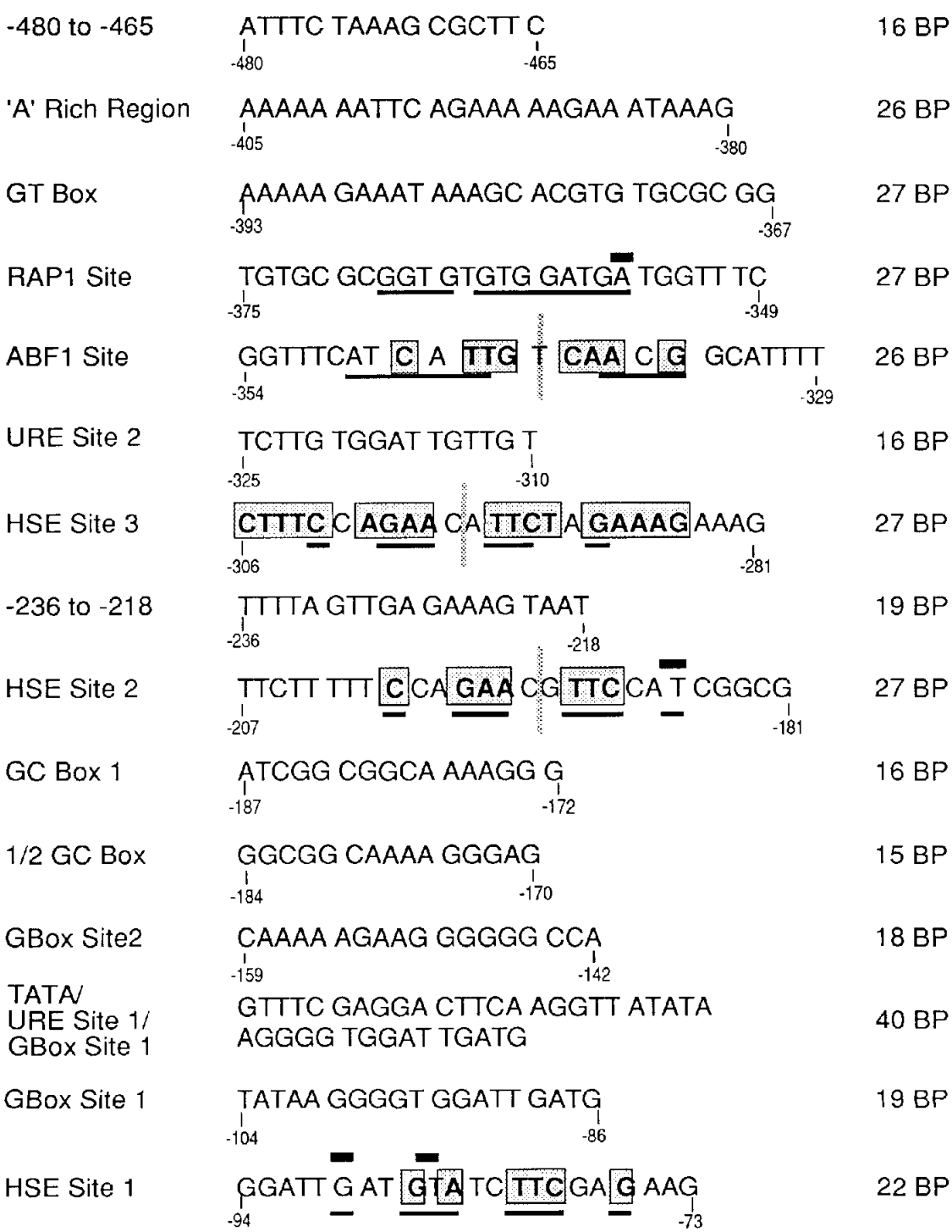

-480 to -465
ATTTC TAAAG CGCTT C
-480
-465
16 BP
'A' Rich Region
AAAAA AATTC AGAAA AAGAA ATAAAG
-405
-380
26 BP
GT Box
AAAAA GAAAT AAAGC ACGTG TGCGC GG
-393
-367
27 BP
RAP1 Site
TGTGC GCGGT GTGTG GATGA TGGTT TC
-375
-349
27 BP
ABF1 Site
GGTTTCAT C A TTG T CAA C G GCATTTT
-354
-329
26 BP
URE Site 2
TCTTG TGGAT TGTTG T
-325
-310
16 BP
HSE Site 3
CTTTC C AGAA CA TTCT A GAAAG AAAG
-306
-281
27 BP
-236 to -218
TTTTA GTTGA GAAAG TAAT
-236
-218
19 BP
HSE Site 2
TTCTT TTT C CA GAA CG TTC CA T CGGCG
-207
-181
27 BP
GC Box 1
ATCGG CGGCA AAAGG G
-187
-172
16 BP
1/2 GC Box
GGCGG CAAAA GGGAG
-184
-170
15 BP
GBox Site2
CAAAA AGAAG GGGGG CCA
-159
-142
18 BP
TATA/
URE Site 1/
GBox Site 1
GTTTC GAGGA CTTCA AGGTT ATATA
AGGGG TGGAT TGATG
40 BP
GBox Site 1
TATAA GGGGT GGATT GATG
-104
-86
19 BP
HSE Site 1
GGATT G AT G TA TC TTC GA G AAG
-94
-73
22 BP

Figure 6A.

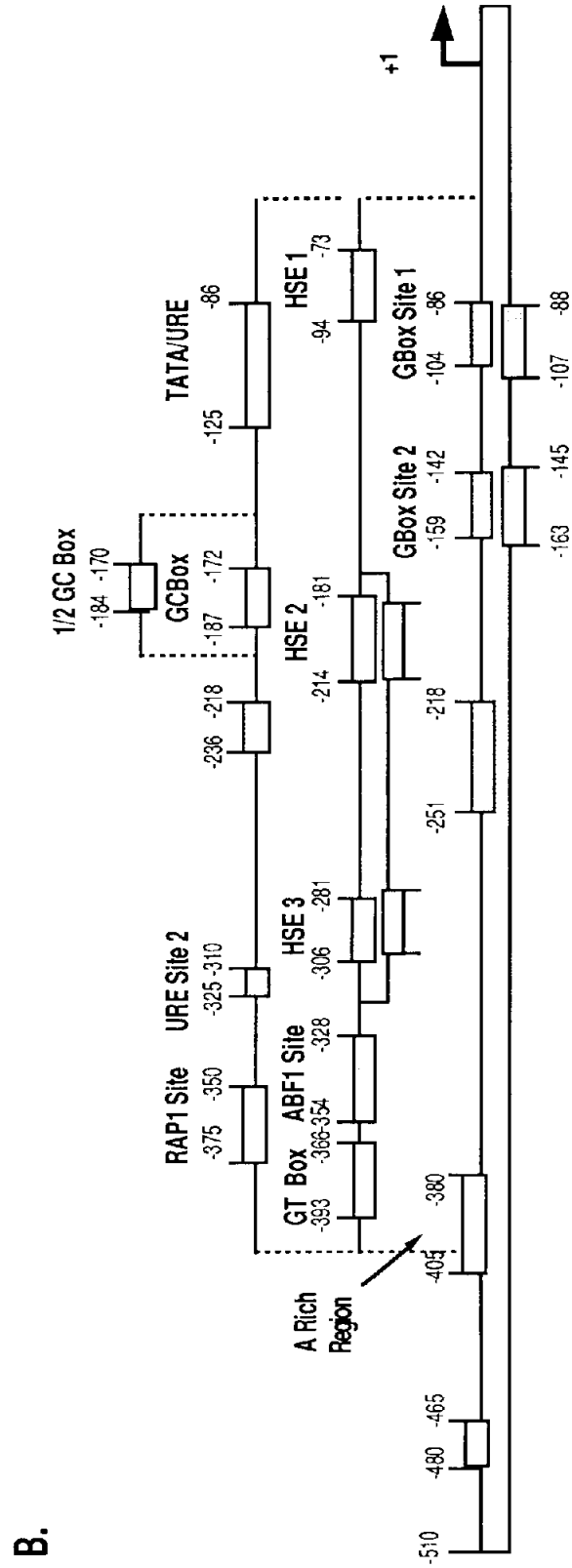

B.
+1
TATA/URE
-86
-125
HSE 1
-73
-94
GBox Site 1
-86
-104
-88
-107
GBox Site 2
-142
-159
-145
-163
1/2 GC Box
-170
-184
GCBox
-172
-187
HSE 2
-181
-214
-218
-236
-218
-251
HSE 3
-281
-306
URE Site 2
-310
-325
RAP1 Site
-350
-375
ABF1 Site
-328
-354
-366
GT Box
-380
-393
-404
A Rich Region
-465
-480
-510

Figure 6B.

Figure 2. Footprint showing the DNAse I protection of the -480 to -465 binding site, the 'A' Rich Region, and the ABF1 binding site. The protein used in lane 1 was prepared by taking the 1.0 M KCl pool from heparin-Sepharose, loading it onto double stranded DNA-Sepharose, eluting at 0.3 and 0.6 M KCl, and pooling the 0.6 M KCl fractions. The fraction used in lane 2 was prepared by pooling the fractions from the 0.3 M KCl eluted heparin-Sepharose material, loading it onto double stranded DNA-Sepharose, and assaying a pool from the 0.3 M KCl step. Lane 3 is the minus protein control. R and Y are purine and pyrimidine sequence lanes, respectively.

Figure 3. DNAse I protection of the 'A' rich region and RAP1 binding site. Both activities elute at high salt (1.0 M KCl) from heparin-Sepharose. 20 µl of the high salt fraction was used in lane 2; whereas 20 µl of buffer was used in lane 1 as a control. Other activities were present in the fractions but were not resolved until further in the purification (see Figure 1a for summary of the factors present in this fraction). R and Y are purine and pyrimidine sequence lanes, respectively.

Figure 4. Protection of the GT-box site, RAP1 site, and HSE3. Pooled fractions of material eluting at 0.25 and 0.6 M KCl from double stranded DNA-Sepharose. The 1.0 M KCl heparin-Sepharose eluted fractions were pooled and loaded onto the dsDNA-Sepharose column. RAP1, HSE3, and GT-box sites are protected, with HSTF split between the two fractions (HSTF elutes at about 0.25 M KCl from dsDNA-Sepharose). Lane 1 is the minus protein control. 5 µl and 20 µl of the 0.25 M KCl dsDNA-Sepharose eluant was used to protect the DNA fragment in lanes 2 and 3, respectively. Lane 5 contained 5 µl and 6 contained 20 µl of the 0.6 M KCl dsDNA-Sepharose fraction. R and Y are purine and pyrimidine sequence lanes, respectively.

Figure 5. Protection profile across double-stranded DNA-Sepharose. The elution profile of the GC-box Factor, 1/2GC-box Factor, and G-box Factor is shown. The first lane contains no protein, lanes I1 and I2 contain 2 and 5 microliters of input material, which is the 1.0 M step from heparin-Sepharose. Lanes FT through 37 assay the footprinting activity of 20 microliters of the labeled fractions eluting from the column. The GC-box binding activity elutes early in the profile, starting in fraction 19, whereas the G-box and 1/2GC-box binding activities elute starting in fractions 29 and 33, respectively. Affinity-purified G-box Factor does not bind to the 1/2GC-box sequence. The concentration of KCl in mM was determined for each fraction, and the protein concentration of the odd fractions was determined using the Bradford protein assay (Bradford 1976).

Figure 6. Sequences and location of the various DNAse I protection sites found in the *SSA1* promoter. Protection regions were defined by DNAse I footprinting on the top strand in all cases, and the bottom strand for the G-box sites.

A. The distances given are the distance from the start point of transcription (+1). Bars over the sequence denote mismatches between the site and the consensus. Bars under the sequence denote the bases included in the consensus. Boxed sequence shows regions of dyad symmetry with the vertical line showing the axis of symmetry.

B. Schematic of the *SSA1* promoter showing the relative position of the binding sites. The exploded view of the same sequence allows the depiction of all of the sites without overlap.

Activity of the binding sites as assayed by a 5' deletion set

The lacZ fusion constructs were assayed in a centromere vector derived from pSEYC-102. Centromere vectors are maintained in yeast as a mini-chromosome, holding the copy number of the plasmid at 1 or 2 per haploid genome. This was done to insure that important low abundance transcription factors were not titrated out by overabundance of binding sites. This

duplicates the cellular concentration of the *SSA1* gene present in wild type yeast.

The results of assaying the level of ß-galactosidase produced by a series of 5' deletions are summarized in Figure 7. Two unusual effects were observed in the deletions. First, the deletion of G-box site 2 reduced the basal and heat-shocked expression to background, and second the deletion of sequences upstream of HSE3 had little affect on the expression of the constructs. As expected, the deletion of the various HSEs lowered the heat-shock inducibility of the constructions. Based upon the 5' deletion series data, HSE2 is the primary element that drives the heat-shock response of the *SSA1* gene. Deletion of HSE2 (deletion at -181) dropped the level of heat-shocked activity from 220 units to 55 units while the basal or non-shocked expression of the construct only changed from 14 to 9 units (see Figure 7). The removal of an element between HSE2 and G-box site 2 (1/2GC-box), resulted in the further reduction of the level of the promoter, causing a decrease in the basal activity to one third its activity, and reducing the shocked level from 55 to 30 units (see Figure 7). This suggests that either 1/2GC-box or an unidentified site could play a role in the transcription of *SSA1*. G-box site 2, closer to the TATA, is also important since the deletion of this site drops the activity of the promoter to essentially background level, from 30 units to 3 units at 39°C (see Figure 7).

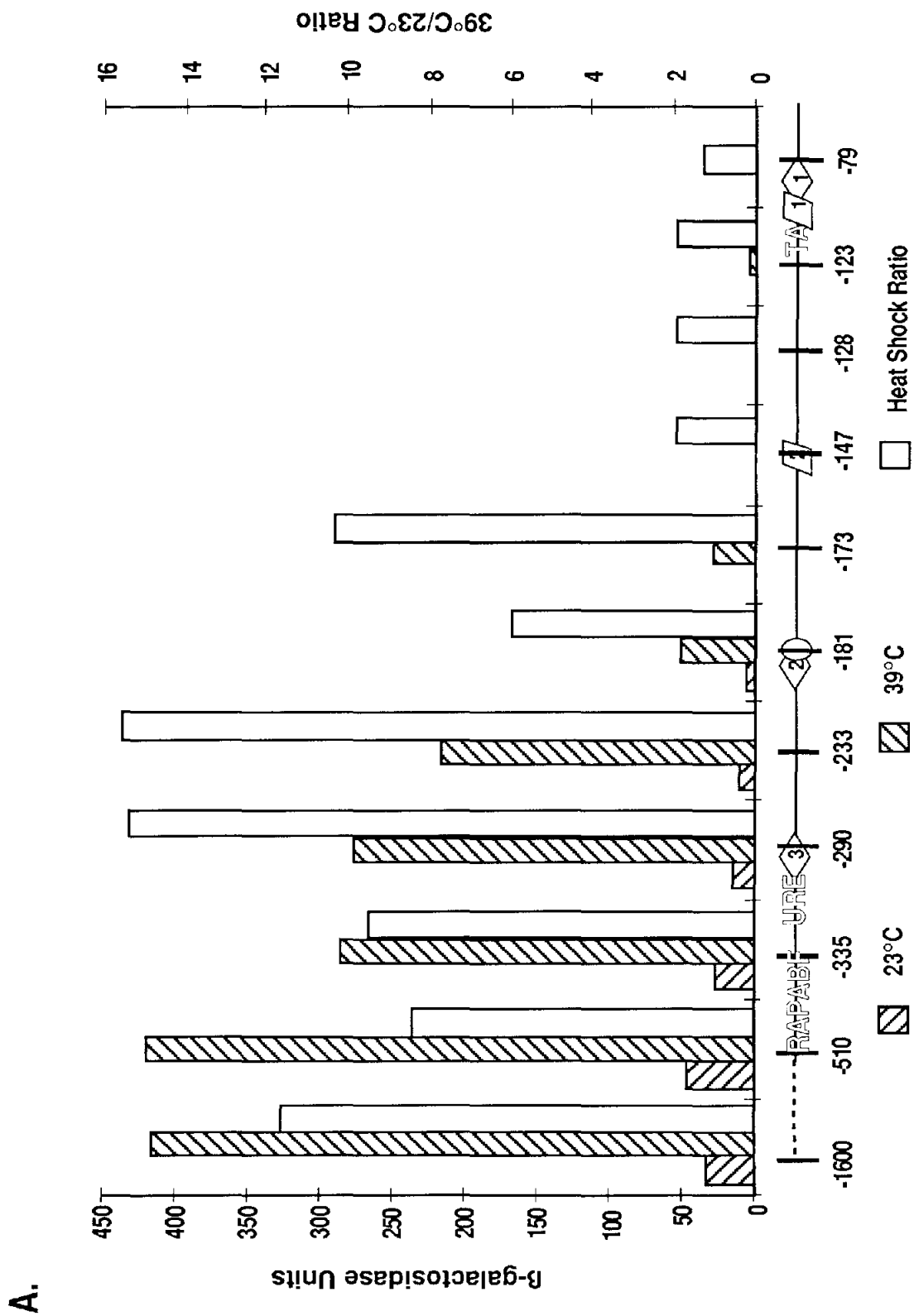

A.
β-galactosidase Units
39°C/23°C Ratio
Heat Shock Ratio
23°C
39°C
TATA
RAP/ABF
URS
-1600
-510
-335
-290
-233
-181
-173
-147
-128
-123
-79

Figure 7A.

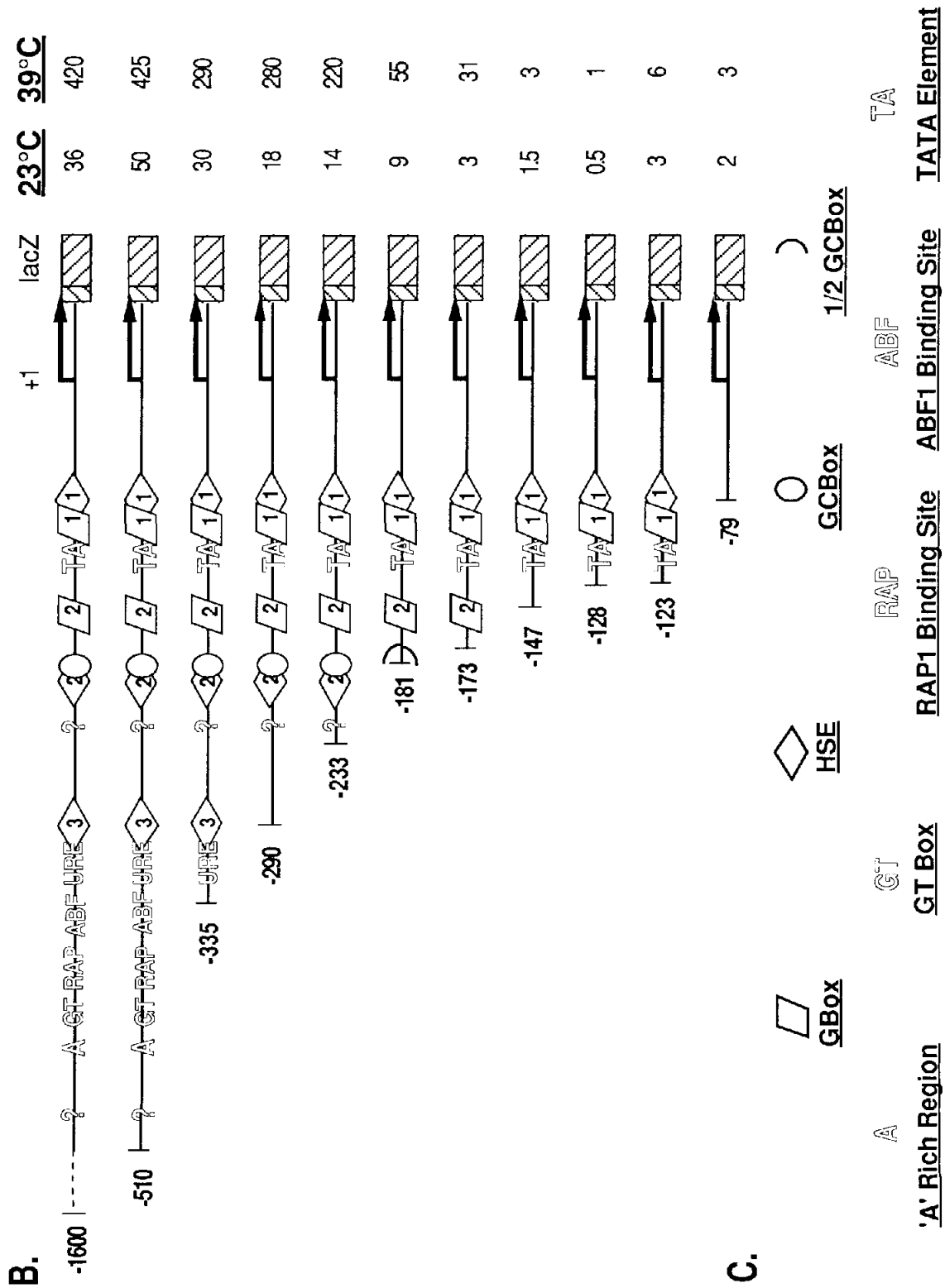


Figure 7, B and C.

Figure 7. ß-galactosidase levels produced by a series of 5' deletions of the *SSA1* promoter. The assays were performed under basal (nonshocked) conditions (23°C) and under heat-shocked conditions (39°C).

A. Graphic display of the data in Figure 7B. The promoter is not drawn to scale. The graphic compares the ß-galactosidase levels produced by a given construction (endpoint is listed underneath the display of its activity) under basal and heat-shocked conditions. The heat-shock ratio is the number of units of activity at 39°C divided by the number of units at 23°C.

B. A schematic representation of each 5' deletion, with the endpoint listed as the distance from the startpoint of transcription. Units of ß-galactosidase under basal and heat-shocked conditions are written to the right of each construction. Units are nanomoles/minute at 30°C.

C. Legend for translating the identity of each icon used in A and B. The ? is used to depict the unnamed binding sites at -480 to -465 and the other site at -251 to -218.

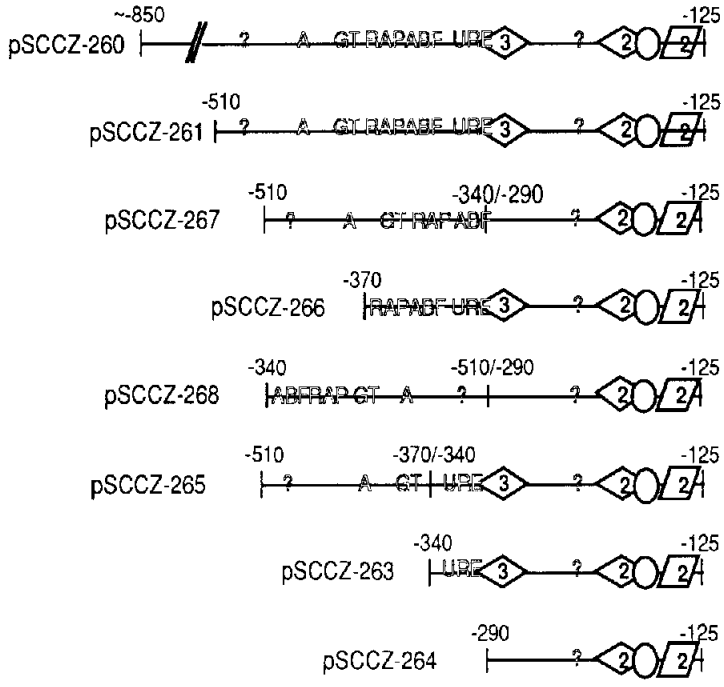
Identification of a transcriptional "blocking" activity

A series of subclones containing different regions of the *SSA1* promoter were placed in both orientations upstream of a UAS deleted *CYC1*/lacZ fusion construct. The vector, named pSCCZ-103RHB or pSCCZ-103BHR, depending on the orientation of the polylinker, contains a *CYC1*/LacZ fragment from pLG-669-Z (Guarente and Ptashne 1981) in a CEN IV-containing plasmid. The construction of the vector is described in detail in *Materials and methods*. The constructions all have the same 3' end (-125 relative to the *SSA1* start-point of transcription) and include between 160 to 700 base pairs of upstream sequence. All of these constructions contain G-box site 2 and HSE2 and the -218 to -236 binding site. The longer constructions, pSCCZ-260 and pSCCZ-261,

responded as expected to heat-shock in the "normal" orientation, but in an inverted orientation displayed a complete loss of heat-shock inducibility (see Figure 8). The shorter construction pSCCZ-263 was essentially identical in either orientation, suggesting that one or more elements that "block" the effect of upstream elements had been removed.

The deletion of the region of DNA from -340 to -370 was sufficient to remove the blocking activity (see construction pSCCZ-265, Figure 8). This region contains the ABF1 and the RAP1 binding sites. A deletion up to -370 but including the RAP1 and ABF1 site still showed some difference in activity, but when compared with pSCCZ-261 the blocking activity was substantially reduced (see pSCCZ-266). Two further constructions have been made to identify the positional requirements for the blocking region, pSCCZ-267 and 268. The region from -340 to -510 was placed in the wild type orientation at -290 (deleting HSE3 and URE site 2) in construction pSCCZ-267, and in the opposite orientation in pSCCZ-268. The results were somewhat surprising. The level of heat-shocked activity of pSCCZ-267 was considerably higher than the level of the -290 endpoint, and in the 'B' orientation of the insert the level of activity was nearly identical to pSCCZ-264 in the 'B' orientation. The reverse orientation of the -340 to -510 region in this same construction gave similar results to construction pSCCZ-265 in both the 'A' and 'B' orientation, suggesting that the 'blocking' activity exerts its function either positionally (requiring close proximity) or directionally on the HSE-mediated heat-shock induction of transcription.

A.



	'A' Orientation		'B' Orientation	
	23°C	39°C	23°C	39°C
pSCCZ-260	2.5	140	1.2	2.3
pSCCZ-261	0.8	125	6.5	13
pSCCZ-267	0.4	170	0.1	6.0
pSCCZ-266	1.3	140	2.0	50
pSCCZ-268	0.1	75	10	75
pSCCZ-265	0.3	75	20	75
pSCCZ-263	0.1	80	1.1	80
pSCCZ-264	0.2	30	0.1	6.5

B.

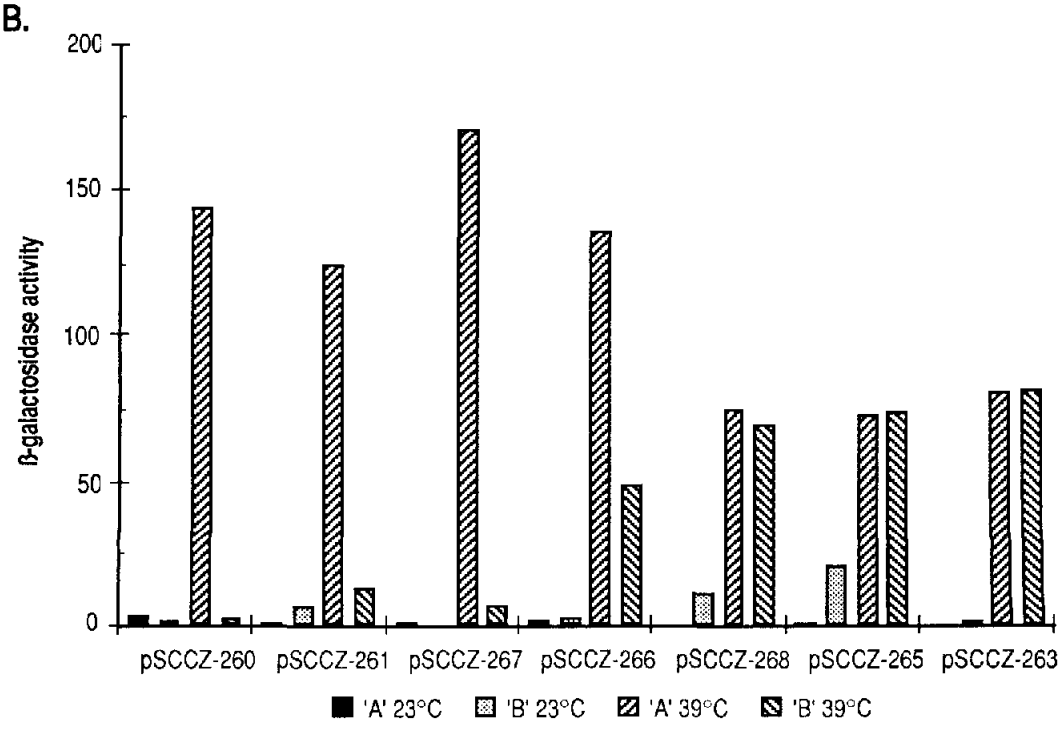


Figure 8.

Figure 8. Constructions used to define the region containing the transcriptional "blocking" activity found upstream of HSE2. Constructions were placed into a *CYC1* promoter fused to lacZ in either the wild type 'A' orientation or in the opposite, 'B' orientation. The activity of the constructs were measured in the yeast strain TD28. Units are in nanomoles/minute/milligram at 30°C and the deviation for the assays was less than 10% except for activities less than 1 unit, in which case the error was ± 0.1 unit. Panel A shows a schematic representation of the promoter fragment placed in the *CYC1/LacZ* fusion, and panel B plots the activity of the various constructions.

The position dependent repression of transcription is clearly not similar to other known yeast repressor sites, such as URE (see Chapter 2) or URS1 (Sumrada and Cooper 1987), which are relatively insensitive to position. However, the need for a "blocking" effect can be postulated from several yeast gene clusters where two genes are divergently transcribed and are in close proximity to each other but are not concomitantly expressed, such as the *SSA1/FUN15* locus or the *HIS3/DDE1* locus. The regulation of this type of promoter is obviously distinct from that of co-regulated divergently transcribed loci such as the *GAL1/GAL10* pair.

Competition of two non-overlapping sites

ABF1 was purified by heparin-Sepharose chromatography (Figure 1), pooling the 0.3 M KCl eluted material, loading it onto dsDNA-Sepharose, and eluted with 0.3 M KCl. This fraction was then loaded onto ssDNA-Sepharose, eluted at 0.3 and 1.0 M KCl, and the 1.0 M KCl fraction used in the competition experiment. GT-box factor was purified by heparin-Sepharose chromatography (Figure 1), pooling the 1.0 M KCl eluted material, loading it onto dsDNA-Sepharose, and eluted with 0.6 M KCl. This material was then affin-

ity-purified on a ligated-GT-box-Sepharose column. This GT-box factor fraction was used in the competition experiment.

The regions protected by the factors GT-box factor and ABF1, as assayed by DNAse I, do not overlap; however, it was found that the order of addition of the fractions containing the respective activities was important: a factor (either ABF1 or GT-box) added to the labeled DNA fragment binds to its site and prevents the factor added later from binding (Figure 9). This competition was reciprocal, showing that at least *in vitro* only one of these factors can bind to this promoter at a time.

Concomitant binding of HSTF and a SP1-like activity

HSTF has been shown to bind to the HSE recognition site in yeast (Wiederrecht, et al. 1987). Using affinity-purified HSTF and GC-box binding factor, the two proteins were shown to bind simultaneously to generate a single, large footprint spanning the region from -214 to -172 (see Figure 10). The presence of both factors was required to generate this extended protection region (data not shown). In a similar experiment, partially purified fractions containing TATA binding activity, G-box binding factor and perhaps other factors protected over 80 bp from G-box site 2 to HSE1 (see Figure 10). Whether these interactions are co-operative or not was not examined.

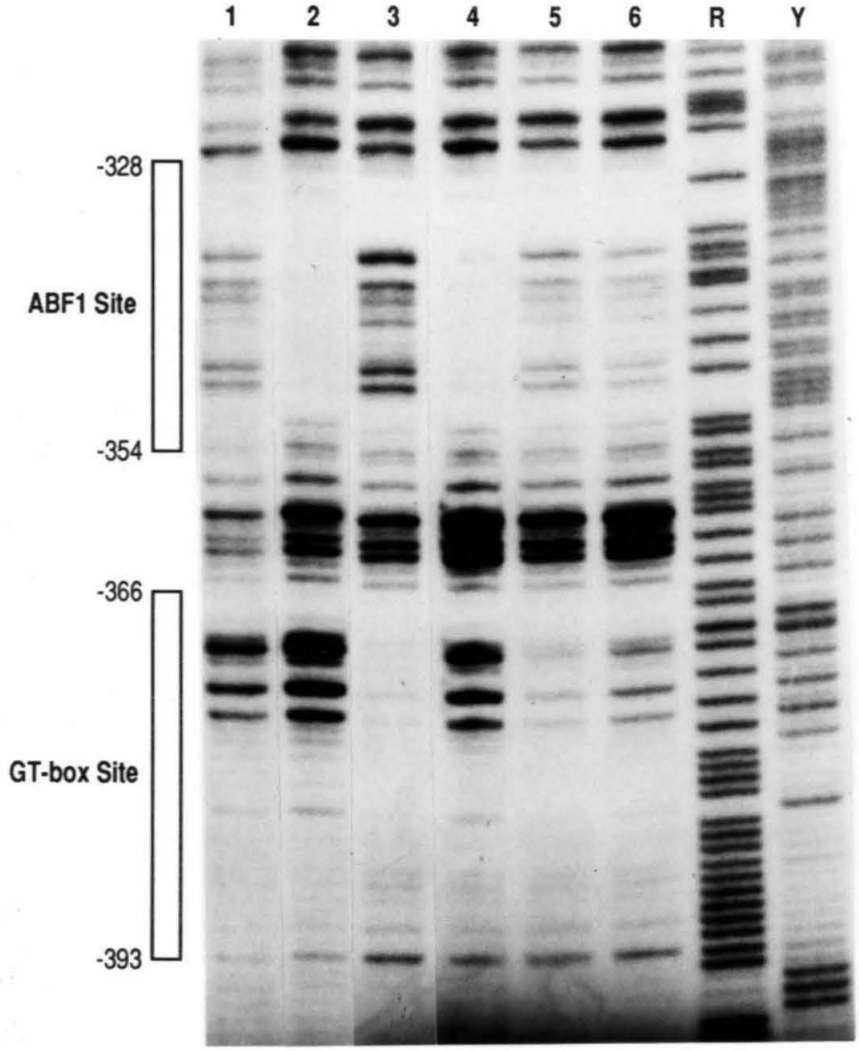


Figure 9.

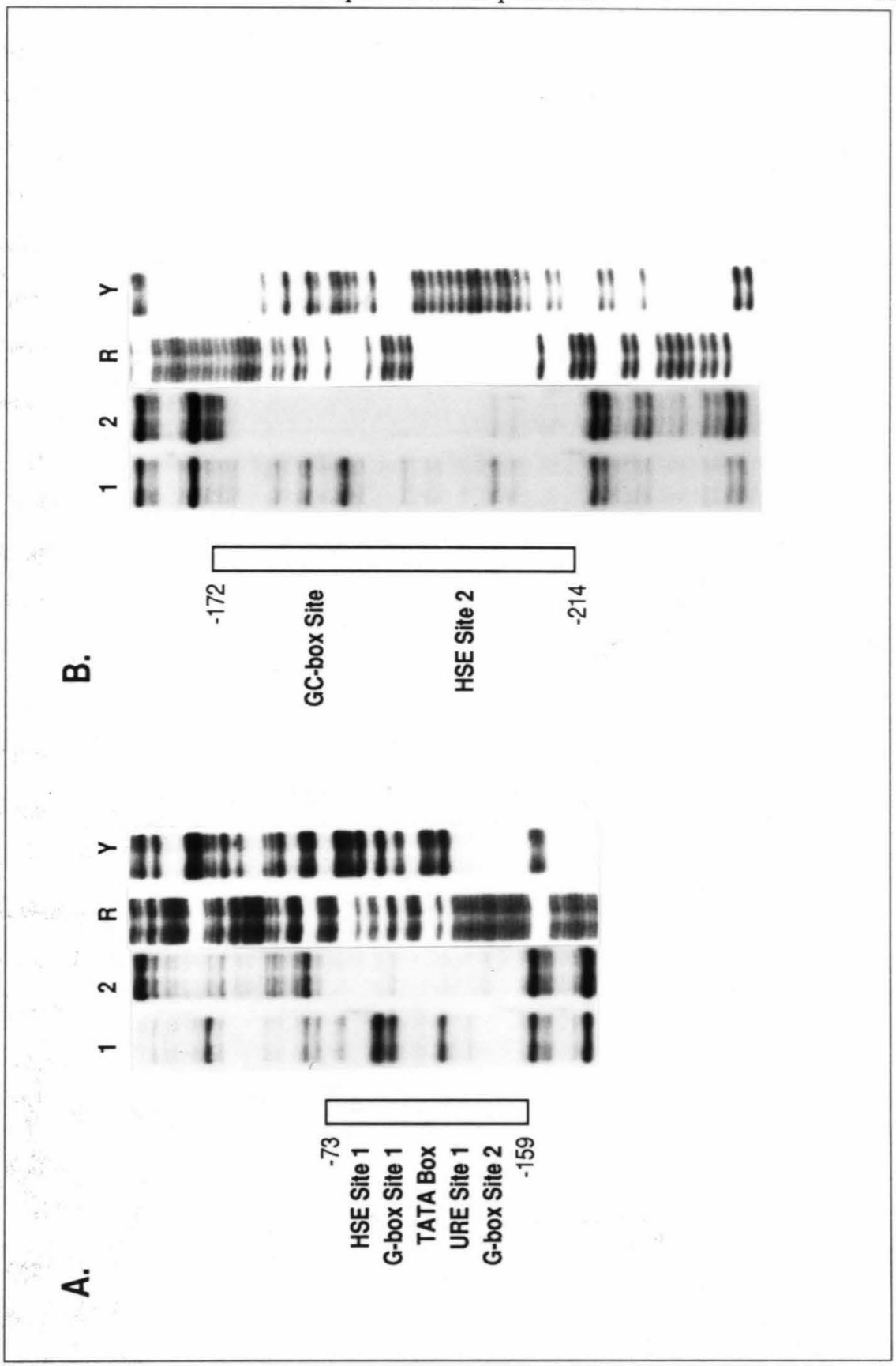

A.
1
2
R
Y
-73
HSE Site 1
G-box Site 1
TATA Box
URE Site 1
G-box Site 2
-159
B.
1
2
R
Y
-172
GC-box Site
HSE Site 2
-214

Figure 10.

Figure 9. ABF1 and the GT-box binding factor competition. Affinity-purified GT-box binding factor and ABF1 purified over three columns were used. Lane 1 is the minus protein control, lane 2 contained 10 μl of the ABF1 containing fraction, and lane 3 contained 10 μl of the affinity-purified GT-box factor. In lane 4, 10 μl of ABF1 was prebound to the promoter fragment; in lane 5, 10 μl of GT-box factor was prebound; and in lane 6, 10 μl of the two fractions were mixed before adding the DNA. R and Y are purine and pyrimidine sequence lanes, respectively.

Figure 10. Simultaneous protection of multiple sites in the *SSA1* promoter. Lane 1 in both panels is the minus protein control, and lane 2 contained chromatographic fractions. R and Y are purine and pyrimidine sequence lanes, respectively.

A. This panel shows the clearing of a region from G-box site 2 to HSE1, or approximately 85 bases. Protein was prepared by concentrating the 1.0 M KCl step from heparin-Sepharose on dsDNA-Sepharose, eluting with a single 1.0 M KCl step. 10 μl of this material was used to form the footprint in lane 2.

B. This panel shows a footprint generated using a mixture of affinity-purified GC-box factor and HSTF.

Discussion

Heat-shock control of *SSA1*

The yeast heat-shock gene *SSA1* is one of at least eight members of the yeast hsp70 gene family. The SSA gene subfamily are highly homologous, although they are divergent in their respective promoter regions (Slater and Craig 1989). Each of the SSA subfamily members have their own transcriptional response patterns to changing and steady-state conditions (Werner-Washburne, et al. 1989).

SSA1 has three heat-shock elements (HSE sites 1-3) that have been shown to be bound by HSTF (Wiederrecht, et al. 1987). HSE3 appears to be responsible for only a modest induction of transcription upon heat-shock in the intact *SSA1* promoter when assayed in a centromere vector (see 5′ promoter deletion data, Figure 7). There is a weak homology to the heat-shock binding consensus upstream of HSE3 (Slater and Craig 1987) that does not appear to play a part in the heat-shock regulation of *SSA1* by the 5′ promoter deletions data. The site is not bound *in vitro* by HSTF at a concentration 50 times higher than that required to clear HSE3 (data not shown).

Based on the 5′ deletion series data, HSE2 is the primary element that drives the heat-shock response of the *SSA1* gene. Deletion of HSE2 dropped the level of heat-shocked activity from 220 units to 55 units while the basal or non-shocked expression of the construct changed from 14 to 9 units (see Figure 7). A construction with only HSE1 left upstream of the start point of transcription does not promote transcription, indicating that there is either a spatial constraint affecting the activity of a single HSE, such as being too close to the start point, must be upstream of the TATA factor, or its binding affinity is too low for *in vivo* activity. It is likely that a single site is insufficient to promote transcription without another element present, and it is possible that in this position the HSE is functioning as a heat-shock TATA element (Chen and Struhl 1989).

HSE2 overlaps with a consensus to the SV40 GC-box element required for binding the mammalian sequence specific DNA binding protein Sp1. In yeast, it has been found that this site can act as a repressor (Park and Craig 1989; Sumrada and Cooper 1987). We have demonstrated that the HSTF and the yeast GC-box binding factor can bind simultaneously (Figure 10). This, coupled with the data on the expression of these sites described in this paper

and elsewhere (Park and Craig 1989; Slater and Craig 1987) leads us to postulate that the effect of the GC-box is to lower the level of non-shocked expression, while maintaining the normal high level of activity of the HSE during heat-shock.

The removal of an element between HSE2 and G-box site 2 (1/2GC-box), resulted in the further reduction of the activity of the promoter, causing a decrease in the basal activity to one third its activity, and reducing the shocked level from 55 to 30 units (see Figure 7). This suggests that either 1/2GC-box or an unidentified site could play a role in the transcription of *SSA1*. G-box site 2, closer to the TATA than either the GC-box or 1/2GC-box, is also necessary for conveying a high degree of heat-shocked activity in a series of 5′ deletions, although when assayed as a single unit in a *CYC1*/lacZ fusion it is only a weak activator. Thus, in the *SSA1* promoter G-box site 2 appears to be working synergistically with a site still present in the 5' deletion, perhaps G-box site 1 or HSE1, both of which are 3' of the TATA element (see Figure 6). The G-box binding factor has been affinity-purified, and will be discussed further in Chapter 4.

Homology of the protected regions with other binding sites

We report in this paper multiple protection sites in the *SSA1* promoter that contain homology to reported binding or consensus sequences, such as the HSE (Park and Craig 1989; Slater and Craig 1987; Sorger and Pelham 1987; Wiederrecht, et al. 1987), the ABF1 binding site (Kimmerly, et al. 1988; Sweder, et al. 1988), the TATA element (Buratowski, et al. 1988; Chen and Struhl 1988) and two overlapping RAP1 binding sites (Buchman, et al. 1988; Diffley and Stillman 1989; Shore and Nasmyth 1987). Three additional protected regions identified in this paper have been previously identified as

upstream repression sites (URSs); the GC-box, which has been identified in this (Park and Craig 1989) and other yeast promoters (Sumrada and Cooper 1987) and is homologous to the SV40 Sp1 binding site (Jankowski and Dixon 1987; Johnson and McKnight 1989; Kadonaga, et al. 1986) and two URE sites (bound by URF, see Chapter 2).

The RAP1 site, identified in the *SSA1* promoter, contains two matches to the previously published consensus, each with a 2 base mismatch to the *RAP1* protein consensus recognition element, 5' $\frac{A}{G}\frac{A}{C}$ACCCANNCA$\frac{T}{C}\frac{T}{C}$ 3'. This consensus has been identified using multiple sites, with many of these sites having quite different functions (Buchman, et al. 1988; Diffley and Stillman 1989; Shore and Nasmyth 1987). Using this consensus, we identified two RAP1 sites in the *SSA1* promoter about 8 base pairs apart, each *SSA1* site containing two mismatches from the consensus. However, based on the footprinting data, only the distal site is bound *in vitro*. Using this information, and carefully going back through the binding sites previously identified, we defined a modified consensus sequence for the binding of RAP1: 5' $\frac{A}{G}\frac{A}{C}$A$\frac{T}{C}$CCA$\frac{T}{C}$NCA$\frac{T}{C}\frac{T}{C}$ 3'. The *SSA1* distal RAP1 site has one mismatch when compared with the modified consensus, whereas the proximal site has three mismatches. This modified consensus was used in Figure 6.

The ABF1 binding site in the *SSA1* promoter is a perfect match to either Halfter's BAF1 consensus $\frac{A}{G}$TC$\frac{A}{G}\frac{T}{C}\frac{T}{C}$NNNNACG (Halfter, et al. 1989) or to Dorsman's GF1 consensus $\frac{A}{G}$TC$\frac{A}{G}\frac{T}{C}$NNNNNACG (Dorsman, et al. 1989). Apparently, the designations ABF1, GF1, SUF, TAF, and BAF1 are either different names for the same factor, or members of a family of proteins that recognize the same sequence. ABF1 has recently been cloned (Diffley and Stillman 1989; Rhode, et al. 1989). The absence of sequences in yeast that are highly homologous to the ABF1 clone indicates that all of these designations

are for a single factor (P.R. Rhode and J.L. Campbell, personal communication).

The 'A' rich region that is protected between -405 and -380 is similar to the dA•dT tracts in the intergenic regions between the *PET56/HIS3/DED1* genes (Struhl 1985). Recently, Winter and Varshavsky have identified and cloned a yeast protein that recognizes dA•dT tracts and called the protein Datin, and the gene *DAT* (Winter and Varshavsky 1989). It apparently can promote transcription weakly, and may also repress transcription when assayed in the proper context (Winter and Varshavsky 1989).

The *SSA1* GC-box is bound by a factor that can also recognize the SV40 GC-box, and can be affinity-purified using the SV40 GC-box consensus sequence. A site overlapping the GC-box was also found. However, unlike the GC-box binding factor, the factor binding this site was unable to bind the SV40 GC-box (data not shown). The site was named 1/2 GC-box, because it shares the GC homology with the GC-box. Based on the deletion data in Figure 7, it is possible that this site plays a role in the transcriptional activity of *SSA1*.

Another yeast repressor element, the URE, was found in two different locations in the *SSA1* promoter. The URE is homologous to the SV40 "core" consensus sequence and has been shown to function as a repressor in yeast (see Chapter 2). One site was identified upstream of HSE3 and the other site overlaps the TATA element. A clear activity for either URE site in the *SSA1* promoter has not been identified.

Factor binding: competition versus co-binding

Two distinct site-specific DNA binding factors, ABF1 and GT-box binding factor, bind to the SSA1 promoter in such a way that the binding of one factor precludes the binding of the other factor. This competition was shown to be

dependent on the order of addition of the two factors - the factor added first precludes the other factor from binding. This type of interaction has been recently shown with the factors AP-2 and AP-3 on the SV40 enhancer (Mercurio and Karin 1989) as well as on the yeast promoter *CYC1* with the factors RC2 and HAP1 (Pfeifer, et al. 1987). This type of interaction results in a very strict binary switch - if either (or both) factor is present in the cell at sufficient concentration to bind its site, then the promoter will be bound by one or the other factors, but never both. At present, the competition between the GT-box binding factor and ABF1 is interesting from a mechanistic rather than functional standpoint, although both the GT-box and ABF1 site are implicated in the transcriptional "blocking" activity found in the *SSA1/FUN15* intergenic region. It is possible that this competition contributes to this activity.

In contrast, many of the sites on this promoter can be protected simultaneously; thus their activity may be co-operative or synergistic, such as HSE2/GC-box, and the region including from G-box site 1 to G-box site 2 spanning a URE homology, the TATA element and perhaps one other binding site (see Figure 10). An anomaly in the competition between the GT-box factor and ABF1 when compared with competition between other DNA binding factors is that the ABF1 site and the GT-box are non-overlapping: there are at least 12 bases of unprotected DNA between the two sites defined by DNAse I protection. The DNA contact sites of these two proteins is undoubtedly even further separated. One possible explanation is that one (or both) of the proteins has a very large domain that extends out into the surrounding environment and blocks the approach of the other protein without affecting the approach of DNAse I. Another possibility is that the binding of one (or both) of the proteins may induce an unusual DNA conformation. Recently, several researchers have postulated that repeating purine/pyrimidine tracts of suffi-

cient length can induce the formation of unusual structures, especially in the presence of supercoiling (Dickerson 1988; Wells, et al. 1988). The GT-box site contains a stretch of 13 alternating purines-pyrimidines, and it is possible that the GT-box binding factor could induce a structural change in the DNA at this site, which would be propagated far enough along to prevent the recognition of the ABF1 site by ABF1 (Wang and Glaever 1988). Likewise, the presence of ABF1 could 'lock' the DNA into a structure that the GT-box factor could not recognize with a high affinity, or perhaps greatly increase the off-rate of the GT-box factor. Further experiments are needed to differentiate between these alternatives.

"Blocking" activity between the *SSA1/FUN15* genes

Another activity of note identified in this promoter is the "blocking" of transcriptional signals between the divergent *SSA1* and *FUN15* promoters. *FUN15* does not respond to heat-shock, even though the promoter is sufficiently close to the *SSA1* HSEs to be driven by the HSEs. Further, the HSEs are symmetric and promote transcription in either direction. Thus, there appears to be an element(s) between the two promoters that acts to block the sharing of transcriptional information in a spatially-directed manner that is distinct from the action of a silencer. A silencer acts at a distance on the transcription of a gene, whereas this "blocking" activity only blocks transcriptional signals of elements that are located further upstream of the "blocking" element. Interestingly, two elements found in silencers are also present in the "blocking" element: the RAP1 and ABF1 binding sites. Another protection site seen adjacent to the RAP1 site, the GT-box, seems to also play a role in the activity of this region.

This transcriptional "blocking" activity may be spatially constrained and/or dependent on orientation. The *SSA1/FUN15* intergenic region and the transcriptionally-active elements present in the *FUN15* promoter are "blocked" from affecting the SSA1 promoter by this region, and likewise the SSA1 promoter elements are "blocked" from affecting the *FUN15* promoter because of the intervening "blocking" element. However, in a construction where the "blocking" region was moved proximal to the TATA in the opposite orientation it had no affect on the activity of the fusion under heat-shocked conditions.

In order to examine other promoters for these sequences, the yeast sequences present in GenBank were examined for homologies to the ABF1 and RAP1 binding sites. Sequences that contained at least one homology to the ABF1 binding site were then examined for matches to the modified RAP1 consensus. Sequences containing matches to both consensus sequences were found, and the orientation of the identified sequences was compared with the orientation of the site in the *SSA1* promoter. A (+) indicates that the identified site is in the same orientation as the *SSA1* site, and a (-) indicates the opposite orientation. 26 genes were found with the ABF1 site (+) orientation. 14 of these 26 genes also contained RAP1 sites in the (+) orientation, and another 4 contained the RAP1 binding site in the (-) orientation. Forty-six genes were found with the ABF1 site in the (-) orientation, and 16 of these had the RAP1 site in the (+) orientation, versus 15 in the (-) orientation. Interestingly, only 6 sequenced genes were found with the RAP1 and ABF1 binding sites within 60 base pairs, and of these 6, all were either (++) or (--) in orientation (referring to the orientation of both sites in the gene). Furthermore, in 4 of these 6 genes the RAP1 and ABF1 sites were located in the pro-

moter (*ENO2, DFR1, SNR17A, UGCSR1*), and in one of the six the 2 sites were found in an ARS.

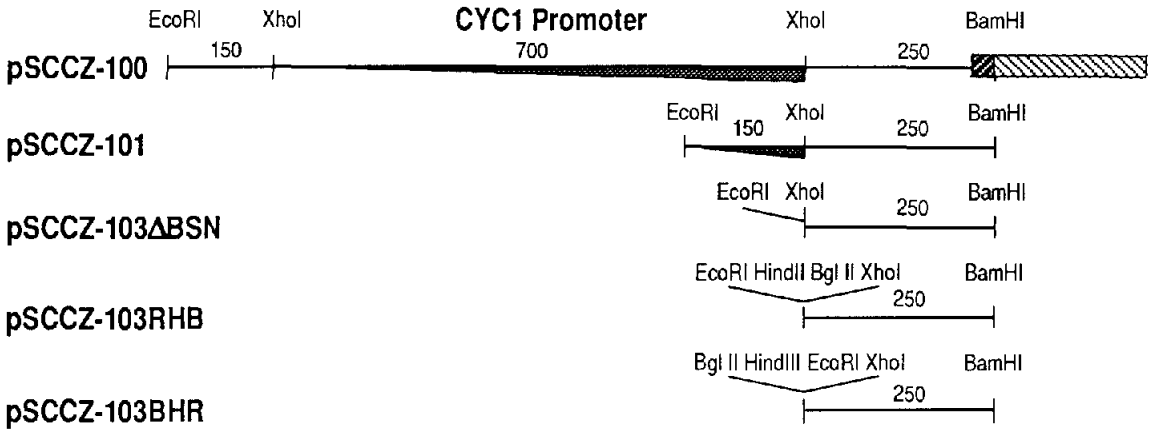
We have looked for other gene clusters that are not co-ordinately controlled and have found that the *HIS3/DDE1* intergenic region also contains an ABF1 binding sequence. *HIS3* is under amino-acid control (general control), whereas *DDE1* is not. This further implicates the ABF1 sequence as important in this context. Apparently, the ABF1 protein is highly responsive to context cues - it has been previously implicated as an important element in ARS elements, silencers, and can also function as a transcriptional activator. How it accomplishes all of these tasks will be the subject of further investigation.

Acknowledgements

We would like to especially thank Dr G. J. Wiederrecht for the kind gift of the HSE3 oligonucleotide, the HSE-Sepharose resin, and affinity-purified HSTF. We would also like to thank Dr. W.S. Moye-Rowley for the ARE oligonucleotide, Dr. K.D. Harshman for the SV40 GC-box oligonucleotide and SV40 GC-box-Sepharose resin and Dr. Z. Y. Zhang-Keck for the URE oligonucleotide. Also, the members of the Parker laboratory for helpful discussions regarding this manuscript. pSEYC-102 was the kind gift of Dr. Scott Emr at Caltech.

Materials and methods

Construction of the pSCCZ series of vectors



Derivation of pSCCZ-103BHR and pSCCZ-103RHB. pSCCZ-100 is a CEN IV vector with the EcoRI to SacI fragment inserted from pLG-669-Z (Guarente and Ptashne 1981) into pSEYC-102 SacI to EcoRI vector sequence.

The pSCCZ-260 through 268 constructions were made by placing the appropriate *SSA1* promoter fragment (always cutting the *SSA1* promoter at the -125 SauIIIA site) into pUC-19, and subsequently excising the fragment using EcoRI and HindIII. This fragment was then placed into pSCCZ-103RHB and pSCCZ-103BHR cut with EcoRI and BamHI, giving rise to the 'A' (wild type orientation of the -125 *SSA1* promoter) and the 'B' orientation.

Preparation of the *Saccharomyces cerevisiae* extracts

The yeast strain EJ926 was grown in modified minimal media (yeast minimal supplemented with 0.2 gm/liter Yeast Extract) to O.D. 3 in a 350 liter fermenter. The cells were harvested in an upright Sharples centrifuge at 8K, and resuspended in a 1 gram yeast/1 ml of 'Y' Buffer (1 M Sorbitol, 50 mM

Tris (pH 7.8), 10 mM $MgCl_2$) and 30 mM DTT slurry. The slurry was quickly frozen in liquid nitrogen as drop-sized beads.

The frozen yeast were thawed in a 30°C water bath in 500 gm aliquots, washed once in 'Y' buffer, and resuspended in 500 mls 'Y' buffer and 6 mM DTT with 50 mgs Zymolyase-100T. The cells were agitated gently at 30°C for 1 hour, and the percentage of Thaw 11 500 ml Beckman J10 bottles until spheroplasting was ≥ 80%. Cells were harvested and washed twice with 500 mls ice cold 'Y' buffer, and resuspended in 500 mls ice cold 'A' buffer (15 mM KCl, 10 mM Hepes (pH 7.6), 5 mM $MgCl_2$) with 3 mM DTT and 1 mM PMSF. The slurry was then homogenized with a Bellco 'B' pestle for 3 to 4 strokes, harvested and the supernate discarded. The pellicle was resuspended in 300 mls 'A' with 3 mM DTT and 1 mM PMSF, mixed for 5' at 4°C. The slurry was brought to 0.30 M $(NH_4)_2SO_4$ and 0.2 mM DIFP and mixed at 4°C for at least 30 minutes. The material was spun at 35K for 60 minutes in a Beckman 45Ti rotor. The resulting pellicle was discarded, 0.22 gm $(NH_4)_2SO_4$/ml added to the supernate, and mixed on ice for 15 minutes. The material was respun at 35K for 15 minutes in a Beckman 45Ti rotor, the supernate discarded and the pellicle rinsed in 'D' buffer (25 mM Hepes (pH 7.6), 10% glycerol, 100 mM KCl, 0.1 mM EDTA, and 0.2% Triton X-100), then resuspended in 100 mls 'D' buffer with 1 mM DTT and 1 mM PMSF. The extract was dialyzed versus 'D' buffer with 1 mM DTT and 1 mM PMSF for ~ 4 hours.

Purification of the binding factors

All purification was carried out at 4°C.

Standard ion-exchange chromatography

Prior to loading onto any column, the input fraction was dialyzed versus 0.1 M KCl until the concentration of KCl was below 120 mM, and the fraction

spun at 10K for 10 minutes in a Beckman JA-21 rotor. Loading and low salt wash for all columns was 0.1 M KCl and were done at 3 to 4 column volumes per hour.

Affinity chromatography

Affinity columns were prepared as previously described (Wiederrecht, et al. 1987), and the flow rate for loading and washing was between 5 and 10 column volumes per hour. The fractions were loaded at 200 mM KCl and washed at 350 mM KCl for at least 10 column volumes.

Footprinting reactions

DNA templates were prepared by calf-alkaline-phosphatase treatment of an appropriately restriction enzyme endonuclease cleaved plasmid, the subsequent γ-^{32}P labeling of the plasmid with T4 polynucleotide kinase, cleaving the plasmid a second time with another endonuclease, and purifying the labeled fragment by agarose electrophoresis. DNAse I footprints were performed on ice. From 0.5 to 5 ng of the DNA fragment was preincubated for 5 minutes on ice with the protein in 5 mM $MgCl_2$, 20 or 100 µg/ml dA•dT (20 for affinity-purified fractions, 100 otherwise), 25 mM Hepes (pH 7.8) buffer. DNAse I was added to a final concentration of 50 µg/ml for affinity-purified protein fractions or to 250 µg/ml for other fractions. After 60 seconds, the DNAse I digestion was stopped and prepared for electrophoresis as previously described (Wiederrecht, et al. 1987).

ß-galactosidase assays

Yeast strain TD-28 was transformed with the various constructions discussed in this paper, and at least three single isolates of each construction were assayed for ß-galactosidase activity at both basal (23°C) and heat-shocked (39°C) temperatures. 100 mls of cells in yeast minimal were grown at 23°C to

an $O.D._{600}$ of 0.5 to 0.8, 50 mls of cells harvested and prepared as previously described (Harshman, et al. 1988). The remaining 50 mls of cells were incubated in a 39°C water bath with shaking for 60 minutes, then placed at 23°C and allowed to recover for 60 minutes. The cells were then harvested and prepared in the same manner as the non-shocked cells.

Oligonucleotide sequences

Top strand only is shown: for a listing of both strands see Appendix A.

G-box TCGA Overhangs
TCGAC CCAAA AAGAA GGGGG GCCAT TTAG

GT-box TCGA Overhangs
TCGAC AAATA AAGCA CGTGT GCGCG GTGTG

GC-box (*SSA1*) GATC Overhangs
GATCA TCGGC GGCAA AAGGG AGA

SV40 GC-box GATC Overhangs
GATCC GATGG GCGGA GTTAG GGGCG GGACT A

SV40 ARE GATC Overhangs
GATCC ATGGT TGCTG ACTAA TTGA

HSE (*SSA1* HSE3) GATC Overhangs
GATCC TTTCC AGAAC ATTCT AGAAA

RHB (Linker) Blunt ends
AATTC AAGCTT AGATCT

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Chapter 1 Appendix

Summary

The further characterization of the yeast heat-shock gene *SSA1* includes some data not included in the body of Chapter 1. This appendix includes results of ß-galactosidase assays not presented in Chapter 1, further DNAse I protection data, and the purification of the yeast homologue of Sp1, ySp1, as well as the purification of the GT-box binding factor. Also, a section on work in progress has been included at the end of the appendix.

Results

Examining the activity of individual sites

The HSE (Park and Craig 1989; Sorger and Pelham 1987; Wiederrecht, et al. 1987); URE, or SV40 "core" sequence (Chapter 2); and GC-box (Park and Craig 1989; Sumrada and Cooper 1987) cis-acting transcription activities have been examined individually elsewhere. However, the effect of the TATA and "TATA-like" elements on other upstream elements has not been previously addressed. In order to address the possible interaction of the upstream elements with G-box site 1, the URE homology at the TATA element, and/or HSE1, two series of fusion vectors were made. Both vectors contained the selectable marker *URA3* and the yeast replication sequence CEN IV. The oligonucleotide constructions were inserted into either a *CYC1*/lacZ fusion or a *SSA1*/lacZ fusion and were assayed at 23°C and 39°C in the *Saccharomyces cerevisiae* strain TD28. A summary of the results is shown in Table 1. Some of these sites are also compared with the same construction placed in a 2µ vector. In the case of the G-box site 2, a noticeable increase in the heat-shock

response is seen in the *SSA1*-containing CEN vector when compared to the *CYC1*-containing CEN vector. It should be noted that this difference is most likely due to the differences in the TATA proximal region - in the SSA1-containing vector, the TATA, URE, G-box site 1, and HSE site 1 are all present in the upstream region of the *SSA1* promoter from -75 to -120. The control plasmid (without an insert) shows no heat-shock response in spite of the presence of these elements. The promoter region, startpoint of transcription and startpoint of translation are the only changes between the *CYC1* CEN and *SSA1* CEN constructions, indicating that either negative elements exist in the *CYC1* promoter to repress the activity mediated by the G-box site, or that the sequence elements present in the *SSA1* construction assist in the function of the G-box site even though they are incapable of function in the control construct.

DNAse I protection analysis of *SSA1*

The DNAse I protection pattern obtained using different partially purified yeast protein fractions demonstrated that the *SSA1* promoter has multiple regions of protection (see Chapter 1). Nine chromatographically distinct factors were identified that interact with the region from -73 to -480 bases, relative to the start point of transcription. Figure 1 shows the protections of all of the sites between HSE2 at -181 to -207 to the 'A' rich region at -380 to -405, except for URE site 2. Some of these activities are separable by gradient elution on heparin-Sepharose: Figure 2 shows the footprinting activity and plots the salt and protein concentration of the fractions from a gradient elution on heparin-Sepharose. Crude yeast nuclear extract was prepared by the method described in *Materials and methods* in Chapter 1, and was loaded onto the heparin-Sepharose column.

	CYC1 2μ		*CYC1* CEN		*SSA1* CEN	
Designation	**23°C**	**39°C**	**23°C**	**39°C**	**23°C**	**39°C**
Control	1	1	0.1	0.1	1.2	1.2
HSE3	40	150	1	8		
HSE2/GC-box	20	160				
G-box site 2	4	14	0.5	3.0	3	30
GT-box	14	14				
ARE	60§		1.1	0.9		

Table 1. Activity of some of the individual binding sites placed upstream of a *CYC1*/lacZ fusion. All of the data points represent the average of at least 3 independent isolates from the original transformation plates. The columns labeled *CYC1* 2μ were constructed by placing the designated sites upstream of the TATA element in a *CYC1/LacZ* fusion vector containing a 2μ replication sequence. The vector was a derivative of pLG-669-Z (Guarente and Ptashne 1981) that had the UAS sequences deleted by the removal of the Xho I fragment. The *CYC1* CEN constructions were made by placing the designated oligonucleotide sequences in the plasmid pSCCZ-103RHB (see *Materials and methods* in Chapter 1 and Appendix A). The *SSA1* CEN constructions were made by placing the plasmids in the vector pSCCZ-205RHB. This centromere-containing vector is identical to pSCCZ-103RHB except for the substitution of the *SSA1* promoter from -123 to +90 relative to the startpoint of transcription for the *CYC1* promoter and coding region (see Appendix A for more details).

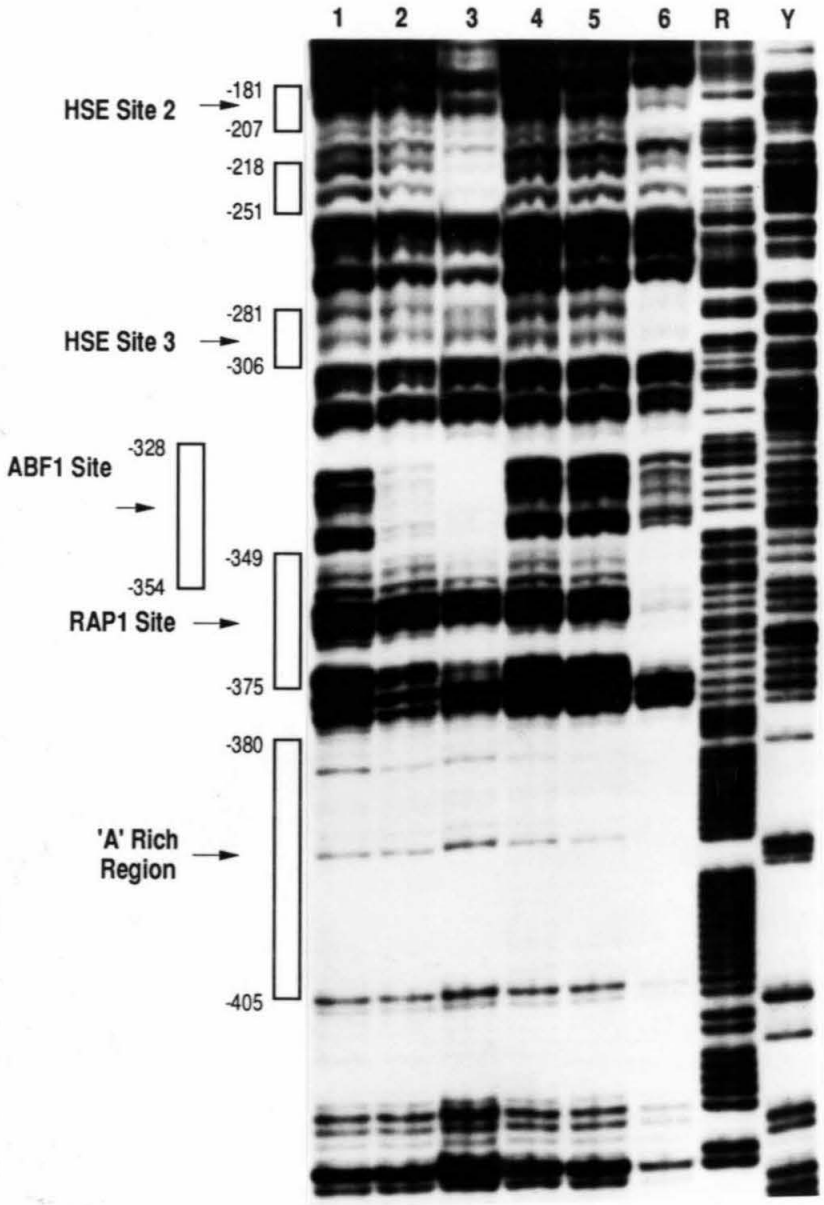

1
2
3
4
5
6
R
Y
HSE Site 2
-181
-207
-218
-251
HSE Site 3
-281
-306
ABF1 Site
-328
-354
RAP1 Site
-349
-375
'A' Rich Region
-380
-405

Figure 1.

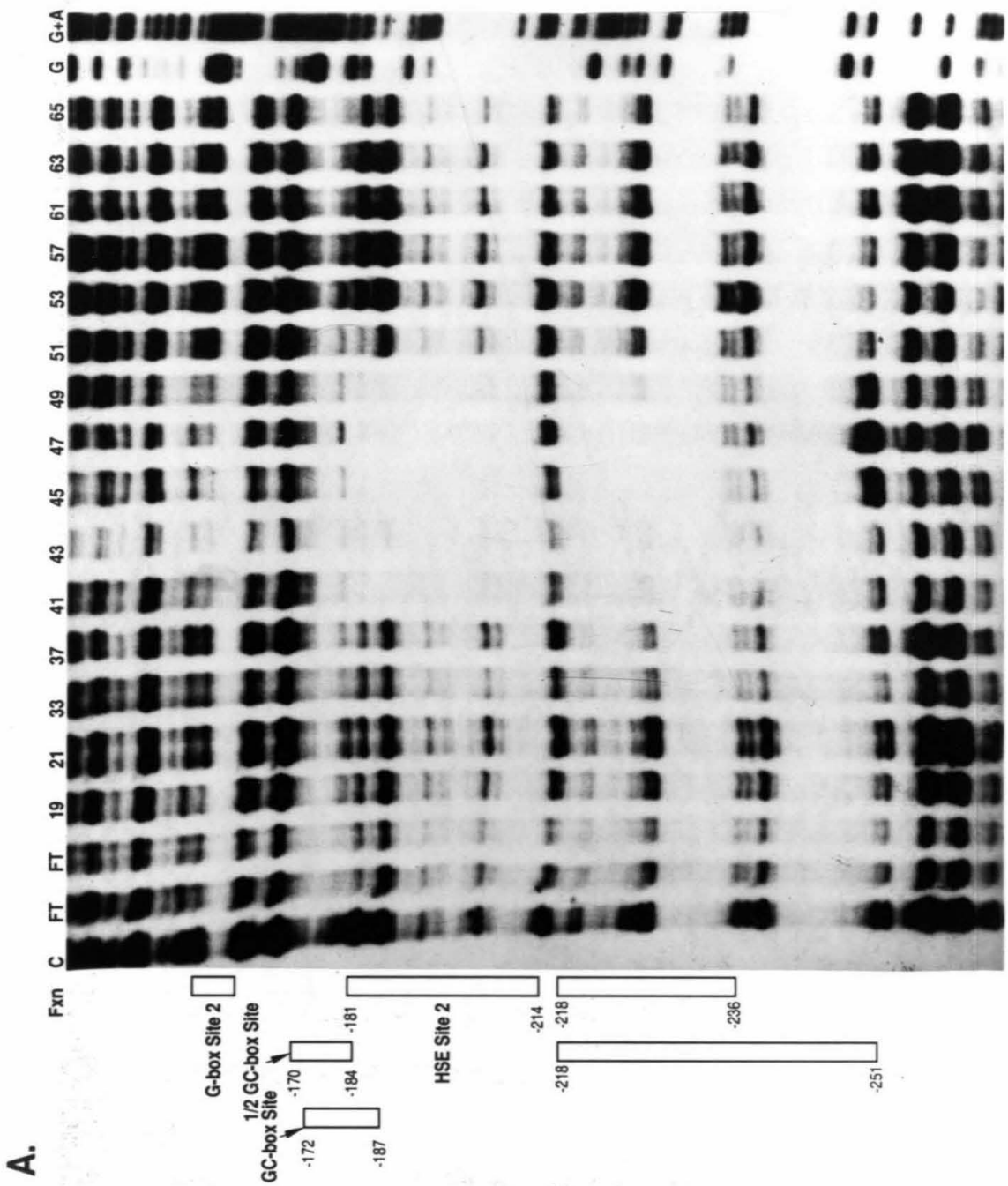

A.
C
FT
FT
19
21
33
37
41
43
45
47
49
51
53
57
61
63
65
G
G+A
Fxn
G-box Site 2
1/2 GC-box Site
GC-box Site
-170
-184
-172
-187
-181
HSE Site 2
-214
-218
-236
-218
-251

Figure 2A.

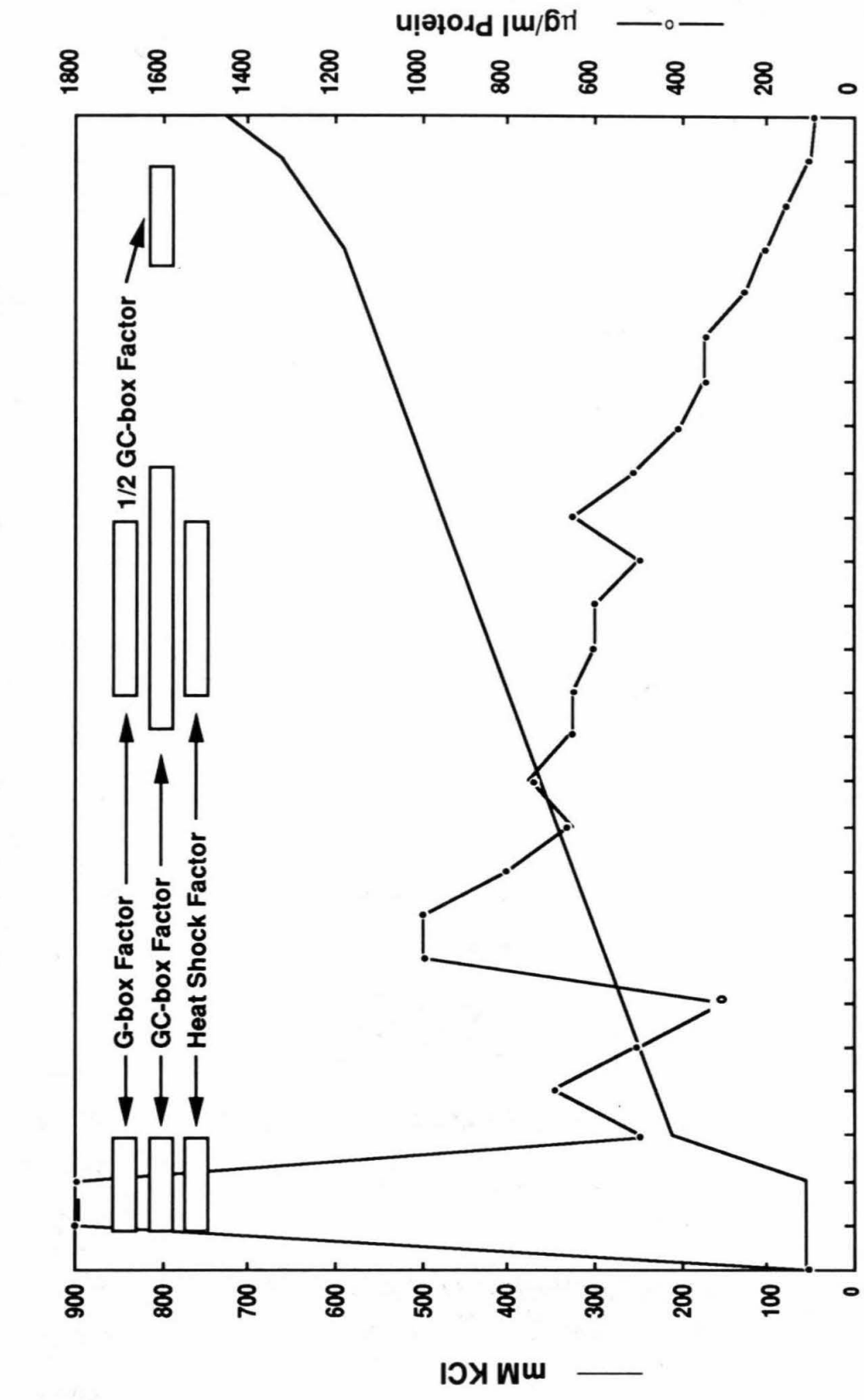
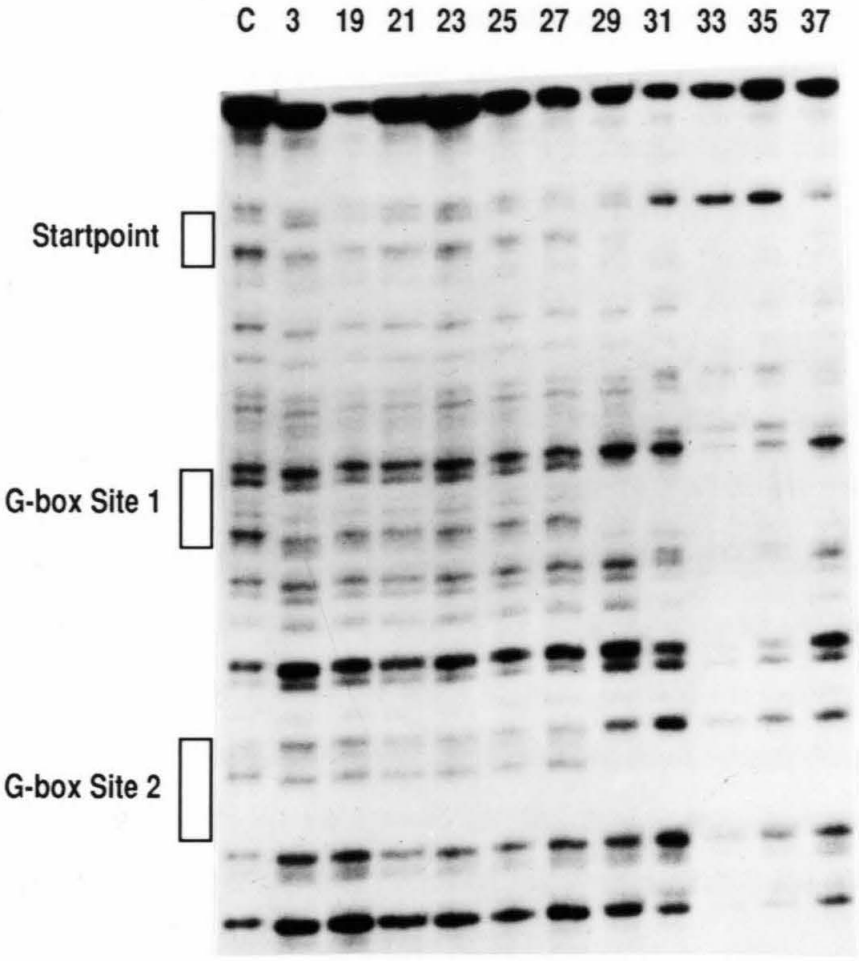

B.
mM KCl
μg/ml Protein
G-box Factor
GC-box Factor
Heat Shock Factor
1/2 GC-box Factor
900
800
700
600
500
400
300
200
100
0
1800
1600
1400
1200
1000
800
600
400
200
0
1 5 7 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57 59 61 63 65

Figure 2B.


C 3 19 21 23 25 27 29 31 33 35 37
Startpoint
G-box Site 1
G-box Site 2

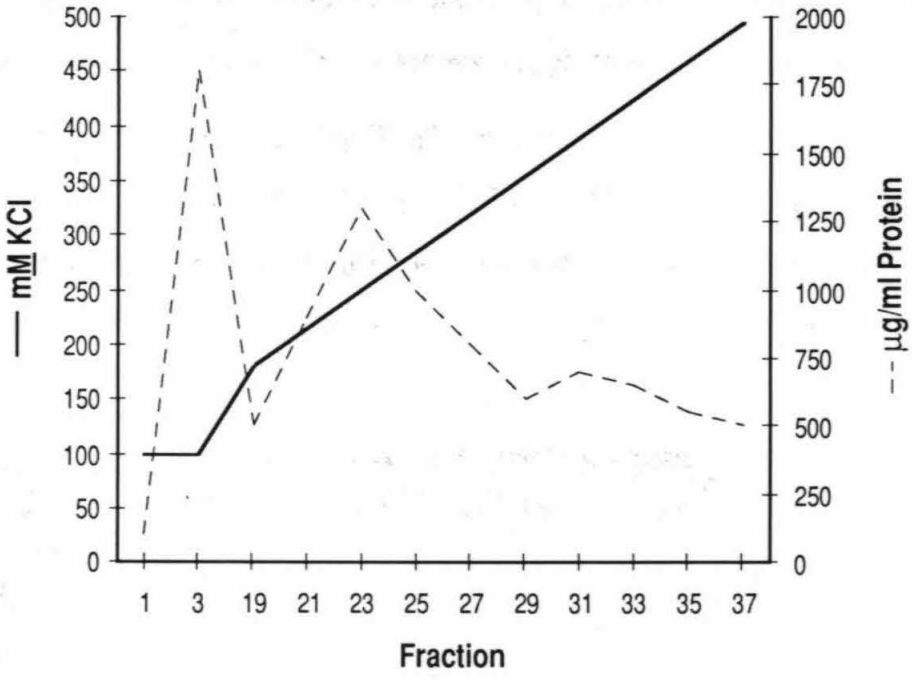

500
450
400
350
300
250
200
150
100
50
0
mM KCl
2000
1750
1500
1250
1000
750
500
250
0
µg/ml Protein
1 3 19 21 23 25 27 29 31 33 35 37
Fraction

Figure 3.

Figure 1. DNAse I protection of the *SSA1* promoter by heparin-Sepharose step-eluted fractions. Lanes 1 and 4 are minus protein controls, lanes 2 and 3 are 5 and 20 µl, respectively, of a pooled 0.32 M KCl step from heparin-Sepharose. Lanes 5 and 6 are 5 and 20 µl, respectively, of a pooled 1.0 M KCl step from heparin-Sepharose.

Figure 2. Footprint, protein, and mM KCl concentration assay of a KCl gradient elution on heparin-Sepharose. Yeast nuclear extract was loaded on the column at 50 mM KCl, washed at 50 mM KCl, and eluted in a 12 column volume gradient from 100 to 800 mM KCl. Each fraction was 0.2 column volumes, and every other fraction was assayed.

A. DNAse I protection analysis of the gradient-eluted fractions. Lane C is the minus protein control, and the 2 lanes labeled FT are 20 µl of the pooled and protein peak fractions of the flow through fractions of the column. Some G-box binding factor, GC-box factor and HSTF (data not shown for HSTF) routinely flows through this column. Lanes labeled 19 through 65 are the assayed fractions of the heparin-Sepharose gradient elution. Lanes labeled G and G+A are purine sequence lanes of G and G+A, prepared by standard methods (Maxam and Gilbert 1977).

B. Plot of the protein and KCl concentration profiles of the gradient elution from heparin-Sepharose. The concentration of the fractions in mM KCl (left X axis) was determined by determining the conductivity of each fraction, and converting the conductivity into mM KCl by a standard curve of known KCl concentrations. The concentration of the fractions in µg/ml protein (right X axis) was determined by standard methods (Bradford 1976), again normalized to a standard curve.

Figure 3. Footprint on the startpoint-proximal region of the *SSA1* promoter using heparin-Sepharose gradient-eluted fractions. The graph depicts the protein and mM KCl concentrations of the KCl gradient elution on heparin-Sepharose. Yeast nuclear extract was loaded on the column at 50 mM KCl, washed at 50 mM KCl, and eluted in a 12 column volume gradient from 100 to 600 mM KCl. Each fraction was 0.2 column volumes, and every other fraction was assayed for footprinting activity, protein concentration and KCl concentration.

Identification of a startpoint of transcription binding activity

Along with the identification of numerous other binding activities, a protection of the sequences flanking the startpoint of transcription was noted in crude fractions prior to Affigel-Blue fractionation. Although this activity was not characterized further, it is interesting to speculate that this activity is due to specific RNA Polymerase II interactions with the startpoint in these extracts. This hypothesis is supported by the co-elution of this activity with RNA Polerymerase II-containing fractions rather than with other DNA binding proteins on the Affigel-Blue column. An example footprint over this region is shown in Figure 3 and it includes footprints on G-box site 1 and G-box site 2 for comparison.

Purification of yeast Sp1

The *SSA1* GC-box is bound by a factor that can also recognize the SV40 GC-box, and can be affinity-purified using the SV40 GC-box consensus sequence. The purified protein, GC-box Factor or ySp1, was affinity-purified using either the GC-box oligonucleotide or using the SV40 GC-box consensus oligonucleotide described further in *Oligonucleotide sequences* in Chapter 1 and Appendix A. Loading the 0.30 M KCl dsDNA-Sepharose fraction onto either the GC-box-Sepharose column or the SV40 GC-box-Sepharose column

resolved a factor that elutes at high salt from either resin (see Figure 4) and binds to *SSA1* GC-box site specifically (data not shown). The factor from the two columns gave identical footprints (data not shown) and after a second pass shows three prominent bands at 150, 130, and 85 kD (see Figure 5). If the 0.30 M KCl dsDNA-Sepharose fraction is loaded onto a Pharmacia FPLC MonoS column, ySp1 elutes at approximately 0.45 M KCl (see Figure 6). The fractions containing activity have prominent bands at 150, 95, 85, and 40 kD (see Figure 6a). If this material is loaded onto the GC-box affinity resin, the factor is further purified with two bands at 85 and 95 kD remaining that co-elute with the GC-box binding activity (see Figure 8a, and D). The GC-box has been shown to act as a repressor in the *SSA1* promoter (Park and Craig 1989), in other yeast promoters (Sumrada and Cooper 1987), and also in other organisms (Jankowski and Dixon 1987).

The sequence of the GC-box oligonucleotide used was either from the SV40 GC-box consensus or from the *SSA1* promoter GC-box. Identical results were obtained with the two columns. The oligonucleotide top strand sequences are shown below. Both strands are displayed in Appendix A.

GC-box (*SSA1*) GATC Overhangs

GATCA TCGGC GGCAA AAGGG AGA

SV40 GC-box GATC Overhangs

GATCC GATGG GCGGA GTTAG GGGCG GGACT A

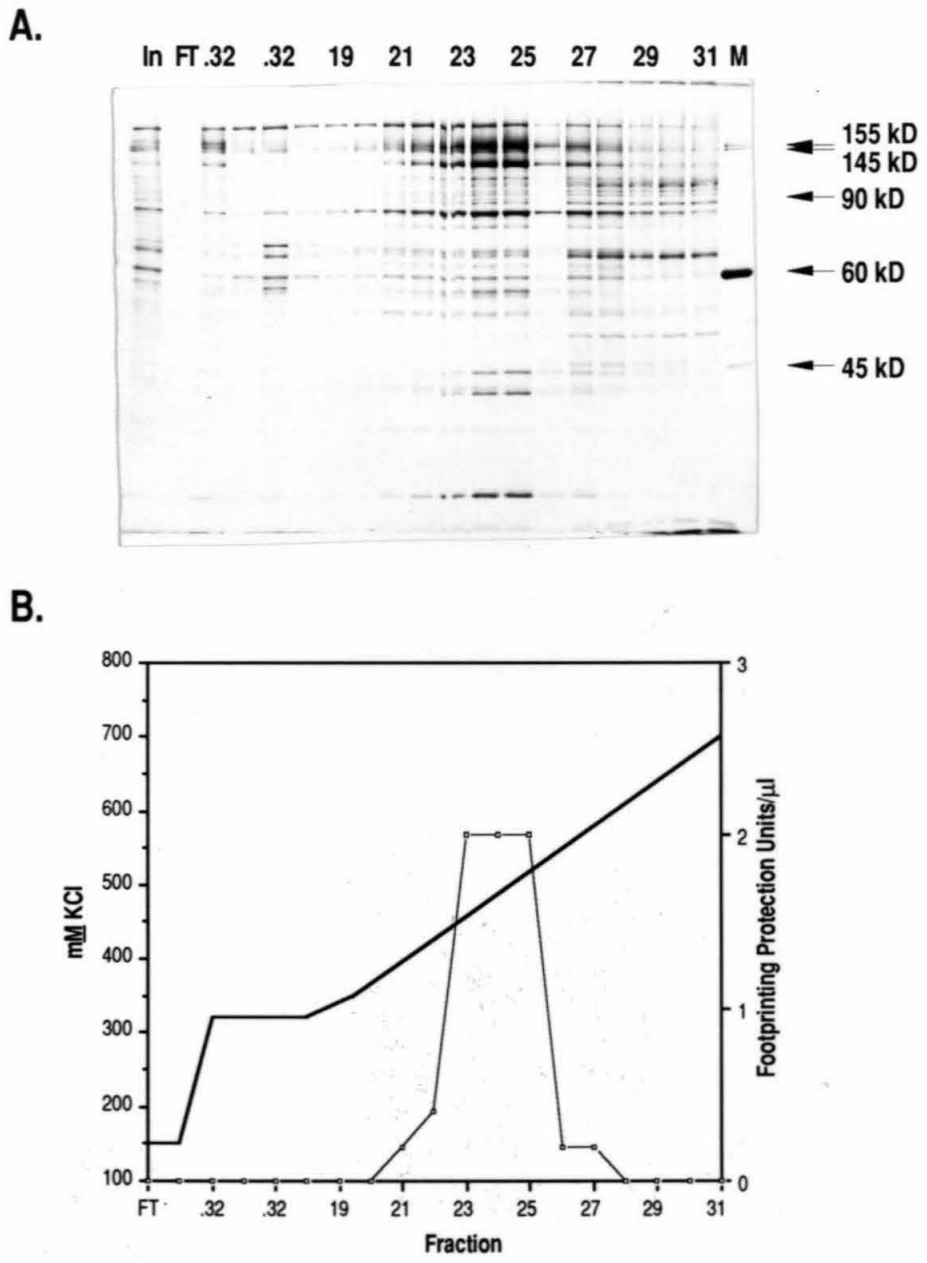

A.
In FT .32 .32 19 21 23 25 27 29 31 M
155 kD
145 kD
90 kD
60 kD
45 kD
B.
mM KCl
800
700
600
500
400
300
200
100
3
2
1
0
Footprinting Protection Units/µl
FT .32 .32 19 21 23 25 27 29 31
Fraction

Figure 4.

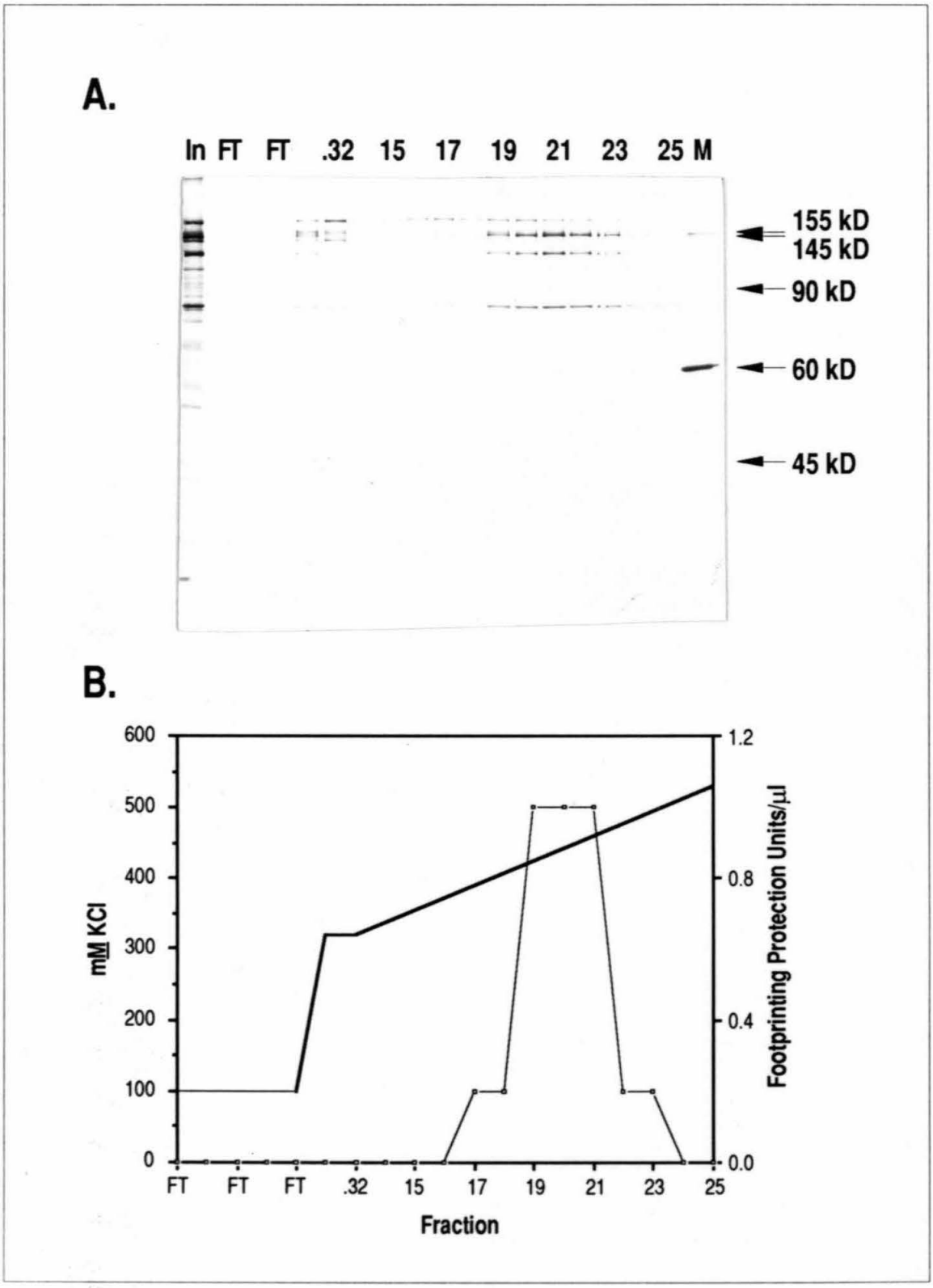

A.
In FT FT .32 15 17 19 21 23 25 M
155 kD
145 kD
90 kD
60 kD
45 kD
B.
600
500
400
300
200
100
0
mM KCl
1.2
0.8
0.4
0.0
Footprinting Protection Units/µl
FT FT FT .32 15 17 19 21 23 25
Fraction

Figure 5.

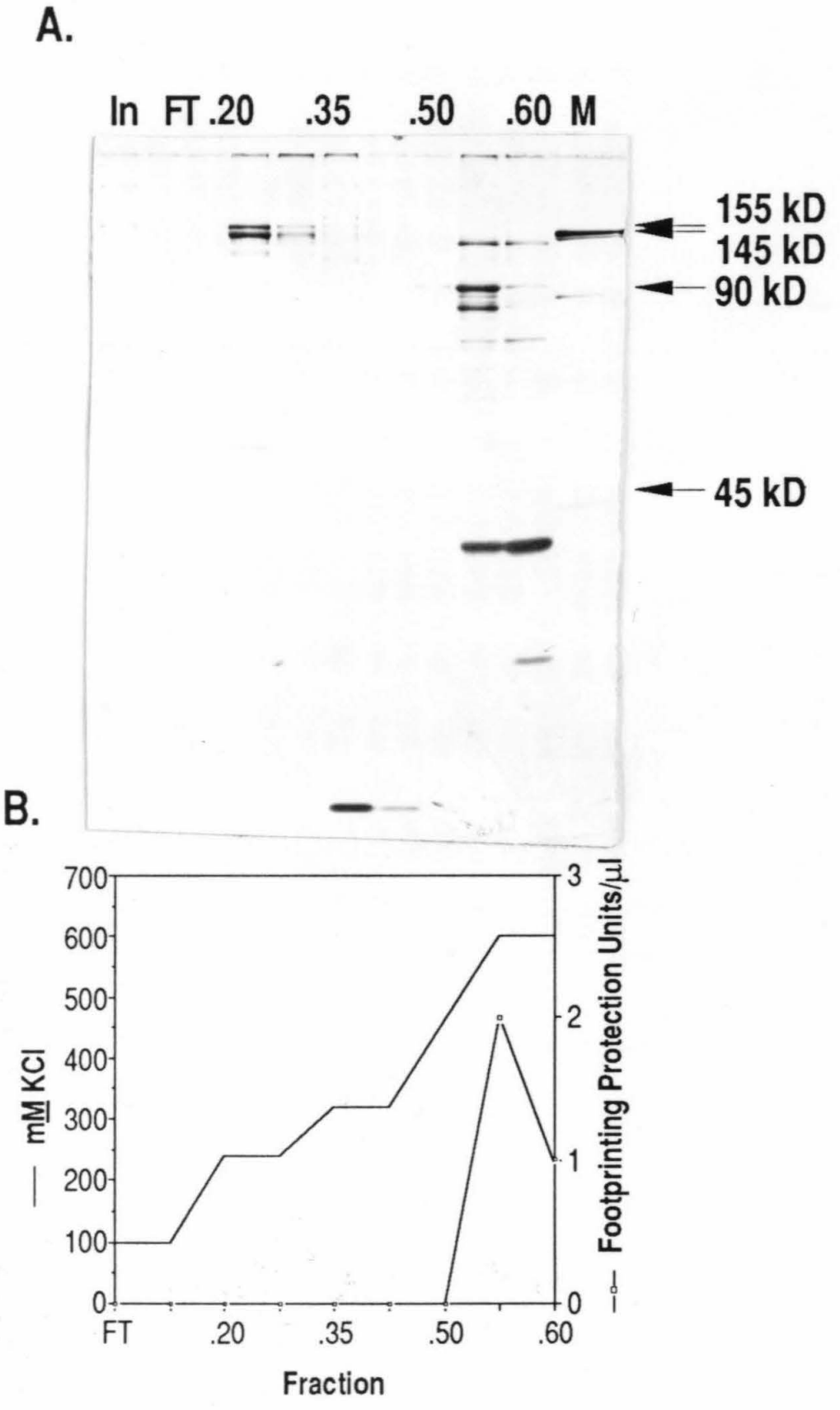

A.
In FT .20 .35 .50 .60 M
155 kD
145 kD
90 kD
45 kD
B.
700
600
500
400
300
200
100
0
mM KCl
3
2
1
0
Footprinting Protection Units/µl
FT .20 .35 .50 .60
Fraction

Figure 6.

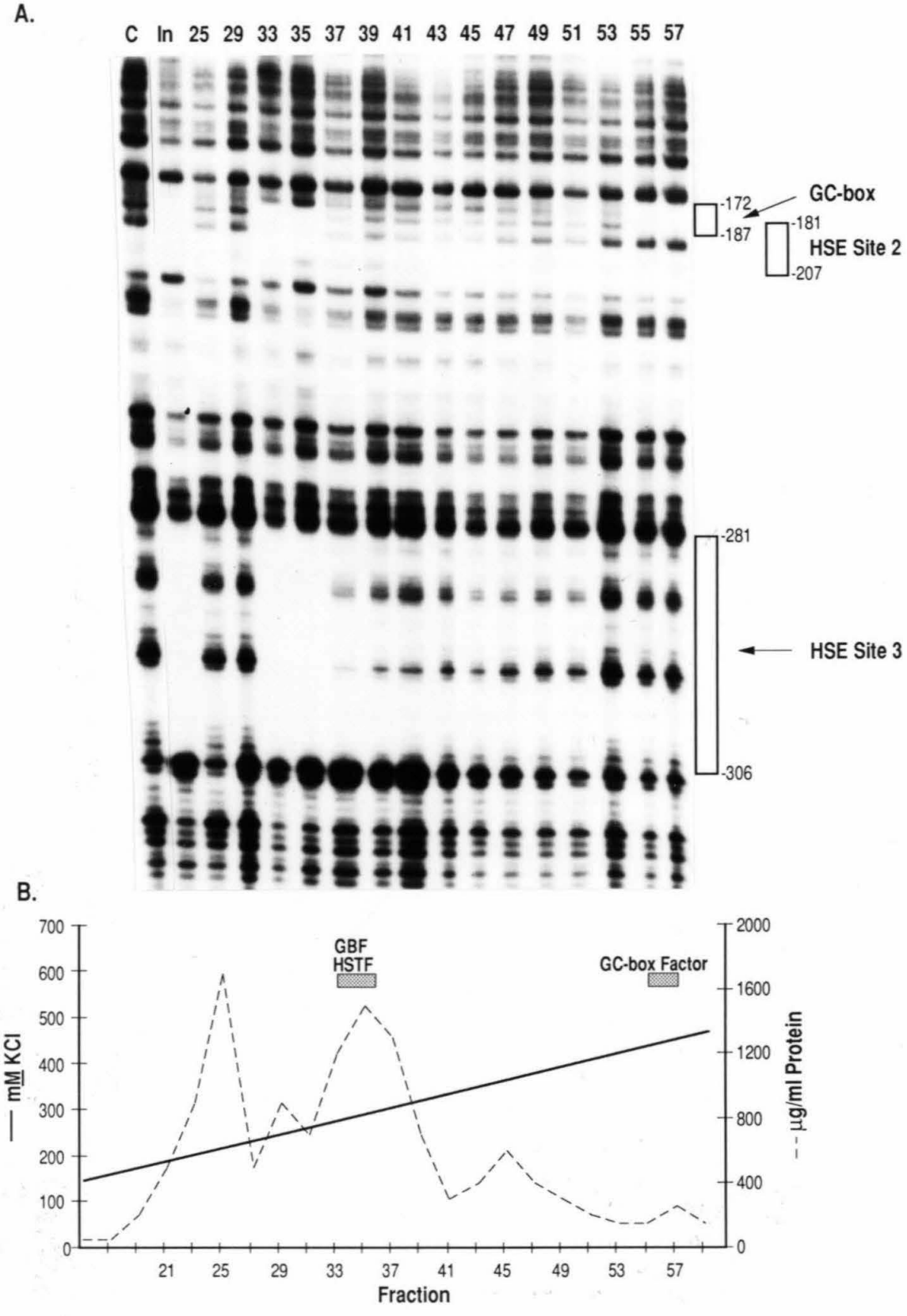

A.
C In 25 29 33 35 37 39 41 43 45 47 49 51 53 55 57
-172
-187
GC-box
-181
-207
HSE Site 2
-281
-306
HSE Site 3
B.
700
600
500
400
300
200
100
0
mM KCl
GBF
HSTF
GC-box Factor
2000
1600
1200
800
400
0
µg/ml Protein
21 25 29 33 37 41 45 49 53 57
Fraction

Figure 7.

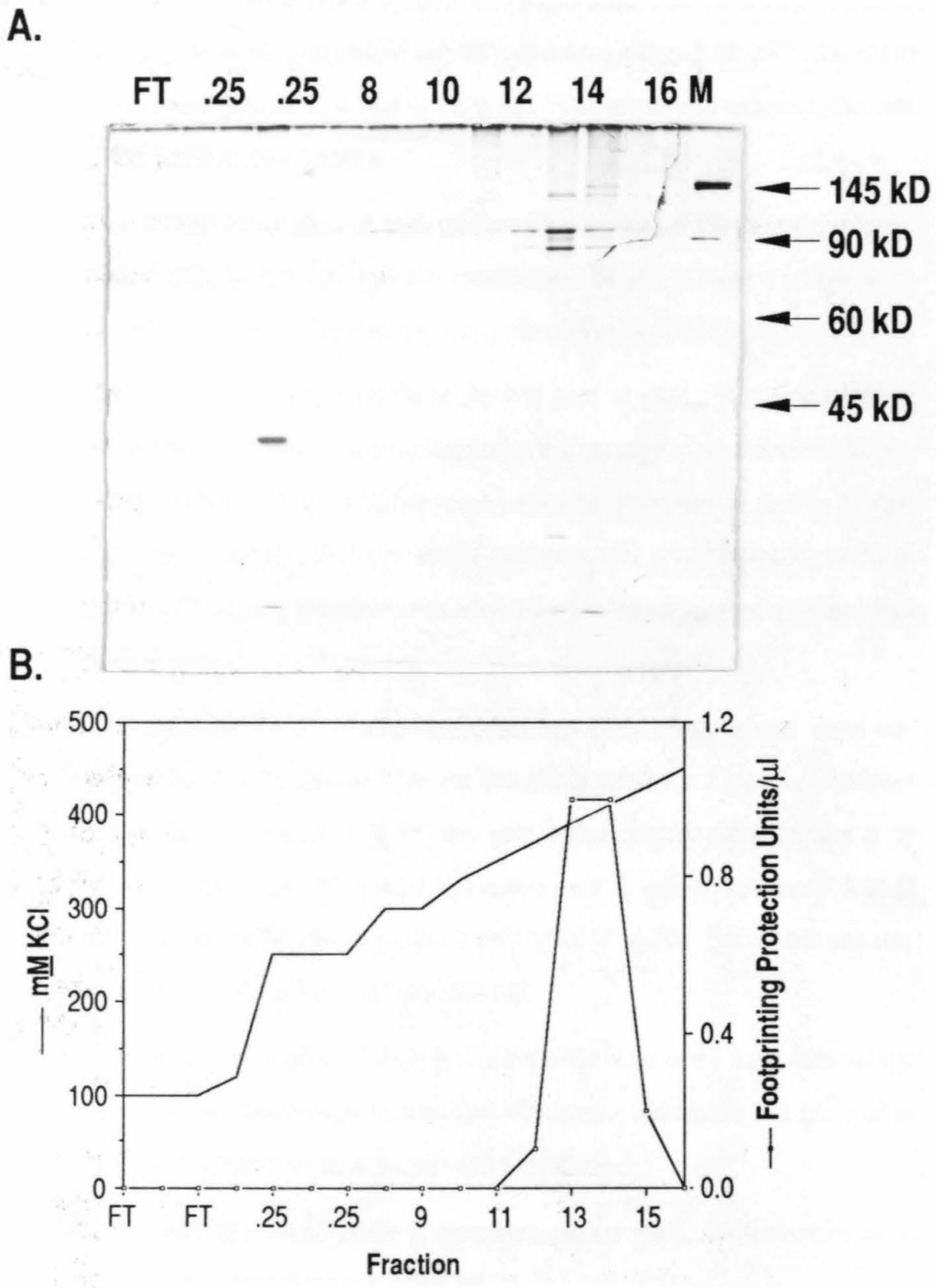

A.
FT .25 .25 8 10 12 14 16 M
145 kD
90 kD
60 kD
45 kD
B.
500
400
300
200
100
0
mM KCl
1.2
0.8
0.4
0.0
Footprinting Protection Units/µl
FT FT .25 .25 9 11 13 15
Fraction

Figure 8.

Figure 4. First pass of ySp1 on a ligated-GC-box-Sepharose affinity column. Input was from a 0.3 M KCl step from dsDNA-Sepharose and was loaded onto the affinity column at 0.15 M KCl. The column was washed with 100 column volumes of 0.32 M KCl, and eluted with a 20 milliliter gradient from 0.32 to 1.0 M KCl. The column size was 1 milliliter, and 1.5 milliliter fractions were collected.

A. Silver-stained gel of 50 µl of each gradient fraction, 5 µl of the input (labeled In), flow through (labeled FT), and 5 µl of 4 consecutive 0.32 M KCl eluted fractions (each fraction was 5 milliliters). Silver-staining was performed as described in Appendix C.

B. Concentration and activity profile of the first pass of ySp1. KCl concentrations were determined by conductance and comparison to a standard curve of known KCl concentrations. Activity in footprinting protection units was determined by titration of each fraction containing activity. Minimum activity required a 50% or better clearing with 5 µl of a fraction. Footprinting protection units were defined in the same manner as described in Chapter 1.

Figure 5. Second pass of ySp1 on a ligated-GC-box-Sepharose affinity column. Input was from pooled fractions 21 through 27 of the first affinity column in Figure 4. The input fractions were dialyzed to 100 mM KCl and were loaded onto the affinity column at 10 column volumes per hour. The column was washed with 20 column volumes of 0.32 M KCl, and eluted with a 20 milliliter gradient from 0.32 to 1.0 M KCl. The column size was 1 milliliter, and 1.5 milliliter fractions were collected.

A. Silver-stained gel of 50 µl of each gradient fraction, 5 µl of the input (labeled In), several consecutive flow through fractions (5 milliliters each and labeled FT), and 5 µl of 0.32 M KCl eluted fractions (each fraction was 5 milliliters).

B. Concentration and activity profile of the second pass of ySp1. Determinations were made by the same methods listed in Figure 4B.

Figure 6. FPLC MonoS chromatography of ySp1. Input was from a 0.3 M KCl step from dsDNA-Sepharose and was loaded onto the FPLC column at 0.10 M KCl. The column was washed and step eluted at 200, 350, 500, and 600 mM KCl. Fractions at 500 and 600 mM KCl were pooled and loaded onto a ligated GC-box-Sepharose affinity column. The GC-box-Sepharose column was run using identical conditions to Figure 5.

A. Silver-stained gel of 5 µl of each fraction eluting from the FPLC column prior to the 500 mM KCl step, and 50 µl of each fraction eluting on or after the 500 mM KCl step.

B. Concentration and activity profile of the FPLC fractions. Determinations were made by the same methods listed in Figure 4B.

Figure 7. *SSA1* promoter footprint using FPLC MonoS-purified fractions. A Pharmacia MonoS column was loaded with 10 milligrams of GBF, HSTF, and GC-box factor-containing heparin-Sepharose fractions. The column was run at 1 megaPascal (MPa) with a flow rate of 1.5 milliliters/minute. A 20 mM KCl/column volume gradient was applied to the column from 0.05 to 0.55 M KCl, and then a 1.0 M KCl step was applied (not shown). The HSTF and GBF activities elute at approximately 0.30 M KCl, and the GC-box factor elutes at approximately 0.45 M KCl.

A. Footprint on the *SSA1* promoter using 5 µl of each fraction eluting from the FPLC column, and 10 µl of the input fraction loaded onto the FPLC column.

B. Concentration and activity profile of the FPLC fractions. Determinations were made by the same methods listed in Figure 4B.

Figure 8. *Affinity purification* of the FPLC MonoS ySp1-containing fractions.

A. Silver-stained gel of 50 µl of each fraction eluting from the ligated GC-box-Sepharose affinity column loaded with active FPLC fractions.

B. Concentration and activity profile of the affinity fractions loaded with the active FPLC fractions. Determinations were made by the same methods listed in Figure 4B.

Purification of the GT-box binding factor

The GT-box site contains a stretch of 13 alternating purine-pyrimidine nucleotides. This type of sequence has been demonstrated to take on an unusual three dimensional structure if the level of supercoiling is high enough. The GT-box binding site is bound by a site-specific DNA binding factor. Since the region recognized by the GT-box binding factor includes a GC-repeat similar to the GC-box element, an oligonucleotide was synthesized to examine whether or not the DNAse I protection observed on this site was attributable to the GC-box binding factor. DNA competition experiments indicated that the GC-box and the GT-box were not bound by the same factor, and when a ligated GC-box oligonucleotide column was constructed, the factor binding to this column would not bind the *SSA1* GC-box site or the SV40 GC-box (data not shown). The material eluting from this column at high salt did bind to the *SSA1* GT-box (see Figure 9 in Chapter 1).

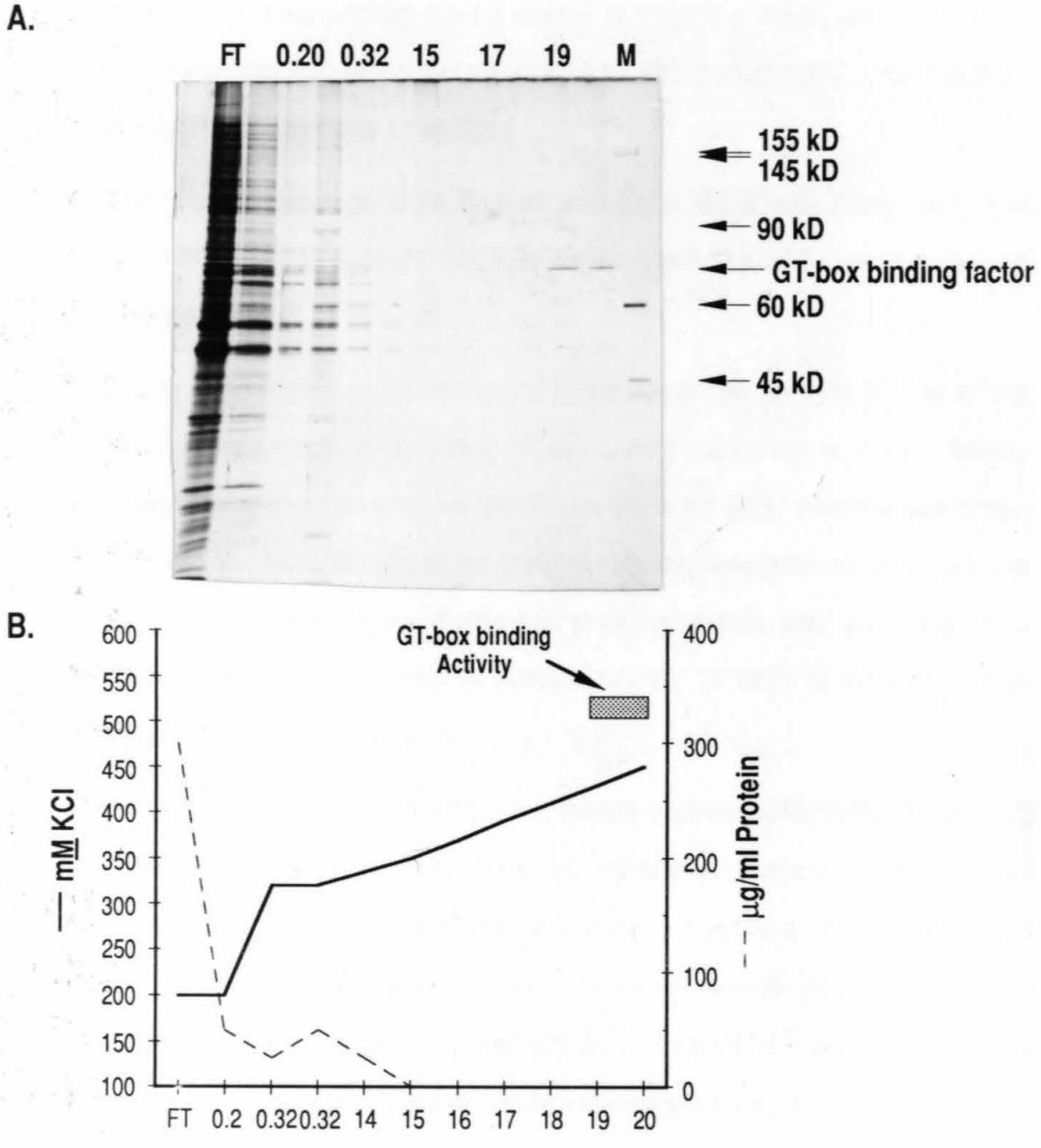

A.
FT
0.20
0.32
15
17
19
M
155 kD
145 kD
90 kD
GT-box binding factor
60 kD
45 kD
B.
GT-box binding
Activity
mM KCl
µg/ml Protein
600
550
500
450
400
350
300
250
200
150
100
400
300
200
100
0
FT
0.2
0.32
0.32
14
15
16
17
18
19
20

Figure 9.

Figure 9. Purification of the GT-box factor on ligated GT-box oligonucleotide-Sepharose resin. Affigel-Blue 2.0 M KCl-eluted fractions were dialyzed to 200 mM KCl and loaded at 10 milliliters per hour onto a column packed with 1 milliliter of GT-box-Sepharose affinity resin. The column was washed with 0.2 HGKET (0.2 M KCl in HGET) and 0.32 HGKET and the protein was then eluted with a gradient from 320 to 1000 mM KCl. GT-box binding activity eluted in fractions 19 and 20.

A. Silver-stained protein gel of the fractions eluting from the GT-box affinity resin. 5 µl of the flow-through (FT), 0.20 and 0.32 M KCl washes and 50 µl of the gradient fractions were loaded on the gel.

B. Profile of the protein concentration and KCl concentration from the GT-box affinity chromatography. Fractions containing GT-box binding activity are indicated. Activity was defined by DNAse I protection of the GT-box site in the *SSA1* promoter (see Chapter 1, Figure 9). 10 µl aliquots of the gradient fractions were assayed for footprinting activity. The GT-box oligonucleotide top strand sequence that was coupled to Sepharose was 5' TCGAC AAATA AAGCA CGTGT GCGCG GTGTG 3'. Both strands are displayed in Appendix A.

The affinity-purified GT-box binding factor was exceptionally clean. Affinity columns for yAP1 (K.D.Harshman, personal communication), HSTF (Chapter 3), and GBF (Chapter 4) did not cleanly resolve a single predominant protein on a silver-stained protein gel. However, purification of the GT-box factor on a ligated GT-box oligonucleotide affinity resin gave an unusually clean result (see Figure 9). One possible explanation for this result is that general DNA-binding proteins that apparently co-chromatograph on many affinity resins do not recognize an alternating purine-pyrimidine structure of this column. Another possibility discussed in Chapter 1 is that when the GT-box factor binds to the GT-box site, the DNA assumes an unusual conformation

and this prevents the binding of other DNA binding proteins, although this is unlikely because the column is run in DNA excess.

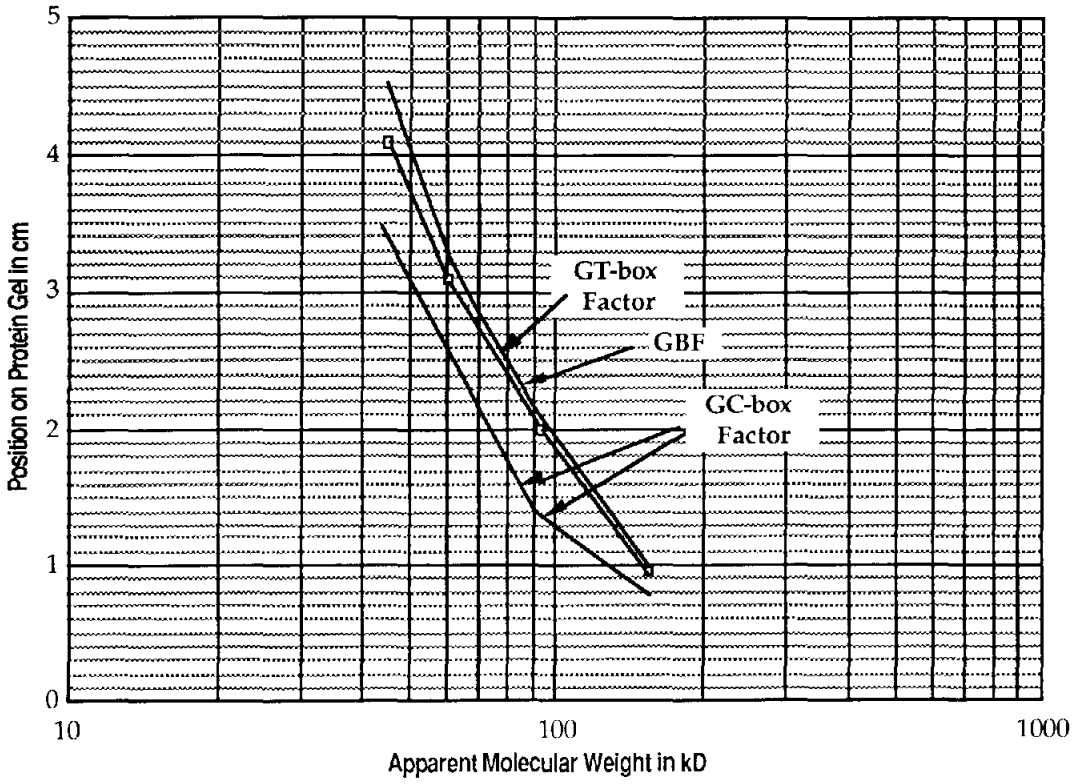


Figure 10. Plotting the size of various affinity-purified proteins. Positions of the protein size markers in Figure 9A (GT-box factor) and Figure 6a (GC-box factor or ySp1) plotted on a semilog plot versus the size of the markers. The size and position of the markers in Figure 2A (GBF) from the Chapter 4 appendix were also plotted. The migration position of these factors has been indicated and shows that the apparent molecular weight of these factors is 75 kD for GT-box factor (plus or minus 5 kD because a 70 kD marker was not included in the gel), 85 and 95 kD for the GC-box doublet, and 85 kD for GBF.

Estimating the size of the GT-box factor

Standard curves for the markers in the protein gel were plotted and from the position of the prominent band eluting from the GT-box affinity column

the size of the GT-box factor was calculated to be 75 kD, plus or minus 5 kD. Figure 10 shows a plot of the apparent size of the GT-box factor, GC-box factor (85 and 95 kD), and GBF (85 kD).

Work currently in progress

Further constructions assaying the activity of the 'blocking' activity described in this chapter are currently in progress. Ideally, a fully synthetic blocking region can be made consisting of short oligonucleotide sequences that can be constructed in various orders and orientations so that the sites necessary to make a functional 'blocking' region can be determined. These constructions will also determine if the orientation of these sites is important for their function. The question of orientation of the individual sites in this case may be important, since the three binding regions identified, the GT-box, the ABF1 site, and the RAP1 site are not symmetric, although the ABF1 site in the *SSA1* promoter does have an axis of symmetry.

An interesting question that is made relevant by the competition between the non-overlapping GT-box and ABF1 site is whether or not the GT-box binding factor conveys an unusual structure on this DNA region. The GT-box has a run of 13 alternating purine-pyrimidine bases followed by two purines, then a run of 8 more alternating purine-pyrimidines. An alternating sequence of purine-pyrimidine bases has been implicated as having the potential to form an unusual DNA structure (Dickerson 1988; Wells, et al. 1988). We would like to examine whether or not the GT-box DNA has an unusual structure, either with or without the GT-box binding factor.

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Chapter 2

The SV40 "Core" Sequence Binding Factor from yeast is a UAS Repressor

Zhen-Yong Zhang-Keck, Warren A. Kibbe, W. Scott Moye-Rowley and Carl S. Parker

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The SV40 "Core" Sequence Binding Factor from yeast is a UAS Repressor

Abstract

We have identified a yeast transcription repressor that recognizes the SV40 enhancer core sequence TGTGG$\frac{AAA}{TTT}$G. The core sequence (known as URE for upstream repressor element) can repress the activity of a GCN4 recognition element (GCRE) when present adjacent to the GCRE. This repression is orientation independent and is maintained when the GCRE and URE sequences are separated by up to 214 bp. However, separating the two sites by 396 bp attenuates the repression. Repression of the GCRE by the URE can be overcome by the addition of another GCRE (a total of two copies of the GCRE and one copy of the URE). Biochemical analysis of the proteins that bind specifically to the URE from yeast demonstrate that two protein-DNA complexes are formed in gel retention analysis using a URE oligonucleotide. Additionally, two major bands of dissimilar molecular weight were detected by Southwestern analysis. The experimental evidence suggests that the repression associated with the URE sequence is mediated by a direct, one to one interaction between the proteins recognizing the URE and GCRE sequences.

Keywords:

URE, GCRE, GCN4, yAP1, transcription regulation, transcription repressors, DNA binding proteins, yeast transcription, UAS, URS.

Introduction

The regulation of transcription in eukaryotes is largely controlled by the interaction of multiple trans-acting proteins with various promoter elements. In addition to binding DNA, these proteins must interact with other components comprising the transcriptional machinery to elicit their regulatory function. Currently, many sequence-specific DNA-binding transcription factors have been identified and characterized from evolutionarily diverse organisms (see reviews: Guarente 1988; Johnson and McKnight 1989; Jones, et al. 1988; Maniatis, et al. 1987; McKnight and Tjian 1986; Parker 1989; Struhl 1989). Most of the factors that have been identified so far are constitutive or pathway-specific activators of transcription.

It is becoming evident that *trans*-acting repressors are also fundemental components in the regulation of transcriptional processes. Several negative control elements, known as upstream repression sites (URSs) in yeast, have been identified in eukaryotic promoters (Bariahmad, et al. 1987; Jankowski and Dixon 1987; Kuhl, et al. 1987; Laimins, et al. 1986; Simpson, et al. 1986; Sumrada and Cooper 1987). The mechanisms by which silencers and URSs work may indeed be different. Silencers can repress transcription in an orientation and position indepenedent fashion, whereas URSs act in a more constrained manner, usually functioning only when positioned between the TATA homology and the enhancer element. Some experiments have suggested that the repression activity is mediated by trans-acting factors (Brent and Ptashne 1984; Gorman, et al. 1985; Sassone-Corsi, et al. 1987), as well as silencers of transcription (Brand, et al. 1987; Kuhlemeier, et al. 1987). However, there are only a few examples of these trans-acting factors that have been identified (Fujita, et al. 1988; Hen, et al. 1985; Johnson and Herskowitz

1985; Thierry and Yaniv 1987) and purified (Buchman, et al. 1988; Shore and Nasmyth 1987).

An examination of the transcriptional activity of various sequence elements of the SV40 enhancer in yeast revealed that the SV40 core sequence was not able to activate transcription on its own. When the core sequence was placed next to the binding site for GCN4 (GCRE) we observed that GCRE-dependent transcription was greatly attenuated. In this report we show that in yeast the SV40 core sequence represses the activation mediated by the GCN4 recognition element (GCRE) in a truncated *CYC1* promoter. The core sequence can therefore be designated as an upstream repression element (URE) in yeast. Repression mediated by this URE is orientation and position independent. Furthermore, this repression is relatively distance independent (up to 214 bp), but not as independent as expected for silencers. DNAse I protection data and gel-shift analysis provide evidence that at least one site-specific DNA-binding protein recognizes the URE. From a detailed analysis of how the URE interacts with activation elements we have gained new insight into how transcriptional activators and repressors function to regulate gene expression.

Results

The URE represses transcriptional activation by GCN4

Our previous studies showed that the AP-1 recognition element (ARE) present within the SV40 72 base pair enhancer will activate transcription in yeast when placed upstream of a truncated *CYC1* promoter (Harshman, et al. 1988). The SV40 core enhancer sequence (TGTGGAAAG) when placed into the same position in the *CYC1* promoter, however, was unable to activate transcription of the reporter gene (Figure 1, pURE). To determine whether

the core sequence could function synergistically with a known activator element, a single GCN4 recognition element (GCRE) was inserted next to the core sequence. The GCRE-containing control plasmid (pGCRE) significantly activated transcription of the reporter gene in both orientations, as expected (Figure 1, pGCRE). When the core sequence was inserted next to the GCRE in either an upstream or a downstream position, a dramatic inhibition of transcription was observed (Figure 1; pURE/GCRE). This was a surprising result in that one would expect the core sequence to be an activator of transcription as it is in mammalian cells, rather than acting as a repressor of transcription.

These experiments demonstrate that a single core sequence (now referred to as the URE, for upstream repressor element) can repress GCRE-mediated activation 18 to 21 fold. Similar levels of repression were also observed when the reporter genes were placed on CEN plasmids, indicating that these results were not limited to high copy number plasmids (data not shown). The ability of the core element to repress other yeast UAS sequences was also investigated by substituting the AP-1 recognition element (ARE) in place of the GCRE (Figure 1, pARE, pURE/ARE). As observed for the GCRE, the presence of the core element repressed ARE-dependent activation. From this functional analysis of the core sequence in yeast it is apparent that the sequence can function as a repressor of activator-dependent transcription. It remains possible, however, that under some conditions or in conjunction with other promoter elements the URE may be able to activate transcription in yeast.

URE repression of GCN4 activation is relatively distance independent

To further understand the mechanism by which the URE carries out repression of UAS-dependent transcription in yeast, we carried out a series of experiments examining the effects of distance between the activation ele-

ment, the GCRE, and the URE. This was done by constructing two sets of reporter plasmids, each containing one of a series of defined length DNA fragments inserted between the URE and GCRE of pURE/GCRE (4, 12, 36, 46, 105, 141, 214, and 396 bp; Figure 2; see *Materials and Methods*). Figure 2A shows that the starting plasmid used in this experiment (pURE/GCRE, which contains the URE and GCRE) was repressed by more than 90% compared to the control that lacked the URE. Separating the GCRE from the URE by 4, 12, 36, 46, 105, 141, and 214 bp caused essentially no loss of repressor function. Efficient repression occurred at these distances when the URE was positioned on either side of the GCRE as indicated in Figure 2A by the 5′ URE and 3′ URE designations. Placing the URE 396 bp away from the GCRE either 5′ or 3′ eliminated the ability of the repressor to block GCN4 activation. Moving the GCRE/URE sites upstream of the TATA homology in tandem did not result in a loss in repression (Figure 2B). These results suggest that the repressor may interact in a direct way with the activator, and that this interaction can be attenuated at a distance.

Evidence for a possible direct interaction between the proteins bound to the URE and the GCRE

Further evidence supporting the idea that the URE is bound by a protein(s) that interacts directly with the GCRE/yAP-1 complex is given by experiments where additional activation or repression sites are added to a promoter containing one copy each of the URE and GCRE (pURE/GCRE, Figure 1). The pURE/GCRE construction is repressed approximately 20-fold and this activity can be used as the control to which the effects of introducing additional activation or repression elements can be compared. Insertion of 1, 2 and 3 extra copies of the GCRE into parental plasmid pURE/GCRE generated new plasmids pURE/$GCRE_2$, pURE/$GCRE_3$, and pURE/$GCRE_4$, each containing one

copy of the URE but a total of 2, 3, and 4 copies of the GCRE, respectively. The levels of ß-galactosidase activity were increased by 17, 21, and 28 fold over the level of activity in the parental plasmid. Interestingly, the addition of a single activator site restores the normal level of single-site GCRE/GCN4 activation when the repression site is placed upstream of the GCRE (orientation A, Figure 1). However, the addition of two activation sites are required to restore the normal levels of transcriptional activation when the URE is positioned between the GCRE and the TATA homology (orientation B, Figure 1). It is important to note that the level of activation brought about by the addition of 2 or 3 activator sites to the URE/GCRE construction in orientation A is equivalent to what one would observe with two or three GCRE sites in the absence of the repressor element (data not shown). The data indicates that the URE can offset the effects of only one activator binding site, suggesting that the URE-bound repressor interacts directly with the activator in some manner. Additional support for this model is given by the lack of further repression of a single GCRE by multiple UREs (Figure 1, $pURE_2/GCRE$, $pURE_3/GCRE$, and $pURE_4/GCRE$).

A second line of evidence supporting a direct interaction between the URE and the UAS comes from experiments where the URE is placed downstream from the start point of transcription. Under these circumstances we do not observe a reduction in the levels of ß-galactosidase (Figure 1, pGAL and pGAL/URE). In these constructions a portion of the *GAL1-10* promoter had been inserted upstream of the *CYC1* TATA homology and the addition of the URE did not repress transcription when the cells were grown on glucose (Figure 1, pGAL and pGAL/URE). The effect of the repressor site when these constructions are grown on galactose has not been determined. From these results, however, it is clear that the repressor cannot block the transcriptional

apparatus from initiating or elongating transcription when the cells are grown on glucose. It seems likely, therefore, that the URE mediates transcriptional repression by the suppression of a UAS-binding factor.

Purification of a yeast protein that binds to the URE

To identify and characterize the protein(s) that may be responsible for the transcriptional repression observed with the URE, a factor was purified from yeast nuclear extracts that was capable of specifically recognizing the URE. The factor was isolated from yeast nuclear extracts by heparin-Sepharose chromatography, followed by DNA affinity chromatography (Wiederrecht, et al. 1987). Shown in Figure 3, the affinity-purified protein binds specifically to the SV40 core enhancer sequence as determined by DNAse I footprinting analysis.

The URE-binding factor (referred to as the upstream repressor factor or URF even though the isolated protein has not yet been formally shown to be responsible for the transcriptional repression observed *in vivo*) protects a larger region of the SV40 core sequence than the core binding protein AP3 from HeLa cells (Mercurio and Karin 1989; Mitchell, et al. 1987) and from calf thymus (Harshman, et al. 1988) To ensure that all essential sequences were present in the URF binding site we repeated the repression experiments by inserting a 35 bp-long oligonucleotide containing the entire DNAse I protected region in place of the smaller URE. The repression obtained with these constructs was identical to the repression obtained with the shorter URE sequence (data not shown). Therefore, the 25 nucleotide URE (core sequence) contains all the sequences necessary for efficient protein binding and function of this site *in vivo*.

URF binds specifically to the URE

To assess the DNA binding properties of the affinity-purified fractions for the URE, we performed DNA competition experiments using the gel-shift assay (Figure 4). When affinity-purified URF was incubated with labeled URE oligonucleotide, we observed the formation of two specific protein-DNA complexes. This unexpected result indicates that there are multiple forms of the URF in yeast. The specificity of these two complexes was tested by incubating affinity-purified URF with labeled URE oligonucleotide and a 100-fold molar excess of the following unlabeled competitor oligonucleotides: URE; GCRE; yAP1 recognition element (ARE); and the heat shock transcription factor recognition element (HSE). When the unlabeled URE was added in the mixture, the formation of the two complexes was completely inhibited (Figure 4). No significant inhibition was observed in the mixture when GCRE, ARE, or HSE were added. When the GCRE, ARE, and HSE were separately labeled and used to further test the sequence specificity of the binding, URF did not form a complex with any of these sequences (data not shown). The data strongly indicates that the two complexes are URE sequence-specific.

Determination of the molecular weight of URF by Southwestern Analysis

The apparent molecular weight of the URF was determined by Southwestern analysis (see *Materials and Methods*). Affinity-purified URF was electrophoresed on an SDS-polyacrylamide gel and then transferred to nitrocellulose. The filter was probed with labeled URE oligonucleotide in the presence of non-specific carrier DNA. The URF activity contains two major URE-binding polypeptides with approximate sizes of 68 and 30 kD. The two complexes seen in the gel-shift experiments could be due to the different molecular weight proteins; the larger URF protein forming the larger gel-shift

complex, and the smaller URF protein forming the smaller gel-shift complex. The lack of an intermediate size gel-shift complex indicates that the URE/URF interaction consists of either a monomer of URF recognizing one binding site, or that the small and large molecular weight proteins do not bind as a heteromeric unit. Alternatively, the large complex may be due to a heteromeric dimer consisting of one small and one large subunit, and no dimerization can occur between the large subunits. Further studies will be needed to clarify this issue.

Discussion

Our studies indicate that in yeast, UAS-dependent transcription can be specifically repressed by the presence of an adjacent site whose sequence corresponds to the SV40 core element (URE). The URE sequence can repress the transcriptional activiation mediated by the GCRE/GCN4 complex or by the ARE/yAP-1 complex when placed on either side of the recognition elements. Transcriptional repression mediated by the URE was relatively distance independent. However, separating the repressor and activator sites by 396 bases abrogated the repression. The possibility exists that the loss of repression is due to the presence of an activator's binding site present fortuitously in the 396 bp DNA fragment. However, the 396 bp DNA fragment does not drive the *CYC1* promoter on its own, nor does it affect the activity of the promoter when placed between the GCRE and the TATA homology (data not shown). The data further suggests that repression mediated by the URE is attenuated by distance from the activator's site and not by distance from the TATA homology.

The addition of another GCRE to a promoter containing one GCRE and one URE effectively neutralizes the action of the URE. It is important to note

that the activity of this construction is virtually the same as the activity of a construction containing only a single GCRE without a URE. Furthermore, the addition of more copies of the URE to a construction containing one GCRE and one URE does not further increase the level of repression. These observations suggest that a single URE-bound URF can neutralize a single activator (in this case GCN4) bound to its recognition site (the GCRE). Therefore, we propose that there is a one-to-one interaction between the repressor URF and the activator GCN4 in these constructions.

The question remains of why is there a distance effect. It is likely that a UAS-associated activator interacts with the TATA homology to enhance the assembly or initiation rate of the transcriptional machinery. The activation domain(s) of the activator may be blocked by the repressor so that the activator cannot interact with the transcriptional machinery. In this model, there is a competition between the transcriptional machinery/TATA homology and the URF for the bound activator. The association of the repressor with the activator is favored when the repressor is positioned closely to the activator. When this distance becomes too great, interaction with the transcriptional apparatus is favored. This model suggests that the repressor interferes directly with the activator's association with the transcriptional apparatus. This model has many testable parameters that can be readily carried out once the gene encoding the URF(s) is cloned.

The mechanism of repression that the URE utilizes is likely to be different from the silencing of enhancers as described by Weissman and his colleagues (Kuhl, et al. 1987), where the simple placement of a tetrameric sequence between the SV40 enhancer and TATA homology repressed the activity of the enhancer, but this sequence did not repress transcription when placed upstream of the SV40 enhancer. The repression mediated *by the URE*

sequence is similar to the action of yeast repression sequence (URS1) found in many yeast promoters (Park and Craig 1989; Sumrada and Cooper 1987). The URE and URS1 both function either upstream of a UAS or between the UAS and TATA homology (Sumrada and Cooper 1987). URS1 was demonstrated to interact with the arginine induction system in the *CAR1* promoter in such a way that URS1-mediated repression is removed on induction with arginine. However, if URS1 is placed upstream of the TATA homology in *CYC1*, the repression meditated by URS1 is not sensitive to arginine induction. These observations suggest that the URS1 function can be neutralized by the action of *cis*-acting elements present in the *CAR1* promoter. Similarly, we have created an artificial promoter where the repression of URE can be overcome by multiple UASs. Thus, the presence of one URE was neutralized, or overcome, by two copies of the UAS, suggesting that the mechanism by which the arginine induction occurs in the *CAR1* promoter is by the neutralization the URS1 by an arginine-specific activator.

By DNAse I protection analysis, the existence of a factor that specifically binds the URE was demonstrated. This binding factor (URF) was affinity-purified on a ligated-URE oligonucleotide affinity column. Gel-shift experiments revealed that two distinct protein-DNA complexes can form on the URE using the affinity-purified preparations. Southwestern analysis of affinity-purified material revealed that two proteins of approximately 68 kD and 30 kD were able to independently and specifically bind to the URE. These observations suggest that multiple forms of URFs exist and perhaps can function independently in response to different control signals. Further definition of the roles played by these proteins in transcriptional regulation in yeast will require the identification of genes controlled by these factors.

Materials and Methods

Oligonucleotides

URE	GATCT	GGGGA	CTTTC	CACAC	CTACG	
GCRE	GATCC	GGATG	ACTCT	TTTTT	TA	
ARE	GATCC	ATGGT	TGCTG	ACTAA	TTGA	
HSE	AGCTT	ATAGA	ACGTT	CTAGA	AGCTT	GAGA

Strains

The *S. cerevisiae* protein-deficient strain EJ926 (a kind gift of E. Jones); CG219 (Harshman, et al. 1988); SM9 (Moye-Rowley, et al. 1989) and the *E. coli* strain DH5 (Hanahan 1985) were used in this work. *E. coli* and yeast were transformationed using standard procedures (Ito, et al. 1983; Maniatis, et al. 1982).

Plasmid Constructions

All plasmids were constructed using standard procedures (Maniatis, et al. 1982). The parent plasmid pLGΔBS (Harshman, et al. 1988) was derived from pLG-669-Z (Guarente and Ptashne 1981) and contains approximately 250 nucleotides of the *CYC1* promoter including the TATA homology, start point of transcription and coding sequence is fused in frame to *lacZ*. The *CYC1* UASs were excised by Xho I and replaced with a Bgl II linker, generating pLGΔBS.

Plasmids containing the URE were constructed by insertion of the synthetic URE directly into pLGΔBS at the Bgl II site upstream of *CYC1/lacZ* fusion gene using the designated GATC overhangs. The synthetic GCRE sequence was derived from the promoter proximal GCRE of the yeast *HIS3* gene, and the oligonucleotide inserted directly into Bgl II site of pLGΔBS

using GATC overhangs. Plasmids pGCRE, A and B orientation, were constructed by K. D. Harshman (Harshman, et al. 1988).

Other plasmids used in this work which contain a series of defined-length DNA sequences either in between or downstream of GCRE/URE were generated by inserting gel-purified Sau 3AI-digested pUC19 fragments: 36 bp, 46 bp, 105 bp, 141 bp; and Hinf I-digested pUC19 fragments 214 bp and 396 bp (which were filled in with DNA polymerase Klenow fragment prior to ligation).

To generate small base changes, the Bgl II site was filled in with DNA polymerase Klenow fragment and then either self-ligated (4 bp) or ligated in the presence of XhoI linker (8 bp). In all of the new constructions, the precise copy number and the orientations of the inserts were verified by restriction mapping including the loss of Bgl II site (fill-in) as well as regeneration of the XhoI restriction site. The gel purification of oligonucleotides were performed as described in detail previously (Wiederrecht, et al. 1987).

ß-Galactosidase Analysis

Single colonies from the transformation plates were grown in minimal culture medium containing glucose. The assay of ß-galactosidase levels, determination of protein concentration and the calculation of the ß-galactosidase units have been previously described (Harshman, et al. 1988). All of the ß-galactosidase assays shown in this paper represent the results obtained from at least three individual isolates from the original yeast transformation plate.

Yeast Nuclear Extracts and Chromatography

The yeast strain EJ926 was grown in minimal medium containing 2% glucose. When the culture reached an O.D. 600 between 1 and 3, the yeast were harvested and quick frozen as described (Wiederrecht, et al. 1987). Nuclear extracts were prepared as described (Wiederrecht, et al. 1987), beginning *with*

400 gm of the frozen material. Nuclear extracts were fractionated on heparin-Sepharose equilibrated with 0.05 HGKE. HGKE is 25 mM Hepes, pH 7.6 at 20°C; 10% glycerol; K stands for the molar concentration of KCl; and 0.1 mM EDTA. 0.3 HGKE step fractions were pooled, dialyzed against 0.1 HGKET (T denotes 0.2% Triton X-100) and applied to a 1.5 ml ligated-URE affinity column. The column was washed with 100 volumes of 0.3 HGKET, and the bound proteins were step eluted with 10 volumes of 2 HGKET. The method for the preparation of ligated oligonucleotide-Sepharose resins has been previously described (Wiederrecht, et al. 1987).

DNAse I Protection Analysis

DNAse I protection experiments were performed as described (Wiederrecht, et al. 1987). The template DNA pENH 1 X 72 containing a single copy of SV40 72 repeat (a kind gift from Dr. Winship Herr) was 5'-end labeled by polynucleotide kinase either at the BamHI end (top strand) or at the EcoRI site (bottom strand).

Gel-shift Analysis

Gel-shift analysis was performed basically as previously described (Wu and Crothers 1984). DNA-protein complexes were formed in 20 µl binding reaction that contained 1 µl of partially affinity column purified protein samples, approximately 20,000 cpm (about 0.3 ng) of end-labeled double stranded URE oligonucleotides, 20 µg/ml sonicated salmon sperm DNA, 10 mM Tris-HCl, pH 7.4, 50 mM KCl, 5% glycerol 0.05% NP40, and 1 mM EDTA.

After incubation at 25°C for 10 min, the reaction was directly loaded on a 5% polyacrylamide gel (acrylamide:bisacrylamide weight ratio 80:1 of dimensions 19 X 16.5 X 0.15 mm) made in 0.5 X TBE. Following electrophoresis at 150 volts, gels were dried and autoradiographed with Kodak XAR-5 film.

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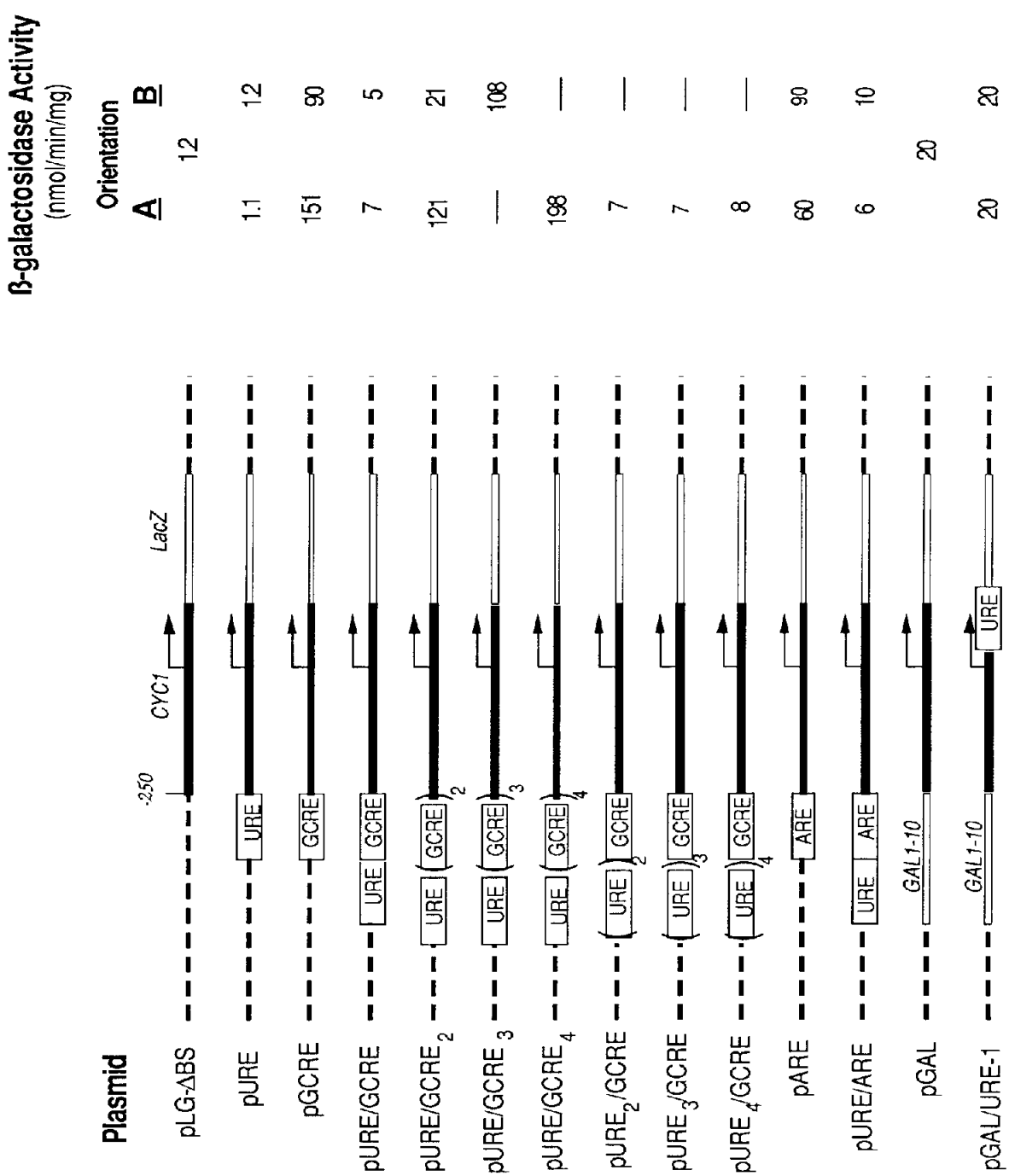
ß-galactosidase Activity
(nmol/min/mg)
Orientation
A
B
Plasmid
-250
CYC1
LacZ
pLG-ΔBS
1.2
pURE
URE
1.1
1.2
pGCRE
GCRE
151
90
pURE/GCRE
URE GCRE
7
5
pURE/GCRE2
URE (GCRE)2
121
21
pURE/GCRE3
URE (GCRE)3
—
108
pURE/GCRE4
URE (GCRE)4
198
—
pURE2/GCRE
(URE)2 GCRE
7
—
pURE3/GCRE
(URE)3 GCRE
7
—
pURE4/GCRE
(URE)4 GCRE
8
—
pARE
ARE
60
90
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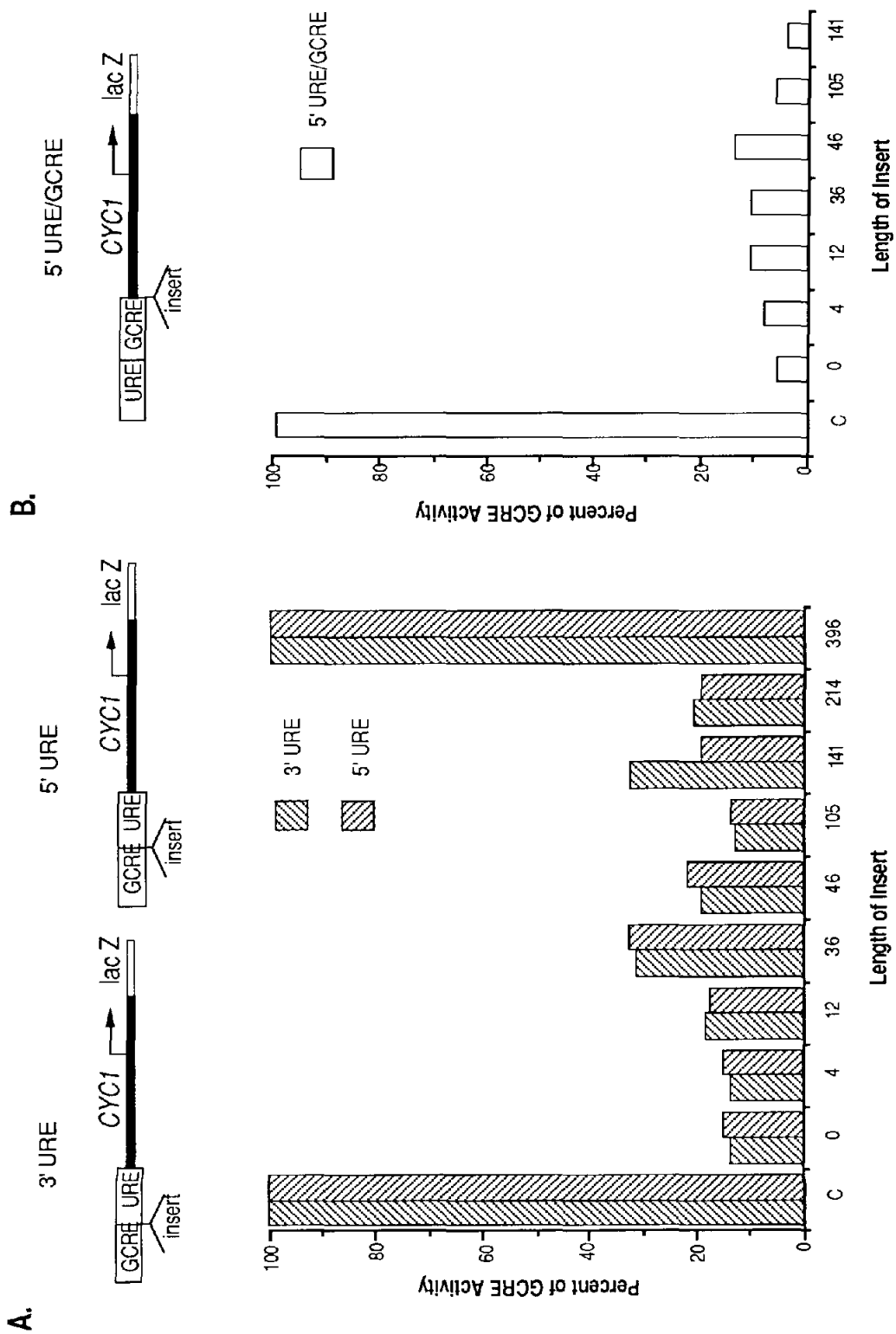
URE
20
20

Figure 1.


A.
3' URE
CYC1
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Percent of GCRE Activity
100
80
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12
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46
105
141
214
396
Length of Insert
B.
5' URE/GCRE
CYC1
lac Z
URE GCRE
insert
5' URE/GCRE
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Figure 2.

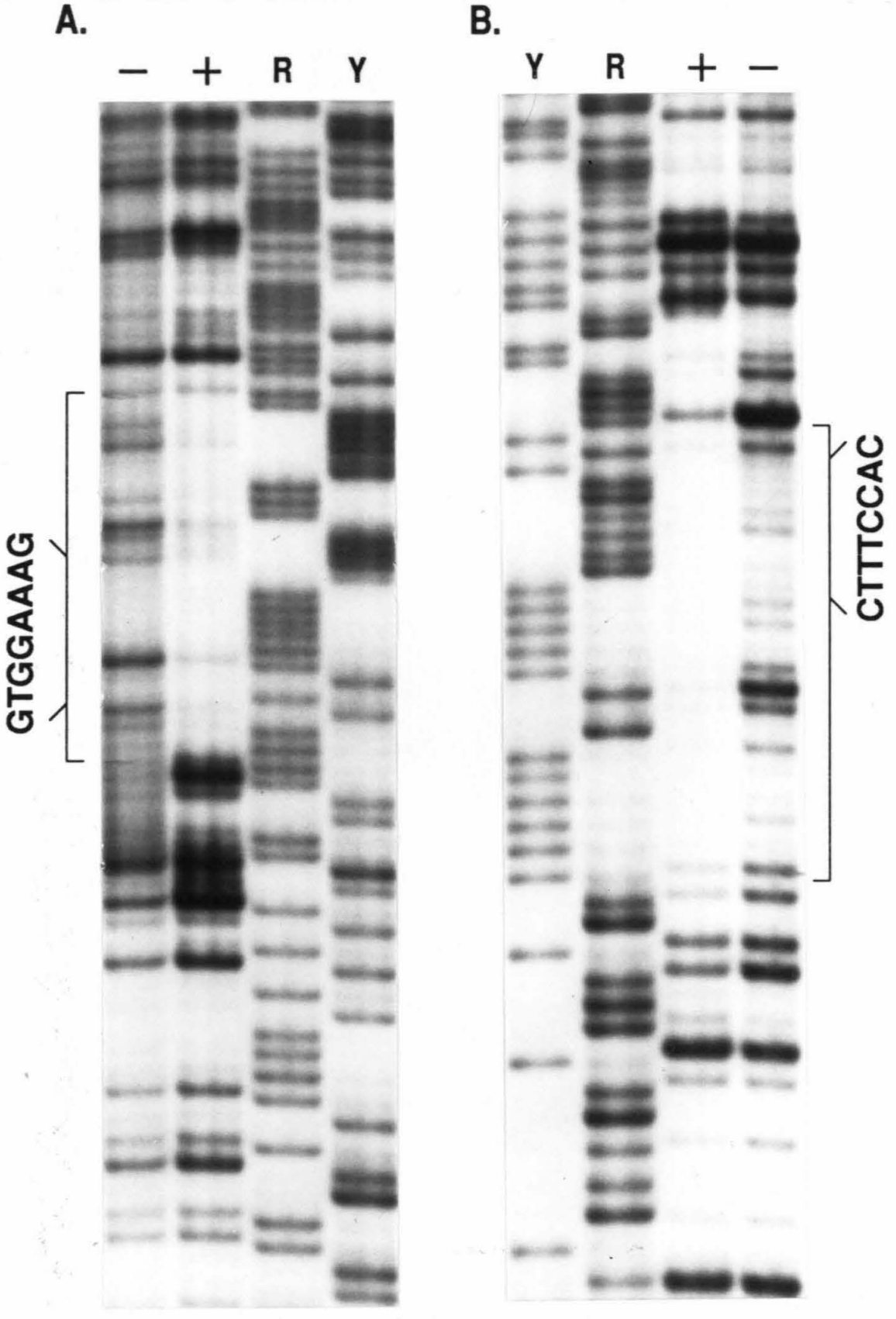

Figure 3.

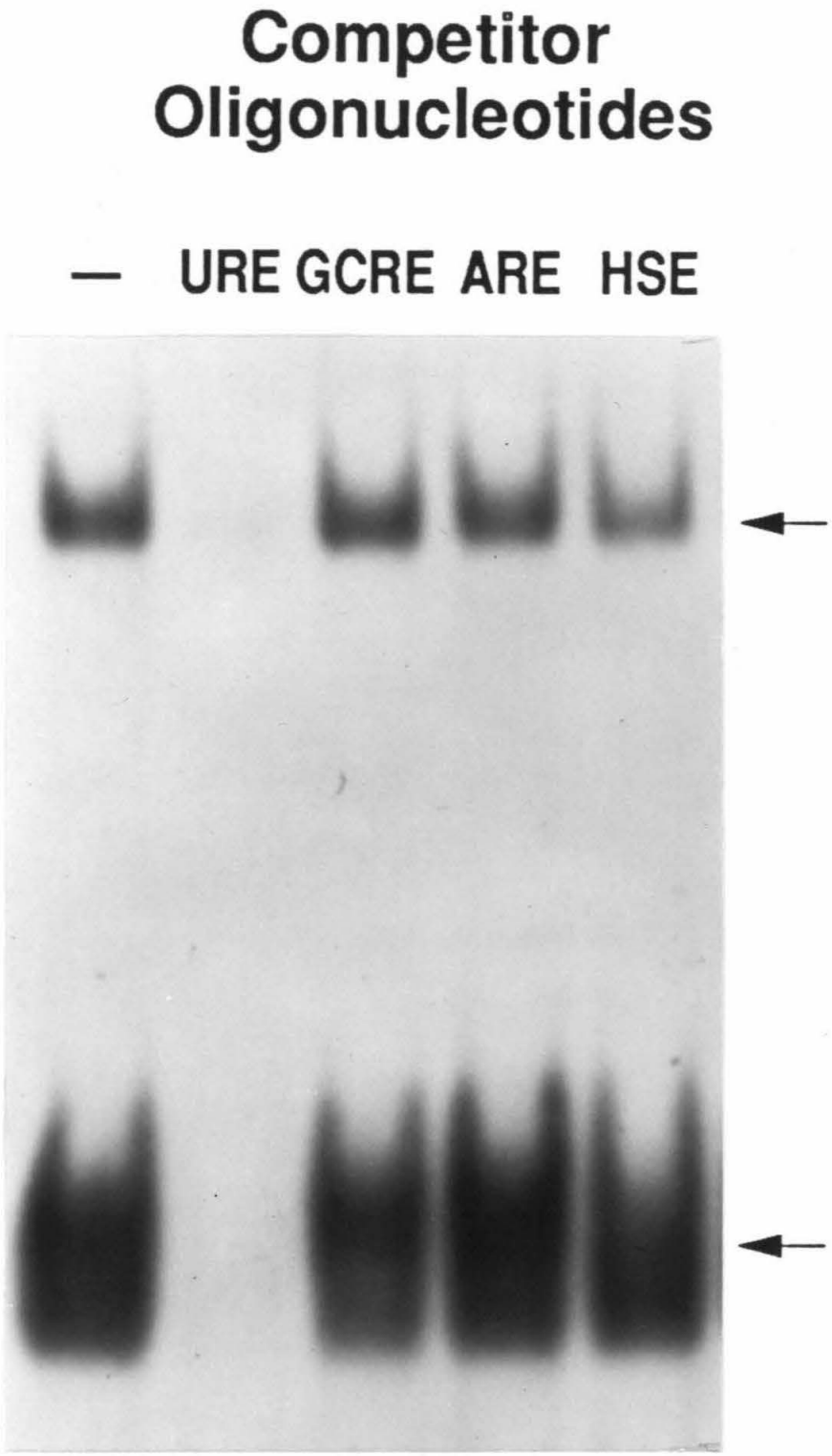

Figure 4.

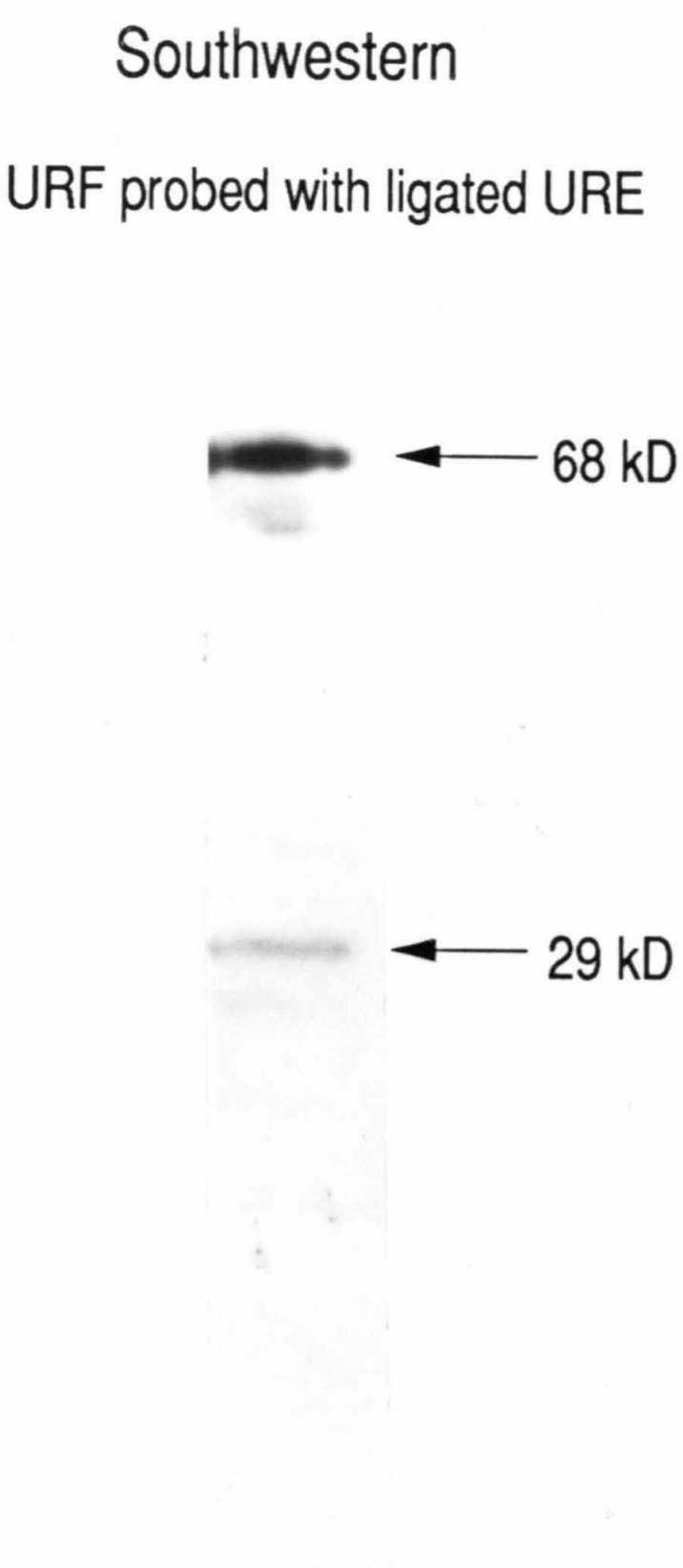

Figure 5.

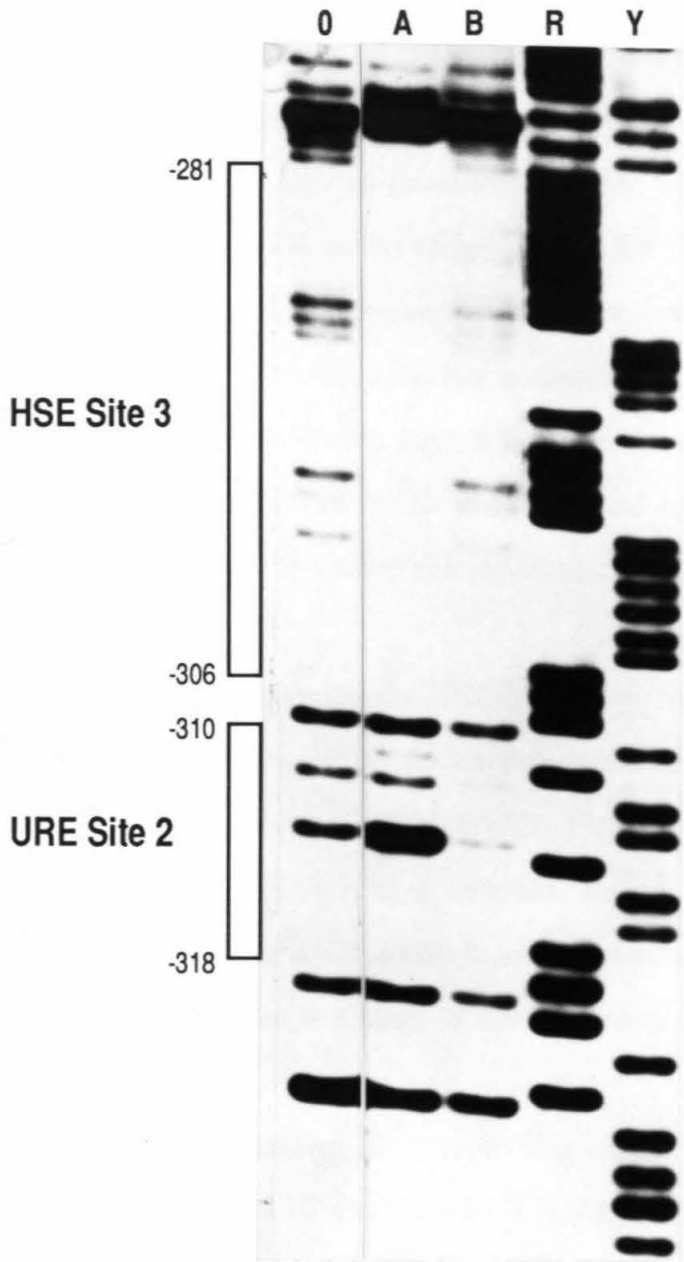

Figure 6.

Figure Legends

Figure 1. URE represses transcriptional activation mediated by GCN4

The dashed line represents plasmid sequence. The open box indicates the β-galactosidase portion of the *CYC1*-lacZ fusion gene. The solid line represents *CYC1* sequences. The direction of transcription is indicated by the arrow above the line. Oligonucleotides inserted into the plasmids are boxed and shown on the left side. URE is the abbreviation for the upstream repression recognition element and GCRE is the abbreviation for the GCN4 recognition element. The orientation of the inserted recognition elements are indicated by A for the orientation as drawn and B for the opposite orientation. The average value of ß-galactosidase levels are indicated in nmol/min/mg on the right, which are obtained from several independent single colony assays

Figure 2. Effect of distance on the URE-mediated repression

(Top) This portion of the figure shows the constructions used to assess the effects of distance on repression. The constructions are described in detail in *Materials and Methods.* The position labeled 'insert' indicates the position where the defined-length DNA fragments were inserted. The length of insertion (bp) was plotted versus % GCN4 enhancer activity measured by ß-galactosidase expression.

(A) The location of the insert is between the GCRE and the URE sites, and the URE is either upstream of the GCRE (5′ URE) or downstream (3′ URE). The activity of the various constructions is shown relative to a construction containing only a GCRE (100% activity). The URE-mediated repression is nearly insensitive to distance until the 396 bp insertion is made.

(B) The GCRE and URE are moved as a unit away from the TATA homology. The activity of the various constructions are shown relative to a construction containing only a GCRE (100% activity). Moving the two sites together had little effect on the URE-mediated repression.

Figure 3. DNAse I footprint of URF isolated from yeast

(-) is control without protein added in the reactions; (+) has 10 µl of protein factor in the reaction. Brackets indicate the protein binding sites. The end labeled fragments were treated to purine and pyrimidine-specific chemical cleavage (Maxam and Gilbert 1980) and electrophoresis along with the DNAse I reaction in lanes marked R and Y, respectively.

(A) The DNA fragments which contain a single SV40 72 repeat were γ-^{32}P 5'-end labeled at EcoRI end and then cut with BamHI (bottom strand). The protein URF used in this experiment was partially purified by the DNA affinity column chromatography (fraction 2) as described in *Materials and Methods*. The sequence protected by the URF is:

5'-GTGTGGAAAGTCCCCAGGCTCCCCA-3'.

(B) The same DNA fragment was labeled at BamHI end and then cut with EcoRI (top strand). The same protein fraction was used in the DNAse I reaction. The sequence protected by the URF is:

5'-GGGAGCCTGGGGACTTTCCACAC-3'

Figure 4. Gel-shift analysis of the URF binding activity

γ-^{32}P labelled URE oligonucleotide was used in the gel-shift analysis. The binding conditions were as described in *Materials and Methods*. The arrows indicate the specific protein-DNA complex.

1 µl of affinity-purified URF was added to a standard binding reaction which contains either no competitor or a 100-fold molar excess of unlabeled competitor. The competitors used are the oligonucleotides URE, GCRE, ARE, and HSE as indicated above each of the lanes.

Figure 5. Southwestern analysis of URF

The filter shown was probed with ^{32}P labelled URE oligonucleotide. 50 µl of URF was applied to a 7% SDS polyacrylamide gel, and transferred to nitrocellulose and processed for Southwestern analysis as described in *Materials and Methods.*

Figure 6. DNAse I footprint of URE site 2 on the *SSA1* promoter

(0) is control without protein added in the reactions; (A) has 10 µl of the 1.0 M KCl step from heparin-Sepharose in the reaction; (B) has 10 µl of the 0.32 M KCl step from heparin-Sepharose in the reaction. Boxes indicate the protein binding sites. The end labeled fragments were treated to purine and pyrimidine-specific chemical cleavage (Maxam and Gilbert 1980) and electrophoresis along with the DNAse I reaction in lanes marked R and Y, respectively.

The sequence protected by the URF is on the *SSA1* URE site 2 is:

5'-GTGGATTGTTGT-3'.

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Chapter 3

The *Saccharomyces* and *Drosophila* Heat Shock Transcription Factors are Identical in Size and DNA Binding Properties

G. Wiederrecht, D.J. Shuey, W. A. Kibbe, and C. S. Parker

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The *Saccharomyces* and *Drosophila* Heat Shock Transcription Factors are Identical in Size and DNA Binding Properties

Abstract

The heat-shock transcription factor (HSTF) has been purified to apparent homogeneity from *Saccharomyces cerevisiae* and *Drosophila melanogaster* by sequence-specific DNA-affinity chromatography. A synthetic oligonucleotide containing an *hsp83*-like heat-shock element (HSE) was prepared and ligated into concatamers and covalently linked to Sepharose. This DNA-affinity resin allowed the rapid isolation of a yeast and *Drosophila* protein with the same apparent molecular weight (70 kD). The yeast HSTF will bind both to its own and the *Drosophila* HSEs. Similarly, the *Drosophila* HSTF will bind both to its own and the yeast HSEs. The yeast and *Drosophila* HSTFs were subjected to preparative SDS gel electrophoresis, and the 70 kD polypeptides were eluted, renatured, and observed to generate the identical footprint pattern as the native HSTFs. Affinity-purified *Drosophila* HSTF was further shown to stimulate specific HSE-dependent transcription from a *Drosophila* hsp70 gene *in vitro*.

Keywords:

HSTF, HSE, heat-shock, heat-shock transcription factor, DNA-affinity chromatography.

The Saccharomyces and Drosophila Heat Shock Transcription Factors Are Identical in Size and DNA Binding Properties

Greg Wiederrecht, David J. Shuey, Warren A. Kibbe, and Carl S. Parker
Division of Chemistry
California Institute of Technology
Pasadena, California 91125

Summary

The heat shock transcription factor (HSTF) has been purified to apparent homogeneity from S. cerevisiae and D. melanogaster by sequence-specific DNA-affinity chromatography. A synthetic oligonucleotide containing an *hsp83*-like heat shock element (HSE) was prepared and ligated into concatamers and covalently coupled to Sepharose. This DNA-affinity resin allowed the rapid isolation of a yeast and a Drosophila protein with the same apparent molecular weight (70 kd). The yeast HSTF will bind to both its own and the Drosophila HSEs. Similarly, the Drosophila HSTF will bind to both its own and the yeast HSEs. The yeast and Drosophila HSTFs were subjected to preparative SDS gel electrophoresis, and the 70 kd polypeptides were eluted, renatured, and observed to generate the identical footprint pattern as the native HSTFs. Affinity-purified Drosophila HSTF was further shown to stimulate specific HSE-dependent transcription from a Drosophila *hsp70* gene in vitro.

Introduction

Transcription of eukaryotic protein-coding genes requires the presence of one or more distinct promoter elements. RNA polymerase II promoters often have a TATA homology segment located 25–30 bp upstream of the transcription start point (Goldberg, 1979). A transcription factor has been identified from both Drosophila and man that specifically binds to these sequences (Parker and Topol, 1984a; Sawadogo and Roeder, 1985). Other identified promoter-proximal elements include the CCAAT sequence and the GC motif (GGGCGG), for which *trans*-acting factors have been identified that specifically bind to each (Jones et al., 1985; Dynan and Tjian, 1984). These sequences are usually located at various positions upstream of the TATA homology or an AT-rich element. Elements of this kind are found associated with a large number of promoters and may represent general promoter elements.

In addition, a few gene-specific transcription factors have been identified by in vitro studies. These include the heat shock transcription factor (HSTF; Parker and Topol, 1984b) and the adenovirus major late gene transcription factor (USF, Sawadogo and Roeder, 1985; and MLTF, Carthew et al., 1985). The HSTF recognizes a specific element, the heat shock element (HSE, C __ __ GAA __ __ TTC __ __ G) in the promoters of a family of heat shock protein genes, whereas the USF or MLTF recognizes a specific sequence found on a few apparently unrelated genes. In vivo studies have identified what seem to be other activators and their target sequences. These include the *GCN4* and *GAL4* gene products in yeast whose target elements are the TGACT motif and UAS_G, respectively (Guarente, 1984; Bram and Kornberg, 1985; Giniger et al., 1985; Keegan et al., 1986; Hope and Struhl, 1986). Additionally, in mammalian cells the glucocorticoid and metal response elements (GRE and MRE, respectively) may be gene-specific targets for possible transcriptional activators (Miesfeld et al., 1986; Richards et al., 1984).

The heat shock response is a particularly useful system for studying the coordinate activation of a specific set of genes. All of the identified major heat shock genes of Drosophila contain multiple HSEs at various locations on their promoters (Pelham, 1985; Parker and Topol, unpublished observations). A comparison of heat shock gene promoters in species ranging from yeast to man also reveals the presence of very similar HSEs in analogous positions. This remarkable similarity of eukaryotic HSTF binding sites, as well as the highly conserved group of proteins induced, is probably a reflection of the importance of the response to the organism.

The HSTF was originally discovered in Drosophila (Parker and Topol, 1984b), but has subsequently been identified in yeast (this paper) and in HeLa cells (D. Shuey, G. Wiederrecht, and C. Parker, unpublished data). An activity similar to the HSTF has also been observed by in vivo chromatin studies (Wu, 1984). The Drosophila HSTF binds cooperatively to the two contiguous HSEs (sites 1 and 2) nearest the TATA homology of a Drosophila *hsp70* gene (Topol et al., 1985). Both contiguous elements were shown to be required for *hsp70* transcriptional activation in vivo (Dudler and Travers, 1985) and in vitro (Topol et al., 1985). Detailed analysis of the HSTF interactions with sites 1 and 2 revealed that a potential conformational change occurs upon cooperative HSTF binding to these sites (Shuey and Parker, 1986a). Further studies also revealed that the HSTF induces DNA bending upon binding to the *hsp70* promoter (Shuey and Parker, 1986b). These conformational changes may be an important component of a molecular switch that turns on the hsp genes.

All of the previous studies have been performed with partially purified Drosophila HSTF. These studies were valuable for demonstrating sequence-specific DNA binding and in vitro transcriptional activation. To understand the precise molecular mechanisms involved in heat shock gene activation and transcription, it is essential that the HSTF be purified to homogeneity. To accomplish this we have utilized the sequence-specific DNA binding properties of the HSTF and performed affinity chromatography using synthetic oligonucleotides of that sequence. We were able to rapidly and efficiently purify a protein from S. cerevisiae and D. melanogaster that bound specifically and tightly to the affinity resin. Interestingly, the Drosophila and yeast proteins are identical in size (70 kd) and possess indistinguishable footprint boundaries. Furthermore, the affinity-purified Drosophila HSTF was shown to acti-

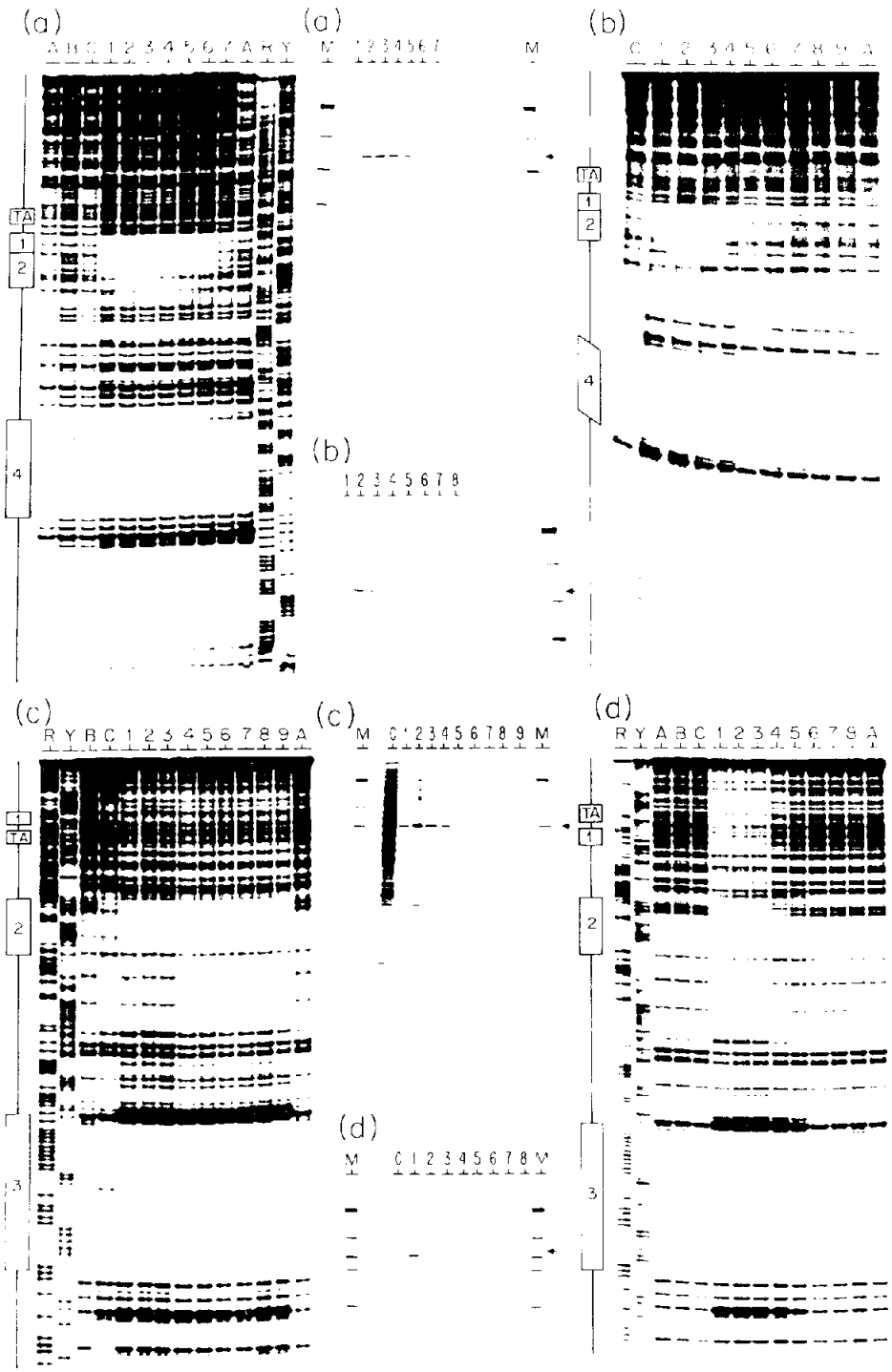

Figure 1. Chromatography of HSTF from Drosophila and Saccharomyces on the Sequence-Specific Oligonucleotide Column

Partially purified Drosophila and Saccharomyces HSTF were cycled twice on the DNA affinity column. Each panel shows the footprint analysis of the fractions eluting from the column and the accompanying protein gel. Lane A: minus protein; lane B: flow-through; lane C: 0.35 M KCl step (0.375

Table 1. Purification Table of the HSTF

Fraction	Volume (ml)	Protein Concentration (mg/ml)	Total Protein (mg)	HSTF Binding Units[a]	Specific Activity U/μg	Yield	Overall Purification[b]
Drosophila HSTF Purification							
Nuclear Extract	35	17	595	ND[c]	ND[c]	ND[c]	–
Phosphocellulose	35	14	490	3,500	0.007	–	–
Heparin Agarose	20	1	20	20,000	1	100%	30
Oligonucleotide	2.5	800 ng/ml	2.0 μg	16,550	8,225	83%	250,000
Saccharomyces HSTF Purification							
Nuclear Extract	90	20	1,800	ND[c]	ND[c]	ND[c]	–
Heparin Agarose	50	3.6	180	29,000	.105	–	–
Affigel Blue	214	0.2	42.8	81,000	1.89	100%	42
Oligonucleotide	50	300 ng/ml	15 μg	58,000	3,866	72%	86,000

[a] HSTF Binding Unit = 1 fmole binding site protected.
[b] Taking the yield into account.
[c] Not determined, footprint analysis in nuclear extracts was not quantitative.

vate *hsp70* transcription in vitro in an HSE-dependent fashion. The striking similarity in molecular weight and DNA binding properties of the HSTF from both sources is very intriguing, yet consistent with the known highly conserved nature of the heat shock response.

Results

HSTF Isolation and Purification by Sequence-Specific DNA Affinity Chromatography

The Drosophila HSTF activity was monitored by DNAase I footprinting on a cloned Drosophila *hsp70* promoter fragment modified to contain binding sites 1, 2, and 4 (Topol et al., 1985). Binding site 4, located between nucleotides −228 and −255, has the highest affinity for the HSTF, thus in footprinting assays this site is occupied first (at lower HSTF concentrations) followed sequentially by sites 1 and 2. A heat-shocked nuclear extract prepared from Drosophila K_c cells (similar results have been obtained for Schneider line 2 cells) was applied to a phosphocellulose (P11) column equilibrated with the standard chromatography buffer (HGE) with 0.1 M KCl. The HSTF flows through the P11 column under these conditions and was directly applied to a heparin-Sepharose column. The column was washed with HGE and 0.1 M KCl followed by step elution of the HSTF with HGE and 0.4 M KCl. The eluted HSTF was dialyzed to 0.1 M KCl in HGE and subjected to sequence-specific DNA-affinity chromatography as described below. At this point in the purification the HSTF had been purified 30-fold (see Table 1 for details).

The activity assay used to follow the elution of the yeast HSTF was also DNAase I footprinting. The promoter used was derived from a yeast *hsp70* gene (YG100; Ingolia et al., 1982) and contains three separate HSEs. The location of these binding sites is shown in Figure 1. Site 1, which has the poorest match to the consensus sequence, also possesses the lowest affinity for the HSTF. (This site is compressed at the top of the DNAase I ladder and is difficult to visualize.) Sites 2 and 3 have approximately equal affinities for the HSTF as expected because both have a good match to the consensus sequence. A yeast extract (prepared as described in Experimental Procedures) was applied to a heparin-Sepharose column equilibrated with 0.1 M KCl in HGE. The column was washed successively with 0.1 M KCl and 0.32 M KCl; the HSTF was eluted with 0.8 M KCl. The HSTF was dialyzed to 0.1 M KCl and applied to an Affigel Blue column. After washing the column with 0.05 and 0.5 M KCl, the yeast HSTF was eluted with 1.5 M KCl. Table 1 shows that the yeast factor was estimated to be purified 42-fold after chromatography on these two resins.

A DNA affinity column was constructed by ligating synthetic oligonucleotides containing the heat shock consensus element. The HSE sequence chosen was a modified form of the *hsp83* element (see Experimental Procedures for details). The *hsp83* HSE contains three overlapping heat shock consensus sequences within a 35 bp region. The HSTF will bind to these elements in a highly cooperative way, resulting in a very stable protein–DNA complex (D. Shuey and C. Parker, unpublished

M step when yeast HSTF was on column); numbered lanes: 1.5 M KCl step fractions; lanes R and Y: chemical cleavage reactions of purines and pyrimidines, respectively. Numbered boxes show locations of HSTF binding sites and arrows show the location of the 70 kd protein. (a) Drosophila HSTF cycled once on the column. Footprint reactions (volume, 25 μl) contained 20 μl of the indicated column fraction and 4.8 ng of the *hsp70* fragment designated d21[1] described in Experimental Procedures. Masses of protein gel markers are: 165 kd, 155 kd, 90 kd, 60 kd, and 40 kd.
(b) Drosophila HSTF cycled twice on the column. Footprint reactions and protein gel markers are as in (a).
(c) Saccharomyces HSTF cycled once on the column. Footprint reactions (volume, 25 μl) contained 20 μl of the indicated column fraction and 1 ng of the pYHS-1 HindIII–EcoRI fragment labeled at the HindIII site. Protein gel markers are as in (a) except that a 66 kd marker has been added.
(d) Saccharomyces HSTF cycled twice on the column. Footprint reactions and protein gel markers are as in (c).

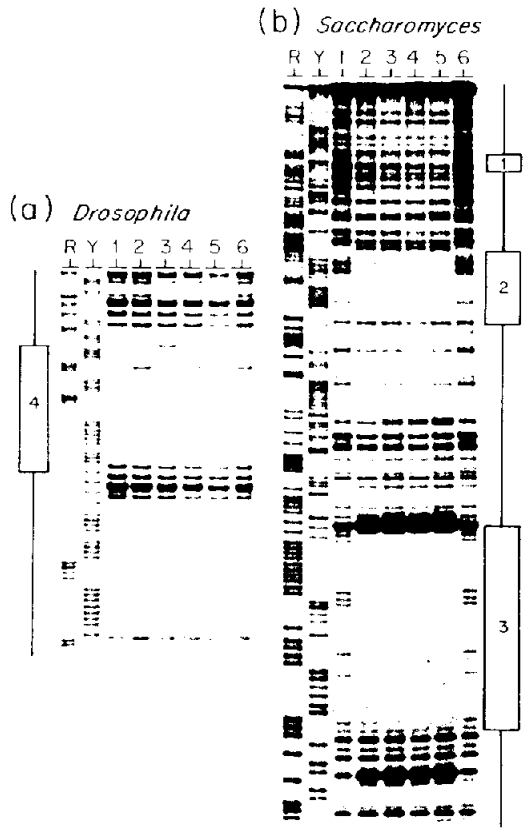

Figure 2. Renaturation of HSTF Binding Activity

(a) Footprint of renatured Drosophila HSTF. Footprint reactions (volume, 50 μl) contained 17 ng of HSTF and 4.8 ng of the d21+ fragment labeled at the EcoRI site. Lane 1: native Drosophila HSTF cycled twice on the DNA affinity column; lanes 2, 4, and 6: no protein; lane 3: renatured 25 kd polypeptide; lane 5: renatured 70 kd polypeptide.

(b) Footprint of renatured Saccharomyces HSTF. Footprint reactions (volume, 25 μl) contained 1 ng of the pYHS HindIII–EcoRI fragment labeled at the HindIII site. Lanes 1 and 6: no protein; lanes 2–5: 8, 16, 32, and 64 ng of renatured Saccharomyces 70 kd HSTF, respectively. Numbered boxes show locations of binding sites.

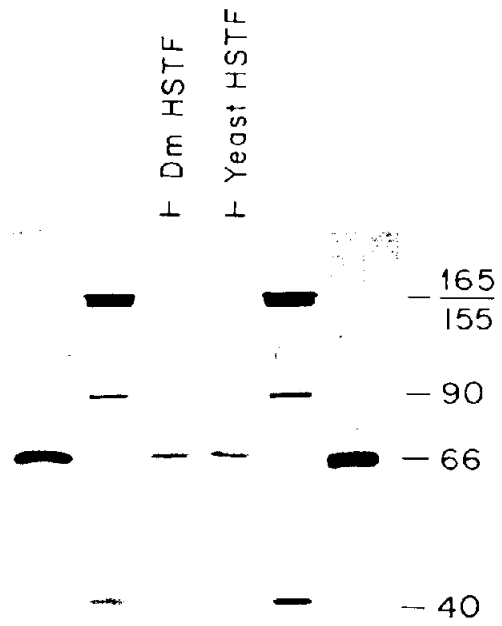

Figure 3. Comparison of Purified Drosophila and Saccharomyces HSTF

Thirty nanograms of affinity-purified HSTF from both yeast and Drosophila was subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel.

observations). It was determined experimentally that the unligated synthetic oligonucleotide served as a poor HSTF binding site. Ligating this oligonucleotide into concatamers, however, allowed high affinity binding by the HSTF. Thus we coupled ligated concatamers of synthetic *hsp83*-like HSEs to Sepharose following the procedure of Arndt-Jovin et al. (1975).

Drosophila or yeast HSTF, partially purified as described above, was applied to the affinity column and washed extensively with HGE containing 0.1 M KCl. This was followed by a second extensive wash with 0.35 M KCl for the Drosophila HSTF or 0.375 M KCl for the yeast HSTF. Both HSTFs were eluted from the column with 1.5 M KCl. A portion of each fraction was assayed by DNAase I footprinting and the protein composition was determined by SDS-polyacrylamide gel electrophoresis. The results of these analyses are shown in Figure 1a for the Drosophila HSTF and in Figure 1c for the yeast HSTF. Binding activity to both *hsp70* promoters co-chromatographs with an abundant 70 kd protein from both organisms (indicated by the arrows in Figure 1). A minor 25 kd protein is observed on both protein gels, as well as some minor higher molecular weight polypeptides. The purification resulting from chromatography on the affinity column is remarkable; the Drosophila HSTF is purified 8300-fold and the Saccharomyces HSTF is purified 2000-fold in a single chromatographic step. The overall purification of the Drosophila HSTF is 250,000-fold and 86,000-fold for the yeast HSTF (Table 1).

To remove minor impurities, the final step in the purification was to rechromatograph the pooled active fractions on the affinity resin. Footprinting assays and SDS protein gel analysis of the second chromatographic cycle are shown in Figure 1b for Drosophila and in Figure 1d for yeast. In the case of Drosophila a major polypeptide of 70 kd and a minor 25 kd polypeptide (barely visible in the experiment shown) are the only proteins observed. Greater than 90% of the protein in the yeast HSTF preparation is present in the 70 kd polypeptide. The yeast HSTF still contains two very minor contaminants of 130 kd and 100 kd and in a few preparations the 25 kd protein is also observed. (Estimates of HSTF mass after affinity chromatography were made by comparison to known amounts of standard proteins on silver-stained gels.)

SDS Gel Electrophoresis and Renaturation of the HSTF

To rigorously demonstrate that the identified 70 kd proteins were responsible for the specific DNA binding activity observed, we further purified the Drosophila and yeast HSTFs by preparative SDS-polyacrylamide gel electrophoresis. The proteins were visualized by KCl staining. The only two bands observed in the Drosophila preparations were 70 kd and 25 kd. Each of these proteins was excised from the gel and electroeluted. Only the 70 kd protein was clearly identifiable in the yeast HSTF preparations, and it was also cut from the gel and electroeluted. To renature these proteins we followed, fundamentally, the procedure of Hager and Burgess (1980) as completely described in Experimental Procedures. Briefly, we fully denatured the eluted proteins in 6 M guanidine hydrochloride and renatured them by slow dialysis to remove the denaturant.

The ability of each of the renatured proteins to bind with sequence-specificity was assessed by DNAase I footprinting. Figure 2b shows a titration of the renatured yeast 70 kd protein on the yeast *hsp70* promoter. The pattern of protection and dimensions of the footprints observed are indistinguishable from those of native HSTF (compare Figure 2b with Figure 1c or 1d). Similarly, the renatured Drosophila 70 kd protein also generated a footprint on site 4 of its own promoter identical to that of native HSTF (Figure 2a, compare lanes 1 and 5). (Note that sites 1 and 2 are not shown in Figure 2a, because the concentration of renatured Drosophila HSTF was insufficient to protect these sites.) Interestingly, the 25 kd Drosophila protein yielded a weak footprint on site 4 (Figure 2a, lane 3). This suggests that it contains the same DNA binding domain as the 70 kd protein and that is derived by proteolysis of the 70 kd HSTF.

A direct comparison of the sizes of affinity purified Drosophila HSTF and yeast HSTF is shown in Figure 3. Here we observe two apparently homogeneous proteins of identical molecular weights. (In these preparations no contaminating proteins were observed.)

Cross Species Binding of the Drosophila and Yeast HSTFs

When the Drosophila *hsp70* gene is transfected into mouse cells, monkey cells, or sea urchin embryos, it retains transcriptional heat-inducibility (for a review see Pelham, 1985). This implies that components of the transcriptional apparatus, such as the HSTF, are conserved between species. This idea is supported by our observation that the HSTFs from two widely divergent species have nearly identical mass. Further evidence that the two 70 kd HSTFs are homologous comes from a comparison of "cross-species" footprints with "same-species" footprints. Figure 4 reveals that the DNAase I footprints formed by binding of affinity-purified yeast and Drosophila HSTF to the yeast *hsp70* promoter have identical boundaries (compare lanes 2 and 3 to lanes 8 and 9). On site 3, the footprints from both HSTFs extend over a 30 bp region between nucleotides −278 and −309. On site 2, the footprints cover 27 bp between nucleotides −183 and

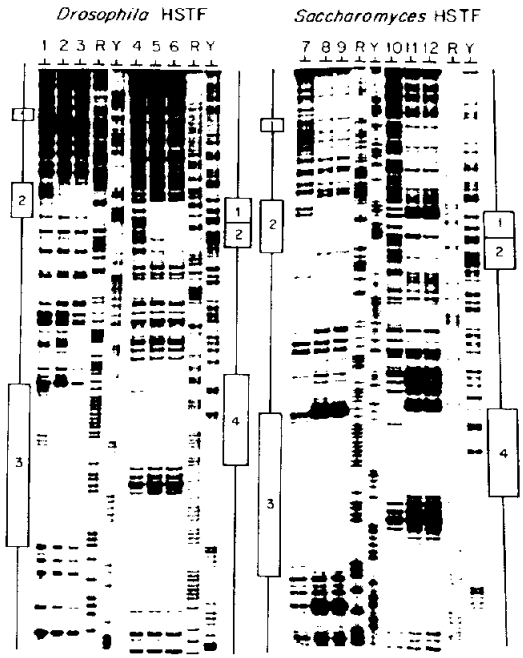

Figure 4. Cross-Species Footprints of Drosophila and Saccharomyces HSTFs

Composition of footprint reactions has been described in the legends to Figures 1 and 2. Lanes 1–3 and lanes 7–9 show footprints on the Saccharomyces *hsp70* promoter and lanes 4–6 and lanes 10–12 on the Drosophila *hsp70* promoter. Lanes 1, 4, 7, and 10: no protein; lanes 2 and 5: 15 ng Drosophila HSTF; lanes 3 and 6: 30 ng Drosophila HSTF; lanes 8 and 11: 16 ng Saccharomyces HSTF; lanes 9 and 12: 32 ng Saccharomyces HSTF; lanes R and Y: chemical cleavage reactions of purines and pyrimidines, respectively. Numbered boxes show locations of binding sites.

−211. Similarly, the footprints formed by binding of affinity-purified yeast and Drosophila HSTF to the Drosophila *hsp70* promoter also have identical boundaries (compare lanes 5 and 6 with lanes 11 and 12). On site 4, 26 bp between nucleotides −228 and −255 are protected from DNAase I cleavage by both HSTFs. On sites 1 and 2, a 54 bp region between nucleotides −37 and −92 is protected. To summarize, the lengths of the footprints vary slightly in a manner dependent upon their location on the promoter. However, at any one binding site, both the yeast and Drosophila HSTFs always generate footprints with identical boundaries.

The only apparent difference between the footprint patterns is that the yeast HSTF induces a higher degree of DNAase hypersensitivity at the footprint boundaries than does the Drosophila HSTF. For example, the hypersensitivity induced by binding of the yeast HSTF to site 4 of the Drosophila DNA fragment extends 15 bases 3′ from the border of the footprint. Another difference is that binding of the yeast HSTF to the contiguous sites 1 and 2 on the Drosophila *hsp70* promoter leaves two bases accessible to DNAase cleavage that are not accessible when Dro-

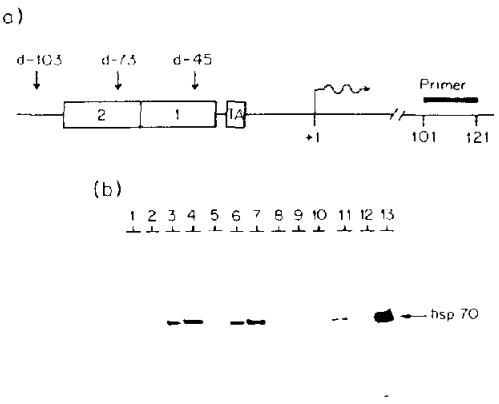

Figure 5. Reconstituted Transcription of the Drosophila *hsp70* Gene with Highly Purified Drosophila HSTF

Transcription reactions (volume, 50 μl) contained 45 μg protein from the heparin-Sepharose flow-through, 30 μg protein from the phosphocellulose 0.8 KCl step, and 250 ng circular template containing the *hsp70* gene, 56H8, deleted to varying degrees at the 5′ end. The deleted templates have been previously described (Topol et al., 1985).
(a) The deletion endpoints, locations of binding sites 1 and 2, the TATA box, the transcription startpoint and the location of the primer.
(b) Lanes 1–7: transcription from the d-103 template; lanes 8–11: transcription from the d-73 template; lane 12: transcription from the d-45 template; lane 13: in vivo *hsp70* RNA. Transcription reactions were reconstituted with HSTF cycled once (lanes 1–4, 8, 9) or twice (lanes 5–7, 10–12) over the sequence-specific DNA column. Increasing amounts of HSTF were added to the transcription reactions as follows. Lane 1: no HSTF; lanes 2 and 5: 1.6 ng HSTF; lanes 3, 6, 8, and 10: 8 ng HSTF; lanes 4, 7, 9, 11, and 12: 16 ng HSTF. Transcription was measured by primer extension assay.

sophila HSTF binds (Figure 4, compare lanes 5 and 6 to lanes 11 and 12). It is not clear what significance should be assigned to the minor differences in the footprints. What is clear, however, is that based upon three criteria, mass equivalence, conserved DNA recognition sequences, and identity of footprint boundaries, the HSTF is a highly conserved transcriptional regulatory protein.

HSTF-Dependent Transcription of *hsp70*

In addition to showing that affinity-purified Drosophila HSTF binds to the correct sequences on the *hsp70* promoter, it was important to determine if affinity-purified HSTF could also stimulate specific transcription of the *hsp70* gene *in vitro*. Most of the previous HSTF preparations used for binding and in vitro transcription studies were purified less than 1% when compared to the purity of HSTF used in this study. Therefore, it is conceivable that the binding activity and the transcriptional activity assigned to the HSTF in previous preparations were, in fact, the activities of two different proteins. In the present study we used our most highly purified Drosophila HSTF to reconstitute transcription in vitro in an HSTF-dependent Drosophila extract. A 0.8 M KCl step from the initial P11 column and the heparin-Sepharose flow-through contain all factors required, except the HSTF, for transcription of the Drosophila *hsp70* gene. Figure 5 shows that when low levels of Drosophila HSTF are added back to these complementing fractions, there is only a low basal level of *hsp70* RNA transcribed from a template containing binding sites 1 and 2. (Transcription was monitored using circular deletion templates as indicated and primer-extension assays; see Experimental Procedures for complete details.) As increasing amounts of HSTF are added back, there is a 25- to 50-fold stimulation of *hsp70* transcription. As a control, templates lacking binding site 2 and lacking both HSTF binding sites were tested. It is known from previous in vivo (Dudler and Travers, 1984) and in vitro (Topol et al., 1985) analyses that both binding sites must be present for maximal *hsp70* transcription. Figure 5 shows that the same amount of pure HSTF which maximally stimulates transcription from a template containing both binding sites does not reconstitute transcription on templates lacking one or both binding sites (compare lanes 3, 4, 6, and 7 with lanes 8–12).

These results provide compelling evidence that DNA binding and transcriptional stimulation are performed by the same protein. We were not able to test the transcriptional activity of the yeast HSTF because a yeast in vitro RNA polymerase II transcription system does not currently exist. However, preliminary results suggest that the yeast HSTF will not reconstitute the Drosophila HSTF-dependent transcription of *hsp70*. Because the yeast HSTF binds to the HSEs on the Drosophila *hsp70* promoter (this paper), it suggests that binding alone is not sufficient to activate transcription.

Discussion

Sequence-specific DNA-affinity chromatography has been successfully used by Rosenfeld and Kelly (1986) to purify nuclear factor 1 from HeLa cells. In their procedure, multiple copies of the DNA binding site were cloned into a plasmid that was then coupled to cellulose. In the procedure described here, concatamers of a synthetic oligonucleotide containing overlapping HSEs were coupled to Sepharose in order to purify the HSTF 250,000-fold from Drosophila and 86,000-fold from yeast. We have successfully applied this technology to the purification of other sequence-specific DNA binding proteins including an enhancer binding protein (K. Harshman, S. Sogo, and C. S. Parker, unpublished observations). A similar procedure has been developed by Kadonaga and Tjian (1986; also Briggs et al., 1986) to purify the Sp1 transcription factor 30,000-fold. It is probable that DNA-affinity chromatography will aid in the purification of many different sequence-specific DNA binding proteins in the future.

The remarkable similarity in size between the Drosophila and yeast HSTF is suggestive of a great deal of evolutionary conservation. Two caveats to this conclusion, however, must be considered. It is possible that the 70 kd species are actually derived from a larger protein of unknown molecular weight. Although we have made use of strong protease inhibitors and shortened the time of purification to just 48 hr, we cannot rule out the possibility of

limited proteolysis generating identically sized proteins in both Drosophila and yeast. The other issue concerns the yeast HSTF, which has not been formally shown to be a transcription factor. The data presented in this paper show that the yeast protein is identical in size and DNA binding properties to the Drosophila factor. Until it can be rigorously shown in vivo or in vitro that the yeast protein is the HSTF by activity criteria we cannot make any definitive claims at this time. Because the apparent molecular weights of the Drosophila and yeast HSTFs are 70 kd we tested the possibility that they are related to *hsp70* or one of its cognates. Using monoclonal antibodies directed against *hsp70* and its cognates (a gift from Dr. Susan Lindquist), we determined that there was no cross-reactivity with the HSTF.

We have shown that both the Drosophila and yeast HSTFs can be further purified through SDS protein gels, renatured, and observed to footprint identically compared to the native proteins. This method, originally developed by Hager and Burgess (1980), should also be generally applicable to many DNA binding proteins. Indeed, we have also applied this technique to an enhancer binding protein and unambiguously identified the protein responsible for the observed activity (K. Harshman, unpublished data). Should a preparation always contain multiple species, this technique may be very useful to determine which of the proteins can bind with sequence-specificity.

The affinity-purified Drosophila HSTF stimulates specific transcription from an *hsp70* template in an HSE-dependent fashion. This data suggests that a native full-length HSTF has been isolated. One concern with this conclusion is that the number of active templates in the reconstitution reaction is less than 10%. Hence it is formally possible that a minor component in the preparation is actually responsible for the transcriptional stimulation observed. For this to be true, however, the minor species would have to possess a significantly higher binding constant than the 70 kd protein. This is because the 70 kd protein is present in at least a 10-fold excess over the putative minor species (based on silver staining) and the 70 kd protein is present in sufficient quantities to saturate all of the templates in the transcription reaction.

One of the goals of our studies on the heat shock response has been to clone the gene for the HSTF. Cloning and characterizing the HSTF gene will help to confirm the native molecular weight of the factor. We recently were successful in obtaining an N-terminal sequence of a proteolytic fragment of the yeast HSTF. With this sequence we have generated several sets of oligonucleotides which will allow us to clone the gene from a yeast λgt10 library (G. Wiederrecht and C. S. Parker, unpublished observations). Additionally, monoclonal antibodies that we have raised against the HSTF will be useful for studying the pre- and post-heat shock cellular and chromosomal locations of the factor. Clearly, having the HSTF gene and the antibodies will allow a large number of different and interesting experiments to be performed, leading to a more complete understanding of the molecular mechanism of heat shock gene activation.

Experimental Procedures

Yeast Strain, Growth of Yeast, and Processing

The protease-deficient Saccharomyces cerevisiae strain EJ926

$$(MAT\ \frac{a}{a}\ \frac{trp1}{+}\ \frac{\ldots}{his1}\ \frac{+}{prc1-126}\ \frac{pep4-3}{pep4-3}\ \frac{prb1-1122}{prb1-1122}\ \frac{can1}{can1})$$

obtained from E. Jones (Carnegie-Mellon) was the strain used for the isolation of the HSTF. The yeast were grown in a 350 liter fermenter at 30°C (heat-shocked) in medium containing Bacto-yeast nitrogen base supplemented with 2% dextrose, 50 μg/ml histidine, and 50 μg/ml tryptophan. When the culture reached an $O.D._{600}$ of 5.0, they were harvested with a Sharples continuous flow centrifuge. The yeast paste was resuspended in a buffer solution containing 1 M sorbitol, 50 mM Tris (pH 7.8), 10 mM $MgCl_2$, and 30 mM DTT. This buffer solution minus the DTT is designated Y buffer. The suspension ratio was 1 kg yeast/liter buffer. The suspension was quick-frozen as small droplets in liquid nitrogen and stored at −80°C.

Nuclear Extract Preparation

Typically, the amount of starting material for a yeast extract was 1.1 kg of the frozen suspension described above. The frozen material was thawed and then subjected to centrifugation (2200 × g; 5 min) in order to pellet the yeast. The pellet was resuspended in three volumes of Y buffer plus 3 mM DTT. Zymolyase 100 T (Miles) was added (100 mg/kg yeast) to the suspension and was then incubated for 1 hr at 37°C. After the zymolyase treatment, PMSF (Sigma) was added to a concentration of 1 mM. The zymolyase-treated cells were subjected to centrifugation (2200 × g; 5 min) and the pellet was resuspended in Y buffer plus 1 mM PMSF. This material was subjected to centrifugation (2200 × g; 6 min). The soft pellet was resuspended in A buffer (10 mM Hepes [pH 7.6], 15 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA) plus 1 mM PMSF and then placed in an ice bath for 20 min. The suspension was homogenized (4–5 strokes) in a large Dounce homogenizer. The homogenate was subjected to centrifugation (18,000 × g; 10 min) and the pellet was resuspended in A buffer plus PMSF such that the final volume was about 1200 ml. The solution was divided equally among eighteen 45 Ti polycarbonate tubes. Ammonium sulfate was added to each tube to a final concentration of 0.25 M and diisopropyl-fluorophosphate (DIFP; Sigma) was added to a final concentration of 0.4 mM. The tubes were rotated on a rotation apparatus for 15 min and then subjected to centrifugation in a 45 Ti rotor (35,000 rpm, 2 hr). Ammonium sulfate was added to the supernatant (0.2 g $[NH_4]_2SO_4$ added per ml supernatant) and the precipitated protein was pelleted by centrifugation (18,000 × g; 45 min). The protein pellet was resuspended in 90 ml of a buffer solution containing 25 mM Hepes (pH 7.6), 10% (v/v) glycerol, 50 mM KCl, 0.1 mM EDTA, and 0.2% Triton X-100 (abbreviated as 0.05 HGKET where 0.05 is the molarity of KCl). The solution was dialyzed overnight against 4 liters of the same buffer plus 1 mM PMSF. Insoluble material was removed by centrifugation (10,000 × g, 10 min) and the supernatant was subjected to the chromatographic techniques described below.

Nuclear extracts derived from K_c cells were prepared essentially as described previously (Parker and Topol, 1984a) with the addition of protease inhibitors as described above.

Chromatographic Procedures

The yeast nuclear extract (total volume, 90 ml) was applied to a column (volume, 100 ml) of heparin-Sepharose equilibrated with 0.05 HGKE. The column was washed with three column volumes of 0.05 HGKE, five column volumes of 0.32 HGKE, and then three column volumes of 0.8 HGKE. Individual fractions (volume, 15 ml) were assayed for HSTF binding activity and the active fractions from the 0.8 step were dialyzed against 0.1 HGKET. Following dialysis, insoluble material was pelleted by centrifugation (12,000 × g; 10 min) and the supernatant was applied to a column (volume, 20 ml) of Affigel Blue at a flow rate of 20 ml/hr. The column was washed with 10 column volumes of 0.05 HGKE, 10 column volumes of 0.5 HGKE, and 20 column volumes of 1.5 HGKET. The fractions from the 1.5 HGKET step were combined, dialyzed against 0.1 HGKET, and then applied to the sequence-specific oligodeoxyribonucleotide column as described below.

The dialyzed K_c nuclear extract (total volume, 35 ml) was applied to a column (volume, 50 ml) of phosphocellulose (P11; Whatman) equilibrated in 0.1 HGKET, and then washed with the same buffer. Protein-containing fractions in the flow-through were combined and applied to a column (volume, 10 ml) of heparin-Sepharose equilibrated with 0.1 HGKET. The column was washed with three column volumes of 0.1 HGKET and HSTF was then step-eluted with three column volumes of 0.4 HGKET. The step-eluted material was dialyzed against 0.1 HGKET, subjected to centrifugation (12,000 × g) to remove insoluble material, and the supernatant applied to the sequence-specific DNA column as described below.

The procedures for chromatography of the partially purified yeast and Drosophila HSTF preparations on the sequence-specific DNA column were nearly identical. The partially purified preparations were loaded onto a 1.1 ml column (constructed as described below) at a flow rate of 15 column volumes per hr. The column was washed with 10 volumes of 0.1 HGKET, 100 volumes of 0.35 (0.375 for yeast HSTF) HGKET, and the HSTF was step-eluted with 10 column volumes of 1.5 HGKET. The HSTF was dialyzed overnight against 0.05 HGKET and loaded onto a 0.2 ml sequence-specific DNA column. Chromatography was performed as described for the larger column.

SDS Gel Electrophoresis and Renaturation

HSTF-containing fractions from the sequence-specific DNA column were combined and the protein concentrated by the addition of 3 volumes acetone. The mixture was frozen in a dry ice–ethanol bath and the protein pelleted by centrifugation (12,000 × g; 30 min) at 0°C. The supernatant was discarded and the protein pellet was vacuum-dried. The pellet was resuspended in SDS protein gel loading buffer, heated at 70°C for 5 min, and applied to a 10% SDS polyacrylamide gel (Laemmli, 1970). Electrophoresis was performed at 3°C. The gel was soaked in 250 ml of 0.25 M KCl for 1–2 min. Staining for longer periods only raised the background. Gel slices containing potassium-SDS-protein precipitates were excised and placed into small dialysis bags containing 600 μl of SDS gel running buffer. Protein was electro-eluted for 3 hr at 250 V by placing the dialysis bags on a horizontal gel box filled with SDS gel running buffer. The dialysis bags containing the HSTF were dialyzed for 3 hr against 0.05 HGKET to remove glycine. The protein was precipitated by the addition of 3 volumes of acetone followed by freezing in a dry ice–acetone bath. The precipitate was pelleted by centrifugation (12,000 × g; 30 min). The pellet was washed with ice-cold 80% acetone to remove residual SDS. Typically, all of the HSTF from a yeast preparation was resuspended in 600 μl 0.1 HGKET containing 6 M guanidine hydrochloride. All of the HSTF from a K_c cell preparation was resuspended in 150 μl of the denaturing buffer. The guanidine hydrochloride was dialyzed out overnight against 0.1 HGKET. We estimate the yield of specific binding activity resulting from this procedure to be approximately 15%.

Preparation of the Sequence-Specific Oligodeoxyribonucleotide Column

Two complementary synthetic oligodeoxyribonucleotides modeled after the arrangement of overlapping HSEs in the Drosophila *hsp83* promoter were prepared at Caltech's Core Microchemical Facility. The sequence of strand 1 was:

5′-AGCTTCTAGAACGTTCTAGAAGCTTCGAGA-3′

The sequence of strand 2 was:

5′-AGCTTCTCGAAGCTTCTAGAACGTTCTAGA-3′

The strands were separately suspended in 7 M Urea, 1× TBE, and dyes and purified on a 20% polyacrylamide–7 M Urea gel. The DNA was visualized under shortwave UV light, over a fluorescent silica gel TLC plate. The band containing the DNA was excised from the gel, the gel slice was crushed, and the DNA was eluted into a 5 ml solution containing 0.5 M sodium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% (w/v) SDS for 6 hr at 37°C. This elution was repeated twice and the pooled eluate was concentrated to 1.5 ml by extraction with sec-butanol. The concentrated DNA was desalted on a Sephadex G-25 column. Equimolar amounts of DNA-containing fractions (determined by absorbance at 260 nm) were combined, frozen, and lyophilized. The dry pellet was resuspended in 0.5 ml of a solution containing 100 mM $NaHCO_3$ (pH 8) and 330 mM NaCl. This solution was heated to 90°C for 10 min, and allowed to cool slowly for several hours. The annealed strands (5 mg) were precipitated with 95% ethanol, washed with 70% ethanol, and resuspended in 0.5 ml of 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 1 mM ATP. The annealed DNA was phosphorylated with T4 polynucleotide kinase (100 units, BMB) at 37°C for 1 hr. Two subsequent additions of 1 mM ATP were made at 30 min intervals. Following this 2 hr incubation, a final 1 mM ATP addition was made and 20 μg of T4 DNA Ligase (gift from S. Scherer) was added. Ligation was performed at 14°C for 12 hr. The ligated DNA was covalently attached to Sepharose CL-4B as described by Arndt-Jovin et al. (1975). Activated Sepharose was neutralized with 0.1 M ethanolamine. We calculate that 250 μg of sequence-specific DNA was coupled per ml of resin.

Drosophila and Saccharomyces *hsp70* Promoter Constructions

Footprinting studies performed on the Drosophila *hsp70* promoter employed a fragment lacking HSE 3. The fragment, $d21^L$, contains an upstream BglI (EcoRI)–XhoI (HindIII) fragment extending from −358 to −184 on the *hsp70* gene, 56H8 (Artavanis-Tsakonas et al., 1979), ligated to a 5′ Bal31 deletion construct extending from −103 (HindIII) to a BglI site at −384.

The yeast *hsp70* promoter fragment used in the footprinting studies is derived from YG100 (Ingolia et al., 1982) and was a gift from E. Craig. A HincII–RsaI fragment derived from YG100 and containing HSEs 1, 2, and 3 was cloned into the polysite linker of pUC9. This clone was designated pYHS-1.

Preparation of Labeled Fragments and Footprint Reactions

The Drosophila $d21^L$ fragment was labeled on the coding strand with T4 polynucleotide kinase and γ-^{32}P-ATP near the BglI site (where an EcoRI linker was ligated and thus labeled by cutting with EcoRI) 358 bp upstream of the transcription startpoint. The pYHS-1 fragment was labeled on the coding strand by phosphorylation near the HincII site (at the HindIII site of the pUC9 polylinker) 337 bp upstream of the transcription startpoint. Footprinting reactions contained, in a total volume of 25 μl, 1–5 ng of labeled fragment, 50 μg/ml poly dA · dT (Pharmacia), 5 mM $MgCl_2$, and HSTF as indicated. Footprinting reactions were performed at 0°C and DNAase I (Cooper) was added to a final concentration of 50 μg/ml. After 30 sec of DNAase digestion, the reaction was terminated by the addition of 100 μl of transcription reaction-termination buffer which also contained 100 μg/ml proteinase K (EMS Labs) and 25 μg/ml calf thymus DNA (Sigma) and treated as previously described (Parker and Topol, 1984a).

In Vitro Transcription Assays

The composition of the transcription reactions has been described (Parker and Topol, 1984b). The plasmid templates used were 5′ Bal31 deletions of 56H8 and are designated d-103, d-73, and d-45. These templates have been previously described (Topol et al., 1985) and contain binding sites 1 and 2, site 1, and no HSTF binding sites, respectively. The transcription reactions were allowed to proceed for 45 min at 22°C, terminated with transcription reaction-termination buffer, extracted with phenol, and the nucleic acids precipitated with 90% ethanol, 0.1 M NaOAc. The nucleic acids were resuspended in 10 μl TE (10 mM Tris [pH 8], 1 mM EDTA) containing a 7.1 picomoles of 5′ end-labeled synthetic primer homologous to RNA sequences between 100 and 121 nucleotides downstream of the *hsp70* cap site. Two microliters of a buffer solution containing TE and 1.25 M KCl was added, the reaction mixture was heated to 65°C and allowed to anneal by slowly cooling to room temperature. Primer extension was performed by adding 25 μl of a buffer solution containing 20 mM Tris (pH 8.7), 10 mM $MgCl_2$, 5 mM DTT, 300 μM deoxyribonucleotides, 10 μg/ml actinomycin D, and 0.5–2 U AMV reverse transcriptase (Boehringer-Mannheim). The reaction was incubated at 37°C for 30 min. Nucleic acids were precipitated with 110 μl 90% ethanol, 0.1 M NaOAc, and analyzed on an 8% polyacrylamide–6 M Urea sequencing gel.

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We wish to thank Dr. Suzanna Horvath of the California Institute of Technology Microchemical Facility for her expert preparation of the oligonucleotides used in these experiments. We are also indebted to

Dr. Susan Lindquist for her gift of the anti-hsp 70 and hsp 70 cognate monoclonal antibodies and to Dr. Elizabeth Craig for the YG100 clone and unpublished sequence data. We thank Drs. Norman Davidson, Eric Davidson, Judy Campbell, Joan Kobori, and Susan Manly for their critical readings and Phoebe Ray for her expert preparation of the manuscript.

G. W. is a recipient of a Helen Hay Whitney postdoctoral fellowship. D. J. S. and W. A. K. are National Institutes of Health predoctoral trainees (GM 07616). This research was supported by a grant from the ACS (NP-0482) and NIH (GM 29430) to C. S. P. C. S. P. is a Rita Allen Foundation Scholar.

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Chapter 3 Appendix

Summary

The optimization of two different yeast extracts, a yeast spheroplast-derived nuclear extract and a whole-cell extract, was carried out in order to efficiently purify the HSTF. The chromatographic behavior of HSTF has been determined on a variety of columns and the results of these experiments presented in this appendix. This data was used to develop the purification scheme used in Chapter 3 for the purification of HSTF. Southwestern analysis of the purified protein with labeled HSE revealed that the HSTF is a 130 kD protein, and that the inclusion of protease inhibitors during the preparation of the extract and subsequent chromatography was necessary to maintain a high yield of intact protein.

Results

Development of a yeast extract for the isolation of HSTF

The HSTF in *Saccharomyces cerevisiae* was initially identified by DNAse I protection of the heat shock elements (HSEs) present in the promoter for the yeast heat-shock gene *SSA1*. The factor used to protect this DNA template was derived from spheroplast-derived extracts from the *S. cerevisiae* strain EJ101 (a protease mutant obtained from E. Jones). It was first noted in these initial experiments that there were multiple HSEs present in a single promoter, and the presence of more than one HSE in a promoter was quickly found to be a common feature of many heat-shock promoters (W.A. Kibbe, unpublished results, Topol, et al. 1985).

The initial conditions used for the yeast extract were those worked out by Dr. C.S.Parker and optimized for RNA Polymerase III transcription. This extract system proved to be very efficient, transcribing nearly 1 transcript per template molecule. After the identification of a putative RNA Polymerase II transcription factor, HSTF, an optimization of the ammonium sulfate lysis and precipitation of the extract for the yield of HSTF was developed. Table 1 shows the results of these titrations, and Figure 1 depicts these results graphically.

Other extraction methods

The large amount yeast cells that were necessary to obtain antibodies and sequence information from HSTF and other factors prompted an investigation into cheaper methods for extracting the factors from yeast. The standard Zymolyase-100T method of making spheroplast extracts is quite expensive when compared to the cost of growing the cells alone (1 kilogram of yeast cells can be grown in supplemented minimal media for about 60 dollars as of 1990, and 120 dollars of Zymolyase-100T and 40 dollars of dithiothreitol are needed to lyse the cells). Thus, we used a glass bead homogenizer available from Glenn Mills (trade name Dynomill) to make whole-cell extracts (the use of this machine was graciously permitted by Dr. J. L. Campbell).

YNE	Lysis	PPT	Protein	HSTF
1	0.10	0.20	500	0
2	0.10	0.25	500	1
3	0.10	0.30	1500	1
4	0.20	0.20	1000	5
5	0.20	0.25	2500	6
6	0.20	0.30	3000	2
7	0.25	0.15	500	0
8	0.25	0.20	1300	5
9	0.25	0.25	2500	6
10	0.25	0.30	3500	2
11	0.30	0.15	500	1
12	0.30	0.20	1500	2
13	0.30	0.25	2200	3
14	0.30	0.30	4000	2
15	0.35	0.15	1000	1
16	0.35	0.20	1800	2
17	0.35	0.25	3000	5
18	0.35	0.30	4500	2
19	0.40	0.20	2000	2
20	0.40	0.25	2500	2
21	0.40	0.30	3500	2
22	0.50	0.20	2200	1
23	0.50	0.25	3000	1
24	0.50	0.30	5000	1

Table 1. Yeast nuclear extract optimization. All extracts are derived from one batch of spheroplasted yeast. The cells were lysed with from 0.10 to 0.50 ammonium sulfate, and the supernate from the lysed cells was precipitated with 0.15 to 0.30 grams ammonium sulfate per milliliter of extract. The column labeled YNE is the extract designation number, the column Lysis is the molar concentration of ammonium sulfate used to lyse the nuclei, the column labeled PPT is the amount of ammonium sulfate added to the extract in grams per milliliter of extract, the column labeled Protein is the number of micrograms of protein precipitated in the 1 milliliter extract, and the column labeled HSTF is the activity of HSTF in the extract defined by the number of HSE sites protected with 100 µl of extract.

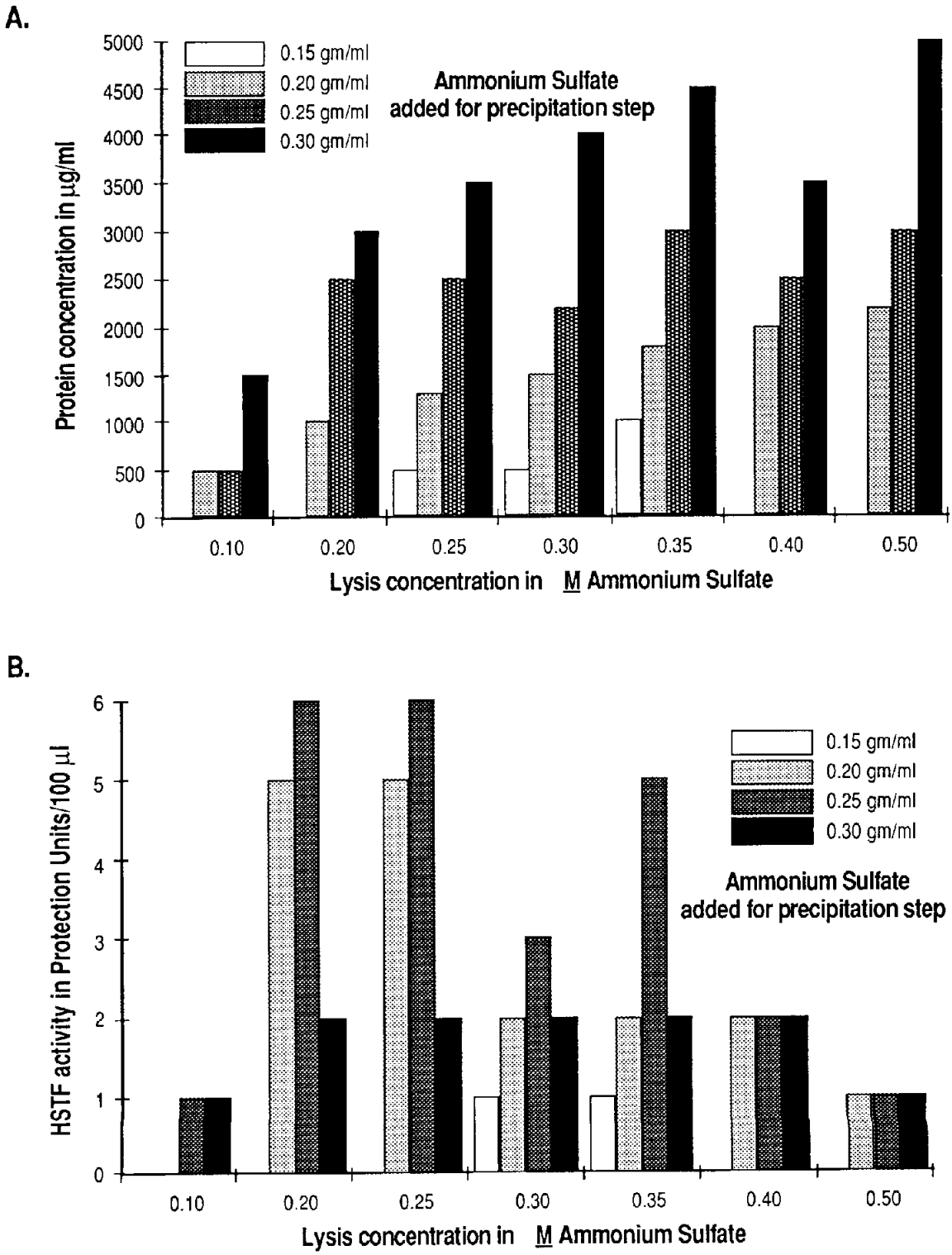

Figure 1. Graphic representation of the activity data presented in Table 1.

The whole-cell yeast extracts were prepared as described in Appendix C, and were used to purify HSTF and GBF (see Chapter 4) by the same columns used in the nuclear extract purification. These extracts were somewhat more viscous and at the final stages of purification were not as homogeneous as the spheroplast-derived factors. Whether the lack of homogeneity was due to proteolysis or to the presence of extra contaminating proteins in the whole-cell extract was not examined.

Chromatographic behavior of HSTF

The resins listed in Table 2 were tested for recovery and purification of HSTF using yeast nuclear extract fractions: heparin-Sepharose, heptyl-Sepharose, DE52, A-25, and P-11. The other resins listed were tested using HSTF that had been partially purified on heparin-Sepharose, with the exception of MonoS, which was used following heparin-Sepharose and Affigel-Blue chromatography. P-11 was tried after heparin-Sepharose fractionation and resulted in both poor recovery and purification.

Resin	Capacity	Load & Wash	Elution	Purification
Heptyl-Sepharose	15 mg/ml	1.2 M AS	0.5-0.7 M AS	5 fold
Hydroxyl-Apatite	10 mg/ml	100 mM KCl	0.12-.20 M $(PO_4)^{-3}$	4-5 fold
Heparin-Sepharose	15 mg/ml	100 mM KCl	350-400 mM KCl	>10 fold
DE52	10 mg/ml	50 mM KCl	80-120 mM KCl	2-3 fold
ACA34	-	50 mM KCl	-	none
ACA34	-	600 mM KCl	-	5 fold
P-11*	10 mg/ml	100 mM KCl	400-500 mM KCl	*
A-25	10 mg/ml	50 mM KCl	70-120 mM KCl	2-3 fold
dsDNA-Sepharose	10 mg/ml	100 mM KCl	300-400 mM KCl	5 fold
ssDNA-Sepharose	10 mg/ml	100 mM KCl	250-300 mM KCl	3-5 fold
Affigel-Blue §	8 mg/ml	200 mM KCl	1.5 M KCl	5-10 fold
MonoS	25 mg/ml	100 mM KCl	300-320 mM KCl	2 fold

* Inconsistent recovery from P-11.

§ Affigel-Blue must be loaded in the absence of Triton X-100 or other detergents, and the elution buffer should contain 0.2% Triton X-100 or other non-ionic detergent for optimal recovery.

Table 2. Chromatographic behavior of HSTF on several columns. Heptyl-Sepharose, heparin-Sepharose, dsDNA-Sepharose and ssDNA-Sepharose were coupled to Sepharose by cyanogen bromide activation (see Appendix C this thesis for experimental details). Commercially obtained (Sigma) heptylamine, heparin, and calf thymus DNA were coupled directly to cyanogen bromide activated Sepharose. The other resins were commercially obtained from Whatman (DE52 and A-25), Pharmacia (ACA34) or Biorad (Hydroxy-Apatite, ssDNA-Sepharose and Affigel-Blue). Abbreviations used in the table are: AS = ammonium sulfate, $(PO_4)^{-3}$ = phosphate concentration, and capacity is the binding capacity of the resin in milligrams of bindable protein per milliliter of wet resin.

In order to determine the molecular weight of the HSTF, several methods were used. The native size was determined by gel filtration (see Figure 6), and the SDS-denatured size was determined by gel renaturation (see Chapter 3) as well as by southwestern (see Chapter 4, Figure 3). The gel filtration size is

approximately 150 kD, corresponding closely to the molecular weight determined by southwestern. However, the gel renaturation data indicates as smaller, approximately 70 kD protein. The apparent discrepancy between this data may be due to differences in the original extracts used for the gel renaturation experiments, in which protease inhibitors were not used. Later, when the potent protease inhibitor diisopropyl fluorophosphate (DIFP) was included during the lysis of the nuclei, and the protease inhibitor phenylmethylsulfonyl flouride (PMSF) was added to all the chromatography and dialysis buffers, the southwestern data indicates that the HSTF exists predominantly as a 130 kD protein. The gel filtration experiment used extracts that were not treated with protease inhibitors, suggesting that the 150 kD apparent size of the native HSTF in this experiment is due to a dimerization of the 70 kD form of the HSTF. The dimerization of the HSTF in solution without DNA is a distinct possibility in view of other work with DNA binding proteins, such as cro (Ptashne 1986) and fos/jun (O'Shea, et al. 1989). In fact, GCN4 was also present in the HSTF sample loaded on the gel-filtration column, and the GCN4 footprinting activity eluted with an apparent size of 80 kD , exactly twice the size of the denatured protein (Arndt and Fink 1986).

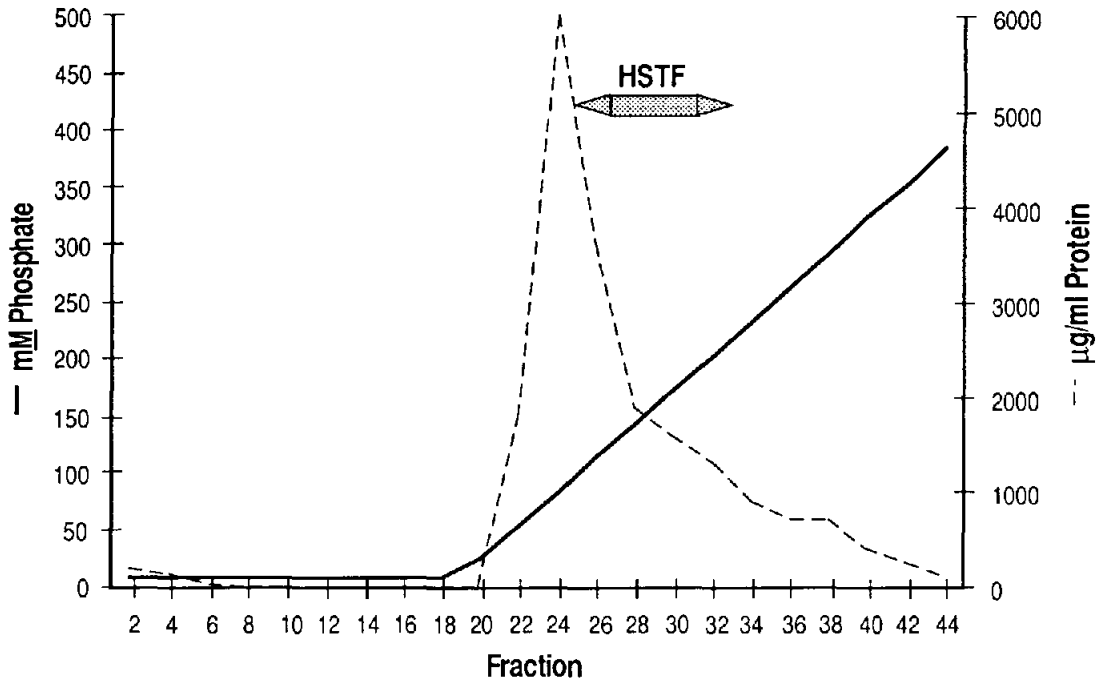

Figure 2. HSTF purification on Hydroxy-Apatite. A 1 ml column was poured and equilibrated with 50 mM KCl in HGE (see Appendix C). 10 milligrams of heparin-Sepharose fractions containing HSTF were loaded on this column, and the proteins were eluted in 0.20 ml fractions with a gradient of 0.01 to 0.40 M sodium phosphate buffered to pH 7.8. HSTF eluted between 0.12 and 0.20 M sodium phosphate. The fractions from the column (not shown) contained approximately 5 milligrams of protein. The HSTF fractions contained 2 milligrams of protein for a 5 fold purification of the HSTF on this column.

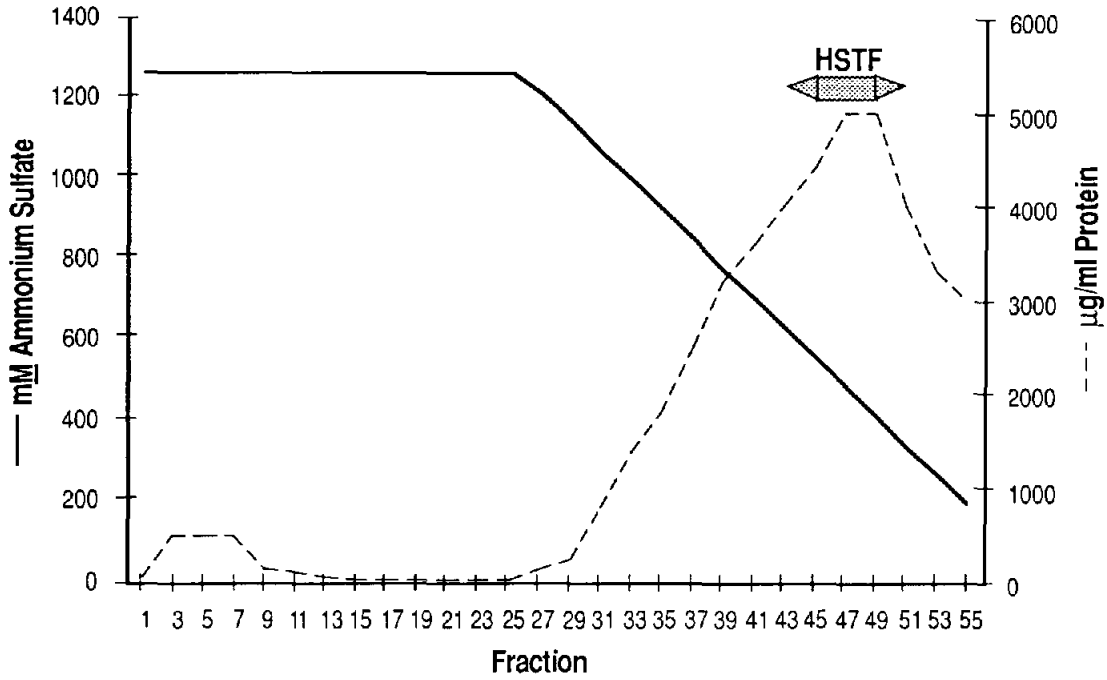

Figure 3. HSTF purification on heptyl-Sepharose. Five milliliters of resin were poured, equilibrated and then loaded with 100 milligrams of yeast nuclear extract in 1.2 M ammonium sulfate. The column was washed with buffer (HGE) containing 1.2 M ammonium sulfate, and a 40 milliliter gradient from 1.2 M ammonium sulfate to 40 mM KCl was used to elute the protein. One milliliter fractions were collected. HSTF eluted from about 0.7 - 0.5 M ammonium sulfate and those fractions contained 20 milligrams of protein for a 5 fold purification of the HSTF on this resin.

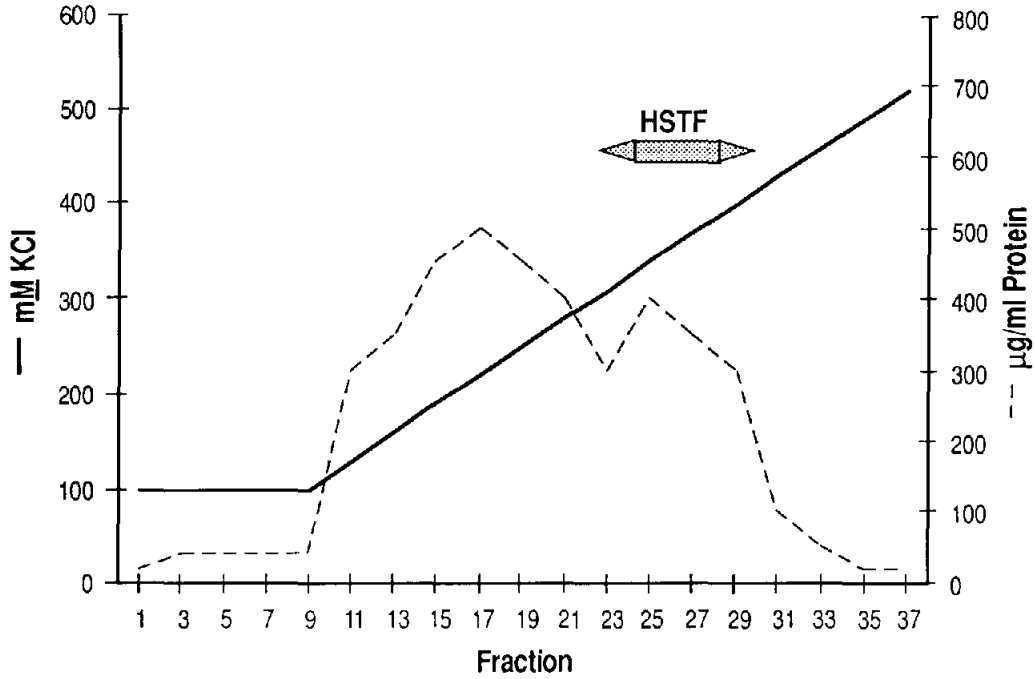

Figure 4. HSTF purification on dsDNA-Sepharose. Three milliliters of resin were poured, equilibrated and then loaded with 10 milligrams of a HSTF-containing heparin-Sepharose fraction. After a 10 milliliter wash with 'D' buffer (see Appendix C) the protein was eluted with a 30 milliliter gradient from 0.1 to 0.6 M KCl in HGE. One milliliter fractions were collected and assayed for protein concentration, conductivity and HSTF activity. HSTF eluted from 0.3 to 0.4 M KCl and the fractions containing HSTF had a total of 2 milligrams of protein, for a 5 fold purification of HSTF on this column.

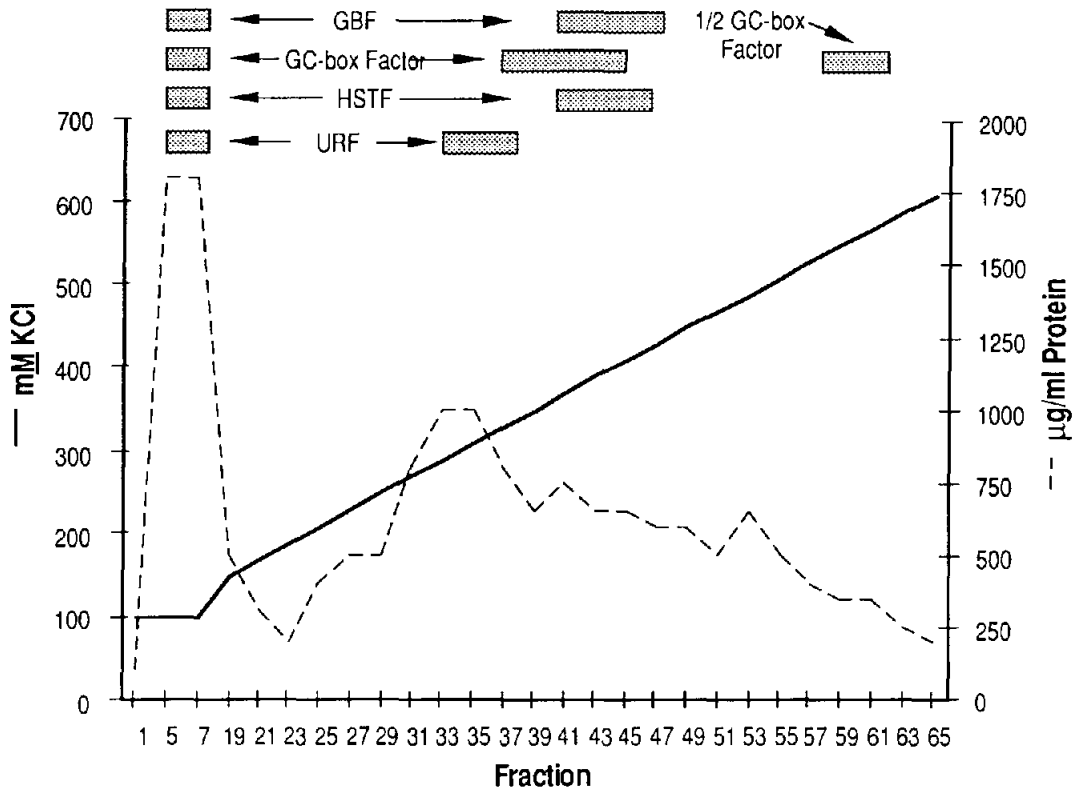

Figure 5. HSTF purification on heparin-Sepharose. Fifty milliliters of resin were poured and equilibrated in 'D' buffer. One gram of extract was loaded on the column, the column washed and eluted with a 600 milliliter gradient from 0.1 to 0.6 KCl in HGET (see Appendix C). Ten milliliter fractions were collected and assayed for protein concentration, conductivity and footprinting activity on a variety of sites. URF elutes at approximately 0.3 M KCl with 60 milligrams of protein, HSTF and GBF elute from 0.36 to 0.40 KCl with 60 milligrams of protein, GC-box Factor (ySp1) elutes from 0.32 to 0.38 with 75 milligrams of protein, and 1/2 GCBox Factor elutes from 0.5 to 0.6 with 15 milligrams of protein. A close to 20 fold purification of the HSTF was achieved on this resin. The reapplication of the does not result in the binding of the footprinting activity found in these fractions to heparin-Sepharose, indicating that a separate form of these activities exist that will not bind to heparin-Sepharose.

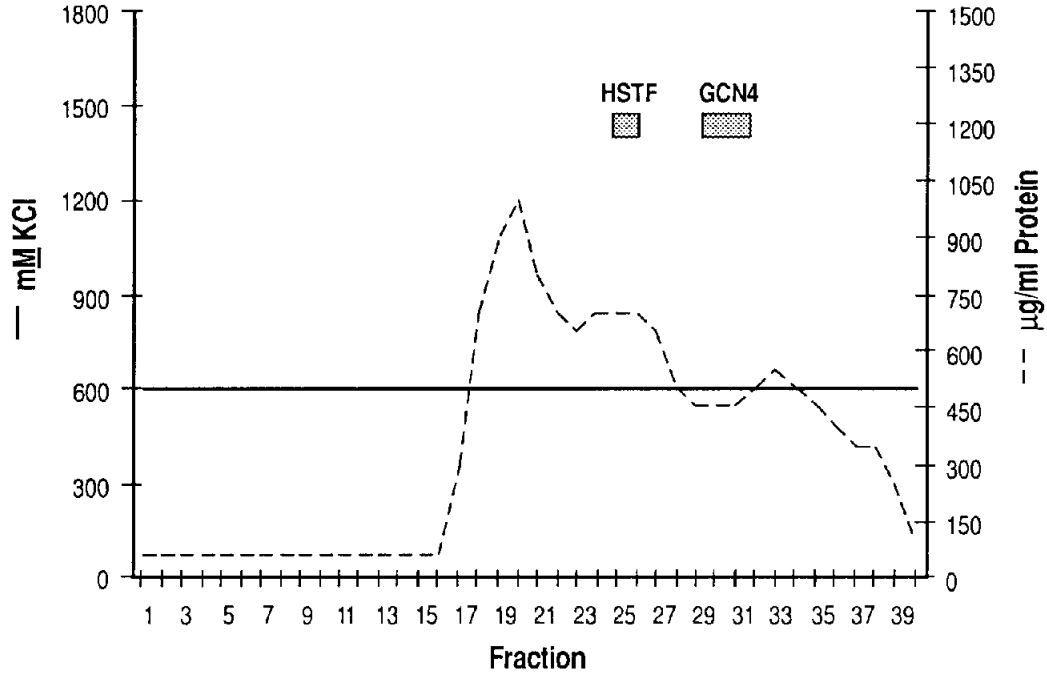

Figure 6. HSTF purification on ACA34. A 35 ml ACA34 column was degassed and poured and equilibrated for two days with 250 milliliters of 0.6 HGKE (0.6 M KCl in HGE, see Appendix C for a description of HGE). The column was then standardized with a sample containing protein standards. After standardization, the column was loaded with 1.5 milliliters of an 8 mg/ml fraction containing HSTF. The heparin-Sepharose purified HSTF sample was concentrated by the reapplication of the protein to heparin-Sepharose and the subsequent step-elution of the protein. The sample was centrifuged at 10,000 rpm for 10 minutes to remove any large precipitate, and then filter through a 0.45 micron filter. The column was loaded and run in 0.6 M KCl to prevent aggregation. One milliliter fractions were collected and assayed for protein concentration, HSTF and GCN4 binding activity. HSTF elutes with an apparent molecular weight of 150 kD, and GCN4 with an apparent molecular weight of 80 kD under these conditions.

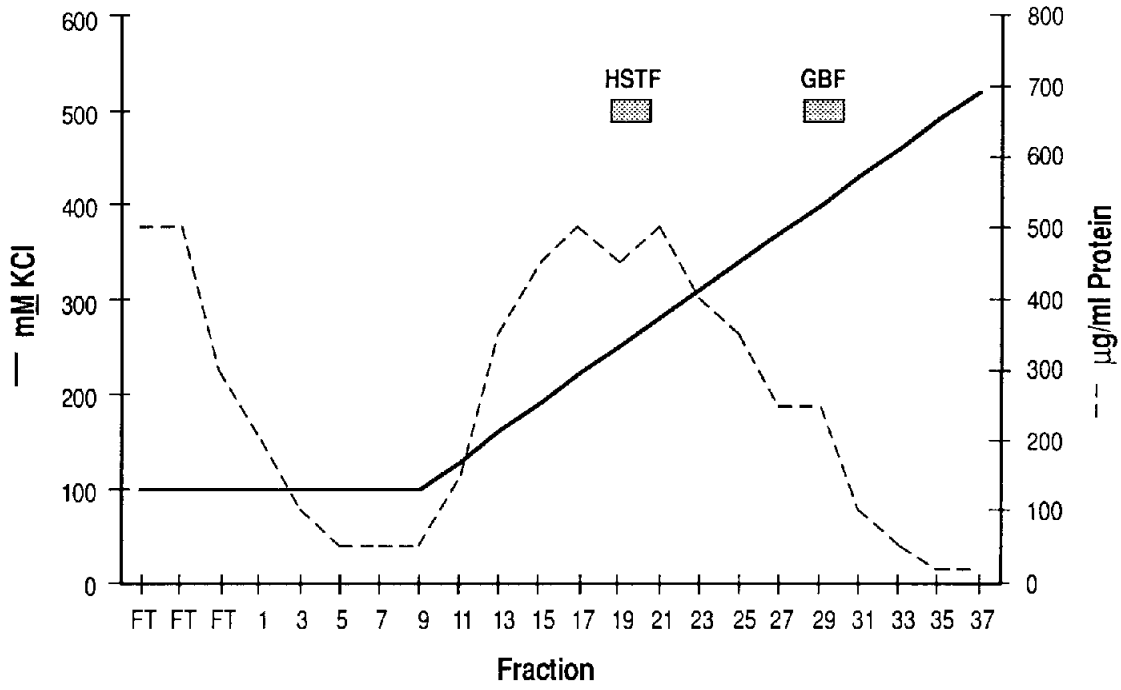

Figure 7. HSTF purification on ssDNA-Sepharose. Three milliliters of resin was poured, equilibrated and then loaded with 10 milligrams of HSTF-containing heparin-Sepharose fractions. The contained approximately 2 milligrams of protein. A 30 milliliter gradient of 0.1 to 0.5 M KCl in HGET was applied to the resin and one milliliter fractions were collected. HSTF eluted from 0.25 to 0.3 M KCl and those fractions contained about 2 milligrams of protein, yielding a 5 fold purification of HSTF on this resin. GBF eluted from 0.4 to 0.45 M KCl and the GBF-containing fractions had approximately 0.8 milligrams of protein, for a greater than 10 fold purification.

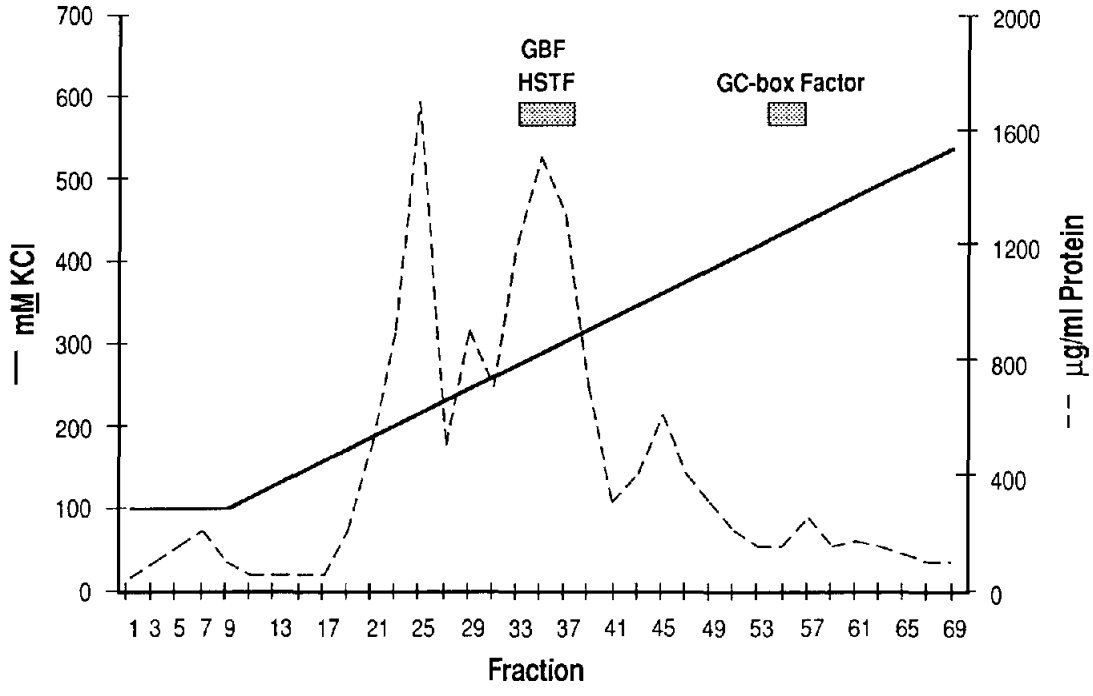

Figure 8. HSTF purification on MonoS. A Pharmacia MonoS column was loaded with 10 milligrams of GBF, HSTF and GC-box factor-containing heparin-Sepharose fractions. The column was run at 1 megaPascal (MPa) with a flow rate of 1.5 milliliters/minute. A 20 mM KCl/column volume gradient was applied to the column from 0.05 to 0.55 M KCl, and then a 1.0 M KCl step was applied (not shown). The HSTF and GBF activities elute at approximately 0.30 M KCl, and the GC-box factor elutes at approximately 0.45 M KCl (see Chapter 1 Appendix for more details on GC-box factor).

Extract freezing conditions

In order to easily obtain large amounts of cells in a convenient manner, an efficient method of freezing the intact cells was developed. Four different buffers and two different cell weight to buffer volume ratios were tested for HSTF recovery. The results of these tests are shown in Table 3. It was determined that the standard 'Y' buffer with 30 mM DTT was adequate for obtain-

ing active HSTF from frozen yeast. The cell slurry concentration was apparently not important to the recovery of active HSTF.

		Recovery	
	Buffer	**1:1 slurry**	**1:4 slurry**
10%	Sucrose	0.2	0.1
50 mM	Tris (pH 7.8)		
1 mM	EDTA		
10%	glycerol	0.5	0.5
	'Y' Buffer		
30 mM	DTT		
	'Y' Buffer	1	1
30 mM	DTT		
	'Y' Buffer	1	1
30 mM	DTT		
7%	DMSO		
Unfrozen		1	1

Table 3. Recovery of HSTF activity from cells quick-frozen in liquid nitrogen. Recovery is measured by the footprinting activity of extracts made from the cells in FPU per 10 µl of extract. All extracts were performed identically, with the same weight of cells used for each extract as measured prior to freezing, and the final volume from each extract was identical.

Conclusion

The lysis and ammonium sulfate precipitation conditions for a *S. cerevisiae* spheroplast-derived nuclear extract and a whole-cell extract were optimized so that the HSTF could be efficiently purified from yeast. The chromatographic behavior of HSTF has been determined on a variety of columns and this data used to develop the purification scheme in Chapter 3. During the development of the purification scheme, it was determined that the yield of HSTF (and subsequently the yield of many of the DNA binding proteins

examined in this thesis) can be increased by the inclusion of a non-ionic detergent, such as Triton X-100 or NP-40. HSTF has been identified and isolated from yeast (this thesis, Chapter 3, and Sorger and Pelham, 1987), *Drosophila* (Chapter 3), and HeLa cells (Sorger and Pelham 1987). The HSTF is a heat stable and reasonably abundant protein that is approximately 130 kD in size and encoded by the *HSF* gene (Wiederrecht, et al. 1988). Experiments that investigate the nature of the transcriptional activation mediated by the HSTF are currently under way in Dr. Parker's lab, as are experiments to determine the nature of the HSTF/HSE interaction as well as the structural components of the HSTF that are involved in transcriptional activation.

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Chapter 4

Identification and Isolation of a Factor Binding to a Novel Heat-shock Responsive Element.

Warren A. Kibbe and Carl S. Parker

Table of Contents

Identification and Isolation of a Factor Binding to a Novel Heat-shock Responsive Element.

Abstract

A novel sequence that is not homologous to the heat shock consensus element (HSE) has been found to convey heat-shock inducibility in *Saccharomyces cerevisiae* when placed in the promoter of a *CYC1*/lacZ fusion. The sequence, $A\frac{A}{T}N\frac{A}{T}AGGGG\frac{A}{T}G$, has been identified in the yeast hsp70 gene *SSA1* promoter and the *HSF* promoter and this sequence has been designated **G-box**. We have isolated a protein, G-box binding factor (GBF), which recognizes this novel heat-shock promoter sequence and have characterized its DNA binding interactions by DNAse I, DMS, and hydroxyl-radical cleavage protection. The binding affinity of GBF for *SSA1* G-box site 2 has also been determined using gel-shift analysis. In addition, we have characterized the molecular weight of GBF by guanidinium-HCl renaturation from a denaturing protein gel, as well as by Southwestern analysis.

Introduction

The heat-shock response is ubiquitous and triggers mechanisms that are necessary for the survival of a cell at heat-shock temperatures and its recovery from heat-shock (see review: Lindquist and Craig, 1988). Heat-shock induces the transcription of a set of genes that encode heat shock proteins (hsps). There are several classes of highly conserved hsp genes, such as the hsp70 class (Ingolia, et al. 1982). Recently, many genes have been identified as hsps by sequence homology to known hsp genes. Some of these highly homologous genes are not induced by heat-shock, and have been named heat-shock cognate genes. Indeed, the function of the heat-shock cognate proteins may be to perform activities necessary for survival of the cell under normal conditions, while the hsps are needed to survive and recover from heat-shock (Lindquist and Craig 1988). Studies have shown that the yeast hsps and hsp cognates respond differently to various physiological conditions, including heat-shock (Werner-Washburne, et al. 1989). Furthermore, the presence of functional hsps and hsp cognates in yeast has been shown to be required for survival under shocked and non-shocked conditions (Craig and Jacobsen 1985; Werner-Washburne, et al. 1987).

At least one member of the hsp70 class of hsps (most genes in this class encode approximately 70 kD proteins) has been identified in virtually every organism examined. *Saccharomyces cerevisiae* has at least 8 hsp70-related genes, including cognates. These genes have been divided into 4 subfamilies by homology and complementation: SSA, SSB, SSC, and SSD, for Stress Seventy subfamily A through D (Craig, et al. 1987). The presence of at least one functional gene product from each of the SSA and SSC subfamilies has been shown to be necessary for cell viability under a variety of growth condi-

tions (Werner-Washburne, et al. 1989; Werner-Washburne, et al. 1987). The SSA subfamily proteins have been shown to direct the translocation of secretory proteins (Deshaies, et al. 1988).

A careful examination of the transitory and steady state RNA levels transcribed from the yeast hsp70 genes under various physiological conditions has been published. This study shows that there are 6 distinctive transcription patterns for the eight hsp70 genes under different physiological conditions. Based on the current understanding of transcription, it can be surmised that the differences in the transcriptional regulation of the hsp genes are due to the presence of different upstream activator sites (UASs) and/or upstream repressor sites (URSs) in each of the promoters. Currently, yeast promoters containing multiple UASs and URSs, such as the Ty1 transposon, have been shown to respond to a variety of signals, and each unrelated response has been shown to be mediated by a different UAS or URS.

The induction of the transcription of hsps by heat-shock has been shown to be mediated by a DNA element containing the consensus C__GAA__TTC__G (Pelham 1982), referred to as the heat-shock element (HSE). The heat-shock transcription factor (HSTF), a site-specific DNA binding factor, has been shown to bind specifically to this site in *Drosophila*, HeLa and *Saccharomyces cerevisiae* cells (Sorger and Pelham 1987; Wiederrecht, et al. 1987). The *Drosophila* HSTF has been shown to stimulate transcription *in vitro* (Wiederrecht, et al. 1987). The presence of functional HSTF in yeast has been shown to be necessary for the transcription induction mediated by the HSE *in vivo* (Sorger and Pelham 1988; Wiederrecht, et al. 1988). However, the induction of transcription on heat-shock appears to occur by different mechanisms in yeast than in *Drosophila* or mammalian cells. In yeast, HSTF can bind the HSE at shocked or non-shocked temperatures, and the activation

of transcription appears to be mediated by the extensive phosphorylation of HSTF (G.J.Wiederrecht and C.Schoenherr, personal communication). In contrast, HSTF derived from mammalian (HeLa) cells requires heat-shock in order to bind the HSE (Sorger and Pelham 1988). HeLa-derived HSTF is also phosphorylated and this phosphorylation may modulate the HSTF-HSE binding (Sorger and Pelham 1988).

The yeast HSTF is encoded by the *HSF* gene, which has been cloned and sequenced (Sorger and Pelham 1988; Wiederrecht, et al. 1988). Transcription of *HSF* has been shown to be heat-induced, even though the promoter does not contain an HSE (G.J.Wiederrecht, personal communication). We have identified two G-rich regions in the *HSF* promoter that are bound *in vitro* by a factor, G-box binding factor (GBF). We have also identified two sites in the yeast hsp70 *SSA1* promoter that are bound by GBF *in vitro*. Deletion of one of the *SSA1* G-box sites, G-box site 2, resulted in the reduction of heat shock inducibility of the promoter by nearly 10 fold, as assayed in a series of 5′ deletions (Chapter 1).

In this paper, we show that a single G-box site placed upstream of a *CYC1*/lacZ fusion can convey heat shock inducibility on the expression of the fusion in yeast. The purification and characterization of GBF is also described.

Results

Purification of GBF

The G-box binding factor, or GBF, has been purified using the chromatography scheme in Figure 1. GBF also binds tightly to dsDNA-Sepharose (eluting at ~320 mM KCl) and to ssDNA-Sepharose (eluting at ~400 mM KCl). GBF has a significant binding affinity for ssDNA, as shown in the gel-shift using ssDNA as a competitor.

GBF was loaded onto the ligated-G-box-Sepharose affinity column a second time and eluted with a KCl gradient rather than steps. DNAse I protection data along with the KCl column profile are shown in Figure 2. GBF starts to elute at ~420 mM KCl. The second passage of GBF on the affinity resin did not substantially improve the purification (data not shown) and was omitted from the standard purification scheme. The amount of GBF in the various fractions was determined by titrating the factor in either a DNAse I protection analysis or a gel-shift analysis.

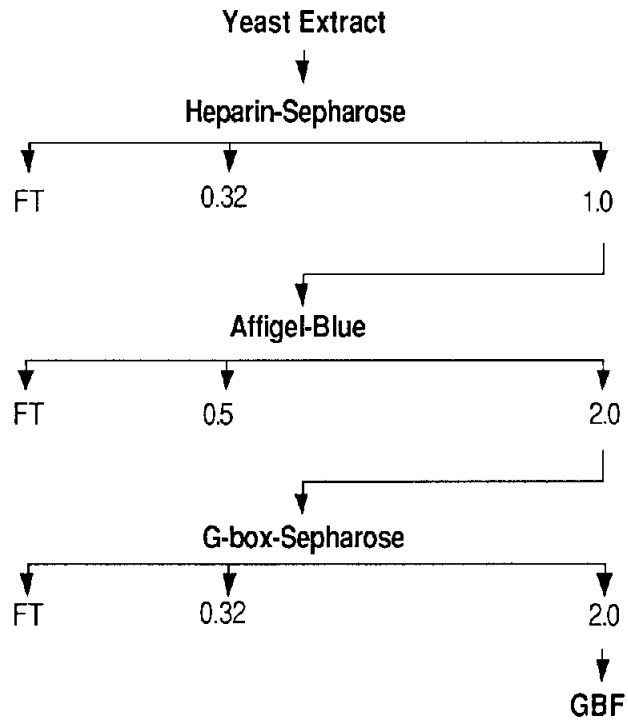

Figure 1. Purification Scheme for obtaining the G-box Binding Factor (GBF). The yeast extract and heparin-Sepharose fractions do not contain Triton X-100. The Affigel-Blue wash and 0.4 M KCl fractions also do not contain Triton X-100, but the high salt elution buffer as well as the G-box-Sepharose buffers all contained 0.1% Triton X-100.

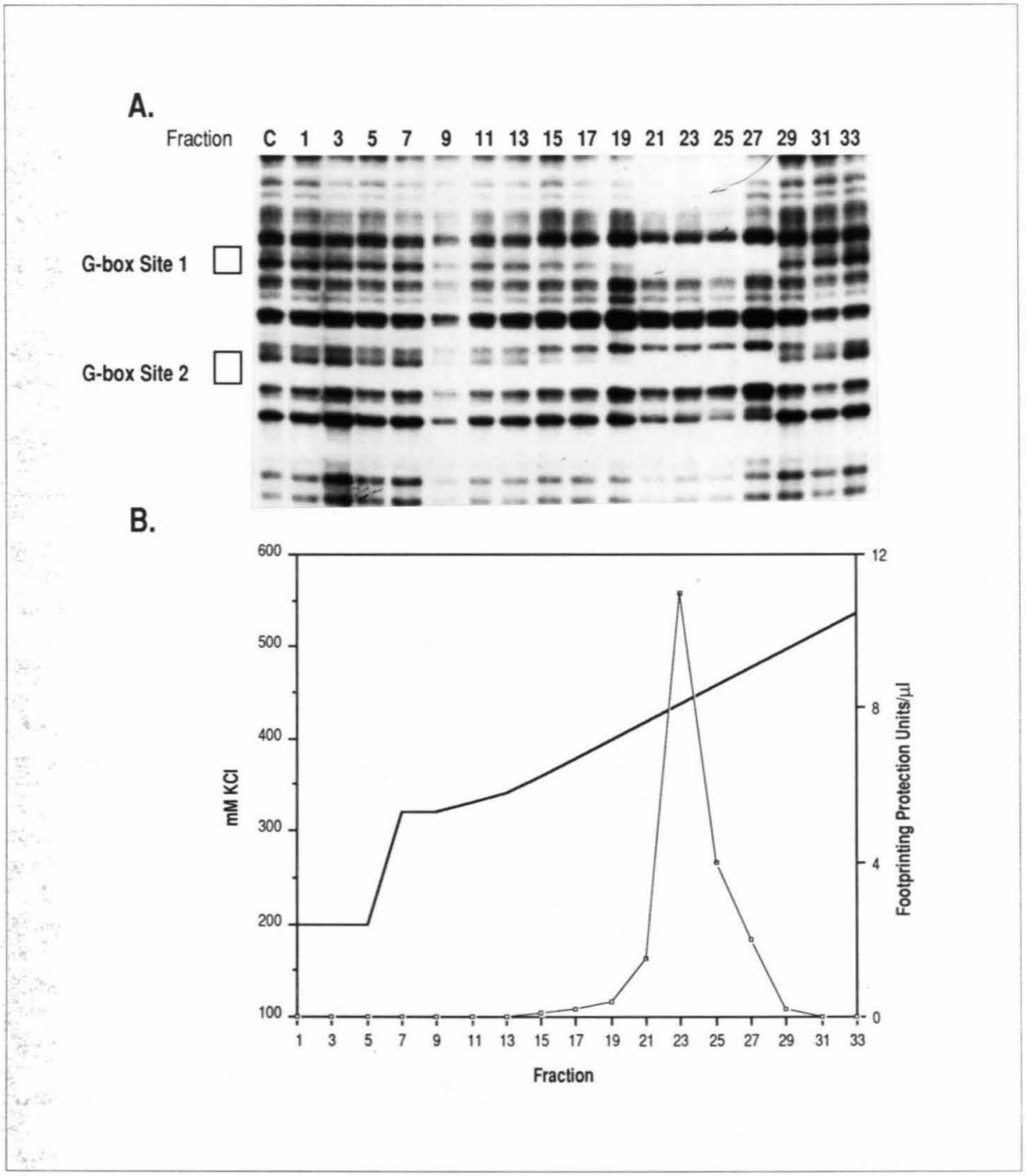
A.
Fraction C 1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33
G-box Site 1
G-box Site 2
B.
mM KCl
600
500
400
300
200
100
12
8
4
0
Footprinting Protection Units/µl
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33
Fraction

Figure 2.

Figure 2. Gradient elution of GBF from G-box-Sepharose. The volume of each fraction was 1.5 mls and 10 µl of every other fraction was assayed by DNAse I protection for G-box specific DNA binding. The fractions containing activity were further titrated. Panel A shows the result of DNAse I protection on the top strand of the SSA1 promoter. Panel B plots the concentration of KCl in mM and the activity of the fractions in footprinting protection units (FPU)/ µl. One FPU was defined as the amount of protein necessary to protect 50% of 10^{-14} moles (10 femtomoles) of a single G-box site.

Identification and quantitation of GBF

The presence of G-box binding activity was determined by DNAse I protection analysis. The template used in both analyses was an oligonucleotide made to the *SSA1* G-box site 2 sequence and inserted into the *E. coli* vector pUC-9.

Calculating the amount of protein present

The molar concentration of GBF present in fractions eluted from the G-box-Sepharose resin was determined using gel-shift titrations. The concentration of G-box site 2 oligonucleotide was known exactly, and the amount of GBF present in the sample could be determined by titrating the GBF/labeled G-box site 2 with unlabeled G-box site 2 oligonucleotide. The concentration of GBF was determined independently by titrating the amount of GBF necessary to clear 50% of 10 femtomoles (approximately 0.5 ng) of labeled fragment containing G-box site 2 in a DNAse I footprint. 50% clearing was defined as the amount of GBF necessary to reduce the major DNAse I hypersensitive site in the protection region to 1/2 of its normal value, as determined by densitometry scans on a titration series. In addition, the DNAse I protection method was used to determine the number of protection units (FPU) of GBF by constructing a standard titration curve of labeled G-box site 2 oligonucleotide

versus unlabeled G-box site 2. Determining the concentration of G-box in terms of FPU by either the gel-shift or DNAse I protection methods gave the same result within experimental error.

Size analysis

The molecular weight of the protein has been estimated using Southwestern analysis, where a denaturing protein gel is run, transferred to nitrocellulose, and probed with labeled binding site. See Figure 3A for the Southwestern result using labeled G-box site 2 as the probe. The G-box probed nitrocellulose shows reactivity with bands at 110 and 85 kD in the lane containing G-box affinity-purified material, and no reactivity with affinity-purified HSTF (see Figure 3A). The identically prepared nitrocellulose bound fractions that were probed with the HSE showed no reactivity to the proteins in the G-box affinity-purified lane (see Figure 3B, lane 2) but recognized two bands at 130 kD and 110 kD. The 130 kD band is intact HSTF and the 110 kD band is a proteolytic product (G.J.Wiederrecht, personal communication). The 110 and 85 kD G-box reactive bands may be either two distinct proteins recognizing the G-Box, or the 85 kD band may be a proteolytic product of the 110 kD band. In other Southwesterns, a prominent 90 kD band is also seen, which again may be a proteolytic product of the 110 kD band (see Chapter 4 Appendix).

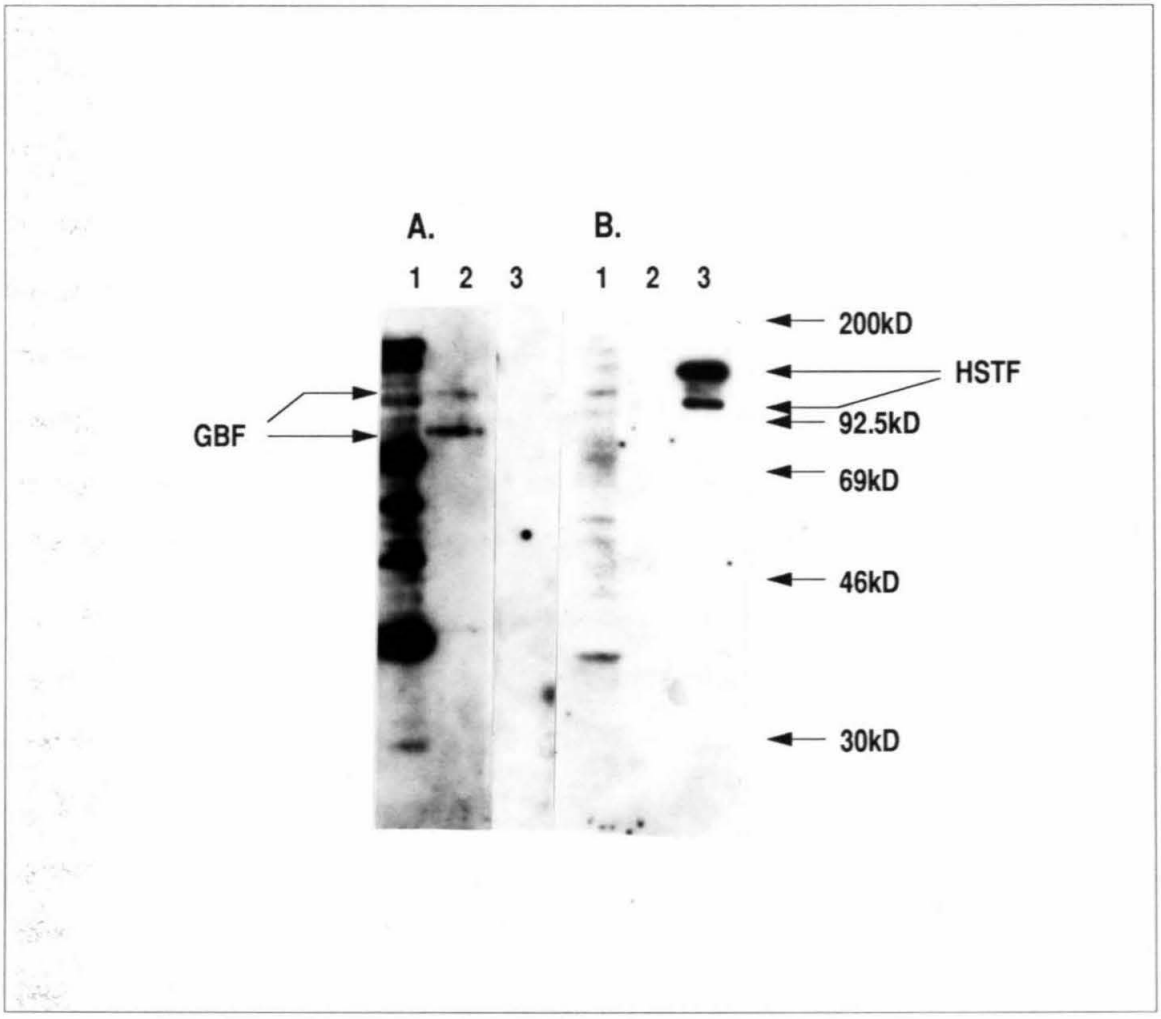

Figure 3.

Figure 3. Determination of the molecular weight by Southwestern. Panel A is the Southwestern blot probed with γ-^{32}P kinased G-box site 2 oligonucleotide. Panel B is the Southwestern blot probed with γ-^{32}P kinased HSE Site 3 oligonucleotide. In each experiment, Lane 1 was loaded with 50 µg of a pool of 1.0 M KCl-eluted heparin-Sepharose fractions, lane 2 was loaded with 1000 footprint protection units of GBF, and lane 3 with approximately 50 footprint protection units of HSTF.

Renaturation of GBF

GBF's DNA binding activity was capable of renaturation following treatment with 6 M guanidinium-HCl and dialysis of the solution overnight to gradually remove the guanidinium-HCl. More specifically, the renaturing ability was shown by precipitating the 1.0 M KCl fraction eluting from heparin-Sepharose with four volumes of acetone, then resuspending the pellet in 6 M guanidinium HCl. The solution was then dialyzed overnight against 100 mM KCl without Triton X-100 to gradually remove the guanidinium, and the fraction analyzed for protection activity on a fragment of the *SSA1* promoter (see Figure 4).

Utilizing the ability of GBF to renature from guanidinium-HCl, the approximate molecular weight of GBF was determined by loading 5,000 footprint protection units of affinity-purified GBF onto a denaturing protein gel. After the gel was electrophoresed, the protein in the gel was visualized by immersion into 200 mls of 0.25 M KCl. The gel was then cut into 8 slices and electroeluted in standard denaturing electrophoresis buffer, then allowed to renature in the same manner as the heparin-Sepharose fraction in the previous paragraph. Figure 5 shows the results. The presence of GBF activity in the renatured fractions containing the 85 kD proteins is consistent with the Southwestern results, where an 85 kD band was strongly recognized by the G-box probe.

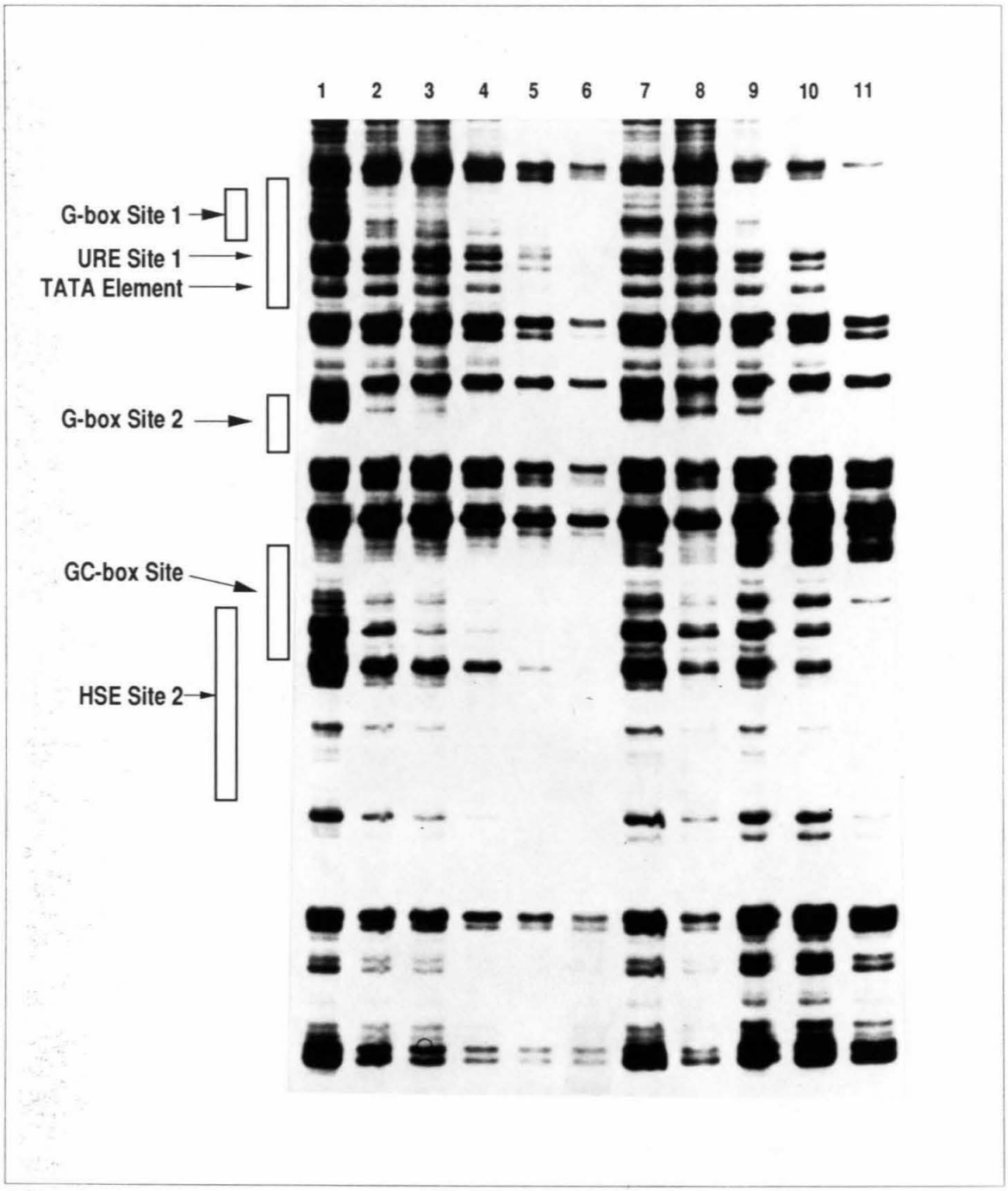
1
2
3
4
5
6
7
8
9
10
11
G-box Site 1
URE Site 1
TATA Element
G-box Site 2
GC-box Site
HSE Site 2

Figure 4.

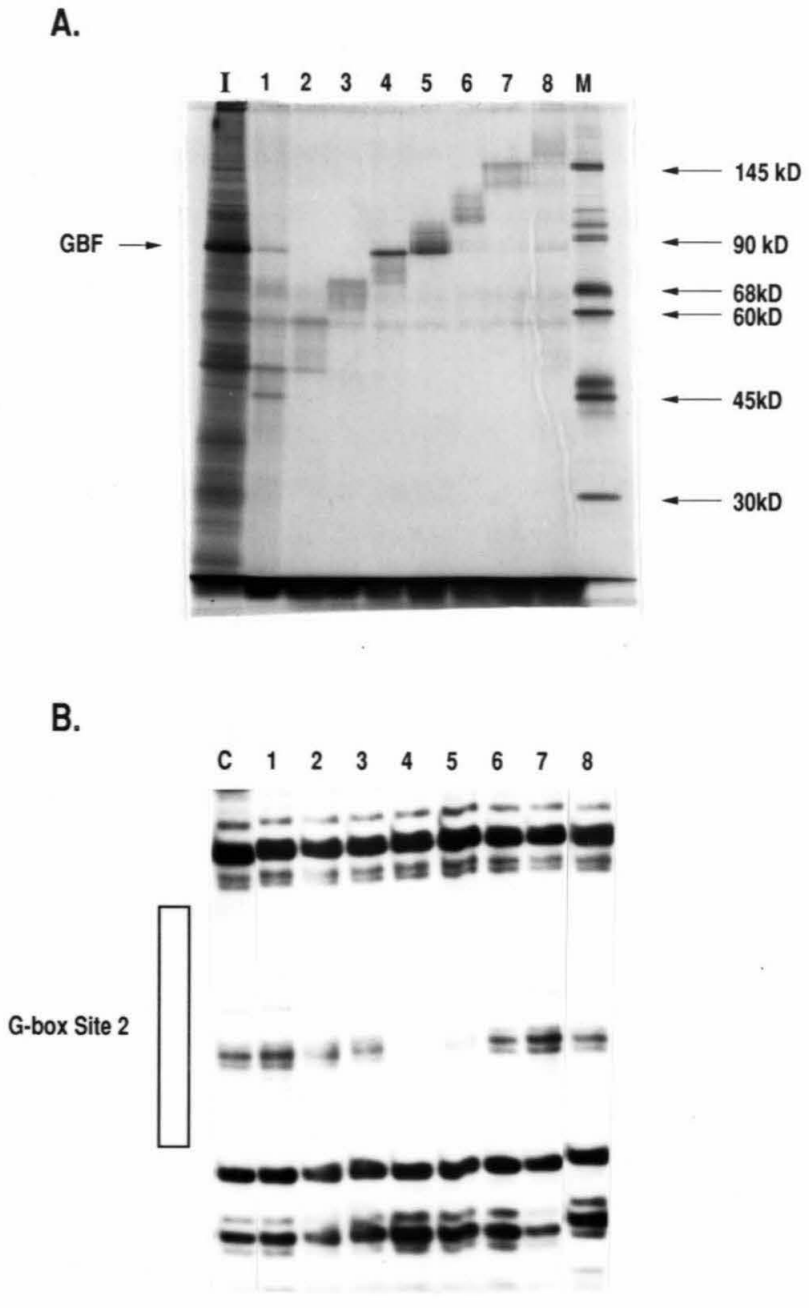
A.
I 1 2 3 4 5 6 7 8 M
GBF
145 kD
90 kD
68kD
60kD
45kD
30kD
B.
C 1 2 3 4 5 6 7 8
G-box Site 2

Figure 5.

Figure 4. Titration of native (non-denatured) or renatured 1.0 M KCl heparin-Sepharose pooled fractions by DNAse I protection. Denaturation was performed by acetone precipitating an aliquot of the material, resuspending it in the original volume of 6 M guanidinium-HCl, and dialyzing it overnight versus 100 mM KCl without Triton X-100. Lane 1 is the minus protein control, lanes 2 through 6 use the non-denatured protein, and lanes 7 through 11 use the renatured protein. One μl of protein was used in lanes 2 and 7, 2 μl were used in 3 and 8, 5 μl in 4 and 9, 10 μl in 5 and 10, and 20 μl were used in lanes 6 and 11.

Figure 5. Elution and activity profile of gel isolated and renatured GBF fractions. Panel A shows a silver stained gel of fractions eluted from a denaturing protein gel. Lanes 1 through 8 are eluted fractions 1 through 8, with lane M containing protein standards and lane I was the input, i.e., the fraction loaded onto the elution gel. Panel B shows the DNAse I protection assay using the isolated, renatured fractions 1 through 8. Lane C is the minus protein control reaction.

The G-box Element Conveys Heat-shock Inducibility

The transcriptional activity of the *SSA1* G-box site 2 was assayed in either a *CYC1*/lacZ fusion or in a *SSA1*/lacZ fusion. 5′ deletions have indicated that the G-box element can act to activate transcription upon heat-shock (Chapter 1). The constructions were placed in a CEN IV containing plasmid, which regulates the copy number of the plasmid to 1 copy per haploid genome. The recorder gene was then assayed at 23°C and at 39°C to determine if the isolated G-box element could induce activation in either of the fusions.

	SSA1 CEN		*CYC1* CEN		*CYC1* 2μ	
Site	23°C	39°C	23°C	39°C	23°C	39°C
G-box	3	30	0.5	3	3.5	14
HSE			1	8	40	150

Table 1. Activity of the G-box and HSE elements in centromere and 2-micron based vectors. *CYC1* plasmids were constructed by the deletion of UAS1 and UAS2 from a *CYC1/LacZ* fusion, and subsequent introduction of either the G-box or HSE oligonucleotide into that site. *SSA1* plasmids were constructed by placing the oligonucleotide in the EcoRI site of pSCCZ-255RHB, shown in Appendix A.

The G-box element in a 2μ plasmid could stimulate the activity of the *CYC1*/lacZ fusion by 3.5 fold by shifting the cells from 23°C to 39°C, causing roughly the same induction upon heat-shock as the HSE (see Table 1). However, the number of units of ß-galactosidase produced by the HSE under both non-shocked and shock conditions was ten fold greater than the G-box element. Thus, although the G-box element conveys a significant level of transcriptional induction upon heat-shock, the activity of an HSE would clearly dominate a promoter with both an HSE and a G-box element.

Footprint Analysis of GBF on the G-box element

The synthetic double-stranded G-box site 2 sequence was inserted into pUC-9. This plasmid was used as the template for DNAse I, DMS, and hydroxyl-radical cleavage protection experiments; these assays were performed on both strands. The results are shown in Figures 6 and 7.

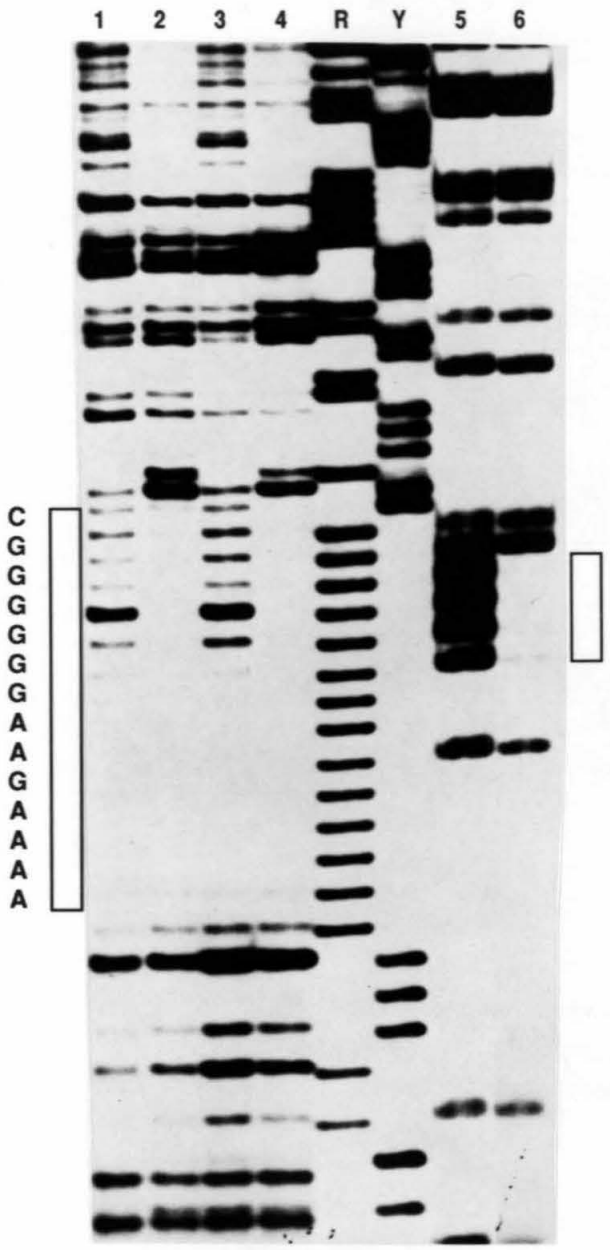

Figure 6A.

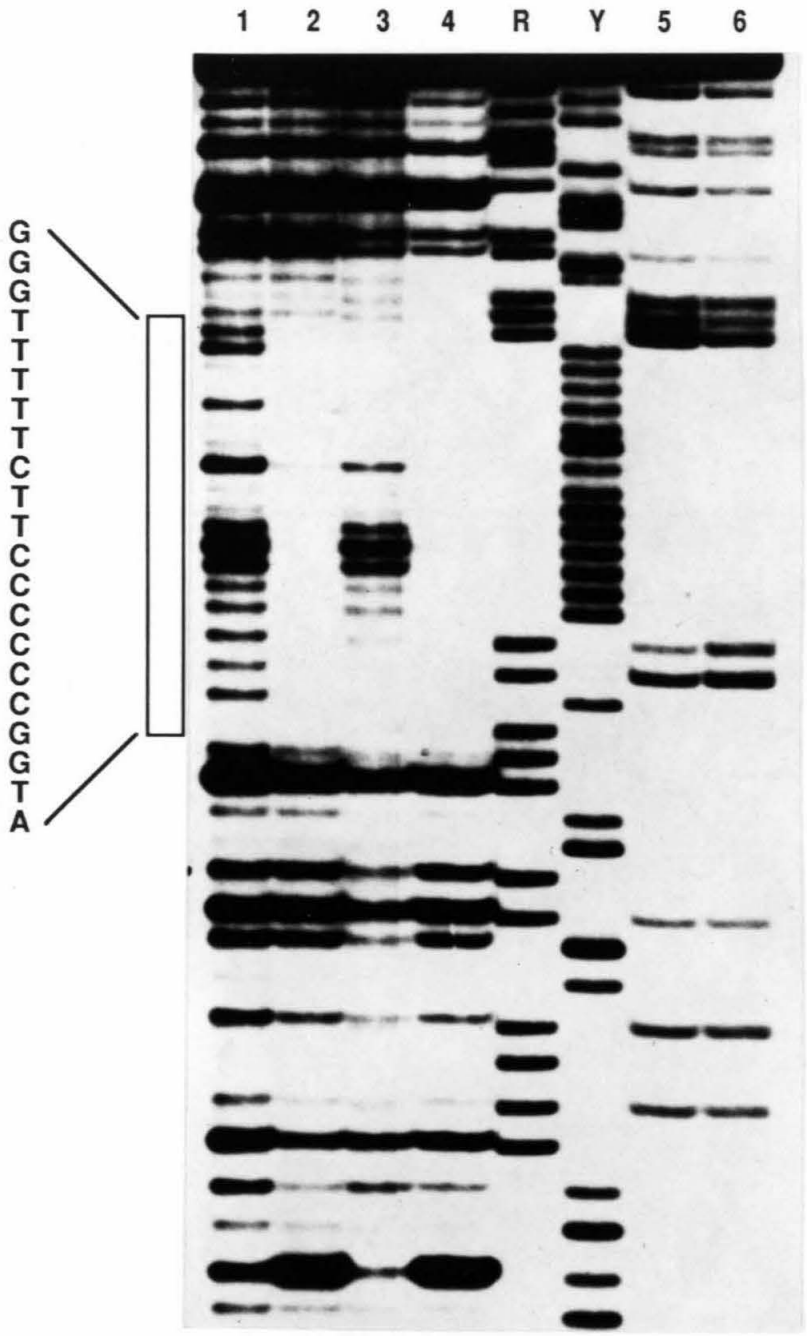

Figure 6B.

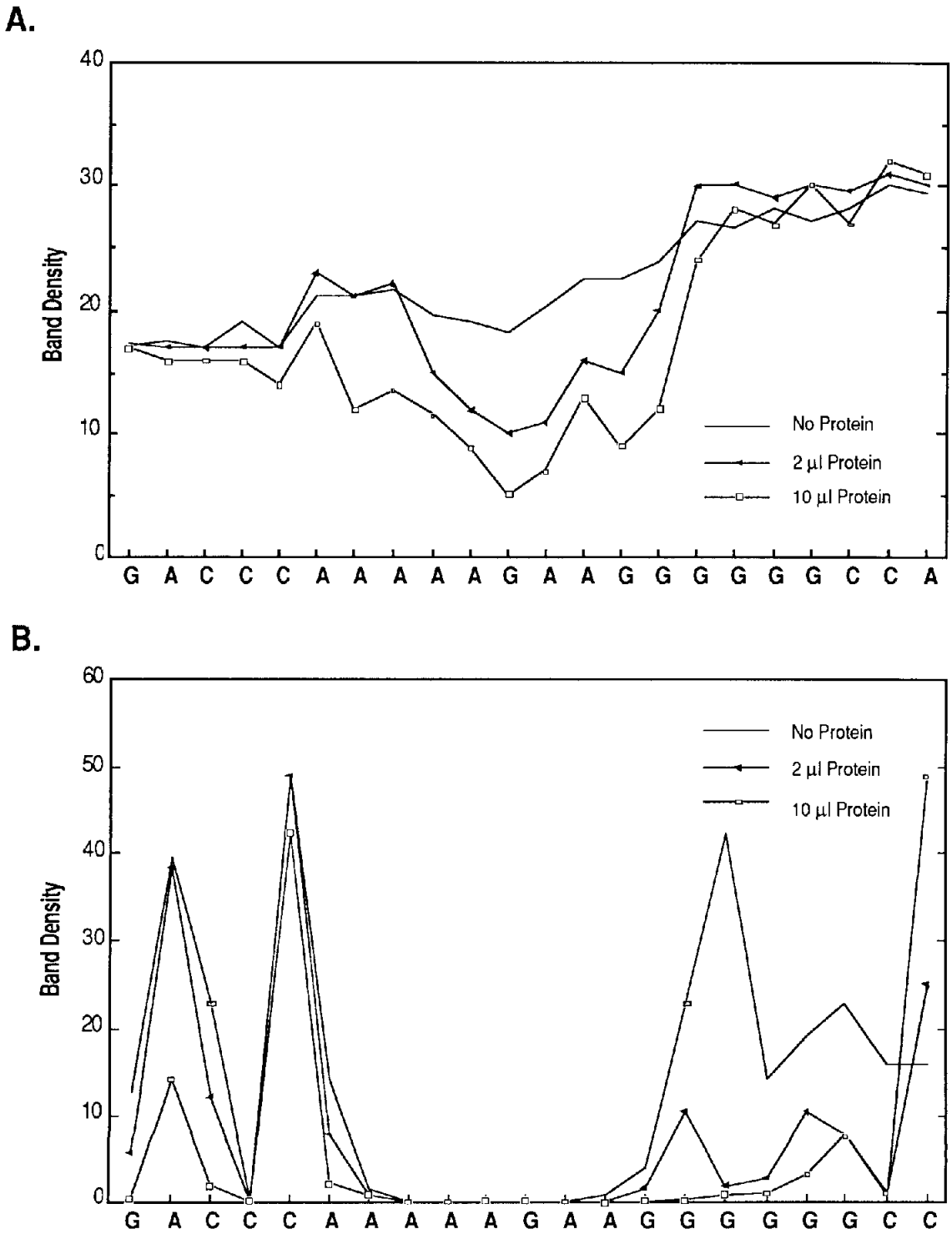
A.
Band Density
40
30
20
10
0
No Protein
2 µl Protein
10 µl Protein
G A C C C A A A A A G A A G G G G G G C C A
B.
Band Density
60
50
40
30
20
10
0
No Protein
2 µl Protein
10 µl Protein
G A C C C A A A A A G A A G G G G G G C C

Figure 7.

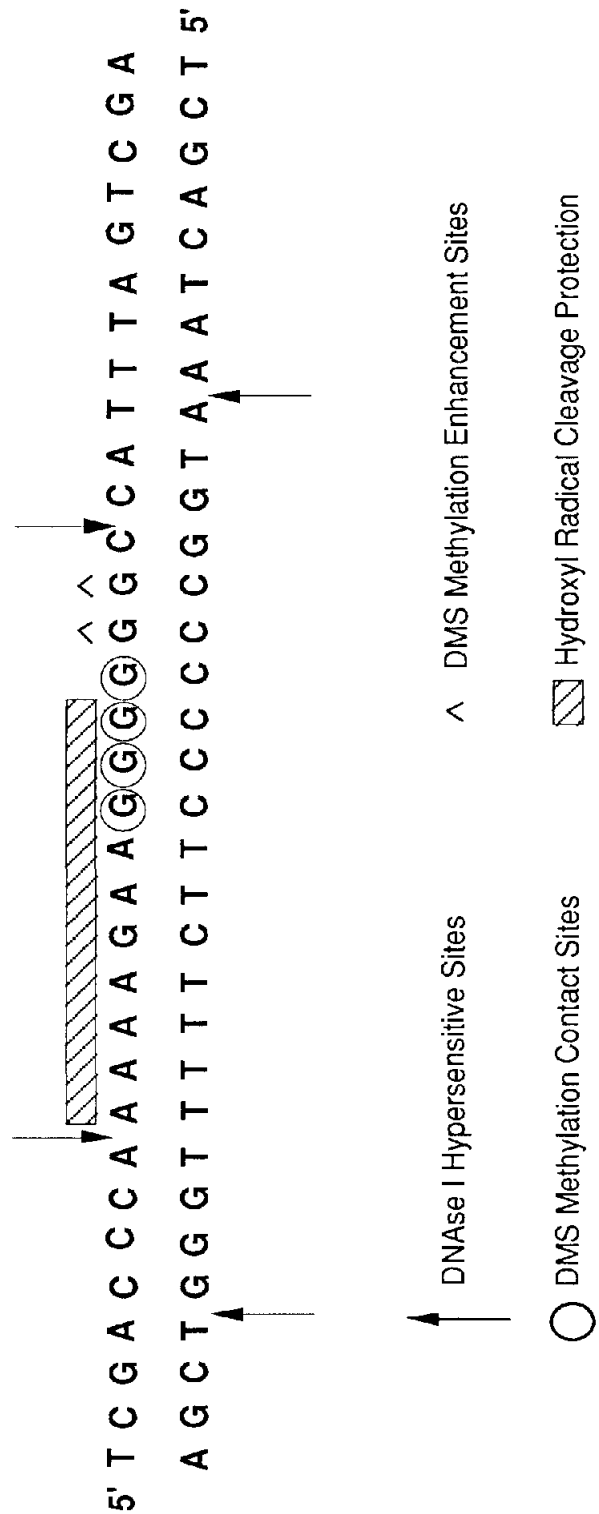
5' T C G A C C C A A A A A G A A G G G G C C A T T T A G T C G A
A G C T G G G T T T T T C T T C C C C C G G T A A A T C A G C T 5'
DNAse I Hypersensitive Sites
DMS Methylation Contact Sites
DMS Methylation Enhancement Sites
Hydroxyl Radical Cleavage Protection

Figure 8.

Figure 6A. Top strand DNAse I and DMS protection of G-box site 2. Lanes 1,3, and 5 were the minus protein controls, and 2,4, and 6 included GBF. DNAse I was performed at 0°C under the standard conditions listed in *Materials and methods* (lanes 1 and 2) and at 25°C for 15 minutes to duplicate the DMS conditions (lanes 3 and 4). DMS protection was performed at 25°C in 50 mM DMS for 15 minutes (lanes 5 and 6). All reactions were prebound for 5 minutes on ice. The lanes labeled R and Y are purine and pyrimidine sequence lanes, respectively.

Figure 6B. Bottom strand DNAse I and DMS protection of G-box site 2. Lanes 1,3, and 5 were the minus protein controls, and 2,4, and 6 included GBF. DNAse I was performed at 0°C under the standard conditions listed in *Materials and methods* (lanes 1 and 2) and at 25°C for 15 minutes to duplicate the DMS conditions (lanes 3 and 4). DMS protection was performed at 25°C in 50 mM DMS for 15 minutes (lanes 5 and 6). All reactions were prebound for 5 minutes on ice. The lanes labeled R and Y are purine and pyrimidine sequence lanes, respectively.

Figure 7. Densitometry plots of DNAse I and hydroxyl-radical cleavage protection experiments. Protection is plotted for the top strand of *SSA1* G-box site 2. Panel A is the hydroxyl-radical cleavage data, panel B is the DNAse I data.

Figure 8. Summary of the DNAse I, DMS, and hydroxyl-radical cleavage protection results on G-box site 2.

Specificity of GBF binding to the G-box element

Affinity-purified GBF was used to assay the equilibrium constant of GBF for G-box site 2 as well as to dA•dT and a mutant G-box site, G-box DPM 1, double-stranded salmon sperm DNA (dsDNA), single-stranded (ss) salmon-sperm DNA, and the HSE. The effects of adding increasing amounts of unlabeled G-box element on a G-box/GBF complex is shown in Figure 9. A com-

parison lane showing a lack of competition with HSE compared to G-box element is included in this figure.

A similar experiment to the gel-shift competition was performed by DNAse I protection. A protein fraction containing G-box and GC-box binding factors (GBF and ySp1) was used to show that the addition of excess unlabeled G-box sites specifically removed binding from G-box sites 1 and 2 without removing the GC-box footprint (see Figure 10).

G-box sites on the *HSF* promoter

We have examined the *HSF* promoter for GBF binding sites using affinity-purified GBF. We have found two sites in the promoter region near the TATA (see Figure 11), and another site at about -400 bp (data not shown). It is likely that the transcriptional induction of the *HSF* gene upon heat-shock is due to the presence of the G-box sites, because the *HSF* promoter does not contain an HSE homology (G.J.Wiederrecht, personal communication).

GBF Activity in HeLa Cells

The human HeLa cell line was examined for GBF binding activity. Nuclear extracts were prepared and chromatographed on heparin-Sepharose, and step eluted at 0.3 and 0.8 M KCl. An activity that recognized the *SSA1* G-box site 2 was found in the 0.8 M step, which was identical in protection with the yeast-derived GBF (see Figure 12).

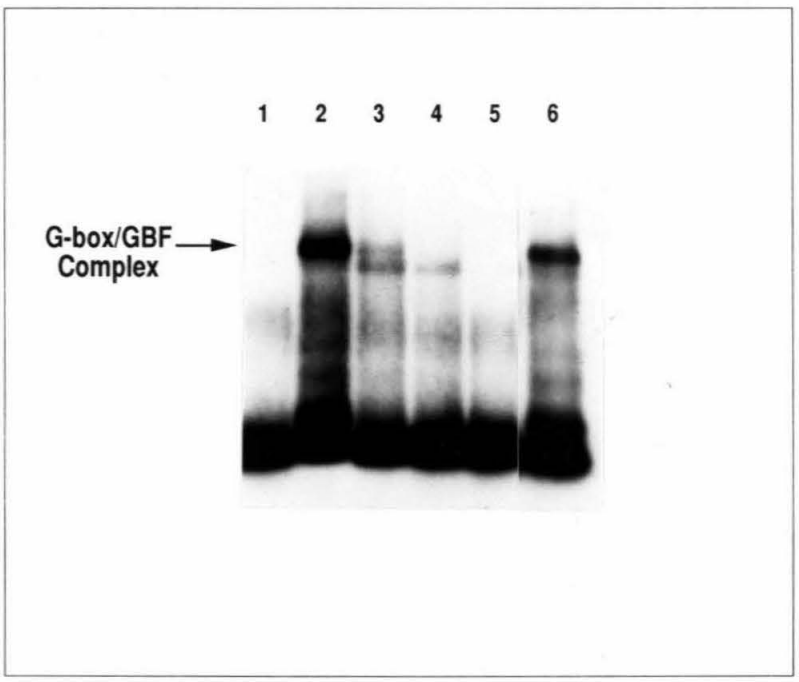

Figure 9.

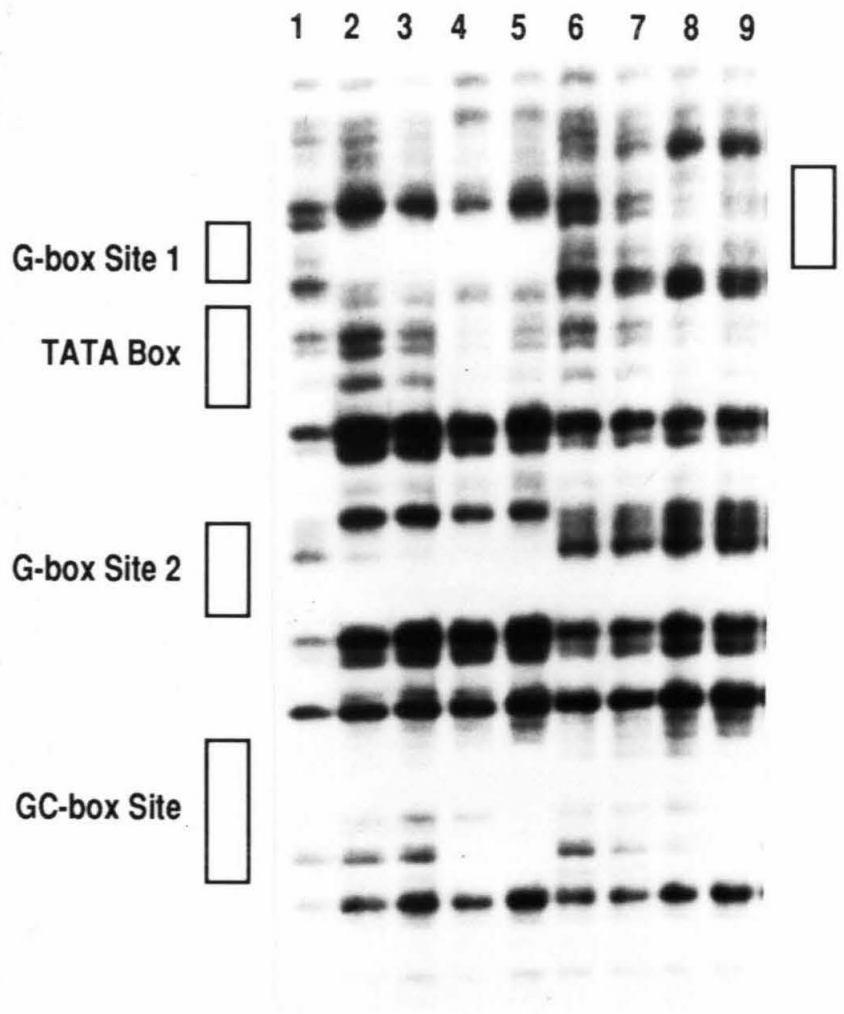

Figure 10.

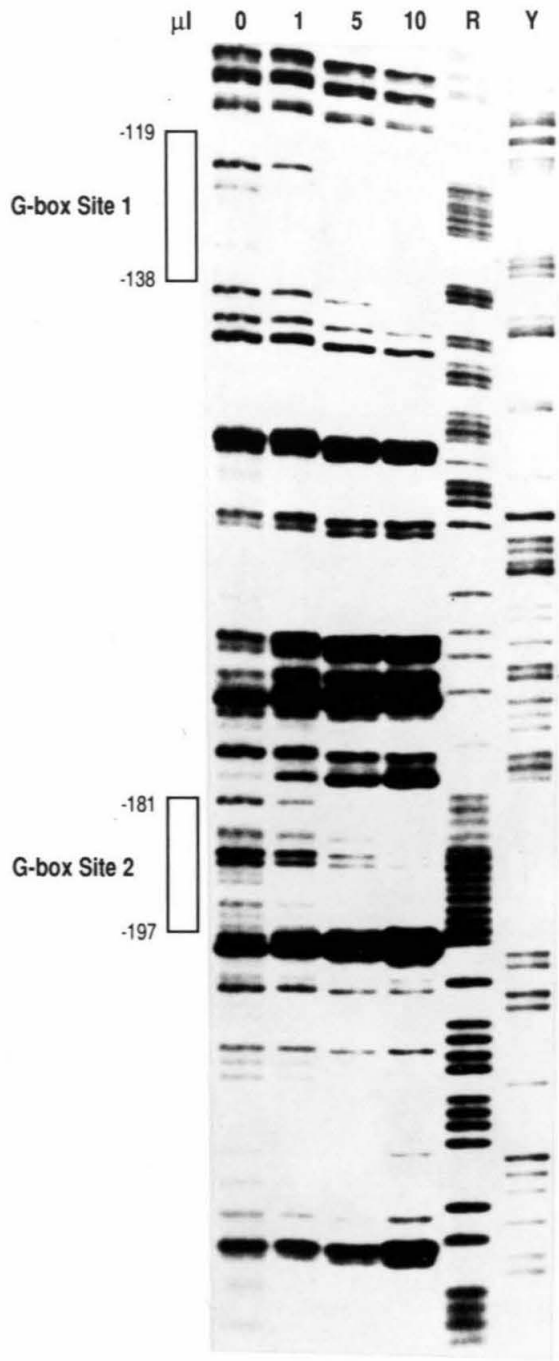

Figure 11.

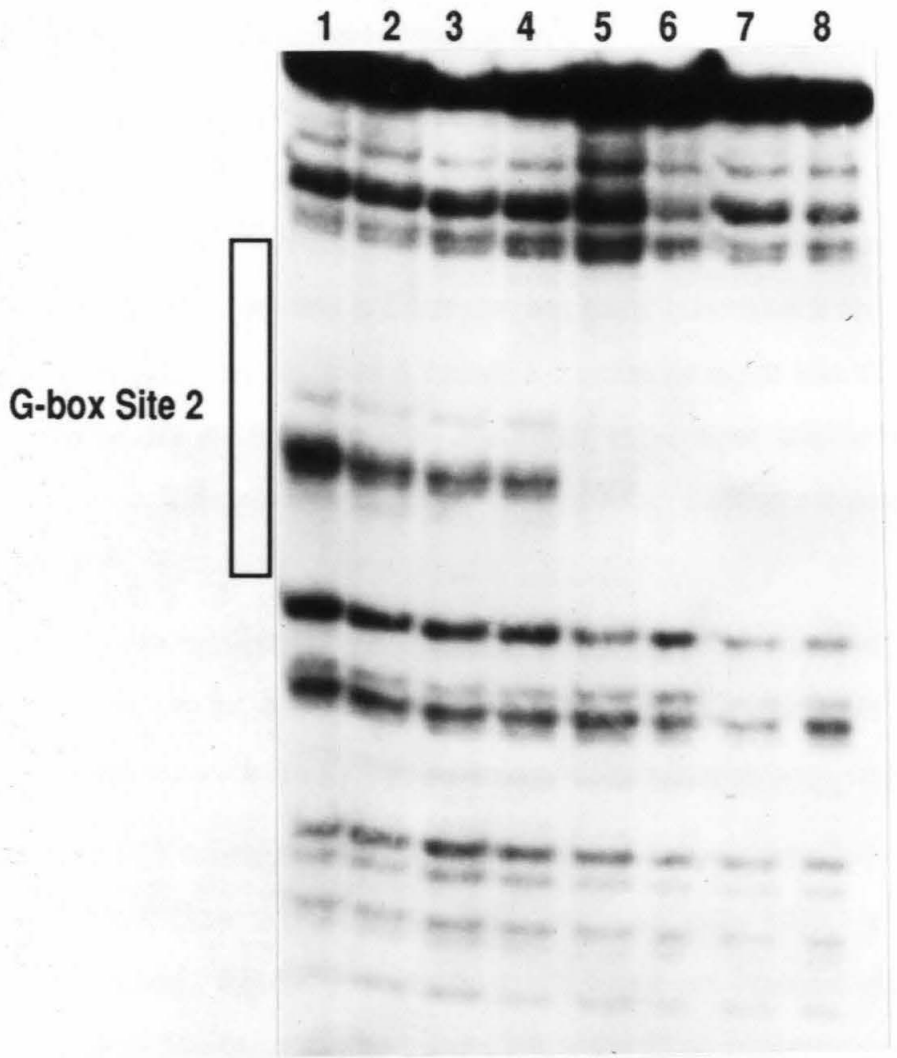

Figure 12.

Figure 9. Gel-shift analysis showing the competition of affinity GBF with various oligonucleotides. 20 femtomoles of labeled G-box site 2 is present in every lane. The first lane does not contain GBF, lanes 2 through 6 contain 1 footprinting protection unit (FPU) of GBF. Lane 2 contains GBF only, lane 3 has 200 femtomoles of unlabeled G-box competitor, lane 4 has 1 picomole G-box competitor, lane 5 has 5 picomoles G-box competitor, and lane 6 has 10 picomoles HSE competitor.

Figure 10. Competition of GBF on the *SSA1* promoter using excess unlabeled G-box sites. Lane 1 is the minus protein control, and lanes 2 and 6 have 1 μl, 3 and 7 have 2 μl, 4 and 8 have 5 μl, and 5 and 9 have 20 μl of a pool of 1.0 M KCl eluant from heparin-Sepharose. One picomole of G-box sites was added to reactions 6 through 9 prior to the addition of protein. In lanes 6 through 9 a protected region was observed that only occurred after the removal of GBF. The identity of this region and the factor responsible for the protection remains uncharacterized, however, it appears to overlap both G-box site 1 and HSE1.

Figure 11. GBF protection of two sites on the *HSF* promoter. A titration of GBF on the HSF is shown, with the number of microliters of GBF fractions used listed above the lane. The lanes labeled R and Y are purine and pyrimidine sequence lanes, respectively.

Figure 12. GBF activity in HeLa cells. Extracts were made as described in *Materials and methods*, and then loaded onto heparin-Sepharose and eluted with 0.3 M KCl and then 0.8 M KCl. Lane 1 was the minus protein control, lane 2 was incubated with 20 μl heparin-Sepharose flow-through, lanes 3 and 4 with 5 and 20 μl, respectively, of the 0.3 M KCl pooled step, lanes 5 and 6 with 5 and 20 μl of the first fraction eluting from the 1.0 M KCl step from heparin-Sepharose, and lanes 7 and 8 with 5 and 20 μl of the second fraction eluting from the 1.0 M KCl step from heparin-Sepharose.

A A A G A A G G G G G
A T A T A A G G G G T G
G A A A T A G G G T T G
A A A G A A G G A G A A
T T T T T A G G A G A G

$$A\,\frac{A}{T}A\,N\,\frac{A}{T}A\,G\,G\,G\,G\,\frac{A}{T}G$$

Figure 13. Determination of a binding concensus sequence for GBF. Five sites which affinity-purified GBF will bind to are shown, and a consensus is derived from these sites.

Discussion

We have identified a site-specific DNA binding protein, G-box binding factor or GBF, that recognizes a novel heat-shock element. This heat-shock element has been placed upstream of a *CYC1*/lacZ fusion and shown to convey a heat-shock inducible response, assayed by the production of ß-galactosidase in yeast. The mediation of the transcriptional activation by GBF has not been shown directly, but by analogy to many other systems, it can be surmised that the heat-shock activity conveyed by the DNA element, G-box, is mediated by a protein interacting with the DNA.

HSTF and GBF are separate proteins

GBF is not the HSTF by several important criteria. One is that affinity-purified GBF does not recognize an HSE, which is the HSTF binding site. Likewise, affinity-purified HSTF does not recognize a G-box, which is the GBF binding site. Furthermore, probing nitrocellulose bound affinity-purified HSTF and GBF with labeled HSE and G-box oligonucleotides demonstrates that different size proteins are responsible for the respective binding, and that

the GBF fraction does not contain HSE binding factors, and likewise the HSTF does not contain G-box binding factor. A final demonstration that HSTF and GBF are separate factors lies in the Western data, which shows that affinity-purified GBF is not recognized by the anti-HSTF antibody (data not shown).

GBF is highly conserved in evolution

The GBF encoding gene has not yet been cloned, although the cloning is presently in progress. However, we can still make statements regarding the conservation of this protein in diverse organisms. We have identified a binding protein from both HeLa cells and yeast that will recognize a yeast G-box site and can be affinity-purified. Furthermore, we have identified a binding site that can be bound by the yeast G-box binding factor *in vitro* in a *Drosophila* hsp70 gene promoter.

Interestingly, the consensus sequence that we have defined for GBF contains a great deal of homology with the bacterial sigma 32 recognition sequence. The GBF consensus sequence is $A\frac{A}{T}N\frac{A}{T}AGGGG\frac{A}{T}G$, and the sigma 32 consensus is $AT\frac{A}{T}ATGGGG$ (Niedhart, 1987). This is in strong contrast with the binding site for the HSTF and the sigma 32 recognition sequence, suggesting that GBF may be an older, primitive heat-shock responsive factor. Additionally, the G-box site is found overlapping the TATA sequence in a number of eukaryotic heat-shock promoters, further indicating that the role of GBF in eukaryotic cells may be similar to that of sigma 32 in bacteria; that is, GBF may function as a TATA factor replacement during heat-shock.

GBF interacts with one strand

The DMS and hydroxyl radical cleavage data provide strong evidence that the GBF only interacts with one strand, the G and A rich strand. This indi-

cates that the DNA binding motif of GBF will not be a zinc-finger or a helix-turn-helix structure, because both of these structures are associated with symmetrical binding elements and thus recognize both strands of the helix. The estrogen receptor has been recently demonstrated to interact selectively with the coding strand of the estrogen responsive element (ERE) by competition with single-stranded and double-stranded DNAs (Lannigan and Notides 1989). The ERE can act as a transcriptional activator when the estrogen receptor is bound by estrogen (Maurer and Notides 1987).

It is possible that GBF may recognize DNA in several modes, and that one aspect of its function is to open the double helix for the entry of polymerase or some other component of the transcriptional machinery. In contrast with the estrogen receptor, TATA factor, or TFIID, has been examined unsuccessfully for single-stranded binding capability and the ability to 'melt' DNA (see reviews: (Mermelstein 1989; Parker 1989; Saltzman and Weinmann 1989)). Likewise, a constitutive activation region found upstream of many genes (Struhl 1985) has been postulated to activate transcription by conferring an unusual structure on DNA (Chen, et al. 1987; Struhl 1986). The constitutive activation region is characterized by an oligo(dA)•oligo(dT) tract and is bound by Datin (Winter and Varshavsky 1989). This region has been determined to stably unwind rather than melt, at least when it is not bound by Datin (Kowalski, et al. 1988).

The G-box binding site has been found in the TATA proximal position of many heat-shock and non-heat-shock genes by sequence homology. The sequence homology used was : $A\frac{A}{T}N\frac{A}{T}AGGG\frac{G}{A}\frac{A}{T}G$. Several of the human hsp70 genes have this homology located in this position (Pelham 1982). Whether or not GBF recognizes the G-box sequence by melting the DNA or by

interacting with only one strand of a double-stranded site has yet to be determined.

Materials and methods

Yeast nuclear extracts

The yeast strain EJ926 was grown in modified minimal media (yeast minimal supplemented with 0.2 gm/liter Yeast Extract) to O.D. 3 in a 350 liter fermenter. The cells were harvested in an upright Sharples centrifuge at 8K, and resuspended in a 1 gram yeast/1 ml of 'Y' Buffer (1 M Sorbitol, 50 mM Tris (pH 7.8), 10 mM $MgCl_2$) and 30 mM DTT slurry. The slurry was fast frozen in liquid nitrogen.

The frozen yeast were thawed in a 30°C water bath in 500 gm aliquots, washed once in 'Y' buffer, and resuspended in 500 mls 'Y' buffer and 6 mM DTT with 50 mgs Zymolyase-100T. The cells were agitated gently at 30°C for 1 hour, and the percentage of Thaw 11 500 ml Beckman J10 bottles until spheroplasting was ≥ 80%. Cells were harvested and washed twice with 500 mls ice cold 'Y' buffer, and resuspended in 500 mls ice cold 'A' buffer (15 mM KCl, 10 mM Hepes (pH 7.6), 5 mM $MgCl_2$) with 3 mM DTT and 1 mM PMSF. The slurry was then homogenized with a Bellco 'B' pestle for 3 to 4 strokes, harvested and the supernate discarded. The pellicle was resuspended in 300 mls 'A' with 3 mM DTT and 1 mM PMSF, mixed for 5' at 4°C. The slurry was brought to 0.30 M $(NH_4)_2SO_4$ and 0.2 mM DIFP and mixed at 4°C for at least 30 minutes. The material was spun at 35K for 60 minutes in a Beckman 45Ti rotor. The resulting pellicle was discarded, 0.22 gm $(NH_4)_2SO_4$/ml. added to the supernate, and mixed on ice for 15 minutes. The material was respun at 35K for 15 minutes in a Beckman 45Ti rotor, the supernate discarded and the pellicle rinsed in 'D' buffer (25 mM Hepes (pH 7.6), 10% glycerol, 100 mM KCl,

0.1 mM EDTA, and 0.2% Triton X-100), then resuspended in 100 mls 'D' buffer with 1 mM DTT and 1 mM PMSF. The extract was dialyzed versus 'D' buffer with 1 mM DTT and 1 mM PMSF for ~ 4 hours.

Chromatography and column preparation

All purification was carried out at 4°C.

Standard ion-exchange chromatography

Prior to loading onto any column, the input fraction was dialyzed versus 0.1 M KCl until the concentration of KCl was below 120 mM, and the fraction spun at 10K for 10 minutes in a Beckman JA-21 rotor. The rate for loading and low salt wash for all columns was 3 to 4 column volumes per hour and the salt concentration was 0.1 M KCl.

Affinity chromatography

Affinity columns were prepared as previously described (Wiederrecht, et al. 1987), and the flow rate for loading and washing was between 5 and 10 column volumes per hour. The fractions were loaded at 200 mM KCl and washed at 350 mM KCl for at least 10 column volumes.

Oligonucleotides and plasmid constructions

Plasmids used in this paper were constructed by inserting the G-box and HSE oligonucleotides into pUC-9, the excision of the fragment with EcoRI and HindIII, and inserting the fragment into pSCCZ-103RHB and pSCCZ-103BHR cut with EcoRI and HindIII. The plasmids pSCCZ-103RHB and pSCCZ-103BHR are described in detail elsewhere (this thesis, Chapter 1).

Gel-shift assays

Gel-shift conditions were essentially as per Fried and Crothers. The gel system used was 3% low EEO Agarose in 50 mM Tris-HCl, 50 mM Boric Acid,

and 0.5 mM EDTA, all from Sigma Chemicals. The reaction (loading) buffer was 2% glycerol, 3% ficoll, 100 μg/ml BSA, and 20 mM K_2PO_4 plus the protein and DNA components added for each experiment.

DNA was labeled by polynucleotide kinase with γ-^{32}P-ATP. Concentrations of labeled DNA were determined by A_{260} or by titration of a gel-shift complex with a known amount of unlabeled G-box site (Letovsky and Dynan 1989). The number of units of G-box binding factor were determined by titrations with labeled and unlabeled DNA (Baker, et al. 1986). The number of units of G-box binding factor derived from this method was then compared with the number of units of GBF required to give a 50% DNAse I protection footprint by titrating 0.2 ng of labeled footprint template with a known GBF standard.

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Chapter 4 Appendix

Summary

Additional work that has been done with GBF that is supplemental in nature has been included in this appendix. Examples of affinity chromatography, as well as purification calculations have been included. Work that is still under progress, such as the cloning of *GBF* gene, is also mentioned at the end of the appendix.

Results

Gel-shift analysis

The gel-shift or gel-retardation assay has been used to examine the specificity of affinity-purified GBF for various DNA binding sequences. Some of these experiments were duplicated in DNAse I protection experiments to verify that the presence of a gel-shift complex correlated exactly with the appearance of a site-specific footprint. Figure 1 shows several gel-shift experiments.

Affinity chromatography

GBF was affinity-purified on a ligated-G-box-oligonucleotide-Sepharose affinity resin. This resin was constructed by cyanogen bromide activation of the Sepharose and subsequent coupling of the ligated oligonucleotide as described in Chapter 3 using the protocol described in Appendix C. Several G-box affinity resins were constructed with slightly different sequences. The following sequence (of the sequences tried) gave the best purification and yield of GBF: 5′ TCGAC CCAAA AAGAA GGGGG GCCAT TTAG 3′, with TCGA

overhangs on both ends (complementary strand is not shown). An example of the resolution of this column when loaded with heparin-Sepharose and Affigel-Blue purified factor is shown in Figure 2. Multiple rounds of affinity chromatography did not substantially improve the purification.

Gel renaturation

One of the key experiments that indicated the molecular weight of GBF was approximately 85 kD was the demonstration that affinity-purified GBF could be electrophoresed on a SDS-denaturing protein gel and a protein of about 80 to 90 kD electroeluted and renatured to give a DNAse I footprint on G-box site 2. One example of this experiment is shown in Chapter 4 Figure 5, and another example is shown in Figure 3.

Southwestern

The molecular weight of GBF on a denaturing gel was also determined by Southwestern analysis. The molecular weight indicated by this experiment was also 85 kD, corroborating with the gel-renaturation experiments and indicating that a major band at 85 kD when the affinity-purified GBF was silver-stained was GBF. An example of a Southwestern experiment with the HSTF as internal controls is shown in Figure 3 of Chapter 4. A similar experiment using the URF as an internal control is shown in Figure 4.

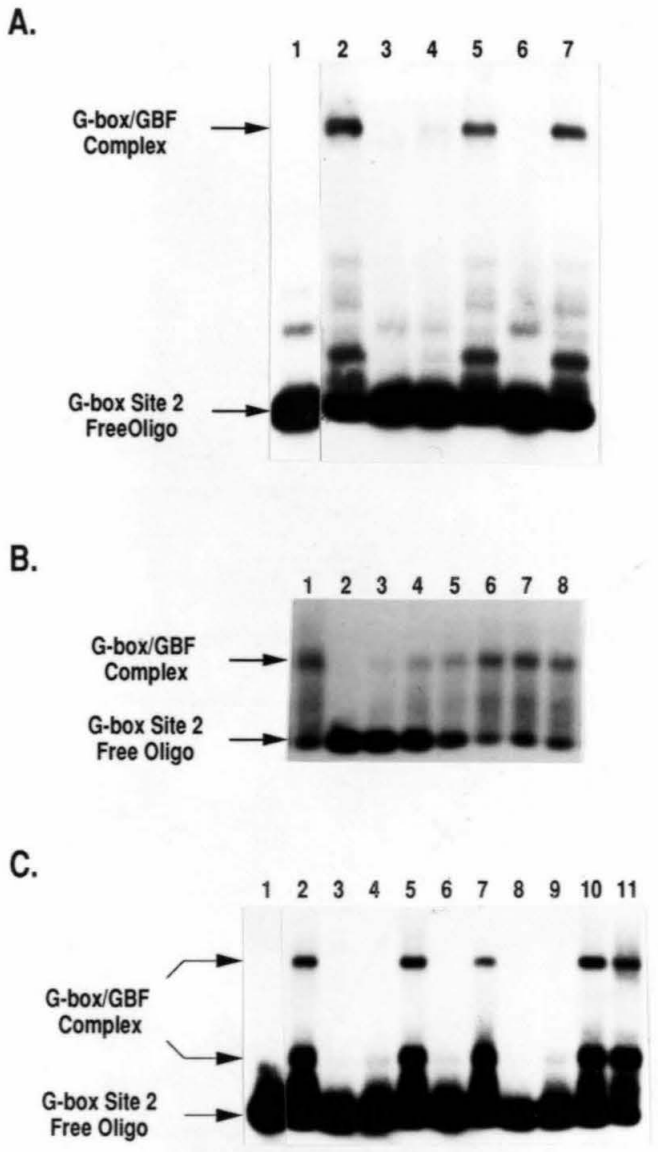
A.
1 2 3 4 5 6 7
G-box/GBF Complex
G-box Site 2 FreeOligo
B.
1 2 3 4 5 6 7 8
G-box/GBF Complex
G-box Site 2 Free Oligo
C.
1 2 3 4 5 6 7 8 9 10 11
G-box/GBF Complex
G-box Site 2 Free Oligo

Figure 1.

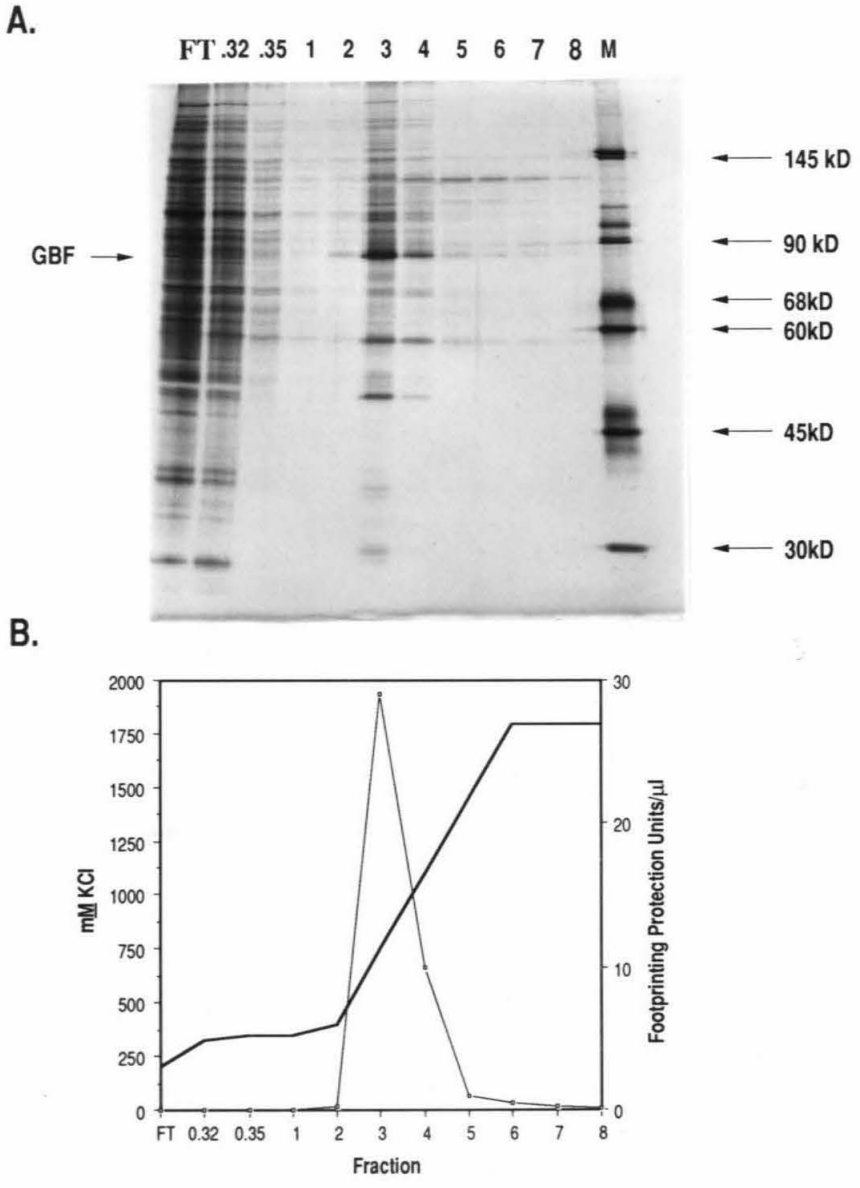
A.
FT .32 .35 1 2 3 4 5 6 7 8 M
GBF
145 kD
90 kD
68kD
60kD
45kD
30kD
B.
mM KCl
Footprinting Protection Units/µl
Fraction
FT 0.32 0.35 1 2 3 4 5 6 7 8

Figure 2.

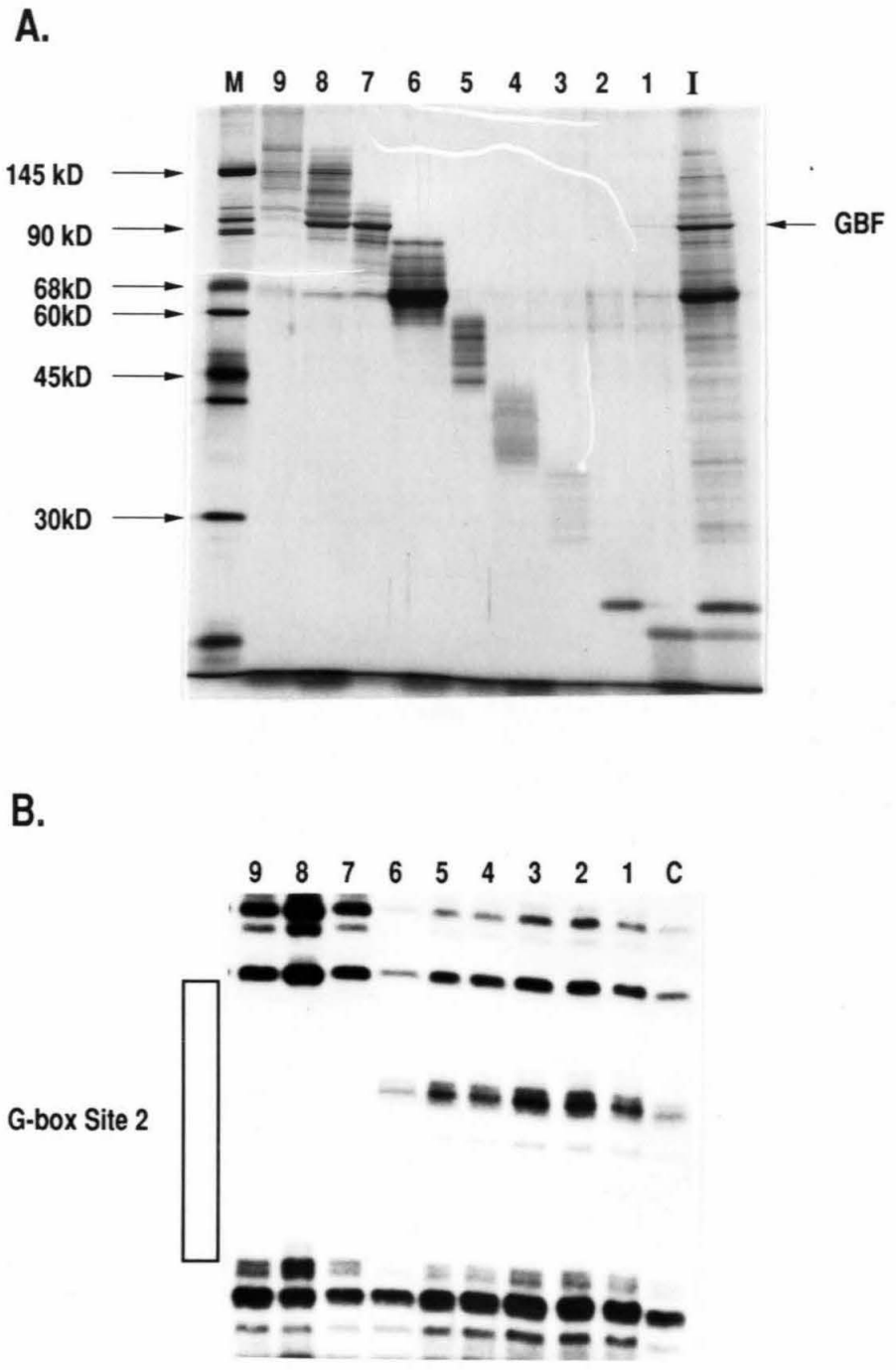
A.
M 9 8 7 6 5 4 3 2 1 I
145 kD
90 kD
68kD
60kD
45kD
30kD
GBF
B.
9 8 7 6 5 4 3 2 1 C
G-box Site 2

Figure 3.

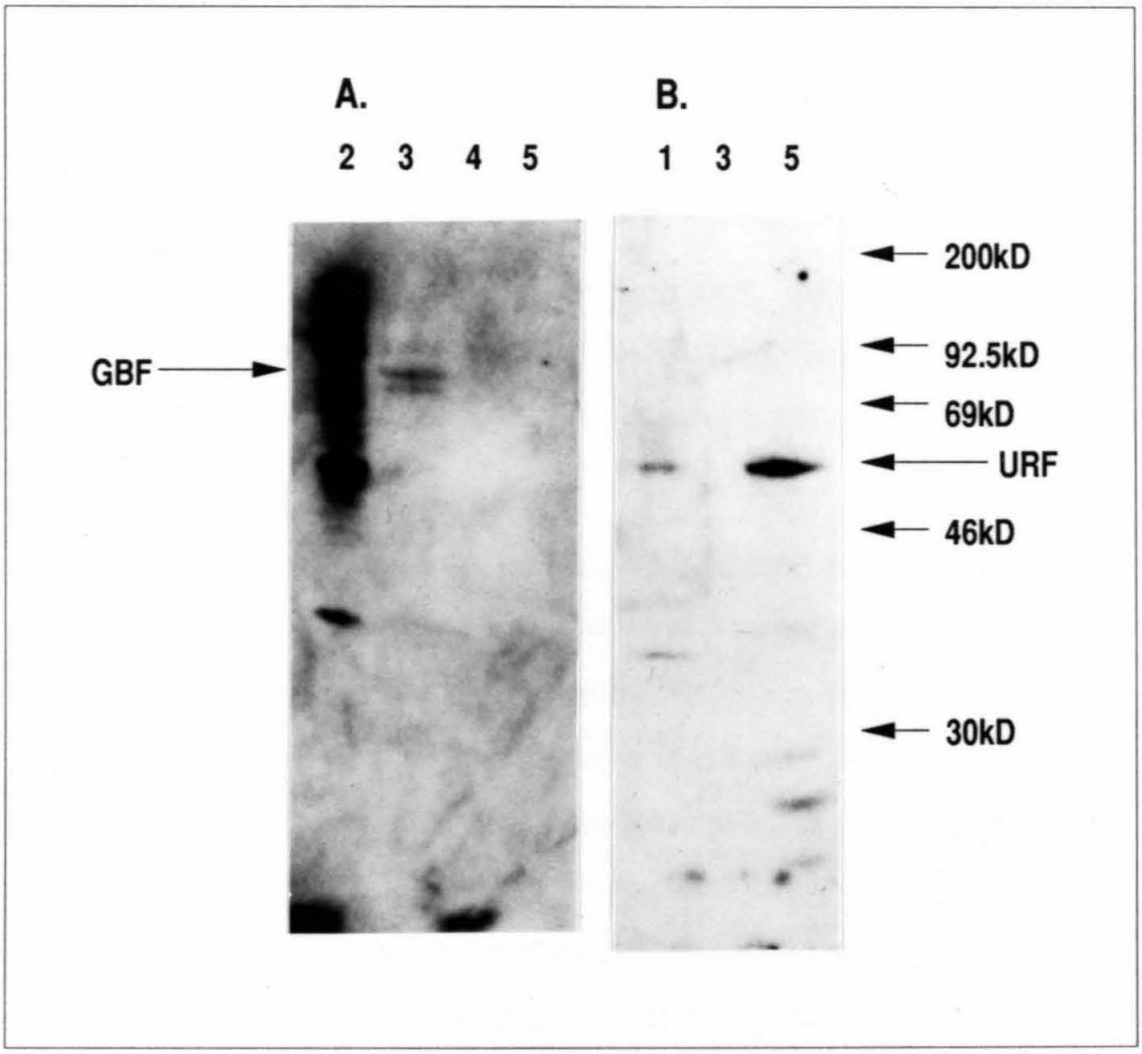

Figure 4.

Figure 1. Gel-shift experiments using a kinase-labeled G-box site 2 cloned fragment. Specific activity of the fragment was approximately 80,000 cpm/ng and 40,000 cpm of the fragment was used per lane.

A. Gel-shift using 0.5 footprinting unit (FPU) per lane of GBF (defined in Chapter 4) in lanes 2 through 7. GBF was preincubated with 100 ng of the following DNAs: G-box site 2 fragment, G-box DPM oligonucleotide, alternating dAdG oligonucleotide, denatured salmon-sperm DNA, and HSE oligonucleotide in lanes 3 through 7, respectively.

B. Titration of the GBF/G-box site 2 complex with dAdT. 0.5 FPU per lane of GBF was used in each lane, and 5 mg, 2 mg, 1 mg, 500 ng, 200 ng, 100 ng, and 50 ng of dAdT in lanes 2 through 8, respectively, were preincubated with GBF prior to the addition of labeled G-box site 2 fragment.

C. Titration of the GBF/G-box site 2 complex with monoclonal antibodies. 0.5 FPU of GBF was used in lanes 2 through 11. 1, 5, and 20 μl of the monoclonal line 4C2 raised against affinity-purified GBF was added to lanes 3 through 5, respectively. 2 and 10 μl of a subclone of this line, 4C2-4F2, was added to lanes 6 and 7, and 1, 5, and 20 μl of another subclone, 4C2-C4, was added to lanes 8 through 10. 1, 5, and 20 μl of preimmune control were identical, and the 5 μl control is shown in lane 11. Note that 20 μl of these monoclonals did not remove or shift the complex. This is consistent with the result of other workers that an excessive amount of MAb can interfere with the monoclonal antibody's ability to recognize antigen, presumably by a mechanism of self-recognition or self-aggregation at high concentration (S. Ou, personal communication). Other titrations with 4C2-C4 showed that 0.02 μl of this monoclonal was capable of inhibiting the formation of the complex. These results suggest that this monoclonal is recognizing an epitope at or near the DNA-binding domain.

Figure 2. GBF affinity chromatography on a ligated-G-box-oligonucleotide-Sepharose resin. Affigel-Blue 2.0 M KCl-eluted fractions were dialyzed to 200 mM KCl and loaded at 10 milliliters per hour onto 1 milliliter of G-box-oligonucleotide-Sepharose affinity resin. The column was washed extensively (50 milliliters) with 0.32 HGKET, with 10 milliliters of 0.35 HGKET, and then eluted with a gradient from 350 to 1000 mM KCl. GBF activity eluted primarily in fractions 3 and 4.

A. Silver-stained protein gel of the fractions eluting from the G-box site 2 affinity resin. 5 µl of the flow-through (FT), 0.32 and 0.32 M KCl washes, and 50 µl of the gradient fractions were loaded on the gel. The silver-staining was performed as described in Appendix C.

B. Plot of GBF activity and the KCl concentration of the chromatographic fractions. Fractions containing G-box binding activity (GBF) are indicated. The fractions containing at least 1 footprinting unit (FPU) per 10 µl were further titrated. Footprinting and activity calculations were done as described in the main body of Chapter 4.

Figure 3. Elution and activity profile of gel isolated and renatured GBF fractions. Panel A shows a silver stained gel of fractions eluted from a denaturing protein gel. Lanes 1 through 8 are eluted fractions 1 through 8, with lane M containing protein standards and lane I was the input, i.e., the fraction loaded onto the elution gel. Panel B shows the DNAse I protection assay using the isolated, renatured fractions 1 through 8. Lane C is the minus protein control reaction.

Figure 4. Determination of the molecular weight of GBF by Southwestern. Lane 1 was loaded with 50 µg of heparin-Sepharose 0.32 M KCl pooled fractions, lane 2 was loaded with 50 µg of heparin-Sepharose 1.0 M KCl pooled fractions, lane 3 was loaded with 1000 footprint protection units of GBF, lane 4 with approximately 50 footprint protection units of HSTF, and lane 5 with approximately 50 µl of affinity-purified URF.

A. The Southwestern blot is probed with γ-^{32}P kinased G-box site 2 oligonucleotide.

B. The Southwestern blot is probed with γ-^{32}P kinased URE oligonucleotide.

Purification calculations

GBF was purified by the chromatography scheme presented in Figure 1 of Chapter 4 several times. The recovery and overall purification of GBF was calculated and the results are shown in Table 1. The recovery percentages were similar, and only in the case of one of the Affigel-Blue runs was there an appreciable loss of GBF activity, and in that case the loss was due to a higher than normal step (the step was at 0.5 M KCl rather than 0.4). This column was somewhat variable in other runs as well, with the primary variable being the amount of residual detergent left in the fractions that are loaded onto the Affigel-Blue resin. Apparently the detergent has a high affinity for the resin, excluding proteins from binding when detergent is present.

	A		B		C	
Fraction	**Protein**	**Activity**	**Protein**	**Activity**	**Protein**	**Activity**
YNE	1,000	>200,000	250	>20,000	1,000	>200,000
Heparin	100	>300,000	25	~40,000	100	>200,000
Affigel-Blue FT&0.4	80§	200,000	20	<10,000	80	<10,000
Affigel-Blue 2.0	20	250,000	5	~40,000	20	>200,000
Affinity FT	20	<10,000	5	<5,000	20	<10,000
Affinity 1.0	<1	>100,000	<1	~40,000	<1	>100,000

§This column was washed with 0.5 M KCl rather than 0.4 M.

Table 1. Purification of GBF from yeast spheroplast-derived nuclear extracts. The column labeled Protein was measured using the method of Bradford (see Appendix C) and represents the total number of milligrams of protein in the pooled fractions. The column labeled Activity was the footprinting activity in total footprinting units in the pooled fractions measured by titrating the pools on G-box site 2 and determining the 50% protection point by densitometry (see Chapter 4 for more details). A, B, and C are separate purification runs.

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Appendix A

Vectors, Plasmid Constructions and Subclones

Table of Contents

Results

ZDØ subclone series of the *SSA1* promoter

A series of constructions subcloning various fragments of the *SSA1* promoter were constructed to facilitate the footprinting of sites distal to 6 base-pair restriction endonuclease ('6 cutters') recognition sites. Many of these subclones were also inserted into a yeast shuttle vector and the ability of that section of the promoter to activate transcription of a *CYC1/LacZ* fusion assayed. All of the ZDØ subclones were inserted into convenient sites in the bacterial subcloning vectors pUC-9 or pUC-18. Figure 1 graphically displays the regions subcloned, and Table 1 lists the orientation and restriction sites used to clone the various fragments into the vector.

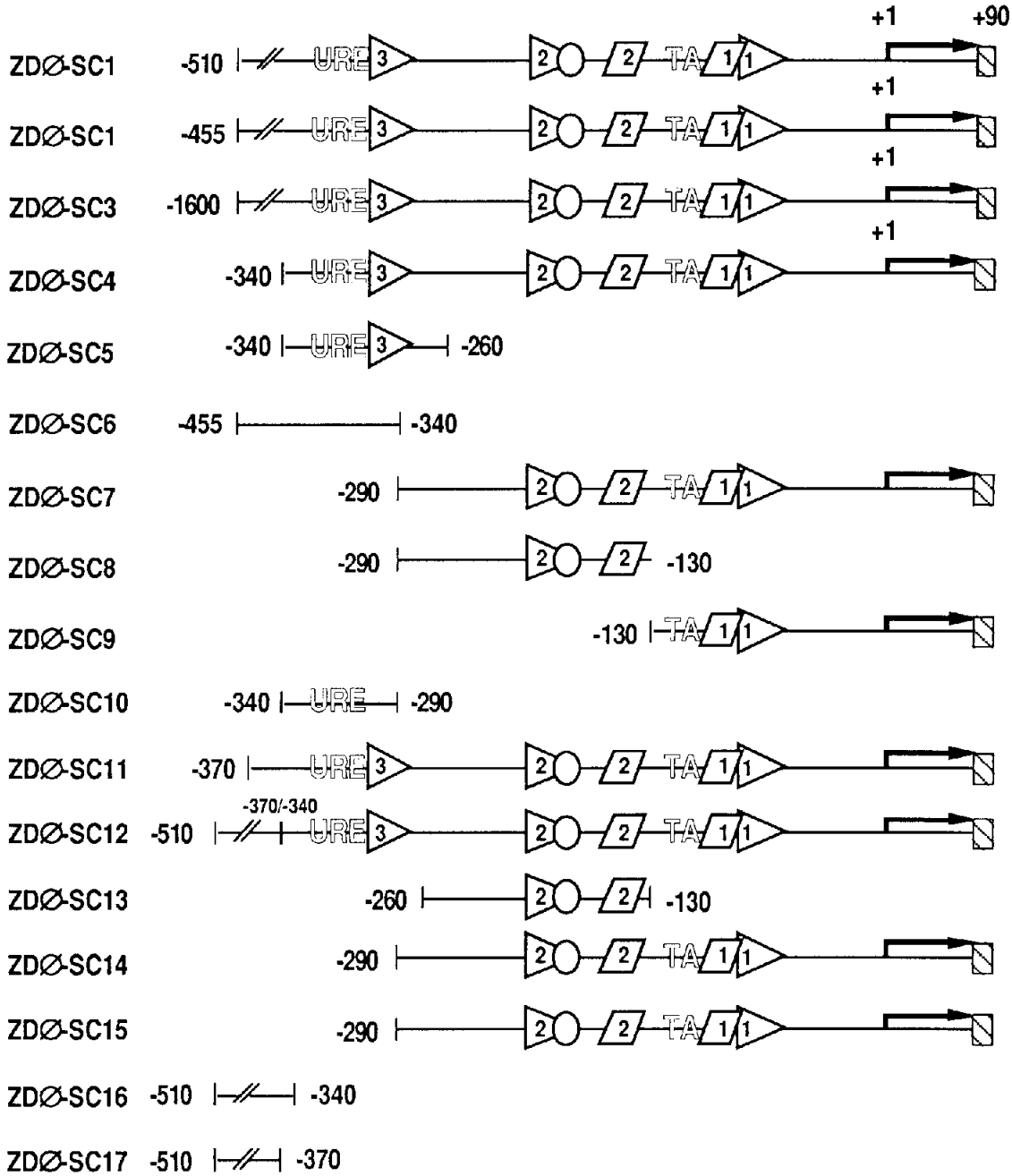

Figure 1. *SSA1* fragments inserted into pUC-9 or pUC-18. Numbers written to the left and right of the graphical representation of the fragment refer to the distance from the startpoint of transcription in the intact *SSA1* gene (Slater and Craig 1987).

ZDØ#	WAK#	Vector	Cell	X Sites	In Sites	Insert Size
SC1	51	pUC-9	HB101	Sal I, BamHI	Sal I, BamHI	600 bp
SC2	52	pUC-9	HB101	PstI, BamHI	PstI, BamHI	550 bp
SC3	53	pUC-9	HB101	EcoRI, BamHI	EcoRI, BamHI	1600 bp
SC4	64	pUC-9	HB101	Hinc II, BamHI	Hinc II, BamHI	430 bp
"	99	"	TB1	"	"	"
SC5	71	pUC-9	TB1	Hinc II, Alu I	Hinc II	90 bp
SC6	85	pUC-9	HB101	PstI,Hinc II	PstI, Hinc II	120 bp
SC7	86	pUC-18	TB1	XbaI, BamHI	XbaI, BamHI	380 bp
SC8	87	pUC-18	TB1	XbaI, SauIIIA	XbaI, BamHI	160 bp
SC9	88	pUC-18	TB1	SauIIIA, BamHI	BamHI	230 bp
SC10	84	pUC-9	TB1	Hinc II, XbaI	Hinc II, XbaI	50 bp
SC11	251	pUC-9	XL1-Blue	FnuDII, SauIIIA	HincII, BamHI	200 bp
SC12	252	ZDØ-SC4	XL1-Blue	Hind III, FnuDII from ZDØ-SC1	Hind III, Hinc II	150 from SC1 560 total
SC13	253	pUC-9	XL1-Blue	Alu I	Hinc II	130 bp
SC14	254	ZDØ-SC8	XL1-Blue	Hinc II	Hinc II	160 from SC1, 320 total
SC15	255	ZDØ-SC8	XL1-Blue	Hinc II	Hinc II	160 from SC1, 320 total
SC16	266	pUC-9	XL1-Blue	Hinc II	Hinc II	160 bp
SC17	267	pUC-9	XL1-Blue	Hind III, FnuDII	Hind III, Hinc II	140 bp

Table 1. Pertinent information on the cloning of each of the ZDØ subclones. The column labeled **WAK#** is the reference number of the plasmid transformed into the bacterial cell type listed in the column **Cell**, and frozen at -70°C. The column labeled **X Sites** refers to the restriction site or sites used to excise the fragment from the *SSA1* promoter, and the column labeled **In Sites** lists the site or sites in the pUC polylinker that the fragment was inserted into. **Insert Size** is the size fo the *SSA1* fragment inserted into the vector. All clones were derived from the master plasmid ZDØ-2 (Slater and Craig 1987).

The pSCCZ-100 series of yeast shuttle vectors

The pSCCZ-100 series of plasmids were constructed using pSEYC-102 (Bankaitis, et al. 1986; obtained from Dr. S. Emr), a CEN IV-containing shuttle vector, and pLG-669-Z (Guarente and Ptashne 1981). The *CYC1/LacZ* fusion in pLG-669-Z was excised by cutting the plasmid with Sal I, DNA Polymerase I Klenow fragment filling the 5′ overhang, and cutting with Sst I. This frag-

ment was then inserted into pSEYC-102 cut with Sma I and Sst I. This vector was designated pSCCZ-100 (for *Saccharomyces cerevisiae* CEN IV-containing vector with a *LacZ* fusion) and was the master plasmid used to derive all other 100 series plasmids.

The plasmid pSCCZ was made by taking pSCCZ-100 and excising the 700 base pair Xho I fragment. This was accomplished by subcloning the EcoRI site to BamHI site *CYC1* fragment into the EcoRI and BamHI sites of pUC-9, digesting the subclone with Xho I, ligating, re-isolating transformants which were missing the fragment, and cloning the EcoRI and BamHI fragment derived from this subclone back into pSCCZ-100 cut with EcoRI and BamHI. This intermediate step was necessary because of the two Xho I sites present in the pSCCZ-100 vector. pSCCZ-103ΔBSN was made by the digestion of pSCCZ-100 with Xho I, filling the 5′ overhang with DNA Polymerase I Klenow fragment ligating EcoRI linkers (Pharmacia) onto the blunt end, and cutting with EcoRI. The vector was ligated and retransformed, and a transformant with the XhoI to BamHI distance reduced was isolated and the presence of a Xho I site adjacent to the EcoRI verified. This plasmid was then cured of the single sites for Bgl II, Sal I and Nru I by restriction and subsequent DNA polymerase I filling and ligation, and re-isolation of the plasmid from transformants after the removal of each site. Plasmids pSCCZ-103RHB and pSCCZ-103BHR were constructed by inserting the RHB oligonucleotide into the EcoRI site of pSCCZ-103ΔBSN.

RHB oligonucleotide: AATTC AAGCTT AGATCT. The opposite strand was made complementary to this sequence with AATT 5′ overhangs on both ends.

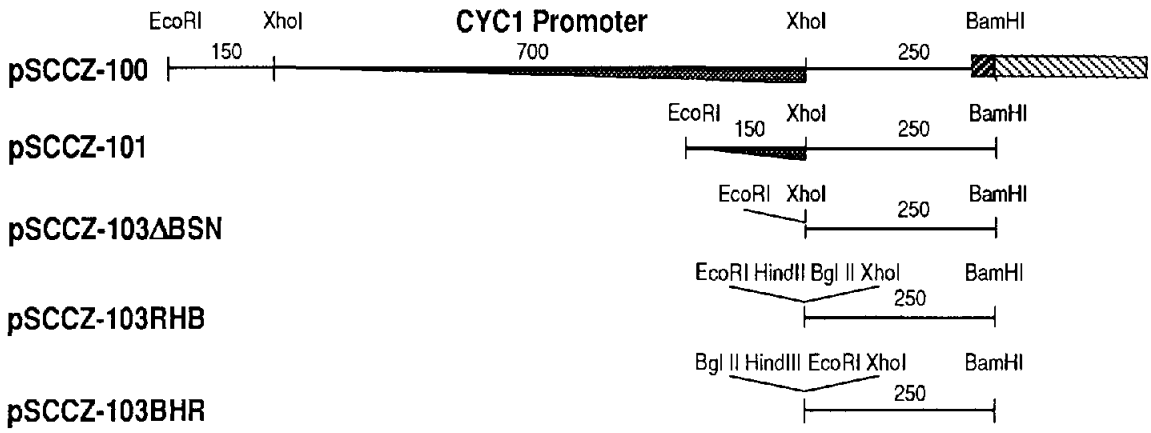

Figure 2. The pSCCZ-100 series of yeast shuttle vectors. The derivation of pSCCZ-103RHB and pSCCZ-103BHR are described in the text. These two plasmids have been used to assay oligonucleotide sequences for transcriptional activity after the introduction of the oligonucleotide and transformation into yeast. Several of the ZDØ subclones have also been tested in this vector.

The pSCCZ-200 series of yeast shuttle vectors

pSCCZ-200 Series: pSCCZ-200 was constructed by joining the *SSA1* 1.6 kilobase fragment derived from ZDØ-2 into pSEYC-102 cut with EcoRI and BamHI. The entire series was constructed so that an EcoRI site is present at the upstream side of the *SSA1*-derived sequence. The activity of these plasmids was determined in the yeast strain TD28 and the results are in Chapter 1.

A set of plasmids have been constructed for assaying inserts in the *SSA1* promoter and are designated pSCCZ-255RHB, pSCCZ-205RHB and pSCCZ-205RB. The RB designation indicates that the RB oligonucleotide was inserted at the EcoRI site of the parent plasmid pSCCZ-205. The RB oligonucleotide has the sequence of AATTC AGATCT and the opposite strand is complementary to it: AGATCT GAATT, giving rise to blunt ends on both sides of the hybridized oligonucleotide.

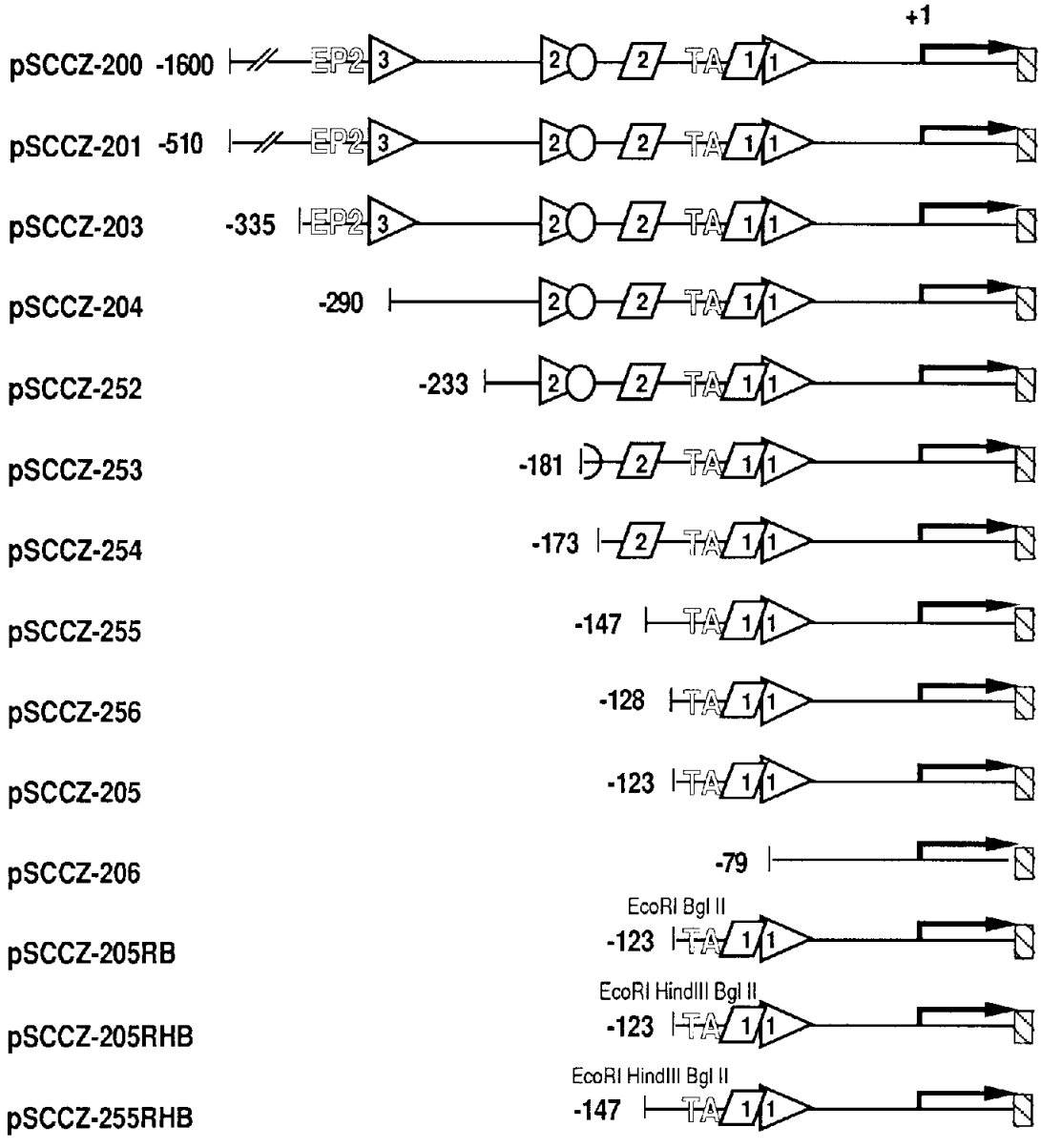

Figure 3. The pSCCZ-200 series of yeast shuttle vectors. The derivation of the series is described *in the text*. The last two plasmids, pSCCZ-205RHB and pSCCZ-255RHB, have been used to assay oligonucleotide sequences for transcriptional activity after the introduction of the oligonucleotide and transformation into yeast in the same manner as the pSCCZ-100 series.

Vectors used

A schematic representation of the pSCCZ-100, pSCCZ-200 and pUC-9 vectors are shown in Figure 4, 5 and 6, respectively.

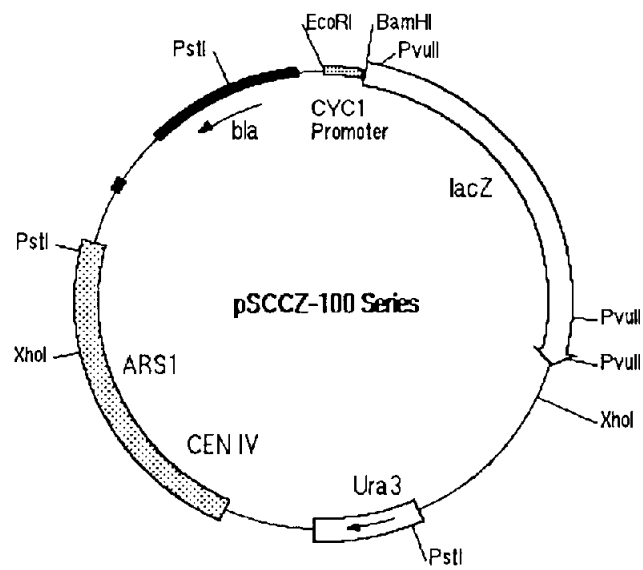

Figure 4. pSCCZ-100 series shuttle vector.

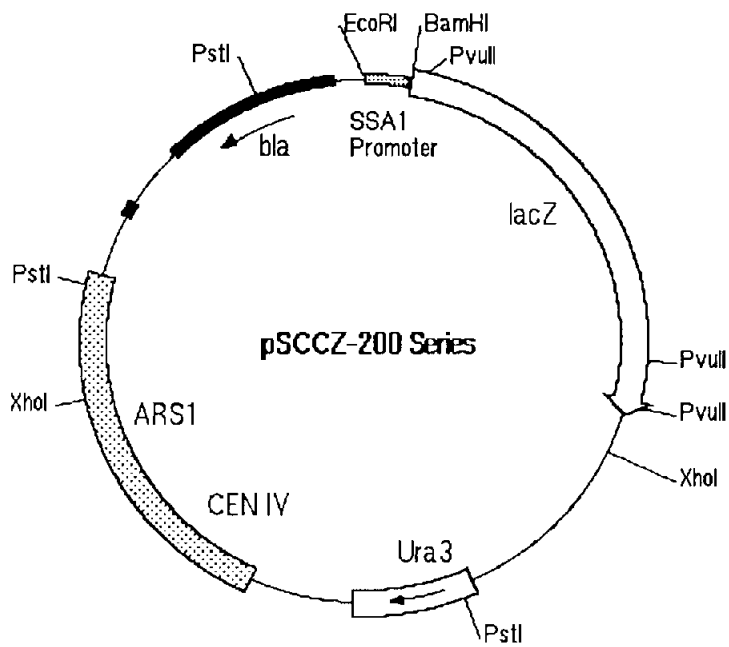

Figure 5. pSCCZ-200 series shuttle vector.

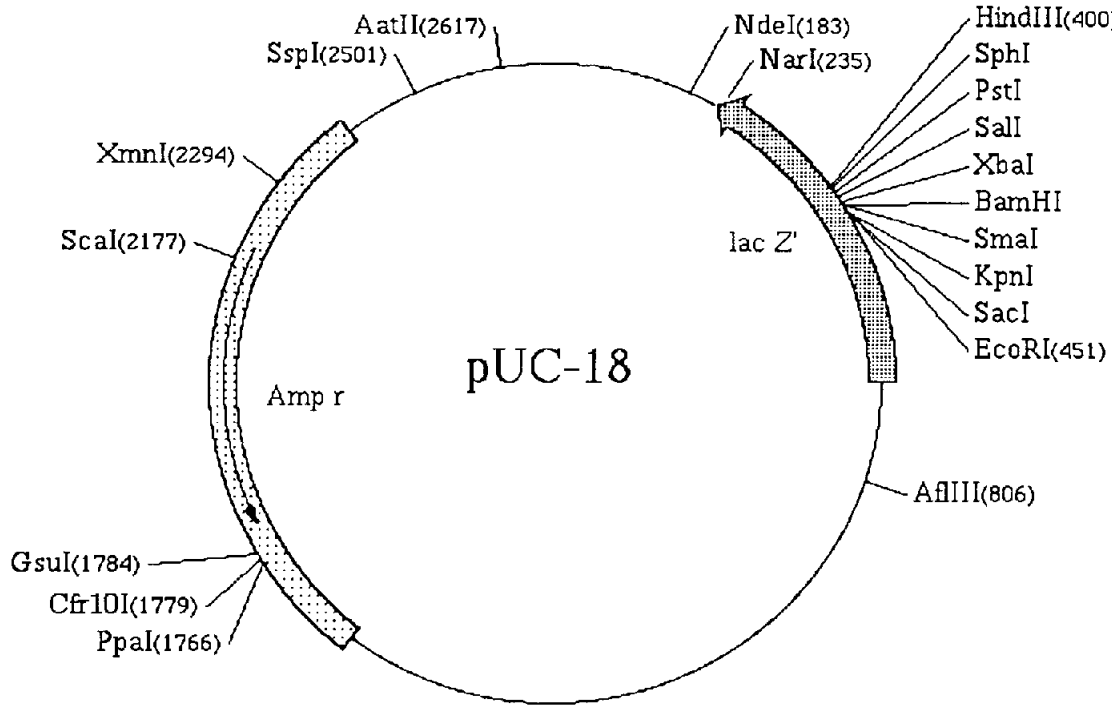

Figure 6. Diagram of pUC-18.

Oligonucleotides used in this thesis

A summary of all of the oligonucleotides used during the construction of shuttle vectors, assayable sites, gel-shift experiments and oligonucleotide affinity columns are listed below.

G-box 1

```
    -Sal I-
5'  TCGAC    CCAAA    AAGAA    GGGGG    GCC
    ----G    GGTTT    TTCTT    CCCCC    CGGAGCT 5'
                                          -Xho I-
```

23 Base pair Oligonucleotide

G-box 2 or GC 1

```
    -Sal I-
5'  TCGAC    CCAAA    AAGAA    GGGGG    GCCAT    TTAG
    ----G    GGTTT    TTCTT    CCCCC    CGGTA    AATCAGCT 5'
                                                  -Sal I-
```

29 Base pair Oligonucleotide

G-box 3

```
    -Sal I-
5'  TCGAC    CCAAA    AAGAA    GGGGG    GCCAT    TTAC
    ----G    GGTTT    TTCTT    CCCCC    CGGTA    AATGAGCT 5'
                                                  -Xho I-
```

29 Base pair Oligonucleotide

G-box 4

```
    -BamH I-
5'  GATCC    AAAAA    GAAGG    GGGGC    CATTA
    ----G    TTTTT    CTTCC    CCCCG    GTAATCTAG 5'
                                          -Bgl II-
```

25 Base pair Oligonucleotide

G-box DPM

```
    -Sal I-
5'  TCGAC    CCAAA    AAGAA    CCGGG    GCCAT    TAC
    ----G    GGTTT    TTCTT    GGCCC    CGGTA    ATGAGCT 5'
                                                  -Xho I-
```

28 Base pair Oligonucleotide

GT-box

```
    -Sal I-
5'  TCGAC    AAATA    AAGCA    CGTGT    GCGCG    GTGTG
    ----G    TTTAT    TTCGT    GCACA    CGCGC    CACACAGCT 5'
                                                    -Sal I-
```

30 Base pair Oligonucleotide

GC-box 3

```
    -Bcl I-
5'  GATCA    TCGGC    GGCAA    AAGGG    AGA
    ----T    AGCCG    CCGTT    TTCCC    TCTCTAG 5'
                                          -Bgl II-
```

23 Base pair Oligonucleotide

ARE (SV40)

```
    -BamH I-
5'  GATCC    ATGGT    TGCTG    ACTAA    TTGA
    ----G    TACCA    ACGAC    TGATT    AACTCATG 5'
                                         -Bgl II-
```

24 Base pair Oligonucleotide

HSE (YG100 Site 3)

```
    -BamH I-
5'  GATCC    TTTCC    AGAAC    ATTCT    AGAAA
    ----G    AAAGG    TCTTG    TAAGA    TCTTTCATG 5'
                                             -Bgl II-
```

25 Base pair Oligonucleotide

TATA1 (Yeast Consensus)

```
    -BamH I-
5'  GATCC    AGGTA    TATAA    ATGCA
    ----G    TCCAT    ATATT    TACGC    TCTAG 5'
                                -Bgl II-
```

20 Base pair Oligonucleotide

RHB (Linker)

```
    -Eco RI-   -Hind III-      -Bgl II-
5'  AATTC      AAGCTT      AGATCT
    ----G      TTCGAA      TCTAGA    TTAA 5'
                                  -Eco RI*-
```

17 Base pair Oligonucleotide

RB (Linker)

```
    -Eco RI-    --Bgl II-
5'  AATTC       AGATCT
    ----G       TCTAGA   TTAA 5'
                         -Eco RI*-
```

11 Base pair Oligonucleotide

References

Bankaitis, V. A., Johnson, L. M. and Emr, S. D. 1986. Isolation of yeast mutants defective in protein targeting to the vacuole. *Proc. Natl. Acad. Sci.* **83:** 9075-9079.

Guarente, L. and Ptashne, M. 1981. Fusion of *Escherichia coli lac*Z to the cytochrome c gene of *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci.* **78:** 2199-2203.

Slater, M. R. and Craig, E. A. 1987. Transcriptional regulation of an hsp70 heat shock gene in the yeast *Saccharomyces cerevisiae. Mol. Cell. Biol.* **7:** 1906-1916.

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Appendix B

Purification of RNA Polymerase I, II and III from yeast

Table of Contents

Results

RNA Polymerase purification from spheroplasts

RNA Polymerase II is primarily found in the supernate of the initial 10,000 gravity spin after lysing the cells (S10). This is shown by the purification of RNA polymerase from the S100 (S10-derived) as well as the P100 (P10-derived) portion of a yeast spheroplast extract (see Appendix C for experimental details). In all yeast extracts that have been prepared using this method, more than 80% of the RNA polymerase II is in the S100 portion of the extract. In the example purification in this section, the level of RNA polymerase II in the P100 was undetectable.

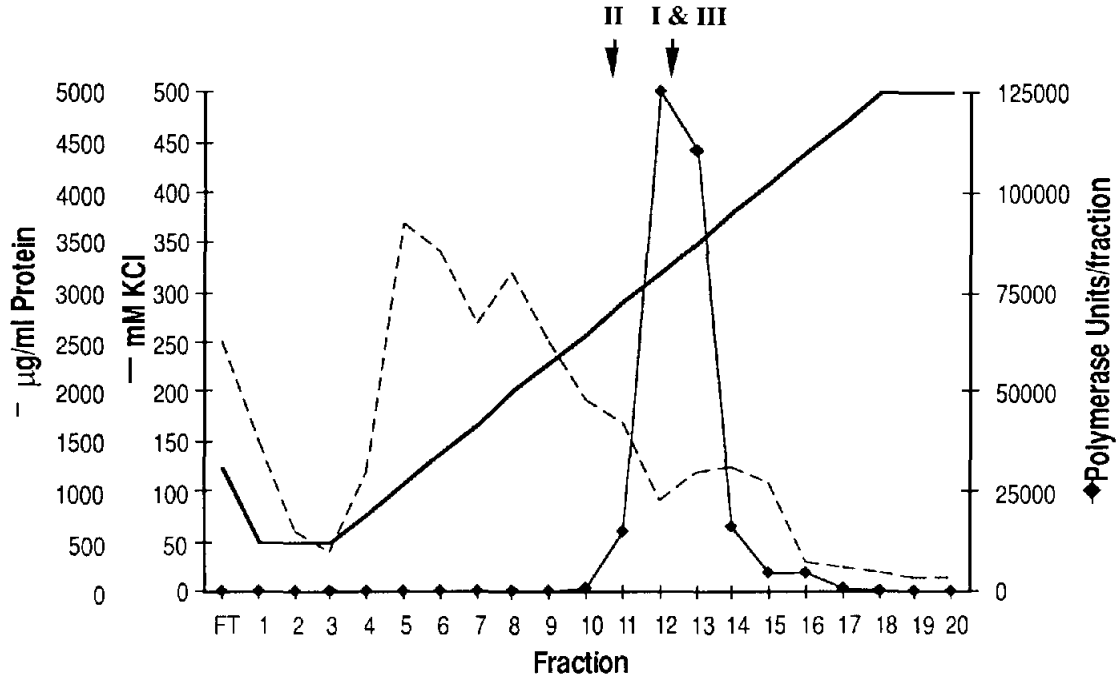

Figure 1. RNA Polymerase I and III behavior on a heparin-Sepharose column. Yeast nuclear extract was prepared by the method described in Appendix C, loaded and gradient eluted from heparin-Sepharose. Activity units are picomoles UMP incorporated per 20 minutes per fraction.

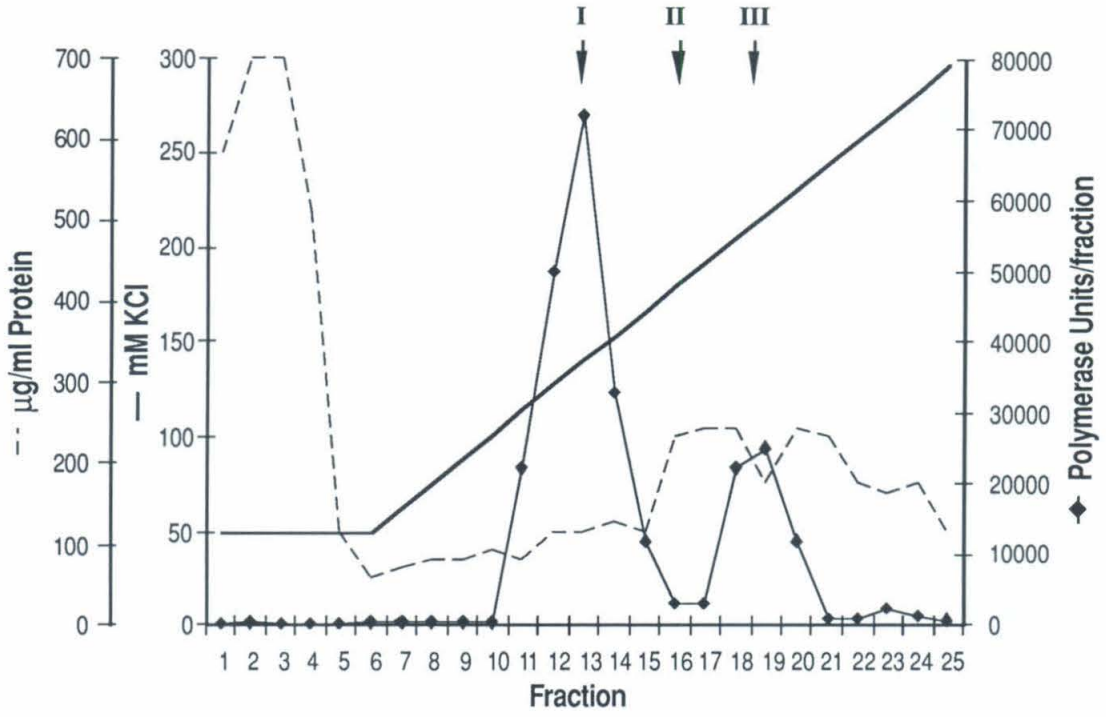

Figure 2. RNA Polymerases I and III loaded onto A-25 after heparin-Sepharose chromatography. Fractions 11 through 14 of the column shown in Figure 1 was loaded, washed and gradient eluted from A-25.

Stage	pmoles activity	milligrams Protein	% Recovery	pmoles/mg
YNE	175,000	250	-	700
Heparin	250,000	40	140	6,000
A-25,Pol I	190,000	4	?	50,000
A-25,Pol III	60,000	4	?	15,000
A-25, total	250,000	8	100	-
P-11, Pol I	210,000	1	110	210,000
P-11,Pol III	40,000	0.5	66	80,000

Table 1. Recovery and purification data from the yeast nuclear extract purification of RNA Polymerases I and III. Isolated RNA Polymerase I and III have values of 200,000 and 250,000 pmoles incorporation of UMP per 20 minutes at 30°C per milligram of enzyme. respectively (Hager, et al. 1977).

RNA polymerase II purification from yeast nuclear extract S100 fraction.

A large percentage of the RNA Polymerase II activity is found in the S10 portion of the extract. This material is precipitated by the same method as the lysed nuclei (S100, see Appendix C), dialyzed and run on heparin-Sepharose, A-25, P-11 and finally sized using either gel-filtration or gradient centrifugation. Examples of each of these steps is shown in Figures 3 through 6 and a compilation of recovery is shown in Table 2.

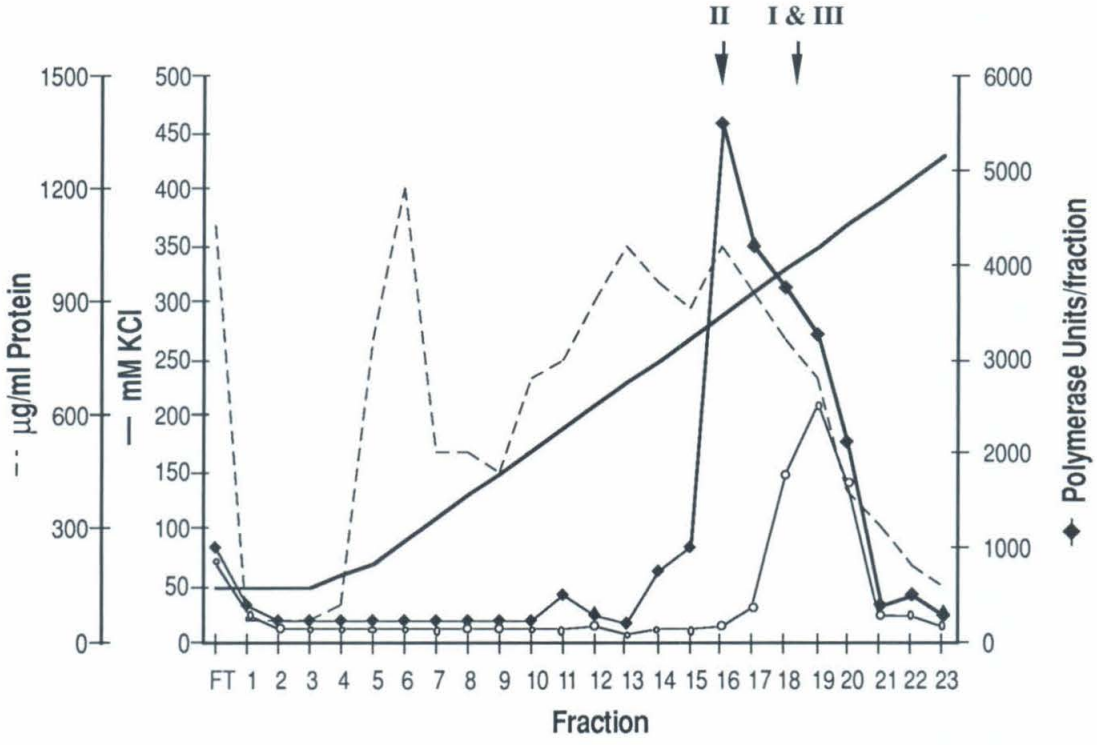

Figure 3. RNA Polymerase II behavior on heparin-Sepharose. One hundred milliliters of yeast S100 extract (YS100) was loaded onto a 60 milliliter heparin-Sepharose column. 15 milliliter fractions were collected. Filled diamonds represent the polymerase activity of a fraction without α-amanitin, and the open circles represent the polymerase activity of a fraction when assayed in 50 µg/ml α-amanitin.

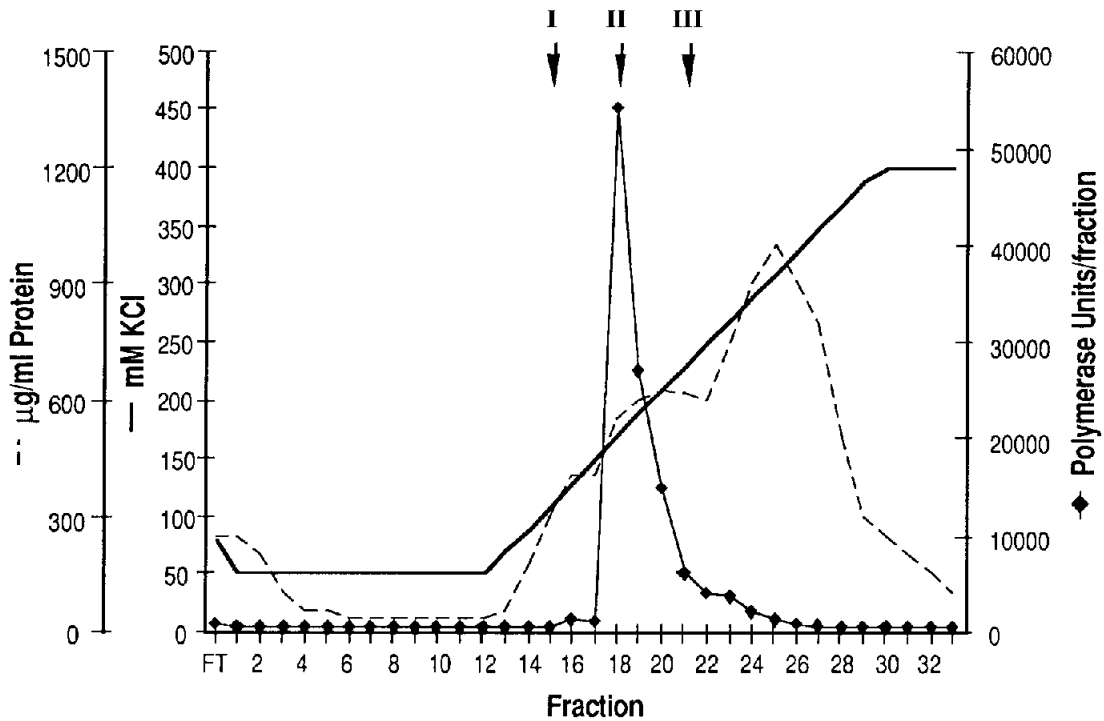

Figure 4. Behavior of RNA Polymerase II on A-25. Ten milliliters of A-25 were poured and equilibrated and then loaded with fractions 16 and 17 from the previous heparin-Sepharose column. 5 milliliter fractions were collected.

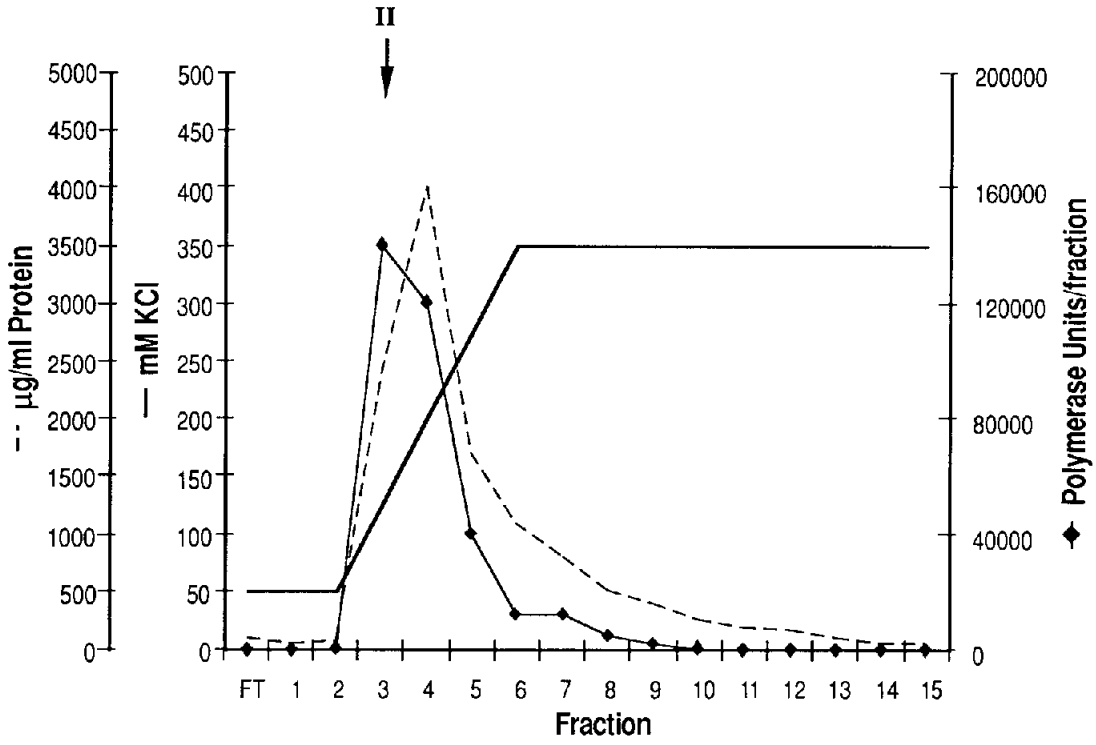

Figure 5. RNA Polymerase II behavior on P-11. Fractions 18 through 20 from the previous A-25 column were loaded onto a 1 milliliter P-11 column. A 0.35 M KCl step was applied to the column and collected in 0.5 milliliter fractions.

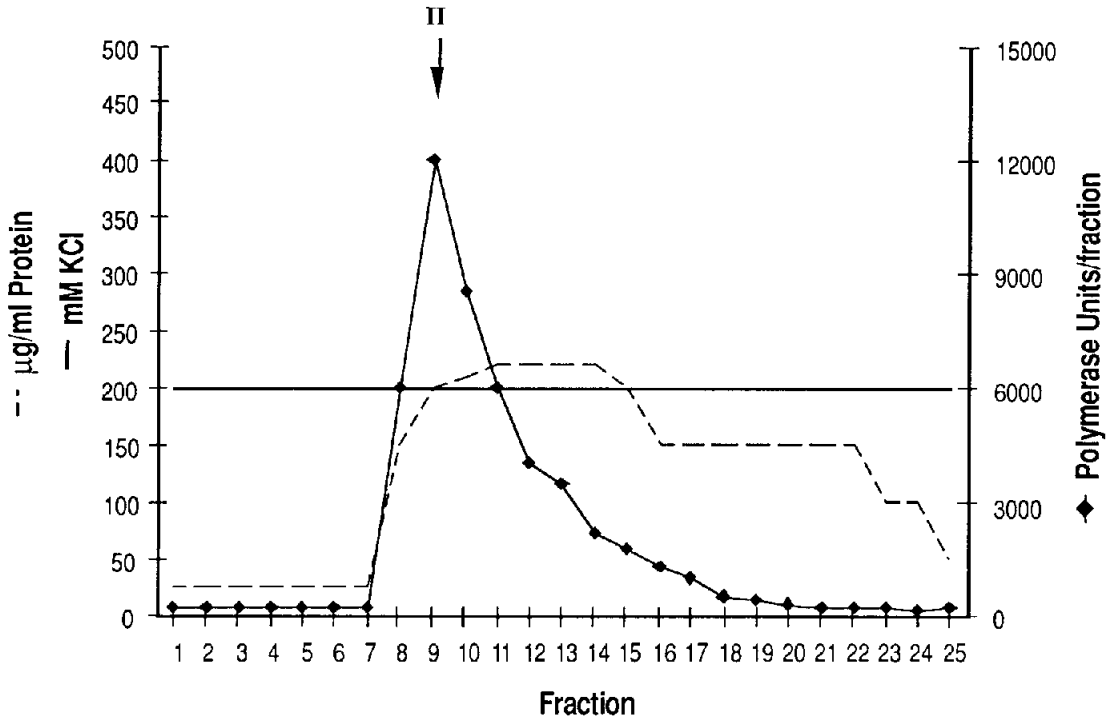

Figure 6. RNA Polymerase II on A1.5M. Fraction 3 from the previous P-11 column was loaded onto a 20 milliliter Biogel A1.5M column and collected in 1 milliliter fractions. The KCl concentration used for this purification results in the polymerase chromatographing as an aggregate. Higher concentrations of KCl resolve allow the polymerase to enter the gel matrix and resolve as an approximately 450 kD complex.

Stage	pmoles activity	milligrams Protein	% Recovery	pmoles/mg
YS100	200,000	400	-	500
Heparin, Pol II	100,000	27	-	4,000
"",Pol I and III	60,000	32	-	2,000
"",Pol II in 18-20	35,000	32	-	1,000
"", Total	195,000		98	-
A-25, Pol II	100,000	7	100	14,000
P-11, Fxn 3	70,000	1	-	70,000
"", Fxn 4	60,000	2	-	30,000
"", Fxn 5	21,000	0.85	-	25,000
"", Fxn 6	6,000	0.50	-	12,000
P-11, Total	160,000	4.4	160	35,000
A1.5M, Fxn 8	12,000	0.15	-	80,000
"", Fxn 9	24,000	0.20	-	125,000
"", Fxn 10	18,000	0.23	-	80,000
"",Fxn 11	12,000	0.23	-	50,000
A1.5M, Total	66,000	0.81	94	82,000

Table 2. Recovery and purification data from the yeast nuclear extract purification of RNA Polymerase II. Isolated RNA Polymerase II has an activity of 200,000 pmoles incorporation of UMP per 20 minutes at 30°C per milligram of enzyme (Hager, et al. 1977).

Polymin P precipitation of RNA Polymerase II

Yeast S100 fractions were made according to the protocol in Appendix C and the fractions were subjected to polyethyleneimine precipitation (Polymin P, purchased from Sigma). The conditions necessary for polymerase precipitation in both the S100 extracts and also Dynomill produced whole-cell extracts were virtually identical, and the optimal conditions determined for YS100 fractions work equally well for the precipitation of polymerase from the whole-cell extract described in Appendix C. The Polymin P concentration necessary for the precipitation of polymerase was determined by adding differing amounts of 10% Polymin P (pH 7.6) to YS100 fractions, mixing for 30 minutes at 4°C, spinning the extracts at 10,000 rpm for 30 minutes, and assay-

ing the supernate for polymerase activity. Table 3 shows the results of these experiments.

From the results in Table 3 and Table 4, the conditions for the precipitation of RNA Polymerase II (the primary factor responsible for the incorporation of UMP into RNA under the conditions tested) were determined. The final protocol used a 0.1% Polymin P precipitation step, a 0.2 M NaCl wash step to remove much of the protein without removing the majority of the polymerase, and a 1.5 M NaCl elution which results in the recovery of more than 50% of the polymerase activity and purifies the YS100 about 4 fold but the whole-cell extract more than 10 fold.

Extract	% Polymin P	mg/ml Protein	cpm
YS100	0.00	15	17,000
YS100	0.01	11	11,000
YS100	0.02	10.5	6,000
YS100	0.04	10	4,100
YS100	0.06	8	3,800
YS100	0.08	5	2,700
YS100	0.10	4.5	900
YS100	0.12	4	550
YS100	0.15	3.5	450
YS100	0.20	3	400
YS100	0.30	3	800
YS100	0.40	4.5	1330
YS100	0.50	6	1400
YS100	0.60	8	3500
WCE	0.00	20	45,000
WCE	0.10	10	1,700

Table 3. Polymin P precipitation of polymerase. Polymerase activity is given by the raw cpm incorporation in a standard polymerase assay (see Appendix C). At very high percentages of Polymin P (0.3% total and higher) the Polymin P either inhibited the precipitation of protein or interfered with the reliability of the assay.

mM NaCl	mg/ml Protein	cpm
0	0.5	1100
40	1.1	1500
200	3.4	1600
400	7.5	3000
600	7.5	3000
800	8.0	3200
1000	8.5	5000
1200	8.5	6000
1500	8.5	7000
2000	9.0	7500

Table 4. Extraction of the Polymin P pellet with sodium chloride. UMP incorporation is given in cpm, and volumes recovered were the same as the original volume of extract used in the above experiment. The YS100 fraction was precipitated with 0.1% Polymin P, mixed for 30 minutes in the cold room, centrifuged for 30 minutes at 10K, and resuspended and mixed for 30 minutes. The suspension was spun at 10K for 30 minutes to remove the remaining precipitate, and the supernate was assayed under the identical conditions as Table 3.

Extract freezing conditions

In order to easily obtain large amounts of cells in a convenient manner, an efficient method of freezing the intact cells was developed. Four different buffers and two different cell weight to buffer volume ratios were tested for HSTF recovery but not for polymerase activity. The results of these tests are shown in Table 5. It was determined that the standard 'Y' buffer with 30 mM DTT was adequate for obtaining active HSTF from frozen yeast. The cell slurry concentration was apparently not important to the recovery of active HSTF.

Buffer		Recovery 1:1 slurry	1:4 slurry
10%	Sucrose	0.2	0.1
50 mM	Tris (pH 7.8)		
1 mM	EDTA		
10%	glycerol	0.5	0.5
	'Y' Buffer		
30 mM	DTT		
	'Y' Buffer	1	1
30 mM	DTT		
		1	1
	'Y' Buffer		
30 mM	DTT		
7%	DMSO		
Unfrozen		1	1

Table 5. Recovery of HSTF activity from cells quick-frozen in liquid nitrogen. Recovery is measured by the footprinting activity of extracts made from the cells in FPU per 10 µl of extract. All extracts were performed identically, with the same weight of cells used for each extract as measured prior to freezing, and the final volume from each extract was identical.

Reference

Hager, G. L., Holland, M. J. and Rutter, W. J. 1977. Isolation of Ribonucleic Acid Polymerases I, II and III from *Saccharomyces cerevisiae*. *Biochemistry* **16**: 1-8.

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Appendix C

Laboratory Protocols

Table of Contents

Abbreviations

LSDB	Low Salt Digestion Buffer, also LSREB.
LSREB	Low Salt Restriction Enzyme Buffer.
MSDB	Medium Salt Digestion Buffer.
HSDB	High Salt Digestion Buffer.
DMF	Dimethyl Formamide.
NH_4Ac	Ammonium acetate.
NaAc	Sodium acetate
TE	10 mM Tris (pH 7.8), 1 mM EDTA
EDTA	Ethylene diamine tetraacetic acid.
Oligo	Oligonucleotide
dsDNA	double-stranded DNA
DNA	deoxyribose nucleic acid.
RNA	Ribose nucleic acid
ssDNA	single-stranded DNA
PMSF	Phenylmethylsulfonyl Flouride
$NaHCO_3$	Sodium carbonate
DIFP	Diisopropyl fluorophosphate
NBT	Nitro Blue Tetrazolium, an alkaline phosphatase color reagent, Biorad.
BCIP	5-Bromo-4-chloro-3-indoyl phosphate.
DMF	Dimethyl formamide
K_2HPO_4	Potassium phosphate, dibasic
NaN_3	Sodium azide
NaOH	Sodium hydroxide
$NaHCO_3$	Sodium carbonate
Na_2HPO_4	Sodium phosphate, dibasic
NaH_2PO_4	Sodium phosphate, monobasic
BSA	Bovine serum albumin

Media and Buffers

Chromatography Buffers

'A' Buffer

15 mM KCl
10 mM Hepes (pH 7.6)
5 mM $MgCl_2$

0.1 HGKET or 'D' Buffer

25 mM Hepes (pH 7.6)
10% Glycerol (Mallincrodt)
100 mM KCl
0.1 mM EDTA
0.2% Triton X-100

0.5 M Hepes,(pH 7.6)

1191 gms Hepes Acid
150 gms KOH
up to 10 liters w/ G-D H_2O
pH to 7.6 @ 25•C, 25 mM

Yeast Media

YPD

980 mls G-D H_2O
10 gms Yeast Extract
20 gms Bacto-Peptone
After autoclaving, add glucose to 2% final.
Autoclave 50% glucose separately.

YM : yeast minimal

1.7 gms Diffco YNB
5 gms $(NH_4)_2SO_4$
20 gms Glucose
1000 mls G-D H_2O

Autoclave, then add filter sterilized supplements.
50 µg/ml Amino Acids

YP Plates

10 gms Yeast Extract
20 gms Bacto-Peptone
20 gms Bacto-Agar
950 mls G-D H_2O

Sporulation Plates

980 mls G-D H_2O
2.5 gms Yeast Extract
2.5 gms KOAc
20 gms Bacto-Agar
2 mls 50% glucose

After autoclaving, add glucose.

BTB Plates

10 gms Yeast Extract
20 gms Peptone
20 gms Bacto-Agar
1000 mls G-D H_2O
pH to 8.0 with 2 N NaOH (~4 mls)
3.3 mls 1% BTB in ethanol

40 mls 50% sugar
pH, autoclave, then add filter sterilized sugar and Bromo Thymol Blue.

X-gal Plates

1.7 gms YNB
5 gms $(NH_4)_2SO_4$
30 gms Bacto-Agar
950 mls G-D H_2O
Autoclave

6.8 gms KH_2PO_4

50 mls G-D H_2O
pH to 7.0 with KOH (~2 gms)

Autoclave
Mix Agar, phosphate and 40 mls sterile 50% glucose.
Cool, add 40 mgs X-gal and pour plates.

Yeast Buffers

'Y' Buffer

1 M Sorbitol
50 mM Tris (pH 7.8)
10 mM $MgCl_2$

Warren Alden Kibbe

Large Scale (400 liters) Yeast Media

Autoclave in Fermentor

7.5 Kg Glucose

1 Kg Yeast Extract

2 Kg Ammonium Sulfate

~400 liters deionized H_2O

Autoclave Separately

1 liter 400 gms Potassium Phosphate, monobasic

1 liter 200 gms Magnesium Chloride

40 gms Sodium Chloride

40 gms $CaCl_2$

Filter Sterilize

in 500 milliliters G-D H_2O

Vitamins

1 mg	Biotin
160 mgs	Pantothenate
1 mg	Folic Acid
800 mgs	Inositol
160 mgs	Niacin
80 mgs	Riboflavin
160 mgs	Thiamine

Minerals

200 mgs	Boric Acid
16 mgs	Copper Sulfate
40 mgs	Potassium Iodide
80 mgs	Iron (III) Chloride
160 mgs	Manganous Sulfate
80 mgs	Sodium Molybdate
160 mgs	Zinc Sulfate

E. coli Media

L-Broth

980 mls	G-D H_2O
5 gms	Yeast Extract
10 gms	Bacto-Tryptone
10 gms	NaCl
10 mls	1 M Tris (pH 7.8)
1 ml	1 M $MgSO_4$

10X M9 Salts : 1 liter

58 gms	Na_2HPO_4
30 gms	NaH_2PO_4
5 gms	NaCl
10 gms	NH_4Cl

M9 Completion Mix

Enough for 10 liters

100 mls	50% Glucose
10 mls	4 mg/ml Thiamine (B1)
10 mls	1 M $MgSO_4$
10 mls	0.1 M $CaCl_2$

Autoclave components separately.

Filter Sterilize B1 solution.

M9CA : 1 liter

100 mls	10x M9 Salts
5 gms	Casamino Acids
890 mls	G-D H_2O

Autoclave, then add 13 mls M9 Completion Mix and antibiotics.

Super Broth

24 gms	Yeast Extract
12 gms	Bacto-Tryptone
3.8 gms	KH_2PO_4
12.5 gms	K_2HPO_4
10 mls	50% Glycerol
980 mls	G-D H_2O

Note : Add Sterilized Glycerol after Autoclaving

L-Plates

980 mls	G-D H_2O
5 gms	Yeast Extract
10 gms	Bacto-Tryptone
5.8 gms	NaCl
13 gms	Bacto-Agar

After autoclaving and ≤60°C, add drugs

Ampicillin	75 µg/ml
Tetracycline	10 µg/ml

Top Agarose

100 mls	L-Broth
8 gms	Bacto-Agar

Autoclave.

lambda dilution Buffer

100 mM	NaCl
5 mM	$MgSO_4$
50 mM	Tris (pH 7.5)

Other Buffers

TE

1 ml	1 M Tris (pH 7.8)
200 µl	0.5 M EDTA

Make up to 100 mls

1 M Tris, pH 7.8

121 gms	Trizma Base
500 mls	G-D H_2O

pH with 12 N HCl

Take up to 1 liter

100x TE

121 gms	Trizma Base
38 gms	EDTA

pH with 12 N HCl

Take up to 1 liter

Enzyme Buffers

Restriction Enzyme Buffers

10x Low Salt (10x LSDB)
- 100 µl 1.0 M Spermine
- 1 ml 1.0 M Tris (pH 7.8)
- 1 ml 1.0 M $MgSO_4$
- 100 µl 1 M DTT
- 7.8 mls G-D H_2O

10x Medium Salt (10x MSDB)
- 1 ml 5 M NaCl
- 1 ml 1.0 M Tris (pH 7.8)
- 1 ml 1.0 M $MgSO_4$
- 7 mls G-D H_2O

10x High Salt (10x HSDB)
- 2 ml 5 M NaCl
- 5 ml 1.0 M Tris (pH 7.8)
- 1 ml 1.0 M $MgSO_4$
- 2 mls G-D H_2O

Other Buffers

10x CIP Buffer
- 100 µl 1.0 M Tris (pH 7.8)
- 10 µl 1.0 M $MgCl_2$
- 1 µl 1.0 M $ZnCl_2$
- 100 µl 0.1 M Spermine
- 789 µl G-D H_2O

10x Kinase Buffer
- 100 µl 1.0 M Tris (pH 7.8)
- 100 µl 1.0 M $MgCl_2$
- 50 µl 1.0 M DTT
- 100 µl 0.1 M Spermine
- 650 µl G-D H_2O

5x Ligation Buffer
- 250 µl 1.0 M Tris (pH 7.8)
- 50 µl 1.0 M $MgCl_2$
- 10 µl 1.0 M DTT
- 20 µl 0.5 M ATP
- 670 µl -D H_2O

10x Pol I Buffer
- 100 µl 1.0 M Tris (pH 7.8)
- 10 µl 1.0 M $MgCl_2$
- 10 µl 1.0 M DTT
- 30 µl 10 mM dNTPs
- 850 µl G-D H_2O

Protein Extracts

Saccharomyces cerevisiae Extract Procedure from Frozen Cells

- Thaw 11 500 ml Beckman J10 bottles full of Yeast pellets frozen at -80°C in a water bath at 30°C. (approximately 1 kg cells). Frozen in a 1:1 slurry of cells to 'Y' buffer with 30 mM DTT.
- Harvest cells in Beckman J21, 5 K for 5 minutes.
- Resuspend in 1 liter 'Y' buffer with 6 mM DTT and 1 mg Zymolyase 100T per 10 grams Yeast cell weight. (100 mgs Zymolyase, 1 ml 'Y' Buffer per gm of cells)
- Stir for 1 hour at 30°C.
- Harvest cells in Beckman J21, 5 K for 5 minutes.
- Resuspend in 1 liter 'Y' buffer with 3 mM DTT. (1 ml 'Y' Buffer per gm of cells)
- Harvest cells in Beckman J21, 5 K for 5 minutes.
- Resuspend in 1 liter 'A' buffer with 3 mM DTT and 1 mM PMSF. (1 ml 'Y' Buffer per gm of cells)
- Let equilibrate on ice for 20 minutes.
- Homogenize with a Bellco 'B' pestle for 3 to 4 strokes.
- Harvest cells in Beckman J21, 10 K for 10 minutes.
- Discard supernate and resuspend pellicle in 600 mls 'A' with 3 mM DTT and 1 mM PMSF, mix for 5 minutes and split into 18 45 Ti tubes, 60 mls each. Add 4.0 mls 4 M Ammonium Sulfate to a final of 0.30 M AS. Add 2 drops DIFP to each tube and seal tubes. Mix in the cold room for at least 30 minutes. (shorter times diminish yield)
- Centrifuge in the 45 Ti at 35 K for 60 minutes.
- Discard pellicle. To the supernate add 0.22 gm AS/ml. Mix in the cold room for 15 minutes.
- Centrifuge for 15 minutes @ 35 K.
- Resuspend pellicle in 200 mls 'D' buffer +1 mM DTT, dialyze versus 'D' buffer + 1 mM DTT + 1 mM PMSF for ≥ 4 hours.

Notes:

Cells must be pretreated with 30 mM DTT or 100 mM ßME prior to Zymolyase treatment for 30 minutes at RT if they were not frozen in DTT.

Saccharomyces cerevisiae Dynomill Extract Procedure

- 1 Kilogram Cells :

  Frozen cells are thawed in 11 500 ml Beckman J10 bottles full of Yeast pellets frozen at -80°C in a water bath at 30°C. (approximately 1 kg cells). Cells are frozen in a 1:1 slurry of cells to 'Y' buffer + 7% DMSO or 30 mM DTT.

  Fresh cells can be resuspended in 1 ml G-D H_2O per gram cell weight.

- Harvest cells in Beckman J21, 5 K for 5 minutes.
- Resuspend in 1 liter 'A' buffer with 1 mM DTT and 1 mM PMSF.
- Pass through Dynomill (Glenn Mills, Inc.) three to six times. Estimate lysis percentage under 400x magnification with a light microscope after each pass. 70% lysis is sufficient.
- Add DIFP (3 drops/100 mls), seal in 500 mls bottles.
- Centrifuge 10 K for 30 minutes.
- Add 10% Polymin P (pH 8,0) to 0.15% Polymin P final concentration.
- Mix for 30 minutes.
- Centrifuge 10 K for 30 minutes.
- Resuspend pellet in 500 mls 0.2 M NaCl in 'A' Buffer, stir for 15 minutes.
- Centrifuge 10 K for 30 minutes.
- Resuspend pellet in 500 mls 1.5 M NaCl in 'A' Buffer, stir for 15 minutes.
- Centrifuge 10 K for 30 minutes, keep supernate.
- Add .22 gms Ammonium Sulfate per milliliter of supernate, mix for 30 minutes.
- Centrifuge 10 K for 30 minutes,rinse pellet with G-D H_2O.
- Resuspend pellicle in 200 mls 'D' buffer +1 mM DTT, dialyze versus 'D' buffer + 1 mM DTT + 1 mM PMSF for ≥ 4 hours.

Notes:

Other concentrations of Polymin P may be optimal for other factors. Conditions given work for HSTF and GBox Factor isolations.

Schizosaccharomyces pombe Extract Procedure from Frozen Cells

- Thaw cells in 500 ml Beckman J10 bottles frozen at -80°C in a water bath at 30°C. (approximately 1 kg cells). Frozen in liquid nitrogen as pellets in a 1:1 slurry of cells to 'Freezing Buffer'.
- Harvest cells in Beckman J21, 10 K for 10 minutes.
- Resuspend in a 1:1 slurry with 20 mM Citrate (pH 5.6)/1.2 M Sorbitol/5 mg/ml Novozyme.
- Stir at 32°C, checking for the completion of spheroplasts by taking a 1:10 dilution of the slurry in a final volume of 2 mls containing 1 M Sorbitol/1% SDS, and measuring the A_{650} at 0 minutes and at 15 minute intervals until the A_{650} reading is 1/10th the initial reading.
- Harvest cells in Beckman J21, 10 K for 10 minutes.
- Wash 3 times in 1:1 slurry of 'Y' buffer with 3 mM DTT.
- Resuspend in 1:1 slurry 'A' buffer with 3 mM DTT and 1 mM PMSF.
- Let equilibrate on ice for 20 minutes.
- Homogenize with a Bellco 'B' pestle for 3 to 4 strokes.
- Harvest cells in Beckman J21, 20 K for 15 minutes.
- Discard supernate and resuspend pellicle in 1:0.6 slurry 'A' buffer with 3 mM DTT and 1 mM PMSF, mix for 5 minutes and place into 45 Ti tubes, 60 mls each (if possible). Add 4 mls 4 M Ammonium Sulfate to a final of 0.3 M AS. Add 2 drops DIFP to each tube **in the hood** and seal tubes. Mix in the cold room for at least 30 minutes. (shorter times diminish yield)
- Centrifuge in the 45 Ti at 35 K for 60 minutes.
- Discard pellicle. To the supernate add 0.30 gm AS/ml.
- Mix in the cold room for 30 minutes.
- Centrifuge for 15 minutes @ 35 K.
- Resuspend pellicle in 1/20th original volume of cells in 'D' buffer +1 mM DTT, dialyze versus 'D' buffer + 1 mM DTT + 1 mM PMSF for ≥ 4 hours.
- Spin at 10 K for 10 minutes to remove precipitate.

HeLa cell Extract Procedure

- Harvest cells in Beckman J6, 4 K for 6 minutes.
- Resuspend in 20 mls 'A' Buffer per liter of cells harvested.
- Harvest cells in Beckman J21, 5 K for 10 minutes.
- Measure volume of cell pellet.
- Resuspend in 2.5 volumes of ['A' buffer with 1 mM DTT and 1 mM PMSF].
- Let equilibrate on ice for 20 minutes.
- Homogenize with a Bellco 'B' pestle for 3 to 4 strokes.
- Harvest nuclei in Beckman J21, 10 K for 10 minutes.
- Separate into pellet (P100) and supernate (S100).

S100

- Add 4 M KCl to a final concentration of 100 mM, and spin to clear supernate.
- Add 0.33 gms Ammonium Sulfate / ml supernate, mix for 30 minutes at 0°C.
- Spin at 35 K for 15 minutes.
- Resuspend pellicle in 1/10th original cell volume in 'D' buffer +1 mM DTT, dialyze versus 'D' buffer + 1 mM DTT + 1 mM PMSF for ≥ 4 hours.
- Spin at 10 K for 10 minutes to remove precipitate.

P100

- Measure volume of nuclei pellet.
- Resuspend nuclei in 2.5 volumes of ['A' Buffer + 1 mM DTT and 1 mM PMSF].
- Place in 45 Ti tubes, and add 1/10th volume 4 M Ammonium Sulfate to each tube.
- Mix in 45 Ti tubes for 30 minutes at 0°C.
- Centrifuge in the 45 Ti at 35 K for 60 minutes.
- Discard pellicle. To the supernate add 0.33 gm AS/ml.
- Mix in the cold room for 30 minutes.
- Centrifuge for 15 minutes @ 35 K.
- Resuspend pellicle in 1/4th volume of nuclei in 'D' buffer +1 mM DTT, dialyze versus 'D' buffer + 1 mM DTT + 1 mM PMSF for ≥ 4 hours.
- Spin at 10 K for 10 minutes to remove precipitate.

Drosophila Kc cell Extract Procedure

- Harvest cells in Beckman J6, 4 K for 6 minutes.
- Resuspend in 20 mls 'A' Buffer per liter of cells harvested.
- Harvest cells in Beckman J21, 5 K for 10 minutes.
- Measure volume of cell pellet.
- Resuspend in 2.5 volumes of ['A' buffer with 1 mM DTT and 1 mM PMSF].
- Let equilibrate on ice for 20 minutes.
- Homogenize with a Bellco 'B' pestle for 3 to 4 strokes.
- Harvest nuclei in Beckman J21, 10 K for 10 minutes.
- Separate into pellet (P100) and supernate (S100).

S100

- Add 0.33 gms Ammonium Sulfate / ml supernate, mix for 30 minutes at 0°C.
- Spin at 35 K for 15 minutes.
- Resuspend pellicle in 1/10th original cell volume in 'D' buffer +1 mM DTT, dialyze versus 'D' buffer + 1 mM DTT + 1 mM PMSF for ≥ 4 hours.
- Spin at 10 K for 10 minutes to remove precipitate.

P100

- Measure volume of nuclei pellet.
- Resuspend nuclei in 2.5 volumes of ['A' Buffer + 1 mM DTT and 1 mM PMSF].
- Place in 45 Ti tubes, and add 4 M Ammonium Sulfate to give a final concentration of 0.25 M.
- Mix in 45 Ti tubes for 30 minutes at 0°C.
- Centrifuge in the 45 Ti at 35 K for 60 minutes.
- Discard pellicle. To the supernate add 0.25 gm AS/ml.
- Mix in the cold room for 30 minutes.
- Centrifuge for 15 minutes @ 35 K.
- Resuspend pellicle in 1/4th volume of nuclei in 'D' buffer +1 mM DTT, dialyze versus 'D' buffer + 1 mM DTT + 1 mM PMSF for ≥ 4 hours.
- Spin at 10 K for 10 minutes to remove precipitate.

Cell Growth and Transformation

Saccharomyces cerevisiae Large Scale Growth

400 liter fermentor

Components:	Minimal Media - Diffco Manual 5 kg Glucose 500 grams Yeast Extract - Diffco
Media Sterilization:	Glucose, Ammonium Sulfate and Yeast Extract are autoclaved in the fermentor. PO_4^{-3} is autoclaved separately from the Chloride salts. Trace Minerals and Vitamins are Filter Sterilized in a 800x solution.
Yeast Strain:	EJ926
Innoculation:	1 liter of YPD saturated culture
Growth Time:	10 to 12 Hours at 30° Centigrade
Harvest:	OD_{600}= 3 to 6
Yield:	2 to 2.5 kg Yeast by cell weight
Storing cells:	Cells are resuspended in 'Y' Buffer containing 30 mM DTT in a 1:1 slurry. The slurry is then added dropwise to liquid Nitrogen and stored at -80°C.

Competent E. coli Cells

Preparation of Competent Cells

Grow an overnight of the culture in L Broth.

Innoculate 300 mls of L Broth with 5 mls of the overnight.

Shake at 250 rpms at 37°C to an A_{650} = 0.5.

Harvest cells in a sterile 500 ml Centrifuge Bottle at 5 K for 5 minutes. Let cells sit on ice for 20 minutes before spinning the cells down.

Spin at 5 K for 10 minutes in a Beckman JA-10 rotor that has been precooled to 4°C.

Discard the supernate and thoroughly resuspend cells in 150 mls Wash Buffer.

Spin at 5 K for 10 minutes in a Beckman JA-10 rotor.

Resuspend in 30 mls of Freezing Buffer.

Let sit on ice for 1 hour.

Aliquot into freezer tubes (1 ml/tube) and store at -70°C.

Note : Prechill Buffers on ice before using. Make sure Buffers are Sterile!

Transformation

Add DNA for the transformation to TE (pH 7.8) to a total volume of 100 µl.

Set DNA/TE solution on ice.

Thaw an aliquot of the competent cells on ice.

Add 200 µl of the competent cells to the DNA in TE.

Incubate on ice for 30 minutes.

Heat shock cells @ 37°C for 3 minutes.

Add 0.3 ml 37°C L Broth to each transformation.

Incubate at 37°C for 20 minutes to 45 minutes.

Plate onto 100 mm Selective Plates.

Let plates grow overnight at 37°C.

Solutions

1 M Mops	Wash Buffer	Freezing Buffer
21 gms MOPS	10.5 gms $MgCl_2$	1.5 gms $CaCl_2$
~50 mls G-D H_2O	~350 mls G-D H_2O	40 mls 50% Glycerol
pH to 8.0	pH with MOPS to 7.2	pH with MOPS to 7.2
100 mls final with G-D H_2O	500 mls final with G-D H_2O	100 mls final with G-D H_2O

Note: This procedure works well for HB101 & TB1. It gives efficiencies ~10^6 / µg supercoiled DNA.

All Buffers should be sterile!

Competent E. coli Cells:2

Preparation of Competent Cells

Grow an overnight of the culture in L Broth.
Innoculate 300 mls of L Broth with 5 mls of the overnight.
Shake at 250 rpms at 37°C to an A_{650} = 0.15-0.2.
Harvest cells in a sterile 500 ml Centrifuge Bottle at 5 K for 5 minutes.
Spin at 5 K for 10 minutes in a Beckman JA-10 rotor that has been precooled to 4°C.
Discard the supernate and thoroughly resuspend cells in 150 mls Wash Buffer.
Spin at 5 K for 10 minutes in a Beckman JA-10 rotor.
Resuspend in 150 mls of Permeabilizing Buffer.
Let sit on ice for 30 minutes.
Spin at 5 K for 10 minutes in a Beckman JA-10 rotor.

For Freezing

Resuspend in 15 mls of Freezing Buffer.
Aliquot into freezer tubes (500 µl / tube), freeze in liquid nitrogen and store at -70°C.

For Immediate Use

Resuspend in 15 mls of Permeabilizing Buffer.
Ready for use : can be stored for up to 24 hours with little or no loss of efficiency.

Transformation

Thaw an aliquot of the competent cells on ice, or use cells before freezing.
Place DNA samples into sterile tubes - DNA should be in a small volume of TE. Keep on ice.
Add 100 µl of the competent cells to the DNA in TE.
Incubate on ice for 30 minutes.
Heat shock cells @ 42°C for 1 minute.
Add 0.3 ml 37°C L Broth to each transformation.
Incubate at 37°C for 20 minutes to 45 minutes.
Plate onto 100 mm Selective Plates.
Let plates grow overnight at 37°C.

Solutions

Wash Buffer (pH 7.0)	Permeabilizing Buffer (pH 6.5)	Freezing Buffer (pH 6.5)
1.15 gms MOPs	3.68 gms $CaCl_2$	3.68 gms $CaCl_2$
225 µl HCl, 12 N	1.15 gms MOPs	1.15 gms MOPs
0.61 gms RbCl	325 µl HCl, 12 N	325 µl HCl, 12 N
~350 mls G-D H_2O	0.61 gms RbCl	0.61 gms RbCl
500 mls final with G-D H_2O	500 mls final with G-D H_2O	200 mls 50% Glycerol
Adjust pH if necessary	Adjust pH if necessary	500 mls final with G-D H_2O

Notes:

This procedure works well for HB101 & TB1. It gives efficiencies ~10^6 / µg supercoiled DNA.
All Buffers should be sterile!
From Promega Corporation - Catalogue and Applications Guide 1985/1986.
Prechill Buffers on ice before using.

Competent E. coli Cells:3

Preparation of Competent Cells

Innoculate 500 mls of L Broth with 5 mls of a fresh overnight.

Shake at 250 rpms at 37°C to an $A_{650} = 0.5$.

Harvest cells in two sterile 500 ml Centrifuge Bottles at 5 K for 5 minutes. Let cells sit on ice for 20 minutes before spinning the cells down.

Spin at 5 K for 10 minutes in a Beckman JA-10 rotor that has been precooled to 4°C.

Discard the supernate and thoroughly resuspend cells in 125 mls Wash Buffer.

Spin at 5 K for 10 minutes in a Beckman JA-10 rotor.

Resuspend in 125 mls of Permeabilizing Buffer.

Let sit on ice for 20 minutes.

Spin at 5 K for 10 minutes in a Beckman JA-10 rotor.

Resuspend in 25 mls of Freezing Buffer.

Aliquot into freezer tubes (500 µl /tube), freeze in LN_2 and store at -70°C.

Transformation

Add DNA in TE for the transformation to tube (as small a volume as possible) and place on ice.

Thaw an aliquot of the competent cells on ice.

Add 100 µl of the competent cells to the DNA in TE.

Incubate on ice for 20 to 40 minutes

Heat shock cells @ 37°C for 3 minutes.

Add 0.3 ml 37°C L Broth to each transformation.

Incubate at 37°C for 20 to 45 minutes.

Plate onto 100 mm Selective Plates.

Let plates grow overnight at 37°C.

Wash Buffer (100 mM $MgCl_2$)	Permeabilizing Buffer (100 mM $CaCl_2$)	Freezing Buffer (100 mM $CaCl_2$)
20.3 gms $MgCl_2 \bullet 6(H_2O)$	7.35 gms $CaCl_2 \bullet 2(H_2O)$	7.35 gms $CaCl_2 \bullet 2(H_2O)$
~450 mls G-D H_2O	500 mls final with G-D H_2O	150 mls 50% Glycerol
500 mls final with G-D H_2O		500 mls final with G-D H_2O

Note: This procedure works well for XL1-Blue. It gives efficiencies ~10^7/µg supercoiled DNA.
Prechill Buffers on ice before using.
All Buffers should be sterile!

Yeast Lithium-Acetate Transformation

Grow Yeast to OD_{600} = 0.5-1 in 100 mls YPD (2 ml inoculation takes 4-6 hours, 100 µl takes 8-12).
Harvest Cells Sterilely : 5 K for 10 minutes.
Resuspend cells in 10 mls TE.
Harvest Cells Sterilely : 5 K for 10 minutes.
Resuspend Cells in 5 mls TE.
Harvest Cells Sterilely : 5 K for 10 minutes.
Resuspend Cells in 20 mls Lithium Acetate Solution.
Incubate for 30 minutes at 30°C with occasional agitation.
Harvest Cells Sterilely : 5 K for 10 minutes.
Resuspend Cells in 2 mls 0.1 M LiAc Solution.
Cells are ready to use - can be stored overnight.
Place 100 µl aliquots of the Cell resuspension in sterile 1.5 ml Eppendorf tubes.
Add 0.2 - 1 µg DNA to 100 µl Cell resuspension. Mix Gently.
Incubate for 30 minutes at 30°C.
Add 900 µl Filtered PEG Solution.
Incubate for 1 hour at 30°C, agitate every 15 minutes.
Heat Shock Cells for 4-8 minutes at 42°C.
Pellet Cells gently in a Microcentrifuge.
Resuspend in 300 µl G-D H_2O.
Spread cells on the appropriate selective plate.
Grow at 30°C for 48-72 hours.

LiAc Solution

10 gms Lithium Acetate (100 mM final)
0.5 mls 1 M Tris, pH 7 to 8 (5 mM final)
40 µl 0.5 M EDTA (0.1 mM final)
make up to 100 mls with G-D H_2O.

PEG Solution

4 gms PEG 4000 (40% final)
Make up to 10 mls with G-D H_2O

Notes:

Cells can be used for up to one week if stored at 4°C - efficiency drops by 50% every 2 days.
Transformation efficiency can be as high as 1000 colonies/ ug.
Some cell lines are more efficient with 5% Ethylene Glycol included in the PEG Solution.
Do not keep the PEG solution for longer than 3 weeks.

Yeast Spheroplast Transformation

Grow Yeast to OD_{600} = 1-3 in 100 mls YPD (2 ml inoculation takes 4-6 hours, 100 µl takes 10-12).

Harvest Cells Sterilely : 5 K for 10 minutes.

Resuspend cells in 10 mls 1 M Sorbitol. Repeat Harvest and Resuspension.

Harvest Cells Sterilely : 5 K for 10 minutes.

Resuspend cells in 5 mls 'Y' Buffer.

Add 5 µl ß-mercaptoethanol and 50 mgs of ß-glucoronidase.

Incubate at 30°C with gently agitation (60 rpm). Check the extent of Spheroplasting by comparing the A_{600} of 50 µl of cells in 1 ml of 1 M Sorbitol and 100 µl 10% SDS at time 0, 15 minutes, 30 minutes,45 minutes and 60 minutes. The enzymatic digestion of the yeast cell wall is complete when the A_{600} is reduced to one tenth the original value.

Harvest Cells Sterilely : Clinical Centrifuge for 10 minutes.

Wash cells twice in 10 mls 1 M Sorbitol (Harvest Cells Sterilely : Clinical Centrifuge for 10 minutes).

Resuspend cells in 1 ml 1 M Sorbitol, 10 mM Tris (pH 7.5).

Aliquot 100 µl of the cell resuspension into tubes containing .5 to 2 µg of DNA.

Gently agitate for 15 minutes at room temperature.

Add 1 ml 20% PEG4000/10 mM $CaCl_2$/10 mM Tris (pH 7.5).

Gently agitate for 15 minutes at room temperature.

Harvest Cells Sterilely : Clinical Centrifuge for 10 minutes.

Resuspend cells in 100 µl 1.2 M Sorbitol/10 mM $CaCl_2$ and 50 µl YPD/1.2 M Sorbitol.

Gently agitate for 20 minutes at 30°C.

Add 8 mls Regen Agar at 50°C.

Plate immediately onto the appropriate selective plate containing 1.2 M Sorbitol.

Regen Agar

15 gms Bacto-Agar
90 gms Sorbitol
8.5 gms Diffco YNB
25 gms Ammonium Sulfate
10 gms Dextrose
Supplements for auxotrophs
Bring to 500 mls with G-D H_2O
Autoclave, pour 100 mm plates

Notes :

Cells can be used for up to one week if stored at 4°C - efficiency drops by 50% every 2 days.
Transformation efficiency can be as high as 1000 colonies/ ug.
20 µl of rapid lysate DNA (DNA from 0.2 mls culture) gives 10-50 colonies (pSEYC-102).

DNA purification / Oligonucleotide techniques

Plasmid Prep

Grow up cells in L-Broth (5 ml overnights).

Grow up cells in Culture.
- -Amplified cells : 1 liter M9 media, induce with 50 mgs chloramphenicol and add 1 gm pf Uridine at an OD of 1.0, grown o/n.
- -L-broth grown cells : 500 mls of media, cells grown 8-12 hrs. (saturated)
- -Super Broth grown cells : 500 mls of media, cells grown 8-12 hrs. (saturated)

Harvest cells (either JA-10 5 K for 5 minutes or SW4.2 4 K for 6 minutes).

Resuspend pellet in 20 mls Solution I containing 2 mgs/ml lysozyme (Sigma), made fresh.

Let sit on ice 15 minutes.

Add 40 mls Solution II, mix gently and let sit on ice 10 minutes.

Add 20 mls Solution III, mix vigorously and let sit on ice 30 minutes.

Centrifuge 10 K for 30 minutes.

Filter supernate through cheesecloth, add 0.6 Volume isopropanol, mix and freeze at -20°C for 30 minutes.

Spin down precipitate 10 K for 10 minutes.

Resuspend in 6 mls TE (3 mls, transfer, then wash tube with another 3 mls).

Centrifuge 10 K for 20 minutes, change tubes.

Precipitate DNA with 2 volumes ethanol.

Centrifuge 10 K for 5 minutes.

Wash with 5 mls 70% ethanol.

Centrifuge 10 K for 5 minutes.

Resuspend in 3 mls TE, then add 4.80 gms CsCl, 50 µl 20 mg/ml ethidium bromide, place in 5.5 ml quick seal tubes. Tube should weigh 9.5-9.6 gms when fully filled.

Spin in vTi 80 6 hours at 65 K.

Pull plasmid band. If RNA contamination is not a problem, rebanding is not necessary, otherwise reband by putting cesium band in a new tube and making up the volume with a cesium chloride solution having an index of refraction of 1.395, and reband.

Remove the Ethidium Bromide by repeatedly extracting with 1 Volume of Isopropanol saturated with 4.5 M CsCl in water, or Butanol saturated with 4.5 M CsCl.

The CsCl/DNA solution can be treated in two ways:
- -Dialyze versus 1 liter of TE, 3 changes for 8 hrs each time.
- -Add 2 volumes G-D H_2O, then precipitate with 2.5 volumes 95% ethanol. Let sit at -20° for 30 minutes, then centrifuge for 10 K for 10 minutes. Wash pellet with 1 ml 70% ethanol, centrifuge, and resuspend in TE.

Solution I
- 22.5 gms Glucose (50 mM)
- 10 mls 0.5 M EDTA (10 mM)
- 12.5 mls Tris,pH 7.8 (25 mM)
- 500 mls Total

Solution II
- 4 gms NaOH (0.2 M)
- 5 gms SDS (1%)
- 500 mls Total

Solution III 3 M KOAc (pH 4.8)
- 147 gms Potassium Acetate
- 210 mls Glacial Acetic Acid
- ~150 mls G-D H_2O
- 500 mls total

Rapid Lysate DNA Isolation from E. coli

Grow 1.5 mls of cells in L-Broth over night (saturated).

Pellet cells by spinning in Eppendorf Centrifuge for 20".

Resuspend Cells in 100 µl Solution I.

Let sit for 5 minutes at room temperature.

Add 200 µl Solution II and vortex.

Let Sit on ice for 15 minutes.

Add 75 µl Solution III and vortex.

Let sit on ice for 30 minutes.

Centrifuge 5 minutes, transfer supernate to new tube.

Add 200 µl Phenol-Chloroform, vortex.

Centrifuge 3 minutes, transfer top layer (aqueous) to new tube.

Add 500 µl of Ether or Butanol, vortex.

Centrifuge 3 minutes, remove top layer (organic solvent) and discard.

Add 2 volumes of 95% ethanol and vortex.

Let sit 2-5 minutes at room temperature.

Spin 3 minutes to precipitate DNA, remove supernate.

Add 500 µl 70% ethanol, vortex.

Spin 3 minutes, remove supernate.

Dry.

Redissolve pellet in 100 µl TE (10 mM Tris (pH 7.8), 1 mM EDTA).

Note: This DNA is clean enough to use to Klenow fill for sequencing. It can also be used to transform yeast (20 µl of pSEYC102 Rapid Lysate DNA gives about 100 colonies when transformed into spheroplasted TD28 yeast cells) or to prepare subcloning fragments. They yield of pUC plasmids with this method is from 2 to 5 µg of plasmid.

Solution I	Solution II	Solution III 3 M KOAc (pH 4.8)
22.5 gms Glucose (50 mM)	4 gms NaOH (0.2 M)	147 gms Potassium Acetate
10 mls 0.5 M EDTA (10 mM)	5 gms SDS (1%)	210 mls Glacial Acetic Acid
12.5 mls Tris,pH 7.8 (25 mM)	500 mls Total	~150 mls G-D H_2O
500 mls Total		500 mls total

Preparing Ligated Oligonucleotide Columns

About **250 µg/ml of ds-oligonucleotide** can be coupled (average size DNA > 300 bp).

Preparing oligonucleotide : 2 different methods : Gel elution or FPLC purification.

Oligonucleotide Preparation via Gel Elution:

Resuspend all of the oligo in 200 µl denaturing loading buffer - 1xTBE, 7 M Urea, 0.02%dyes.

Load onto gel. Gel consists of two layers, the lower layer is 20% acrylamide, the upper layer, which is only necessary to insure that the comb can be removed, is only a few mm deep before the comb. I use a two well comb, each well 5 cm wide, 1 cm deep and 4.5 mm thick.

Size : 4.5 mm x 13 cm x 15 cm gel

Lower Buffer : 125 mls 20%acrylamide (1:28 bis), 1xTBE, 8 M Urea, plus polymerizers.

Upper Buffer : 30 mls 8% acrylamide (1:28 or 1:38 bis), 1xTBE, 8 M Urea plus polymerizers.

Run the gel at 300 volts for 15 hours to get the bromophenol blue to the bottom.

Ethidium bromide stain or UV shadow the bands.

Cut bands, chop acrylamide into small slices with a sharp razor blade.

Elute with 5 mls of acrylamide Elution Buffer for 8 hours at 37°C: the first elution may require more buffer.

Combine elutions and concentrate to 3 mls with isobutanol.

Precipitate with 9 mls 95% ethanol.

Wash the pellet with 70% ethanol.

Resuspend the pellet in 200 µl of TE.

Measure the DNA concentration.

If the oligonucleotide is not self-complementary, pool both strands.

Ethanol precipitate with 90%ethanol/0.1 M NaOAc and then 70% ethanol wash the combined oligos.

Oligonucleotide Annealing

Resuspend the pellet containing both strands in 200 µl of TE or 1x LSDB (or enough buffer to keep the concentration below 5 mgs/ml oligo).

Heat in a well sealed Eppendorf tube to 90°C for 10 minutes.

Transfer to a 70°C temp block for 30 minutes.

Transfer the temp block and tube to a 42°C incubator, making sure the temp block is insulated from metal contact.

Allow the temp block to cool for 2 hours.

Preparing Ligated Oligonucleotide Columns

Continued

Kinase and Ligate the Oligonucleotides

Use 1 milligram of the annealed oligo mix. This is sufficient for 5 mls of resin.

Add enough 10x Low Salt Restriction Enzyme Buffer to make the solution 1x.

Add 0.5 M ATP, (pH 7.8) to 1 mM.

Add 50 units of kinase for 1 mg of oligo.

Let the kinase incubate for 1 hour at 37°C.

Add another aliquot of ATP, same amount as before.

Add 20 more units kinase.

Add 50 units ligase.

Ligate overnight at 14°C.

Check on gel. The oligo is sufficiently ligated when the average ligation is around 10 monomers. Repeat as necessary. See notes.

Couple to resin

Ethanol precipitate with 90% ethanol/0.4 M NH_4OAc and then 70% ethanol wash the oligos.

Resuspend the oligos in 10 mM K_2HPO_4.

Couple to Sepharose by Jovin's method. (See miscellaneous techniques in this Appendix)

Uncoupled DNA elutes at the first elution. I normally keep this elution and precipitate it to calculate the coupling efficiency.

Notes:

The A^{260} reading for oligonucleotides with different composition and self-complementarity can be extremely different. I use a 1:1 mix of purified oligos, starting with the same amount of each strand according to the synthesis results.

The annealing buffer used is not critical. I have used TE, but 1x LSDB works just as well.

If the ligation has worked very poorly the first time, then a 90% ethanol/0.4 M NH_4OAc precipitate, followed by a 70% ethanol wash can remove inhibiting compounds. Resuspend the pellet in 400 µl of TE and repeat the kinasing and ligation procedure until a satisfactory ligation has been achieved. I have found that it is sometimes necessary to precipitate an oligonucleotide with 90% ethanol/0.4 M NH_4OAc multiple times before getting adequate ligations.

NH_4OAc should not be used as a precipitating salt if the oligonucleotides have not yet been annealed. NH_4OAc precipitations should be done at -80°C for 1 hour or at -20°C overnight to insure good recovery. NH_4OAc will not bring down small single-stranded (approximately 70 nucleotides or less) oligonucleotides or mononucleotides, whereas NaOAc will bring down nucleotides quantitatively. Double-stranded oligonucleotides less than 20 bp show poor recovery with NH_4OAc precipitations.

I find that 200-250 µgs of well-ligated double-stranded oligonucleotides (average size at least 300 bp) will couple per milliliter of Sepharose beads.

Protein Techniques

Laemmli Protein Gel

Stacking Protein Gel : Laemmli, Nature 227:680. (1970).

Solutions:

30% acrylamide, 0.8% Bisacrylamide in G-D H_2O.

Separating Buffer : 4x : 1.5 M Tris-HCl (pH 8.8), 0.4% SDS.
200 mls : 36 gms Tris Base, .8 gm SDS, pH with 12 N HCl (requires approximately 5 mls), up to 200 mls with G-D H_2O.

Stacking Buffer : 4x : 0.5 M Tris-HCl (pH 6.8), 0.4% SDS.
200 mls : 12 gms Tris Base, .8 gm SDS, pH with 12 N HCl, up to 200 mls with G-D H_2O.

Electrode Buffer : 10x : 0.25 M Tris base, 1.9 M Glycine, 1% SDS.
1 liter : 144 gms Glycine, 30 gms Tris Base, 10 gms SDS up to 1 liter in G-D H_2O.

Sample Buffer : 5x : 50% Glycerol, 2x Stacking Buffer + 10% SDS + 5 mM ßME+ 0.05% BPB.
10 mls : 5 mls Glycerol, 1 gm SDS, 5 mls 4x Stacking Buffer + 5 µl ßME + 500 µl 1% BPB.

Preparing Gel Plates: Clean plates by cleaning with 409 detergent, then thoroughly rinse in 95% ethanol and again in Acetone. Use Kimwipes to wipe the plates, do not use a lesser quality paper towel which will leave particulates on the glass. Prepare the spacers and combs in a similar manner, being sure to wipe in between all the teeth on the comb.

Separating Gel : 5% to 20% acrylamide in 1x Separating Buffer, 10% is Standard for 50- 100 kD proteins. Add 1 µl TEMED per ml of Gel, and 5 µl 10% APS per ml of Gel just before pouring gel. For a 20 cm x 20 cm x 5 mm gel, 25 mls of Separating Gel is adequate. When pouring the gel, only fill to 1 cm below the bottom of the comb. Place G-D H_2O on top of the poured gel and allow to polymerize for 30 minutes.

Stacking Gel : 3% acrylamide in 1x Stacking Buffer. Add 1 µl TEMED per ml of Gel, and 5 µl 10% APS per ml of Gel just before pouring gel. For a 20 cm x 20 cm x 5 mm gel, 5 mls of Stacking Gel is adequate. Remove the G-D H_2O before pouring the Stacking Gel. Allow to polymerize for 30 minutes before using.

Running the gel : Place the gel in a clean electrophoresis apparatus, seal the gel in place with a 1% Agarose solution, and fill the buffer wells with 1x Running Buffer. Run the gel at a constant current of 25 milliAmps, or at a constant power of 3 Watts. Run until Bromophenol Blue is at the bottom of the gel.

Note: The most critical part of running a protein gel, especially if it will be Silver Stained, is to be sure that the plates and comb are clean. Be sure to thoroughly clean the plates in a detergent solution such as 409, and only use high quality paper towels to wipe the plates or comb with. Some paper towels will leave behind paper residue, which can lead to a high background when stained.

Laemmli Gel Silver Stain

*Silver Stain method derived from the method of Wray, et al, **Analytical Biochemistry,** 118:197-203 (1981).

Run gel using the Laemmli protein gel system.

Options:

1. Soak gel overnight in 300 mls of 50% methanol, 10% glacial acetic acid aqueous solution. Soak gel in a 50% methanol/G-D H_2O solution approximately 1 hour before staining.
2. Soak gel overnight in 300 mls of 50% methanol/G-D H_2O solution.

Make up stain Solution:

A: 1.6 gms $AgNO_3$ in 10 mls G-D H_2O.

B: 0.8 mls 5 N NaOH and 2.8 mls 14.8 M NH_4OH in 20 mls G-D H_2O.
-Add A to B dropwise with constant stirring.
-If solution becomes cloudy and stays cloudy add more 14.8 M NH_4OH dropwise until the solution clears (continue stirring during additions).
-Fill to 200 mls with G-D H_2O.

USE STAIN IMMEDIATELY!

Stain gel for 15 minutes.

Soak in G-D H_2O for 5 minutes.

Soak the gel in Developer solution until bands reach the desired intensity. Time will be dependent on the length of the previous washes, the protein concentration in the gel and the acrylamide percentage.

Developer:
1 ml 2.5% Citric Acid
0.25 mls 38% Formaldehyde
bring up to 400 mls in G-D H_2O.

Rinse gel in G-D H_2O.

Stop development by placing gel in a 50% methanol/10% Glacial Acetic Acid/G-D H_2O solution for at least 4 hours.

Rinse gel in G-D H_2O.

Soak gel in G-D H_2O for 2 to 4 hours in G-D H_2O.

Dry Gel.

Note:

It is important to presoak the gel without glacial acetic acid before attempting to stain the gel with silver. The acidic pH will inhibit staining.

Combination Commassie Blue/Silver Stain

*Silver Stain method derived from the method of Wray, et al, **Analytical Biochemistry,** 118:197-203 (1981).

Run gel using the Laemmli protein gel system.

Soak gel overnight in 300 mls of 50% methanol, 7% trichloroacetic acid aqueous solution.

Stain for 30 minutes in Commassie Blue Stain solution:

0.625 grams Commassie Brilliant Blue G-250
125 mls methanol
30 grams trichloroacetic acid
make up to 250 mls in G-D H_2O.

Destain three times for 10 minutes with a 5% trichloroacetic acid aqueous solution.

Destain in the following buffers until background is reduced to a faint blue:

50% methanol for 10 minutes, then G-D H_2O for 10 minutes, repeat as necessary.

Make up stain Solution:

A: 1.6 gms $AgNO_3$ in 10 mls G-D H_2O.

B: 0.8 mls 5 N NaOH and 2.8 mls 14.8 M NH_4OH in 20 mls G-D H_2O.
-Add A to B dropwise with constant stirring.
-If solution becomes cloudy and stays cloudy add more 14.8 M NH_4OH dropwise until the solution clears (continue stirring during additions).
-Fill to 200 mls with G-D H_2O.

USE STAIN IMMEDIATELY!

Stain gel for 15 minutes.

Soak in G-D H_2O for 5 minutes.

Soak the gel in Developer solution until bands reach the desired intensity. Time will be dependent on the length of the previous washes, the protein concentration in the gel and the acrylamide percentage.

Developer:
1 ml 2.5% Citric Acid
0.25 mls 38% Formaldehyde
bring up to 400 mls in G-D H_2O.

Rinse gel in G-D H_2O.

Stop development by placing gel in a 50% methanol/10% Glacial Acetic Acid/G-D H_2O solution for at least 4 hours.

Rinse gel in G-D H_2O.

Soak gel in G-D H_2O for 2 to 4 hours in G-D H_2O.

Dry Gel.

Note:
Allow destaining of the Commassie blue to continue until the background of the gel is slightly blue. The gel will destain further during the soaking step with silver.

TCA Protein Precipitation

Under 50µl Protein Sample

Place sample in a 1.5 ml Eppendorf tube.
Add 400 µl 7% TCA, vortex vigorously.
Leave on ice for 10 minutes.
Spin for 2 minutes, discard supernate.
Wash with 500 µl 100% Acetone.
Spin for 2 minutes.
Dry.

Over 50 µl Protein Sample

Place sample in a 1.5 ml Eppendorf tube.
Add the proper volume of 100% TCA to give a final concentration of 7% TCA, vortex vigorously.
Leave on ice for 10 minutes.
Spin for 2 minutes, discard supernate.
Wash with 500 µl 100% Acetone.
Spin for 2 minutes.
Dry.

Acetone Protein Precipitation

Precipitate Protein with 4 volumes 100% Acetone at -20°C.
Place at -20°C for at least 30 minutes.
Spin for 5 minutes.
Wash with 400 µl 80% Acetone at -20°C.
Dry.

Guanidinium HCl Renaturation

Precipitate Protein with Acetone above above.
Resuspend in 6 M Guanidinium-HCl in 'C' buffer.
Dialyze in BioRad microdialysis apparatus overnight versus 'C' Buffer.
Assay activity of protein.

Bradford Protein Assay

Samples from 200 µg/ml to 4 mg/ml

Set up a sufficient number of 5 ml glass tubes, include 4 for blanks.

Aliquot out 1.50 mls G-D H_2O into 5 ml glass test tubes.

Add 12 µl of the protein sample to each tube (none to blanks).

Add 370 µl of the Biorad Bradford protein solution (phosphoric acid and commassie blue).

Mix by hand or vortex for 15 seconds.

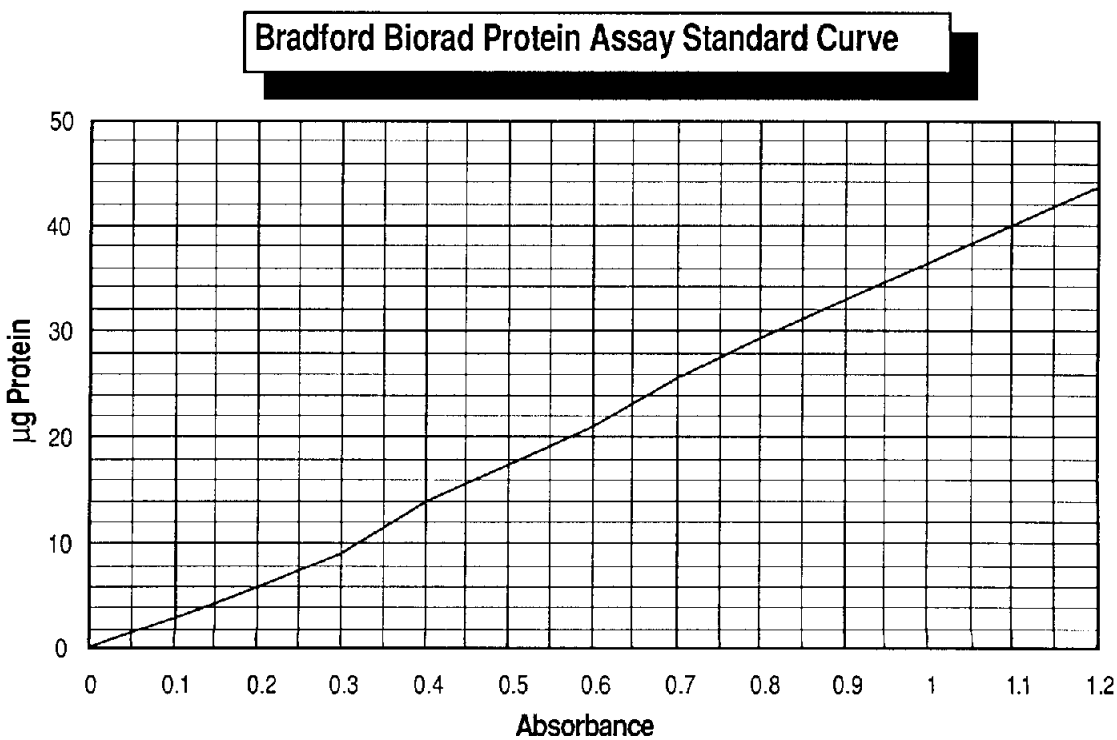
Let solution sit for 2 to 5 minutes.

Read the A_{595} of the samples, using the blanks to set zero absorbance.

Compare on the scale shown below.

Note:

The absorption coefficient of various proteins using this method is somewhat varied. Therefore, it should only be used for the determination of the protein concentration of heterogeneous protein fractions, not highly purified proteins.



Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Bioch.* **72:** 248-254.

DNA/Protein Techniques

RNA Polymerase general transcription assay

Reaction cocktail	Number of polymerase assays						
Stock	10	20	25	30	40	50	100
0.5 M Tris (pH 7.8)	25	50	62.5	75	100	125	250
1.0 M $MnCl_2$	0.5	1	1.25	1.5	2	2.5	5
0.03 M GTP, CTP, ATP	5	10	12.5	15	20	25	50
0.003 M UTP	5	10	12.5	15	20	25	50
10 mCi/ml $^{32}\alpha$-UTP	0.5	1	1.25	1.5	2	2.5	5
10 mg/ml Calf Thymus DNA	2	4	5	6	8	10	20
G-D H_2O	112	224	280	336	448	560	1120

α-amanitin may be added to inhibit specific polymerases.

90% Inhibition	organism	RNA Polymerase I	RNA Polymerase II	RNA Polymerase III
	yeast	600 µg/ml	50 µg/ml	>2000 µg/ml

Steps:

-Make sufficient reaction cocktail for samples and controls.

-If desired, make duplicate cocktails with and without α-amanitin.

-add 15 µl reaction cocktail with 10 µl sample.

-mix thoroughly.

-incubate at 30°C for 20 minutes.

-spot onto labeled DEAE paper (1 cm by 1 cm).

-let dry at room temperature for 5 minutes.

-rinse in 5% (w/v) K_2HPO_4, 10 mls solution/DEAE square).

-repeat rinse a total of 3 times, 5 minutes each.

-microwave to dryness for ~1 minute.

-place in scintillation vials with scintillation fluid and count radioactivity.

Notes:

Include the following controls: 2 µl of cocktail spotted onto a square and not rinsed, 1 blank, 1 15 µl cocktail with rinses and input to column, if chromatographic fractions.

Calculating Polymerase yield

$$\text{Factor} = \frac{\text{pmoles UTP}}{\text{cocktail cpm}}$$

$$\text{Factor} = \frac{50\,\text{mM UTP} \times 25\mu\text{l}}{\text{cocktail cpm}}$$

$$\text{Factor} = \frac{1250}{2\mu\text{l cocktail cpm} \times \frac{15\mu\text{l}}{2\mu\text{l}}}$$

$$\text{Units} = \text{Factor} \times \text{cpm in fraction}$$

$$\frac{\text{Units}}{\text{ml}} = \text{Factor} \times \text{cpm in fraction} \times \frac{\#\text{ aliquots}}{\text{ml}}$$

$$\frac{\text{Units}}{\text{ml}} = \text{Factor} \times \text{cpm in fraction} \times 100$$

The total yield of RNA polymerase II in micrograms can be calculated by assuming that there 1 µg of RNA Polymerase has 200 units of activity.

Note: Concentration of DNAse required depends on the composition of the fragment and its length.

Yeast Affinity Purified Protein *in vitro* Footprinting

5x Cocktail

Volume (µl)	Chemical	Stock Concentration	Final Concentration
1.5	$MgCl_2$	1 M	5 mM
3	dAdT	2 mg/ml	20 µg/ml
	Kinase labeled DNA		30 Kcpm/rxn
	G-DH_2O		
total 65			

Sample Reaction Set

Reaction	Protein Fxn	'D' (µl)	Cocktail	DNAse
1	----	20	5 µl	70µg/ml
2	0.1 µl GBF	20	"	Final
3	0.2 µl "	20	"	"
4	0.5 µl "	20	"	"
5	1 µl "	19	"	"
6	2 µl "	18	"	"
7	5 µl "	15	"	"
8	10 µl "	10	"	"
9	1 µl yAP-1	19	"	"
10	10 µl "	10	"	"
11	20 µl "	-	"	"
12	20 µl GC-Box factor	-	"	"

Steps:

-Chill all tubes to 0°C in a Temp Block on ice.

-Add 'D' buffer, then Protein, then add Cocktail.

-Allow protein to bind for 3 minutes @ 0°C.

-DNAse samples for 1 minutes @ 0°C : Add 5 µl 400 µg/ml DNAse I and mix.

-Stop with 100 µl of Sarkosyl Solution:

1% Sarkosyl,

0.1 M Tris (pH 7.8),

0.1 M NaCl,

0.01 M EDTA,

10 µl 1 mg/ml carrier DNA.

-Add 400 µl 90% ethanol / 0.1 M NaAc, chill @ -20°C for 5 minutes or longer.

-Spin 5 minutes.

-400 µl 70% ethanol wash, 2 minutes Spin.

-Dry samples 3 minutes or longer in vacuum desiccator.

-Resuspend in 6 µl (or 10 µl for 40 to 80 cm gels) 95% formamide + Dyes.

-Vortex lightly, spin.

-Heat @ 90°C - 3 minutes.

-Place on ice.

-Load onto gel.

Notes:

Assaying affinity resins does not require Proteinase K or Phenol Extractions.

When making 2 mg/ml dAdT stocks, make it in TE and store it in 100 µl aliquots @ -20° C.

When making 5 mg/ml DNAse I stocks, make it in either 'D' Buffer or 50% Glycerol 1x LSDB, and store it in 100 µl aliquots @ -80°C.

Prepare Protein sample to be footprinted by dialyzing vs 'D' buffer until KCl concentration is below 150 mM.

All tubes are Eppendorf 1.5 ml tubes, except for the tubes used to store the DNAse I stock, which is kept in 700 µl Eppendorf tubes.

Reaction Volumes can be changed without changing DNAse I conditions. For example, if 50 µl reactions are desired, a 10x Cocktail can be made that still gives a final concentration of 20 µg/ml dAdT, and a DNAsed with a final DNAse concentration of 70 µg/ml.

Note: Concentration of DNAse required depends on the composition of the fragment and its length.

DMS *in vitro* Footprinting

5x Cocktail

Volume (µl)	Chemical	Stock Concentration	Final Concentration
1.5	$MgCl_2$	1 M	5 mM
2	dAdT	2 mg/ml	14 µg/ml
	Kinase labeled DNA		30 Kcpm/rxn
	G-DH_2O		
total 65			

Sample Reaction Set

Reaction	Protein Fxn	'D' (µl)	Cocktail	DMS
1	----	20	5 µl	50 mM
2	0.1 µl GBF	20	"	Final
3	0.2 µl "	20	"	"
4	0.5 µl "	20	"	"
5	1 µl "	19	"	"
6	2 µl "	18	"	"
7	5 µl "	15	"	"
8	10 µl "	10	"	"
9	1 µl yAP-1	19	"	"
10	10 µl "	10	"	"
11	20 µl "	-	"	"
12	20 µl GC-Box factor	-	"	"

Steps:

-Let cocktail and buffers reach room temperature.

-Add 'D' buffer, then Protein, then add Cocktail.

-Allow protein to bind for 3 minutes @ RT.

-DMS treat samples for 15 minutes @ RT : Add 2.5 µl 500 mM DMS and mix.

-Stop with 100 µl of DMS Stop Solution:

-Add 400 µl 95% ethanol chill @ -20°C for 5 minutes or longer.

-Spin 5 minutes.

-Resuspend in 120 µl 50 mM Tris(7.8)/50 mM EDTA.

-Precipitate with 90% ethanol/0.1 M NaOAc. Let sit at -20°C for at least 5 minutes.

-Spin 5 minutes.

-400 µl 95% ethanol wash.

-Dry samples for at least 20 minutes.

-Resuspend in 100 µl 0.1 M NaOH or a freshly prepared 1/10 dilution of piperdine.

-Heat samples for 30 minutes-90°C.

-Add 150 µl HZ Stop, add 750 µl 95% ethanol.

-Let sit at -20°C for 5 minutes, spin 5 minutes.

Steps (continued):

-400 µl 70% ethanol wash, spin 2 minutes.

-Dry samples 3 minutes or longer in vacuum dessicator.

-Resuspend in 6 µl (or 10 µl for 40 to 80 cm gels) 95% formamide + Dyes.

-Vortex lightly, spin.

-Heat @ 90°C - 3 minutes.

-Place on ice.

-Load onto gel.

Notes:

Assaying affinity resins does not require Proteinase K or Phenol Extractions.

When making 2 mg/ml dAdT stocks, make it in TE and store it in 100 µl aliquots @ -20° C.

DMS stock is 10 M - make a fresh dilution before using.

Prepare Protein sample to be footprinted by dialyzing vs 'D' buffer until KCl concentration is below 150 mM.

All tubes are Eppendorf 1.5 ml tubes.

See Maxam-Gilbert protocol for HZ Stop and DMS Stop.

MPE *in vitro* Footprinting

From Harshman et al, Cell 53:321-330 (1988).

5x Cocktail

Volume (µl)	Chemical	Stock Concentration	Final Concentration
1.5	$MgCl_2$	1 M	5 mM
2	dAdT	2 mg/ml	14 µg/ml
	Kinase labeled DNA		30 Kcpm/rxn
	G-DH_2O		
total 65			

Sample Reaction Set

Reaction	Protein Fxn	'D' (µl)	Cocktail	MPE	DTT
1	----	20	5 µl	25 µM	4 mM
2	0.1 µl GBF	20	"	Final	Final
3	0.2 µl "	20	"	"	"
4	0.5 µl "	20	"	"	"
5	1 µl "	19	"	"	"
6	2 µl "	18	"	"	"
7	5 µl "	15	"	"	"
8	10 µl "	10	"	"	"
9	1 µl yAP-1	19	"	"	"
10	10 µl "	10	"	"	"
11	20 µl "	-	"	"	"
12	20 µl GC-Box factor	-	"	"	"

Steps:

-Let cocktail and buffers equilibrate to room temperature.

-Add 'D' buffer, then Protein, then Cocktail.

-Allow protein to bind for 3 minutes @ RT.

-Add MPE and allow to bind for 3 minutes @ RT.

-Activate with DTT (4 mM Final).

-React for 15 minutes at RT.

-Stop with 0.5 µl EDTA and 100 µl of Sarkosyl Solution:

1% Sarkosyl,
0.1 M Tris (pH 7.8),
0.1 M NaCl,
0.01 M EDTA,
10 µl 1 mg/ml carrier DNA.

-Add 400 µl 90% ethanol / 0.1 M NaAc, chill @ -20°C for 5 minutes or longer.

-Spin 5 minutes.

-Wash with 400 µl 70% ethanol, 2 minutes Spin.

-Dry samples 3 minutes or longer in vacuum dessicator.

-Resuspend in 6 µl (or 10 µl for 40 to 80 cm gels) 95% formamide + Dyes.

-Vortex lightly, spin.

-Heat @ 90°C - 3 minutes.

-Place on ice.

-Load onto gel.

Notes:

Assaying affinity resins does not require Proteinase K or Phenol Extractions.

When making 2 mg/ml dAdT stocks, make it in TE and store it in 100 µl aliquots @ -20° C.

MPE stock is 100 µM MPE and 200 µM $Fe(II)[(NH_4)_2\ SO_4]_2$. Fresh MPE stock is made by adding the 200 µM FE(II) solution to powdered MPE.

Prepare Protein sample to be footprinted by dialyzing vs 'D' buffer until KCl concentration is below 150 mM.

All tubes are Eppendorf 1.5 ml tubes.

Hydroxyl Radical Cleavage *in vitro* Footprinting

From Tullius et al, Methods in Enzymology, 155:537-558, (1987).

Binding Buffer	10x Cocktail	3x Reaction Cocktail
1 M K_2HPO_4, 50 mM final	0.75 µl 1 M $MgCl_2$,50 mM in cocktail	30 µl 1 mM Fe(II)[$(NH_4)_2SO_4]_2$, 330 µM in cocktail
0.5 M KCl, 120 mM final	1 µl 2 mg/ml dAdT, 100 µg/ml in cocktail	30 µl 2 mM EDTA, 660 µM in cocktail
20% Triton, 0.2% final	? µl labeled DNA, 20-30 Kcpm/rxn.	15 µl 20 mM sodium ascorbate, 3 mM in cocktail
	up to 20 µl with G-D H_2O	15 µl 0.06% H_2O_2, 0.01% in cocktail

Sample Reaction Set

Reaction	Protein Fraction	Binding Buffer(µl)	10x Cocktail	3x Reaction Cocktail
1	---	20	3 µl	10 µl
2	0.1 µl GBF	20	"	"
3	0.2 µl "	20	"	"
4	0.5 µl "	19.5	"	"
5	1 µl "	19	"	"
6	2 µl "	18	"	"

Steps:

-Protein is dialyzed for 4 hours versus Binding Buffer.

-Let cocktails and buffers reach room temperature.

-Allow protein to bind for 3 minutes @ RT.

-Mix Reaction Cocktail, then add to samples : treat samples for 5 minutes @ RT.

-Stop with 1 µl 0.5 M EDTA and 100 µl of DMS Stop Solution.

-Add 400 µl 95% ethanol chill @ -20°C for 5 minutes or longer.

-Spin 5 minutes.

-Resuspend in 120 µl 50 mM Tris(7.8)/50 mM EDTA.

-Precipitate with 90% ethanol/0.1 M NaOAc. Let sit at -20°C for at least 5 minutes.

-Spin 5 minutes.

-400 µl 95% ethanol wash.

-400 µl 70% ethanol wash.

-Dry samples 3 minutes or longer in vacuum dessicator.

-Resuspend in 6 µl (or 10 µl for 40 to 80 cm gels) 95% formamide + Dyes.

-Vortex lightly, spin.

-Heat @ 90°C - 3 minutes.

-Place on ice.

-Load onto gel.

Notes:

Assaying affinity resins does not require Proteinase K or Phenol Extractions.

When making 2 mg/ml dAdT stocks, make it in TE and store it in 100 µl aliquots @ -20° C.

DMS stock is 10 M - make a fresh dilution before using.

Prepare Protein sample to be footprinted by dialyzing vs 'D' buffer until KCl concentration is below 150 mM.

All tubes are Eppendorf 1.5 ml tubes.

See Maxam-Gilbert protocol for HZ Stop and DMS Stop.

Burgess Gel - Protein Renaturation

Pour a 10% Laemmli Protein gel with a 4 or 5 well comb, let sit overnight to partially remove polymerization agents.

Acetone precipitate sample if volume is greater than 400 µl. (use 4 volumes of -20°C Acetone, let sample sit at -20°C for 30 minutes)

Equilibrate gel and buffers to 4°C.

Briefly (30") dry in vacuum dessicator, resuspend in 250 µl 1x loading buffer.

Load with 10 µg insulin per lane, including marker lanes.

Run gel at 150 volts at 4°C.

Run Bromophenol Blue to the bottom (25 kD at the front).

Visualize the protein bands by immersing the gel in 0.25 M KCl + 1 mM DTT.

Immerse for 60" with constant agitation, then rinse in cold G-D H_2O.

Take out of solution and lay on moistened, cold clear glass with a black surface underneath.

Visualize with a strong light source directly over the gel.

Cut out the bands, and place them in small dialysis bags.

Place the sealed dialysis bags in a horizontal gel apparatus filled with 4°C Laemmli Electrode Buffer.

Electrophorese for 3 hours at 75 milliAmps.

Back electrophorese for 30".

Remove acrylamide, dialyze vs 1 liter HGE + 0.1 M NaCl for 6 to 8 hours at 4°C.

Remove solution, and if any cloudiness or precipitate has formed, spin samples to remove it.

4x ice cold Acetone precipitate samples, let sit a -20°C for 30 minutes. There should be a very small pellet. Centrifuge, rinse with 80% ice cold Acetone.

Resuspend pellet in 250 µl 6 M Guanidinium HCl in HGET + 0.1 M NaCl.

Dialyze versus 0.1 HGKET overnight.

Yeast 'Crude Extract' *in vitro* Footprinting

5x Cocktail

Volume (µl)	Chemical	Stock Concentration	Final Concentration
1.5	$MgCl_2$	1 M	5 mM
15	dAdT	2 mg/ml	100 µg/ml
	Kinase labeled DNA		30 Kcpm/rxn
	Pol I labeled DNA		20 Kcpm/rxn
	G-DH_2O		
total 65			

Sample Reaction Set

Reaction	Protein Fxn	'D' (µl)	Cocktail	DNAse I
1	----	20	5 µl	250µg/ml
2	1 µl YNE 'A'	19	"	Final
3	2 µl "	18	"	"
4	5 µl "	15	"	"
5	10 µl "	10	"	"
6	20 µl "	-	"	"
7	1 µl YNE 'B'	19	"	"
8	2 µl "	18	"	"
9	5 µl "	15	"	"
10	10 µl "	10	"	"
11	20 µl "	-	"	"
12	----------	20	"	"

Steps:

-Chill all tubes to 0°C in a Temp Block on ice.

-Add 'D' buffer, then Protein, then add Cocktail.

-Allow protein to bind for 3 minutes @ 0°C.

-DNAse samples for 1 minutes @ 0°C : Add 5 µl 1500 µg/ml DNAse I and mix.

-Stop with 100 µl of Proteinase K solution :

150 µl 1 mg/ml Proteinase K in Sarkosyl
1000 µl 1% Sarkosyl in TE
150 µl 1M Tris (pH 9.5)
10 µl 1 mg/ml carrier DNA

-Heat samples @ 37°C -15 minutes.

-Add 100 µl Phenol (pH 7 to 8), vortex.

-Spin 3 minutes.

-Transfer Aqueous phase, discard phenol phase.

-Add 400 µl 90% ethanol / 0.1 M NaAc, chill @ -20°C for 5 minutes or longer.

-Spin 5 minutes.

-400 µl 70% ethanol wash, 2 minutes Spin.

-Dry samples 3 minutes or longer in vacuum dessicator.

-Resuspend in 6 µl (or 10 µl for 40 to 80 cm gels) 95% formamide + Dyes.

-Vortex lightly, spin.

-Heat @ 90°C - 3 minutes.

-Place on ice.

-Load onto gel.

Notes:

Heparin Sepharose & Affigel Blue fractions can be assayed with 50 µg/ml dAdT final and 150 µg/ml DNAse I final concentrations.

Assaying affinity resins does not require Proteinase K or Phenol Extractions.

When making 2 mg/ml dAdT stocks, make it in TE and store it in 100 µl aliquots @ -20° C.

When making 5 mg/ml DNAse I stocks, make it in either 'D' Buffer or 50% Glycerol 1x LSDB, and store it in 100 µl aliquots @ -80°C.

Prepare Protein sample to be footprinted by dialyzing vs 'D' buffer until KCl concentration is below 150 mM.

All tubes are Eppendorf 1.5 ml tubes, except for the tubes used to store the DNAse I stock, which is kept in 700 µl Eppendorf tubes.

Reaction Volumes can be changed without changing DNAse I conditions. For example, if 50 µl reactions are desired, a 10x Cocktail can be made that still gives a final concentration of 100 µg/ml dAdT, and a DNAsed with a final DNAse concentration of 250 µg/ml.

Note: Concentration of DNAse required depends on the composition of the fragment and its length.

Yeast Gel Shift Protocol

5x Cocktail - Affinity purified material

1.5 µl 1M $MgCl_2$, 25 mM in cocktail
3 µl 2 mg/ml dAdT, 100 µg/ml in cocktail
? µl labeled DNA, 30 Kcpm/reaction
15 µl 40% Ficoll, 10% in cocktail
~40 µl "D" Buffer

5x Cocktail - 'Crude' material

1.5 µl 1M $MgCl_2$, 25 mM in cocktail
15 µl 2 mg/ml dAdT, 500 µg/ml in cocktail
? µl labeled DNA, 30 Kcpm/reaction
15 µl 40% Ficoll, 10% in cocktail
~28 µl "D" Buffer

Sample Reaction Set

Reaction	Protein Fxn	'D' (µl)	Cocktail
1	----	5	20 µl
2	0.1 µl G-Box	5	"
3	0.2 µl "	5	"
4	0.5 µl "	4.5	"
5	1 µl "	4	"
6	2 µl "	3	"
7	5 µl "	-	"
8	1 µl yAP1	4	"
9	2 µl "	3	"
10	5 µl "	-	"
11	2 µl GC-Box	3	"
12	5 µl "	-	"

Steps:

Chill tubes on ice.
Add cocktail to protein.
Bind 3 minutes on ice.
Load onto Gel.
Load one lane of dyes.
Run Gel for 1.5 - 2 hrs @ 100 volts.
Dry gel and expose.

Notes:

One protection unit (footprinting unit) should give complete binding to 20,000 cpm of a 100 bp kinased fragment.

Crude extract reactions can be bound for up to 5 minutes.

Gels should be run in the cold room initially.

4% acrylamide 1:80 bis 0.25x TBE vertical Gel OR 2% Agarose 0.25x TBE horizontal Gel can be used. 0.5x TBE may give sharper bands if the complex is stable in the buffer.

DNA Sequencing

Maxam-Gilbert Sequencing

G	G+A	C+T	C
1 µl carrier DNA	1 µl carrier DNA	1 µl carrier DNA	1 µl carrier DNA
Labeled DNA	Labeled DNA	Labeled DNA	Labeled DNA
200 µl DMS Buffer	up to 21 µl w/ H_2O	up to 21 µl w/ H_2O	20 µl 5 M NaCl
0.5 µl DMS	50 µl Formic Acid	30 µl Hydrazine	30 µl Hydrazine
RT - 6'	RT - 8'	RT - 8'	RT - 8'
50 µl DMS Stop	200 µl HZ Stop	200 µl HZ Stop	200 µl HZ Stop

Add 750 µl ice cold 95% ethanol. Let sit @ -20°C for 5 minutes. Spin 5 minutes. Discard Supernate.

Add 250 µl 50 mM Tris (pH 7.8), 50 mM EDTA, vortex and heat @ 70°C - 2 minutes.

Pipet solutions into new tubes.

Add 750 µl ice cold 90% ethanol/ 0.4 M Ammonium Acetate. Let sit @ -20°C for 5 minutes. Spin 5 minutes. Discard Supernate.

Wash pellet with 750 µl 95% ethanol. Spin. Discard Supernate.

Dry tubes at least 20 minutes in a vacuum dessicator.

Resuspend in a freshly made 1 M piperidine.

Heat @ 90°C for 30 minutes.

Lyophilize or add 150 µl HZ Stop and precipitate with 750 µl ice cold 95% ethanol.

Resuspend in formamide running dye.

Notes:

Times are for a 100 bp fragment with 65% AT content. Length and composition will affect reaction times.
piperidine Stock solution is 10 M and should be clear. discard if yellowed or brown.
Use 88% Formic Acid.

DMS Buffer

50 mM Sodium Cacodylate (pH 8)
10 mM $MgCl_2$
0.1 mM EDTA

DMS Stop

1 M ß-mercaptoethanol
1.5 M Sodium Acetate
50 mM Magnesium Acetate
1 mM Magnesium Chloride
20 µg/ml Carrier DNA

HZ Stop

1 ml 3 M NaOAc
5 µl 0.5 M EDTA
50 µl 5 mg/ml Carrier DNA
9 mls G-D H_2O

Yeast Assays

Yeast ß-galactosidase Assay

Grow 50 mls of cells in minimal media plus necessary supplements to an $A_{650} \approx 1$.

If grown at 23°C, doubling in YM media is ~4 hours.

Weigh 50 ml centrifuge bottles.

Spin down cells at 5 K for 5 minutes, weigh total cell weight.

Resuspend cells in a volume of Suspension Buffer that gives 100 mgs cell weight per ml of solution.

Freeze cells in a dry ice/ethanol bath.

Thaw at room temperature, Repeat Freeze/Thaw twice.

Cells are now permeabilized for ß-galactosidase assay.

ß-galactosidase Activity Assay

Z-Buffer	650 µl	pre-equilibrate to 30°C
4 mg/ml ONPG	150 µl	pre-equilibrate to 30°C
Cell Suspension	100 µl	(prepare one sample with 100 µl of suspension buffer as a blank)

Mix tube with the above solution and let sit at 30°C for 3 minutes.

Stop reaction with 600 µl Stop Buffer (1.0 M Na_2CO_3).

Spin in a clinical centrifuge for 10 minutes to pellet cells.

Measure A_{420}.

Yeast Protein Assay

Cell Assay	No Protein control	1 mg BSA	2 mgs BSA
0 µl BSA	0 µl BSA	100 µl BSA	200 µl BSA
100 µl cells	100 µl Suspension Buffer	100 µl Suspension Buffer	100 µl Suspension Buffer
250 µl 5 N NaOH	250 µl 5 N NaOH	250 µl 5 N NaOH	250 µl 5 N NaOH
750 µl G-D H_2O	750 µl H_2O	650 µl H_2O	550 µl H_2O

Heat for 5 minutes at 90°C.

Cool on ice to room temperature.

Add 375 µl 2.5% $CuSO_4$.

Mix, let sit at room temperature for 5 minutes.

Spin in a clinical centrifuge for 10 minutes.

Measure A_{555}.

ß-galactosidase Activity Calculation:
Units/mg = $2.0 * A_{420} / (5.3 * 10^6 * (\text{mgs protein}) * (\text{time in minutes}))$

Suspension Buffer

0.1 M Tris (pH 7.8)	60 mM	Na_2HPO_4	1 mM	$MgSO_4$
0.05% Triton X-100	40 mM	NaH_2PO_4	30 mM	ß-mercaptoethanol
	10 mM	KCl		

Miscellaneous Techniques

DNA Fragment Isolation onto DE81

Set up a horizontal Agarose gel.

Run the gel so that the fragment(s) of interest is resolved from other fragments by at least 3 mm.

Insert DE81 paper either to the side of the band and rotate the gel 90° and electrophorese into the DE81 paper, or if the fragment is not well resolved place DE81 paper ahead of and the behind the fragment of interest and continue driving the gel in the same direction.

After driving the fragment onto the DE81 paper, remove the DE81 paper, roll it into a ball and place in a 0.7 ml eppendorf tube with 2 - 20 gauge needle holes in the bottom of the tube. The holes should not be in the axial plane of the tube, but rather should be perpendicular to it.

Wash the DE81 paper with 100 µl of 0.1 M NaCl / 10 mM Tris (pH 7.8), first pushing the DE81 paper to the bottom of the tube, then pipetting the Wash Buffer on top of the DE81 paper. Remove the Wash Buffer by placing the tube inside a 1.5 ml eppendorf tube and spinning at a low rate in a microcentrifuge. Spin the microcentrifuge for no longer than 10 seconds at a time or above 1000 rpm.

Discard the Wash Buffer.

Pipet 100 µl Elution Buffer [1 M NaCl / 50 mM arginine (pH 11)] into the eppendorf tube containing the DE81 paper with the DNA fragment.

Elute the fragment by capping the 0.7 ml tube, placing it inside a 1.5 ml tube, and heating on a 70°C Temp Block for 5 minutes or until the Elution Buffer is driven through the DE81 paper into the 1.5 ml tube.

For a DNA fragment smaller than 500 bp elute twice. For a DNA fragment larger than 500 bp elute three times.

Combine the eluant and Phenol/Chloroform Extract (0.75 volumes Phenol/Chloroform per volume eluant).

Ethanol precipitate with 3 volumes 95% ethanol.

Wash with 400 µl 70% ethanol.

Dry pellet.

Resuspend in TE.

Notes:

When inserting and removing DE81 paper from the agarose gel, be sure to remove the gel from the running buffer and drying the surface of the gel. Handling the DE81 paper when it is excessively wet or trying to insert it into the gel after it is wet will cause it to tear.

When washing the DE81 paper in Wash Buffer or in Elution Buffer, do not spin the microcentrifuge too fast - DE81 paper debris will come through the needle holes in the bottom of the 0.7 ml eppendorf tube and could interfere with enzymatic treatment of the isolated DNA fragment.

Antibody Screening by Dot Blot

Clean dot blot apparatus thoroughly with 409 and deionized water.

Wet nitrocellulose in a pan of G-D H_2O. Make sure the nitrocellulose filter is evenly wet.

Place nitrocellulose in the BioRad Dot blot apparatus. I mark the upper left hand corner with a TL to orient the filter in the apparatus.

Screw top of the manifold down evenly. I tighten opposing corners simultaneously, the first time barely snug, then change corners and repeat, so that the top is tightened evenly.

Allow apparatus to chill to 4°C in the cold room.

Apply sample to the wells, I use a minimum of 40 µl of sample. Triton, NP-40 and SDS should be minimized since detergents can interfere with protein binding to Nitrocellulose. 5 ng of a 130 kD protein gives a good signal with undiluted supernates.

After the samples have been applied, allow the antigen to absorb to the nitrocellulose for 1 hour, then suck the sample through the filter. At this point, I remove the top of the apparatus, and dry the bottom lane of the filter carefully, and circle the imprint of the leftmost and rightmost wells in the nitrocellulose with a pen. This simplifies the alignment of the filter later.

Remove the nitrocellulose from the apparatus and "Block" in either 3% BSA in Wash Buffer or 2.5% Non-fat dry milk in Wash Buffer. Block for at least 1 hour, up to 16 hours has been tried without adverse affects. Note : All washes should be done at 4°C, and the Wash Buffer should be premade and equilibrated to 4°C.

Rinse the filter in Wash Buffer, and then replace in the Dot Blot apparatus. It is very helpful to offset the antigen spots so that they only partially realign with the Dot blot wells, so that the antibody-antigen reaction forms a half moon, antigen background forms a full circle, and nonspecific antibody absorption forms a different circle. The individual supernates or antibody dilutions can now be applied to the wells.

Allow the antibodies to incubate for 1 hour, then disassemble the Dot Blot and wash the filter twice for 10-15 minutes in Wash Buffer plus either 0.2% BSA (if BSA was used in the Block) or 0.5% nonfat milk (if nonfat milk was used in the Block). Finally wash the filter in Wash Buffer for 5 to 10 minutes.

Place the filter in a seal-a-meal bag or other appropriate containment system and incubate with the secondary antibody. For our mouse antibodies, we use Goat anti-Mouse Alkaline Phosphatase Conjugate as the second antibody, and use a 1:5000 dilution in 20 mls Wash Buffer. The filter is then rocked at 4°C for 1 hour.

The second antibody is saved (it can be used for up to three filters) and the filter is washed twice for 10-15 minutes in Wash Buffer plus 0.2% BSA (if BSA was used in the Block) or 0.5% nonfat milk (if nonfat milk was used in the Block). Wash the filter in Wash Buffer for 5 to 10 minutes.

The filter is now placed in another seal-a-meal bag and developed in 20 mls of Carbonate Buffer plus 0.25 ml of NBT Stock and 0.25 ml BCIP Stock.

Wash Buffer

1x PBS
0.2% Tween-20
0.1% BSA
0.1% Sodium Azide

Carbonate Buffer

0.1 M $NaHCO_3$
1.0 mM $MgCl_2$
adjust pH to 9.8 w/ NaOH

10x PBS

60 mM K_2HPO_4 (pH 7.5)
30 mM KCl
1.4 M NaCl
pH with H_3PO_4 to 7.2

BCIP Stock

3.75 mg BCIP
250 µl DMF

NBT Stock

7.5 mg NBT
75 µl G-D H_2O
175 µl DMF

Protein Iodination

Prepare Eppendorf Tubes:
100 µl of 1 mg/ml Iodogen™ from Pierce in Chloroform.
Allow chloroform to dry out @ RT in hood.
Cap, and store at RT for up to 2 weeks.

Rinse Iodogenized tubes with G-D H_2O just prior to using.

Add Protein in 250 µl of 100 mM Na_2PO_4 (7.0).

Add 2.5 µl (0.1 µCi/µl) ^{125}I.

Mix, let sit at RT for 30 minutes.

Place on a 2 ml prepared Sephadex G-25 quick-spin column.

Spin column for 2 minutes in a clinical centrifuge.

Take the eluant and check on a ^{125}I radiation counter.

Acetone precipitate with 4 volumes ice cold acetone, let sit at -20°C for at least 30 minutes.

Spin for 5 minutes in an Eppendorf centrifuge.

Vacuum dry.

Recount on a ^{125}I radiation counter.

Preparing Sephadex G-25 Quick-Spin Columns:

Take 2 mls preswollen Sephadex G-25 (swollen in TE) and place in a Quick-Spin column.

Add 1 ml TE to the top of the column.

Spin in a clinical centrifuge for 2 minutes.

If the top of the column is nearly dry, or there is not any buffer left on top of the resin, then the column is prepared. Otherwise repeat spin step until the resin is nearly dry.

Note: Prepare a 'blank' sample along with the protein samples to be iodinated. Treat the blank as another sample to be iodinated, but do not add any protein to the sample. When the spin column is run, the level of ^{125}I eluting from the blank will give the free ^{125}I level in the other reactions. A typical ratio for background to labeled protein is 1:100, using 10 µg of E. coli Pol I.

ImmunoPrecipitations (IPs)

Wash Staph A cells :
Fully resuspend cells, take 100 µl of suspension per IP.
Wash three times in IP Wash Buffer.
Wash twice in IP Binding Buffer.
Bring up to original volume with IP Binding Buffer.

To 100 µl of washed Staph A cell suspension add 20 µg of Goat Anti-Mouse IgG (20 µl of a 1 mg/ml stock).

Mix in cold room for 1 hour.

Add 500 µl of mouse supernate.

Mix in cold room for 1 hour.

Wash twice in 300 µl of IP Tween-20 Wash Buffer.

Resuspend in 50 µl of 'D' Buffer.

Add 100 µl of Heparin (2.0) or another source of crude material with at least 50 Footprinting Units.

Mix in cold room for 1 hour.

Wash twice in 300 µl IP Tween-20 Wash Buffer.

Wash once in 300 µl IP Urea Wash Buffer (thoroughly remove supernate).

Elute twice with 30 µl 6 M Guanidinium HCl in 'C' Buffer + 10 mM DTT. Vortex gently, let sit @ RT for 5 minutes.

Pool both elutions, heat at 70°C for 3 minutes, microdialyze overnight versus 'D' Buffer + 10 mM DTT.

Note: During washes, add buffer and resuspend with a P1000, spin for 15" @ RT [Wash,Binding, & Tween-20], 30" @ RT [Urea and guanidinium-HCl].

Note: It is extremely important to include DTT in the Guanidinium HCl so that the antibodies are completely denatured and do not renature during dialysis. According to Dorrington & Tanford, Advanced Immunology, 12:333-381 (1969), antibodies denatured in 6 M guanidinium-HCl recovery 75% of their activity upon dialysis. Furthermore, antibodies denatured in 6 M guanidinium-HCl in reducing conditions and dialyzed under oxidizing conditions, recover 25-50% of their activity, versus no activity under continued reducing conditions.

IP Wash Buffer
10 mM Tris (7.8)
0.1 mM EDTA
0.5% Tween-20
100 µg/ml BSA

IP Binding Buffer
20 mM Hepes (7.6)
0.5 M NaCl

IP Tween-20 Wash
50 mM Tris (7.8)
150 mM NaCl
0.1 mM EDTA
0.5% Tween-20

IP Urea Wash
100 mM Tris (7.8)
200 mM NaCl
2 M Urea
0.5% Tween 20

Guanidinium HCl
6 M Guanidinium HCl
50 mM Hepes (7.6)
50 mM KCl
0.1 mM EDTA

ImmunoPrecipitations II (IPs)

Wash Protein A -Sepharose CL-4B :
Fully resuspend Sepharose, take 50 µl of suspension per IP.
Wash twice times in IP Wash Buffer.
Bring up to 100 µl per IP with IP Tween-20 Buffer.

Take 500 µl of mouse supernate, add 150 µl Heparin (2.0) Pool (150 µ) and 350 µl Tween-20 Buffer.

Mix in cold room for 1 hour.

Add 100 µl Protein A -Sepharose suspension per IP.

Mix in cold room for 30 to 60 minutes.

Spin.

Wash twice in 1000 µl IP Tween-20 Wash Buffer.

Wash once in 1000 µl IP 'D' Buffer (thoroughly remove supernate).

Elute twice with 50 µl 6 M guanidinium-HCl in 'C' Buffer + 10 mM DTT. Vortex gently, let sit @ RT for 5 minutes.

Pool both elutions heat at 70°C for 3 minutes, microdialyze overnight versus 'D' Buffer + 10 mM DTT.

Note: During washes, add buffer and resuspend with a P1000, spin for 1 minute @ RT [Wash & Tween-20], 2 minutes @ RT [Urea and guanidinium-HCl].

Note: It is extremely important to include DTT in the Guanidinium HCl so that the antibodies are completely denatured and do not renature during dialysis. According to Dorrington & Tanford, Advanced Immunology, 12:333-381 (1969), antibodies denatured in 6 M guanidinium-HCl recovery 75% of their activity upon dialysis. Furthermore, antibodies denatured in 6 M guanidinium-HCl in reducing conditions and dialyzed under oxidizing conditions, recover 25-50% of their activity, versus no activity under continued reducing conditions.

IP Wash Buffer
10 mM Tris (7.8)
0.1 mM EDTA
0.5% Tween-20
100 µg/ml BSA

'D' Buffer
25 mM Hepes (7.6)
10% glycerol
100 mM KCl
0.1 mM EDTA
0.2% Triton X-100

IP Tween-20 Wash
50 mM Tris (7.8)
150 mM NaCl
0.1 mM EDTA
0.5% Tween-20

IP Urea Wash
100 mM Tris (7.8)
200 mM NaCl
2 M Urea
0.5% Tween 20

Guanidinium HCl
6 M Guanidinium HCl
50 mM Hepes (7.6)
50 mM KCl
0.1 mM EDTA

Activation of Sepharose with Cyanogen Bromide

Derived from the method of Jovin : **Eur. J. Biochem. 54:411-418 (1975).**

Obtain Sepharose CL-4B or CL-6B from Pharmacia or Sigma.

Prepare Resin:

-: Wash 10 mls of resin with 10 volumes of G-D H_2O in a medium frit glass filter.
-: Make a 1:1 Slurry of Sepharose in G-D H_2O.

Activate Resin with Cyanogen Bromide:

-: Prepare cyanogen bromide by making a 1 gm cyanogen bromide/ 2 ml DMF solution.
-: Add 1 gm cynaogen bromide to 6 mls Sepharose CL-4B beads (12 mls slurry).
-: Put the Sepharose slurry in a plastic beaker and mix the reaction with a plastic spatula or pipet.
-: Keep temperature between 15 - 20° C by adding ice to reaction. For small volumes (less than 50 mls Sepharose beads) put entire reaction on ice.
-: Keep pH between 10.5 and 11.5 by adding more 5 N NaOH.
-: Activate for 10 minutes or until pH of the reaction stabilizes.

Terminate Activation:

-: Wash Sepharose with 15 volumes G-D H_2O.
-: Wash Sepharose with 15 volumes 10 mM K_2HPO_4 (pH 8.0).

Couple ligand to Sepharose:

-: I have coupled dsDNA, ssDNA, Heparin, and Teterotoxin to Cyanogen Bromide Activated Sepharose prepared by this method.
-: Couple at RT with agitation for 8 to 20 hours. I place the slurry in a plastic bottle and put the bottle on a rocker.

Inactivating Resin:

-: Add 1 M ethanolamine (pH 8.0) to a final concentration of 0.1 M.
-: Mix resin as above for 4 hours at RT.

Preparing Resin for Chromatography:

Pour the coupled, inactivated resin into the column that it will be used in and perform the following washes in the column.

-: Wash resin with 15 volumes 1 M K_2HPO_4 (pH 8.0).
-: Wash resin with 15 volumes 10 mM K_2HPO_4 (pH 8.0).
-: Wash resin with 15 volumes of G-D H_2O.
-: Wash resin with 15 volumes 'D' Buffer.
-: Wash resin with 5 volumes 'D' Buffer + 1 M KCl + 0.1% NaN_3.
-: Store at 4°C.

Notes:

'D' Buffer is a standard chromatography buffer containing 10% Glycerol (Mallincrodt), 25 mM Hepes (pH 7.6) (Calbiochem), 0.1 mM EDTA, 100 mM KCl and 0.2% Triton X-100 (Sigma).

We use CL Sepharose rather than uncrosslinked Sepharose because it has superior resistance to compaction when run at high flow rates and pressures.

It is important to be sure that the ligand to be bound to the activated Sepharose matrix has been cleaned and resuspended in 10 mM phosphate buffer - Tris, high salt and other pH buffers have been shown to inhibit coupling.

If DNA is being ligated to the resin, be sure to remove as much ATP as possible by precipitating in 90% ethanol/0.4 M NH_4OAc before resuspending in the 10 mM Phosphate buffer.

The Phosphate buffer is made up of K_2HPO_4 and KH_2PO_4 so that the final pH is 8.0 and the final PO_4^{-3} is 1 M.

The final wash buffers that are used to prepare the resin are **not** the ones used in the Jovin paper. They are designed so that if DNA is coupled to the Sepharose, any uncoupled DNA comes out in the first two washes, rather than the third wash, as in the Jovin method.

Southwesterns

Pour a 10% Laemmli Protein gel.

Load 50 - 100 Footprinting Units of highly purified factor on gel with 10 µg Insulin.

If desired, load a similar number of Footprinting Units of less purified fractions as comparisons.

Run gel : minigel - 100 volts - 1.5 hours; 20 cm gel - 150 volts for 3 hours.

Transfer gel to nitrocellulose filter in electroblot apparatus for 4 hours at 80 volts in 1x Laemmli Electrode buffer w/o SDS. The transfer may also be done overnight at 50 volts. The transfer is done in prechilled buffer and at 4°C.

Block for 2 hours in 'D' buffer + 3% BSA or 2.5% dried nonfat milk in the cold room.

Wash in 'D' Buffer for 10 minutes in the cold room.

Cut the nitrocellulose filter into strips if multiple DNA probing solutions will be used.

Place the nitrocellulose filter in a seal-a-meal bag with 20 mls of 'D' buffer containing 100 µg/ml of denatured salmon sperm DNA and 300,000 cpm/ml of hot probe. Smaller amounts of Probe buffer may be used for smaller sheets of nitrocellulose.

Mix sample for 1 hour in the cold room.

Wash the nitrocellulose filter in 200 mls 'D' buffer for 15 minutes.

Wash the nitrocellulose filter twice in 200 mls 0.3 HGKET for 15 minutes.

Rinse in 'D' buffer and expose with X-ray film overnight.

Notes:

If a specific signal is **not** found using this protocol, several additions can be made :

1. Adding a series of guanidinium-HCl washes to renature the protein on the nitrocellulose filter. I have found that this is necessary to get a signal from G-Box factor from S. cerevisiae. I transfer and block as normally, and then place the filter in a seal-a-meal bag with 15 mls of 6 M guanidinium-HCl in 'C' buffer, mix for 30 minutes, and dilute to 3 M, mix for 30 minutes. Repeat dilutions and mixing times until the filter is in 0.375 M guanidinium-HCl, then remove from the seal-a-meal bag and reblock in 'D' Buffer + 2.5% dried milk for 30 minutes.
2. Rather than running a denaturing protein gel, run a native protein gel and transfer to nitrocellulose. This is necessary for heterologous complexes that recognize DNA and even then may not give good signals. If it works, it does allow one to estimate the number of multimers present in the complex, if the complex is composed of only one subunit, and a denaturing gel Southwestern gives the size of the monomer.
3. A combination of all of these approaches may be necessary to achieve a good signal.

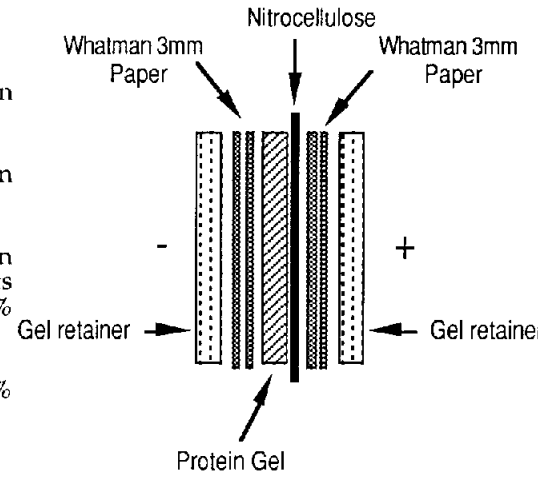
Westerns

Pour 10% Laemmli Protein gel.

Load 500 - 1000 Footprinting Units of factor on gel with 10 µg Insulin.

Run gel : minigel - 100 volts - 1.5 hours; 20 cm gel - 150 volts for 3 hours.

Transfer gel to nitrocellulose filter in electroblot apparatus for 4 hours at 80 volts in 1x Laemmli Electrode buffer w/ 20% methanol.



Block for 2 hours in Western Wash buffer + 3% BSA or 2.5% dried nonfat milk.

Wash in Wash Buffer for 10 minutes.

Either cut the nitrocellulose filter into strips or place filter in a miniWestern device (e.g., Immunetics)

Strips: Add 1 ml supernates to the filters, let mix for 1 hour.

Miniblot : Add 150 µl 5:1 concentrated supernates, let sit for 2 hours.

Remove from primary antibody, rinse filter in Wash Buffer.
Wash twice in Wash Buffer + 0.5% nonfat dried milk or 0.2% BSA for 10 minutes.

Rinse in Wash Buffer.

Place in a seal-a-meal bag with 20 mls of Wash buffer + 4 µl Goat Anti-mouse IgG Alkaline Phosphatase Conjugate (1:5000 dilution).

Mix sample for 1 hour.

The second antibody is saved (it can be used for up to three filters) and the filter is washed for 10-15 minutes in Wash Buffer plus 0.2% BSA (if BSA was used in the Block) or 0.5% nonfat milk (if nonfat milk was used in the Block). Wash the filter in Wash Buffer for 5 to 10 minutes.

The filter is now placed in another seal-a-meal bag and developed in 20 mls of Carbonate Buffer plus 0.25 ml of NBT Stock and 0.25 ml BCIP Stock.

Wash Buffer
1x PBS
0.2% Tween-20
0.1% BSA
0.1% Sodium Azide

Carbonate Buffer
0.1 M $NaHCO_3$
1.0 mM $MgCl_2$
adjust pH to 9.8 w/ NaOH

10x PBS
60 mM K_2HPO_4 (pH 7.5)
30 mM KCl
1.4 M NaCl
pH with H_3PO_4 to 7.2

BCIP Stock
3.75 mg BCIP
250 µl DMF

NBT Stock
7.5 mg NBT
75 µl G-D H_2O
175 µl DMF