Regulatory Elements in ColE1 DNA Replication in *Escherichia coli*

Mutants of *Saccharomyces cerevisiae* DNA Polymerase I Resistant to Nucleotide Analogs: dNTP Binding Site Definition

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To my grandmother
whom I hold forever close to my heart

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ABSTRACT

Control of ColE1-type plasmid DNA replication in *Escherichia coli* was investigated. The initiation of DNA replication in ColE1-type plasmids is regulated by two *trans*-acting negative control elements: RNA I and the *rop* protein. RNA I is a transcript 108 nucleotides long made off the L-strand (lagging strand) of the plasmid and is complementary to the 5' portion of the preprimer, RNA II. The direct base-pairing interaction between the two RNA species precludes the formation of an RNA II:template DNA hybrid, which is processed by RNase H at the origin of replication to create the 3'-OH end of the mature primer. Another *trans*-acting regulatory element is a 63 amino acid plasmid-encoded protein, the *rop* gene product. By providing the *rop* gene product in *trans* on a compatible plasmid, suppression of the runaway replication phenotype of pJN75 was observed. Utilizing this property of the regulatory mechanism, we proceeded to select pJN75 derivatives that are insensitive to *rop*-mediated suppression. These mutant plasmids were designated pJN75*nsr* for non-suppressible by *rop*. Sequence analyses of 7 *nsr*, showed disruptions of base-pairing within the stem of loop structure III of RNA II and loop structure III' of RNA I, implying that *rop* mediates its action via the region comprising loop structures III and III'. We were also interested in the presence of a dnaA protein binding site about 90bp downstream of the *ori*-pBR322. We investigated the role of dnaA protein in ColE1-type DNA replication by purifying the dnaA protein to homogeneity from an overproducing strain and examining its effect on various mutant DNA templates in an *in vitro E. coli* DNA replication extract developed in the Campbell lab. We found that the combination of dnaA protein binding at the dnaA consensus sequence can substitute for the lack of primosome assembly site (pas) on the H-strand (leading strand), which is postulated to be the point of transition between DNA polymerase I and polymerase III-dependent DNA synthesis. In the absence of the H-strand pas, dnaA protein may direct

other essential proteins to form a replication complex at the dnaA site, functionally acting as proteins i, n, n' and n" at pas.

In an effort to identify and characterize the nucleotide and/or pyrophosphate binding site(s) of yeast DNA polymerase I, we have attempted to isolate *pol1* mutants that are resistant to nucleotide/pyrophosphate analogs. We successfully constructed a *Saccharomyces cerevisiae* strain that depends on exogenous thymidine for survival and also contains a temperature-sensitive DNA polymerase I allele (*pol1-17*). Using this strain, 167-pol1-17, we screened for *pol1* mutants that are resistant to nucleotide/pyrophosphate analogs, e.g., AraT or PAA, which are normally impermeable to the cell wall of yeast. Our strategy was to introduce a plasmid containing a mutagenized *pol1* gene into 167-pol1-17, which contains a ts DNA polymerase. By screening for survivors at 37°C (non-permissive temperature of *pol1-17* allele) on plates containing 1 mM AraT and 40 mM PAA, we hoped to isolate DNA polymerase I mutants on the transformed plasmid. To our disappointment, we were unable to isolate such DNA pol I mutants. We offer two explanations for the failure of our strategy: 1) the presence of another essential polymerase, *CDC2* gene product and 2) perturbation of pyrimidine nucleotide pool. Finally, we propose to conduct site-directed mutagenesis of DNA polymerase I at putative dNTP/PP_i binding domains and to analyze the mutant polymerases *in vitro* for resistance to dNTP/PP_i analogs. Site-directed mutagenesis experiments are now in progress, and we are hopeful that these mutants will provide structural and functional information regarding the nucleotide/pyrophosphate binding site(s) of yeast DNA polymerase I.

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PART I

Regulatory Elements in ColE1 DNA Replication in *Escherichia coli*

INTRODUCTION

Bacterial plasmids, as extrachromosomal elements, are dispensable for cell viability and replicate independently of the bacterial chromosome. They are popular as model systems for studies regarding regulation of DNA synthesis. In a given host under defined growth conditions, each specific plasmid is maintained at its individual characteristic number per cell, its "copy number". Both the low- and high-copy number plasmids share a number of common replication features. Unlike the bacterial chromosome, plasmids can replicate in the absence of *de novo* protein synthesis. In the presence of chloramphenicol or other drugs at levels that inhibit protein synthesis, the bacterial chromosome is prevented from undergoing new rounds of replication. However, since plasmids do not require any plasmid-encoded proteins for replication *in vivo* and the necessary host proteins are stable, the plasmid continues to replicate and accumulates >1000 copies per cell. Hence, these plasmids are popular vectors for *in vitro* cloning of genes that are desired in large quantities.

The colicin E1 plasmid of *Escherichia coli* (ColE1) is considered the prototype of the multicopy plasmids, normally existing at 10-15 copies per chromosome. ColE1 is a closed, circular DNA molecule of approximately 6600 base pairs (molecular weight = 4.2×10^6) (Helinski, 1976; Tomizawa, 1978). ColE1 replicates *in vitro* as well as *in vivo* in the absence of plasmid-specified proteins (Donoghue and Sharp, 1978; Kahn and Helinski, 1978). All members of the ColE1 group of replicons require the host-encoded enzymes DNA polymerase I (*polA* gene product) (Kingsbury and Helinski, 1973) and DNA-dependent RNA polymerase (Staudenbauer, 1975) as well as DNA polymerase III (*dnaE* gene product) (Staudenbauer, 1976) for DNA replication. The products of *Escherichia coli dnaB*, *dnaC*, *dnaG* and *dnaZ* are also needed. Plasmids belonging to this group include pMB1, CloDF13, p15A and RSF1030 (RSF1030 is the same as NTP1) (Conrad *et al.*, 1979; Grindley and Nakada, 1981; Som and Tomizawa, 1982; Yamada *et al.*, 1979). By a combination of genetics and *in vitro* biochemistry,

two major players have been revealed in the regulation of replication initiation: the *cis*-acting RNAI and the *trans*-acting *rop* protein, both acting on a 600 base pairs (bp) region mainly upstream of the origin and known to contain all the information necessary for replication (Backman *et al.*, 1978; Oka *et al.*, 1979).

Within the 600bp region, there are two start sites for transcription by RNA polymerase *in vitro* (Itoh and Tomizawa, 1980). Figure 1 is the *Fnu*DII fragment isolated from pMB1 that comprises most of the information needed for replication of ColE1 plasmids. Transcription from one of the sites, 555 bp upstream of the replication origin, produces a transcript called RNAII. This nascent transcript forms a persistent hybrid with the template DNA near the origin. The DNA-RNA hybrid is then specifically processed by RNaseH at the origin and serves as a primer for L-strand DNA synthesis by DNA polymerase I (Itoh and Tomizawa, 1980). Transcription from the other site, 450 nucleotides upstream, occurs in the opposite direction of RNAII to give the major regulator of DNA replication, RNAI. RNAI, a 108 nucleotide transcript, made off the L-strand of the plasmid and complementary to the 5' portion of RNAII, interferes with RNaseH-catalyzed processing of RNAII (Tomizawa *et al.*, 1981). From the sequences of inhibitory RNAI and the preprimer RNAII, it is deduced that both can form 3 stem-and-loop structures (see Fig. 2) (Cesareni *et al.*, 1982; Lacatena and Cesareni, 1981; Lacatena and Cesareni, 1983; Ohmori and Tomizawa, 1979; Oka *et al.*, 1979; Tomizawa and Itoh, 1981). Physical evidence for this cloverleaf structure has also been obtained (Morita and Oka, 1979; Tamm and Polisky, 1983). Direct base-pairing interaction between the two RNA species interferes with RNaseH processing of the primer (Tomizawa, 1984; Tamm and Polisky, 1985). However, addition of RNAI after the primer precursor transcription is complete has no effect on subsequent RNaseH cleavage. These results indicate that inhibition involves interaction of RNAI with newly transcribed primer before the primer assumes some conformation critical for hybridization with DNA template (Tomizawa *et al.*, 1981). By

studying the altered secondary structures of the RNAII in a variety of mutants, a great deal is now known of the complex formed between the preprimer, RNAII and RNAI.

Masukata and Tomizawa (1986) have studied the conformation of wild-type RNAII in the presence or absence of RNAI by partial digestion of RNAII with various RNases. The cleavage patterns of RNAII by various RNases were very much altered by the presence of RNAI during transcription. It is evident that association with RNAI alters preprimer conformation in structural domains distant from the RNA-RNA duplex domain (Masukata and Tomizawa, 1986). These structural changes of RNAII can also be induced by *pri7*, a point mutation within the DNA coding region for both RNAI and RNAII. The RNase cleavage patterns of *pri7* RNAII were different from those of wild-type RNAII but almost identical to those of RNAII made in the presence of RNAI. Thus, *pri7* mutation causes alteration of RNAII structure similar to that produced by RNAI binding. Genetic analyses of mutations and biochemical data on RNases structural probing were used as constraints in computer-assisted modeling of primer folding. The predicted secondary structures of the wild-type and *pri7* RNAII suggest specific domains likely to be involved in the regulation of processing by RNAI. One such domain in the nascent primer is a palindromic structure known as stem-loop IV, whose formation is important for subsequent primer hybridization to the template (Fig. 3A, 2). Mutations that destabilize stem-loop IV are phenotypically replication-deficient but can be suppressed by second-site changes that restore stem formation (Masukata and Tomizawa, 1984). Interaction of the nascent primer with RNAI prior to stem-loop IV formation actually prevents stem-loop formation. Because RNAI is partially complementary to the 5' side (α) of this structure (Fig. 3B), the β domain is then free to interact with a downstream γ domain, resulting in conformational changes that preclude hybrid formation between RNAII and the DNA template (Fig. 3B, 3).

The kinetics of duplex formation between between RNAI and primer has also been investigated. Tomizawa (1984) postulated that the association of the two RNAs occurs

in at least 3 stages: 1) an initial reversible stage involving "kissing" between the two RNAs via the three loops (Fig. 4, I); 2) nucleation of the 5' terminus of RNAI with its complement in the primer (Fig. 4, II); and 3) final annealing of the two RNAs to form an RNA-RNA duplex (Fig. 4, III to VI). At the first reversible stage of interaction between RNAI and the primer, a *trans*-acting regulatory element is known to act, the *rop* protein.

Rop gene product (repressor of primer) or rom protein (RNA one modulator) is the sole plasmid-encoded protein involved in control of DNA replication in ColE1. It is located within the *Hae*II-C restriction enzyme fragment of ColE1 (Twigg and Sherrat, 1980) and has been identified as a 63 amino acid protein (Lacatena *et al.*, 1984). It exists in solution as a dimer and is acidic. This protein is dispensable: deletions of the gene increase copy number by four- to sixfold but do not alter other plasmid characteristics (Twigg and Sherrat, 1980). The *rop* gene product has been reported to reduce the extent of expression of *lacZ* or *galK* when these genes are placed under RNAII promoter control (Cesareni *et al.*, 1982; Som and Tomizawa, 1983). Som and Tomizawa (1983) have determined that a region of RNAII ~50 to 135 bp downstream from the 5' end is essential for interaction with the *rop* gene product. Chapter 1 describes our effort to identify the site at which the *rop* gene product mediates its effect. Briefly, the *rop* gene product is able to suppress the runaway replication of an NTP1 derivative, pJN75. By isolating second-site mutations in pJN75, which render the plasmid non-suppressible by rop (*nsr*), we hope to gain insight into the mechanism of *rop*-mediated regulation of plasmid replication. All the *nsr* mutants isolated were found to be single-base mutations within positions -429 to -449, and they act by disrupting base-pairing interactions in stem structures III and III' of RNAII and RNAI, respectively. We have suggested that the *rop* gene product acts by enhancing the RNAI:RNAII interaction and disfavoring the RNAII:DNA hybrid formation. The study was completed in 1984, and since then Tomizawa and Som (1984) have shown

that rop protein accelerates the initial reversible interaction between RNAI and the nascent primer, the "kissing' stage. Recently, the rop protein has been demonstrated to interact with the stem sequences of RNAI and the primer, based on RNase protection experiments (Helmer-Citterich *et al.*, 1988). A consequence of the binding is that the 5' terminal single-stranded region of RNAI is more accessible to nuclease, suggesting that the protein makes the region more available for interaction with the complementary sequence in RNAII. The model proposed by Helmer-Citterich *et al.* (1988) suggested that the rop protein acts as an adaptor between the two RNAs. Each subunit of the symmetric dimer of rop binds to the stem(s) of one RNA molecule and if the two RNA molecules possess complementary loops, these are then positioned correctly for the "kissing" action. The rop protein has been crystallized and its structure solved to 1.7 Å by X-ray diffraction (Banner *et al.*, 1987). The rop dimer is a bundle of four tightly packed α-helices that are held together by hydrophobic interactions. Systematic site-directed mutagensis of the rop protein reveals that rop folding is rather insensitive to amino acid substitutions and to other drastic deletion and insertion mutations (Castagnoli *et al.*, 1989). However, a small number of side chains clustered at the extremeties of the rop cylinder play an important role in promoting the interaction between RNAI and RNAII and thus plasmid number control. These essential side chains all belong to the first helix and reside on one of the two surfaces of the protein perpendicular to its twofold axis. It is still unclear how these structural features facilitate the interactions between the protein and the 2 RNAs. Cocrystallization of rop protein with the 2 RNAs will be helpful in resolving the mystery.

Another aspect of ColE1 DNA replication that I have investigated is the role that dnaA protein plays in replication of this plasmid. DnaA protein is essential for the viability of wild-type *E. coli*. Temperature-sensitive *dnaA* mutants show a slow stop phenotype, usually associated with defective origin initiation. DnaA protein was first isolated by using transducing phages that carry the *dnaA* gene (Chakraborty *et al.*,

1982) or by placing the cloned *dnaA* gene into plasmids under control of the λ P_L promoter (Fuller and Kornberg, 1983). Subsequently, dnaA protein was purified to homogeneity by Fuller and Kornberg (1983) by assaying its ability to promote the replication of *oriC* minichromosome plasmids in a soluble enzyme system (Fuller *et al.*, 1981). Purified dnaA protein is a soluble polypeptide of 52,000 daltons, active as a monomer but has a strong tendency to aggregate into multimeric forms of reduced specific activity.

Filter-binding assays (Fuller and Kornberg, 1983) showed dnaA protein to specifically bind the minimal 245bp *oriC* sequence identified by Oka *et al.* (1980) (Fig. 5). Footprinting studies of dnaA protein revealed strong binding interactions at four conserved 9 base pair sequences termed dnaA boxes, each of which contains the consensus (Fuller *et al.*, 1984):

$$5'\text{-}TTAT^{C}_{A}CA^{C}_{A}A\text{-}3'$$

The interaction of dnaA protein with *oriC* DNA was also shown to be highly cooperative. Starting with the binding of dnaA monomers to the 9bp consensus sequences, the addition of monomers in both directions creates a mass of 20 to 40 protein monomers interacting with 200-250 bp of DNA. This complex has been confirmed by electron microcospy (Funnell *et al.*, 1987) yet the structure of this nucleoprotein complex is still largely unknown.

DnaA protein binds ATP with a high affinity (K_D = .03 μM) and in the presence of DNA, slowly hydrolyzes ATP to ADP. The ADP bound form of dnaA protein exchanges very slowly with ATP. Both the ATP and ADP form of dnaA protein bind the dnaA boxes in *oriC* and protect them from DNase I cleavage, but only the ATP form is active in replication (Sekimizu *et al.*, 1987). However, ATPγS, a non-hydrolyzable analog of ATP, is capable of replacing ATP in the prepriming reaction, suggesting that the ATP function is allosteric rather than energy-providing. It has been

postulated that fluctuating levels of nucleotides during the cell cycle regulates the activity of the initiator protein, dnaA protein, and henceforth, indirectly regulates the initiation of chromosomal replication. Recently, cAMP has been implicated in this kind of control mechanism. DnaA protein interacts with cAMP with a K_D of 1μM. The interaction releases ADP but not ATP bound to dnaA protein and thus restores DNA replication activity to inactive ADP-dnaA protein preparations (Hughes *et al.*, 1988). The mechanism of reactivation of ADP-dnaA protein has yet another intriguing aspect. In the presence of ATP and the components of the reconstituted replicative system, cardiolipin (an acidic membrane phospholipid), has been shown to displace ADP from dnaA protein, rejuvenating the inactive ADP form (Sekimizu and Kornberg, 1988). This observation together with the fact that active ATP-dnaA protein is recovered from particular fractions of non-overproducing strains (Sekimizu *et al.*, 1988) have prompted the suggestion that membrane attachment of dnaA protein is essential to its function in the initiation of chromosomal replication in *E. coli* . Yung and Kornberg (1988) have further substantiated the claim of membrane dependence of the initiation of replication by reporting that membrane phospholipids must contain an unsaturated fatty acid to facilitate the exchange of ADP and ATP-bound forms of dnaA protein. The presence of the *cis* double bond in the unsaturated fatty acid disrupts the regular stacking of the saturated fatty acids and moderates the fluidity of the lipid bilayer. Why dnaA protein needs to react with acidic phospholipids in a fluid phase of a bilayer requires further investigation.

The mechanism of initiation by dnaA protein at *oriC* has been thoroughly examined and the following model derived from these investigations (Fig. 6). DnaA protein binds tightly to the four conserved dnaA boxes (9 mers showed in Fig. 6) in *oriC* to form an initial complex. By cooperative binding, a nucleoprotein complex is formed with a core of 20-40 dnaA monomers surrounded by *oriC* DNA. In the presence of 5mM ATP and high temperature, 38°C, the stepwise duplex opening of the AT-rich 13-

mer repeats occurs to form an open complex. DnaB and dnaC then enter this complex via protein-protein interactions to form the prepriming complex (Bramhill and Kornberg, 1988a). The unwinding of AT-rich region requires ATP and dnaA protien acting at a temperature above 22°C. Deletion studies indicate that *oriC* DNA retaining only a single 13-mer (the rightmost one, closest to the dnaA boxes) can form an open complex, but *oriC* DNA templates lacking all 13-mers are inactive in replication. DnaA protein involvement in initiation of chromosomal replication is not confined to direct interaction with *oriC* DNA. Extragenic suppressors of *dnaA* have been isolated over the past two decades and map to two major genes known to be involved in the initiation of replication, namely, *rpoB* (coding for β-subuit of RNA polymerase) and *rnh* (RNaseH).

RNA polymerase (RNAP) has long been implicated in *E. coli* chromosomal replication because of sensitivity of initiation to rifampicin, a known RNAP inhibitor (Lark, 1972). Mutations in *rpoB*, the structural gene for the β subunit of RNA polymerase, suppress the temperature sensitivity of given $dnaA_{ts}$ alleles. Analysis of the suppression pattern reveals allele specificity (Atlung, 1981; Atlung, 1984;, Bagdasarian *et al.*, 1977; Schaus *et al.*, 1981) and indicates that specific mutations of RNAP can assist defective dnaA proteins in initiation. Further, dnaA protein and RNAP probably interact directly with each other. Several promoters have been identified in or near the 245bp minimal *oriC* replication sequence (Lother and Messer, 1981; Morelli *et al.*, 1981; Rokeach and Zyskind, 1986; Schauzu *et al.*, 1987) (Fig. 7), and studies have been done to relate the level of transcript synthesis to intracellular dnaA protein concentration. Transcription from the *mioC* gene promoter (modulator of initiator at *oriC*; *mioC* encodes a 16kd protein of unknown function) is blocked by dnaA protein *in vitro*, and transcriptional activity increases *in vivo* if the cellular dnaA protein is inactivated (deWind *et al.*, 1987; Lother *et al.*, 1985). Transcription of *mioC* specifically enhances the stability and copy number of *oriC* plasmids (Stuitje and

Meijer, 1983; Stuitje *et al.*, 1986). These studies also established that a substantial fraction of the *mioC* gene transcript enters and terminates at the origin and that some sites of termination of RNA correspond to RNA·DNA junctions previously identified (Fig. 7) (Junker *et al.*, 1986; Kohara *et al.*, 1985; Rokeach and Zyskind, 1986; Schauzu *et al.*, 1987). In light of the transcription studies, suggestions were made that the *mioC* gene transcript may be required for initiation, perhaps by providing RNA primers for the leading strand synthesis. However, the *mioC* promoter can be deleted from both plasmid and chromosome without abolishing *oriC*, dnaA-dependent replication. An alternate explanation is that by controlling the frequency of the *mioC* gene transcription, dnaA protein in turn regulates the frequency of transcriptional activation of the origin. This model is supported by *in vitro* studies. The crude *in vitro* system that specifically replicates *oriC* plasmids can be inhibited by rifampicin, suggesting a role for RNAP (Fuller *et al.* 1981), which agrees with genetic results. In contrast, the reconstituted system with purified proteins has obviated the need of RNAP except under conditions of high concentration of histone like protein HU or topoisomerase I, or low temperature (Ogawa *et al.*, 1985; van der Ende *et al.*, 1985). In each case, lack of initiation seems to be related to failure by the dnaA protein to open the duplex DNA for the dnaB helicase to enter the complex. Further investigation showed that transcription by RNAP towards *oriC* assists dnaA protein in opening the DNA duplex at AT-rich 13-mer repeats (Baker and Kornberg, 1988). Thus, dnaA protein facilitates the formation of open complex at *oriC* by: 1) binding the 9-mer dnaA boxes leading to stepwise duplex opening of the AT-rich 13-mer repeats, and 2) playing a regulatory role in transcriptional activation of the origin.

The second major class of extragenic suppressors of *dnaA* is *rnh* mutants defective in RNaseH activity. Ogawa *et al.* (1984) have demonstrated that RNaseH enhances the specificity of dnaA protein-dependent replication initiated at *oriC* in a partially purified enzyme system. RNA transcripts hybridized to template DNA outside *oriC* are

eliminated by RNaseH to abolish abnormal priming of DNA replication. Interestingly, *rnh* mutants display a dnaA, *oriC*-independent pathway of replication called *sdr*^{c}, constitutive stable DNA replication (Kogoma, 1978). This pathway was first observed when thymine-starved *E. coli* cells developed the ability to replicate DNA without *de novo* protein synthesis (Pritchard and Lark, 1964). Subsequently, it was shown that this *recA*$^{+}$-mediated response can also be induced by other treatments similar to those that induce SOS response in *E. coli* (Kogoma and Lark, 1975). Besides *rnh* mutants, there are two other types of mutants that are part of this inducible SDR pathway, namely, *dnaT* and *sdrT* mutants. *DnaT* mutants fail to assemble the pathway after being induced, whereas *sdrT* showed SDR as the only pathway of replication. Inducible SDR has been shown not to require dnaA protein (Lark and Lark, 1979; Lark *et al.*, 1981). However, certain *dnaA* mutations can suppress the severity of the phenotype displayed by *dnaT* and *sdrT* mutants (Lark *et al.*, 1981). Recently, *dnaT* has been identified to encode protein i, a protein necessary for primosome assembly in ϕX174 replication (Arai and Kornberg, 1981; Arai *et al.*, 1981) and in ColE1 replication (Masai and Arai, 1988). One interesting interpretation is that dnaT and dnaA protein actively cooperate in the correct assembly of a primosome structure, which is different from the traditional role envisioned for dnaA protein binding to its consensus sites at replication origins.

The presence of the 9bp consensus sequence for dnaA protein binding is not confined to minimal *oriC*. The dnaA site is also present at the promoters of the *mioC* gene described above and at its own promoters for autoregulation of dnaA protein levels. In both cases, it has been shown that dnaA protein regulates transcription (Atlung *et al.*, 1985; Junker *et al.*, 1986). The conservation of both dnaA protein and the dnaA binding sequences at bacterial origin is widespread throughout eubacteria (Fujita *et al.*, 1989; Zyskind *et al.*, 1983) (Fig. 5). It is likely that dnaA protein and its analogous protein in other eubacteria will be involved in the same kind of regulatory

mechanism as found in *oriC*. At least one copy of the 9bp consensus sequence is found in or near origins of plasmids pSC101, F, P1, R1 and ColE1. For pSC101, concerted action of dnaA protein and plasmid-encoded rep protein is postulated (Frey *et al.*, 1979). Rep protein binds tightly to repeated sequences in one portion of the origin and creates a nucleoprotein complex. DnaA protein then binds to its consensus sequence followed by DNA duplex melting at the 13-mer repeats similar to those present in *oriC*. This process enables the origin to open up for the entry of dnaB helicase. P1 and F (Hansen and Yarmolinsky, 1986; Klein *et al.*, 1986) and also R1 plasmids (Masai and Arai, 1987; Ortega *et al.*, 1986) have been demonstrated to be dnaA-dependent. Because of the striking similarity in the organization of the basic replicons in all these plasmids (Chattoraj *et al.*, 1985; Linder *et al.*, 1985) (Fig. 8), it was suggested that initiation of replication of these plasmids may involve similar mechanisms. However, ColE1 plasmid replication is different in replicon organization from all the plasmids above and undoubtedly utilizes a different initiation mechanism (Masukata *et al.*, 1987; Minden and Marians, 1985). In spite of ColE1's unique initiation mechanism, a dnaA site is present ~90bp downstream of the origin (origin defined as the site of first nucleotide incorporation by DNA polymerase I). Since the presence of dnaA protein binding sites in all the plasmids, chromosomal origins and promoters of genes have without exception revealed a major role for dnaA protein in replication, we are interested in discovering the role played by dnaA protein in ColE1 replication. In Chapter 2, we describe our effort to purify dnaA protein and to define its role in ColE1 DNA replication. Our results support Seufert and Messer's view (1987) that the combination of dnaA protein and dnaA site can participate in primosome assembly during ColE1 replication independent of primosome assembly sites.

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Figure 1. Nucleotide Sequence of the *Fnu*DII Fragment from Plasmid pMB1. ORI indicates the ColE1 replication origin. RNAI and RNAII are transcription initiation sites and direction of elongation are indicated by arrows. Terminator region corresponds to position of RNA I transcript termination. Figure from Backman *et al.* (1978).

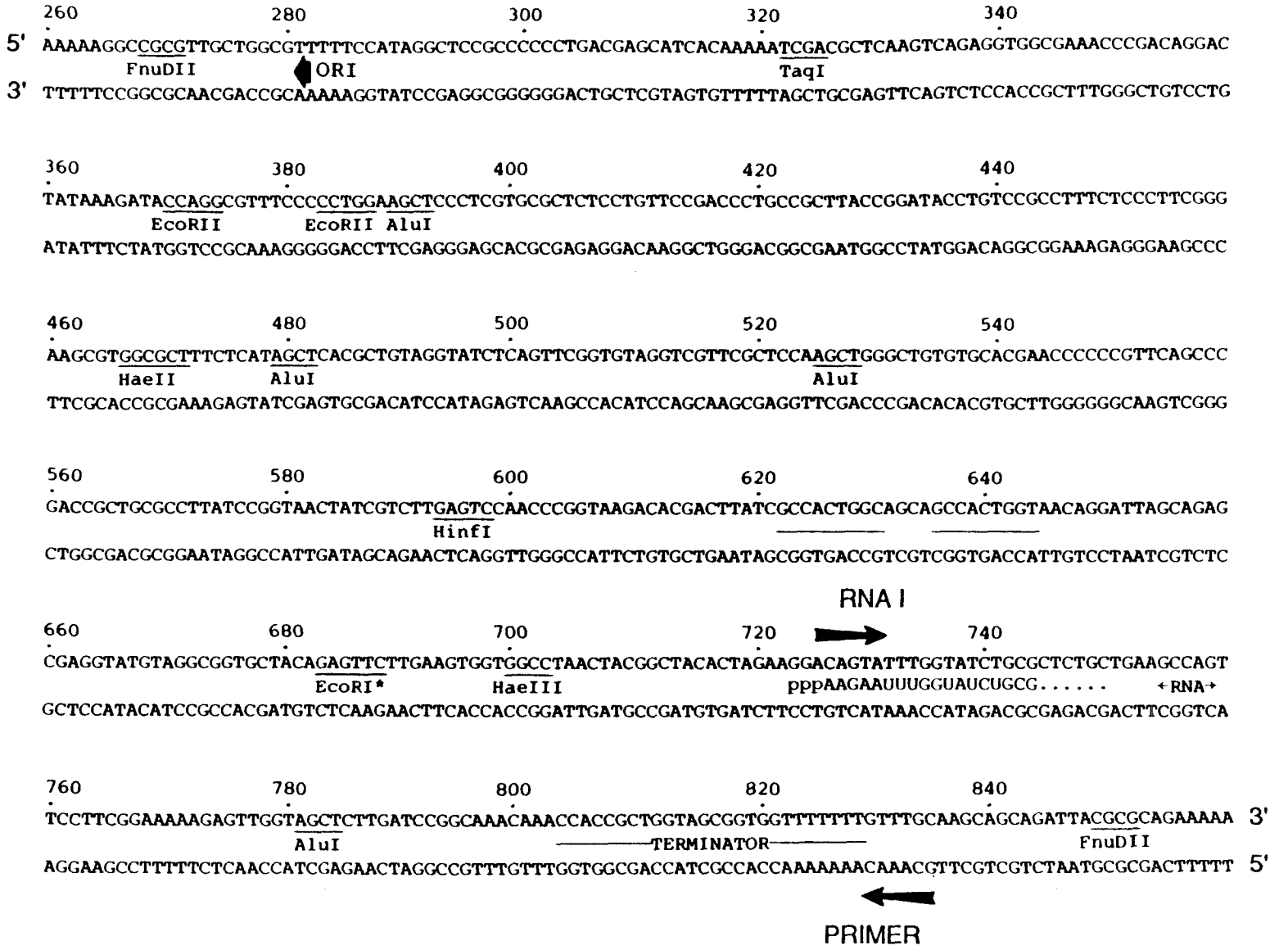

260
280
300
320
340
5'
AAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGAC
FnuDII
ORI
TaqI
3'
TTTTTCCGGCGCAACGACCGCAAAAAGGTATCCGAGGCGGGGGGACTGCTCGTAGTGTTTTTAGCTGCGAGTTCAGTCTCCACCGCTTTGGGCTGTCCTG
360
380
400
420
440
TATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGG
EcoRII
EcoRII
AluI
ATATTTCTATGGTCCGCAAAGGGGGACCTTCGAGGGAGCACGCGAGAGGACAAGGCTGGGACGGCGAATGGCCTATGGACAGGCGGAAAGAGGGAAGCCC
460
480
500
520
540
AAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCC
HaeII
AluI
AluI
TTCGCACCGCGAAAGAGTATCGAGTGCGACATCCATAGAGTCAAGCCACATCCAGCAAGCGAGGTTCGACCCGACACACGTGCTTGGGGGGCAAGTCGGG
560
580
600
620
640
GACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAG
HinfI
CTGGCGACGCGGAATAGGCCATTGATAGCAGAACTCAGGTTGGGCCATTCTGTGCTGAATAGCGGTGACCGTCGTCGGTGACCATTGTCCTAATCGTCTC
RNA I
660
680
700
720
740
CGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGT
EcoRI*
HaeIII
pppAAGAAUUUGGUAUCUGCG.....
+RNA+
GCTCCATACATCCGCCACGATGTCTCAAGAACTTCACCACCGGATTGATGCCGATGTGATCTTCCTGTCATAAACCATAGACGCGAGACGACTTCGGTCA
760
780
800
820
840
TCCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAA
3'
AluI
TERMINATOR
FnuDII
AGGAAGCCTTTTTCTCAACCATCGAGAACTAGGCCGTTTGTTTGGTGGCGACCATCGCCACCAAAAAAACAAACGTTCGTCGTCTAATGCGCGACTTTTT
5'
PRIMER

Figure 2. Proposed Interactions between Nascent Primer Transcript and RNA I. Both the primer and RNAI transcripts are proposed to form three stem-loop structures, designated I to III and III' to I' reading from the 5' end of the primer and RNAI transcripts, respectively. The ellipsis marks indicate that the primer transcript continues to the right, but its sequence is not shown in Figure 2.

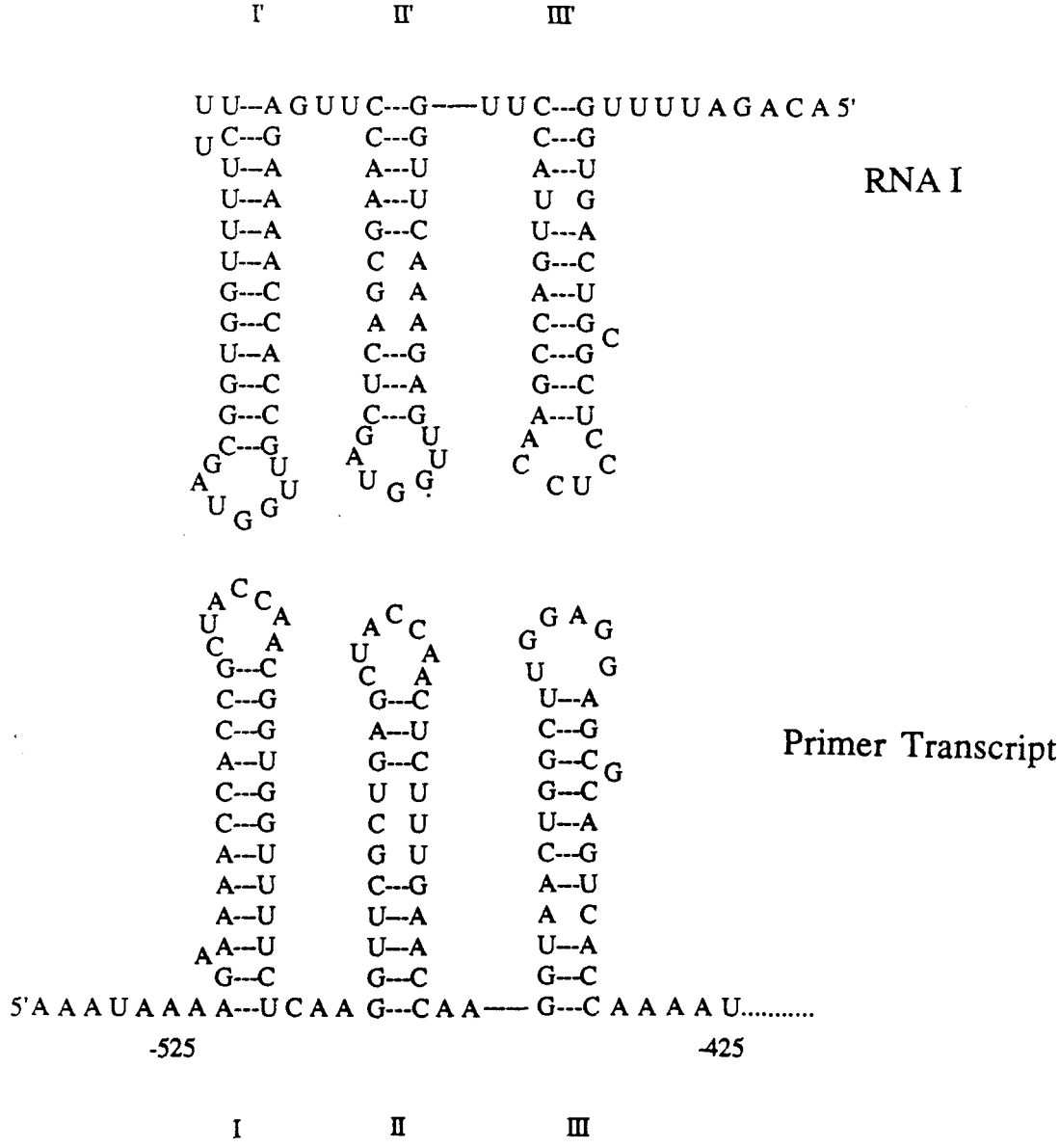
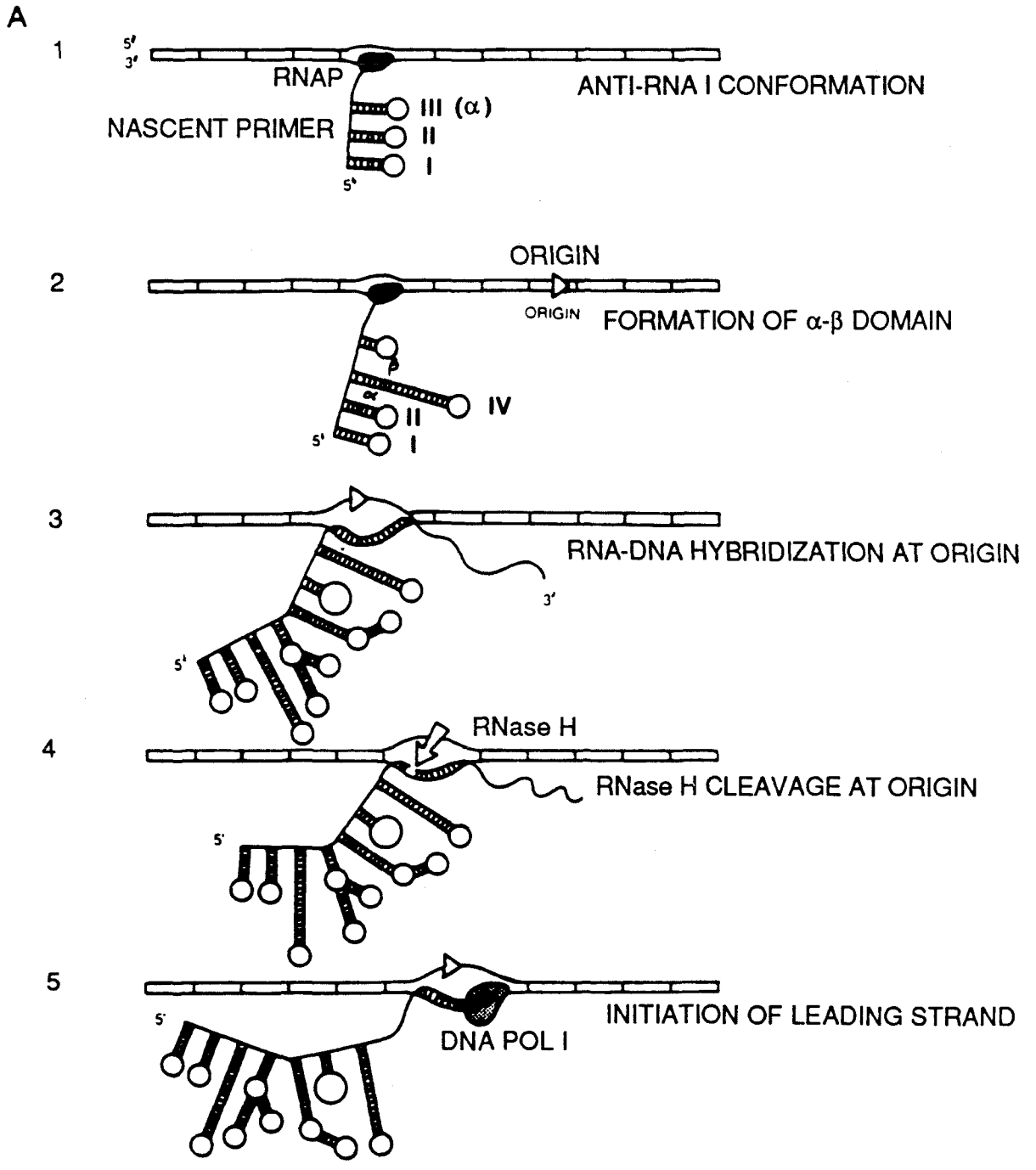
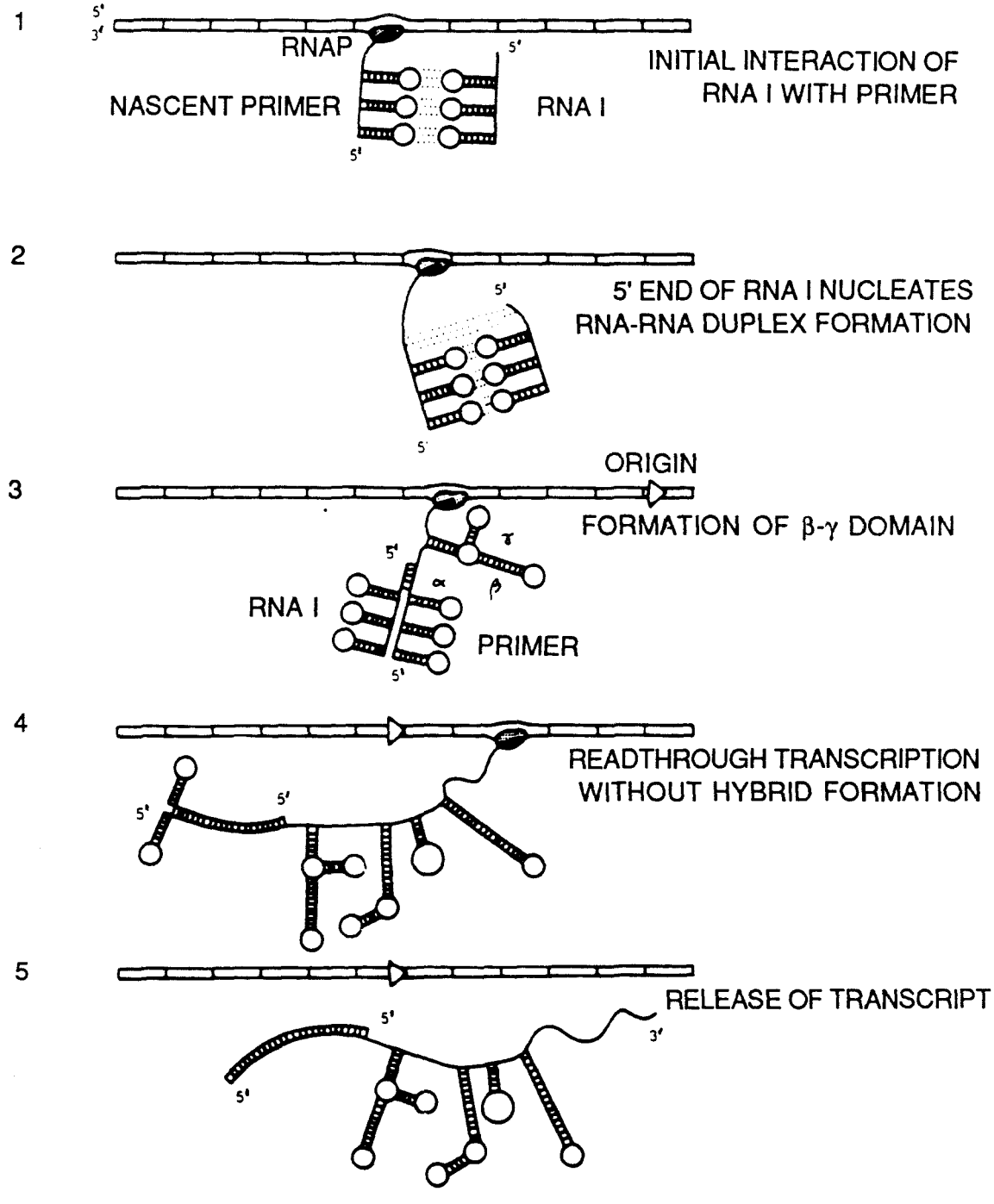

I'
II'
III'
RNA I
Primer Transcript
I
II
III
-525
-425

Figure 3. Major Secondary Structural Features of RNAs Involved in ColE1 Replication Control. (A) Primer formation and initiation of DNA synthesis on leading strand. 1) Transcription of the preprimer produces the three stem-loop structures, an anti-RNA I conformation. The stem-loop III is also known as the α domain; 2) Continued elongation of the primer results in an altered structure with α domain pairing with the β domain (formation of stem-loop IV). The formation of stem-loop IV effectively precludes the β domain from interacting with a downstream γ domain; 3) RNA primer forms a persistent hybrid with the DNA; 4) RNaseH cleaves the RNA-DNA hybrid at the origin, yielding a primer for DNA synthesis; 5) DNA polymerase I utilizes the primer and initiates DNA synthesis. (B) Inhibition of primer processing by RNA I. 1) RNA I interacts with the nascent primer between the loops of their folded structures; 2) Hybridization of RNA I and primer starts at the 5' end of RNA I indicated by the dotted lines; 3) Pairing prevents the β domain from interacting with the α domain and allows the β domain to interact with the downstream γ domain; 4) Failure to form stem-loop IV leads to read-through transcription of the primer without hybrid formation; 5) Primer transcript is released from DNA template with no initiation of DNA replication. Figures from Polisky, 1988.


A
1
5'
3'
RNAP
ANTI-RNA I CONFORMATION
NASCENT PRIMER
III (α)
II
I
5'
ORIGIN
2
ORIGIN
FORMATION OF α-β DOMAIN
β
α
IV
II
I
5'
3
RNA-DNA HYBRIDIZATION AT ORIGIN
3'
5'
RNase H
4
RNase H CLEAVAGE AT ORIGIN
5'
5
5'
INITIATION OF LEADING STRAND
DNA POL I

B


1
RNAP
NASCENT PRIMER
RNA I
INITIAL INTERACTION OF
RNA I WITH PRIMER
2
5' END OF RNA I NUCLEATES
RNA-RNA DUPLEX FORMATION
ORIGIN
3
FORMATION OF β-γ DOMAIN
RNA I
PRIMER
4
READTHROUGH TRANSCRIPTION
WITHOUT HYBRID FORMATION
5
RELEASE OF TRANSCRIPT

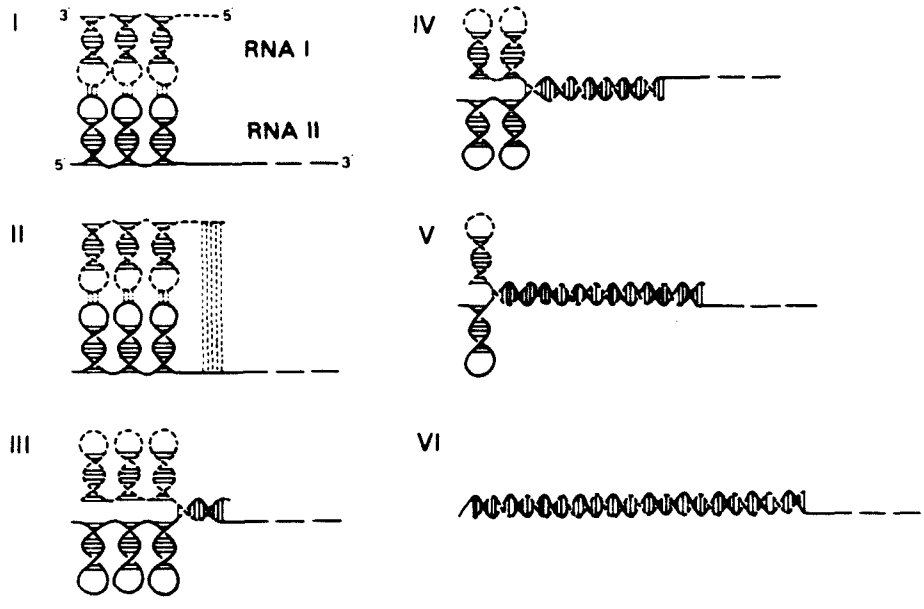


Figure 4. Schematic Diagram of the Pairing Process between RNA I and the Nascent Primer Transcript. I) RNA I and the primer transcript interact with each other through complementary base-pairing at the loop structures in the initial, reversible stage of the interaction. II) Complete pairing between the two RNA species then starts at the 5' end of RNA I. Pairing continues to propagate (III to V) until a complete RNA duplex (VI) has formed. This prevents the primer transcript from forming the RNA-DNA hybrid required for RNase H primer processing. Figures from Tomizawa, 1984.

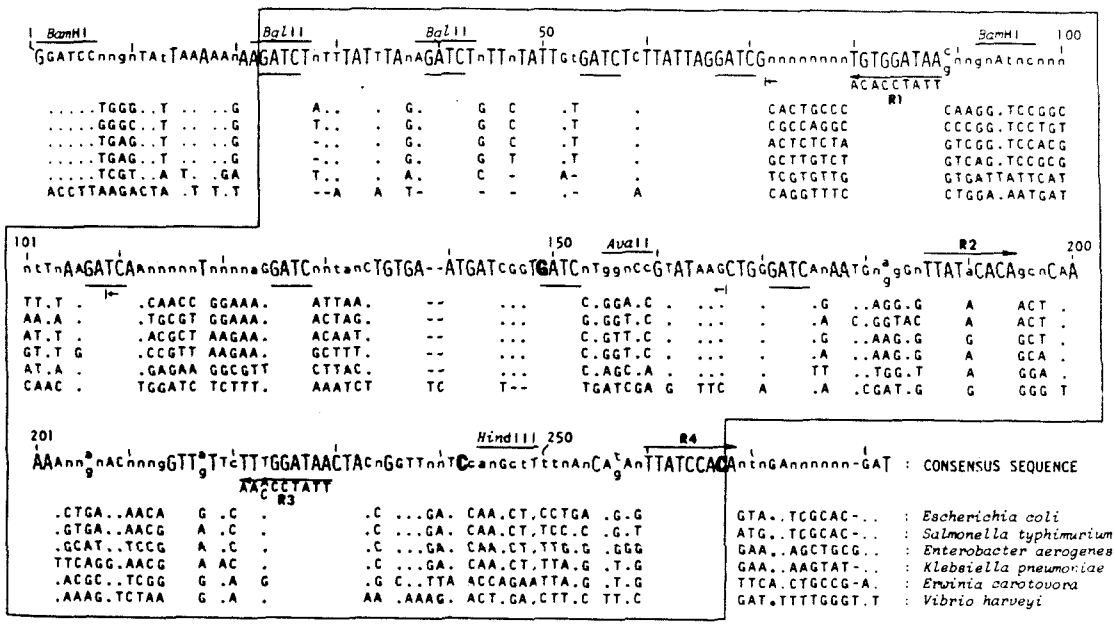


Figure 5. Consensus Sequence of the Minimal Replication Origin of the Bacterial Chromosome. Six different bacterial origins (as listed in the figure) were used to derive the consensus sequence. In the consensus sequence, a large capital letter denotes complete conservation of that nucleotide in all six origins; a small capital letter indicates that the same nucleotide occurs in five out of six origins; a lowercase letter represents the same nucleotide appearing in at least 3 origins and that only 2 different nucleotides appearing at that position. R1 to R4 are the 9bp dnaA consensus binding sequences. In the individual sequences, '-' denotes a deletion in the sequence and '.' represents the same nucleotide as consensus. Figure from Zyskind *et al.*, 1983.

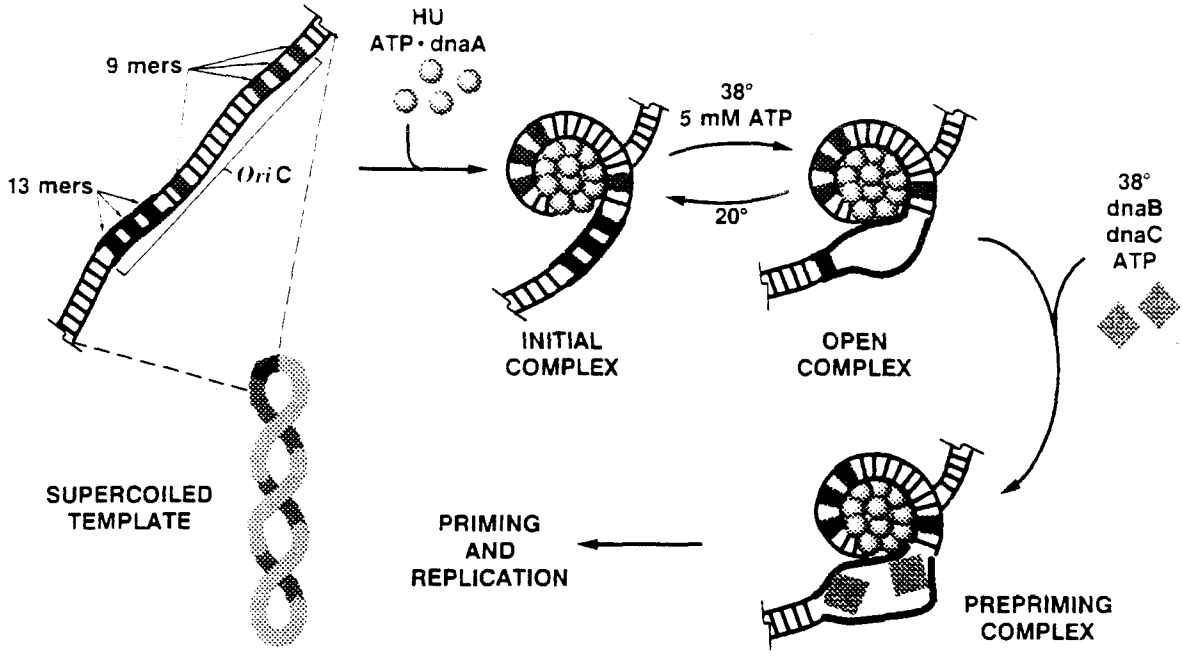
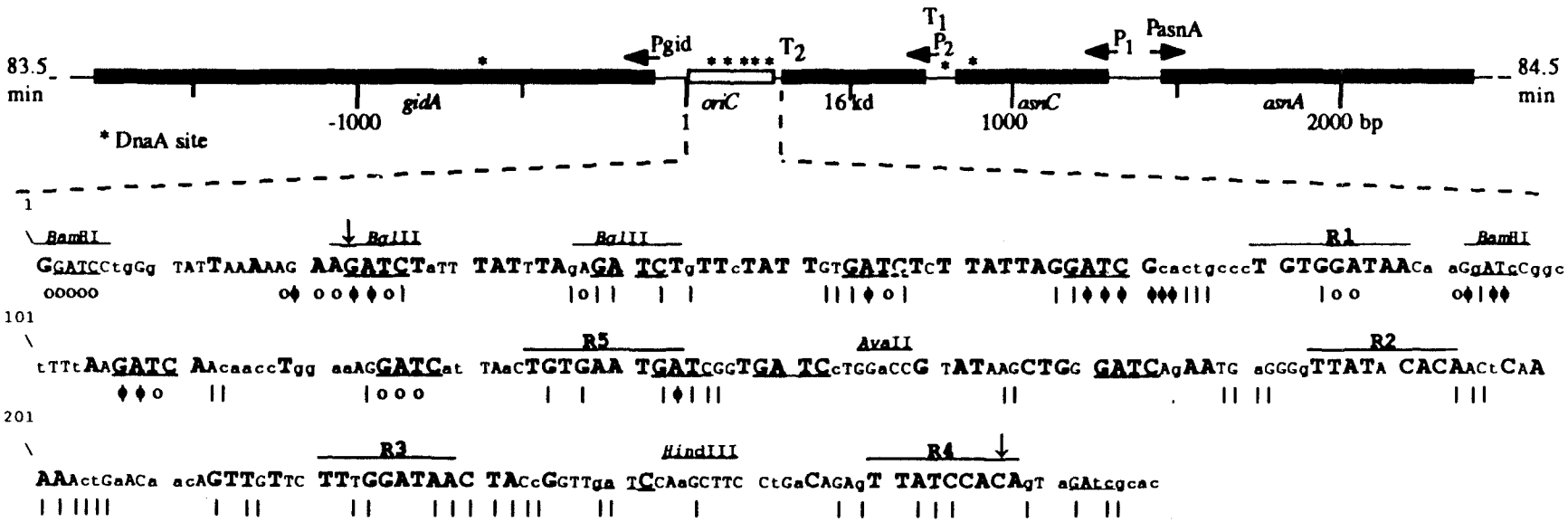


Figure 6. Model of dnaA Binding and Initiation of DNA Replication at *oriC*. Refer to text for details. Figure from Bramhill and Kornberg (1988).

Figure 7. *E. coli* Replication Origin Region and Nucleotide Sequence of the Minimal Origin, *oriC*. Within the region of replication origin, filled arrows indicate promoter signals and the direction of transcription. asnA, asparagine synthetase A; asnC plays a regulatory role in asparagine metabolism with P_1 as its promoter. The 16 kD protein is equivalent to the *mioC* gene product with the promoter of transcription at P_2. DnaA binding sites are indicated by asterisks. Within the minimal *oriC* sequence, RNA-DNA transition points are indicated by 'o'. 3'-ends of counterclockwise-moving transcripts (leftward in this linear map) terminate in *oriC* at positions indicated by vertical lines. Figures from Zyskind and Smith, 1986.


83.5 min
84.5 min
Pgid
T2
T1
P2
P1
PasnA
gidA
oriC
16 kd
asnC
asnA
-1000
1
1000
2000 bp
* DnaA site
BamHI
BglII
BglII
R1
BamHI
R5
AvaII
R2
R3
HindIII
R4

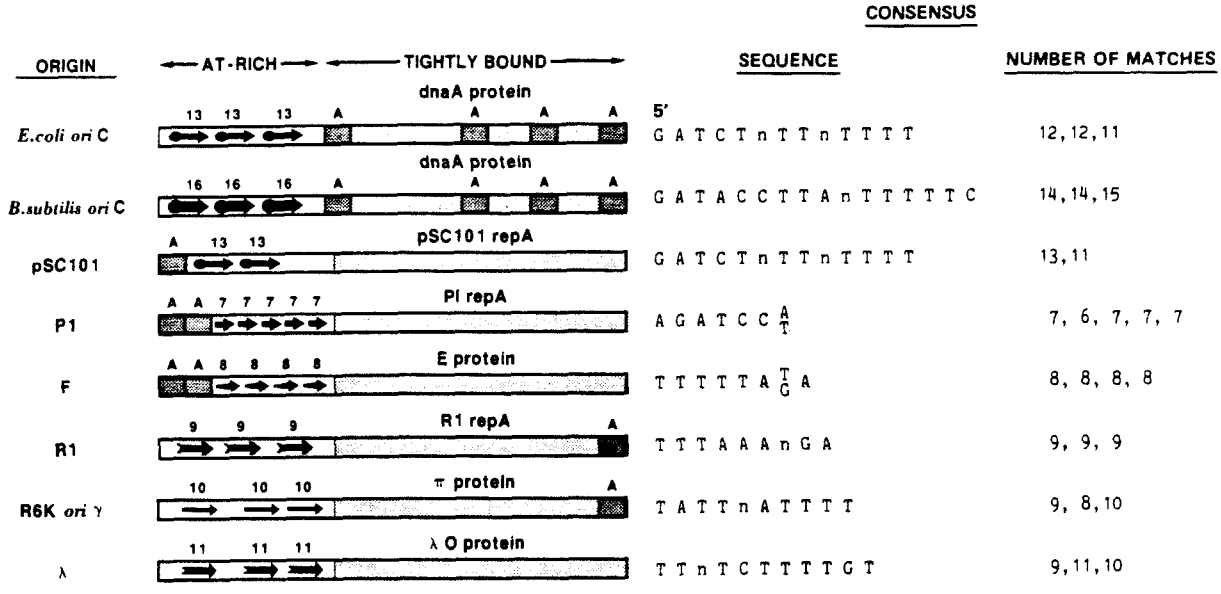


Figure 8. Basic Organization of Different Prokaryotic Replicons. Arrows indicate the AT-rich repeats with the length of the repeat specified above each arrow. Stippled boxes represent DNA segments that bind tightly to the specific initiator protein involved. The stippled boxes with "A" denote the 9bp dnaA consensus sequence. Sequence and the number of matches to consensus of the AT-rich repeats are listed on the right. Figure from Bramhill and Kornberg, 1988b.

CHAPTER 1

Cis-acting Mutations that Affect Rop Protein Control of Plasmid Copy Number

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Genetics

cis-acting mutations that affect rop protein control of plasmid copy number

(regulation of DNA replication/temperature-sensitive mutant plasmid/small plasmid-encoded protein)

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ABSTRACT **A number of pMB1 derivatives provide a *trans*-acting function that can suppress lethal runaway replication of a temperature-sensitive copy-number mutant of NTP1. Deletion analysis indicates that the region of the pMB1 genome that contains the *rop* gene is required for this suppression. Mutant derivatives of the temperature-sensitive copy-number mutant plasmid whose conditional lethal phenotype is not suppressed in *trans* by the region encoding the *rop* gene have been isolated. These *rop*-insensitive derivatives contain single nucleotide changes within the RNA I coding region.**

Initiation of replication of ColE1-type plasmids is regulated by two *trans*-acting negative control elements (Fig. 1) (1–5). One of these elements, RNA I, acts as a replicon-specific inhibitor of plasmid replication and is responsible for plasmid incompatibility (2, 7, 8). Purified RNA I inhibits the processing of a second plasmid transcript, RNA II, *in vitro*. This processing is necessary to form the primer for initiation of DNA synthesis. The target of RNA I inhibition is believed to lie within a region of RNA II that is complementary to RNA I (7, 9, 10). Both RNA I and its complementary region on RNA II may adopt a secondary structure with three stem-loop structures. Genetic studies indicate that the single-strand loops are involved in the inhibitory activity of RNA I, which occurs by base-pairing with the complementary sequence of RNA II (7, 10). The hybridization of the two transcripts prevents the formation of an RNA·DNA hybrid structure between RNA II and its template near the replication origin (2). The RNA·DNA hybrid is a substrate for ribonuclease H, which cleaves the transcript at the origin of replication to create the 3'-OH end of the primer (11).

The second element in the copy control system is the product of a gene located 500 base pairs (bp) downstream from the replication origin of ColE1 and pMB1 (3–5). Twigg and Sherratt (3) observed that deletions of this region of ColE1 and pMB1 derivatives caused an increase in the plasmid copy numbers. They also showed that the elevated copy numbers could be reduced to wild-type levels by providing the deleted function in *trans* from a second compatible plasmid in the cell. Cesareni *et al.* (4) reported that *in vivo* expression of the *lacZ* gene directed by the primer promoter was reduced in the presence of a plasmid carrying this "repressor." They proposed that the repressor is a 63 amino acid polypeptide that is conserved in both ColE1 and pMB1. They speculated that this repressor, which they designated rop (for repressor of primer), acted independently of RNA I to regulate plasmid replication by limiting transcription initiation of RNA II, the primer precursor.

We have observed that a ts runaway replication mutant plasmid fails to express its conditional lethal phenotype in the presence of certain compatible plasmids. Genetic evidence suggests that this suppression is mediated by the *rop* gene encoded on the second plasmid. We have taken advantage of this activity of rop to select mutant derivatives of the ts plasmid that are insensitive to rop suppression. DNA sequencing of a number of these rop-insensitive derivatives provides information about the mechanism of rop-mediated regulation of plasmid replication.

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MATERIALS AND METHODS

Bacterial Strains and Plasmids. All of the transformation and plasmid studies were carried out in *Escherichia coli* HB101 *pro gal hsdR hsdM recA1*. Plasmid pJN75 is a 5.4-kilobase ampicillin-resistant derivative of NTP1 (12, 13). pJN75 exhibits a ts mutant copy-number phenotype. The presence of the plasmid is lethal to the host cell at the restrictive temperature, 37°C. The lethality is a result of runaway plasmid replication. A single nucleotide difference from the wild-type NTP1 sequence within the region required for replication appears to be responsible for the ts mutant phenotype (12). This mutation is a G·C to A·T transition located 398 bp upstream of the origin of DNA synthesis. Plasmid pMB9 is a tetracycline-resistant derivative of pMB1. Other plasmids are described in Tables 1 and 2.

Isolation of pJN75 Mutants Whose ts Runaway Replication Phenotype Is Not Suppressed by the *rop* Gene Product. Twenty cultures of *E. coli* HB101 containing pJN75 were grown at 30°C to an A_{590} of 0.3 in 5 ml of L broth. Chloramphenicol (100 μg/ml) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (20 μg/ml) were added and incubation was continued overnight. Plasmid DNA was isolated from each of the cultures used to transform individual cultures of *E. coli* HB101 containing pMB9 by the procedure of Dagert and Ehrlich (17). Cells were spread onto L plates containing tetracycline (15 μg/ml) and a high concentration of ampicillin (4 mg/ml) and incubated at 34°C overnight. Under these conditions, cells containing pJN75, which is at high copy number, survive. However, cells containing both pJN75 and pMB9 do not form colonies under these conditions, presumably because *rop* suppression of pJN75 replication reduces the copy number of pJN75 to a level below what is needed to confer resistance to 4 mg of ampicillin per ml. Mutants of pJN75 that are insensitive to the *rop* suppression are able to grow in the presence of pMB9 at 34°C on 4 mg of ampicillin per ml. Several colonies grew up from each of the 20 transformations carried out with mutagenized DNA, whereas no colonies were obtained when nonmutagenized DNA was used. Colonies from each of the 20 plates were then tested for expression of the ts lethal phenotype by replica-plating colonies onto two sets of plates containing tetracycline (15 μg/ml) and a low concentration of ampicillin (50 μg/ml) and growing one set at 30°C and the other set at 42°C overnight. Approximately 25% of the colonies grew at 30°C but did not grow at the higher temperature. Plasmid DNA was purified from seven ts colonies, each derived from a separate mutagenized DNA to insure independence of mutants examined.

Abbreviations: ts, temperature sensitive; bp, base pair(s).

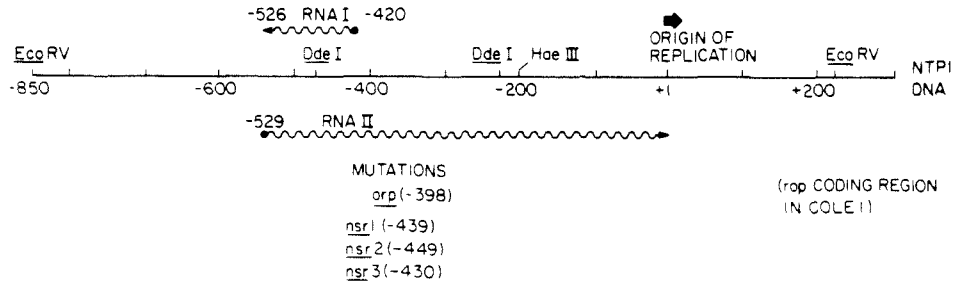

FIG. 1. DNA replication origin of plasmid NTP1 (RSF1030). The origin region of NTP1 has a sequence identical to that of RSF1030 (6). The locations of the coding region for RNA I and RNA II are shown relative to the site of initiation of DNA synthesis. This plasmid does not contain a *rop* gene. The original temperature-sensitive (ts) mutation (*orp*) maps at −398, within the sequence for RNA II, whereas the mutations identified in this study map at −430, −439, and −449.

DNA Sequence Analysis. The nucleotide sequence surrounding the replication origin of pJN75 has been reported (13, 14). The sequence of the origin region of seven pJN75 mutants was determined by the chemical degradation method of Maxam and Gilbert (21). Approximately 800 nucleotides were sequenced, encompassing a region extending from 700 nucleotides upstream of the origin of DNA synthesis to 100 nucleotides downstream of the origin. Some of the sites used for sequencing are indicated in Fig. 1.

RESULTS

Suppression of pJN75 Runaway Replication by the *rop* Gene Product. Plasmid pJN75 is a ts copy-number mutant derivative of plasmid NTP1. (12, 13). [Although isolated independently, NTP1 is apparently identical to plasmid RSF1030 (6, 14, 15).] Bacteria containing pJN75 grow normally at the permissive temperature (30°C) but cease growth and die one or two generations after a shift to the nonpermissive temperature (37°C). Loss of viability appears to be a consequence of runaway replication, which results in an increase in plasmid synthesis from about 8% of chromosomal DNA synthesis to 150% in the first 10 min after the temperature shift (13).

We have previously identified the mutation in pJN75 responsible for the ts mutant replication phenotype (12). This mutation is a single base pair change, a G·C to A·T transition, located 398 bp upstream of the origin of DNA synthesis (see Fig. 1). The temperature-induced over-replication phenotype is most likely the consequence of thermolability of a secondary structure within the primer precursor that is normally essential for regulation, though this has not been shown conclusively (12, 22). The inability of this structure to form at the restrictive temperature may render replication of the plasmid insensitive to normal regulation through RNA I inhibition.

We have observed that runaway replication of pJN75 at the nonpermissive temperature is suppressed in the presence of certain compatible plasmids. This suppression was first observed in double transformants of pJN75 and pMB9, a tetracycline-resistant derivative of plasmid pMB1. The double transformants grew normally at 42°C as well as at 30°C in the presence of both ampicillin and tetracycline. Plasmid DNA levels were analyzed from lysates of cells grown in liquid cultures before and several hours after a shift to the restrictive temperature. Although this analysis was somewhat complicated by the similarity in molecular weights of pMB9 and pJN75, we were still able to observe that with pMB9 present in the cell, there was very little temperature-induced amplification of pJN75 DNA (data not shown). Suppression of the conditional lethal phenotype of pJN75 was also observed with the pMB1 derivatives, pBR322 and pBR325 (Table 2). The electrophoretogram shown in Fig. 2 illustrates the effect of a plasmid derived from pBR325 on the temperature-induced amplification of pJN75 DNA.

NTP1 is compatible with pMB1-derived plasmids—i.e., NTP1 and pMB1 derivatives can stably coexist in the same cell. This is apparently a consequence of the fact that the RNA I species encoded by NTP1 is not identical to the pMB1 RNA I species. *In vitro* experiments described by Tomizawa and Itoh (7) indicate that purified NTP1 (RSF1030) RNA I does not inhibit primer formation of a ColE1 template

Table 1. Major plasmids used in these studies

Plasmid	Characteristic	Compatibility	Drug resistance	Refs.
pJN70	Wild type	RSF1030	Ampicillin	6, 14, 15
pJN75	ts	RSF1030	Ampicillin	12, 13
pMB9	rop⁻	pMB1	Tetracycline	16

Other plasmids used are found in Table 2. *E. coli* HB101 *pro gal hsdR hsdM recA1* (1) was used for all of the transformation and plasmid studies.

Table 2. *trans*-suppression of pJN75 runaway replication by various compatible plasmids

Plasmid	Incompatibility class	Presence of *rop* gene	Suppression of ts lethal phenotype of pJN75-containing cells
pMB9	pMB1	+	+
pBR322	pMB1	+	+
pBR325	pMB1	+	+
pAT153	pMB1	−	−
pKO1	pMB1	−	−
pUC8	pMB1	−	−
pACYC184	p15A	(−)?	−
pDM254	p15A, pMB1	(−)?	−

E. coli HB101 cells containing pJN75 were transformed with the indicated plasmids following the transformation procedure of Dagert and Ehrlich (17). Cells were spread onto L plates containing ampicillin (50 μg/ml), which were then incubated overnight at 42°C. Plasmids that produced temperature-resistant colonies with a high transformation efficiency (>10^6 per μg of plasmid DNA) are indicated by a + sign. A − sign indicates those plasmids that did not give temperature-resistant colonies. Plasmids pMB9, pBR322, and pBR325 are all pMB1 derivatives that contain the region of the plasmid genome encoding the *rop* gene. Plasmid pAT153 (3) is identical to pBR322 except for deletion of a 622-bp *Hae* II restriction fragment that contains the entire *rop* gene (4). Plasmids pKO1 (18) and pUC8 (19) are also derived from pBR322 and contain only the portion of the *rop* gene that codes for the last 9 amino acids of the 63 amino acid polypeptide. Plasmid pACYC184 contains the replication origin from p15A (20). There is no evidence of a *rop* gene encoded on pACYC184 or p15A. Plasmid pDM254 (8) is a pACYC184 derivative with four copies of the pMB1-RNA I gene cloned in tandem at the *Bam*HI site.

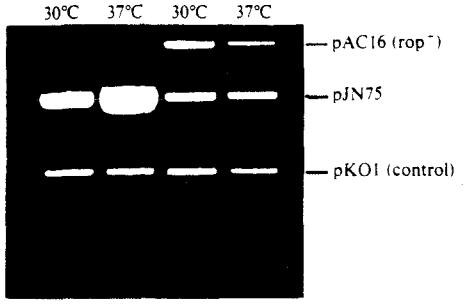

FIG. 2. Suppression of temperature-induced amplification of pJN75 DNA by a pMB1 derivative carrying the *rop* gene. HB101 cells carrying either pJN75 (ampicillin resistant) or pJN75 and plasmid pAC16 (tetracycline resistant, chloramphenicol resistant) were grown in L broth containing the appropriate antibiotics at 30°C to an A_{590} of 0.15. Half of each culture was then transferred to 37°C and all cultures were incubated for an additional 3 hr. A portion of each culture was then mixed with a portion of another cell culture containing an equal number of HB101 cells carrying plasmid pKO1 (18). The pKO1-containing cells were added immediately prior to cell harvest to provide a control plasmid for standardizing plasmid DNA recoveries. After harvesting by centrifugation, the cells were lysed and total plasmid DNA was isolated as described (8). The covalently closed circular plasmids were analyzed by electrophoresis through a 1.0% agarose gel. Plasmid pAC16 (23) is a pBR325 derivative that contains a 700-bp rat actin cDNA insert cloned into the *Pst* I site and therefore does not confer ampicillin resistance to its host.

and that ColE1 RNA I does not inhibit NTP1 primer formation. Since there is apparently no cross-inhibition of plasmid replication by the two wild-type RNA I species, we speculated that a negative control element other than RNA I was responsible for the suppression of pJN75 replication by the pMB1 derivatives. The inability of plasmid pDM254 (8), which contains multiple pMB1 RNA I genes, to suppress pJN75 runaway replication supports this notion (Table 2). Since the pMB1 derivatives that provide suppressor activity also encode the *rop* gene described by Cesareni *et al.* (4), the *rop* gene product seemed a likely candidate for the *trans*-acting suppressor. Therefore, we looked at the effect of cocultivation of pJN75 with several pMB1-derived plasmids that contained deletions of all or part of the *rop* gene (Table 2). No suppression was observed with the pBR322 derivative pAT153, pKO1, or pUC8. Plasmid pAT153 differs from pBR322 by a deletion of the 622-bp *Hae* II restriction fragment that encodes the entire *rop* gene. Plasmids pKO1 and pUC8 contain a deletion of the 3' end of the *rop* gene. These results imply that the *rop* gene product is responsible for the suppression of the runaway replication phenotype of pJN75.

Isolation of Mutant Derivatives of pJN75 Insensitive to *rop* Suppression. These observations suggested a way to identify the site where *rop* interacts in suppressing pJN75 runaway replication—namely, by isolating mutants of pJN75 that are insensitive to *rop*-mediated suppression. The rationale for selecting such mutants depends on the idea that, at temperatures intermediate between permissive (30°C) and nonpermissive (37°C), the copy number of the mutant plasmid should be elevated but not lethal to the cells. To test this idea, cells carrying pJN70 or pJN75 were plated at 34°C on L agar plates containing a high concentration of ampicillin (4 mg/ml). As expected, cells carrying pJN75 were able to grow because the copy number was high, whereas cells carrying the wild-type plasmid did not produce enough β-lactamase to survive. Furthermore, cells carrying pJN75 plus pMB9 did not form colonies under these conditions, because *rop*-mediated suppression of pJN75 replication reduces the copy number to a level below what is required to confer resistance to ampicillin at 4 mg/ml (Table 2). To isolate a *rop*-insensitive plasmid, mutagenized pJN75 DNA was used to transform cells that contained pMB9 at 34°C. Only pJN75 derivatives insensitive to replication inhibition by *rop* function were expected to grow. Several such colonies were isolated and shown to retain the ts lethal phenotype at 42°C, indicating that the original mutation in pJN75 was still present. Seven of the colonies able to grow at 34°C but not at 42°C were selected for further characterization. Analysis of the DNA content of these cells revealed that both plasmids (pMB9 and the pJN75 derivative) were present as monomers, suggesting the phenotype was due to a mutation of the pJN75 genome. The plasmids exhibiting the non-suppressed phenotype in the presence of *rop* were designated pJN75*nsr* for nonsuppressible by *rop*.

Mapping of the *nsr* Mutations. The nucleotide sequence surrounding the replication origin of the *nsr* derivatives of pJN75 was determined following the procedure of Maxam and Gilbert (21). Each of the mutant plasmids was found to contain the original pJN75 alteration and a single additional base pair change within the 800-bp region extending from 100 bp downstream of the origin of DNA synthesis to 700 bp upstream of the origin. One of the mutants contained a G·C to A·T transition at position −439 (*nsr1*). (Position +1 is defined as the site of incorporation of the first deoxynucleotide during the initiation of plasmid DNA replication.) Five other mutants contained a G·C to A·T transition 10 bp away at position −449 (*nsr2*). An additional mutant appeared at −430 (*nsr3*). All of these mutations cause disruption of base-pairing within the stem of loop-structure III in the primer transcript and loop-structure III′ of RNA I (Fig. 3). This result is consistent with the *galK* fusion studies of Som and Tomizawa (5), which indicate that sequences important for rop function do not fall in the promoter for RNA II, but instead lie further downstream. The mutations we have identified define this region to be within the coding sequnce for both RNA I and RNA II, near the 5′ terminus of RNA I.

DISCUSSION

In this paper we have shown that the *rop* gene provides a *trans*-acting function that can suppress runaway replication of the NTP1 ts copy-number mutant pJN75. This conclusion is based on the observation that plasmids with deletions of part or all of the *rop* gene lack the suppressor activity. This interpretation is also supported by previous reports that the *rop* gene acts in *trans* to lower plasmid copy number (3, 5). We have used the ability of the *rop* gene product to suppress the ts lethal phenotype of the runaway replication mutant pJN75 to further investigate the general mechanism by which *rop* modulates plasmid copy number. Although the molecular details of this inhibition are not yet understood, one model has been that the *rop* gene product may be inhibiting transcription of RNA II and therefore controlling replication by limiting the amount of precursor RNA available for primer formation. This idea is supported by the observations of Cesareni *et al.* (4) and Som and Tomizawa (5) that the *rop* gene product inhibits production of β-galactosidase or galactokinase when the *lacZ* or *galK* genes are fused to the primer downstream of the primer promoter.

To investigate how such inhibition might occur, we looked for mutants resistant to inhibition by the *rop* gene product as described above. We have identified mutants of pJN75 designated *nsr*, which express the lethal runaway replication phenotype at 42°C even in the presence of the *rop* gene encoded on a second plasmid in the cell. The nucleotide sequence of seven *nsr* plasmids revealed that none of the mutations falls within the promoter for RNA II, as might have been expected from the model just described. The mutations disrupt base pairs within stem-loop structure III (see Fig. 3)

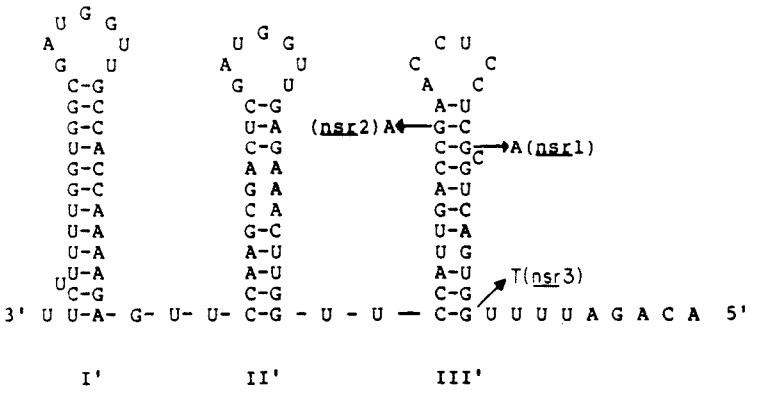

FIG. 3. RNA I transcript from plasmid pJN75. Mutations *nsr1*, *nsr2*, and *nrs3* are those identified within the mutant pJN75 derivatives that were selected by their ts phenotypes in the presence of pMB9. The RNA I secondary structure shown is that proposed by Som and Tomizawa (6) based on the secondary structure of ColE1 RNA I proposed by Morita and Oka (24).

present in both RNA I and RNA II. It is perhaps worth noting that the two nucleotide pairs affected by the mutations *nsr1* and *nsr2* are conserved in the RNA I species from ColE1, p15A, RSF1030, and CloDF13 (25). In addition, on the same stem structure immediately adjacent to these paired bases is an unpaired nucleotide that is also conserved in these plasmids. It is imaginable that this "spur" on the stem could be a part of a recognition site for the binding of a small protein. Our findings, along with those of Som and Tomizawa (5), that a region within the coding sequence of RNA II, rather than in the primer promoter, was necessary for inhibition suggests that the rop protein does not act by binding to an operator site near or within the promoter and competing with RNA polymerase binding. This is also consistent with the fact that the rop protein from pMB1 can inhibit replication of plasmids such as NTP1, which have virtually no sequence homology to pMB1 in the DNA immediately 5′ to the primer RNA, in the primer promoter.

Som and Tomizawa (5) have recently suggested that the rop protein enhances hybrid formation between RNA I and its complementary region of RNA II. Our results are consistent with, though by no means prove, this interpretation, in that all of the *nsr* mutations fall in the region of RNA I and RNA II overlap. Thus, one possibility is that the rop protein may recognize one or more of the stem-loop structures on either RNA I or RNA II and bind them. Binding of the rop protein might then favor the interaction of RNA I and its target region of RNA II.

However, there is one important caveat in interpreting the *nsr* mutations as identifying a rop–nucleic acid interaction site. Analysis of the *nsr* mutations is complicated by the fact that the *rop*-insensitive mutations described here may also alter the structure of RNA I or RNA II such that the inherent ability of these RNAs to interact with each other is diminished. In other words, an altered copy-number phenotype associated with a mutation in the RNA I coding region can be a consequence of either a defective (unstable) RNA I inhibitor, an altered RNA I target site within the primer transcript, increased RNA II transcription, or the inability of the rop protein to interact with either of the mutant transcripts. It is quite likely that all of these functions could be affected by a single point mutation. The mutation *nsr1* that we have identified affects a nucleotide at the same position in pJN75 RNA I as the *svir19* mutation of pMB1 described by Lacatena and Cesareni (10). The latter mutation was obtained by using a "phasmid" selection designed to isolate mutants exhibiting reduced sensitivity to the wild-type replication inhibitor RNA I. When released from the λ chromosome, the mutant plasmid containing the *svir19* mutation was lethal for the host cell. We have also found that *nsr1* and *nsr2* mutants are lethal to the host cell in the absence of pMB9 or other compatible rop⁻ plasmids (data not shown). This lethality is independent of temperature and is assumed to result from uncontrolled or runaway plasmid replication, although this has not been demonstrated either for *svir19* or for the *nsr* mutants. Thus, *nsr1* and *nsr2* appear to retain at least some sensitivity to rop protein. These results are consistent with the possibility that the mutations *nsr1* and *nsr2* in NTP1 and *svir19* in pMB1 affect both the RNA I–RNA II interaction and the rop protein function. The rop protein may enhance the hybridization of the two RNAs as proposed by Som and Tomizawa (5). However, the possibility that rop may affect transcription or stability of RNA II or stability of RNA I is not ruled out by our data.

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CHAPTER 2

The Effect of DnaA Protein and n' Sites on the Replication of Plasmid ColE1

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The Effect of dnaA Protein and n′ Sites on the Replication of Plasmid ColE1*

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The role of the dnaA protein in the replication of plasmid ColE1 and its derivatives was examined. Wild-type and mutant ColE1 plasmids were compared as to their ability to replicate in an *in vitro* replication system supplemented with ammonium sulfate fractionated extracts from a dnaA-overproducing strain. Synthesis on plasmid templates containing the wild-type origin of replication was stimulated 1.3-fold by addition of the dnaA-overproducing extract. A larger effect was observed after deletion of the primosome assembly site, the n′ site, on the leading strand. On the latter template, synthesis was only about one-half that observed with the wild-type templates, but synthesis could be restored to normal levels by addition of the dnaA-overproducing fractions. When the n′ site on the lagging strand of pBR322 was deleted, synthesis in the *in vitro* replication system was reduced to less than 10% of levels seen with intact templates. dnaA-overproducing extract did not restore activity since the dnaA site was also deleted on these plasmids. To verify that the observed stimulation of wild-type and leading strand n′ site mutants was due to the dnaA protein, dnaA protein was purified to greater than 50% homogeneity, and antiserum was prepared. The purified protein stimulated synthesis on the plasmid templates to the same extent as the overproducing extracts, and dnaA antiserum blocked stimulation both by extracts and by the purified protein. Thus, dnaA protein, and, by inference, the dnaA recognition site at the ColE1 origin of replication seem to be important for ColE1 replication. The effect of dnaA protein is enhanced when the n′ site is defective, suggesting that the dnaA protein plays a role similar to that of the proteins i, n, n′, and n″ in directing primosome assembly, as proposed by Seufert, W., and Messer, W. ((1987) *Cell* 48, 73–78).

The dnaA protein of *Escherichia coli* is essential for initiation of DNA replication at *oriC*, the chromosomal origin of replication. DNase I footprinting data indicate that the dnaA protein binds to a 9-bp[1] consensus sequence, 5′-TTAT$^{C}_{A}$CA$^{C}_{A}$A which is repeated four times within the minimal *oriC* sequence (Fuller *et al.*, 1984; Hansen *et al.*, 1982; Fuller and Kornberg, 1983). By recognizing and binding to the 9-bp consensus sequence, dnaA protein directs the dnaC and dnaB proteins to the origin. This complex unwinds the DNA, with the dnaB protein acting as a helicase. Current models suggest that the binding of dnaA protein to the origin induces a change of DNA conformation in that region, and the dnaB and C proteins may either recognize that structure or be involved in direct protein-protein interactions with the dnaA protein. Other proteins have also been suggested to interact specifically with dnaA protein based on genetic studies. Mutations in the β-subunit of RNA polymerase (*rpoB*) suppress the temperature sensitivity of some *dnaA* alleles (Bagdasarian *et al.*, 1977; Atlung, 1984). The products of *dnaZ* and perhaps *dnaB*, *dnaC*, *dnaE*, *dnaG*, *gyrB*, and the *rnh* gene product, RNase H, may also interact with the dnaA protein (Blinkowa and Walker, 1983; Filutowicz and Jonczyk 1983, 1984; Horiuchi *et al.*, 1984; Lindahl and Lindahl, 1984; Ogawa *et al.*, 1984; Torrey *et al.*, 1984).

The 9-bp dnaA recognition consensus sequence is found within or near the replication origins of many plasmids and phages as well as at *oriC*. For many years, among all plasmids that possess this site, only pSC101 could be shown to be dnaA-dependent. Integrative suppression was cited as the major genetic evidence against the participation of dnaA protein in the replication of other plasmids. Recently, however, P1 and F (Hansen and Yarmolinsky, 1986; Klein *et al.*, 1986) and also R1 plasmids (Ortega *et al.*, 1986; Masai and Arai, 1987) have been demonstrated to be dnaA-dependent. Therefore, there has been renewed interest in the occurrence of the dnaA binding site at the ColE1 origin and the possible participation of the dnaA protein in ColE1 replication, a matter which has received much attention over the years. dnaA protein was shown to be nonessential for replication of ColE1-like plasmids *in vivo* (Hansen and Yarmolinsky, 1986). In addition, several *in vitro* replication systems have reinforced the notion that dnaA protein is dispensable (Conrad and Campbell, 1979; Fuller *et al.*, 1981; Minden and Marians, 1985). However, the replication of ColE1 DNA proceeds via a rolling circle type intermediate in a *dnaA* mutant, *dnaA* 167 (ts), instead of via the normal θ form (Abe, 1980). Furthermore, Polaczek and Ciésla (1984) demonstrated a reduced rate of replication of plasmids in *dnaA* mutants grown at the restrictive temperature and an apparent increase in the activity of the RNA I promoter as a consequence of the inactivation of dnaA protein. Since RNA I is a negative regulator of ColE1 replication, they proposed that the reduced rate of replication of plasmids in *dnaA* mutants was due to the overproduction of RNA I (Polaczek and Ciésla, 1984).

The first insight into a positive role for the dnaA protein in plasmid replication is the recent suggestion of Seufert and Messer (1987) that binding of the dnaA protein to the pBR322 origin region can initiate primosome assembly by directing the dnaB and dnaC proteins to the origin. Primosome assembly had been thought to be mediated by proteins i, n, n′, and n″ at n′ sites near the ColE1 origin as these proteins are

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[1] The abbreviations used are: bp, base pair(s); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

required for ColE1 replication *in vitro* (Minden and Marians, 1985). A role for dnaA in primosome assembly was inferred from experiments in which a dnaA-overproducing extract stimulated ColE1 replication *in vitro* and from studies with plasmid mutants missing the n′ sites. However, the stimulatory activity was never purified from the extracts, and the evidence for dnaA protein involvement remained somewhat indirect. Furthermore, in the plasmid deletion mutants, no plasmids were studied that retained n′ sites and selectively deleted the dnaA site. This control is important because the deletion of the dnaA site in the set of deletion mutants removed DNA to within 20–80 bp from the origin of replication, which may either have deleted important sites for protein interactions around the origin or juxtaposed sequences that interfere nonspecifically with initiation. Finally, it was not possible to conclude whether dnaA, if it functions at all, acts as an alternative to n′ site-directed initiation or only as an enhancer.

Therefore, it was of interest to more directly investigate the role of the dnaA protein in ColE1-type replication. In this study, we have purified the dnaA protein to greater than 50% homogeneity and also prepared antibody directed against the protein. We have compared the ability of the purified protein to stimulate *in vitro* replication on a set of mutant plasmid templates (kindly provided before publication by H. Masai, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA) and have investigated the inhibition of replication by the antibody. Our results support Seufert and Messer's view (1987) that dnaA protein and the dnaA site can participate in "primosome assembly" during ColE1 replication independent of n′ sites. Furthermore, our results demonstrate participation of the leading strand n′ site in ColE1 replication and substantiate the results of Masai and Arai,[2] who first showed that the n′ site on the lagging strand was essential for ColE1 replication in a different type of *in vitro* replication system.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Phages—*E. coli* W3110 *thy*$^-$ was used as the standard strain for preparation of the *in vitro* ColE1-type plasmid DNA replication system (Conrad and Campbell, 1979). pBR322 deletion derivatives, pBR322Δ8, pHM38, and pHM54 were from H. Masai (DNAX, Palo Alto, CA 94304-1104), pHM38 lacks the primosome assembly site (n′ site) on the leading strand (H-strand) but has both the dnaA site and the n′ site on the lagging strand (L-strand), pHM54 and pBR322Δ8 both have their dnaA site and lagging strand n′ site deleted, but pHM54 retains the leading strand n′ site whereas pBR322Δ8 lacks it.[3]

All strains, plasmids, and phages below were acquired from A. Kornberg's laboratory (Stanford University, Stanford, CA). WM433 (*dnaA204*) was used to prepare the extracts for *oriC* replication that were used to assay the dnaA protein (Tippe-Schindler *et al.*, 1979). M13*oriC26* and M13*oriC26Δ221* (*oriC*$^-$) were DNA templates used in the dnaA protein assay. The latter has the minimal *oriC* sequence removed by a 330-bp deletion between *Bgl*II and *Bcl*I sites at positions 22 and 352, respectively, in the *oriC* sequence (Kaguni *et al.*, 1981). The *oriC*$^-$ template serves to distinguish between dnaA-dependent initiation of chromosomal replication and randomly initiated replication. Stocks of these chimeric phages were prepared from single plaques by using *E. coli* K37 (Hfr) as the host. Plasmid pBF1509 contains the *dnaA* gene under the control of the phage λ P_L promoter.[4] Briefly, pBF1509 was derived from a *Bal*31 deletion of pBF110 (Fuller and Kornberg, 1983) cloned into the large *Bam*HI/*Ava*I fragment of pBF221. pBF221 is identical to pAD329 (Hoyt *et al.*, 1982) except that it lacks the *Pvu*II fragment containing the *galK* gene and the *Hpa*I/*Bam*HI fragment containing the phage λ *N* gene. Strain N4830 (λ cI857) carries pBF109 for overproduction of dnaA protein (Gottesman *et al.*, 1980).

Materials—Acrylamide, bisacrylamide, dithiothreitol, Hepes, and sodium dodecyl sulfate were from United States Biochem. Heparin agarose was prepared by the method of Davison *et al.* (1979). EDTA, EGTA, Tris base, sodium chloride, potassium chloride, sucrose, creatine phosphate, creatine phosphokinase (type I, from Rabbit muscle) and polyvinyl alcohol (type II) were from Sigma. Yeast extract, Bacto-Tryptone, complete and incomplete Freund's adjuvants were purchased from Difco. Nitrocellulose was from the Millipore Corporation. All nucleotides were from Pharmacia LKB Biotechnology Inc. [^{3}H]dTTP (50–80 mCi/mmol) was from Du Pont-New England Nuclear. Affinity purified goat anti-rabbit IgG horseradish peroxidase conjugate second antibody and horseradish peroxidase color development agent were from Bio-Rad.

Protein Assay—Protein concentration was determined by method of Bradford (1976).

Polyacrylamide Gel Electrophoresis—The procedure for polyacrylamide gel electrophoresis was similar to the method of Weber and Osborn (1969). Samples were run on a 12.5% acrylamide, 0.6% methylene-bisacrylamide slab gel (20 × 25 × 0.1) at 30 mA until the tracking dye (bromphenol blue) reached the bottom of the gel. Gels were stained by the method of Wray *et al.* (1981).

Antibody Production—dnaA protein (Fraction IV) (see Table I) was purified for injection by polyacrylamide gel electrophoresis in the presence of SDS. The band corresponding to dnaA protein was cut out of the gel and prepared for injection as described by Nelson and Lazarides (1983). Antiserum was raised in a New Zealand White rabbit, 6 weeks of age, by injecting 50 μg of denatured dnaA protein in complete Freund's adjuvant at multiple intradermal sites. A boost of 50 μg of denatured dnaA protein in incomplete adjuvant was given twice.

Protein Blotting and Detection of Antigen-Antibody Complex—Proteins were transferred from SDS-PAGE to nitrocellulose paper by electrophoresis toward the anode in 25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20% (v/v) methanol at 0.17 A for 6 h as described by Towbin *et al.* (1979). Nitrocellulose blots were rinsed in phosphate-buffered saline (PBS, 5 mM potassium phosphate, pH 7.4, 150 mM NaCl) for 10 min and soaked in Blotto (5% non-fat dry milk in PBS) for 2 h and then incubated with Blotto containing dnaA antiserum or preimmune serum overnight. The nitrocellulose blots were then rinsed with four changes of Blotto. The antigen-antibody complexes on the nitrocellulose papers were localized using goat anti-rabbit IgG horseradish peroxidase conjugate as described by Huru and Chantler (1980).

Assay for the dnaA Protein—The *in vitro* complementation assay developed by Fuller and Kornberg (1983) was used to measure dnaA activity. *In vitro* replication extracts dependent on *oriC* templates and the dnaA protein were prepared from *dnaA* mutant WM433 as described by Fuller *et al.* (1981). The standard replication reaction mixture, 25 μl, contained Hepes·KOH, pH 7.6, 40 mM; GTP, CTP, UTP, each at 0.5 mM; ATP, 2 mM; dGTP, dCTP, [^{3}H]dTTP (60 cpm/pmol), and dATP, each at 100 μM; magnesium acetate, 11 mM; polyvinyl alcohol, 7% (w/v); creatine phosphate, 40 mM; creatine kinase, 100 μg/ml; WM433 extract, 300 μg; and M13*oriC26* RFI or M13*oriC26Δ221* RFI DNA, 200 ng. Components, except for creatine kinase and WM433 extract were assembled at 0 °C, mixed thoroughly, and centrifuged for 10 s in an Eppendorf microcentrifuge. The last two components were then added and centrifugation repeated. The dnaA protein fraction to be assayed was next added to the supernatant and the reaction initiated by addition of template DNA and incubation at 30 °C. After 30 min at 30 °C, reactions were terminated by placing tubes at 0 °C followed by addition of 1 N HCl containing 0.1 M sodium pyrophosphate. The precipitate was collected on Whatman GF/C filters. The filters were washed, dried, and radioactivity determined in a toluene-based fluid in a scintillation counter. The extent of DNA synthesis with M13*oriC26Δ221* (*oriC*$^-$) template was subtracted from that with M13*oriC26* (*oriC*$^+$) template to yield dnaA-dependent *oriC* replication. One unit of dnaA protein activity is 1 pmol of dNMP incorporated per min (Fuller and Kornberg, 1983).

ColE1 DNA Replication in Vitro—We have previously found, as did Fuller *et al.* (1981), that the extracts prepared for *oriC* replication as described above are relatively inactive for ColE1 replication, probably due to low levels of DNA polymerase I. Therefore, to study ColE1 replication, extracts were prepared from strain W3110 as described by Conrad and Campbell (1979) with the following modifications. The ammonium sulfate precipitate was resuspended in a minimal amount of 10 mM Hepes, pH 8, 1 mM dithiothreitol, 1 mM

[2] H. Masai and K. Arai, personal communication.

[3] H. Masai, unpublished results.

[4] A. Kornberg, unpublished results.

EDTA, 100 mM KCl, 10% ethylene glycol (v/v), and dialyzed against the same buffer until conductivity was equivalent to ~200 mM KCl. The dialysate, with a protein concentration of 80–140 mg/ml (Fraction II), was distributed as 250-μl aliquots, frozen in liquid nitrogen, and stored at −70 °C. The standard replication assay was as described by Conrad and Campbell (1979) using 1 mg of extract.

RESULTS

Stimulation of ColE1 Replication by Partially Purified Protein from a dnaA-overproducing Strain—The presence of a dnaA protein recognition sequence near the origin of replication on ColE1 plasmids, as well as several studies using $dnaA_{ts}$ mutants, suggest that the dnaA protein may participate in ColE1 DNA replication *in vivo*. In contrast, we have previously shown that extracts of $dnaA_{ts}$ strains exhibit no defects in ColE1 DNA replication *in vitro*. This implies that dnaA either plays a regulatory role that is not mimicked *in vitro* or that the dnaA protein can be replaced by another protein or proteins. We have investigated these possibilities as follows. Strain N4830 carrying the high copy number plasmid pBF1509 $dnaA^+$ overproduces the dnaA protein. Overproduction is increased by 100-fold relative to wild-type strains when the λ repressor is inactivated by induction at high temperature (Fig. 1*A*). Extracts were prepared from both induced and uninduced cultures of N4830/pBF1509, and their effect on DNA replication in the ColE1 replication system was tested. The *in vitro* replication system was prepared from strain W3110. Although this strain carried a single copy wild-type *dnaA* gene, dnaA protein was undetectable either by protein blotting (see Fig. 1) or by assay for replication activity in the *oriC* system. pBR322 DNA and its derivatives, pHM38, pHM54, and pBR322Δ8 were compared as templates in the ColE1 *in vitro* system in the presence of added Fraction II (see Table I) from the dnaA-overproducing strain. For pBR322 DNA, both induced and uninduced Fraction II stimulated synthesis 1.35-fold over the basal level in W3110 extracts (Fig. 2*A*). The amount of dnaA protein in extracts of strains carrying the dnaA plasmid is elevated over wild-type as shown in Fig. 1*A* even without induction, and apparently this level is sufficient to stimulate the reaction. Another possibility is that some other limiting factor in the W3110 extracts, such as DNA polymerase I, is being provided by both of the added extracts, leading to stimulation. In any case, the stimulation was not specific for dnaA induction and allowed no conclusion about the role of dnaA.

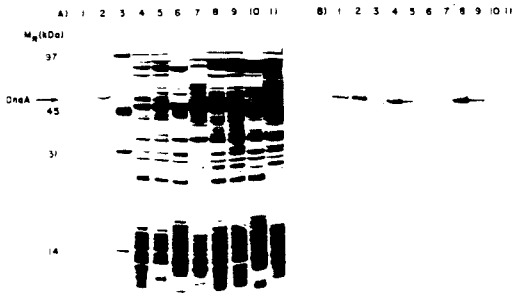

FIG. 1. **SDS-PAGE and protein blot of dnaA protein.** *A*, SDS-PAGE electrophoresis and silver staining were performed as described under "Experimental Procedures." *B*, protein blot of the gel shown in *A*, with dnaA antiserum diluted 1:250 for blotting. Bands were visualized using goat anti-rabbit IgG horseradish peroxidase and horseradish peroxidase color development agents. *Lanes 1* and *2* contain 0.5 and 1.0 μg, respectively, of dnaA protein, Fraction IV. *Lane 3* contains protein molecular weight standards. *Lanes 4* and *8* contain 40 and 80 μg, respectively, of Fraction II from induced cultures of N4830/pBF1509. *Lanes 5* and *9* contain 40 and 80 μg of Fraction II from uninduced N4830/pBF1509. *Lanes 6* and *10* contain 40 and 80 μg of WM433 Fraction II. *Lanes 7* and *11* contain 40 and 80 μg, respectively, of W3110 Fraction II. All fractions were prepared as described under "Experimental Procedures."

TABLE I

Summary of purification of the dnaA protein

Fraction number	Protein	Activity	Specific activity	Yield
	mg	*units* × 10^{-5}	*units/mg*	%
I. Cleared lysate	4,540			
II. $(NH_4)_2SO_4$	857	2.2	256.7	(100)[a]
III. Heparin-agarose (300–540 mM KCl)	55.5	1.66	3,000	75.7
IV. Heparin-agarose (540–700 mM KCl)	0.48	0.2	41,750	9.0

[a] Yield and purification are based on Fraction II because activity could not be reliably measured in Fraction I.

A second plasmid, pHM38, consistently showed a 50% reduction in replication in these extracts compared to pBR322. pHM38 is a pBR322 derivative that lacks the primosome assembly site (n′ site) on the leading strand (H-strand) but has both the dnaA site and the n′ site on the lagging strand (L-strand). When induced pBF1509 $dnaA^+$-Fraction II was added, synthesis of pHM38 was restored to the level observed with pBR322 (Fig. 2*B*). All synthesis was inhibited by 5 μg/ml rifampicin, confirming that Fraction II specifically stimulated replication but not repair. Since uninduced extracts did not stimulate pHM38, it seemed likely that the dnaA protein was responsible for stimulation.

pHM54 and pBR322Δ8 are two other derivatives of pBR322. Both lack the dnaA site and lagging strand n′ site, but pHM54 retains the n′ site on the leading strand whereas pBR322Δ8 does not. pBR322Δ8 and pHM54 showed extremely low levels of DNA replication compared to pBR322 and pHM38 (Fig. 2, *A* and *B*). The low levels are probably due to the lack of an n′ site on the lagging strand, which has recently been shown by Masai and Arai[2] to be essential for *in vitro* DNA replication. Addition of induced or uninduced Fractions II of N4830/pBF1509 did not restore the ability of these plasmids to replicate (Fig. 2, *A* and *B*). Thus, in the absence of a dnaA recognition site, dnaA protein has no effect on pBR322 replication.

We conclude from these studies, in particular with the pHM38 template, that the dnaA protein may participate in ColE1 replication when n′ sites are deleted. This was originally proposed as an interpretation of similar studies by Seufert and Messer (1987). However, since all experiments were carried out in extracts, crucial proof was lacking as to whether it was the dnaA protein or other factors in the induced extracts that caused the stimulation. This was especially true because even uninduced extracts contain relatively large amounts of dnaA protein (see Fig. 1*A*). To verify the involvement of dnaA protein, we have purified the dnaA protein by a simple, three-step procedure and prepared antibody against the protein.

Purification of dnaA Protein—Strain N4830 (λ*cI857*) carrying plasmid pBF1509 with the *dnaA* gene under the control of λ P_L promoter was grown at 30 °C (uninduced) or at 42 °C (induced) and the dnaA protein activity measured as described under "Experimental Procedures." Induction at 42 °C led to a 50 to 100-fold overproduction of dnaA protein relative to the uninduced (30 °C) level in various trials as measured in the standard replication assay described under "Experimental Procedures." Since the dnaA protein is absolutely required for the replication of *oriC*, the *E. coli* chromosomal origin of replication, a template containing *oriC* was used in this assay.

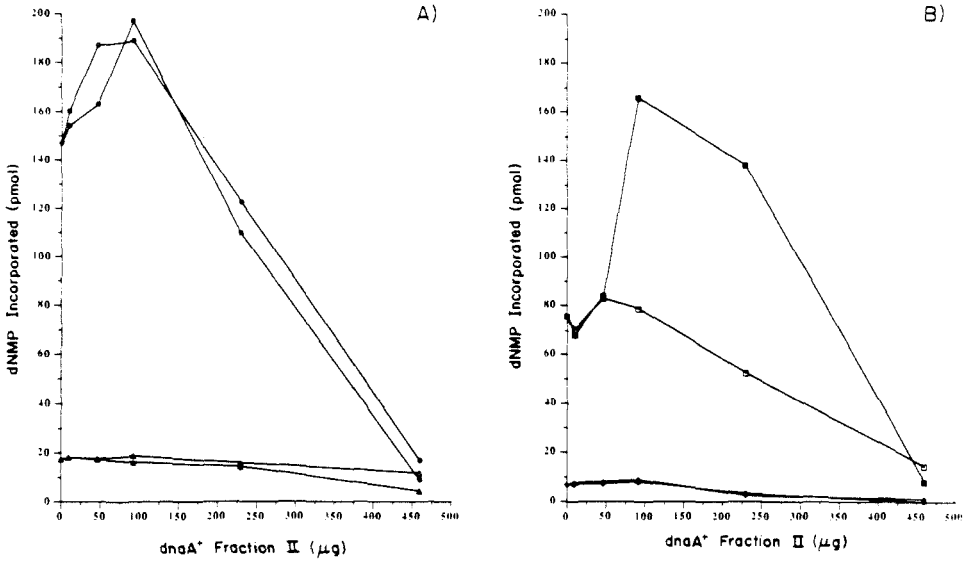

FIG. 2. **Stimulation of replication of pBR322 and its derivatives by Fraction II from induced or uninduced N4830/pBF1509.** Induced and uninduced N4830/pBF1509 Fractions II were prepared as described under "Experimental Procedures." Reactions were carried out in the standard reaction mixture and the amount of Fraction II was varied as shown. *Closed symbols* represent induced Fraction II; *open symbols* represent uninduced Fraction II. *A:* ●, pBR322; ▲, pBR322Δ8. *B:* ■, pHM38; ◆, pHM54.

Initial steps in the purification of dnaA protein (preparation of Fractions I and II) were based on those of Fuller and Kornberg (1983). Strain N4830/pBF1509 was grown at 30 °C in 20 liters of L-broth containing 25 μg/ml thymine, 0.2% glucose, and 25 μg/ml ampicillin until A_{595} reached 0.8. The temperature was shifted to 42 °C for 40 min and returned to 30 °C for 50 min at which time A_{595} was 1.6–2.0. Cells were harvested by centrifugation and resuspended in buffer A (50 mM Hepes·KOH, pH 7.6, 1 mM EDTA, 2 mM dithiothreitol, 20% sucrose) containing 250 mM KCl at A_{595} = 400, frozen in liquid nitrogen, and stored at −70 °C.

Cells were thawed at 0 °C and an equal volume (80 ml) of buffer A containing 250 mM KCl and 40 mM spermidine was added. Lysozyme was added to a final concentration of 250 μg/ml. The mixture was kept on ice for 30 min followed by 4 min at 37 °C. Lysates were chilled to 0 °C and insoluble material removed by centrifugation at 30,000 rpm in a Beckman Ti60 rotor for 40 min at 4 °C. The supernatant was Fraction I (121 ml). Ammonium sulfate (34 g) was added to Fraction I, and the suspension was stirred for 30 min at 4 °C. After centrifugation of the suspension, pellets were resuspended in 5 ml of buffer A to a final volume of 7 ml and dialyzed against 1 liter of buffer A for 3 h. The fraction was diluted with buffer A to give a conductivity equivalent to that of buffer A containing 50 mM KCl. The dialyzed fraction was designated Fraction II (34 ml). All subsequent chromatographic steps were performed at 4 °C.

Fraction II was loaded onto a 30-ml heparin agarose column equilibrated with buffer A containing 50 mM KCl. The column was washed with 300 ml of buffer A containing 165 mM KCl. Protein that remained bound to the column was eluted with 300 ml of linear gradient from 165 mM to 1 M KCl in buffer A (Fig. 3). Fractions eluting between 300 and 540 mM KCl (84 ml) were pooled and designated Fraction III. The protein was concentrated by precipitation with 29.4 g of ammonium sulfate. The ammonium sulfate suspension was centrifuged at 30,000 rpm in a Beckman Ti60 rotor for 30 min. The pellets were resuspended in buffer A and dialyzed against 1 liter of buffer A containing 50 mM KCl. The dialysate contained 90% of the dnaA protein activity but had only 12-fold greater specific activity than Fraction II (Table I).

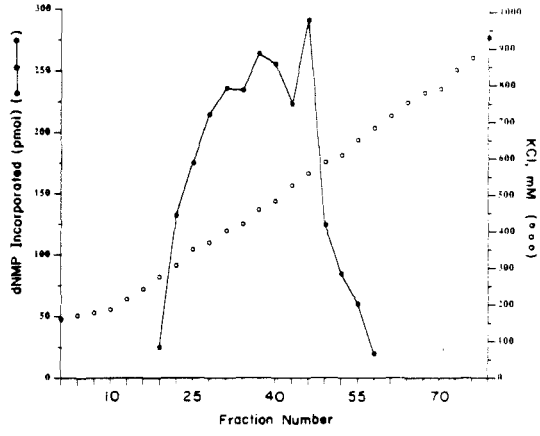

FIG. 3. **Heparin agarose chromatography of dnaA protein.** Samples (5 μl) of individual fractions were included in the dnaA protein replication assays described under "Experimental Procedures."

Fractions eluting from the heparin agarose column between 540 and 700 mM KCl (Fraction IV) were pooled (70 ml) and dialyzed directly against buffer A containing 50 mM KCl. The dialysate contained such a low concentration of dnaA protein that it was undetectable with the *in vitro* complementation assay. Therefore, Fraction IV was concentrated by loading

onto a 1-ml phosphocellulose (P11) column equilibrated with buffer A containing 50 mM KCl. The phosphocellulose column was washed with 15 ml of buffer A containing 50 mM KCl and eluted with 6 ml of buffer A containing 1 M KCl. The eluate was then dialyzed against 1 liter of buffer A containing 50 mM KCl. The dialysate was judged to be greater than 50% dnaA protein by polyacrylamide gel electrophoresis with SDS (see Fig. 1, *A* and *B*) and showed a 160-fold purification over Fraction II (Table I). The purified protein was divided into aliquots and stored at −70 °C.

It should be emphasized that both Fractions III and IV were active in *oriC* replication. Thus, Fraction IV does not represent a minor or inactive form of the protein such as that described recently by Sekimizu *et al.* (1987). Fuller and Kornberg (1983) also observed separation of dnaA activity into several peaks similar to those observed in the heparin agarose elution profile shown in Fig. 3. We propose that the peaks correspond to monomeric and multimeric forms of the dnaA protein, which have been demonstrated by Fuller and Kornberg (1983).

Characterization of dnaA Antiserum—Polyclonal antibody was prepared against the dnaA protein as described under "Experimental Procedures." When the dnaA protein (Fraction IV) was submitted to SDS-PAGE and blotted to nitrocellulose, a single band corresponding to the dnaA protein was observed to react with dnaA antiserum (Fig. 1*B*, *lanes 1* and *2*). The antibody is highly specific and reacts exclusively with the 52-kDa dnaA protein in extracts (Fig. 1*B*, *lanes 4*, *5*, *8*, and *9*). Preimmune serum does not react with any *E. coli* protein on protein blots (data not shown). While Fraction II from induced N4830/pBF1509 gave a more intense dnaA protein band on the protein blots than did the uninduced Fraction II, the apparent ratio is not as high as expected from the replication assays, which showed a 100-fold overproduction of dnaA protein replication activity (Fig. 1*B*, *lanes 4*, *5*, *8*, and *9*). The low ratio was due to failure to achieve quantitative transfer of the overproduced dnaA protein to nitrocellulose, as evidenced by silver staining of the gel after protein transfer (data not shown). For the *dnaA* mutant WM433, which was used in the dnaA protein replication assay, and for strain W3110 from which ColE1 replication extracts were prepared, no bands were visible on the blot. Thus, the levels of dnaA protein are very low even in wild-type *E. coli* extracts prepared as described for *in vitro* replication.

Since the antibody was raised against previously denatured protein, it was essential to evaluate the inhibitory activity of the antiserum in the same dnaA *oriC*-dependent replication assay used for purification (see "Experimental Procedures"). Antisera (50 μg) inhibited replication completely, whereas preimmune serum showed no inhibition up to 200 μg of protein. Thus, the antiserum inhibits the native form of the dnaA protein.

Inhibition of ColE1 in Vitro Replication Activity by dnaA Antiserum—To confirm that the stimulation of ColE1 *in vitro* DNA replication by induced Fraction II of N4830/pBF1509 was due to dnaA protein, we first tested the ability of dnaA antiserum to prevent stimulation of pHM38 replication *in vitro*. Addition of 250 μg of dnaA antiserum reduced synthesis to levels observed in the absence of added induced dnaA$^+$-Fraction II (Fig. 4). Further addition of antiserum failed to reduce synthesis significantly. Preimmune serum had no effect on the stimulation (Fig. 4). The inhibition by dnaA antiserum could be completely reversed by preincubation of antiserum with purified dnaA protein (Fig. 5), verifying that

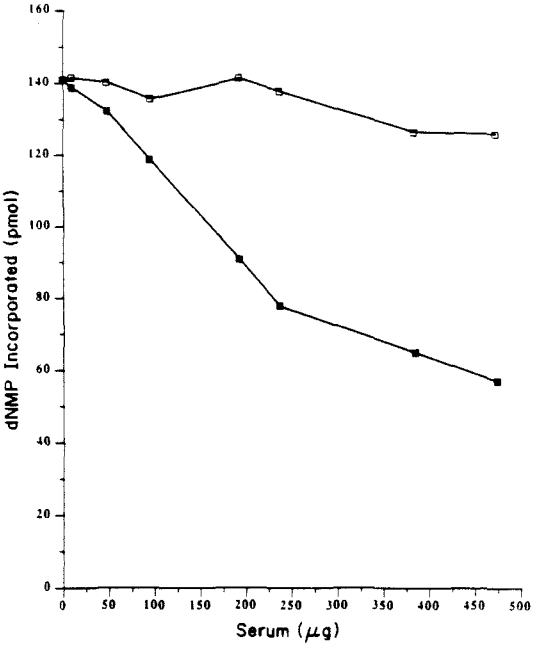

FIG. 4. **Effect of dnaA antiserum on *in vitro* DNA replication of plasmid pHM38.** Reactions were carried out in the standard reaction mixture with a fixed amount of induced Fraction II from N4830/pBF1509 (90 μg), and the amount of dnaA antiserum or preimmune serum was varied as shown. *Closed symbols* denote dnaA antiserum; *open symbols* denote preimmune serum.

the dnaA antiserum is specifically inhibiting the dnaA protein in these reaction mixtures.

Stimulation of ColE1-type Replication by Purified dnaA Protein—Addition of purified dnaA protein to plasmid replication extracts using the pHM38 template provided a direct demonstration of the ability of dnaA protein to stimulate ColE1 replication. As shown in Fig. 6, the same levels of stimulation were attained as with the dnaA-overproducing extract. The observed stimulation was due to replication, as all DNA synthesis was abolished by addition of 5 μg/ml rifampicin. dnaA antiserum inhibited stimulation, whereas preimmune serum had no effect (data not shown). The amount of dnaA protein required to maximally stimulate ColE1 replication is 10-fold higher than that needed in the replication assay used to purify the dnaA protein. The latter assay used *oriC* rather than ColE1 plasmid DNA template, suggesting that dnaA acts very inefficiently in the case of ColE1. In accord with this observation, 250 μg of antiserum was needed to inhibit ColE1-type replication, which is 5-fold more than the amount needed to inhibit in the *oriC* replication assay. The dnaA protein may act relatively inefficiently at the ColE1 origin of replication because there is only one copy of the dnaA consensus recognition sequence in the ColE1 plasmid origin, whereas *oriC* contains four such sites.

Does dnaA Protein Participate in ColE1-type DNA Replication when n′ Sites Are Intact?—As shown in Fig. 2, both induced and uninduced Fraction II of N4830/pBF1509 stimulate pBR322 replication 1.35-fold *in vitro*. dnaA antiserum blocked the effect of either added extract (Fig. 7). Interestingly, the dnaA antiserum reduced DNA synthesis to levels lower than those observed in the absence of any added dnaA-containing extract (*cf.* Figs. 2 and 7). These observations were

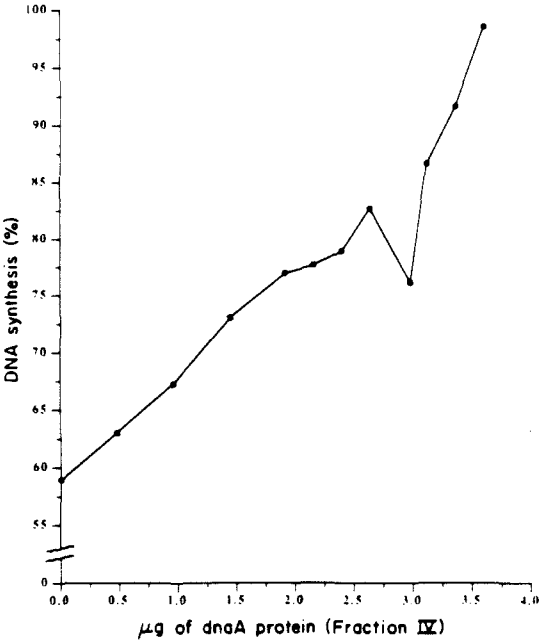

FIG. 5. **Reversal of antibody inhibition with purified dnaA protein (Fraction IV).** Reaction mixtures contained pHM38 as the template and 90 μg of induced Fraction II of N4830/pBF1509. Varying amounts of dnaA protein (Fraction IV) were incubated with 400 μg of dnaA antiserum for 10 min and then added to the reaction mixture. The reaction was initiated by addition of pHM38 and incubated for 1 h at 30 °C. The amount of DNA synthesis was measured as described under "Experimental Procedures." 100% DNA synthesis was equivalent to 158 pmol of dNMP incorporated in the absence of antibody.

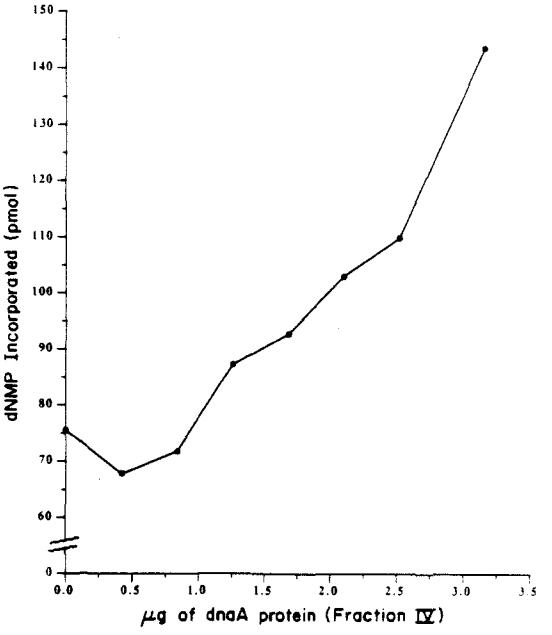

FIG. 6. **Stimulation of plasmid pHM38 replication by purified dnaA protein (Fraction IV).** Reactions were carried out in the standard reaction mixture except that the amount of Fraction IV was varied as shown.

specifically due to effects on the dnaA protein, since, when antiserum was preincubated with purified dnaA protein, DNA synthesis returned to normal levels (Fig. 7). These results suggest that dnaA protein may participate in normal pBR322 replication. Since both n′ sites are intact on pBR322, the dnaA site might function as an accessory "primosome assembly site," or the dnaA protein may play additional roles in ColE1-type DNA replication not revealed by the types of experiments reported here.

DISCUSSION

Although dnaA protein is not essential for ColE1 DNA replication, this investigation suggests that it does participate in the process. A partially purified fraction from a dnaA-overproducing strain showed a 2-fold stimulation of replication with pHM38, a pBR322 derivative that lacks the n′ site on the leading strand. This stimulation was demonstrated to be due to dnaA protein, since it was specifically blocked by dnaA antiserum and since the same degree of stimulation was observed using purified dnaA protein as with extracts. Purification of dnaA protein and preparation of dnaA antiserum have been crucial tools in this investigation.

n′ sites or primosome assembly sites (*pas*) were first described on φX174 phage DNA strands, as protein n′ effector sites (Shlomai and Kornberg, 1980). Subsequently, similar n′ sites, present on both strands of pBR322 and ColE1 near the origin of replication, were shown to support protein n′, single-stranded DNA-dependent ATPase activity (Zipursky and Marians, 1980). The n′ site on the leading strand (H-strand)

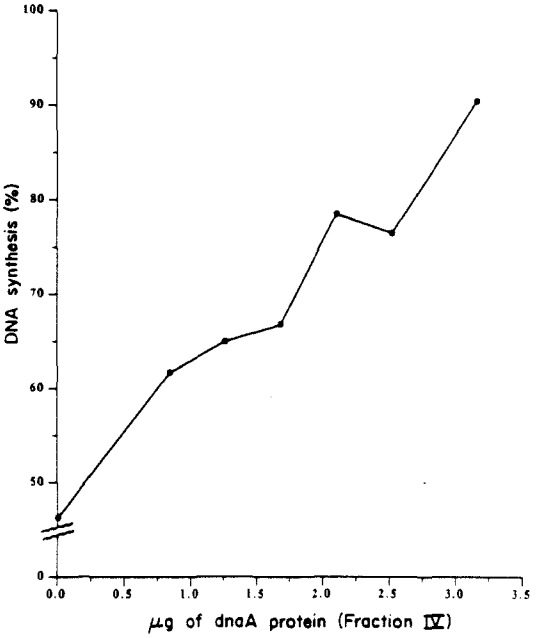

FIG. 7. **Reversal of antibody inhibition with purified dnaA protein (Fraction IV).** Conditions were as described in the legend to Fig. 5 except pBR322 was used as the DNA template. The amount of DNA synthesis was measured as described under "Experimental Procedures." 100% DNA synthesis was equivalent to 180 pmol of dNMP incorporated in the absence of antibody.

of pBR322 (positions 2114 and 2185 (Sutcliffe, 1978)) is ~400 nucleotides downstream of the origin of replication and has been proposed as the point of transition between DNA polymerase I and polymerase III-dependent DNA synthesis. The n′ site on the lagging strand (L-strand) of pBR322 (positions 2353–2416) is 150 nucleotides downstream of the origin and has been considered as the origin of lagging strand DNA synthesis (Zipursky and Marians, 1980). Comparison of the two n′ sites revealed little sequence homology except for a hexanucleotide 5′-AAGCGG that is also present on the n′ site of φX174 (Marians *et al.*, 1982). This hexanucleotide is unlikely to be the sole recognition element for protein n′ as this sequence is present in numerous other positions on both the H and L strands of pBR322 that do not function as primosome assembly sequences. Furthermore, DNase I footprinting and dimethyl sulfate methylation patterns demonstrated that protein n′ was bound to the entire length of n′ sites (Greenbaum and Marians, 1984). Two lines of evidence suggest that n′ sites participate in ColE1 replication. First, protein n′ is one of the seven primosomal proteins required for lagging strand DNA synthesis of pBR322 *in vitro* (Minden and Marians, 1985). The other proteins are gene products of the *dnaB*, *dnaC*, and *dnaG* gene, and proteins i, n, and n″, the same set of proteins necessary for primosome assembly on φX174 templates. Second, Van der Ende *et al.* (1983) found that pBR322 derivatives with deletion of both n′ sites were as stable as the wild-type, but that they had a copy number 2- to 3-fold lower. Thus, n′ sites are important but may not be essential when the rest of the origin of replication is intact.

Our results indicate that the n′ site on the leading strand is not essential for DNA replication *in vitro* but that it is important for optimal replication since a plasmid lacking the site showed a 2-fold reduction in replication efficiency compared to pBR322. In contrast, Seufert and Messer (1987) found no reduction in replication efficiency in a plasmid carrying a partial deletion of this n′ site. One explanation for the discrepancy might be that the partial deletion did not destroy the functional binding site. Abarzúa *et al.* (1984) have shown, however, that base substitutions at position 2122 abolished protein n′ binding. Since position 2122 is absent in the partial n′ site deletion of Seufert and Messer (1987), one would expect that the mutation would affect *in vitro* DNA replication. An alternative explanation for the difference in results is that the extract employed by Seufert and Messer (1987) was specifically designed for *oriC* replication and had relatively low activity with ColE1 (Fuller *et al.*, 1981). Therefore, the extract might not provide as sensitive a measure of required sites as the extracts used in the current study. Regardless of the reason for this slight discrepancy, the reduced levels of synthesis on the n′ mutant template allowed us to demonstrate a role for dnaA protein. The combination of intact dnaA binding site and dnaA protein seems to be able to compensate for the loss of function of the n′ site on the leading strand, because wild-type levels of DNA synthesis could be attained by addition of purified dnaA protein. The amount of dnaA protein needed to maximally stimulate pHM38 is 10-fold higher than that needed to stimulate the *oriC* template in the DNA replication assay of dnaA protein activity. This observation might reflect the fact that there is only one dnaA binding site on ColE1, whereas in *oriC*, there are four such sites available for cooperative binding, making the latter template more efficient in utilizing dnaA protein.

While deletion of the n′ site on the leading strand reduced synthesis in our system by 50%, deletion of the n′ site on the lagging strand completely abolished synthesis (Fig. 2, pBR322Δ8, pHM54). A requirement for the n′ site on the lagging strand during *in vitro* replication has been demonstrated in other *in vitro* replication extracts (Seufert and Messer, 1987 and Footnote 2), and it has also been shown that an intact dnaA site in an extract containing elevated levels of dnaA protein can at least partially compensate for the lack of the n′ site. Since the essential function of n′ sites is to serve as sites for primosome assembly, the dnaA site may be serving the same purpose. To further test this idea, it should be possible to demonstrate that the dnaA protein can compensate for loss of one of the primosomal proteins, *i.e.* proteins i, n, n′, and n″. For instance, Masai *et al.* (1986) have shown that *dnaT* mutants are deficient in protein i and that extracts of *dnaT1* (UT205) strains show reduced ColE1 plasmid replication *in vitro*. It would be of interest to test the ability of purified dnaA protein to complement *dnaT1* extracts.

Even if a role in primosome assembly is accepted for the dnaA protein, it is difficult to evaluate the relative importance of the n′ and dnaA sites, since a mutant template that selectively deletes the dnaA site, but has both n′ sites, has not been constructed. The question of the relative contribution was, however, addressed in our experiments using dnaA antiserum and wild-type templates. Fig. 2 showed that induced and uninduced dnaA$^+$-Fraction II stimulated pBR322 replication. dnaA antiserum inhibited the stimulation, and the inhibition was specifically titrated by preincubation of the antiserum with purified dnaA protein (Fig. 7). The antiserum even inhibited below the basal level of synthesis observed in the absence of added dnaA$^+$ extract. Although this suggests that dnaA protein participates in normal pBR322 replication, it should be kept in mind that inhibition might occur because dnaA protein sits passively at its recognition site at the origin of replication, and the aggregate of the protein and its antibody at the site near the origin interferes nonspecifically with the DNA replication process. This possibility could be addressed by an experimental protocol that depletes dnaA protein from the extracts with antibody before addition of DNA. Preliminary results suggest that removal of dnaA protein in this way inhibits pBR322 replication, strengthening the idea that dnaA plays a direct role in pBR322 replication. Another possible explanation for the inhibition derives from the model of Polaczek and Ciésla (1984) in which dnaA protein interacts with RNA I promoter so as to reduce transcription of RNA I. By titrating dnaA protein with dnaA antiserum, we may have induced the RNA I promoter and thus increased transcription of RNA I, which is known to be an inhibitor of primer synthesis in the ColE1 replication system. Preliminary studies, however, showed no changes in the levels of RNA I synthesized *in vitro* in the presence and absence of antibody. Finally, initiation of the leading strand at *oriC* differs from that at the pBR322 *ori* in that the latter initiates, apparently obligatorily, by synthesis of an RNA primer by RNA polymerase and processing by RNase H to make a primer for DNA polymerase I. At *oriC*, on the other hand, dnaA protein is required to initiate the leading strand where the primer is thought to be made by dnaG primase and synthesis extended by DNA polymerase III. It would be interesting to look at the effect of dnaA antibody on pBR322 replication in an *rnh*$^-$ mutant extract, where the mode of replication might be altered drastically. In such a mutant, the role of dnaA protein in ColE1 replication might be enhanced, and initiation might be more similar to that found at *oriC*.

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PART II

Mutants of *Saccharomyces cerevisiae* DNA Polymerase I Resistant to Nucleotide Analogs: dNTP Binding Site Definition

INTRODUCTION

Replicative DNA polymerases are essential enzymes in DNA replication and are undoubtedly the most extensively studied of all replication proteins. The first DNA polymerase isolated was DNA polymerase I (Pol I) of *E. coli* (Kornberg *et al.*, 1956). At that time, it was believed to be the sole replicative polymerase of *E. coli.* However, the discovery of thermolabile DNA polymerase III (Pol III) (Kornberg and Gefter, 1971) from *dnaE* mutants convincingly demonstrated Pol III to be the replicative polymerase responsible for elongation in *E. coli.* Like *E. coli,* eukaryotes possess multiple classes of DNA polymerases, each performing specific roles. DNA polymerase α (Pol α) and polymerase δ (Pol δ) are involved in chromosomal replication and possibly in DNA repair as well. The low molecular weight DNA polymerase β (Pol β) is believed to function in DNA repair, but there is no direct proof that this is the case. DNA polymerase γ (Pol γ) is localized in the cellular mitochondria and is most likely involved in mitochondrial DNA replication. Among the four classes of eukaryotic polymerases, DNA pol α has been the focus of DNA replication research, because it was isolated first and was long regarded as the sole polymerase responsible for cellular DNA replication until the recent discovery of Pol δ involvement in SV40 DNA replication. Pol δ has prompted a major restructuring of thinking in the eukaryotic DNA replication field. The following account will concentrate on Pol α because part II of this thesis deals with yeast DNA polymerase I, the yeast analog of higher cells Pol α. Pol δ will be mentioned mainly for comparison to Pol α. Similarly, yeast DNA pol III, the analog of Pol δ, will be mentioned at appropriate points to contrast the functional and structural differences between Pol I and Pol III.

DNA polymerase α is located in the nucleus as expected for any enzyme that plays a key role in cellular DNA replication. Immunofluorescence staining of KB cells previously exposed to monoclonal antibodies directed against Pol α showed all immunoreactive material to be confined to the nucleus (Bensch *et al.*, 1982). Many

lines of evidence also support a role for Polymerase α in chromosomal DNA replication. First, the level of Pol α is highest in tissues where there is rapid cell division and lowest in quiescent cells. The relative quantities of DNA pol α and β of chick cells were examined during different stages of the cell cycle by using antibodies specifically directed against Pol α and Pol β. The intensity of immunofluorescence of Pol α, the replicative polymerase, correlated closely with cellular proliferation, while that of Pol β, a presumed repair polymerase, remained constant in both proliferating and resting cells (Matsukage *et al.*, 1983). The cell cycle regulation of yeast DNA polymerase I was investigated. *POL1* transcript levels increased at least 100-fold at late G_1 with a peak around the G_1/S boundary just prior to the start of DNA replication (Johnston *et al.*, 1987). Second, DNA synthesis of eukaryotic cells and certain animal viruses (SV40, herpes viruses and vaccinia viruses) were inhibited by aphidicolin, a potent inhibitor of Pol α *in vitro* (Ikegami *et al.*, 1978; Okashi *et al.*, 1978). Third, monoclonal antibodies directed against Pol α inhibited cellular replication in permeabilized nuclei or when directly injected into cultured mammalian cells (Miller *et al.*, 1985). Also, HeLa cell extracts depleted of Pol α by immunoprecipitation do not support SV40 replication *in vitro* (Murakami *et al.*, 1986). Fourth, temperature-sensitive mutants of Pol α are unable to replicate their DNA at the non-permissive temperature. Examples include both the mouse Pol α and the yeast DNA pol I mutants (Murakami *et al.*, 1985; Budd and Campbell, 1987; Budd *et al.*, 1989). Fifth, the gene for yeast DNA pol I, *POL1*, was isolated by probing a yeast genomic DNA expression library in λgt11 with antibodies prepared against the purified protein (Johnson *et al.*, 1985; Lucchini *et al.*, 1985). The *POL1* gene was sequenced and found to have an open reading frame of 1468 codons, encoding a polypeptide of calculated M_r 166,794 (Pizzagalli *et al.*, 1988), which corresponds closely to the 180 kD DNA polymerse I observed on an SDS-PAGE (Plevani *et al.*, 1985). Gene disruption and Southern hybridization experiments demonstrated that the polymerase subunit of yeast DNA

polymerase I is encoded by a single-copy, essential gene. Spores carrying a disrupted *POL1* gene germinate but arrest after a few cell divisions with a dumbell morphology typical of a defect in medial nuclear division and in DNA replication (Johnson *et al.*, 1985).

DNA polymerase α has been purified from a variety of sources by several laboratories. Recent advances in rapid purification schemes, including immunoaffinity chromatography have minimized proteolysis and allowed the isolation of intact, mutltisubunit enzymes. There is now a general agreement that the enzyme is composed of four distinct subunits, a structural feature shared by Pol α from species as divergent as yeast and human (Campbell, 1986; Fry and Loeb, 1986). The largest subunit of M_r 180,000 (a family of polypeptides ranging from 140-185 kD) contains the polymerase active site. In the case of *Drosophila*, this large subunit harbors a cryptic 3'-->5' exonuclease that serves as a proofreading function during polymerization (Cotterill *et al.*, 1987). In the intact polymerase, the potent 3'-->5' proofreading exonuclease is masked by the 73 kD subunit whose function is unclear in all other α-type polymerases. The proofreading exonuclease activity is likely to be an important general feature of DNA pol α contributing to the fidelity of replication. However, attempts to uncover similar exonuclease activity in DNA pol α from other species have thus far been unsuccessful.

The smallest subunits of DNA pol α (50-60 kD) constitute a primase activity capable of synthesizing short RNA transcripts of defined length to serve as primers for subsequent DNA elongation by the polymerase subunit (Conaway and Lehman, 1982; Singh *et al.*, 1986; Tseng and Ahlem, 1983). The primase subunits associated with yeast Pol I are of M_r 48 and 58 kD. Antibodies directed separately against the 48 and 58 kD subunits inhibit the activity of purified yeast DNA primase, providing direct evidence that both subunits are involved in DNA-priming activity (Lucchini *et al.*, 1987; Plevani *et al.*, 1988). The 48 kD subunit also contains an ATP-binding site,

suggesting that the catalytic site is within this polypeptide. Recently, both the 48 and 58 kD subunits were labeled by *p*-hydroxybenzaldehyde ester of ATP, which selectively label the active site of RNA polymerase (Schaffner *et al.*, 1987). Since primase is a specialized RNA polymerase devoted to the initiation of DNA synthesis, this result indicates that both subunits participate in formation of the active site (Plevani *et al.*, 1988). The yeast genes encoding the 48 and 58 kD subunits have been cloned, sequenced and each shown to be present in a single copy and essential for cell viability (Plevani *et al.*, 1987; Plevani *et al.*, 1988).

The intact DNA pol α complex contains all of the activity required for the initiation and synthesis of nascent DNA chains on single-stranded DNA templates. It is not, however, a highly processive DNA polymerase, since it polymerizes less than 100 nucleotides per binding event under normal assay conditions (Fisher *et al.*, 1979; Hockensmith and Bambara, 1981; Pritchard *et al.*, 1983; Sabatino *et al.*, 1988; Villani *et al.*, 1981). Thus, polymerase accessory factors should exist to enhance DNA polymerase α processivity, allowing more efficient DNA replication. Three kinds of accessory factors have been identified for Pol α, namely, proteins C1 and C2, Ribonuclease H and single-stranded DNA binding proteins.

Proteins C1 and C2 have been purified to homogeneity from HeLa cells by assaying stimulation of DNA synthesis on DNA substrates with a low primer:template ratio (Lamothe *et al.*, 1981; Novak and Baril, 1978; Pritchard *et al.*, 1983). Analogous factors from monkey CV-1 cells (Pritchard and DePamphilis, 1983), *Drosophila* embryos (Kaguni *et al.*, 1983) and mouse FM3A cells (DiFrancesco and Lehman, 1985) have been identified. Interestingly, the C1 C2 complex possesses a high degree of specificity; i.e., they interact only with the Pol α from the same source. These factors stimulate Pol α activity on ss DNA templates by >20-fold, effectively eliminating non-productive binding by Pol α to ss DNA (Pritchard *et al.*, 1983).

Ribonucleases H (RNaseH) that stimulate the homologous DNA pol α have been isolated from yeast and *Drosophila* (Karwan *et al.*, 1983; DiFrancesco and Lehman, 1985). In yeast, RNaseH exerts its stimulatory effect by increasing the affinity of Pol I for the primer terminus (Karwan *et al.*, 1983). In *Drosophila*, the stimulation mechanism is quite different. RNaseH stimulation of DNA synthesis occurs only in coupled priming-chain elongation reactions and appears to be due to an increased rate of recycling the primase-polymerase complex (DiFrancesco and Lehman, 1985).

Single-stranded DNA binding proteins (SSB) in eukaryotes have been shown to play a role in replication equivalent to that played by the *E. coli* SSB. By analogy to the bacteria, the ability of an SSB to stimulate DNA synthesis by DNA polymerase implies SSB involvement in the replication process. Yeast SSB-1 stimulates DNA synthesis on templates with low primer:template densities (Jong *et al.*, 1985), however, recent evidence has indicated a role for SSB-1 in RNA metabolism *in vivo* (Jong *et al.*, 1987). In addition, the predicted amino acid sequence of SSB-1 shows some homology to proteins involved in RNA binding. A mammalian SSB, RP-A (replication protein A), has been identified as an essential component of a reconstituted system that can promote SV40 DNA replication *in vitro* (Dean *et al.*, 1987; Fairman and Stillman, 1988; Wold *et al.*, 1987; Wold and Kelly, 1988). The precise role of the protein is unknown. One possible role is to assist the SV40 T antigen in unwinding of SV40 DNA at the origin, a property shared by *E. coli* SSB in the *E. coli in vitro* system. It is not known whether these accessory proteins actually increase the processivity of Pol α *in vivo*. It may not be necessary to increase Pol α processivity, because Pol α may be responsible for lagging strand synthesis only, which does not require a highly processive polymerase. The discovery that Pol δ is an essential component in the complex of enzymes that promotes SV40 DNA replication *in vitro*, and the possibility of its being the replicative polymerase responsible for leading strand synthesis, have heightened the interest in this polymerase.

Polymerase δ has been isolated from various mammalian sources and like Pol α is sensitive to aphidicolin. Otherwise, however, Pol δ is quite different from Pol α. Pol δ has an intrinsic and readily detectable 3'-->5' exonuclease activity (Byrnes *et al.*, 1976) while the α-type polymerases (except *Drosophila* Pol α as described above) have no such activity (Cotterill *et al.*, 1987). The two types of polymerases also differ from each other in template preferences: Synthetic DNA substrates such as poly dA-$(dT)_{10}$ with low primer:template ratios are the preferred templates for Pol δ, whereas Pol α prefers activated DNA as templates. An inhibitor that dicriminates between Pol α and δ is the nucleotide analog, N^2-(*p*-*n*-Butylphenyl)-2'-dexoxyguanosine triphosphate (BuPdGTP) and its relative, BuPdATP (Wright and Dudycz, 1984). Both inhibit Pol α effectively at 1 μM concentrations, whereas greater than 100 μM are required to inhibit Pol δ (Byrnes, 1985). A known cell-cycle regulated protein required for SV40 DNA replication, proliferating cell nuclear antigen (PCNA), was shown to be an auxiliary protein of Pol δ but has no effect on Pol α (Prelich *et al.*, 1987). Recently, δ-type polymerases have been subdivided into two classes based on their processivity and enhancement of processivity by PCNA. Pol δ_1 is relatively non-processive, but its processivity is enhanced many fold (increased to 1000 nucleotides polymerized per binding event) by addition of PCNA to the *in vitro* replication system. Pol δ_2 is processive in the absence of PCNA and is not stimulated by the addition of this cofactor.

In yeast, two polymerases, Pol II and Pol III, are possible counterparts of Pol δ, judging from the physical properties (Bauer *et al.*, 1988; Budd *et al.*, in press; Burgers and Bauer, 1988). They are both sensitive to aphidicolin, possess or are associated with a 3'-->5' exonuclease activity and are relatively resistant to BuPdGTP and BuPdATP. DNA pol II is highly processive and is apparently unaffected by PCNA, while DNA pol III is stimulated by both mammalian and yeast PCNA (Bauer and Burgers, 1988; Burgers, 1988). Thus, yeast DNA pol III and II are the analogs of Pol

δ_1 and δ_2 of higher cells. The gene for Pol II has not yet been identified but DNA pol III has been shown to be encoded by *CDC2* (Sitney *et al.*, 1989). *CDC2* is an essential gene based on the lethality displayed by temperature-sensitive mutants of *CDC2* when placed at the restrictive temperature. Thus, in yeast, DNA pol I and pol III are conclusively demonstrated to be distinct enzymes as they are encoded by *POL1* [recently discovered to be allelic to *CDC17* (Carson, 1988)] and *CDC2*, respectively. Both Pol I and Pol III are essential for yeast chromosome replication, implying that both α and δ type polymerases are required for DNA replication in eukaryotes. The dual involvement of Pol α and δ can be envisioned as distinct polymerases acting on leading and lagging strand synthesis. The absence of primase activity and the high processivity of nucleotide polymerization with accessory factor PCNA have led to the suggestion that the Pol δ_1 functions on leading strand synthesis, and the Pol α with its associated primase and low processivity functions on lagging strand synthesis.

Both *POL1* (Pizzagalli *et al.*, 1988) and *CDC2* (A. Boulet and G. Faye, pers. commun.) have been sequenced, and comparison of the two polymerases shows conservation of the same regions conserved in human DNA pol α, DNA polymerases of phage T4, PRD1 and ϕ29, vaccinia virus, adenovirus and the herpes family viruses (Bernard *et al.*, 1987; Spicer *et al.*, 1988; Wong *et al.*, 1988). Six conserved regions were found among the polymerases in the same linear spatial arrangements on each polypeptide (Fig. 1). These regions are numbered according to the degree of homology, region I being the most similar and region VI the least. Region I contains the highly homologous YGDTDS domain; region II, the SLYPSII domain and region III, the NS-YG motif. Region IV contains a number of conserved dipeptides separated by conserved spacings. This region is not present in ϕ29 DNA polymerase. The homology present in regions V and VI is much less extensive but still significant, considering the wide range of DNA polymerases surveyed. Regions I, II and III are present in all enzymes surveyed and probably represent a set of basic core sequences

for this DNA polymerase class. The presence of these homologous regions distinguishes this class of polymerase from other eukaryotic DNA polymerases, e.g. DNA pol β, terminal transferase and reverse transcriptase (Matsukage *et al.*, 1987; Zmudzka *et al.*, 1986) and other prokaryotic DNA polymerases, e.g., *E. coli* DNA pol I and III and phage T7 DNA polymerase (Joyce *et al.*, 1982; Tomasiewicz and McHenry, 1987).

The observed sequence similarities suggest conservation during evolution and imply an important functional role for these regions. Highly conserved regions are likely to encode essential domains of polymerases, including DNA interaction domain(s), nucleotide and/or pyrophosphate binding domain(s). Sequence homology confined to only a few polymerases can also be significant if these few polymerases share common accessory proteins or enzymatic properties. For example, α-type polymerases should all possess a DNA primase interaction domain, as all highly purified forms of Pol α are found to associate with a primase activity. On the other hand, δ-type polymerases should contain a 3'-->5' exonuclease domain, which is intrinsic to most of these polymerases. By comparative sequence analysis, these domains can be tentatively assigned by virtue of mutations that have already been located and the phenotypes exhibited by these mutants.

All conserved regions identified thus far are located in the central and C-terminal one-third of the polymerases. This explains observations that extensively proteolyzed preparations of Pol α still retain catalytic activity. Sequence comparison of human DNA pol α and yeast DNA pol I alone demonstrates an overall 31% sequence similarity between these α-type polymerases (Fig. 2). Extensive regions of similarity were found upstream of region IV at the N-terminal third of these proteins, where no homology was found when compared with other polymerases. It is logical to assume that the N-terminal third of the Pol α interacts with protein(s) associated with α-type polymerases only. This view is supported by the analysis of a mutant of yeast DNA pol I, which

displays a slow-stop, temperature-sensitive phenotype *in vivo* and a defect in polymerase-primase interaction *in vitro* (Lucchini *et al.*, 1988; Pizzagalli *et al.*, 1988). This mutant has been sequenced and found to be a point mutation converting glycine to arginine at codon 493 ($glycine_{493}$ is in a stippled box in Fig.2). An attractive hypothesis is that this region is responsible for DNA primase-DNA polymerase interaction, but this remains to be tested by isolation of more mutants.

A cysteine-rich region is located near the carboxyl terminus of both human DNA pol α and yeast DNA pol I. Amino acid residues 1244-1391 in human pol α and the homologous region of yeast Pol I, amino acids 1246-1388, are regions that can potentially form the zinc finger DNA binding motif (Berg, 1986; Evans and Hollenberg, 1988). A secondary structure has been proposed with an extended protein loop containing amino acids, whose side chains have the potential to interact with the phosphate backbone of DNA. This is the putative DNA interaction domain of Pol α (Wong et al., 1988).

As mentioned above, almost all polymerases surveyed were found to possess conserved regions I, II and III. No mutations have been found in region I, presumably because this region is vital for catalytic activity of polymerase. Site-directed mutagenesis in region I was attempted, but all mutations gave an inactive enzyme. However, numerous single amino acid substitution mutations were identified in herpes simplex virus (HSV) DNA polymerase (Coen *et al.*, 1983; Gibbs *et al.*, 1988; Knopf, 1986; Knopf, 1987; Knopf *et al.*, 1981; Quinn and McGeoch, 1985; Tsurumi *et al.*, 1987). These mutations confer altered sensitivities to drugs, including acyclovir (ACV), bromovinyldeoxyuridine (BVdU), ganciclovir (DHPG), vidarabine (AraA), phosphonoacetic acid (PAA) and aphidicolin. ACV (Elion *et al.*, 1977; Furman *et al.*, 1979), BVdU (St. Clair *et al.*, 1984), DHPG (Ashton *et al.*, 1982; Germershausen *et al.*, 1983) and AraA (Coen *et al.*, 1982; Pavan-Langston *et al.*, 1975) are antiviral drugs that resemble deoxyribonucleotides (dNTPs). When these

drugs are phosphorylated *in vivo*, they inhibit replication by inactivating the virus DNA polymerase or by serving as a poor primer, once incorporated (Furman *et al.*, 1984). PAA is an analog of the pyrophosphate (PP_i) moiety and probably acts by occupying the PP_i binding site essential for catalysis (Mao *et al.*, 1975; Reno *et al.*, 1978). Aphidicolin has been demonstrated to be a competitive inhibitor of polymerase (Pedrali-Noy and Spadani, 1980). Mutants with altered sensitivities to these antiviral drugs are potential candidates possessing altered dNTP or PP_i binding site(s). Most mutants resistant to acyclovir also show altered sensitivities to PAA, indicating interacting and/or overlapping binding sites for the nucleotide and pyrophosphate moieties (Gibbs *et al.*, 1988; Knopf, 1987).

An additional mutant was mapped to region V of the HSV DNA polymerase, indicating a role of this region in the three-dimensional structure of the dNTP and/or PP_i binding domain(s). The isolation of antimutator mutants of T4 DNA polymerase just outside region V further corroborates the importance of this region as a possible nucleotide/pyrophosphate binding domain. These antimutators of T4 DNA polymerase exhibit an increased accuracy in nucleotide selection, as well as an elevated nucleotide turnover rate (Gillin and Nossal, 1976a; Gillin and Nossal, 1976b). Among the HSV DNA polymerase mutants, PAA^r5, which mapped to region III, also demonstrated an antimutator phenotype (shown in Fig. 3). This mutant enzyme has altered interactions with nucleoside triphosphates as evidenced by its resistance to ACV and elevated K_M values for normal dNTPs. Hall *et al.* (1985) proposed that reduced affinity of the polymerase for nucleoside triphosphates accounts for the antimutator phenotype by accentuating differences on base-pairing stability, thus facilitating selection of correct nucleotides. Based on these studies of the herpes simplex virus and T4 DNA polymerases, regions II, III and V are proposed to constitute the domain(s) required for dNTP and/or PP_i interaction (Gibbs *et al.*, 1988; Wong *et al.*, 1988). The single amino acid substitutions within the consensus regions show no preference for conserved or

non-conserved amino acids. For example, in region II, the change of a non-conservative arginine to glycine at position 700 of HSV DNA polymerase (mutant PFA^{r}1 in Fig. 3) confers resistance to PAA, ACV and hypersensitivity to aphidicolin. A similar phenotype is conferred by a change of a conservative serine to asparagine at position 724 (mutant PFA^{r}5 in Fig. 3) (Gibbs *et al.*, 1988). Thus, we are as yet unable to establish any rule of interactions between specific amino acids involved in substrate binding or between the amino acids and substrates/drugs.

There are three other mutations that confer altered sensitivities to antiviral drugs that do not map to regions II, III or V. However, they are clustered between regions IV and II and this domain has been designated region A by Gibbs *et al.*, (1988) (see Fig. 3). This region contains little or no sequence similarity to Pol α. Region A does contain sequences conserved among the polymerase genes of the herpes simplex family of viruses and to a lesser extent, the vaccinia virus. Two of the mutations involve substitution of highly conserved amino acids in all herpes virus DNA polymerases. Gibbs *et al.* (1988) proposed that region A is specific to viruses that are sensitive to certain antiviral drugs. This region is probably involved in drug/substrate interactions, or with other regions of the polymerase in a way that sensitizes the viral polymerase to the drugs relative to human DNA polymerase α. Although T4 DNA polymerase does not contain a region homologous to HSV region A, three mutations located between region IV and II are known to reduce the exonuclease activity of T4 DNA polymerase (Reha-Krantz, 1988). Sequence comparison of T4 DNA polymerase to δ-type polymerases, when they become available, may reveal some interesting homology in this region, a potential exonuclease domain.

In an effort to identify and characterize the nucleotide binding site of yeast DNA polymerase I, we have attempted to isolate *pol1* mutants that are resistant to nucleotide analogs with abnormal sugar moieties such as arabinosyl thymidine (AraT). Part II describes our strategy for creating a strain that is both permeable to nucleotide analogs

and contains *pol1*$_{ts}$, and for the screening of Pol I mutants that are resistant to these analogs. Numerous attempts have failed to yield such mutants. We offer possible explanations for the failure of the strategy and discuss an alternate route for identification of the yeast DNA pol I nucleotide and/or PP_i binding site(s), namely, site-directed mutagenesis. Screening for random mutations was preferred over site-directed mutagenesis, because no set rules have yet been established through HSV and T4 DNA polymerase mutants. Further, if this strategy were successful, we would be able to screen for second-site suppression of resistance to analogs, potentially yielding more information about the amino acids involved in nucleotide and/or PP_i binding.

MATERIALS AND METHODS

Strains and Plasmids

Haploid strain 167 was acquired from Robert A. Sclafani (University of Colorado, Denver). The genotype of strain 167 is *Mata leu2-3, 112 ade1, 2 trp1-289 tmp1-6 tut1-2 ura1 lys2* : pJM81 *LEU2 HIS3 TK*$^+$. This strain contains a lesion in the yeast thymidylate synthase gene (*tmp1-6*) and thus requires an exogenous supply of deoxythymidine monophosphate (dTMP) or thymidine (TdR). The *tut1-2* allele (TdR utilization) was derived from a *tup*$^-$ strain by selecting for growth with a low concentration of TdR (2 μg/ml). The *tup*$^-$ strain, like the *tut1* strain, is able to utilize exogenous dTMP or TdR, provided that thymidine kinase is present. However, the *tut1* strain can grow on a non-fermentable carbon source such as glycerol, in contrast with a *tup*$^-$ strain. In fact, when strain 167 is allowed to grow on a solid medium containing glucose and 100 μg/ml TdR, 80-90% of the colonies are ρ$^+$ (Sclafani and Fangman, 1986). 100 μg/ml thymidine is routinely included in all media used to maintain strain 167 and its derivatives. pJM81 is a high-copy number plasmid that possesses a fusion of the herpes simplex type 1 virus thymidine kinase gene to a yeast promoter (McNeil and Friesen, 1981), furnishing the cells with the ability to convert TdR to dTMP. Haploid strains of different *pol1*$_{ts}$alleles were derived from strain 488(α), a genetic background that allows high frequency, synchronous sporulation. The genotypes of 488-pol1-1i are *Matα trp1 leu2 ura3-52 his1-7 can1 pol1-1i*, where i = 1 to 7 (Budd and Campbell, 1987). *E. coli* strain MC1066 *pyrF:Tn50* was used to isolate plasmids containing the *URA3* gene from yeast DNA minipreps. Plasmid pBM150-264 contains a functional *POL1* gene inserted in the *Ba*mHI site of pBM150 and is thus induced in the presence of galactose (unpublished result of C. Gordon and J. L. Campbell). pBM150 also carries the *URA3*, *CEN4* and *ARS1* markers (Johnston and Davis, 1984).

Mutagenesis

Plasmid pBM150-264 was mutagenized according to the procedure of Busby *et al.* (1982) with hydroxylamine, which deaminates cytosine, causing a unidirectional transition of CG to AT in DNA.

Other Methods

Yeast transformations were performed by the method of Ito *et al.* (1983). Tetrad dissection and sporulation were carried out according to Sherman *et al.* (1986).

Media

All bacterial media were made as described by Miller (1972). All yeast media except presporulation media were made according to Sherman *et al.* (1986) . Presporulation media were 1% potassium acid phthalate, 1% potassium acetate, 0.17% yeast nitrogen base without amino acids and without ammonium sulfate, 0.5% ammonium sulfate and concentrations of amino acids are as listed under the synthetic complete medium of Sherman *et al.* (1986).

Materials

1-β-D-Arabinofuranosyl adenine (AraA) and 1-β-D-arabinofuranosyl thymine (AraT) and phosphonoacetic acid (PAA) were purchased from Sigma. Acyclovir (ACV) was kindly provided by Burroughs Wellcome Research Laboratories, Research Triangle Park, NC. Ganciclovir (DHPG) was supplied by Syntex.

RESULTS

Construction of Strain 167-pol1-17

McIntosh *et al.* (1986) had found that 15 mM araCMP but not araC (cytosine arabinoside) inhibits *tup*$^-$ strains (thymidylate permeable) of *S. cerevisiae*. By analyzing the uptake and metabolism of araC in *tup*$^-$ strain, they determined that cells are permeable to araC, but that the compound is quickly degraded to inactive products by deamination and breakage of the glycosidic bond. Thus, araC is not phosphorylated

in vivo to araCTP and is unable to block DNA replication by competitive inhibition of DNA polymerases. They suggested that *S. cerevisiae* lacks not only thymidine kinase but all deoxynucleoside kinase activity. To screen for DNA polymerase I mutants resistant to nucleotide or pyrophosphate analogs, we require a strain that is permeable to these analogs and in the case of nucleoside analogs, is able to phosphorylate them to diphosphates so that yeast cells can utilize their intrinsic nucleoside diphosphate kinases to complete the phosphorylation to triphosphates state and to exert their inhibitory effects. Our investigation started off with construction of a strain that is $pol1_{ts}$ and is able to utilize exogenous thymidine (pol_{ts} *tmp1-6 tut1-2*: pJM81 *TK*$^+$). Our strategy is to introduce the *in vitro* mutagenized DNA polymerase I gene into this constructed strain and to screen for mutants that are resistant to nucleotide and pyrophosphate analogs, provided that the strain is permeable to these analogs. The DNA polymerase I expressed by the chromosomal copy of the gene is temperature-sensitive and would be inactivated at the restrictive temperature of $pol1_{ts}$ alleles, making the DNA polymerase I encoded on the mutagenized plasmid the only functional Polymerase I at 37°C.

We mated strains 167(**a**) and 488-pol1-17(α), because *pol1-17* is the tightest $pol1_{ts}$ allele isolated. *pol1-17* exhibited no residual chromosomal DNA synthesis at 36°C, the restrictive temperature (Budd and Campbell, 1987; Budd *et al.*, 1989). The mating was done at 23°C on a solid medium lacking leucine to ensure that the pJM81 TK$^+$ plasmid was maintained in the diploid. The *tmp1-6* allele is recessive and would be complemented by the wild-type copy of *TMP1* supplied by 488-pol1-17(α). The diploid would have a functional thymidylate synthase, obviating the need for thymidine kinase encoded on the pJM81 TK$^+$ plasmid. The mixture of diploid and haploid cells was then streaked onto medium lacking both uracil and leucine and incubated at 30°C to isolate the diploid. The dipolid would have genotype *Mata/Matα ura1/URA1 URA3/ura3 leu2/leu2 tmp1-6/TMP1 tut1-2/Tut* :pJM81 *LEU2 TK*$^+$. The diploid colony was then patched onto a presporulation medium lacking leucine, and incubation

continued at 30°C. The patches were then replica-plated onto sporulation medium lacking leucine and incubated at 30°C to induce sporulation. We dissected tetrads resulting from the cross, and isolated a strain that possesses the following genotype *Mata leu2 trp1 ura3 can1 tmp1-6 tut1-2 pol1-17* : pJM81 *LEU2 TK+*. Strain 167-pol1-17 exhibited a temperature-sensitive phenotype at 37°C and required TdR for cell growth.

We then screened 167-pol1-17 for sensitivity to an array of antiviral drugs that have proven useful in isolating HSV DNA polymerases with mutations potentially located in dNTP/PP_i binding domain(s). Acyclovir is a guanine derivative with an acyclic side chain, 2-hydroxyethoxymethyl, at position 9. ACV is converted to a monophosphate by the viral thymidine kinase and subsequently to diphosphate and triphosphate. HSV DNA polymerase is 10-30 times more susceptible to inhibition by ACV triphosphates than the cellular HeLa DNA polymerase (Elion *et al.*, 1977). The dosage required for 50% inhibition (ED_{50}) of the HSV DNA pol is 0.1 μM. Ganciclovir (DHPG) is another guanine derivative but with a slightly different side chain than ACV (Ashton *et al.*, 1982). DHPG is more soluble than ACV and is also more efficiently converted to the triphosphate form active in inhibiting DNA polymerase *in vivo*. The ED_{50} of DHPG is in the nM range, 30-60 times more effective than ACV. AraA and AraT are analogs of deoxyadenosine and deoxythymidine, respectively (Coen *et al.*, 1982), and PAA is an analog of PP_i. They are known inhibitors of HSV DNA polymerase, and the ED_{50} of AraA, AraT and PAA are in the 10-20 μM range, much less potent that ACV or DHPG. We screened strain 167-pol1-17(**a**) for sensitivity to these drugs in a range of 1-50 mM, basing our concentrations on McIntosh *et al.* (1986) who observed that at 15 mM AraCMP, cell division was blocked almost immediately and cell viability decreased rapidly.

The initial drug-sensitivity screening was done by pipetting a 100 μl aliquot of a drug at the concentration indicated in Table 1 into a cylindrical well situated in the center

of a plate covered by a lawn of cells, 2-4x10^7/plate. The drug diffuses into the agar forming a radial concentration gradient. The plates were then incubated overnight at 23°C, the permissive temperature of strain 167-pol1-17. Upon incubation, crystals formed in the wells of plates containing ACV, DHPG and AraA, but not with AraT or PAA. ACV, DHPG, AraA and AraT were dissolved in DMSO because of limited solubility in water. Inability of ACV, DHPG and AraA to diffuse through the agar medium probably led them to precipitate out after the DMSO was totally absorbed by the media. Table 1 showed the result of the screening. AraT strongly inhibited strain 167-pol1-17 probably due to the efficient phosphorylation of AraT to AraTMP by thymidine kinase. Both AraA and PAA exerted small but notable inhibitory effect on cell growth. Since AraA precipitated in the well, it could affect only cells that were close to the well, thereby only giving a small area of inhibition. As a control, we tested the parent strains 167(**a**) and 488-pol1-17(α) for their sensitivities to these drugs. As expected, 167(**a**) with the *tut1-2* mutation demonstrated a similar inhibition pattern but 488-pol1-17(a) was totally inert to the drugs because of impermeability. Because of the insolubility of ACV, DHPG and AraA when plated on agar plates, we were unable to use them for mutant screening. We decided to proceed with AraT and PAA ,as they are soluble when plated and exhibit a high degree of inhibition of 167-pol1-17. The well test was useful to determine an approximate concentration of drugs needed for inhibitory activity. We next fine-tuned the concentration by plating 2-4x10^7 cells on plates containing different concentrations of drugs and determined the number of viable colonies after overnight incubation at 23°C. A lawn of cells appeared on plates with 30 mM PAA or 0.75 mM AraT, but only a few colonies survived the treatment of 40 mM PAA or 1 mM AraT. These results provided the basis of our conditions for screening drug-resistant mutants of DNA polymerase I in 167-pol1-17: 40 mM PAA for the pyrophosphate analog and 1 mM AraT for the nucleoside analog.

Screening AraT- or PAA-Resistant Mutants in 167-pol1-17

The plasmid to be mutagenized, pBM150-264, contains a functional *POL1* gene and three other markers, *CEN4*, *ARS1* and *URA3*. The plasmid was treated with hydroxylamine as described in Materials and Methods. The frequency of transformation of 167-pol1-17 with mutagenized and unmutagenized pBM150-264 was ~200 transformants per μg of DNA. The transformation was repeated four times and a total of 2000 URA$^+$ LEU$^+$ transformants were obtained. The URA$^+$ LEU$^+$ transformants were kept on medium lacking both uracil and leucine, to ensure that both the mutagenized plasmid pBM150-264 and pJM81 *LEU2 TK*$^+$ were within the cells. These URA$^+$ LEU$^+$ transformants were then individually streaked on a medium lacking uracil and leucine and were left at 23°C. These transformants were then replica-plated and incubated at 37°C. Transformants that survived at 37°C should be due to a functional DNA polymerase I encoded on the plasmid, as the chromosomal *pol1* allele is temperature-sensitive. 387 transformants were found to survive at 37°C and were replica-plated onto a medium lacking uracil and leucine but with 1 mM AraT or 40 mM PAA. 154 mutants demonstrated resistance to 1 mM AraT at 37°C and surprisingly, none were resistant to 40 mM PAA. Since transformation is a highly recombinogenic and mutagenic process itself, we tested these 154 transformants for their thymidine requirements. If thymidine were no longer required for the strain viability, it might also mean that the strain was impermeable to exogenously supplied nucleosides. Thus, AraT resistance of these strains would mean only loss of permeability but not resistance conferred by a mutant DNA polymerase I. The assay of thymidine requirement is to replica-plate cells on both YPD media and on YPD media supplemented with 100 μg/ml TdR, and to assess growth rates of strain on these plates. TdR requiring strains grow faster on TdR plates than YPD plates and also maintain their grande (ρ^+)phenotype. 96 transformants were found to retain their thymidine requirement. The high frequency of strain 167-pol1-17 to turn petite (ρ^-) was another aspect we were concerned about. 96

transformants were replica-plated onto medium containing a non-fermentable carbon source, glycerol, to determine whether they were ρ^+ or ρ^-. We found that only 37 transformants retained their grande phenotype up to this step, a much lower number than expected based on Sclafani and Fangman (1986). These 37 potential mutants are all grande, TdR-requiring and resistant to 1 mM AraT at 37°C.

Next we attempted to eliminate the *URA3* plasmid by patching the mutants on medium containing 0.1% 5-fluoro-orotic acid. Cells with mutations at the *URA3* locus are able to grow on 5-FOA medium, while *URA3*$^+$ cells are unable to withstand the toxicity of the compound 5-fluoro-UMP, the product of 5-FOA through OMP decarboxylase encoded by *URA3* (Boeke *et al.*, 1984). After the 5-FOA treatment, cells should not contain the *URA3* plasmid, presumably also encoding the drug-resistant polymerase I, and thus the cells should not be resistant to AraT anymore. AraT-sensitive strains would indicate that mutations conferring AraT resistance reside on the plasmid that had been evicted by the 5-FOA treatment. Of the 34 potential mutants, 13 demonstrated AraT sensitivity after 5-FOA treatment, and they were subjected to further investigations. We isolated the plasmids from 6 of the mutant strains and then retransformed the plasmids into the original 167-pol1-17 strain to determine whether the plasmid is solely responsible for conferring resistance to AraT. To our disappointment, none of the plasmids from these strains were able to confer resistance when reintroduced into strain 167-pol1-17. We also tried to transform the plasmids back into the strains they were isolated from but were rid of the plasmid by treatment with 5-FOA. In one case, the cells so transformed regained resistance to AraT. In the other cases, this retransformation was not performed because the strain had turned petite after the 5-FOA treatment.

Construction of 167-pol1-1i and Screening for Drug Resistance

Our unsuccessful attempt to isolate drug resistant DNA polymerase I by the above strategy had led us to try out another possibility. *pol1-11* to *pol1-17* ts mutations of

DNA polymerase I isolated by Budd and Campbell (1987) have recently been mapped by marker rescue to regions conserved between yeast DNA pol I, human DNA pol α and other phage and viral polymerases (Budd *et al.*, 1989; Wong *et al.*, 1988). *pol1-11*, *pol1-13*, *pol1-14* and *pol1-15* were mapped to region II, while *pol1-17* was located in region III or I. Since regions II and III are postulated to constitute the dNTP/PP_i binding site(s), these ts DNA polymerases may harbor altered sensitivities to nucleotide or PP_i analogs. To determine whether these temperature-sensitive DNA polymerase I possess any resistance to AraT or PAA, we needed to construct strains that were both permeable to the drugs and contained the $pol1_{ts}$ alleles. The construction of strains were as described for 167-pol1-17 except that the only phenotypes we needed were 1) temperature sensitivity at 37°C and 2) requirement for exogenous thymidine. We isolated strains 167-pol1-11, 167-pol1-12, 167-pol1-13, 167-pol1-14 and 167-pol1-15 and tested their sensitivities to AraT and PAA compared to the parental strain 167(a) and found no difference.

DISCUSSION

The failure to isolate any DNA polymerase I mutants that are resistant to nucleotide or pyrophosphate analogs has prompted us to reconsider our strategy. The major fault of the strategy is the failure to take into account a second essential DNA polymerase other than Pol I. When this research started, the existence of yeast DNA pol III was unknown. Even after yeast DNA pol III was isolated, it was difficult to prove that DNA pol III was not a modified form of Pol I. For example, the levels of Pol I and Pol III appeared inversely proportional to each other in wild-type and protease-deficient cell types, and Pol III was extremely sensitive to proteolysis which is part of its elusiveness (Bauer *et al.*, 1988). It was not until recently that Sitney *et al.* (1989) established that yeast DNA pol III is distinct from DNA pol I, based on the following observations: 1)

DNA pol III was present in normal amounts and stable at 37°C in a *pol1-17* mutant. 2) The cloned *CDC2* gene of *S. cerevisiae* has been sequenced and found to have homology to DNA polymerases when compared to both yeast DNA pol I and human DNA pol α (Boulet and Faye, pers. commun; Wong *et al.*, 1988). Fractionation of extracts of wild-type and *cdc2-1* mutant cells of *S. cerevisiae* showed that *cdc2-1* lacks DNA pol III activity, suggesting that *CDC2* encodes DNA polymerase III. *cdc2* of *S. cerevisiae* arrests at the restrictive temperature with complete, short spindles in the nuclei situated at the mother-bud neck. While some DNA is made at 36°C (non-permissive temperature), cells remain sensitive to hydroxyurea when returned to 23°C, suggesting that the DNA synthesized is defective or incomplete (Pringle and Hartwell, 1981). The temperature-sensitive *cdc2* mutants are able to initiate DNA synthesis at restrictive tempearture but fail to complete replication (Conrad and Newlon, 1983). We have previously studied *in vitro* DNA replication using yeast cells permeabilized with Brij in the presence of sucrose. This *in vitro* system can continue propagation of replication forks initiated before the Brij-sucrose treatment but cannot initiate new rounds of replication. DNA synthesis in the Brij-sucrose treated *cdc2* mutants ts 328, ts 346 and ts 370 is normal at 23°C but is completely defective at 37°C, consistent with a defect in elongation in *cdc2* mutants (Kuo *et al.*, 1983). Thus, *S. cerevisiae* requires two polymerases, DNA polymerase I and pol III, for chromosomal DNA replication. The failure to acquire any Pol I mutants resistant to dNTP/PP_i analogs is not surprising then. Even if Pol I were mutated and acquired resistance to analogs, it would not be feasible to screen and isolate this mutant because the cell harboring this altered Pol I also contains a wild-type Pol III, which is sensitive to the analogs. The retardation of growth can be due to only one essential DNA polymerase's being inhibited, as is evident in temperature-sensitive mutants of *POL1* and *CDC2*. The temperature sensitive mutants of Pol I isolated by Budd and Campbell (1987) have been mapped to regions II and III, the same regions that contain drug-resistant mutants of HSV DNA

polymerase. As described in the Results section, these $polI_{ts}$ alleles have been introduced into the thymidine permeable strain, but they are not resistant to dNTP/PP_i analogs tested in accordance with the two essential polymerases theory. These temperature-sensitive mutant polymerases have not been tested for drug resistance *in vitro*, because the mutant polymerases are unstable to lysis even when cells are grown at 23°C (M. Budd, K. Sitney and J. Campbell, unpublished results).

Although our attempts to isolate DNA polymerase I resistant to AraT and PAA were unsuccessful, there is still a possibility that differential inhibitors that are nucleotide analogs can be used to screen for such mutants. Different types of polymerases exhibit differential sensitivities towards certain nucleotide analogs, e.g., BuPdGTP, BuPdATP and aphidicolin. DNA pol I is 100 times more sensitive to BuPdGTP and BuPdATP than Pol II and Pol III are *in vitro*. AraCTP was shown to inhibit 50% of the activity of Pol I, Pol II and Pol γ at 25, 100, 50 μM, respectively (Wintersberger, 1974). Aphidicolin, which has long been regarded as a competitor of dCTP, affects polymerases differently compared to AraCTP, an analog of dCTP. Thus, every nucleotide analog inhibits various polymerases at different levels. Maybe the analogs we used, AraT and PAA, were unable to exert this kind of differential sensitivity towards Pol I and Pol III. If Pol I is more sensitive to a certain analog than Pol II or Pol III, we may be able to use an intermediate concentration to screen for nucleotide analog-resistant mutants of Pol I without completely inhibiting the polymerase activities of Pol II and Pol III.

The perplexing result of having AraT resistant mutations that are not plasmid-related is probably due to an increase in spontaneous mutation frequency caused by disturbance in pyrimidine nucleotide pool size. Kunz *et al.* (1980) observed that thymidylate deprivation in yeast induced genetic recombination. This condition can be reversed by provision of deoxythymidine monophosphate (dTMP) to *tup*$^-$ strain. In a later investigation, they found that low concentration of dTMP prompted

recombination, but high concentrations of dTMP increased mutation frequencies (Barclay and Little, 1981). They postulated that by greatly increasing the dTTP:dCTP ratio in the pyrimidine pool size, the fidelity of DNA synthesis decreases with erroneous insertion of thymine residues instead of cytosine, causing GC to AT transitions. An alternate explanation to an increase of mutation frequency that is due to dTMP is that thymidine nucleotides or metabolic derivatives of such nucleotides can affect the DNA replication complex allosterically and thus can decrease the fidelity of DNA synthesis. Thymidine triphosphate (dTTP) occupies a unique position in the biosynthetic pathway of the dNTPs (see Fig. 4). It appears initially as dTMP in the cells, while other deoxyribonucleotides are found at the diphosphate level from reduction of the corresponding ribonucleoside diphosphate by the enzyme ribonucleotide reductase. *De novo* synthesis of dTTP involves the production of dCDP or dUTP, which are all pyrimidine dNTPs potentially available for DNA synthesis. dTTP is also the only terminal pyrimidine dNTP: dCTP can be deaminated to dUTP and dUTP hydrolyzed to dUMP, which is ultimately converted to dTTP. dNTP biosynthesis is coupled to the folate metabolism: N^5, N^{10}-methylene tetrahydrolfolate serves as a methyl donor in the conversion of dUMP to dTMP by thymidylate synthase. This reaction significantly depletes the intracellular tetrahydrofolate pools and thus also subjects the synthesis of dTMP to inhibition by sulfanilamide and methotrexate, drugs that affect folate metabolism. Therefore, the regulation of intracellular concentration of dTTP and maintainence of pyrimidine nucleotide pool size are central in the accuracy of DNA replication.

Further evidence that fluctuation of intracellular concentrations of dTTP affects DNA replication fidelity came from studies of thymidylate analogs. Ross *et al.* (1987) observed that *tmp1* cells can also incorporate analogs of thymidylate, e.g., 5-bromodeoxyuridylate (BrdUMP), which when incorporated into *tmp1* yeast cells is both lethal and mutagenic. The lethality and mutation frequency can be drastically

altered by perturbation of the pyrimidine nucleotide pools: Thymidylate enhances mutagenesis, while deoxycytidylate suppresses it. They found that 500 μM of dTMP induced no toxic or mutagenic effects in the *tmp1-6, tup*⁻ strain. However, 200 μM dTMP in combination with 100 μM of BrdUMP increases the mutation frequency from $34.5/10^6$ with BrdUMP alone to that of $88.3/10^6$, whereas 1 mM dCMP in combination with BrdUMP decreases the frequency to $12.3/10^6$. Our method of using 500 μM TdR to supplement the *tmp1-6, tut1-2* strain to maintain low ρ⁻ frequency and 1 mM AraT to screen for resistant mutants undoubtedly contributed to the imbalance of pyrimidine pool size in the cells. This induction of high mutation frequency led to rapid changes of genotype of the strains. High frequency of mutation was observed when URA⁺ LEU⁺ AraT^r transformants were screened for their TdR requirement: Only 60% retained their TdR requirement. The few transformants that showed AraT resitance related to plasmids may have mutations in both the plasmids and the strains. As described in the results, one of the strains actually regained AraT resistance when transformed with the mutagenized plasmid. Perhaps the combination of mutations on the plasmid and the chromosome interact with each other and contribute to the AraT resistance demonstrated.

Since screening for analog resistant Pol I *in vivo* is infeasible as other polymerase(s) is/are still sensitive to these dNTPs and PP_i analogs, we propose construction of mutant polymerases by site-directed mutagensis and testing their drug resistance *in vitro*. The potential regions responsible for dNTP/PP_i binding site(s) are known to us (Wong *et al.*, 1988), and the HSV DNA polymerase mutations can serve as a guide as to which amino acid to mutate. We would attempt to isolate the mutant DNA polymerases and it is hoped, the polymerases are stable to biochemical manipulations such as lysis and column chromatography, unlike the ts mutations of Pol I. Experiments are already under way to clone a functional *POL1* gene under the *GAL* control of pBM150. An *Xba*I-*Bam*HI fragment of *POL1* was selected because it

contains the entire *POL1* gene with most of the upstream promoter sequence removed. The cloning is complete and we are now testing the overproduction of DNA polymerase I upon galactose induction. The postulated regions of DNA polymerase I responsible for dNTP/PP_i interactions were also cloned into M13mp18 within a *Sac*I-*Xba*I fragment. *In vitro* site-directed mutagenesis can be performed in this phage and the mutated DNA segment reintroduced into the pBM150-*Sac*I-*Xba*I *POL1* plasmid for expression. We intend to test resistance of isolated mutant DNA polymerase I to ACV triphosphate, BuPdATP, BuPdGTP, AraTTP and PAA, using the *in vitro* DNA replication assay (Johnson *et al.*, 1985). The site-directed mutagenesis study will enable us to confirm amino acid residues involved in dNTP or PP_i binding sites, further characterizing DNA polymerase I of *Saccharomyces cerevisiae*. Furthermore, it will lead to a better comprehension of the structure and the mechanism by which polymerase α acts in higher eukaryotes.

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Table 1. Growth Inhibition of *tmp1-6 tut1-2 pol$_{ts}$*: pJM81 *TK*$^+$ Strain by Various Nucleotide/PP$_i$ Analogs.

	Diameter of Inhibition (cm)*				
Strain	500mM ACV	500mM DHPG	250mM AraA	250mM AraT	1M PAA
167(**a**)	0	0	1.5	4.0	1.5
488-pol1-17(α)	0	0	0	0	0
167-pol1-17(**a**)	0	0	2.5	4.0	2.4

* Diameter of inhibition includes the diameter of well in the center which measures 0.6 cm.

Figure 1. Conserved Regions of Yeast DNA Polymerase I, Human DNA Polymerase α and Other Selected Polymerases. Identical residues between polymerases in five or more sequences are boxed. Gaps are represented by dashes, and extensive gaps are marked by the number of amino acids deleted. The conserved regions are marked by dashed lines under the amino acid residues. The six conserved regions are in the same linear spatial arrangement, IV-II-VI-III-I-V, in each polymerase. Figure from Wang *et al.*, 1989.

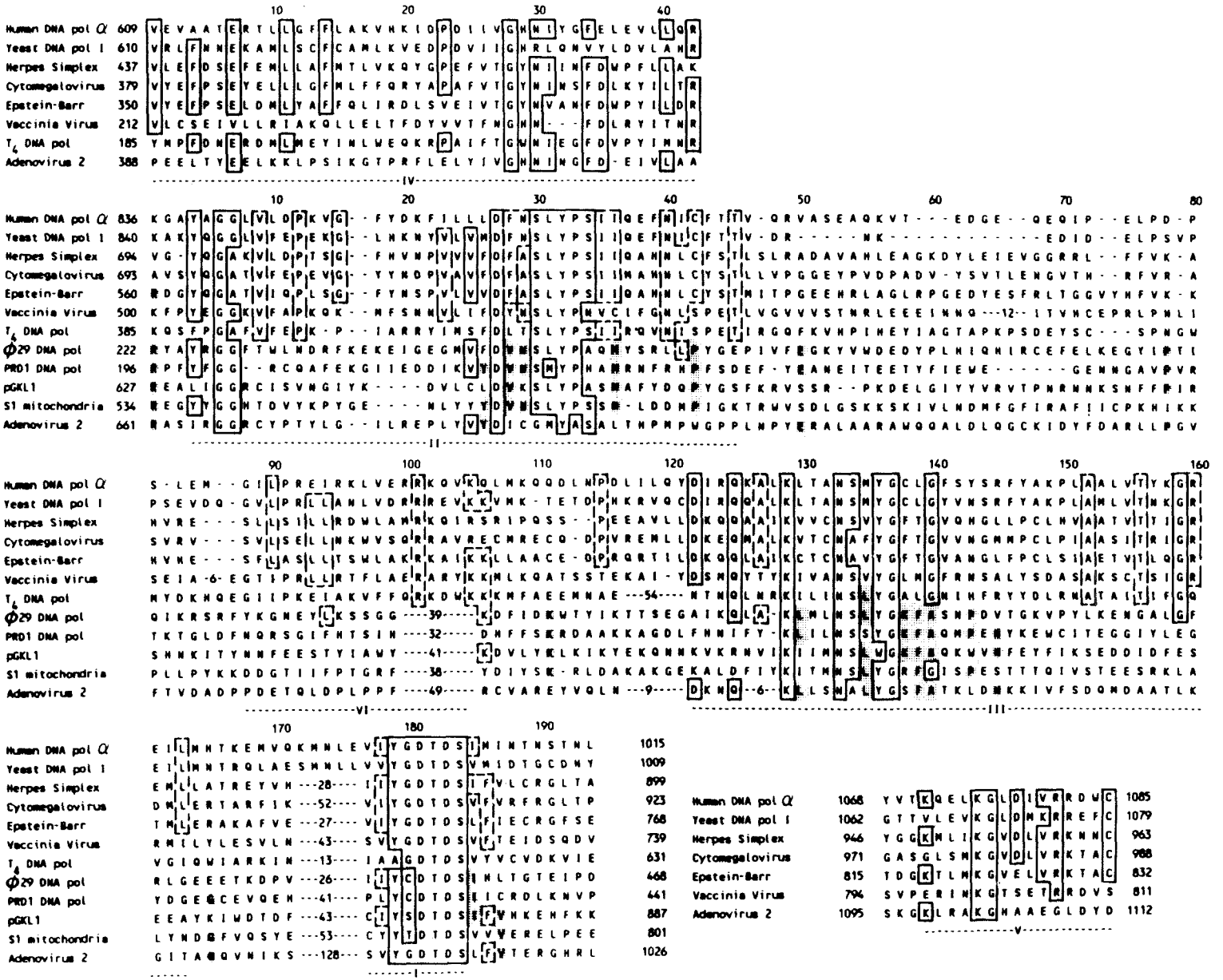

Human DNA pol α
Yeast DNA pol I
Herpes Simplex
Cytomegalovirus
Epstein-Barr
Vaccinia Virus
T4 DNA pol
Adenovirus 2
ϕ29 DNA pol
PRD1 DNA pol
pGKL1
S1 mitochondria
I
II
III
IV
V
VI

Figure 2. Primary Protein Sequence Comparison of Human DNA Polymerase α and Yeast DNA Polymerase I. Identical amino acid residues are boxed and the six conserved regions are marked by dashed lines under the amino acid residues. Glycine$_{493}$ of yeast DNA polymerase I, marked in a stippled box, is the site of a yeast *pol1$_{ts}$* mutation with altered stability of DNA primase association. Figure from Wang *et al.*, 1989.

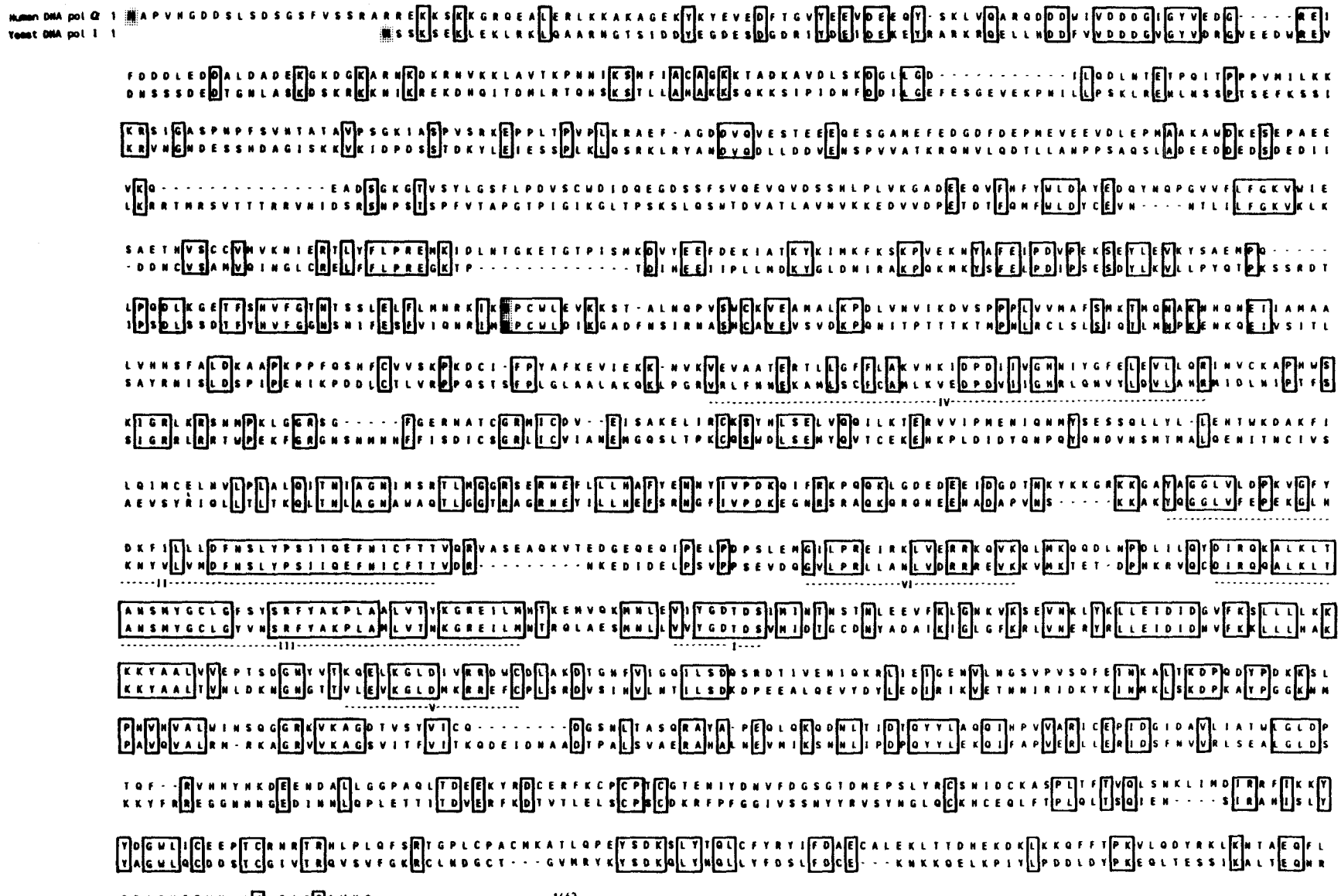

Human DNA pol α 1
Yeast DNA pol I 1
1462
1468

Figure 3. Locations of HSV DNA Polymerase Mutations that Alter Drug Sensitivity. Amino acids identical to that found in HSV are indicated with a colon. The conserved regions are marked by dashed lines above the amino acid residues. The amino acid changes, name of the mutations and the phenotypes conferred by mutations are listed. Figure from Gibbs *et al.*, 1988.

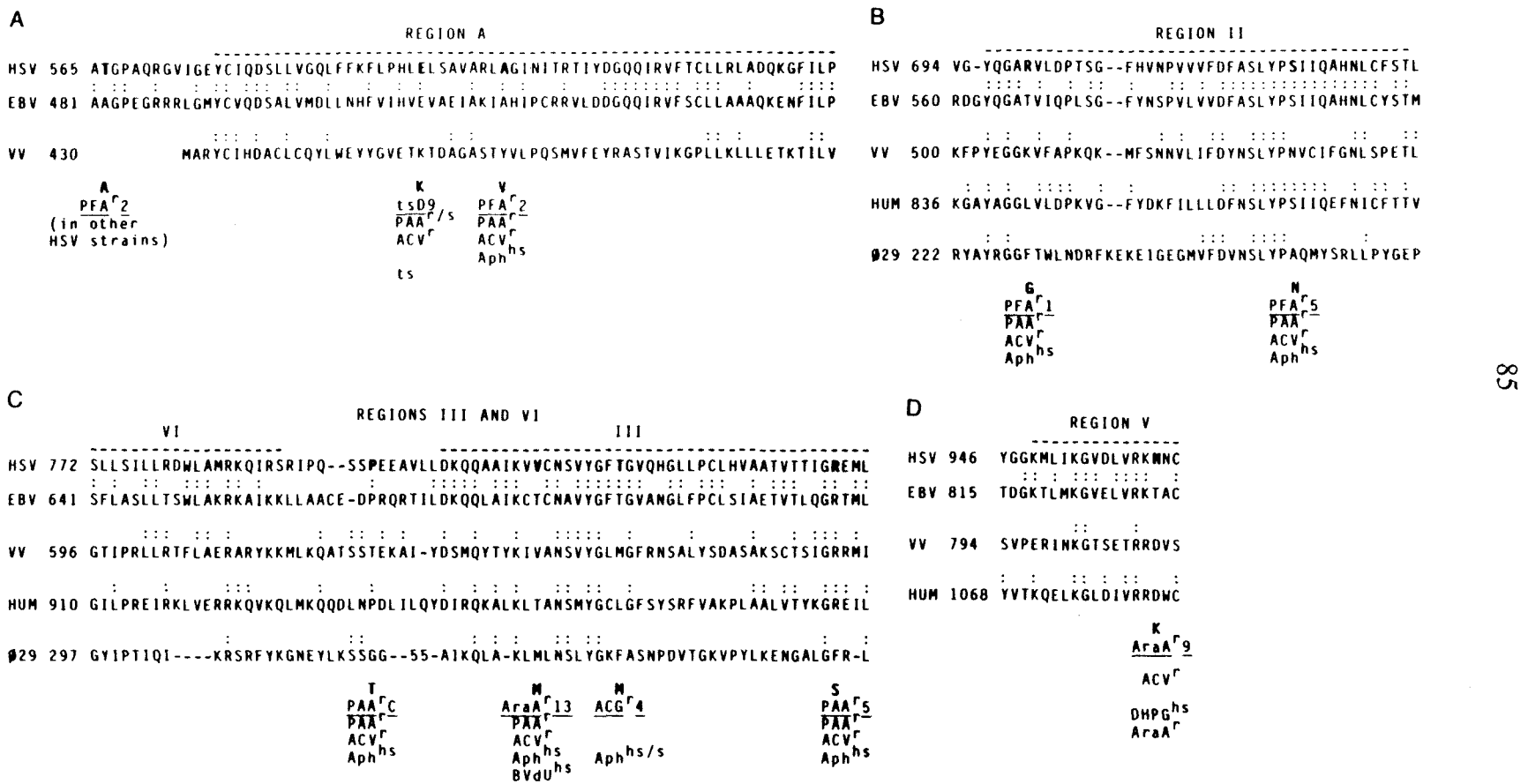
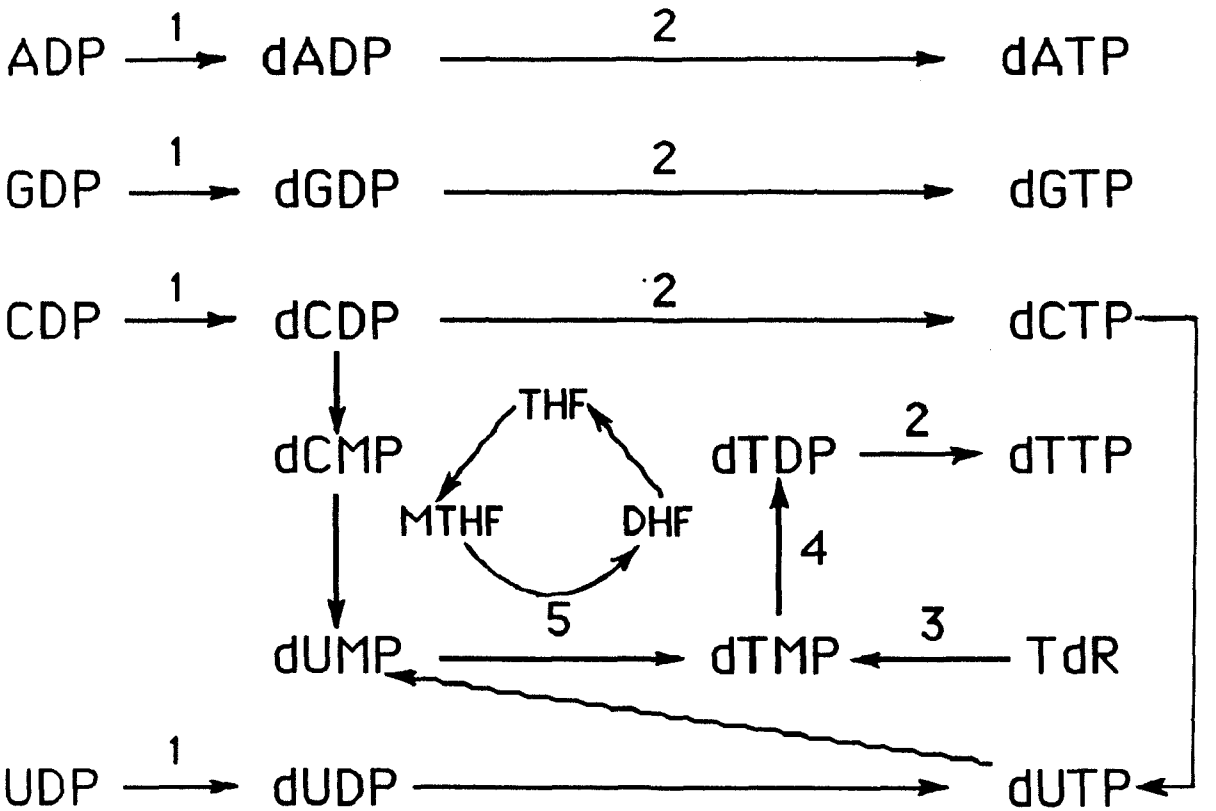

A
REGION A
HSV 565 ATGPAQRGVIGEYCIQDSLLVGQLFFKFLPHLELSAVARLAGINITRTIYDGQQIRVFTCLLRLADQKGFILP
EBV 481 AAGPEGRRRLGMYCVQDSALVMDLLNHFVIHVEVAEIAKIAHIPCRRVLDDGQQIRVFSCLLAAAQKENFILP
VV 430 MARYCIHDACLCQYLWEYYGVETKTDAGASTYVLPQSMVFEYRASTVIKGPLLKLLLETKTILV
A
PFAr2
(in other
HSV strains)
K
tsD9
PAAr/s
ACVr
ts
V
PFAr2
PAAr
ACVr
Aphhs
B
REGION II
HSV 694 VG-YQGARVLDPTSG--FHVNPVVVFDFASLYPSIIQAHNLCFSTL
EBV 560 RDGYQGATVIQPLSG--FYNSPVLVVDFASLYPSIIQAHNLCYSTM
VV 500 KFPYEGGKVFAPKQK--MFSNNVLIFDYNSLYPNVCIFGNLSPETL
HUM 836 KGAYAGGLVLDPKVG--FYDKFILLLDFNSLYPSIIQEFNICFTTV
Ø29 222 RYAYRGGFTWLNDRFKEKEIGEGMVFDVNSLYPAQMYSRLLPYGEP
G
PFAr1
PAAr
ACVr
Aphhs
N
PFAr5
PAAr
ACVr
Aphhs
C
REGIONS III AND VI
VI
III
HSV 772 SLLSILLRDWLAMRKQIRSRIPQ--SSPEEAVLLDKQQAAIKVVCNSVYGFTGVQHGLLPCLHVAATVTTIGREML
EBV 641 SFLASLLTSWLAKRKAIKKLLAACE-DPRQRTILDKQQLAIKCTCNAVYGFTGVANGLFPCLSIAETVTLQGRTML
VV 596 GTIPRLLRTFLAERARYKKMLKQATSSTEKAI-YDSMQYTYKIVANSVYGLMGFRNSALYSDASAKSCTSIGRRMI
HUM 910 GILPREIRKLVERRKQVKQLMKQQDLNPDLILQYDIRQKALKLTANSMYGCLGFSYSRFVAKPLAALVTYKGREIL
Ø29 297 GYIPTIQI----KRSRFYKGNEYLKSSGG--55-AIKQLA-KLMLNSLYGKFASNPDVTGKVPYLKENGALGFR-L
T
PAArC
PAAr
ACVr
Aphhs
M
AraAr13
PAAr
ACVr
Aphhs
BVdUhs
M
ACGr4
Aphhs/s
S
PAAr5
PAAr
ACVr
Aphhs
D
REGION V
HSV 946 YGGKMLIKGVDLVRKMNC
EBV 815 TDGKTLMKGVELVRKTAC
VV 794 SVPERINKGTSETRRDVS
HUM 1068 YVTKQELKGLDIVRRDWC
K
AraAr9
ACVr
DHPGhs
AraAr

Figure 4. Biosynthetic Pathway of Nucleotides in Yeast. Numbers above the arrows symbolize the enzymes catalyzing the particular reactions in the biosynthetic pathway. 1) Ribonucleotide reductase; 2) Nucleoside diphosphate kinase; 3) Thymidine kinase (not intrinsic in yeast, provided by the plasmid pJM81, which contains the HSV *TK* gene); 4) Thymidylate kinase encoded by *CDC8*; 5) Thymidylate synthase encoded by *TMP1* or *CDC21*.


ADP
1
dADP
2
dATP
GDP
1
dGDP
2
dGTP
CDP
1
dCDP
2
dCTP
THF
dCMP
dTDP
2
dTTP
MTHF
DHF
4
5
dUMP
dTMP
3
TdR
UDP
1
dUDP
dUTP