ISOLATION OF THE MURINE INTERLEUKIN 2 GENE AND CHARACTERIZATION OF ITS REGULATORY ARCHITECTURE

Thesis by

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Abstract

Interleukin 2 (IL2) is the primary growth hormone used by mature T cells and this lymphokine plays an important role in the magnification of cell-mediated immune responses. Under normal circumstances its expression is limited to antigen-activated type 1 helper T cells (T_H1) and the ability to transcribe this gene is often regarded as evidence for commitment to this developmental lineage. There is, however, abundant evidence than many non-T_H1 T cells, under appropriate conditions, possess the ability to express this gene. Of paramount interest in the study of T-cell development is the mechanisms by which differentiating thymocytes are endowed with particular combinations of cell surface proteins and response repertoires. For example, why do most helper T cells express the CD4 differentiation antigen?

As a first step in understanding these developmental processes the gene encoding IL2 was isolated from a mouse genomic library by probing with a conspecific IL2 cDNA. The sequence of the 5' flanking region from +1 to -2800 was determined and compared to the previously reported human sequence. Extensive identity exists between +1 and -580 (86%) and sites previously shown to be crucial for the proper expression of the human gene are well conserved in both sequence location in the mouse counterpart.

Transient expression assays were used to evaluate the contribution of various genomic sequences to high-level gene expression mediated by a cloned IL2 promoter fragment. Differing lengths of 5' flanking DNA, all terminating in the 5' untranslated region, were linked to a reporter gene, bacterial chloramphenicol acetyltransferase (CAT) and enzyme activity was measured after introduction into IL2-producing cell lines. No CAT was ever detected without stimulation of the recipient cells. A cloned promoter fragment containing only 321 bp of upstream DNA was expressed well in both Jurkat and EL4.E1 cells. Addition of intragenic

or downstream DNA to these 5' IL2-CAT constructs showed that no obvious regulatory regions resided there. However, increasing the extent of 5' DNA from -321 to -2800 revealed several positive and negative regulatory elements. One negative region that was well characterized resided between -750 and -1000 and consisted almost exclusively of alternating purine and pyrimidines. There is no sequence resembling this in the human gene now, but there is evidence that there may have once been.

No region, when deleted, could relax either the stringent induction-dependence or cell-type specificity displayed by this promoter. Reagents that modulated endogenous IL2 expression, such as cAMP, cyclosporin A, and IL1, affected expression of the 5' IL2-CAT constructs also. For a given reagent, expression from all expressible constructs was suppressed or enhanced to the same extent. This suggests that these modulators affect IL2 expression through perturbation of a central inductive signal rather than by summation of the effects of discrete, independently regulated, negative and positive transcription factors.

TABLE OF CONTENTS

Introduction

The antigen-specific cellular and humoral effector functions of the mammalian immune system are carried out by cytotoxic T cells (CTL) and immunoglobulin (Ig)-secreting B cells, respectively. As a general rule, however, such cells do not reveal their ultimate effector phenotype in response to antigen alone. Thus it was appreciated as early as 1966 that the restoration of an antibody response against sheep red blood cells in lethally irradiated mice required reconstituting these animals with either spleen cells or a combination of bone marrow cells and thymocytes (Claman *et al.*, 1966). Reconstitution of these immunocompromised, irradiated animals with either bone marrow or thymus cells alone was ineffective. The subsequent use of major histocompatibility complex (MHC-disparate thymus and bone marrow cells in similar reconstitution experiments identified marrow-derived B cells as the source of Ig secretion, thereby implicating T cells in the provision of the necessary helper activity (Miller and Mitchell, 1968; Mitchell and Miller, 1968).

Later studies on the *in vitro* generation of CTL revealed the presence of two phenotypically distinct classes of T cells defined by reactivity to antisera specific for Lyt1 or Lyt2/3. Cytotoxic T cells were shown to reside mostly in the Lyt2/3$^+$, Lyt(low)-class, but the efficient expansion of these cells in response to alloantigen also required the presence of a Lyt1(high), Lyt2/3$^-$ helper T cell (T_H) (reviewed in Nabholz and MacDonald, 1983). (The changes in immunological nomenclature have more than kept pace with the increase in knowledge. Suffice it to say that CTL are mostly in the CD8$^+$ subset and T_H cells in the CD4$^+$ subclass. While CD8 is identical to Lyt2/3, CD4 defines a protein distinct from that detected by anti-Lyt1 antisera.)

The discovery of soluble T-cell growth factor activity in the conditioned medium of phytohemagglutinin (PHA)-stimulated bone marrow cells (Morgan *et al.*, 1976) was followed by the demonstration that this activity [subsequently

shown to reside in a single protein and renamed interleukin 2 (IL2)] could be used to maintain cultures of antigen-specific CTL for months, even in the absence of repeated antigenic challenge (Gillis and Smith, 1977). In addition to proving that soluble helper-derived factors provided more than just immunologically uninteresting nutritive support, the availability of IL2-dependent cell lines provided a convenient and sensitive means by which to assay for its activity (Cantrell and Smith, 1984). Utilizing such lines also facilitated the cloning of the IL2 gene. Taniguchi *et al.* (1983) size-fractionated poly(A)$^+$ RNA from lectin-stimulated Jurkat cells (an IL2-producing human leukemia cell line), translated aliquots from each fraction in *X. Laevis* oocytes, and screened supernatants for IL2 activity by assaying on CTLL-2, one of Kendall Smith's original IL2-dependent cell lines. They then made a selected cDNA library from the RNA fraction that contained the IL2 message and screened it by hybrid selection of Jurkat poly(A)$^+$ RNA and oocyte translation. By such a labor-intensive method they were able to isolate a full-length human IL2 cDNA and, subsequently, by using it as a probe in a genomic library screen, they isolated the IL2 gene (Fujita *et al.*, 1983). This group also isolated the mouse IL2 gene in the same manner, starting instead with RNA from a mouse IL2-producing cell line (Fuse *et al.*, 1984).

The murine interleukin 2 gene is single-copy and encodes a protein of 169 amino acids, including a 20 amino acid leader peptide, and the mature protein has a predicted molecular weight of 16 Kd. There are no N-linked glycosylation sites present but by analogy to the human protein, mouse IL2 is probably variably sialylated and O-glycosylated on threonine residue 3. The coding regions of the mouse and human IL2 genes are ~62% identical at the amino acid level if one excludes a stretch of $(Gln)_{12}$ not found in the human protein. There is greater homology between the two genes in the 5' upstream region than in the coding region, as discussed in Chapter 2.

The molecular cloning of the IL2 cDNA and gene meant that not only were hybridization probes available to detect expression of this gene, but also that one could begin to study the stringent regulatory mechanisms by which IL2 expression is controlled.

Functional Characteristics of Mature T Cells

Mature, peripheral T cells of the mouse can be subdivided into two main populations based on reciprocal expression of the differentiation antigens CD4 and CD8. As mentioned previously, T_H cells are predominantly $CD4^+$ whereas CTL are found largely in the $CD8^+$ subset. All T cells, regardless of class, express on their surface a highly polymorphic, clonally-restricted receptor for antigen. There are four T-cell receptor (TcR) chains: α, β, γ, and δ, which are assembled from multiple germline-encoded segments in a manner analogous to Ig rearrangement (for reviews see Davis and Bjorkman, 1988; Marrack and Kappler, 1987). Functional proteins are expressed on the cell surface as $\alpha\beta$ or $\gamma\delta$ heterodimers, in association with the non-polymorphic five-chain CD3 complex. The antigen receptors of T_H and CTL are of the $\alpha\beta$ type and, excepting the clonally unique antigen combining site, are essentially identical.

In the absence of any stimulation, circulating T lymphocytes are small, quiescent cells. Encounter with antigen is required to cause a transition from G_0 to G_1, but antigen alone cannot drive cells into S-phase. The antigen-induced G_0 to G_1 transition is accompanied by the synthesis and secretion of IL2 by T_H cells and by the expression of high-affinity surface receptors for IL2 (IL2R) by both T_H and CTL. Binding and internalization of this secreted IL2 is the G_1 "progression factor" that ultimately induces DNA replication and mitosis. Although an efficient immune response requires the amplification of reactive cells, cell division is not required for the induction of cytolytic activity or lymphokine secretion

(MacDonald and Lees, 1980; Nau *et al.*, 1988).

In addition to differences in effector function, cells of the $CD4^+$ and $CD8^+$ classes also differ in the context in which they recognize antigen. Unlike immunoglobulin molecules, TcR heterodimers do not bind intact, soluble antigen. Rather, the immunogenic entity recognized by T cells consists of processed, nominal antigen complexed with self-MHC molecules on the surface of an auxiallary antigen presenting cell (APC). This limitation in their recognition capabilities ensures that they will not be distracted engaging free antigen, say a virus, to the exclusion of, in this example, virally-infected cells.

$CD4^+$ T cells are restricted to recognizing antigen in a complex with class II MHC molecules while $CD8^+$ cells are restricted by class I molecules. At present we do not know the molecular mechanisms by which the combination of MHC restriction specificity, CD4/8 expression, and effector function is endowed to a particular T cell. This will be considered further in the section on intrathymic T-cell development.

This problem of coordinated gene regulation of T-cell development is made more complex by two additional considerations. The generalization cited above that T_H cells are $CD4^+$ is merely a reflection of their proportionally greater representation in this class than in the $CD8^+$ pool. However, neither $CD8^+$, class I-restricted helpers, nor $CD4^+$, class II-restricted killers are so rare that they cannot be detected and cloned with relative ease (Singer *et al.*, 1987; Morrison *et al.*, 1986). Thus, the problem posed above can be narrowed to consider the more mechanistically-complicated question of how cells that utilize the *same* restricting elements and express the *same* differentiation antigens come to express *different* batteries of response genes.

Another complication regarding the division of labor among T cells arose from the observation of Mosmann *et al.* (1986) that the T_H pool is heterogeneous.

Cloned type 1 helper T cells (T_H1) assist T cell-mediated responses such as cytotoxicity and delayed-type hypersensitivity. These cells secrete IL2, interleukin 3 (IL3), immune interferon (IFN-γ) and lymphotoxin. In contrast, type 2 helper T cells (T_H2) are involved in the regulation of humoral immunity by virtue of their production of the necessary B-cell growth and differentiation factors, IL4 and IL5. These T_H2 cells also make IL3 but not IL2 or IFN-γ (Cherwinski *et al.*, 1987). Obviously, the same questions that exist regarding the divergent development of T_H and CTL can also be asked about T_H1 and T_H2 cells.

Signaling Pathways for IL2 Expression

In their natural environment T_H1 cells are triggered to express IL2 by the combination of antigen plus self-MHC, but this is not usually the most convenient way to study such an event. Aside from problems of accessibility, the study of cell activation *in vivo* is complicated by the low frequency of clones reactive toward a given antigen, the complex composition of cell types of the peripheral lymphoid tissue and the impossibility of knowing with certainty which cells are responsible for an observed effect. It is also difficult to distinguish direct effects from indirect effects. For these reasons the availability of cloned cell lines has been a boon to investigators. In general these lines fall into one of three categories: 1) malignantly transformed cells that can proliferate without any stimulation, 2) "normal" antigen-dependent cell lines that require periodic restimulation with antigen and APC, and 3) factor-dependent lines that constitutively express receptors for some growth-promoting hormone such as IL2 or IL4. It is also possible, and in practice, useful to compare results obtained from the study of cell lines to those gotten from primary T-cell populations such as thymus, spleen or lymph node. The use of primary T cells has the effect of averaging out over the entire population any response being studied and it avoids

the uncertainty inherent in the use of cells artificially frozen in some particular developmental state.

The results of numerous studies on the control of IL2 gene expression have shown that IL2 is transcribed only by T cells that have been activated by antigen or an appropriate surrogate (see below). Unstimulated cells do not transcribe the gene at a detectable rate, nor do they contain detectable transcripts. This result holds not only for primary T cells, but for cell lines as well. However, once triggered, primary T_H1 cells begin to transcribe the IL2 gene within four to six hours and continue for several days (Krönke *et al.*, 1985). It should be noted that these data were obtained from nuclear run-on experiments utilizing human peripheral blood lymphocytes. Somewhat faster kinetics have been reported for mitogen activated mouse spleen T cells (Swoboda *et al.*, 1987). Individual cells may show a more transient expression profile than is seen with an asynchronous population. Results with established cell lines generally show faster and sharper kinetics of response. For example, it is possible to detect IL2 mRNA in Jurkat cells within 45 min of stimulation and maximum steady-levels are achieved by 5 h (Shaw *et al.*, 1988). These results probably reflect the use of a homogeneous population of cells which, being malignantly transformed, are also likely to display more relaxed control of IL2 gene expression.

T-cell triggering by antigen + MHC can be mimicked by ligating the TcR with mitogenic lectins such as concanavalin A (Con A) and PHA or with immobilized anti-TcR antibodies. Perturbation of the TcR with any of these reagents initiates a cascade of events beginning with the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C, liberating the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) (Weiss *et al.*, 1986). This pathway is common to receptor-mediated signal transduction systems in a variety of cell types (reviewed in Berridge, 1987), and

results in activation of protein kinase C (PKC) and an increase in intracellular calcium. Agonists of this PIP_2 pathway are also capable of inducing IL2 expression (Truneh *et al.*, 1985). Exposure of T cells to the tumor-promoting phorbol ester 12-0-tetradecanoylphorbol 13-acetate (TPA) and the calcium ionophore A23187 can activate cells that have lost their TcR and, thus, are incapable of being triggered by more conventional means (Schmitt-Verhulst *et al.*, 1987). This combination of TPA + A23187 also allows one to assay for activation-specific gene expression in immature cells that have not yet expressed a TcR.

Although TcR ligation and phorbol ester/calcium ionophore are thought to work in similar ways, there are likely to be differences in the ultimate signal that each delivers to the cell. We have demonstrated that ~56% of $CD8^+$ splenic T cells can be induced to express IL2 with TPA + A23187 and that this combination is much more effective than TPA + Con A or TPA + anti-CD3 antibody (McGuire *et al.*, 1988). These results are important because they imply that there exist as yet unknown differences between the TcR-mediated signal transduction pathways of $CD4^+$ and $CD8^+$ cells. Thus, the failure of most $CD8^+$ T cells to express IL2 may result not from an intrinsic inability to transcribe the gene but, rather, from a PIP_2 breakdown signal that is subthreshold for IL2 expression.

There is evidence supporting this view from the work of Singer *et al.* (1987) on the activation requirements of $CD8^+$ T_H cells. By using purified populations of primary T cells from appropriate combinations of inbred congenic and mutant mice these investigators were able to compare the response profile (measured by IL2 production) of $CD8^+$ and $CD4^+$ T_H1 cells to defined antigens (for experimental details see Singer *et al.*, 1987). Their results were intriguing. $CD4^+$ T_H1 cells were found to respond to a plethora of antigens, including foreign class I and class II MHC molecules, foreign minor histocompatibility antigens, foreign Mls antigens, viruses and chemically-modified foreign and self MHC molecules. In striking

contrast, $CD8^+$ T_H1 cells responded only to foreign class I MHC. Since it is difficult to envision the evolutionary pressure that would maintain such an exclusive population of alloreactive cells, these authors conclude that the narrow response profile displayed by these cells is more apparent than real. To explain why these cells are unresponsive to most of the tested antigens it was proposed that $CD8^+$ cells have a higher threshold of activation than $CD4^+$ cells and, therefore, require seeing a higher concentration of antigen on the surface of an APC in order to betriggered. APC are continually processing and presenting antigenic fragments (Germain, 1986), including those from as many as 10,000 different endogenous proteins that preferentially associate with class I MHC molecules. Thus, it is likely that the concentration of any given antigen is going to be extremely low. In such a situation too few TcR on a $CD8^+$ cell would be engaged. However, in the case of allorecognition, the foreign class I molecule serves as both the restricting element *and* the antigen and is present in sufficient quantity to ensure triggering. This interpretation would explain why $CD8^+$ splenic T cells are efficiently activated by reagents, such as phorbol ester and ionophore, which bypass the TcR altogether. It is important to point out that this model, if true, implies that under normal circumstances $CD8^+$ T_H1 cells play little if any role in mediating cellular immunity, since allogeneic challenge is limited to cases of experimental manipulation in the lab and organ transplants. A possible naturally-occurring exception would be a viral infection in which large amounts of viral glycoproteins associated with class I moleules on the surface of the infected cell.

T-Cell Development and IL2 Expression in the Thymus

T cells arise from a bone marrow-derived precursor, the prothymocyte, which undergoes a program of expansion and selection within the thymus

(reviewed in Rothenberg and Lugo, 1985). Phenotypic characterization of post-natal thymocytes with antibodies against CD4 and CD8, combined with two-color flow cytometry, reveal four major populations: 1) $CD4^-8^-$ or double negative (DN) thymocytes; 2) $CD4^+8^+$ double positives; 3) and 4) $CD4^-8^-$ and $CD4^-8^+$ single positives. In general, single positives are precursors to mature peripheral T cells but have limited ability to reconstitute a depleted thymus. DN cells, on the other hand, have been shown by their ability to reconstitute an irradiated thymus, to contain the cells with the highest capacity for differentiation (Fowlkes *et al.*, 1985). DP thymocytes comprise 85% of all thymocytes and this pool has recently come under intense scrutiny as they are apparently the population on which selection, both positive and negative, works (Rothenberg, 1990). This pool also contains a poulation of large cycling blast cells and the majority of post-mitotic cells that are presumed destined to die.

The $CD4^+$ single positive cells contain a large fraction of the IL2 producers found within the thymus (Caplan and Rothenberg, 1984), which is not unexpected for cells on the verge of being exported to the periphery. These cells are competent to be triggered by the combination of TPA + Con A, indicative of functional TcR expression. However, these are not the sole IL2 producers found in the thymus.

As early as fetal d13, ~17% of resident thymocytes express IL2 mRNA constitutively as determined by *in situ* hybridization (Carding *et al.*, 1989). By d15 fetal, almost 90% of thymocytes are positive and 45% are positive for IL4. From d16 of gestation until birth, no IL2 mRNA is found. Since almost all d14 and d15 fetal thymocytes express mRNA for IL2R it is tempting to speculate that this combination of growth factor and receptor is driving proliferation. The probe used to detect IL2R in these experiments is specific for the low affinity form of the receptor thus the presence of the high affinity receptors cannot be implied.

These results are still important because they demonstrate that the IL2 locus is not repressed in these cells.

Post-natal DN thymocytes comprise ~5% of the total thymus and contain all of the $IL2R^+$ cells. However, most of these cells are not in cycle. The cycling DP blast population is mostly $IL2R^-$ suggesting that at this stage in an animal's life IL2 does not drive proliferation within the thymus (Lugo *et al.*, 1985).

The DN population represents a cell population that has not yet undergone selection. These cells initially rearrange their γ and δ TcR genes and may emigrate immediately, without acquiring CD4 and CD8. However, in general these cells undergo further rearrangement of their α and β loci and begin to express CD4 and CD8. What is important to note about the DN pool, however, is that it contains many cells capable of being triggered with TPA + A23187 to produce IL2 (Lugo *et al.*, 1988; McGuire and Rothenberg, 1987) and recent work in our lab has shown that these DN IL2 producers are composed of two distinct subsets (Rothenberg *et al.*, 1990). One subset is TcR^+ and expresses IL2 in response to TPA + A23187. It is also unresponsive to the macrophage-derived cytokine interleukin 1. Thus, these TcR^+ DN cells display a response profile identical to mature peripheral T cells.

The second DN subset is TcR^- and differs from mature T cells in that it does not respond to TPA + A23187 at all unless IL1 is present. Thus cells progress from a TcR^- stage, at which they display more stringent requirements for IL2 expression, to a TcR^+ stage at which time they now become unresponsive to this cytokine. In Chapter 3, I examine this question of IL1 responsiveness and consider some possible mechanisms through which it may operate.

Experimental Considerations

A T-cell's ability to express IL2 changes both during the ontogeny of the

animal and, in adults, during the ontogeny of individual T cells - from constitutive fetal expression at d13-15 gestation, to IL1-coinducible, and finally, IL1-independent. Paralleling this change in IL2 gene inducibility is the increase in regulatory restrictions being placed on a given T cell. DP cells whose TcR display an inherent affinity for class I MHC antigens lose expression of CD4 and, in general, become CTL. However, not all of these cells lose the ability to transcribe the IL2 gene (McGuire *et al.*, 1988). On the other hand, cells with TcR displaying affinity for class II tend to become $CD4^+$ T_H cells. What we would like to understand is how this decision to express one differentiation antigen or the other is coupled to adoption of a particular functional phenotype? At what level is this decision imposed, i.e., 1) is the IL2 gene rendered inaccessible in non-producers, or 2) do non-producers lack necessary transcription factors required to express the gene or finally, 3) does expression of CD4 vs. CD8 impose on the cell a signaling capability that ultimately predisposes it to one type of effector function or another? Much data from this lab and others suggest that the third mechanism is most likely. Thus, it suggests that any T cell is likely to be able to express IL2 upon stimulation with TPA + A23187. Data from numerous cell lines, however, suggest that this is not so. In general, cloned CTL do not express IL2 and cloned T_H1 cells do not express killer-specific genes. The data of McGuire ***et al.*** (1988) demonstrated that only about 50% of $CD8^+$ cells were capable of transcribing IL2 in response to phorbol ester and ionophore. Whether CTL cell lines originated from cells that had lost the ability to transcribe this gene, or from cells that never could, is unknown. If they arose from the former, it should be possible to determine if this acquired inability to express IL2 is the result of a repressed chromatin configuration near the transcription start site or rather, whether it is due to a loss of necessary transcription factors (models 1 and 2 from above). Introduction of hybrid gene constructs containing the mouse IL2 promoter linked

to a reporter gene function can resolve this issue. Results presented in Chapter 2 suggest that only T_H1 cells possess the necessary transcriptional apparatus to utilize the IL2 promoter even when present in the cell as a naked supercoiled template. Utilizing such IL2-CAT constructs it has been possible to identify the sequences responsible for high-level expression of this gene and, also, to study the effects of competing signaling pathways, both positive and negative. Only after we have determined at the molecular level what is required for expression can we begin to study the developmental process that combines selection with an assignment of function.

REFERENCES

Berridge, M. J. 1987. Inositol trisphosphate and diacylglycerol: Two interacting second messengers. Ann. Rev. Biochem. 56: 159-193.

Cantrell, D. A. and K. A. Smith. 1984. The interleukin-2 T-cell system: A new cell growth model. Science 224: 1312-1316.

Caplan, B. and E. Rothenberg. 1984. High-level secretion of interleukin 2 by a subset of proliferating thymic lymphoblasts. J. Immunol. 133: 1983-1991.

Carding, S. R., E. J. Jenkinson, R. Kinston, A. C. Hayday, K. Bottomly and J. J. T. Owen. 1989. Developmental control of lymphokine gene expression in fetal thymocytes during T-cell ontogeny. Proc. Natl. Acad. Sci. USA 86: 3342-3345.

Cherwinski, H. M., J. H. Schumacher, K. D. Brown and T. R. Mosmann. 1987. Two types of mouse helper T-cell clone. II. Further differences in lymphokine synthesis between T_H1 and T_H2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. J. Exp. Med. 166: 1229-1244.

Claman, H. N., E. A. Chaperon and R. F. Triplett. 1966. Thymus-marrow cell combinations. Synergism in antibody production. Proc. Soc. Exp. Biol. Med. 122: 1167-1171.

Davis, M. M. and P. J. Bjorkman. 1988. T-cell antigen receptor genes and T-cell recognition. Nature 334: 395-402.

Fowlkes, B. J., L. Edison, B. J. Mathieson and T. M. Chused. 1985. Early T lymphocytes: Differentiation *in vivo* of adult intrathymic precursor cells. J. Exp. Med. 162: 802-822.

Fujita, T., C. Takaoka, H. Matsui and T. Taniguchi. 1983. Structure of the human interleukin 2 gene. Proc. Natl. Acad. Sci. USA 80: 7437-7441.

Fuse, A., T. Jujita, H. Yasumitsu, N. Kashima, K. Hasegawa and T. Taniguchi. 1984. Organization and structure of the mouse interleukin-2 gene. Nucl. Acids Res. 12: 9323-9331.

German, R. N., 1986. The ins and outs of antigen processing and presentation. Nature 322: 687-689.

Gillis, S. and K. A. Smith. 1977. Long-term culture of tumor-specific cytotoxic T cells. Nature 268: 154-156.

Krönke, M., W. J. Leonard, J. M. Depper and W. C. Greene. 1985. Sequential expression of genes involved in human T-lymphocyte growth and differentiation. J. Exp. Med. 161: 1593-1598.

Lugo, J. P., S. N. Krishnan, R. D. Sailor, P. Koen, T. Malek and E. Rothenberg. 1985. Proliferation of thymic stem cells with and without receptors for interleukin 2: Implications for intrathymic antigen recognition. J. Exp. Med. 161: 1048-1062.

Lugo, J. P., S. N. Krishnan, R. D. Sailor and E. V. Rothenberg. 1986. Early precursor thymocytes can produce interleukin 2 upon stimulation with calcium ionophore and phorbol ester. Proc. Natl. Acad. Sci. USA 83: 1862-1866.

MacDonald, H. R. and R. K. Lees. 1980. Dissociation of differentiation and proliferation in the primary induction of cytolytic T lymphocytes by alloantigens. J. Immunol. 124: 1308-1313.

Marrack, P. and J. Kappler. 1987. The T-cell receptor. Science 238: 1073-1079.

McGuire, K. L. and E. V. Rothenberg. 1987. Inducibility of interleukin-2 RNA expression in individual mature and immature T lymphocytes. EMBO J. 6: 939-946.

McGuire, K. L., J. A. Yang and E. V. Rothenberg. 1988. Influence of activating stimulus on functional phenotype: Interleukin 2 mRNA accumulation differentially induced by ionophore and receptor ligands in subsets of mature T cells. Proc. Natl. Acad. Sci. USA 85: 6503-6507.

Miller, J. F. A. P. and G. F. Mitchell. 1968. Cell to cell interaction in the immune response. I. Hemolysis forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. J. Exp. Med. 128: 801-820.

Mitchell, G. F. and J. F. A. P. Miller. 1968. Cell to cell interaction in the immune response. II. The source of hemolysin forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. J. Exp. Med. 128: 821-837.

Morgan, D. A., F. W. Russetti and R. C. Gallo. 1976. Selective *in vitro* growth of T-lymphocytes from normal human bone marrows. Science 193: 1007-1008.

Morrison, L. A., A. E. Lukacher, V. L. Braciale, D. P. Fan and T. J. Braciale. 1986. Differences in antigen presentation to MHC class I- and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. J. Exp. Med. 163: 903-921.

Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Gieldin and R. L. Coffman. 1986. Two types of murine helper T-cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. 136: 2348-2357.

Nabholz, M. and H. R. MacDonald. 1983. Cytolytic T lymphocytes. Ann. Rev. Immunol. 1: 273-306.

Nau, G. J., D.-K. Kim and F. W. Fitch. 1988. Agents that mimic antigen receptor signaling inhibit proliferation of cloned murine T lymphocytes induced by IL2. J. Immunol. 141: 3557-3563.

Rothenberg, E. and J. P. Lugo. 1985. Differentiation and cell division in the mammalian thymus. Dev. Biol. 112: 1-17.

Rothenberg, E. V. 1990. Death and transfiguration of cortical thymocytes: A reconsideration. Immunol. Today, in press.

Schmitt-Verhulst, A.-M., A. Guimezanes, C. Boyer, M. Poenie, R. Tsien, M. Buferne, C. Hua and L. Leserman. 1987. Pleiotropic loss of activation pathways in a T-cell receptor α-chain deletion variant of a cytolytic T-cell clone. Nature 325: 628-631.

Scollay, R., A. Wilson, A. D'Amico, K. Kelly, M. Egerton, M. Pearse, L. Wu and K. Shortman 1988. Developmental status and reconstitution potential of subpopulations of murine thymocytes. Immunol. Rev. 104: 81-120.

Shaw, J.-P., P. J. Utz, D. B. Durand, J. J. Toole, E. A. Emmel and G. R. Crabtree. 1988. Identification of a putative regulator of early T-cell activtion genes. Science 241: 202-205.

Singer, A., T. I. Munitz, H. Golding, A. S. Rosenberg and T. Mizuochi. 1987. Recognition requirements for the activation, differentiation and function of T-helper cells specific for class I MHC alloantigens. Immunol. Rev. 98: 143-170.

Swoboda, R., E. Wecker and A. Schimpl. 1987. Regulation of IL2 expression in mitogen-activated murine T lymphocytes. Immunobiol. 174: 300-312.

Taniguchi, T., H. Matsui, T. Fujita, C. Takaoka, N. Kashima, R. Yoshimoto and J. Hamuro. 1983. Structure and expression of a cloned cDNA for human interleukin-2. Nature 302: 305-310.

Truneh, A., F. Albert, P. Golstein and A.-M. Schmitt-Verhulst. 1985. Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. Nature 313: 318-320.

Weiss, A., J. Imboden, K. Hardy, B. Manger, C. Terhorst and J. Stobo. 1986. The role of the T3/antigen receptor complex in T-cell activation. Ann. Rev. Immunol. 4:593-619.

CHAPTER 1

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Differential Transient and Long-Term Expression of DNA Sequences Introduced into T-Lymphocyte Lines

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ABSTRACT

We have used a protoplast fusion protocol to introduce the genes encoding neomycin phosphotransferase (neo) and chloramphenicol acetyltransferase (CAT) into murine and human T-lymphocyte lines. Plasmid constructs containing the *neo* gene under the control of the promoters from the Rous sarcoma virus long terminal repeat (RSV LTR), the SV40 early region, or the herpes simplex virus thymidine kinase gene (HSV TK) can stably transform each of three T-cell lines to G-418 resistance. The characteristic frequencies for different cell lines can differ by at least two orders of magnitude, although initial DNA uptake and transient expression are similar. In the two murine cell lines, low numbers of gene copies are retained in long-term transformants. Prior to integration, transient expression assays for *cat* or *neo* gene products reveal that the differences in intrinsic promoter strength of different constructs are further influenced by the coding sequences being transcribed. Thus, while transient expression of the neo protein is similar from both the Rous LTR and the SV40 early promoter, the Rous LTR directs synthesis of CAT protein at levels two orders of magnitude higher than those from the SV40 early promoter.

INTRODUCTION

THE ABILITY TO INTRODUCE CLONED GENES into tissue culture cells has provided both a means for manipulating cell behavior and a method for studying the sequence elements involved in the control of expression. Mammalian T lymphocytes should be particularly interesting subjects for gene transfer studies. They express a set of tissue-specific genes that are increasingly well defined, including several that are inducible at the RNA level when the cell is triggered by antigen recognition (Krönke *et al.*, 1985). Both the regulation of these genes and the participation of their products in the tightly regulated T-cell behavioral responses could be explored by gene transfer. In general, however, T cells are inefficiently transformed by exogenous DNA. Most results in the literature have been obtained with a single malignant T-lymphoma line, BW5147, which itself is not competent in any T-cell functional assay (Berman *et al.*, 1984). In this work, we report the factors controlling the efficient introduction and expression of exogenous genes in a variety of T-lymphocyte lines. We have quantitated the ability of different viral promoters to drive expression of two "recorder genes" in recipient T cells: the aminoglycoside 3′-phosphotransferase(II) [APH(3′)II or *neo*[1]] gene of Tn*5* (Colbère-Garapin *et al.*, 1981) and the chloramphenicol acetyltransferase (CAT) gene of Tn*9* (Gorman *et al.*, 1982a). In this sytem, the DNA copies that are taken up are expressed as well in T cells as in fibroblastic L cells. While the optimum transient expression levels per gene are similar, however, our results show marked variations among the T-cell lines in three other parameters affecting long-term transformation: initial DNA uptake, frequency of probable integration, and stability of integrated DNA copies.

[1]Throughout this paper, abbreviations for proteins are in capital letters (*e.g.*, CAT) and abbreviations for the DNA sequences that encode them are in lower-case italics (*e.g.*, *cat*).

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MATERIALS AND METHODS

Cell lines and culture conditions

The mouse T-cell lines, EL4.E1, a thymoma of helper lineage (Farrar *et al.*, 1980) and MTL2.8.2, an H-2^b anti-H-2^d cytotoxic line (Bleackley *et al.*, 1982), were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (HyClone Laboratories, Logan, UT), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, and antibiotics. Mouse interleukin-2 (IL2) produced by phorbol ester-stimulated EL4.E1 cells was added to the MTL2.8.2 growth medium at a final concentration of 30 U/ml. The human T-cell line MJ (Popovic *et al.*, 1983) was grown in the same medium but the 2-mercaptoethanol was omitted and 100 U/ml of human IL2 from stimulated Jurkat cells (Gillis and Watson, 1980) was substituted for the mouse variety. Mouse $Lapr1^-$ tk^- (La^-t^-) (Wigler *et al.*, 1979) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 50 μg/ml 2,6-diaminopurine, and antibiotics.

G-418 concentrations given in this paper refer to the concentration of Geneticin powder (Gibco) per milliliter of medium. The actual antibiotic concentration of this crude preparation varies between 450 and 480 μg/ml.

Plasmids

All of the plasmid constructs used in this study, except pTK-Neo, were provided by Drs. B. Howard and C. Gorman (NIH) and have been described previously (Gorman *et al.*, 1982a,b 1983; Southern and Berg, 1982; and Figs. 1 and 4). The plasmid pTK-Neo (pNeo3) was provided by Dr. B. Wold (Caltech). It consists of the neomycin phosphotransferase gene from transposon Tn*5* flanked 5′ and 3′ by the herpes simplex virus thymidine kinase (TK) gene promoter and downstream polyadenylation sequences, respectively.

Protoplast fusion

T cells were maintained in exponential growth for several days prior to transfection. Protoplasts were produced by the method of Sandri-Goldin *et al.* (1981) with some modifications. Two differences involved the bacterial host strain used (HB101 instead of DH-1) and the size of the amplified plasmid cultures which were converted into protoplasts (100 ml instead of 25 ml). Briefly, 1 day prior to fusion, 2 ml of a saturated overnight culture were inoculated into 100 ml of M9 minimal medium supplemented with 0.4% casamino acids and 1 mg/ml uridine. Bacteria were grown at 37°C to an OD_{590} 0.6–0.7, at which time 170 μg/ml chloramphenicol (for *neo* plasmids) or 300 μg/ml spectinomycin (for *cat* plasmids) was added. Amplification of the plasmid copy number was for 18 hr ± 1 hr. The next day each culture was chilled on ice and pelleted in two 50-ml conical test tubes at 2000 × *g* at 4°C. The two pellets of a given sample were each resuspended in 2.5 ml of 20% sucrose, 50 mM Tris pH 8.0 and pooled. Lysozyme, EDTA, and additional 50 mM Tris pH 8.0 were then added sequentially according to the protocol of Sandri-Goldin *et al.* (1981), with all volumes increased to compensate for the larger initial culture volume. After incubating the suspensions for 7.5 min in a 37°C water bath, the protoplasts were diluted slowly with 40 ml of 10% sucrose, 10 mM $MgCl_2$ in serum-free DMEM without mixing. If the incubation at 37°C was allowed to proceed for even 15 min, an unacceptable amount of lysis occurred during the final dilution step. When using this timetable, we reproducibly converted >90% of the bacteria to protoplasts as determined by phase-contrast microscopy.

Cells to be transfected were resuspended at 1–2 × 10^7 cells/ml in growth medium in 17 × 100-mm polypropylene, round-bottomed tubes (Falcon 2059). Five milliliters of clear protoplast suspension taken from the middle of the tube, was added for each $\sim 10^8$ cells. The tubes were capped, inverted gently, and then spun at room temperature in a tabletop centrifuge for 5 min at 500 × *g*. The medium was carefully aspirated and 3 ml of fusion reagent was added. The fusion reagent consisted of serum-free RPMI 1640 containing 45% fusion-grade polyethylene glycol (PEG 1000, Sigma) and 10% dimethyl sulfoxide. Preparation of fusion-grade PEG by the method of Yoakum (1984) allowed us to increase its concentration from 35% in initial experiments to 45%, while decreasing its toxicity to T cells. After the addition of fusion reagent, tubes were swirled gently at room temperature for 30–60 s. The PEG was then diluted with 10 ml of serum-free RPMI 1640 prewarmed to 37°C. Tubes were inverted to mix and then respun for 3 min at 500 × *g* at room temperature. Cells were resuspended in growth medium, counted, and plated as described below. Recovery of cells ranged from 25% to 100% and was usually greater than 75%. The recovered cells were always greater than 90% viable by eosin dye exclusion.

For long-term selection of stable transformants, newly transfected T cells were plated into the inner 60 wells of four 96-well, flat-bottomed tissue culture plates (Corning 25860), with tissue culture medium in the outer 36 wells. In all fusion experiments cells were plated for the first week in the presence of 200 μg/ml gentamicin (for *neo* selection) or kanamycin (for other experiments) to kill any unconverted bacteria. The adherent MTL2.8.2 cells were plated at 1 × 10^3 cells in 0.2 ml medium/well. Two or three days after transfection, and every 3–4 days thereafter, the medium was removed and replaced with an equal volume of fresh medium containing 1 mg/ml G-418. Control cells consisted of MTL2.8.2 cells that had been transfected with pBR322. Nonadherent EL4.E1 and MJ cells were initially plated in 0.15 ml of growth medium per well, then supplemented 2 or 3 days post-transfection with 50 μl of fresh medium containing 400 μg/ml G-418 (final drug concentration was 100 μg/ml). Subsequent feedings were done after first spinning the plates for 5 min at 500 × *g* in a Sorvall tabletop centrifuge (RT6000) equipped with microtiter plate carriers. Stable MJ transformants could be selected in the presence of 100 μg/ml G-418. EL4.E1 cells, however, often required a progressive increase in concentration over a period of several weeks to final concentrations of 400 μg/ml or 750 μg/ml before the mock-transfected cells died. The frequency of transformation of T cells was calculated from

the null term of the Poisson distribution after determining the percentage of wells which contained no transformed cells.

Mouse L a- t- cells were transfected in suspension as described for T cells and were plated at 2.5 × 10^5 cells/100-mm tissue culture dish. Cells were fed every 4 days with medium containing 400 μg/ml G-418. Transformed colonies were enumerated by staining the plates with hematoxylin and eosin.

Neomycin phosphotransferase assay

The method of Fregien and Davidson (1985) was used with minor modifications. Lysates containi. cellular proteins were separated on a 10% native acrylamide gel instead of a 7.5% gel. A 15 × 15 × 0.1-cm gel was incubated for 60 min in 100 ml of assay buffer which co. d 6 μCi [γ-^{32}P]ATP per milliliter of buffer. An aliquot from a lysate of an overnight culture of bacteria harboring pRSV-Neo was used as a positive control.

CAT assay

CAT assays were performed essentially as described by Gorman *et al.* (1982a) except that reaction time was extended to 2 hr. The assay was calibrated with commercial CAT enzyme (P-L Biochemicals) and all values given fell within the linear range of the assay (McMahon *et al.*, 1984). Molecules of the CAT subunit were estimated by determining the specific activity of a 75% pure preparation of CAT, as previously described (McMahon *et al.*, 1984).

DNA extractions

Stable transformants were grown in bulk culture, washed once in phosphate-buffered saline, and resuspended at 10^7–10^8 cells/ml in 10 m*M* Tris pH 7.5, 0.4 *M* NaCl, 2 m*M* EDTA. $NaDodSO_4$ was added to 0.2% and proteinase K to 100 μg/ml, and the mixture was put at 56°C for 4–5 hr. The DNA was extracted three times with phenol, twice with phenol/chloroform/isoamyl alcohol (50:48:2), and three times with chloroform/isoamyl alcohol (96:4). High-molecular-weight DNA was removed by spooling after addition of 2.5 ml of EtOH, dried under vacuum, and resuspended in 1/10 its original volume in 10 m*M* Tris pH 7.5, 1 m*M* EDTA (TE). RNase A was added to 100 μg/ml and the sample was digested for 3 hr at 37°C. The DNA was phenol/chloroform-extracted as above, then ethanol-precipitated three times, and stored in a small volume of TE pH 8.0. Approximately 12 μg of DNA (2 × 10^6 cell equivalents) was digested to completion with a fivefold excess of *Bam* HI or *Kpn* I. The digested DNA was separated on a 0.8% agarose gel, blotted to a nitrocellulose filter, and probed with nick-translated pSV2-Neo plasmid DNA. Known amounts of *Bam* HI-linearized pSV2-Neo served as copy number standards. Hybridizations were carried out for 16–24 hr at 68°C in 5× SSC, 5× Denhardt's, 0.1% $NaDodSO_4$, 10% dextran sulfate, and 100 μg/ml sheared, denatured salmon sperm DNA (1× SSC = 0.15 *M* NaCl, 0.015 *M* Na citrate pH 7.0). Filters were washed three times for 1 min each at room temperature in 2× SSC, 0.2% $NaDodSO_4$, 0.05% NaPPi, twice for 30 min each at 68°C in 0.2% SSC, 0.1% $NaDodSO_4$, 0.05% NaPPi, and then were exposed to Kodak XAR-5 film with a Cronex intensifying screen at −70°C. Gene copy number was determined by densitometric scanning of the resulting autoradiograms and comparison of the band intensity to that of known amounts of *neo* DNA.

For the determination of gene copy number in transient assays, the pelleted nuclei and cellular debris were resuspended in 0.5 ml of lysis buffer [140 m*M* NaCl, 1.5 m*M* $MgCl_2$, 10 m*M* Tris pH 8.6, and 0.5% NP-40] and layered over an equal volume of lysis buffer containing 24% sucrose and 0.5% NP-40 in a 1.5-ml Eppendorf tube. Nuclei were pelleted in a microfuge for 2 min at 4°C. They were resuspended in 0.1 ml of 10 m*M* Tris pH 7.8, 5 m*M* EDTA, 1% $NaDodSO_4$. Proteinase K was added to 200 μg/ml and samples were digested at 56°C for 3 hr after which the DNA was extracted as described above. RNase A was then added to 100 μg/ml and samples were put at 37°C for 2 hr. The DNA was then reextracted as before and ethanol-precipitated. The precipitated DNA was pelleted by spinning for 30 min in a microfuge in the cold and resuspended in TE pH 8.0. Amounts of DNA representing 1.5 × 10^5, 5 × 10^4, and 1.5 × 10^4 cell equivalents were spotted onto a nitrocellulose filter using a dot blot manifold (Schleicher & Schuell). *neo* or *cat* DNA representing 10^7, 10^6, 10^5, and 10^4 copies were mixed with nontransformed EL4.E1 DNA and spotted as standards. The filters were probed with a nick-translated 2.2-kb *Hind* III–*Bam* HI fragment containing the *neo* sequences or the corresponding 1.6-kb fragment from pSV2-CAT, and gene copy number per cell was determined densitometrically.

Data selection for transient expression assays

The data shown in the tables were taken from experiments in which multiple plasmids and/or cell lines were compared in parallel, and in which both DNA and enzyme activity measurements were available for each sample. These results were only cited if representative of those from three or more independent experiments. While absolute levels of expression varied among experiments, relative levels of expression within experiments fell into the ranges cited in the text in every case.

RESULTS

Stable transformation of T cells

These experiments were undertaken to develop a procedure by which specific genes could be introduced, stably integrated, and expressed in functionally differentiated T cells. To optimize conditions for transformation, the bacterial gene for neomycin/kanamycin resistance was introduced into various murine T-cell lines. MTL2.8.2 is a cytotoxic T-cell line that depends for its growth on the T-cell growth factor IL2. A second line, EL4.E1, is a subline of a thymoma which can be specifically induced to se-

crete IL2 by activation with phorbol esters. The *neo* gene, derived from the Tn5 transposon, encodes an aminoglycoside phosphotransferase which confers on eukaryotic cells the ability to grow in the presence of the antibiotic G-418 (Colbère-Garapin, 1981; Southern and Berg, 1982). Several constructs incorporating *neo* were compared for activity in T cells. These are diagrammed in Fig. 1 and described in the legend. The plasmids pRSV-Neo and pSV2-Neo are identical except for the promoter–enhancer region. Plasmid pTK-Neo, however, utilizes both the herpes simplex virus TK gene promoter and downstream flanking sequences which contain the polyadenylation site. It further differs in that its orientation with respect to the pBR322 vector sequences is opposite that of the other constructs and it lacks a downstream intron. Each of these constructs was previously shown to be active in mammalian cells (Fregien and Davidson, 1984; Gorman *et al.*, 1983; Southern and Berg, 1982).

We were reproducibly successful in transforming T cells using protoplast fusion. Protoplasts were prepared from *Escherichia coli* (strain HB101) harboring the recombinant plasmids, or pBR322 as a negative control, after amplification of the plasmids overnight. Selection was initiated 2 or 3 days after fusion, using concentrations of G-418 that had been determined previously for each cell line (see Materials and Methods). After 2–3 weeks of selection, wells of transformed G-418-resistant cells could be detected at a frequency of ~1 per 10^4 cells for MTL 2.8.2 cells and ~1 per 10^6 cells for EL4.E1 (Table 1). In no case did spontaneous G-418-resistant clones grow out from cultures transfected with pBR322. Once isolated, T-cell transformants grew progressively in G-418 through more than 30 generations, in clear contrast to the arrested growth and lysis of the parent lines. As shown in Table 1, the three *neo* constructs showed similar efficiencies in transforming T cells to G-418 resistance.

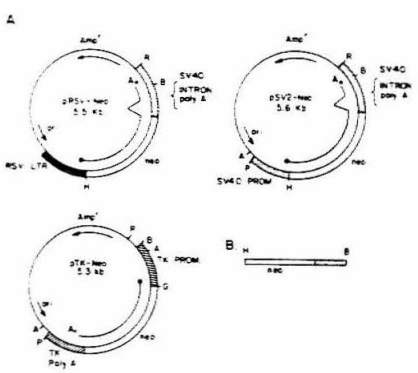


FIG. 1. Diagrammatic representation of the Neo plasmids used. A. These plasmids have been described previously (Fregien and Davidson, 1984; Gorman *et al.*, 1983; Southern and Berg, 1982). Thin lines, pBR322 vector sequences; open box, neomycin phosphotransferase gene (*neo*) from Tn5; filled box, the promoter-containing region of the LTR from RSV; hatched boxes, HSV TK gene promoter and polyadenylation sequences; stippled boxes, SV40 early region promoter and small t intron and polyadenylation site. Filled circles represent the mRNA cap site. Indicated restriction sites are: R, *Eco* RI; B, *Bam* HI; G, *Bgl* II; H, *Hind* III; P, *Pvu* II; A, *Acc* I. Only relevant sites are shown. B. The 2.2-kb *Hind* III–*Bam* HI fragment from pSV2-Neo used as a hybridization probe in some experiments.

The presence of *neo* DNA sequences in eleven G-418-resistant T-cell clones was verified by Southern blotting (Fig. 2 and data not shown). These transformants were expanded for 2-6 months in G-418 prior to DNA extraction. High-molecular-weight genomic DNA from these cells was digested with the endonuclease *Bam* HI (Fig. 2, lanes 1–8 and 14–17), which has one cleavage site in each plasmid, or *Kpn* I (Fig. 2, lanes 9–13), which does not cleave any of the original plasmids. After electrophoretic separation, the resulting DNA fragments were blotted to nitrocellulose and probed with pSV2-Neo DNA. The bands observed in *Kpn* I digests were uniformly larger than unit plasmid length, indicating that the *neo* sequences were incorporated into a high-molecular-weight duplex. Densitometric scanning of the autoradiograms and comparison to known amounts of *neo* DNA (*e.g.*, Fig. 2, lanes 6–8) showed that most of the transformants had integrated one or fewer copies per diploid cell of the *neo* gene. This result is in agreement with the single hybridizing fragment observed in each lane when the *neo*-specific probe shown in Fig. 1B was used instead of the entire pSV2-Neo plasmid (Fig. 2, lanes 9–13 and data not shown). The mobilities of these fragments after *Bam* HI digestion in most cases distinguished them from input plasmid DNA. The presence of two bands which hybridize to the labeled pSV2-Neo probe is consistent with a single integration event yielding two unique junction fragments upon cleavage with *Bam* HI (Fig. 2, lanes 2–4, 16). One transformant, EL4.E1-SV2.1 ("S.1," Fig. 2, lanes 4 and 12), presumably contains several copies in a tandem array, linked head to tail, as evidenced by the generation of unit plasmid-length 5-kb bands after *Bam* HI digestion. Whereas the EL4.E1 transformants examined here had one to three copies, MTL2.8.2-transformed cultures contained *neo* sequences at only 0.1–0.3 copies per diploid cell (Fig. 2, lanes 14–17). G-418 resistance was generally correlated with expression of the phosphotransferase encoded by the *neo* gene, APH(3')II, as shown in Fig. 3. Of six T-cell transformants, all containing approximately one copy of the *neo* gene per cell (Fig. 2, lanes 16 and 17 and data not shown), five showed virtually equivalent levels of phosphotransferase activity. Our selection procedure did allow the survival of one clone, MR5-A1 (Fig. 3, lane 8), which appears to contain a *neo* gene at a transcriptionally inactive site. Transient expression of the *neo* gene prior to its integration may have allowed the outgrowth of this variant with spontaneously elevated G-418 resistance.

The low copy number per transformant is noteworthy for several reasons. While the nominal ratio of protoplasts to cells is high (~$2.5–5 \times 10^3$:1), the number of plasmids actually transferred may be much lower, depending on the

TABLE 1. FREQUENCY OF STABLE TRANSFORMATION OF T CELLS BY PROTOPLAST FUSION

Cell line	Plasmid	Number positive wells/ total numbers wells plated[a]				Number of transformants/ total number cells	Transformation frequency[b]
		Exp #1	#2	#3	#4		
EL4.E1	pBR322	0/240	0/240			0/2.4 × 10^6	0
	pRSV-Neo	2/240	1/240			3/2.4 × 10^6	1.2 × 10^{-6}
	pSV2-Neo	3/106	0/240			3/1.7 × 10^6	1.7 × 10^{-6}
	pTK-Neo	1/195	0/240			1/2.2 × 10^6	4.6 × 10^{-7}
MTL 2.8.2	pBR322	0/240	0/240	0/240	0/240	0/9.6 × 10^5	0
	pRSV-Neo	30/240	39/240	33/240	24/240	126/9.6 × 10^5	1.4 × 10^{-4}
	pSV2-Neo	22/240	50/240	13/240	23/240	108/9.6 × 10^5	1.2 × 10^{-4}
	pTK-Neo	23/240	54/240	24/240	14/240	115/9.6 × 10^5	1.3 × 10^{-4}
MJ	pBR322	0/240				0/2.4 × 10^6	0
	pRSV-Neo	240/240				≥240/2.4 × 10^6	>1 × 10^{-4}
	pSV2-Neo	240/240				≥240/2.4 × 10^6	>1 × 10^{-4}
	pTK-Neo	240/240				≥240/2.4 × 10^6	>1 × 10^{-4}
La$^-$t$^-$	pBR322	0[c]	0	0		0/7.5 × 10^5	0
	pRSV-Neo	132	325	74		531/7.5 × 10^5	7 × 10^{-4}
	pSV2-Neo	108	605	130		843/7.5 × 10^5	1 × 10^{-3}
	pTK-Neo	25	23	10		58/7.5 × 10^5	8 × 10^{-5}

[a]EL4.E1 cells were plated at 5 × 10^3 cells/well; MTL 2.8.2 cells were plated at 1 × 10^3 cells/well. MJ cells were plated at 1 × 10^4 cells/well.

[b]Calculated from the Poisson distribution assuming single-hit kinetics.

[c]Values for La$^-$t$^-$ cells are number of colonies/2.5 × 10^5 cells.

distribution of protoplasts among the phases of the initial suspension. Evidence for a low actual ratio is presented in Table 2, suggesting that donor DNA is stabilized rather efficiently in T cells. Under these conditions, DNA concatenates appear to be generated infrequently as an intermediate in the integration process. These observations allow precise identification of the *neo* gene copy being expressed but could rule out the use of protoplast fusion to cotransfect unlinked genes. The presence of fewer than one copy of *neo* per cell in clonal lines of MTL2.8.2 cells suggests that exogenous sequences are unstable in some T-cell transformants. This interpretation is supported by the growth characteristics of several lines. While most MTL2.8.2 lines remain G-418-resistant throughout several months of culture in selective medium, one pSV2-Neo-transformed line (Fig. 2, lane 15) lost the ability to grow when split to low density (4 × 10^4 cells/ml), and eventually died. EL4.E1 transformants, on the other hand, appear to be stable.

Using these procedures, the human T-cell line MJ could be transformed to G-418 resistance at high frequency (Table 1). This line is T4$^+$, HTLV-1$^+$, and IL2 dependent (Popovic *et al.*, 1983). After 14 days of growth in the presence of 100 μg/ml G-418, all cells transfected with pBR322 were dead, while every cell containing cells transfected with pSV2-Neo, pRSV-Neo, or pTK-Neo showed vigorously growing foci of cells (Table 1). These cultures as a rule contained multiple copies of the *neo* DNA sequence per cell, with some cultures transformed by pRSV-Neo and pSV2-Neo yielding estimates of 30–50 copies of *neo* sequences per cell. The transformation frequency could not be determined more accurately because this line grows poorly below 5 × 10^3 cells per ml (J. Lugo, unpublished observations). Nevertheless, these human cells appear to be considerably more efficient than the two murine lines at taking up DNA, or stabilizing it, or both.

In marked contrast to the results with protoplast fusion, several other transfection techniques consistently failed to transform our T-cell lines. We have never detected functional DNA uptake in these T cells, either by transient expression assays (see below) or by selection of stable transformants, using three different calcium phosphate coprecipitation protocols (Wigler *et al.*, 1978; Graham and Van Der Eb, 1973; Chu and Sharp, 1981). The results were equally poor whether or not the cells were pulsed after transfection with dimethylsulfoxide or glycerol (data not shown). Transfection by polycation facilitation was also unsatisfactory (data not shown). Concentrations of DEAE-dextran and poly-L-ornithine which promote maximal transformation of fibroblasts (10–100 μg/ml) were acutely toxic to our T-cell lines, and lower doses were found to be ineffectual in promoting DNA uptake.

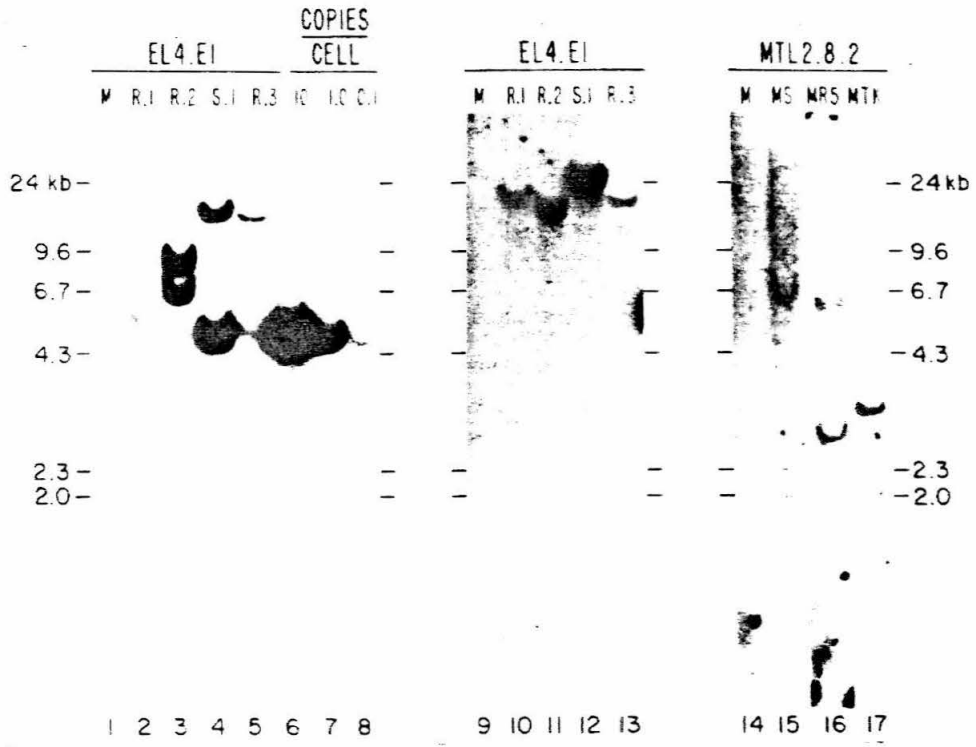


FIG. 2. Southern blot analysis of G-418-resistant murine T-cell lines. Twelve micrograms of genomic DNA was digested with *Bam* HI (lanes 1–8 and 14–17) or *Kpn* I (lanes 9–13), electrophoresed on a 0.8% agarose gel, and blotted onto nitrocellulose. Hybridization probes consisted of a nick-translated pSV2-Neo plasmid for the *Bam* HI-digested samples and the 2.2-kb *Hind* III–*Bam* HI fragment shown in Fig. 1B for the *Kpn* I-digested samples. Lines R.1, R.2, R.3, and MR5 were transformed with pRSV-Neo; lines S.1 and MS with pSV2-Neo and line MTK with pTK-Neo. Lanes 6–8 contain 12 μg of *Bam* HI-digested genomic DNA from nontransformed EL4.E1 cells mixed with *Bam* HI-linearized pSV2-Neo DNA equivalent to 10, 1.0, or 0.1 copies of *neo* per cell, respectively. Densitometry was carried out on exposures that were determined to fall in the linear range. MTL cells (lanes 14–17) carried approximately 0.1–0.3 copies per cell by visual inspection with standards. These lanes are from a longer exposure than lanes 1–8.

The promoter influence on gene expression may not be reflected in transformation frequency

The frequency of stable transformation has often been taken as a relative measure of promoter strength. While this has been verified in some cases (Gorman *et al.*, 1983; Spandidos and Wilkie, 1983; Sodroski *et al.*, 1984), it may not be generally true. For example, the effects of intrinsic promoter strength or site of integration in the host genome can be overlooked any time that the minimum amount of gene product expressed exceeds the amount required for survival under particular selective conditions. We have therefore used the assay for measuring APH(3')II activity (Fregien and Davidson, 1985) to monitor quantitative effects on *neo* expression independently of the ability to survive long-term challenge with G-418.

Mass cultures transfected by protoplast fusion were used to correlate enzyme production with gene copy number. Forty-eight hours after fusion, cytoplasmic extracts were assayed for phosphotransferase activity. DNA isolated from the same cells was assayed in parallel by dot blot hybridization to determine the number of *neo* DNA copies present per cell. To minimize error due to carryover of protoplasts, the DNA was extracted from nuclei that had been isolated by pelleting through sucrose. Results of two representative experiments are presented in Table 2. By this method, the majority of transfected cultures (29/35 in four experiments) were found to contain less than 20 copies of the *neo* gene per cell, on average, at 2 days post-transfection. In the occasional cases where large numbers of copies were found (~100/cell), APH(3')II activity was not correspondingly increased, suggesting that the additional DNA

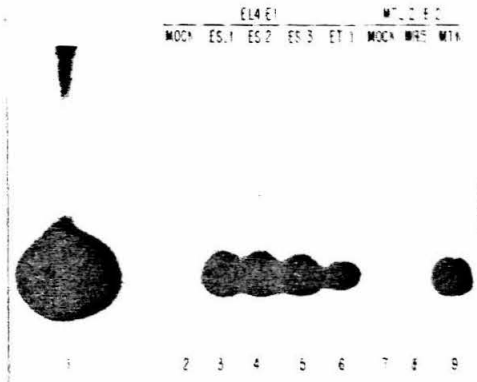


FIG. 3. Detection of APH(3')II (*neo* product) activity in stable transformants. Lysates of total cellular proteins from 1.5×10^6 EL4.E1 or 2.5×10^6 MTL2.8.2 transformants were electrophoresed on a 10% native polyacrylamide gel to separate endogenous ATPase activity from the phosphotransferase. The gel was then incubated with [γ-^{32}P]ATP and kanamycin as described in Materials and Methods. After 60 min incubation at 37°C, the labeled kanamycin was blotted onto phosphocellulose paper and visualized by autoradiography. The positive control (line 1) is an aliquot of a lysate from a saturated culture of HB101 harboring pRSV-Neo. All cell lines contained one copy of *neo* per cell (Fig. 1, lanes 16 and 17, and data not shown). Exposure time was for 2 hr with an intensifer screen.

was not functionally active (data not shown). Thus, the low copy number found in long-term transformants (Fig. 2) may represent only a 5- to 10-fold further loss of plasmid sequences after initial DNA uptake.

The results of these experiments show that the cell lines differ less in their transient expression of a given construct than in their frequencies of long-term transformation. Thus, transfected MTL 2.8.2 cells transiently express all three Neo plasmids to about the same level as EL4.E1 cells (Table 2 and data not shown), in contrast to the 100-fold difference between these cell lines in transformation efficiency (Table 1). The second experiment shown in Table 2 strongly suggests that the levels of APH(3')II activity detected 48 hr after fusion are in fact dependent on transcription of the plasmid templates in the recipient cells and not a reflection of carryover from the protoplasts themselves. In this experiment, aliquots of the same protoplasts used for fusion were harvested on the day of fusion and assayed in parallel with extracts made from the recipient cells 2 days later. The transfected cells in most cases showed an increase over the protoplasts in phosphotransferase activity relative to their content of plasmid DNA. Furthermore, the hierarchy of expression from the different constructs was slightly different in the cells than in the protoplasts. Whereas protoplasts carrying pSV2-Neo contained the highest levels of APH(3')II, the T cells preferentially accumulated phosphotransferase activity after fusion with pRSV-Neo or pTK-Neo. The same general result was also obtained when DNA alone was introduced into the cells by fusion to plasmid-bearing erythrocyte ghosts (data not shown). In two such experiments, the two T-cell lines each expressed similar levels of APH(3')II activity per gene, with the levels per gene expressed from the pRSV-Neo and pTK-Neo constructs about 4- to 10-fold higher than from pSV2-Neo. Thus, the transient assays indicate that the MTL 2.8.2 and EL4.E1 cell lines do not differ appreciably in their ability to express the *neo* gene from each of the three constructs. The cause of the difference in long-term transformation efficiency must lie elsewhere.

Promoter strength is influenced by intragenic sequences

The results from transient assays of APH(3')II activity suggest that the RSV LTR, the herpes TK promoter, and the SV40 early promoter are all about equally effective, with the SV40 construct possibly the weakest of the three in these murine cell lines. Because no pure standard of APH(3')II is available, these results are not easily interpreted in terms of amounts of enzyme synthesized per gene. Recently, we calibrated the assay for a different transient expression marker, CAT, to convert measured CAT activity levels into steady-state concentrations of CAT subunit per cell (McMahon *et al.*, 1984). Parallel experiments with *cat* in place of *neo* could therefore provide an absolute measurement of the efficiencies of expression from the TK, RSV, and SV40 early promoters in T cells. Protoplast fusion was used to transfect mouse T cells and La$^-$T$^-$ cells with each of the *cat* plasmids shown in Fig. 4A. Two days later the cells were lysed and both CAT enzyme activity and gene copy number were measured (Table 3 and Fig. 4C). The levels of expression, presented in Table 3, were similar for each construct whether assayed in the two T-cell lines or in La$^-$T$^-$ cells. Depending on the experiment, maximum levels of expression were from 10 to 100 molecules of CAT subunit per *cat* gene.

The hierarchy of expression from the different *cat* constructs was striking. While pRSV-CAT was highly efficient, pTK-CAT gave no detectable activity at any DNA dosage (data not shown). This result is not directly comparable with *neo* results because the pTK-Neo and pTK-CAT plasmids are not structurally homologous. However, in contrast to the results with transient expression of *neo* (Table 2), the RSV LTR is also at least two orders of magnitude better on a per gene basis than the SV40 early promoter in driving *cat* expression (Table 3, last column). In most cases no activity could be detected after transfection of pSV2-CAT. This is not likely to be due to organizational differences in the plasmids since the *cat* and *neo* derivatives of the SV40 and RSV promoter construct are identical except for the recorder genes. As shown in Fig. 5, the pSV2-CAT construct is not defective, since it is amplified and expressed efficiently when introduced into COS-7 monkey cells by either calcium phosphate precipitation or proto-

TABLE 2. TRANSIENT EXPRESSION OF THE *neo* GENE PRODUCT IN MURINE T-CELL LINES AND L CELLS

Cell line	*Plasmid*	*Relative APH(3')II activity/cell*[a]	*Neo copies/cell*	*Relative APH(3')II activity/gene*[b]
Experiment 1				
EL4.E1	pBR322	1.7[c]	[0]	–
	pRSV-Neo	[100][e]	13.3	7.4
MTL 2.8.2	pBR322	3.6[c]	[0]	–
	pRSV-Neo	88	3.8	22
La$^-$	pBR322	0.2[c]	[0]	–
	pRSV-Neo	36	3.1	12
Experiment 2				
EL4.E1	pRSV-Neo	7.2	0.12	60
	pSV2-Neo	11	0.18	61
	pTK-Neo	4.9	0.025	196
MTL 2.8.2	pRSV-Neo	97.5	1.1	89
	pSV2-Neo	37	1.5	25
	pTK-Neo	86	0.7	123
Protoplasts (HB101)[d]	pRSV-Neo	[100][e,f]	7.3	13.7
	pSV2-Neo	280	7.3	38
	pTK-Neo	12	4.7	2.5

[a]Based on densitometric tracing of ^{32}P-labeled-neomycin autoradiograms; arbitrary units.
[b][(Activity/cell) – (pBR322 activity/cell)]/(neo copies/cell)
[c]Background.
[d]T cells assayed 48 hr after fusion; protoplasts assayed on day of fusion.
[e]Normalization standard for the experiment.
[f]The amount of protoplast suspension assayed was the amount used for fusion with 3 × 10^6 cell equivalents.

plast fusion. Thus, the levels of expression from the SV40 early promoter relative to the RSV LTR are determined, in part, by sequences within the *neo* and *cat* genes.

As discussed with respect to Table 2, several considerations make it most probable that these differences in expression reflect the utilization of plasmid DNA templates by the recipient cell transcription machinery, or differential RNA utilization, and not carryover of protein in the protoplasts. When samples of the HB101 protoplasts used for fusion were assayed for CAT activity directly, pRSV-CAT, pTK-CAT, and pSV2-CAT protoplasts all contained similar amounts of CAT protein (Table 3). This was in sharp contrast to the differences between CAT expression levels found in mammalian cells 2 days after fusion with pRSV-CAT and pSV2-CAT, and the complete absence of CAT activity in every case where cells were fused with protoplasts carrying pTK-CAT (data not shown). There was no detectable bias in the efficiencies with which different protoplasts were taken up by recipient cells, because the numbers of plasmid DNA copies per recipient cell nucleus fell in the same range for all protoplasts. In most cases (Table 3), less than 10 copies of DNA were retained. Thus, the poor expression observed in cells fused with pSV2-CAT or pTK-CAT can be regarded as an upper limit for carryover of CAT protein in the fusion, further reinforcing the superior expression of pRSV-CAT. Finally, at least for the adherent MTL2.8.2 and La$^-$ cells, the presence of CAT activity was dependent on PEG-mediated fusion with the protoplasts. In the absence of PEG treatment, the number of copies of *cat* sequences and the amount of CAT activity were both sharply reduced (Table 4). PEG treatment was not required for optimal expression of CAT in EL4.E1 cells, but in these nonadherent cells the uptake of plasmid sequences into nuclear DNA was also PEG independent (Table 4). The EL4.E1 cells thus appear to be capable of spontaneous fusion with the protoplasts under the condition used for their culture. In every case, expression of cytoplasmic CAT activity was correlated with incorporation of plasmid sequences into cell nuclei. The poor expression of pSV2-CAT relative to pRSV-CAT, and the more similar expression of pSV2-Neo relative to pRSV-Neo, most likely indicate differential influences on the eukaryotic transcription machinery by the bacterial *neo* and *cat* sequences.

DISCUSSION

We report here conditions under which model genes are consistently introduced into T cells, integrated into the T-cell genome, and expressed at levels comparable to their expression in L cells. As discussed below, the system has certain limitations, but nevertheless provides a reliable

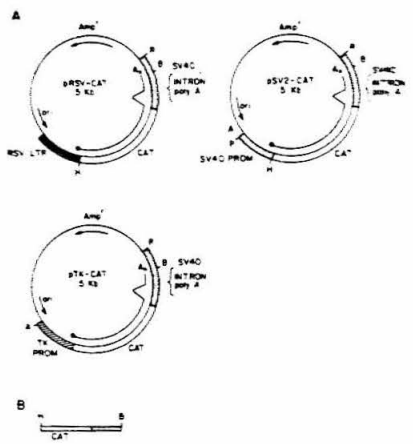
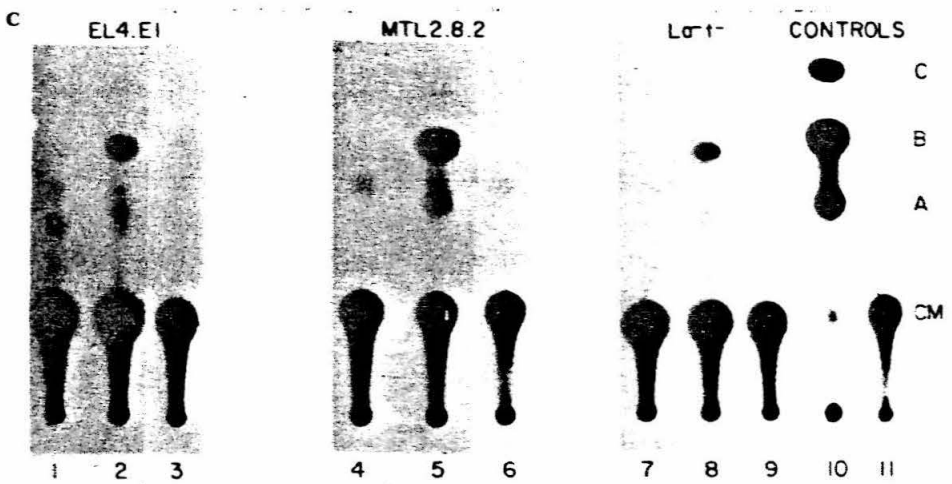


FIG. 4. Diagrammatic representation of CAT plasmids. A. The various plasmid segments and restriction sites are the same as in Fig. 1 except that the open boxes represent the *cat* gene of Tn*9*. B. The 1.6-kb *Hind* III-*Bam* HI CAT-specific probe from pSV2-CAT used for the determination of gene copy number by dot blot hybridizations. C. Assay of CAT activity in T cells and L cells transfected by protoplast fusion. The assay was performed as described by Gorman *et al.* with the modifications given in Materials and Methods. Each sample contained the lysate from 4 × 10^6 EL4.E1 and La$^-$t$^-$ cells or 6 × 10^6 MTL2.8.2 cells transfected with pBR322 (lanes 1, 4, 7), pRSV-CAT (lanes 2, 5, 8), or pSV2-CAT (lanes 3, 6, 9). The raw data is presented in Table 3, Exp. 1 for EL4 and L cells and Exp. 2 for MTL cells. The positive control (lane 10) contained 0.48 units of purified CAT enzyme (P-L Biochemicals); the negative control lacked any cellular lysate or CAT enzyme. CM, unreacted [^{14}C]chloramphenicol; B, 1-acetate chloramphenicol, the primary reaction product; A, 3-acetate chloramphenicol which results from the 1-acetate form by nonenzymatic acyl migration; C, 1,3-diacetate chloramphenicol.



means for manipulating the long-term characteristics of a variety of functional T-cell lines.

The most successful method we have used for transforming T cells is PEG-mediated fusion with protoplasts carrying the desired plasmid. This works reproducibly to import plasmid sequences into the lymphocytes in an expressible form, in distinct contrast to the rare expression and frequency toxicity observed when lymphocytes are treated with naked DNA in the presence of $Ca_3(PO_4)_2$ or polycations. In our hands thus far, IL2-dependent T cells have also been refractory to electroporation (T.J.N., Christine Kinnon, and John Ngai, unpublished). In accord with our results, Hatakeyama *et al.* (1985) and Steinmetz and coworkers (Dembic *et al.*, 1986) have also used protoplast fusion in their recent isolation of T-cell transformants. The difficulty of introducing naked DNA may be related to the presence of a Ca^{2+}-activated endonuclease in many T lymphocytes (Cohen and Duke, 1984). DNA in the protoplasts is likely to be doubly protected: first, by the bacterial membrane, and second, by bacterial proteins forming a nucleoprotein complex with the plasmid DNA. The first of these alone may be adequate, since our preliminary attempts to introduce DNA encapsulated in erythrocyte ghosts have also resulted in fairly high levels of expression (T.J.N., un-

TABLE 3. TRANSIENT EXPRESSION OF THE *cat* GENE PRODUCT IN TRANSFECTED T CELLS

Cell line	*Experiment*	*Plasmid*	*Number of cells*	*Percent acetylation*	*CAT activity (in units) (m units)*[a]	*Number of copies of* cat *gene per cell*[b]	*Number of molecules CAT subunit per cell*[c]	*Number of molecules CAT subunit per* cat *gene*
MTL 2.8.2	1	pBR322	7.5×10^6	<0.01	0	[0][d]	0	–
	1	pRSV-CAT	7.5×10^5	0.3	0.3	1.3	104	80
	2	pRSV-CAT	6×10^6	9.3	3.8	24	165	7
	2	pSV2-CAT	6×10^6	0.01	0.01	4.8	0.4	0.08
EL4.E1	1	pBR322	4×10^6	<0.01	0	[0][d]	0	–
	1	pRSV-CAT	4×10^6	1.3	0.85	0.5	55	110
	2	pSV2-CAT	4×10^6	0.04	0.04	1.3	2.6	2
	2	pRSV-CAT	2×10^6	8.5	3.2	8	424	53
	2	pSV2-CAT	1×10^7	0.01	0.01	2	0.3	0.2
La$^-$t$^-$	1	pBR322	4×10^6	<0.01	0	[0][d]	0	–
	1	pRSV-CAT	4×10^6	1.6	0.95	0.6	62	103
	1	pSV2-CAT	4×10^6	0.04	0.04	3.6	2.6	0.7
	2	pRSV-CAT	5×10^6	6.9	2.8	10.0	144	14.4
HB101	3	pRSV-CAT	10^{10}	63	190	ND[e]	ND	ND
(protoplasts)	3	pSV2-CAT	10^{10}	27	80	ND	ND	ND
	3	pTK-CAT	10^{10}	30	88	ND	ND	ND

[a]Estimated relative to commercial CAT enzyme control, as in McMahon *et al.* (1984).
[b]Calculated by dot blot hybridization as described in Materials and Methods.
[c]Assay calibrated as described in McMahon *et al.* (1984).
[d]Value taken as background.
[e]ND, Not determined.

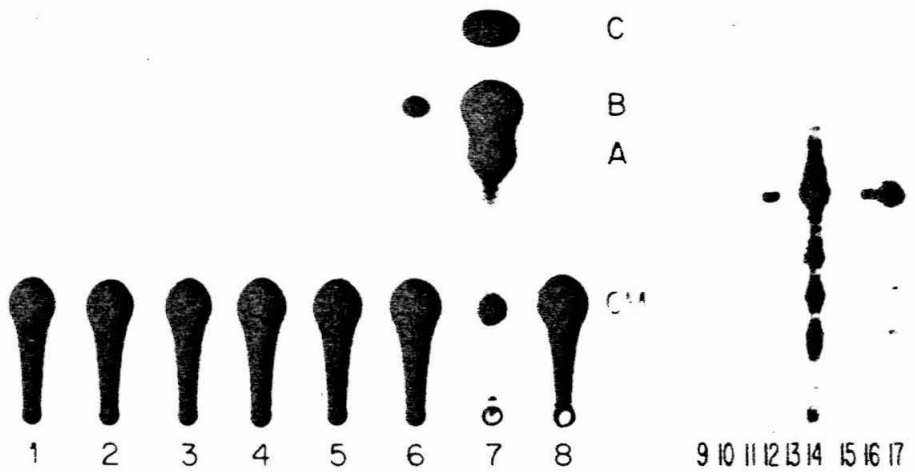


FIG. 5. Expression of pSV2-CAT in COS-7 cells. Left: MTL2.8.2 (lanes 1 and 2), La-1- (lanes 3 and 4), and COS-7 cells (lanes 5 and 6) were transfected by protoplast fusion with pBR322 (lanes 1, 3, and 5) or pSV2-CAT (lanes 2, 4, and 6) and assayed for CAT activity 48 hr later as described in Materials and Methods. The percent acetylation was <0.1 for MTL; 0.2 for La-1- and 0.7 for COS-7. Lanes 7 and 8 are a positive and negative control, respectively, and the products labeled CM, A, B, and C are the same as in Fig. 4. Right: Estimation of the *cat* gene copy number from the samples assayed at left. Lanes 9–14 are identical to lanes 1–6. Unintegrated plasmid DNA isolated by the method of Hirt (1967) from isolated nuclei was linearized with *Bam* HI and electrophoresed on a 1% agarose gel. *Bam* HI-linearized pSV2-CAT DNA corresponding to 10^4, 10^5, and 10^6 copies (lanes 15–17, respectively) was run in parallel to provide copy number standards. The DNA was blotted onto nitrocellulose and probed with a nick-translated pSV2-CAT plasmid. The number of *cat* gene copies per cell as determined by densitometry was 0.4 for MTL; 0.28 for La-1- and 4.7 for COS-7.

published results). Protoplast fusion has substantial drawbacks, particularly the introduction of extraneous or deleterious bacterial macromolecules along with the plasmid DNA. These molecules may even restrict the amount of protoplast DNA that mammalian cells can retain, since L cells transfected by $Ca_3(PO_4)_2$ coprecipitation routinely expressed 10-fold higher levels of CAT activity than L cells transfected by protoplast fusion (data not shown). However, the protoplasts are presently the most accessible source of plasmid DNA in the membrane-bounded form, which seems to be optimal for successful introduction into T cells.

In transient expression assays, T cells can express as much CAT or APH(3')II activity per *cat* or *neo* gene as do L cells. The viral promoters in the RSV LTR and the SV40 early region appear to be utilized just as well by the lymphocytes as by fibroblasts. This suggests that the frequency with which long-term G-418-resistant transformants of T cells can be isolated may be limited primarily by the efficiency of incorporation and stabilization of the exogenous DNA sequences rather than by a lack of T-cell-specific enhancer sequences. Of course, RNA levels may be further increased in constructs with such regulatory sequences. We are currently examining the relative efficiencies of transient expression from the RSV LTR, for which there is no evidence of preferential activity in T cells, and from the LTRs of viruses known to cause T-cell leukemias, as potential sources of T-cell-specific promoter/enhancers (F. Yoshimura, T.J. Novak, and E. Rothenberg, unpublished results).

Different T cell lines do in fact show striking differences in the efficiencies with which they are transformed and the numbers of exogenous DNA copies that they retain. The same preparations of protoplasts yield EL4.E1 transformants at frequencies of $<10^{-5}$, MTL2.8.2 transformants at 10^{-4}, and MJ transformants at frequencies possibly two orders of magnitude higher. In the cases we have analyzed most closely, the low transformation efficiency of EL4.E1 seems to be correlated with high stability of the integrated DNA. Cloned transformant lines appear to carry one integrated *neo* gene per cell genome (Fig. 2), whether analyzed after 2 or 6 months (data not shown). By contrast, the more easily transformed MTL2.8.2 cells rapidly lose *neo* DNA. During expansion of these clones, the APH(3')II produced by a shrinking percentage of the transformed cells appears to be sufficient to preserve the viability of segregants that have lost *neo* sequences, presumably due to leakage or cross-feeding. Both of these murine lines take up

TABLE 4. EFFECT OF POLYETHYLENE GLYCOL-MEDIATED FUSION ON DNA UPTAKE AND CAT SYNTHESIS

Cell	*Plasmid*	*PEG*	*Number of molecules CAT subunit per cell*[a]	*Number of molecules cat gene per cell*[b]
MTL2.8.2	pRSV-CAT	–	35	1.7
		+[c]	165	24.0
EL4.E1	pRSV-CAT	–	392	9.3
		+[c]	424	8.0
La⁻1⁻	pRSV-CAT	–	21	2.0
		+[c]	144	10.0

[a]Determined as described in Table 3.
[b]Determined as described in Table 3.
[c]Raw data shown in Table 3, experiment 2.

relatively low numbers of DNA copies measured after 2 days (Tables 2 and 3). Their different behavior might be explained by a higher level of illegitimate recombination activity in the MTL2.8.2 cells. Experiments using other selectable genes are under way to test the generality of this phenomenon. Note that the MJ line of human T cells appears significantly more permissive for initial DNA uptake, resulting in high transformation frequencies with retention of high copy numbers of the exogenous sequences.

Finally, the quantitative analysis of transient gene expression in T cells has revealed a new example of a more general phenomenon, namely the ability of relative promoter strengths to be influenced by sequences in the genes transcribed from those promoters. Thus, while the RSV LTR and the SV40 early region are equally efficient at driving *neo* expression in murine cells, they differ markedly in their efficiencies for *cat* sequences. Our results do not distinguish between influences on transcription *per se* and on RNA stability or translation efficiency. The CAT mRNAs expressed from the two viral promoters differ in their first 60–70 nucleotides, so that it is conceivable that the SV40 sequences might selectively interfere with the utilization of *cat* sequences in murine cells. Also, because RNA levels are too low to be measured by nuclease protection assays, we cannot distinguish between an effect of *neo* sequences to enhance measured expression from the SV40 promoter and an effect to decrease expression from the RSV promoter. Nevertheless, it is clear that the regulatory sequences can interact with the "recorder" sequences in determining the ultimate level of gene product.

Such a phenomenon has been noted in several other systems, particularly those in which the recorder gene is one which undergoes developmental regulation of its expression *in vivo* (Charnay *et al.*, 1984; Wright *et al.*, 1984; Grosschedl and Baltimore, 1985). In these cases the intragenic sequences that interact with upstream sequences are interpreted to be products of rigorous evolutionary selection for accuracy in gene regulation. The results presented here, by contrast, suggest that such interactions can also take place among sequences in completely ectopic evolutionary contexts, including sequences derived from prokaryotes. This has the practical consequence that particular model genes may behave anomalously when driven by certain promoters. In addition, the ability of bacterial sequences to modulate their own expression from eukaryotic promoters could be a clue that the sequence elements involved in these interactions occur frequently in the absence of selective pressure.

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REFERENCES

BERMAN, J.W., BASCH, R.S., and PELLICER, A. (1984). Gene transfer in lymphoid cells: Expression of the Thy-1.2 antigen by Thy-1.1 BW5147 lymphoma cells transfected with unfractionated cellular DNA. Proc. Natl. Acad. Sci. USA **81**, 7176–7179.

BLEACKLEY, R.C., HAVELE, C., and PAETKAU, V. (1982). Cellular and molecular properties of an antigen-specific cytotoxic T lymphocyte line. J. Immunol. **129**, 758–767.

CHARNAY, P., TREISMAN, R., MELLON, P., CHAO, M., AXEL, R., and MANIATIS, T. (1984). Differences in human α- and β-globin gene expression in mouse erythroleukemia cells: The role of intragenic sequences. Cell **38**, 251–263.

CHU, G., and SHARP, P.A. (1981). SV40 DNA transfection of cells in suspension: Analysis of the efficiency of transcription and translation of T-antigen. Gene **13**, 1979–2002.

COHEN, J.J., and DUKE, R.C. (1984). Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. J. Immunol. **132**, 38–42.

COLBÈRE-GARAPIN, F., HORODNICEANU, F., KOURILSKY, P., and GARAPIN, A.-C. (1981). A new dominant hy-

brid selective marker for higher eukaryotic cells. J. Mol. Biol. **150**, 1–14.

DEMBIĆ, Z., HAAS, W., WEISS, S., McCUBREY, J., KIEFER, H., VON BOEHMER, H., and STEINMETZ, M. (1986). Transfer of specificity by murine α and β T-cell receptor genes. Nature **320**, 232–238.

FARRAR, J.J., FULLER-FARRAR, J., SIMON, P.L., HILFIKER, M.L., STADLER, B.M., and FARRAR, W.L. (1980). Thymoma production of T cell growth factor (interleukin 2). J. Immunol. **125**, 2555–2558.

FREGIEN, N., and DAVIDSON, N. (1984). The LTR of feline leukemia virus enhances the expression of the bacterial Neo^r gene in human cells. In *Molecular Biology of Development*, E.H. Davidson and R.A. Firtel, eds. (Alan R. Liss, New York) pp. 657–665.

FREGIEN, N., and DAVIDSON, N. (1985). Quantitative in situ gel electrophoretic assay for neomycin phosphotransferase activity in mammalian cell lysates. Anal. Biochem. **148**, 101–104.

LIS, S., and WATSON, J. (1980). Biochemical and biological characterization of lymphocyte regulatory molecules. V. Identification of an interleukin 2 producer human leukemia T cell line. J. Exp. Med. **152**, 1709–1719.

GORMAN, C.M., MOFFET, L.F., and HOWARD, B.H. (1982a). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**, 1044–1051.

GORMAN, C.M., MERLINO, G.T., WILLINGHAM, M.C., PASTAN, I., and HOWARD, B.H. (1982b). The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. Proc. Natl. Acad. Sci. USA **79**, 6777–6781.

GORMAN, C., PADMANABHAN, R., and HOWARD, B.H. (1983). High efficiency DNA-mediated transformation of primate cells. Science **221**, 551–553.

GRAHAM, F.L., and VAN DER EB, A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**, 456–467.

GROSSCHEDL, R., and BALTIMORE, D. (1985). Cell-type specificity of immunoglobulin gene expression is regulated by at least three DNA sequence elements. Cell **41**, 885–897.

HATAKEYAMA, M., MINAMOTO, S., UCHIYAMA, T., HARDY, R.R., YAMADA, G., and TANIGUCHI, T. (1985). Reconstitution of functional receptor for human interleukin-2 in mouse cells. Nature **318**, 467–470.

HIRT, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**, 365–369.

KRÖNKE, M., LEONARD, W.J., DEPPER, J.M., and GREENE, W.C. (1985). Sequential expression of genes involved in human T lymphocyte growth and differentiation. J. Exp. Med. **161**, 1593–1598.

McMAHON, A.P., NOVAK, T.J., BRITTEN, R.J., and DAVIDSON, E.H. (1984). Inducible expression of a cloned heat shock fusion gene in sea urchin embryos. Proc. Natl. Acad. Sci. USA **81**, 7491–7494.

POPOVIC, M., LANGE-WANTZIN, G., SARIN, P.S., MANN, D., and GALLO, R.C. (1983). Transformation of human umbilical cord blood T cells by human T-cell leukemia/lymphoma virus. Proc. Natl. Acad. Sci. USA **80**, 5402–5406.

SANDRI-GOLDIN, R.M., GOLDIN, A.L., LEVINE, M., and GLORIOSO, J.C. (1981). High-frequency transfer of cloned herpes simplex virus type I sequences to mammalian cells by protoplast fusion. Mol. Cell. Biol. **1**, 743–752.

SCHÖLER, H.R., and GRUSS, P. (1984). Specific interactions between enhancer-containing molecules and cellular components. Cell **36**, 403–411.

SODROSKI, J.G., ROSEN, C.A., and HASELTINE, W.A. (1984). Trans-acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. Science **225**, 381–385.

SOUTHERN, P.J., and BERG, P. (1982). Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**, 327–341.

SPANDIDOS, D.A., and WILKIE, N.M. (1983). Host-specificities of papillomavirus, Moloney murine sarcoma virus and simian virus 40 enhancer sequences. EMBO J. **2**, 1193–1199.

WIGLER, M., PELLICER, A., SILVERSTEIN, S., and AXEL, R. (1978). Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. Cell **14**, 725–731.

WIGLER, M., PELLICER, A., SILVERSTEIN, S., AXEL, R., URLAUB, G., and CHASIN, L. (1979). DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. USA **76**, 1373–1376.

WRIGHT, S., ROSENTHAL, A., FLAVELL, R., and GROSVELD, F. (1984). DNA sequences required for regulated expression of β-globin genes in murine erythroleukemia cells. Cell **38**, 265–273.

YOAKUM, G.H. (1984). Protoplast-fusion: A method to transfect human cells for gene isolation, oncogene testing and construction of specialized cell lines. BioTechniques **Jan./Feb.**, 24–30.

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CHAPTER 2

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REGULATORY ARCHITECTURE OF THE MURINE INTERLEUKIN-2 GENE

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INTRODUCTION

Perturbation of the antigen receptor/CD3 complex on resting T cells initiates a variety of biochemical responses whose proximal results include phospholipid hydrolysis, production of intracellular second messengers, transmembrane calcium fluxes and activation of protein kinase C (reviewed in Weiss *et al.*, 1986). These common, activation-specific events result in the expression of a preprogrammed set of genes by the responding cells, leading ultimately to proliferation and/or the elaboration of effector function (Crabtree, 1989). Subsets of T cells may be defined by the way they utilize similar stimuli to effect distinct outcomes at the transcriptional level. For example, activated type 1 helper T-cell lines (T_H1) synthesize interleukin 2 (IL2), interferon-γ (IFN-γ) and lymphotoxin (LT) in preference to IL4 and IL5, which are the products of type 2 helper T cells (T_H2) (Mosmann and Coffman, 1989). Both types of helper lines can secrete IL3. Although they evince distinct response profiles, T_H1 and T_H2 cells usually both express the CD4 differentiation antigen and are restricted to recognizing antigen in association with class II major histocompatibility complex (MHC) molecules. Cytotoxic T-lymphocytes (CTL), on the other hand, are usually $CD8^+$, class I-MHC restricted, and when activated synthesize a battery of lytic proteins consistent with their role in cell-mediated immunity. It is generally agreed that the particular combination of cell-surface proteins, MHC restriction, and response potential possessed by any T cell is imposed on it during its passage through the thymus (Chen *et al.*, 1983; von Boehmer *et al.*, 1989), although there is evidence that this is true more for helper T cells than for CTLs (Mizuochi and Singer, 1988).

The molecular mechanisms by which a differentiating thymocyte limits its response repertoire to that of a particular T-cell subset are not entirely understood but presumably involve some combination of transcriptional and

posttranscriptional controls. Transcriptional control can take two forms: 1) selective expression of *trans*-acting transcriptional proteins required for the expression of a particular gene (reviewed in Maniatis *et al.*, 1987) or 2) chromatin-mediated gene shutoff whereby some gene promoters are rendered inaccessible to the cell's transcriptional machinery (reviewed in Brown, 1984). In addition, we have shown in both mature and immature populations of T cells that the frequency of IL2 producers and the level of IL2 mRNA that they accumulate are a function of the activating stimulus used (McGuire and Rothenberg, 1987; McGuire *et al.*, 1988; Rothenberg *et al.*, 1990). This suggests that a T cell's ultimate response phenotype may not be heritably fixed, but may be influenced by the type of external stimulus it receives.

The mechanisms by which cell-type-specific patterns of gene expression are allocated to discrete subsets of cells are obviously a fundamental aspect of T-cell development. To investigate the nature of these mechanisms, we have focused on the regulation of the murine IL2 gene. IL2 is subject to particularly stringent control, both in cell-type specificity and in its requirements for particular activating stimuli. The extreme transience of the kinetics of IL2 expression, and the refractoriness of proliferating normal T cells to IL2 induction, suggest that this gene is negatively as well as positively regulated. In addition, various T-cell subsets differ in the precise activation signals they require for IL2 induction (McGuire *et al.*, 1988; Rothenberg *et al.*, 1990). These considerations indicate that the minimal mechanisms involved in activating IL2 transcription in tumor cells may not fully account for the sophisticated physiological control of this gene.

In this work, we have therefore characterized the sequences and potential regulatory elements in the mouse IL2 gene in regions extending considerably beyond the minimal sequences previously shown to be required for expression.

Some of these sequences appear to have a partial silencer effect, even in tumor cell lines that exhibit minimal negative regulation of IL2. We have used these constructs to explore whether the tissue-specificity and sensitivity to physiological inhibition of IL2 expression are conferred by specific sites in the IL2 regulatory region, or alternatively by the susceptibility to shutoff of the signal transduction pathway necessary for IL2 induction.

MATERIALS AND METHODS

Cloning the Mouse IL2 Gene

A BALB/c genomic library in the vector λ Charon 4A was kindly provided by Dr. L. Hood. The insert DNA consisted of equal masses of sperm DNA that had been partially digested with EcoRI or partially digested with a combination of HaeIII and AluI followed by EcoRI linker addition (Davis *et al.*, 1980). The library was probed with a nick-translated 470 bp BglII-AccI mouse IL2 cDNA fragment from pCD-IL2 (Yokota *et al.*, 1985) (generously provided by Dr. K.-I. Arai, DNAX Corporation). Positive plaques were isolated and the resulting phage purified through two additional rounds of infection. Phage DNA was prepared by standard plate lysate procedures (Maniatis *et al.*, 1982) and the EcoRI restriction fragment pattern was compared to the previously published mouse IL2 map by DNA gel blot analysis using the entire IL2 cDNA insert from pCD-IL2 as a probe. Two clones, λIL2-9.1 and λIL2-2.1, which contain the entire coding region and flanking DNA were selected for further analysis.

Sequencing the IL2 5' Flanking Region

A 618 bp RsaI-PstI fragment extending from -578 to +40 relative to the start site of transcription was subcloned into the SmaI-PstI sites of pSP65 (Promega Biotec) and the sequence determined according to Maxam and Gilbert (1980). A 2700 bp EcoRI-AccI fragment from λIL2-9.1 extending from -2800 to -100 was subcloned into the EcoRI-SmaI sites of pGem7Zf(+) (Promega) and the sequence determined by the dideoxy chain termination method using the Sequenase^{TM} kit as directed by the manufacturer (U.S. Biochemical).

Plasmid Construction

A series of IL2-CAT hybrid genes was constructed in which expression of the bacterial gene for chloramphenicol acetyltransferase (CAT) is under the control of increasing amounts of IL2 5' flanking DNA. These plasmids are designated as pIL2 (-X) where X indicates the 5' terminal nucleotide, relative to the transcriptional start site, that is included in the construct. pIL2 (-2800) was constructed by first subcloning a 2.8 kb EcoRI-PstI (-2800 to +40) fragment from λIL2-9.1 into the identical sites of pSP65, followed by insertion of a 2.25 kb PstI fragment from pTK-CAT (gift of C. Gorman and B. Howard, NIH; Novak and Rothenberg, 1986). This PstI fragment contains 76 bp of HSV TK gene 5' untranslated DNA, the bacterial gene for CAT, and SV40 downstream processing signals. pIL2 (-1890) was constructed in a similar manner but starting with the 1.9 kb EcoRI-PstI fragment from λIL2-2.1 instead of the 2.8 kb fragment. pIL2 (-321) was made from pIL2 (-1890) by first digesting with BglI followed by partial XmnI digestion and isolation of the largest fragment on a low-melt agarose gel. This fragment was recircularized by ligation to an SmaI-BglI fragment from pSP64. Plasmid pIL2 (-103) was generated from pIL2 (-321) by digesting completely with AccI, filling in with Klenow polymerase and religating. pIL2 (-578) was made by cloning a 618 bp RsaI-PstI fragment, extending from -578 to +40, into the SmaI-PstI site of pSP65, followed by digestion with PstI and insertion of the 2.25 kb PstI CAT cassette described above. pIL2 (-232) and pIL2 (-753) were made in the identical manner beginning with, respectively, a 272 bp DdeI-PstI fragment (-232 to +40) and a 793 bp SspI-PstI fragment (-753 to +40). Plasmids pIL2 (-1449), pIL2 (-1332), pIL2 (-1219), and pIL2 (-351) are deletions made by digesting pIL2 (-2800) with StuI (-2205) followed by addition of Bal31 nuclease. Aliquots were removed at 5, 10, and 15 min, treated briefly with Klenow to generate flush ends, and digested to completion with BglI. DNA in the desired size range was isolated on a

low-melt agarose gel and recircularized by ligation to a BglI-SmaI fragment from pSP65. The exact extent of the deletions was determined by sequencing.

Plasmids containing intragenic or 3' flanking DNA were constructed as follows. Genomic fragments (see Fig. 1A) designated A (350 bp PvuII-EcoRI), B (2.2 kb EcoRI-HindIII), C (1.8 kb HindIII), D(0.9 kb HindIII), E(1.1 kb EcoRI-Asp718), F(1.8 kb Asp718-EcoRV), and G(2.0 kb EcoRV-EcoRI) were cloned in both orientations into the unique HindIII site in pIL2 (-1890), either by blunt-end ligation (fragments A, B, E, F, and G) or cohesive-end ligation (fragments C and D). These were designated pIL2 (-1890) Y (+) or pIL2 (-1890) Y (-), where Y denotes the inserted fragment (A-G) and +/- indicates, respectively, the sense and anti-sense orientation of the inserted fragment with respect to the IL2-CAT gene.

All plasmids were purified on CsCl-ethidium bromide density gradients before use.

Drugs and Reagents

The phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) (Sigma) was dissolved in DMSO at a concentration of 10 µg per ml and the calcium ionophore A23187 (Calbiochem) was made up in DMSO to a final concentration of 0.37 mg per ml; both were stored in small aliquots at -20°C. Forskolin, dibutyryl cyclic AMP (dBcAMP), and prostaglandin E_1 (PGE_1) were purchased from Sigma and Calbiochem. PGE_1 was made up in 95% EtOH to a final concentration of 10 mM and was stored desiccated at -20°C. Forskolin was made up as a 10 mM stock in 95% EtOH and was stored desiccated at 4°C. dBcAMP was dissolved in water, neutralized with 0.5 M Tris base, diluted to a final concentration of 0.1 M and stored at -20°C. Cyclosporin A (50 mg per ml; Sandimmune I.V.) was purchased from Sandoz and stored in the dark at room temperature. Immediately before use

it was diluted in cell-growth medium to the desired concentration. In all cases the final organic solvent concentration was 0.1%.

Cell Lines and DNA Transfections

The human T-cell leukemia line Jurkat (kindly provided by Dr. G. Crabtree, Stanford University) and the murine lines EL4.E1, NS-1, P388D1, S49.1, and BW5147 were grown in RPMI 1640 medium supplemented with 10% FBS, 50 μM 2-ME, 2 mM L-glutamine (L-Q), and antibiotics. The IL2-dependent lines HT-2, MTL2.8.2, and CTLL-2 were grown in the same medium supplemented with 5% TPA-stimulated EL4.E1 cell supernatant as a source of growth factor. The 32D cl 5 line was grown in DMEM, which contained 10% FBS, 2 mM L-Q, and 20% WEHI-3B conditioned medium as a source of IL3. Ltk^- cells were grown in DMEM without IL3. D10.G4.1 were passaged every 10 days with antigen and antigen presenting cells as described (Kaye *et al.*, 1983), except that we used 5% EL4.E1 conditioned medium instead of rat Con A supernatant as a source of growth factors. Cells were maintained in logarithmic growth prior to being transfected.

Transfection of hematopoietic cells with plasmid DNA was by DEAE-dextran facilitation. Cells to be transfected were washed in growth medium and counted. They were then rewashed in serum-free DMEM containing 10 mM Hepes, pH 7 (hereafter DMEM). Finally, the washed cells were resuspended to a density of 1×10^7 per ml in a transfection cocktail consisting of DMEM, 0.1 mM chloroquine, 250 μg per ml DEAE-dextran (2×10^6 M.W.), and 10 μg per ml supercoiled plasmid DNA. The cells were put into loosely capped 17 x 100 mm round-bottom polypropylene tubes (Falcon 2059) and placed in a 37°C/7% CO_2 incubator for 30 min (EL4.E1) or 60 min (Jurkat). The tubes were swirled gently every 10 min to prevent extensive cell clumping. Generally, $1\text{-}2 \times 10^7$ cells were transfected with each plasmid in an experiment. After incubation in the

transfection cocktail, 6 ml of DMEM were added to each tube and the cells were pelleted at 500 x g in a room temperature centrifuge. Jurkat cells were then plated into two 60 mm tissue culture plates. EL4.E1 cells were washed an additional time in 3 ml DMEM containing 150 units per ml heparin (sodium salt; Sigma) to reduce clumping (Deans et al., 1984) before being plated. Approximately 24 hr post-transfection, one plate of each pair received TPA and A23187 to final concentrations of 10 ng per ml and 37 ng per ml, respectively. After an additional 18 hr of incubation, cells were harvested for assay of CAT activity as described previously (Novak and Rotherberg, 1986) except that extracts were incubated for 5 hr. In some experiments both plates of each pair received TPA and A23187, but one of them also received cyclosporin A, forskolin, dBcAMP or PGE_1. All comparisons were based on transfections using at least two independent DNA preparations of each plasmid.

Transfections included pBR322 as a negative control. Relative CAT activity was calculated by setting pBR322 to 0% acetylation and subtracting its cpm from the other samples. The percent acetylation per 10^6 cell equivalents was then calculated for each plasmid and normalized with respect to pIL2(-321), which was arbitrarily set to 1.0. Results are presented as mean ± S.E.M.

Ltk^- cells were transfected by calcium phosphate precipitation as described (Wigler *et al.*, 1978). Cells (8×10^5) were seeded in 100 mm tissue culture plates one day prior to transfection. Each plate received 25 µg of plasmid without added carrier DNA.

RNA Extraction and Gel Blot Analysis

Cytoplasmic RNA was extracted after 5 hr of stimulation with TPA and A23187 as described, in the presence of 10 mM vanadyl ribonucleoside complexes (Favaloro *et al.*, 1980). Approximately equalized masses of RNA, usually 5-10 µg,

were electrophoresed on denaturing 1% agarose/formaldehyde gels and stained with acridine orange to visualize the rRNA. The presence of residual vanadyl ribonucleoside complexes led to some variation in the actual amounts of RNA loaded. The RNA was then blotted onto nylon membranes (Nytran, Schleicher and Schuell) according to the manufacturer's instructions. After baking at 80°C for 60 min to fix the RNA, the filters were probed with random primed cDNAs specific for human IL2 (Holbrook *et al.*, 1984a) and mouse IL2 (Yokota *et al.*, 1985) and IL4 (Lee *et al.*, 1986). All filters were also probed with a mouse α skeletal actin cDNA (N. Davidson, Caltech, unpublished) to verify the integrity of the RNA and to allow the signal to be normalized to the amounts of RNA present in each lane. Hybridizations were for 20 hr at 42°C in 5 x SSPE, 50% formamide, 0.2% SDS, 5 x Denhardt's and 10% dextran sulfate. Filters were washed three times for 1 min each at room temperature in 2 x SSC, 0.2% SDS and 0.05% $NaPP_i$, followed by two 30 min washes at 68°C in 0.2 x SSC, 0.1% SDS, 0.05% $NaPP_i$. They were then exposed to film at -70°C with an intensifying screen.

RESULTS

Isolation of the Mouse IL2 Gene

We isolated phage containing the murine IL2 gene from a BALB/c genomic library in λ Charon 4A, using a 470 bp BglII-AccI nick-translated cDNA probe to identify six positive clones representing four different genomic inserts (Fig. 1A). The identities of the resulting clones were verified by comparison with predictions from the genome organization and partial sequence previously reported by Fuse *et al.* (1984). These authors had shown that, in the mouse, the IL2 gene resides on four contiguous EcoRI fragments spanning 8.3 kb of DNA, as shown in Fig. 1A. We identified three genomic clones that contain at least 2 kb of 5' flanking DNA in addition to the entire coding region. One of the clones, λIL2-9.1, contains the intact 3.3 kb genomic EcoRI fragment upon which exons 1 and 2 reside, with at least 2.8 kb of upstream sequence. Clones λIL2-2.1 and λIL2-11.1 appear identical and possess a 2.4 kb EcoRI fragment that hybridizes to a 5' IL2 cDNA probe (not shown). Their 5' termini are at a HaeIII site, modified with EcoRI linkers, 1.9 kb upstream of the transcriptional start site. All of the clones isolated except λIL2-9.1 also contain >7 kb of 3' flanking DNA.

We determined the sequence of the 2.8 kb of 5' flanking sequence present on the 3.3 kb genomic EcoRI fragment, as described in Materials and Methods. Figure 1B shows the sequencing strategy used. The major features of our murine sequence (Fig. 2), in comparison to the human IL2 sequence, are discussed in the following.

Comparison Between Murine and Human Sequences

A dot matrix comparison between the human (Holbrook *et al.*, 1984b) and mouse IL2 5' flanking sequences is shown in Fig. 3. There is extensive sequence

identity extending from -1 to -600 and only scattered islands of similarity further upstream. A direct sequence comparison of the mouse and human 5' flanking regions up to -580 (-585 in the human) is shown in Fig. 4. The identity throughout this region is 86%, which is similar to the conservation between the upstream regions of other mouse and human lymphokine genes. However, the length of the conserved upstream region is longer in the IL2 gene than in IL3, IL6, and GM-CSF (Tanabe *et al.*, 1988; Miyatake *et al.*, 1985; Yang and Clark, 1987). As for other lymphokine genes, this degree of flanking sequence conservation is considerably greater than that of the coding regions of the two genes. Such strong evidence of evolutionary pressure against sequence divergence suggests that sequences throughout this region play a role in DNA-protein interactions required for IL2 gene regulation.

The sequence similarity terminates abruptly within 100 bp of the border of a striking feature of the murine sequence, namely the block of alternating purines and pyrimidines between -759 and -960. There is nothing in the human gene that resembles this murine sequence. On the coding strand, poly(dC-dA) predominates with interspersed islands of poly(dG-dT). There are 20 alternating purine-pyrimidine residues between -944 and -925, 48 between -905 and -858, and 35 between -830 and -796 with several shorter stretches also present (Fig. 2). Poly(dG-dT)•poly(dC-dA) is able to assume a Z-DNA configuration (Haniford and Pulleyblank, 1983) and in some assays shows transcriptional enhancing activity (Hamada *et al.*, 1984). However, it is interesting that while similar sequences are present in the control regions of murine IL6 [$(GT)_{28}$ from -446 to -391 as well as $(CA)_{15}$ in intron 2] (Tanabe *et al.*, 1988) and murine GM-CSF [$(GT)_{14}$ from -1024 to -997)] (Miyatake *et al.*, 1985), in addition to IL2, there is no evidence for corresponding sequences in any of their three human counterparts. It should be noted that the (GT) element in the mouse GM-CSF gene resides in a region that

has not been sequenced in the human version. The alternating Pu-Py tracts may have been introduced into the murine gene, or deleted from the human gene, *en bloc*, because where mouse-human sequence similarities resume further upstream, they are out of register. The murine sequence is usually present at a position about 250 bp further from the RNA cap site than its human counterpart (see Figs. 3 and 5). Of interest is the presence of two contiguous 7 bp direct repeats of the sequence 5'-ACACATA-3' between -746 and -733 of the human IL2 gene, suggesting the possible loss of a larger alternating Pu-Py stretch by recombinational excision.

Figure 5 lists the additional promoter-distal regions of sequence similarity identified by the dot-matrix comparison of the murine and human genes. Some of these matches are impressive; 28/30 identical base pairs with one gap (between -1645 and 1674 in the murine gene); 15/16 identical (-1695 to -1710); 24/27 identical (between -1466 and -1492); and 19 identical in a contiguous run of 21 (between -1400 and -1420). These do not represent obvious repetitions of motifs found in the more proximal regions, nor do the conserved sequences include recognizable binding sites for the DNA-binding proteins considered in the next section. Their functional significance will be discussed in a later section.

Candidate Binding Sites for Transcription Factors

Several DNA binding proteins have been shown to footprint over the region from -1 to -279 in the human IL2 gene (Durand *et al.*, 1988; Brunvand *et al.*, 1988) and these protected regions have been shown by genetic analysis to play a role in its inducible expression (Fujita *et al.*, 1986; Durand *et al.*, 1988; Williams *et al.*, 1988; Shaw *et al.*, 1988). The regions protected by two of these proteins, NF-IL2A and NFAT-1, are indicated in Fig. 4. The proximal NF-IL2A site is perfectly conserved between the two genes, while the distal sites differ at 7 out of 39

nucleotides. The binding site for NFAT-1, a protein restricted to activated T cells (Shaw *et al.*, 1988) and whose appearance precedes IL2 gene transcription, is also well conserved between mouse and human. This is not surprising in view of the fact that nuclear extracts from activated EL4.E1 cells contain a protein that has similar properties to NFAT-1 in a gel retardation assay (Shaw *et al.*, 1988). Binding sites for NF-κB and AP-1 in the human gene (Serfling *et al.*, 1989; Hoyos *et al.*, 1989; Muegge *et al.*, 1989) are conserved in both sequence and position in the mouse 5' flanking region.

A computer-assisted search of the IL2 5' region identified several potential binding sites for known regulatory proteins in the promoter-distal flanking regions of the murine IL-2 gene. These identifications are provisional because they are based solely upon homology to published consensus sequences. We could not locate consensus motifs for Ig octamer (100%), T-cell receptor decamer (>80%), Sp1 (≥85%) or the cAMP response element CREB (at a level of >75% matching). The last is somewhat surprising, for cAMP has clear effects on IL2 expression, albeit negative ones (see below). The failure to identify consensus motifs of course does not exclude other binding sites for any of these factors. However, we did locate four glucocorticoid response element (GRE) core motifs (5'-TGTTCT-3') (Scheidereit *et al.*, 1983) at -1913, -1704, -1194, and -327. The GRE at -1704 falls within an extended region of homology between the mouse and human IL2 genes (Figs. 3 and 4B). Normally, the signals inducing IL2 transcription can be mimicked by the combination of calcium ionophore and TPA (Truneh *et al.*, 1985). The proteins AP-1 and AP-3 are involved in TPA-inducible gene responses, and consensus binding sites have been proposed for both. In addition to the probable AP-1 binding sites at -153 and at -185 (Serfling *et al.*, 1989; Muegge *et al.*, 1989), there are two more potential sites further upstream at -1515 and -1015. Three possible AP-3 sites are present at -2708, -2492, and -2264.

Far Upstream Elements Modulate IL2 Expression in T_H1-Type Lymphomas

Extensive analysis of the human IL2 gene promoter has revealed numerous regions between -60 and -361 which, when deleted, reduce the level of inducible expression of a linked reporter gene following transfection into the Jurkat and EL4 T-cell lines (Fujita *et al.*, 1986; Durand *et al.*, 1988; Williams *et al.*, 1988; Hoyos *et al.*, 1989). At least five regions have been implicated thus in activation. There is no evidence, however, that this catalogue of positive elements is complete. Furthermore, there has been no test of the functional roles of negative regulatory sequences in the maintenance of strict activation and/or tissue specificity of this gene. Because the upstream conservation between mouse and human IL2 genes suggests a functional role for sequences lying 5' to -300, we constructed a series of IL2-CAT plasmids in which the bacterial gene for chloramphenicol acetyltransferase is under the control of the mouse IL2 gene promoter. To minimize non-IL2 regulatory sequences that might elevate basal levels of expression nonspecifically, no heterologous promoter or enhancer sequences were included. The structures of these chimeric molecules, containing between 103 bp and 2800 bp of 5' flanking IL2 DNA, are diagrammed in Fig. 6.

The smallest construct, pIL2 (-103) CAT, contains only the promoter-proximal "NF-IL-2A" enhancer element. The pIL2 (-232) construct includes, in addition, three putative sites for inducible activation factors: NF-κB (at -206) and two proposed AP-1 sites (at -185 and -153). All sites known to be "essential" are included in the pIL2 (-321) construct (Crabtree, 1989).

To test for a role of promoter-distal elements in controlling the magnitude of inducible IL2 expression, this series of plasmids was introduced into Jurkat and EL4.E1 cells, and the resulting CAT activity determined after 18 hr of stimulation with A23187 and TPA (Fig. 6). In all cases, the plasmids were transfected into the

same cell population in parallel, with our shortest expressible construct [pIL2(-321)] serving as a normalization standard for the resulting levels of CAT activity. The results are presented schematically in Fig. 7.

With the exception of pIL2 (-103) and pIL2 (-232), all the IL2-CAT plasmids yielded easily detectable amounts of CAT activity in extracts from transfected cells following stimulation (Fig. 7). There was no detectable CAT activity in any case unless the cells were stimulated, in accordance with the fact that transcription of the IL2 gene is linked tightly to cell activation (Krönke *et al.*, 1985). The failure of pIL2 (-103) to be expressed under any condition indicates that this fragment lacks intrinsic promoter function in the absence of enhancing elements between -103 and -321. Some expression was observed from pIL2 (-232), but this was at least an order of magnitude less than from pIL2 (-321). Thus, the NF-κB and AP-1 candidate sites are not sufficient for efficient induction in these cells.

There were several effects from increasing the amount of IL2 flanking DNA beyond -321. First, the increase in 5' flanking DNA from -321 to -578 was correlated with a substantial increase in CAT activity, in both cell lines. This suggests that additional positive regulatory elements are located between -321 and -578 in the murine gene. It should be noted that a similar increase of human 5' flanking DNA reportedly results in slightly lower expression of a linked indicator gene (Fujita *et al.*, 1986).

The second point is the apparent presence of a negative regulatory region lying somewhere between -578 and -1219. In both cell lines examined, CAT activity was reproducibly about twofold lower with pIL2 (-1219) than with pIL2 (-578). This negative effect was seen with two separate sets of plasmid preparations. It does not represent a nonspecific length effect because constructs with longer extents of 5' flanking sequence yield higher expression (see below).

The pIL2 (-1219) plasmid contains sequences that lie upstream of the longest tested human IL2-CAT construct and it encompasses the block of alternating purine-pyrimidine residues that is not found in the human 5' flanking region. Thus, either the poly d(CA) tract or other sequences in this region appear to act as negative regulators.

To test these possibilities, we cloned a 260 bp HinFI-SspI fragment containing the poly d(CA) region upstream of the IL2 promoter sequences in pIL2 (-103) and pIL2 (-321). These constructs were then transfected into EL4.E1 cells in parallel with the unmodified parental plasmids. That this region lacks intrinsic enhancing activity in EL4.E1 is shown in Table I. The presence of one or two copies of the d(CA) tract was unable to impart transcriptional activity to pIL2 (-103). Furthermore, when one to three copies of this element are inserted upstream of the expressible (-321) construct, the resulting plasmids showed a lower level of CAT activity than did the unmodified parental plasmids. Thus, rather than possessing positive enhancing activity, the poly d(CA) region could account for the lower level of expression seen with pIL2 (-1219). To test alternative sites for the negative element(s) in this region, we examined two additional constructs, pIL2 (-753) and pIL2 (-1219; Δ-1002 to -579). Results (not shown) revealed that both pIL2 (-753) and pIL2 (-1219; Δ-1002 to -579) were expressed as well as pIL2 (-578), thus implicating the poly d(CA) tract as most likely to account for the negative effect on IL2 promoter function.

Third, two novel positive regulatory elements appear further upstream, with their effects detected more clearly in the conspecific EL4.E1 line than in the human Jurkat cells. The negative effect of sequences between -1219 and -578 could be reversed by extending the 5' boundary of IL2 sequences as little as 113 bp upstream, suggesting the presence of a positive element in this interval. Both pIL2 (-1332) and pIL2 (-1449) showed higher levels of expression than pIL2 (-1219)

(Fig. 7). At least one other possible positive regulator of IL2 gene expression lies further upstream. Inclusion of the sequences between -1449 and -1890 reproducibly resulted in the highest level of IL2-promoted CAT activity achieved in EL4.E1 cells. Note that this region includes the highly conserved sequences between -1466 and -1492, between -1645 and -1673, and between -1694 and -1710, as described above. An additional increase to -2800 had a modest negative effect in EL4.E1, which is more substantial in human Jurkat cells.

In general, the positive or negative effects on expression of each increment of 5' flanking sequence were observed both in murine EL4.E1 cells and in human Jurkat cells. However, as shown in Fig. 7, the two lines differ in their relative strengths of recorder gene expression from the IL2(-578)-CAT and IL2(-1890)-CAT constructs, with EL4.E1 showing considerably higher expression from the longer constructs. Whether this reflects a cell-type-specific (or species-specific) positive regulatory element, or alternatively a systematic difference in the utilization of longer fragments of exogenous DNA, is not presently resolved.

Transcriptional enhancers have been discovered 3' to the mouse T-cell receptor $C_\beta 2$ locus (Krimpenfort *et al.*, 1988) and CD3δ gene (Georgopoulos *et al.*, 1988) and the human CD2 gene (Greaves *et al.*, 1989). We therefore tested the possibility that additional transcriptional regulatory sequences reside within or 3' to the IL2 gene by subcloning different fragments of genomic DNA (Fig. 1A) into pIL2 (-1890) as described in Methods. In these constructs, the genomic fragments were inserted 0.6 kb downstream of the two SV40 derived polyadenylation sites, and therefore were not included in the IL2/CAT transcription unit. In this way, 5.0 kb of 3' flanking DNA and all but 100 bp of intragenic DNA were tested. All of these constructs were expressed at a lower level than the parent plasmid from which they were derived (Table II). Clearly there are no positive regulatory elements in these regions that can act in a position-independent way. These

results do not, however, identify sequence-specific negative regulatory regions, because the degree of inhibition seemed primarily related to the length of the inserted DNA. While the relative reductions seen in CAT activity greatly exceed the decreases in the molar concentration of plasmid DNA in the transfection protocol, it remains possible that nonspecific mechanisms were responsible for limiting expression. The more dramatic negative effect of B (second intron) may, however, indicate that some sequences limiting IL2 expression are located outside of the 5' flanking region.

Control of Tissue-Specific IL2 Expression is Mediated Through the 5' Flanking DNA by a T_H1-Specific Signalling Pathway

Normally, IL2 gene transcription appears to be restricted to activated T_H1 cells, even though these cells share a common precursor with all hematopoietic cells. To test the roles of IL2 regulatory sequences in maintaining cell-type specificity, the series of pIL2-CAT plasmids was transfected into a variety of hematopoietic and non-hematopoietic cell lines by DEAE-dextran facilitation, as shown in Table III. The panel included other T-cell types, such as T_H2 cells and CTLs, in addition to non-T cells. To ensure delivery of an activation signal we used agonists of the universal phosphoinositide pathway, namely the calcium ionophore A23187 and the phorbol ester TPA. After an 18 hr stimulation with TPA + A23187, the cells were harvested and assayed for the presence of CAT.

None of the plasmids was expressed in any cell line tested except EL4.E1 and Jurkat, although only the results for pIL2 (-1890) are shown here. This lack of expression by other cell lines is not due to their refractivity to transfection or to an inability to respond to TPA + A23187. All the cells listed express easily detectable amounts of CAT protein when transfected with a control plasmid, pRSV-CAT, and several of them (32D cl 5, S49.1, our subline of BW5147, and

others) possess an activation pathway capable of elevating expression from the RSV LTR upon stimulation. The successful stimulation of the HT-2, 32D cl 5, and D10.G4.1 lines was demonstrated by their synthesis of endogenous IL4 RNA under these conditions (Fig. 8 and data not shown). Table III shows that even the combination of transfectability and inducibility in these cell lines is not sufficient to permit expression from any IL2 promoter fragment, even when introduced as naked DNA. Thus, cell-type specific expression of IL2 appears to depend upon a signalling response specific to T_H1-type lymphomas.

Modulation of IL2 Gene Expression by Competing Signal Transduction Pathways

The positive and negative effects of the distal sequences we have described may result from two general mechanisms. They may be sites for generalized amplification or silencing of the induced response, or they may be sites for mediators of distinct, auxiliary signaling pathways. In the latter case, constructs containing different extents of flanking sequence may behave differentially when cells are treated with A23187 and TPA under conditions that antagonize endogenous IL2 induction.

In general, the different extents of 5' flanking sequence affected the magnitude but not the kinetics of CAT expression from the IL2-CAT constructs after induction. Comparison of the relative levels of CAT activity expressed at 6 hr, 9 hr, and 24 hr of induction showed indistinguishable kinetics with any of the plasmids (data not shown). Thus the positive and negative effects on expression appear to be kinetically subordinate to a common time course of stimulation. We attempt to dissect this stimulatory pathway in the following.

IL2 gene expression is known to be influenced by several physiological and pharmacological agents. Among these, glucocorticosteroids, cyclosporin A (CsA) and elevators of intracellular cAMP have a suppressive effect on IL2 expression

(Gillis *et al.*, 1979; Krönke *et al.*, 1984; Novogrodsky *et al.*, 1983), while interleukin 1 (IL1) and anti-CD28 mAb act to enhance IL2 production (Truneh *et al.*, 1986; Martin *et al.*, 1986). We tested whether such effects were mediated by binding of proteins to discrete regions of upstream DNA by transfecting pIL2-CAT plasmids into EL4.E1 and Jurkat cells, and then stimulating the cells in the presence or absence of these agents.

We were unable to evaluate the potential role of the four upstream GREs. Dexamethasone concentrations up to 10 μM had very little effect on the accumulation of steady-state IL2 mRNA in either Jurkat or EL4.E1 cells (see below, Fig. 10 and data not shown). This may be due to the fact that these lines have been passaged for many years in medium supplemented with steroid-containing serum. The comparison of pIL2 (-321), which has no upstream GREs, to pIL2 (-2800), which has all four, would have been informative. This experiment awaits identification of a Dex-sensitive T-cell line that can express detectable amounts of CAT from the IL2 promoter after transfection.

CsA is a fungal metabolite with potent immunosuppressive activity. It has been shown to block IL2 gene trancription in cells induced through a variety of stimulation pathways (i.e., Con A, anti-TcR/CD3 MAb, and TPA + A23187; Shevach, 1985). At 1 μg/ml CsA prevents accumulation of steady-state IL2 and IL4 RNA in EL4.E1 cells and IL2 RNA in Jurkat (Krönke *et al.*, 1984; Elliott *et al.*, 1984; data not shown). At this same dose CsA completely blocked expression of all pIL2-CAT plasmids in transfected cells, assayed after 18 hr of stimulation with TPA + A23187 (Fig. 9). This block could not be removed by decreasing the amount of IL2 5'-flanking DNA down to -321. This is consistent with studies on the human IL2 promoter in which no discrete CsA-sensitive site could be found between -548 and -42 when a number of internal deletion mutants were tested (Williams *et al.*, 1988). The same concentration of CsA that completely ablated pIL2-CAT

activity, however, further enhanced expression of pRSV-CAT by a factor of two (Fig. 9). Thus, the suppressive effects on the pIL2-CAT plasmids are not attributable to a generalized down-regulation of activation, transcription, or translation. This emphasizes the point that the same intracellular signal in the same cell population can give different results on induction, depending on the inducible gene being studied.

Cyclic AMP is a more selective antagonist of IL2 gene expression that works, presumably, through protein kinase A phosphorylation of an unknown substrate protein. The cAMP agonists forskolin (10 μM), dBcAMP (1 mM), and PGE_1 (1 μM) all prevent accumulation of detectable steady-state IL2 RNA in EL4.E1 and Jurkat cells examined 5 hr after stimulation with TPA + A23187 (Fig. 10). Unlike CSA, these agents have very little effect on the induction of IL4 RNA in EL4.E1 cells (Fig. 10). Thus, the sensitivity of IL2 to cAMP appears to reflect a highly specific activation pathway. Figure 9 shows the results of elevating cAMP levels in three experiments in which pIL2-CAT plasmids were transfected into both cell lines along with pRSV-CAT as a control. Note, again, that in EL4.E1 cells pRSV-CAT expression was <u>increased</u> fourfold in the presence of cAMP agonists. By contrast, expression of all the pIL2-CAT plasmids was suppressed by 50-80%, relative to their drug-free controls.

The greater inhibitory effect seen at 18 hr in the experiment shown with Jurkat (>75% inhibition) as compared to EL4.E1 (~50%) was not due to the relative efficacy of dBcAMP vs. forskolin, but rather reflected the slower kinetics of IL2-CAT induction in Jurkat cells (TJN and EVR, unpublished observations). CAT activity can be detected in EL4.E1 cells as early as 5 hr post-stimulation and reaches a maximum between 15 hr and 18 hr. In Jurkat, however, no CAT can be detected until ~9 hr post-stimulation and maximum activity is not reached until ~20 hr. When extracts of stimulated EL4.E1 cells were assayed at 5 hr rather than

18 hr, 10 μM forskolin also caused more severe inhibition (≥70%; data not shown). Results similar to those in Fig. 9 were also achieved with 1 μM PGE_1 (data not shown).

In spite of the varying degrees of inhibition observed under different conditions, all constructs were affected, within error, to the same extent (Fig. 9). The degree of suppression was neither reduced nor intensified by altering the amount of IL2 5' DNA contained in the constructs. These results indicate that the negative regulatory sites we have identified upstream of -321 are not specific targets of the cAMP pathway blocking IL2 induction. This conclusion is in agreement with the absence of identifiable CREB sites in the entire 2800 bp of 5' flanking region. Conversely, none of the positive regulatory elements upstream of -321 appear to mediate signals that are either unusually cAMP-sensitive or cAMP-resistant. Thus, the effect of cAMP on IL2 transcription *per se* is likely to be mediated by interference with the generation of a central inductive signal, rather than by summation of the effects of discrete, independently regulated negative and positive transcription factors.

DISCUSSION

Structure of the Murine IL2 5' Flanking Region

In order to understand the molecular basis for the restricted expression of the murine IL2 gene we have cloned the gene and determined the nucleotide sequence of the 5' flanking region. Our upstream sequence extends to -2800 relative to the start site of transcription (Fig. 2); this is 2300 bp further upstream than sequences previously reported (Fuse *et al.*, 1984). This region showed no extended homology to any other murine lymphokine gene. Comparison of this sequence to that previously determined for the human IL2 gene (Holbrook *et al.*, 1984b) revealed extensive similarity from -1 to -650 (Fig. 3). The regions between -1 and -580 are 86% identical throughout their length, comparible to the degree of upstream similarity between other mouse and human lymphokine genes. In general, however, the highly similar flanking regions of other pairs of lymphokine genes are less than 350 bp long. Thus, the evolutionarily conserved sequence in the IL2 5' flanking region extends ~350 bp beyond the sequences previously reported to be required for maximal induction of the human IL2 promoter (Fujita *et al.*, 1986; Durand *et al.*, 1988; Williams *et al.*, 1988), with patches of similarity resuming further upstream. The extensive conservation suggests a role for multiple regulatory sequence elements in the relatively complex control of IL2 gene expression.

Deletion analysis of the mouse IL2 upstream region suggests a net positive role for the conserved sequences between of -351 and -578. Transfection experiments utilizing IL2 promoter-CAT constructs demonstrated that an increase in 5' DNA from -351 to -578 was correlated with an increase in inducible CAT activity (Fig. 7). This is in accord with the results of Williams *et al.* (1988) showing that this region could successfully compensate for deletions between -162

and -289. Studies of the human gene, however, have not previously shown this positive effect to be additive with that of sequences up to -321. That sequences upstream of -351 play a role in regulating IL2 gene expression is further supported by experiments of Nabel et al. (1988) using various upstream regions of the human IL2 gene as probes in gel mobility shift assays.

Further upstream, the murine and human sequences diverge, but regulatory effects of specific sequences are still seen. A significant positive element or elements can be correlated with a region where several islands of excellent mouse-human similarity reappear (between -1440 and -1890). Another likely positive element ocurs in the region between -1219 and -1332. On the other hand, a striking aspect of the mouse IL2 upstream region is the 250 bp poly d(CA) motif from -1000 to -750, apparently associated with a negative regulatory element. Stretches of alternating purines and pyrimidines are found upstream of a number of murine lymphokine genes (e.g., IL6, GM-CSF) (Tanabe *et al.*, 1988; Miyatake *et al.*, 1985) and in their large introns (*e.g.*, IL4, IL6; the large IL2 intron remains unsequenced to date) (Otsuka *et al.*, 1987). Their absence from the human counterparts to these genes is intriguing, given that poly d(GT)•poly d(CA) stretches are present at ~10^5 copies per human genome. As yet, no function has been ascribed to these motifs in lymphokine genes. However, synthetic oligodeoxynucleotides and cloned human genomic fragments of the type d(GT)•d$(AC)_{30-130}$ have been shown to possess weak transcriptional enhancing activity in fibroblasts when cloned either upstream or downstream of a truncated SV40 early promoter (Hamada *et al.*, 1984). Because the presence of the poly d(CA) motif is correlated with lower levels of expression from the IL2 promoter [Table I, and compare pIL2 (-578) and pIL2 (-1219), Fig. 7] it is possible that it acts either as an enhancer or as a silencer, in a cell-type specific way. In any event, the absence of such motifs from the upstream regions of all human lymphokine

genes sequenced to date suggests that they may not be indispensible for expression, although the possibility that they can modulate transcription remains open (Santoro *et al.*, 1984).

Role of Regulatory Sequences in Control of IL2 Gene Expression

What is the significance of these multiple sites? One possible answer is that particular sequences in the IL2 regulatory region confer cell-type specificity by interaction with *trans*-acting repressors or cell-type specific activators, while other required sequence elements mediate responses to general cell activation. Physical segregation of the two types of elements might then result in relaxation of induction-dependence or of cell-type specificity. Alternatively, it is possible that mature T cells, regardless of their type, utilize the same *trans*-acting transcriptional factors, and that lineage-specific gene expression results from inactivation of inappropriate genes at the chromatin level. If this were true, it might be expected that the introduction of an IL2-CAT gene as a supercoiled, naked DNA template into an IL2 non-producing T-cell line would result in inappropriate expression from the exogenous IL2 promoter. Finally, if all mandatory activation pathways for IL2 were themselves tissue-specific, then all expressible IL2-CAT constructs should be equally dependent for expression on stimulation and cell type.

The data we present here fail to confirm the first model; refute the second model; and generally support the third. The results in Table III are clearly inconsistent with a common set of T-cell transcription factors, since many T-cell lines could not express any IL2 promoter construct, even though introduced as naked DNA. Even helper T-cell responses to phorbol ester/calcium ionophore stimulation cannot be mediated by a single set of *trans*-acting factors, because the nonexpressors included cell lines capable of expressing other lymphokine

RNAs, *e.g.* IL4, in response to stimulation. The implication is that IL2 and IL4, which have a broader cell-type distribution than IL2, are induced through distinguishable sets of transcriptional regulators. This conclusion is further supported by the pharmacological data, in Fig. 10, that even within the same clonal cell population the two lymphokine RNAs show differential sensitivity to inhibition by cAMP. Altogether the ability to express any of our IL2-CAT constructs appears to be dependent on the presence of a very specific response pathway.

An important result of our investigations was the inability to segregate any of the determinants of induction-dependence or pharmacological sensitivity from expression *per se*. By contrast, Serfling *et al.* (1989) have suggested that when IL2 upstream sequences are combined with a heterologous promoter, the resulting expression is somewhat less induction-dependent when shorter (~300 bp) IL2 sequences are used than with longer (~2000 bp) ones. This relaxed expression with shorter fragments may be due to read-through transcription by RNA Pol II. For example, aberrant transcripts initiating from upstream plasmid sites have been detected with constructs containing smaller but not larger fragments of a mouse rRNA promoter (Lopata *et al.*, 1986). Yet, in our experiments using the natural IL2 promoter with its regulatory sequences, it is striking that all constructs from pIL2 (-321) to pIL2 (-2800) were induced or inhibited, with indistinguishable kinetics, although to different maximal levels, in parallel. These results strongly suggest that the overall progress of IL2 promoter induction is governed by a single rate-limiting mechanism, to which all the specific positive and negative binding these upstream sites are subordinate. With respect to the logic of the triggering signal, these sites appear to be largely redundant.

The notion of distinct regulatory elements for induction and tissue specificity cannot be rejected decisively because of our limited data on more

proximal sites. Our pIL2 (-232) construct was expressed so poorly that the fine points of its regulation could not be assessed. This result shows, at least, that the presence of sites for the activation factors NF-κB and AP-1 (Hoyos *et al.*, 1989; Muegge *et al.*, 1989; Serfling *et al.*, 1989) is not sufficient to drive significant levels of IL2 promoter expression. The lack of data means that unique roles for these sites, for the NFAT-1 site excluded from this construct (around -270), and for the upstream NF-IL2A sites (around -240) cannot be ruled out. All of these "downstream" regulatory sequences have been studied extensively by other laboratories, however. While our 5' deletion analysis might imply that the NFAT-1 site is uniquely potent in conferring inducibility, Williams *et al.* (1988) have shown that this site, too, can be deleted entirely from the human gene with no loss of expression, provided that additional upstream sequences are present (Williams *et al.*, 1988). Thus, NFAT-1 also may be functionally redundant with some of the upstream sequences that seem to lack individually distinct roles.

In conclusion, our analysis shows that the flanking sequences of the IL2 gene contain many sites of potential regulatory significance, extending over 1890 bp upstream of the transcriptional start site. In the two cell types in which we have studied their regulatory significance, these sites appear to be weak silencers and weak, induction-dependent enhancers, fully subordinate to a powerful, IL2-specific induction mechanism. The cell-type specificity of this activation mechanism could plausibly be the major determinant of competence to express the IL2 gene in hematopoietic cells.

SUMMARY

We have cloned the mouse IL2 gene and sequenced 2000 bp of 5' flanking DNA. Comparison to the previously reported human sequence revealed extensive identity (~86%) between the two gene from +1 to -530 with only small islands of homology further upstream. Sites that have been shown to be important in regulation of the human IL2 gene are well conserved in sequence and location. Transfection experiments using hybrid gene constructs containing varying lengths of the mouse 5' flanking DNA linked to a CAT reporter gene have demonstrated the presence of several positive and negative regulatory elements. One negative region lying between -750 and -1000 consists primarily of alternative purines and pyrimidines and is absent from the human gene. All the elements identified affect only the magnitude of the inducible response. No region when deleted had the effect of relaxing any of the following: 1) the need for induction, 2) cell-type specificity of expression, or 3) the suppression due to cAMP or CsA. Therefore we conclude that IL2 gene expression is controlled primarily through a T_H1-specific signaling pathway upon which secondary cis-elements exert their effect. Finally we found no evidence for intragenic or downstream regulatory elements in this gene.

Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, M. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin. 1987. Phorbol ester-inducible genes contain a common Cis element recognized by a TPA-modulated trans-acting factor. Cell **49**:729-739.

Brown, D. D. 1984. The role of stable complexes that repress and activate eucaryotic genes. Cell **37**:359-365.

Brunvand, M. W., A. Schmidt and U. Siebenlist. 1988. Nuclear factors interacting with the mitogen-responsive regulatory region of the interleukin-2 gene. J. Biol. Chem. **263**:18904-18910.

Chen, W. F., R. Scollay, and K. Shortman. 1983. The Ly phenotype of functional medullary thymocytes. Thymus **5**:197-207.

Crabtree, G. 1989. Contingent genetic regulatory events in T lymphocyte activation. Science **243**:355-361.

Davis, M. M., K. Calame, P. W. Early, D. L. Livant, R. Joho, I. L. Weissman, and L. Hood. 1980. An immunoglobulin heavy-chain gene is formed by at least two recombination events. Nature **283**:733-739.

Deans, R. J., K. A. Davis, A. Taylor, and R. Wall. 1984. Expression of an immunoglobulin heavy chain gene translated into lymphocytes. Proc. Natl. Acad. Sci. USA **81**:1292-1296.

Devos, R., G. Plaetinck, H. Cheroutre, G. Simons, W. Degrave, J. Tavernier, E. Remant, and W. Fiers. 1983. Molecular cloning of human interleukin 2 cDNA and its expression in E. coli. Nucl. Acids Res. **11**:4307-4323.

Durand, D. B., J.-P. Shaw, M. R. Bush, R. E. Replogle, R. Belagaje, and G. R. Crabtree. 1988. Characterization of antigen receptor response elements within the interleukin-2 enhancer. Mol. Cell. Biol. **8**:1715-1724.

Elliott, J. F., Y. Lin, S. B. Mizel, R. C. Bleackley, D. G. Harnish, and V. Paetkau. 1984. Induction of interleukin 2 messenger RNA inhibited by cyclosporin A. Science **226**:1439-1441.

Favaloro, J., R. Treisman, and R. Kamen. 1980. Transcription maps of polyoma virus-specific RNA: Analysis by two-dimensional nuclease S1 gel mapping. Meth. Enzymol. **65**:718-749.

Fujita, T., H. Shibuya, T. Ohasi, K. Yamanishi, and T. Taniguchi. 1986. Regulation of human interleukin-2 gene: Functional DNA sequences in the 5' flanking region for the gene expression in activated T lymphocytes. Cell **46**:401-407.

Fuse, A., T. Fujita, H. Yasumitsu, N. Kashima, K. Hasegawa, and T. Taniguchi. 1984. Organization and structure of the mouse interleukin-2 gene. Nucl. Acids Res. **12**:9323-9331.

Georgopoulos, K., P. van den Elsen, E. Bier, A. Maxam, and C. Terhorst. 1988. A T cell-specific enhancer is located in a DNase 1-hypersensitive area at the 3' end of the CD3-δ gene. EMBO J. **7**:2401-2407.

Gillis, S., G. R. Crabtree, and K. A. Smith. 1979. Glucocorticoid-induced inhibition of T cell growth factor production. I. The effect on mitogen-induced lymphocyte proliferation. J. Immunol. **123**:1624-1631.

Greaves, D. R., F. D. Wilson, G. Lang, and D. Kioussis. 1989. Human CD2 3'-flanking sequences confer high-level T cell-specific, position independent gene expression in transgenic mice. Cell **56**:979-986.

Hagiwara, H., T. Yokota, J. Luh, F. Lee, K.-I. Arai, N. Arai, and A. Zlotnik. 1988. The AKR thymoma BW5147 is able to produce lymphokines when stimulated with calcium ionophore and phorbol ester. J. Immunol. **140**:1561-1565.

Hamada, H., M. Seidman, B. H. Howard, and C. M. Gorman. 1984. Enhanced gene expression by the poly (dT-dG)•poly (dC-dA) sequence. Mol. Cell. Biol. **4**:2622-2630.

Haniford, D. B. and D. E. Pulleyblank. 1983. Facile transition of poly [d(TG)•(CA)] into a left-handed helix in physiological conditions. Nature (London) **302**:632-634.

Holbrook, N. J., K. A. Smith, A. J. Fornace, Jr., C. M. Comeau, R. L. Wiskocil, and G. R. Crabtree. 1984a. T-cell growth factor: Complete nucleotide sequence and organization of the gene in normal and malignant cells. Proc. Natl. Acad. Sci. USA **81**:1634-1638.

Holbrook, N. J., M. Lieber, and G. R. Crabtree. 1984b. DNA sequence of the 5' flanking region of the human interleukin 2 gene: Homologies with adult T-cell leukemia virus. Nucl. Acids Res. **12**:5005-5013.

Hoyos, B., D. W. Ballard, H. Böhnlein, M. Siekevitz, and W. C. Greene. 1989. Kappa B-specific DNA binding proteins. Role in the regulation of human interleukin-2 gene expression. Science **244**:457-460.

Kaye, J., S. Porcelli, J. Tite, B. Jones, and C. A. Janeway, Jr. 1983. Both a monoclonal antibody and antisera specific for determining unique to individual cloned helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells. J. Exp. Med. **158**:836-856.

Krimpenfort, R., R. de Jong, Y. Uematsu, Z. Dembić, S. Ryser, H. von Boehmer, M. Steinmetz, and A. Berns. 1988. Transcription of T-cell receptor β-chain genes is controlled by a downstream regulatory element. EMBO J. **7**:745-750.

Krönke, M., W. J. Leonard, J. M. Depper, S. K. Arya, F. Wong-Staal, R. C. Gallo, T. A. Waldmann, and W. C. Greene. 1984. Cyclosporin A inhibits T-cell growth factor gene expression at the level of mRNA transcription. Proc. Natl. Acad. Sci. USA **81**:5214-5218.

Krönke, M., W. J. Leonard, J. M. Depper, and W. C. Greene. 1985. Sequential expression of genes involved in human T lymphocyte growth and differentiation. J. Exp. Med. **161**:1593-1598.

Lee, F., T. Yokota, T. Otsuka, P. Meyerson, D. Villaret, R. Coffman, T. Mosmann, D. Rennick, N. Roehm, C. Smith, A. Zlotnick, and K.-I. Arai. 1986. Isolation and characterization of a mouse interleukin cDNA clone that expresses B-cell stimulatory factor 1 activities and T-cell- and mast-cell-stimulating activities. Proc. Natl. Acad. Sci. USA **83**:2061-2065.

Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb. 1986. RNA polymerase specificity of mRNA production and enhancer action. Proc. Natl. Acad. Sci. USA **83**:6677-6681.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: A Laboratory Manual. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Maniatis, T., S. Goodbourn, and J. A. Fischer. 1987. Regulation of inducible and tissue-specific gene expression. Science **236**:1237-1245.

Martin, P. J., J. A. Ledbetter, Y. Morishita, C. H. June, P. G. Beatty, and J. A. Hansen. 1986. A 44 kDa cell surface homodimer regulates interleukin-2 production by activated human T lymphocytes. J. Immunol. **136**:3282-3287.

Maxam, A. and W. Gilbert. 1980. Sequencing end-labelled DNA with base-specific chemical cleavages. Meth. Enzymol. **65**:499-560.

McGuire, K. L., and E. V. Rothenberg. 1987. Inducibility of interleukin 2 RNA expression in individual mature and immature T lymphocytes. EMBO J. **6**:939-946.

McGuire, K. L., J. A. Yang, and E. V. Rothenberg. 1988. Influence of activating stimulus on functional phenotype: Interleukin 2 mRNA accumulation differentially induced by ionophore and receptor ligands in subsets of murine T cells. Proc. Natl. Acad. Sci. USA **85**:6503-6507.

Miyatake, S., T. Otsuka, T. Yokota, F. Lee, and K.-I. Arai. 1985. Structure of the chromosomal gene for granulocyte-macrophage colony stimulating factor: Comparison of the mouse and human genes. EMBO J. **4**:2561-2568.

Mizouchi, T. and A. Singer. 1988. Intrathymic differentiation and tolerance induction of lymphokine-secreting Lyt-2^+ T helper cells. J. Immunol. **140**:1421-1424.

Mosmann, T. R. and R. L. Cottman. 1989. T_H1 and T_H2 cells: Different patterns of lymphokine secretion lead to different functional properties. Ann. Rev. Immunol. **7**:145-173.

Nabel, G. J., C. Gorka, and D. Baltimore. 1988. T-cell-specific expression of interleukin 2: Evidence for a negative regulatory site. Proc. Natl. Acad. Sci. USA **85**:2934-2938.

Novak, T. J., and E. V. Rothenberg. 1986. Differential transient and long-term expression of DNA sequences introduced into T-lymphocyte lines. DNA **5**:439-451.

Novogrodsky, A., M. Patya, A. L. Rubin, and K. H. Stenzel. 1983. Agents that increase cellular cAMP inhibit production of interleukin-2 but not its activity. Biochem. Biophys. Res. Comm. **114**:93-98.

Otsuka, T., D. Villaret, T. Yokota, Y. Takebe, F. Lee, N. Arai, and K.-I. Arai. 1987. Structural analysis of the mouse chromosomal gene encoding interleukin 4 which expresses B cell, T cell and mast cell stimulating activities. Nucl. Acids Res. **15**:333-334.

Rothenberg, E. V., R. A. Diamond, K. A. Pepper, and J. A. Yang. 1990. Interleukin-2 gene inducibility in T cells prior to T-cel receptor expression: Changes in signaling pathways and gene expression requirements during intrathymic maturation. J. Immunol., in press.

Santoro, C., F. Costanzo, and G. Ciliberto. 1984. Inhibition of eukaryotic tRNA transcription by potential Z-DNA sequences. EMBO J. **3**:1553-1559.

Scheidereit, C., S. Geisse, H. M. Westphal, and M. Beato. 1983. The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of the mouse mammary tumour virus. Nature (London) **304**:749-752.

Serfling, E., R. Barthelmas, I. Pfeuffer, B. Schenk, S. Zarius, R. Swoboda, F. Mercurio, and M. Karin. 1989. Ubiquitous and lymphocyte-specific factors are involved in the induction of the mouse interleukin 2 gene in T lymphocytes. EMBO J. **8**:465-473.

Shaw, J.-P., P. J. Utz, D. B. Durand, J. J. Toole, E. A. Emmel, and G. R. Crabtree. 1988. Identification of a putative regulator of early T-cell activation genes. Science **241**:202-205.

Shevach, E. M. 1985. The effects of cyclosporin A on the immune system. Ann. Rev. Immunol. **3**:397-423.

Tanabe, O., S. Akira, T. Kamiya, G. G. Wong, T. Hirano, and T. Kishimoto. 1988. Genomic structure of the murine IL-6 gene. High degree of conservation of potential regulatory sequences between mouse and human. J. Imunol. **141**:3875-3881.

Taniguchi, T., H. Matsui, T. Fujita, C. Takaoka, N. Kashima, R. Yoshimoto, and J. Hamuro. 1983. Structure and expression of a cloned cDNA for human interleukin-2. Nature (London) **302**:305-310.

Truneh, A., F. Albert, P. Golstein, and A.-M. Schmitt-Verhulst. 1985. Early steps of lymphocyte activation bypassed by synergy between calcium ionophores and phorbol ester. Nature (London) **313**:318-320.

Truneh, A., P. Simon, and A.-M. Schmitt-Verhulst. 1986. Interleukin 1 and protein kinase C activator and dissimilar in their effects on IL-2 receptor expression and IL-2 secretion by T lymphocytes. Cell Immunol. **103**:365-374.

Von Boehmer, H., H. S. Teh, and P. Kisielow. 1989. The thymus selects the useful, neglects the useless and destroys the harmful. Immunology Today **10**:57-61.

Weiss, A., J. Imboden, K. Hardy, B. Manger, C. Terhorst, and J. Stobo. 1986. The role of T3/antigen receptor in T-cell activation. Ann. Rev. Immunol. **4**:593-619.

Wigler, M., A. Pellicer, S. Silverstein, and R. Axel. 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. Cell **14**:725-731.

Williams, T. M., L. Eisenberg, J. E. Burlein, C. A. Norris, S. Pancer, D. Yao, S. Burger, M. Kamoun, and J. A. Kant. 1988. Two regions within the human IL-2 gene promoter are important for inducible IL-2 expression. J. Immunol. **141**:662-666.

Yang, Y.-C., and S. C. Clark. 1987. Molecular cloning and characterization of the human gene for interleukin-3. *In*: Molecular Basis of Lymphokine Action, D. R. Webb, C. W. Pierce, and S. Cohen, eds. Humana Press, Clifton, NJ, pp. 325-337.

Yokota, T., N. Arai, F. Lee, D. Rennick, T. Mossmann, and K.-I. Arai. 1985. Use of a cDNA expresison vector for isolation of mouse interleukin 2 cDNA clones: Expression of T-cell growth-factor activity after transfection of monkey cells. Proc. Natl. Acad. Sci. USA **82**:68-72.

Table I. The poly(dCA)•poly(dGT) tract lacks transcriptional enhancing activity in EL4.E1 cells

Plasmid	Insert[a]	Relative CAT activity[b]
pIL2 (-103)	-	0
"	1 x CA (+)	0
"	1 x CA (-)	0
"	2 x CA	0
pIL2 (-321)	-	1.0
"	1 x CA (+)	1.0±0.3
"	1 x CA (-)	0.78±0.01
"	2 x CA	0.48±0.08
"	3 x CA	0.42±0.01

[a]The number of copies of the 260 bp HinFI-SspI poly(dCA) fragment is indicated. (+) or (-) indicates the orientation of this fragment relative to its normal location in the IL2 upstream region. The orientation was not determined for plasmids containing multiple copies.

[b]Plasmids were transfected into EL4.E1 cells along with pBR322 as a negative control. Relative CAT activity was determined as indicated in Table III after 18 h stimulation with 10 ng/ml TPA + 37 ng/ml A23187. Results are the mean ± range of two experiments.

Table II. Absence of intragenic or 3' enhancer elements in the mouse IL2 gene

Plasmid		Insert size (kb)	Relative CAT activity[a]
pIL2 (-1890)			1.0
"	A(+)	0.35	0.54±0.12
"	B(+)	2.3	0.23±0.08
"	C(+)	1.8	0.40±0.17
"	D(+)	0.9	0.50±0.11
"	E(+)	1.1	0.43±0.08
"	E(-)	1.1	0.53±0.12
"	F(+)	1.8	0.71±0.27
"	F(-)	1.8	0.49±0.16
"	G(+)	2.0	0.48±0.18
"	G(-)	2.0	0.53±0.20

[a]CAT activity in extracts of cells stimulated for 18 h with TPA + A23187 was determined as in Table III except that values were normalized with respect to pIL2 (-1890). Results are given as mean ± range of two experiments for fragments A-D and three experiments for fragments E-G.

Table III. pIL2-CAT expression is cell-type specific and dependent on both cell-type and gene-specific inductive signals

Cell line	Cell type	Endogenous mRNA[a] IL2	IL4	pRSV-CAT activity[b] -	+	Fold induction	pIL2(-1890) CAT activity[b] -	+
NS-1 B-cell		-	-	0.33	0.33	1.0	0	0
32D cl 5	pre-mast cell	-	+	3.8	14.3	3.8	0	0
$p388D_1$	macrophage	-	-	0	0.1	∞	0	0
HT-2	T_H2	-	+	0.55	3.3	6.0	0	0
D10.G4.1	T_H2	-	+	N.D.[c]	0.28	-	0	0
S49.1	Thymoma	N.D.	N.D.	0.012	0.040	3.3	0	0
BW5147	Thymoma	-	-	0.7	4.2	6.0	0	0
MTL2.8.2	CTL	-	-	9.9	9.3	0.9	0	0
CTLL-2	CTL	-	-	9.7	19.3	2.0	0	0
Ltk^-	Fibroblast	-	-	17	22	1.3	0	0
EL4.E1	Thymoma	+	+	0.35	3.4	10	0	0.42
Jurkat	T_H1	+	N.D.	8.7	12.8	1.5	0	4.1

a Cytoplasmic RNA was extracted after 5 h stimulation with 10 ng/ml TPA + 37 ng/ml A23187 and analyzed as described in Materials and Methods.

b CAT activity is presented as % acetylation per $1x10^6$ cell equivalents in a 5 h assay, except for Ltk^- extracts which were assayed for 30 min. - = unstimulated; + = 18 h stimulation with 10 ng/ml TPA + 37 ng/ml A23187, except for D10.G4.1 cells which were cultured at $2x10^5$ per ml for 45 h with $6.7x10^5$ mitomycin C-treated spleen cells and 100 μg conalbumin per ml. 0 = < 0.05

c Not done

Fig. 1. Cloning the mouse IL2 gene and sequencing of its 5' flanking region. (A) (i) Top lines (A-G) show the intragenic and 3' fragments that were tested for the presence of transcriptional regulators. (ii) The organization of the mouse IL2 gene is shown. Thin line denotes chromosome; thick boxes represent exons. Arrow marks start site and direction of transcription. Letters under chromosome represent restriction enzyme sites relevant to the constructions. R = EcoRI, P = PvuII, H = HindIII, As = Asp718 and RV = EcoRV. (iii) Thick lines below exons 3 and 4 indicate the sequences included in the 3' cDNA probe used in library screen. (iv) Regions represented in genomic clones are diagrammed. (B) Expanded view of exons 1 and 2 and 5' flanking DNA showing the sequencing strategy employed. Letters designate restriction enzyme sites. R = EcoRI, Rs = RsaI, A = AccI and Ps = PstI.

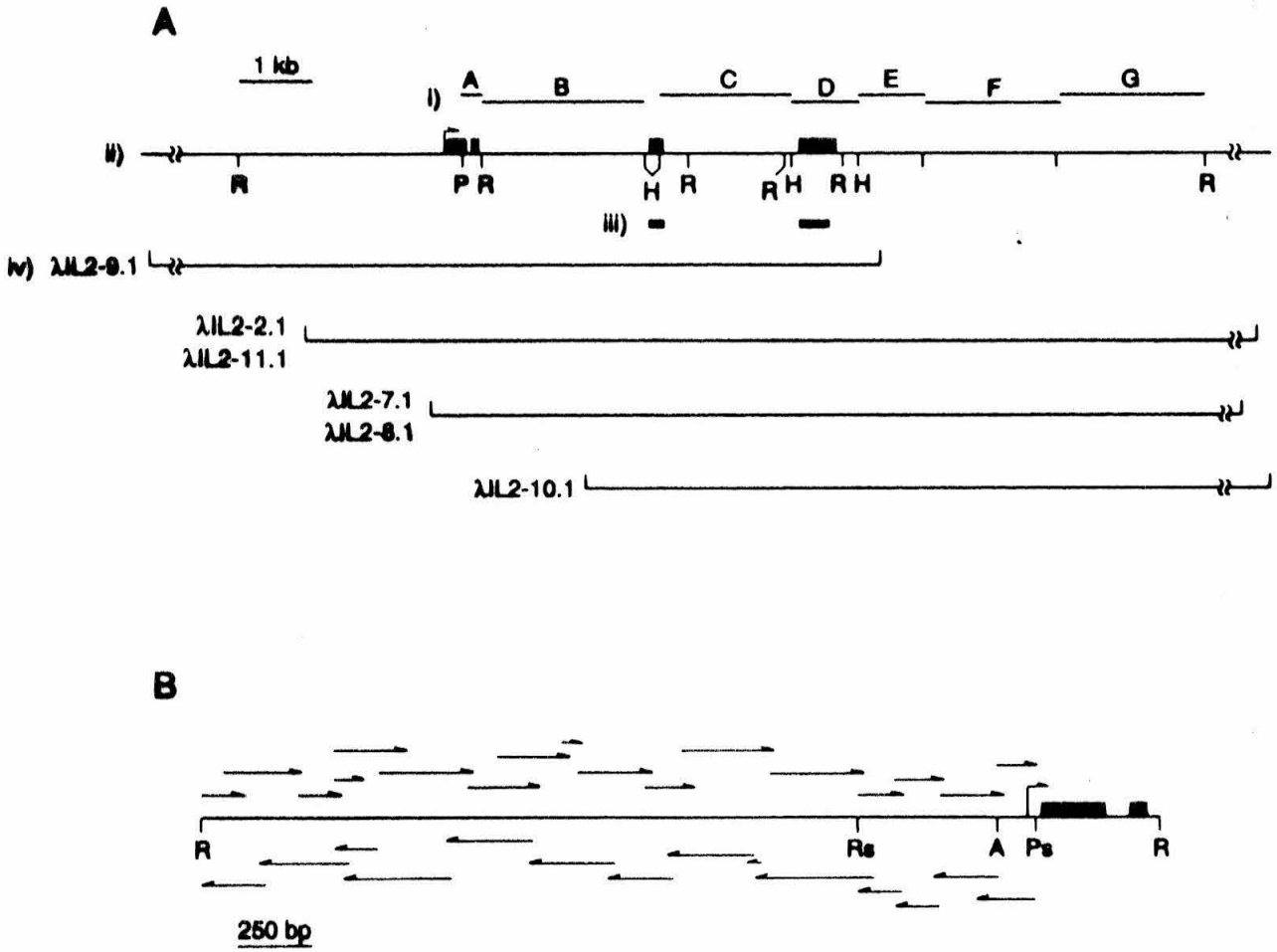

A
1 kb
i)
A
B
C
D
E
F
G
ii)
R
P R
H
R
R H
R H
R
iii)
iv) λIL2-9.1
λIL2-2.1
λIL2-11.1
λIL2-7.1
λIL2-8.1
λIL2-10.1
B
R
Rs
A
Ps
R
250 bp

Fig. 2. Sequence of the mouse IL2 gene 5' flanking region from -2800 to +45. Sequence was determined by both chemical degradation and dideoxy chain termination methods. Regions upstream of -580 that show homology to the human IL2 gene are boxed (see Fig. 4). Underlined and overlined regions show stretches of poly d(CA) and poly d(GT), respectively. Also indicated are the 5' termini of the IL2 sequences in the constructs diagrammed in Fig. 6.

```
                                *-2800
-2800                           GAATTCACAC TGGCCTCACA CCAGACTAAA CTGTAGCTTT

-2760 AATCCCATGA AAGATCAGGC AAAAGAAACT CTTGTGGGAG GTGGAGTTCT CAGTAGTTTG

-2700 CATGAGTGGA CCATGTAAGC AACAGGTGAC AGGTTCCCCG GGCAGCATCT CTAGGGTCTG

-2640 GGCCTGCCTT CACTTCTAGG AGCAGCTGGA ACAGCTAATA CTGAGCAGTA GAGCTCTGTG

-2580 GCTTATGACA CATGTGGTAG TCAGGCAGCG ACTGATTACA TCACCTCTCC AGCCAGAAAC

-2520 CCCAGCTGCT GTGTCAAGGA TGTTAAAAAT GGAAAGTGCA CCACAGCTGT TGGTAGCTTT

-2460 CTCCCCAAGC ATCTTAAGAA ACTGGGAGGC CAACCTTTGT AATGCTGCCA ATTATGCAAT

-2400 GCTCTGTAGG GGTGAACCTT GGCCCCAGGA ACTCATTCTA CAGGCTTTTA GAAACGCGGC

-2340 CATGTAAATT TCTCTAATGG AAACAGCTGG AGAAATAGAG ATGTTGAGCC CATGAGGACA

-2280 TCAAAGGCCC CACTGTGTGG AAAAGCCCCC ACACAGGAAA ATGTGTGGGC CTGACTCCTT

-2220 CCATTCTGGA AGGGGAGGCC TGAGTTGCTT GGGCTGGGAT GAGATGCTGA AGATCTGAGA

-2160 AGAGAGACAA GAGCATTGAG GCTGAGAGTT ATTAGGTAGA TGCCCCTCAC GATGGCATGC

-2100 TTAATCTGAA GGCACAGAAG GTGATGATGA CTAGGAGATT TGTCCCCATC CTGCTTTCCT

-2040 TCAGACATGC CAACAGGGCC ACTATGATAC CAGAAGTTGT CAAGCTTACT GCAAGATACA

-1980 TGCAGAGTTT TTTGTTGTTT TCTAGAATAC TGTCCAATGA TTGGAGAACT ATCCCAGAAC

                                *-1890
-1920 CATTCTCTGT TCTTTGTGAG AGACTTTAGG CCAACATTCC TTACATTGCT TAGTCTTCCT

-1860 GCTTTCACTA TTCCCCAGCA TCAAAATTAC ACTTAAAGTA ACTATCTCAA AACTCTTTAC

-1800 ATTGCCCAAC ATCCAACATT TCTAGGATGT TATCTGTCTC CATCCAAGGA TCAGGTCAAT

-1740 AGGAAGACAG ATGGTGTGAG AGTAGATTCT GGAGTCAGAA CATTCTAGTT TCGAATCTTC
                                ^-1710

-1680 ACCCTACCCT TTACTGGCAA TAAGGCTGAG TAACCTCAAT GATTTATCTA ATCTACCCAC
                                -1645^

-1620 AGTGTGCATG TAGCAGTCAA AGACAACTTG TGGAGTCAGT GAGAACTGAC TTCCACCTTT

-1560 ATGTAAGTTC AGGGATCAGA GAGCAAGGTT TGTATAGCAA GCCCCTTAGT CATCTCATCA

                                                          *-1449
-1500 CCCTACATCC TCAGTTTTCA AATCTACAAA ATGGGGTAGG TGTGTGGTGA GGCTTATGTT
           ^-1492                        -1466^                ^-1451  -1440^

-1440 AATCTGTGTA TTTGCACATA TTTTCTCCCT TTCTATGTAC CCCAGGATGT TTTAGATGGT
                           ^-1420                -1400^-1400

                                                          *-1332
-1380 AGATAAATGT CTGCATAAAG ACTAGACCAG TACAAGTTAT TAGAAATGGG GACACCACAG
     -1377^   ^-1373              -1354^
```

```
-1320 GCAAGCTCCC TAAGAAAGAC CCCGTCTCTA CCAGTTGATT TGGAACTATG TTCCTACTCC

                                                        *-1219
-1260 ATCACGCAGC CAGTGTACTA CACGGAGGAT AAGGAATCCA ATGTATCCTA TTCAGGTGAC

-1200 CCACTGAGAA CACGTGGGAT AGTCCCTAGC TATTACTCTC AGAGTGCCCA GGTACTTTTA
                                                  ^-1162
-1140 GGATAAACCC AAATCTACTG AATTAGGGGG AAGAAGGTTG GCAAGATGCC TCAGTGGTAA
        -1134^
-1080 AGGCTAGGTA GAGGCTAGTA GCAGAGGTAG GCACACAGAC TGGATGACTT TTGTGTTTAG

-1020 ATTTCTGAGT CACACAAGGT GACAGGAGAG AAGTTACTAG CAAGAGTTGG TCTCTGACCT

 -960 CCACAGGTGT ACTGTGGCAC ACACACACAC ACACACTCAT AATACATGTG CACAAATGCA

 -900 TGTACACATA CATACACACA TGCATACATG CACACACACA CACTCATATA CACACGCACA

 -840 TAAACATGCT CACACACACA TACATGTGCA CGCACATGCA CATACTCATA CACACATGAA
                                        *-753
 -780 CACATGTGTG TGTACACACG TGAAAATATT TTTTAAAAAT GAAAGTGCAA CTAGAGACAT

 -720 ATAAAATAAC ACCAACATCC TTAGATGCAA CCCTTCCTGA GAATTTGTTG GACATCATAC

 -660 TCTTTTTAAA AAGCATAATA AACATCAAGA CACTTACACA AAATATGTTA AATTAAATTT
          ^-656                      -629^          ^-616      -604^
                                *-578
 -600 AAAACAACAA CGACAAAATA GTACCTCAAG CTCAACAAGC ATTTTAGGTG TCCTTAGCTT

 -540 ACTATTTCTC TGGCTAACTG TATGAAGCCA TCTATCACCC TGTGTGCAAT TAGCTCATTG

 -480 TGTAGATAAG AAGGTAAAAC CATCTTGAAA CAGGAAACCA ATATCCTTCC TGTCTAATCA

 -420 ACAAATCTAA AAGATTTATT CTTTTCATCT ATCTCCTCTT GCGTTTGTCC ACCACAACAG
              *-351                                    *-321
 -360 GCTGCTTACA GGTTCAGGAT GGTTTTGACA AAGAGAACAT TTTCATGAGT TACTTTTGTG

 -300 TCTCCACCCC AAAGAGGAAA ATTTGTTTCA TACAGAAGGC GTTCATTGTA TGAATTAAAA
             *-232
 -240 CTGCCACCTA AGTGTGGGCT AACCCGACCA AGAGGGATTT CACCTAAATC CATTCAGTCA

 -180 GTGTATGGGG GTTTAAAGAA ATTCCAGAGA GTCATCAGAA GAGGAAAAAC AAAGGTAATG
                          *-103
 -120 CTTTCTGCCA CACAGGTAGA CTCTTTGAAA ATATGTGTAA TATGTAAAAC ATCGTGACAC

  -60 CCCCATATTA TTTTTCCAGC ATTAACAGTA TAAATTGCCT CCCATGCTGA AGAGCTGCCT

   +1 ATCACCCTTG CTAATCACTC CTCACAGTGA CCTCAAGTCC TGCAG
```

Fig. 3. Dot matrix comparison of mouse IL2 upstream region from -2800 to +45 and human sequence from -1370 to +45. The window size was 10 and the number needed to match was 8. Human sequence was from (Holbrook *et al.*, 1984b), with the start site of transcription moved 8 bp to the 3' direction in accord with Taniguchi *et al.* (1983) and Devos *et al.* (1983).

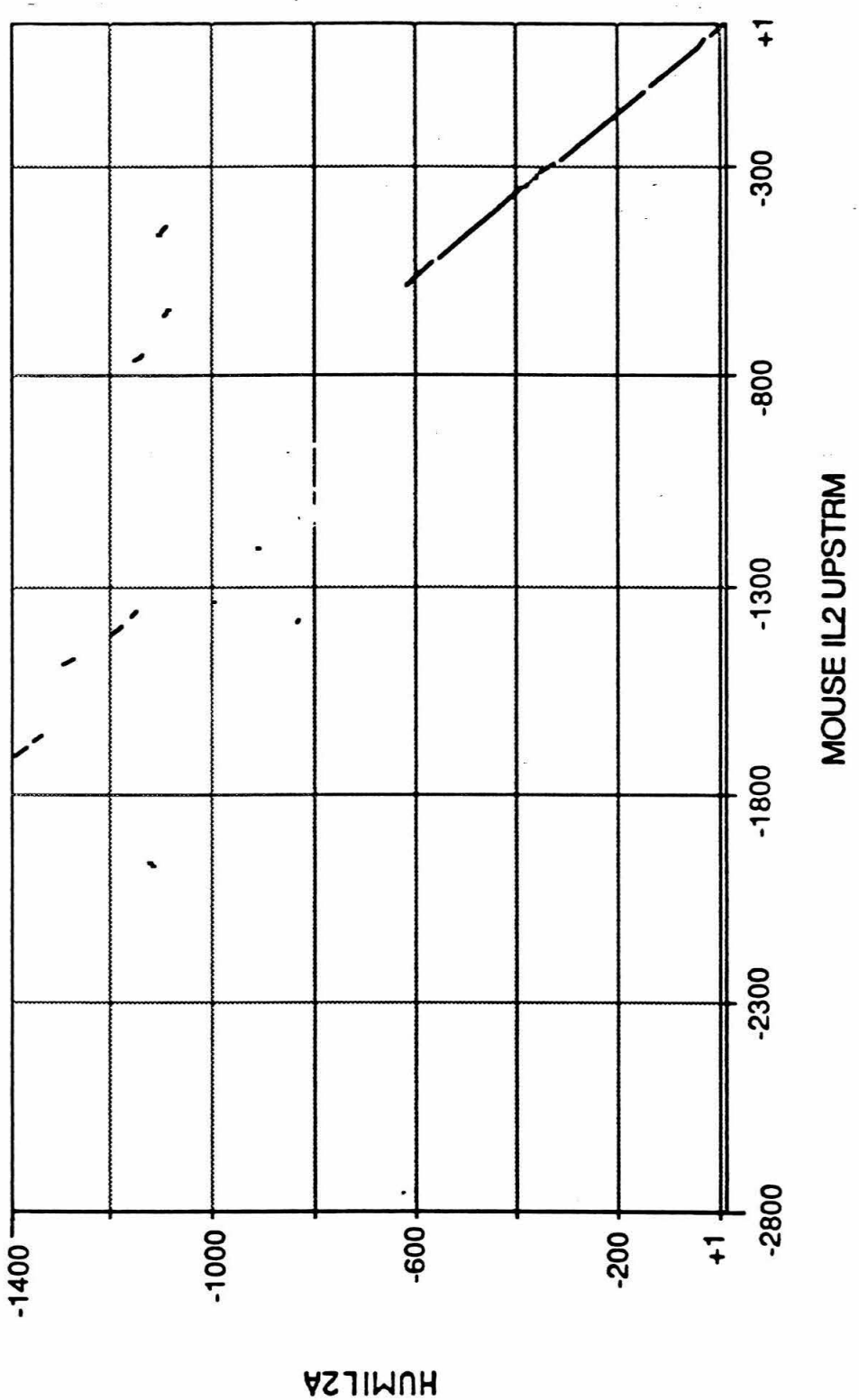

HUMIL2A
-1400
-1000
-600
-200
+1
-2800
-2300
-1800
-1300
-800
-300
+1
MOUSE IL2 UPSTRM

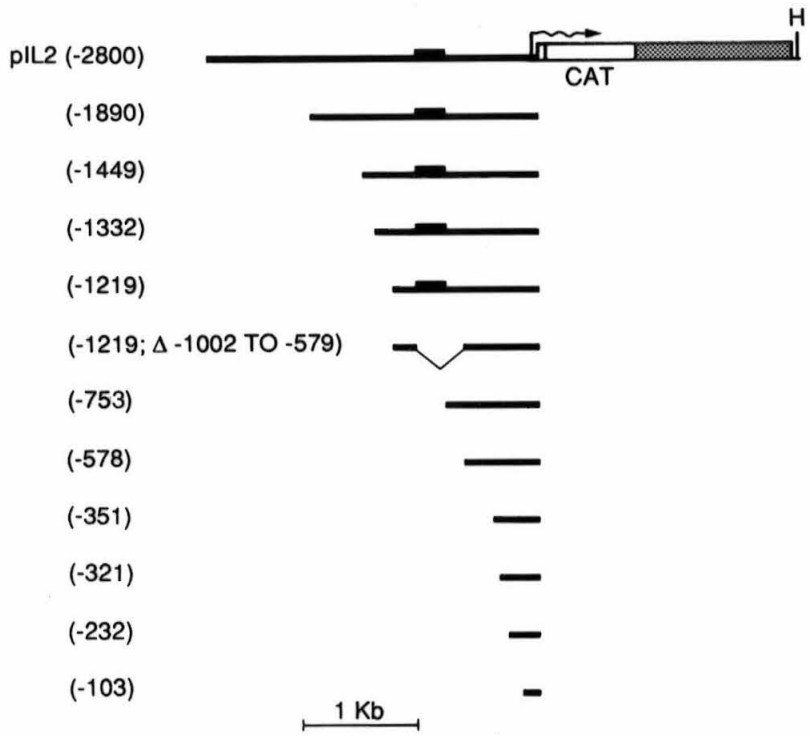
Fig. 4. Comparison of mouse and human IL2 immediate upstream regions from -580 to +45. Dashes indicate identity, and gaps were added by visual inspection to maximize matches. Indicated on the figure are binding sizes for NFAT-1, NF-κB, NF-IL2A and AP-1 (see text). Other proteins footprint over this region but have not been identified (Serfling *et al.*, 1989). Overlines indicate binding sites for proteins demonstrated to interact with the mouse or human IL2 promoter. The extent of each binding site shown is from published consensus sites for AP-1 and NF-κB (Angel *et al.*, 1987; Hoyos *et al.*, 1989), from deletion analysis for NFAT-1 and the proximal NF-IL2A site (Durand *et al.*, 1988) and from footprint analysis for the distal NF-IL2A site (Durand *et al.*, 1988). For sites that were determined by several methods, the minimum size of the site is shown.

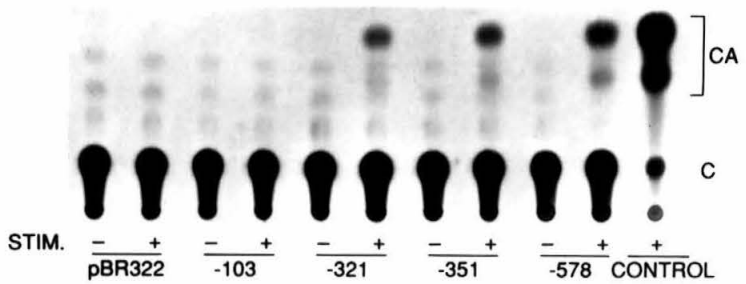
```
-580              GTACCTCAAGCTCAACAAGCATTTTAGGTGTCCTTAGCTTACTATTTCT    M
-585              ---------------T----------A--A-T--A-T----G-------    H

-531  CTGGCTAAC TGTATGAAGCCATCTATCACCCTGTGTGCAATTAGCTCATTGTGTAGATA    M
-536  --A---G--A----A-----A-------TTAT---A-------------------G----    H

-472  AGAAGGTAAAACCATCTTGAAACAGGAAACCAATATCCTTCCTGTCTAATCAACAAATCT    M
-476  -A-------------TC------------------CA--------T--------------    H

-412  AAAAGATTTATTCTT:.TCATCTATCTCCTCTTGCGTTTGTCCACCACAACAGGCTGCTTA    M
-416  - --C-----------..-----G-T-A-------TC-------------T-T---AT-C-    H

-352  CAGGTTCAGGATGGTTTTGACAAAGAGAACATTTTCATGAGTTACTTTTGTGTCTCCACC    M
-357  --T------TG-A-----ATG-C-A----A------ --------------A--C-----    H

                                NFAT-1                  NF-IL2A
-292  CC   AAA     GAGGAAAATTTGTTTCATACAGAAGGCGTTCATTGTATGAATTAAAA    M
-298  --CTT---GAAAG--------AC--------------------A----C--------G-G    H

                                          NF-κB              AP-1
-240  CTGCCACCTAAGTGTGGGCTAACCCGACCAAGAGGGATTTCACCTAAATCCATTCAGTCA    M
-238  --AT------------------TGTA--A-----------------C--------------    H

                                  AP-1
-180  GTGTATGGGGGTTTAAAGAAATTCCAGAGAGTCATCAGAAGAGGAAAAACAAAGGTAATG    M
-178  --C-T---------------------A----------------------TG---------    H

                                          NF-IL2A
-120  CTTTCTGCCACACAGGTAGACTCTTTGAAAATATGTGTAATATGTAAAACATCGTGACAC    M
-118  T--- - T--G--T----A-G--------------------------------TT------    H

-60   CCCCATATTATTTTTCCAGCATTAACAGTATAAATTGCCTCCCATGCTGAAGAGCTGCCT    M
-60   -------A-----------A------------------A--T-T--T-C-----T-C---    H

+1    ATCACCCTTGCTAATCACTCCTCACAGTGACCTCAAGTCCTGCAG                   M
+1    -----T---  --------A--------A-------C------CA                  H
```

Fig. 5. Comparison of nine mouse and human similarity regions located upstream of -580. Top line is mouse sequence. Gaps have been introduced by inspection to maximize similarity. Numbers above and below the sequences indicate their position relative to the transcriptional start sites of the respective genes.


-1710
-1645
GGAGTCAGAACATTCTAGTTTCGAATC TTCACCCTACCCTTTACTGGCAATAAGG CTGAGTAACCT
--------T-------CT----A----C--- T- -G---C---------------G------G----
-1360
-1295
-1492
-1466
CCTCAGTTTTCAAATCTACAAAATGGG
-----------T-----G------A--
-1267
-1241
-1451
-1440
AGGCTTATGTTA
--A-----A---
-1217
-1206
-1420
-1400
TTTTCTCCCTTTCTATGTACC
-------T--------A----
-1175
-1155
-1400
-1373
CCCAGGATGTTTTAGATGGTAGATAAAT
------ --A------ -- ------
-821
-790
-1377
-1354
TAAATGTCTGCATAAAGACTAGAC
--G-------TT-------C----
-1137
-1114
-1162
-1134
TCAGAGTG CCCAGGTACTTT TAGGATAAA
---T----T-------GA---AG--------
-830
-800
-656
-629
TTTAAAAAGCATAATAAACATCAAGACA
-------- --GT---- -----
-659
-630
-616
-604
ATGTTAAATTAAA
--T----------
-611
-599

Fig. 6. (Left) pIL2-CAT constructs. The number in parentheses refers to the 5' terminal nucleotide located in the plasmid. All IL2 sequences terminate downstream at +45 in the 5' untranslated region. Thick line, IL2 5' sequence; black box, poly(dC-dA) tract; open box, CAT gene; stippled box, SV40 sequences containing small t intron and polyadenylation sites. Vector is pSP65 (not shown). Arrow indicates start site of transcription. H indicates location of unique HindIII site in pIL2 (-1890) used in construction of plasmids with intragenic and 3' genomic sequences. (Right) Representative CAT assay of extracts from Jurkat cells transfected with each of the indicated constructs. Unacetylated chloramphenicol and its monoacetylated derivatives are indicated with (C) and (CA), respectively. - = uninduced; + = 18 hr stimulation with TPA + A23187.


pIL2 (-2800)
H
CAT
(-1890)
(-1449)
(-1332)
(-1219)
(-1219; Δ -1002 TO -579)
(-753)
(-578)
(-351)
(-321)
(-232)
(-103)
1 Kb


CA
C
STIM.
– + – + – + – + – + +
pBR322
-103
-321
-351
-578
CONTROL

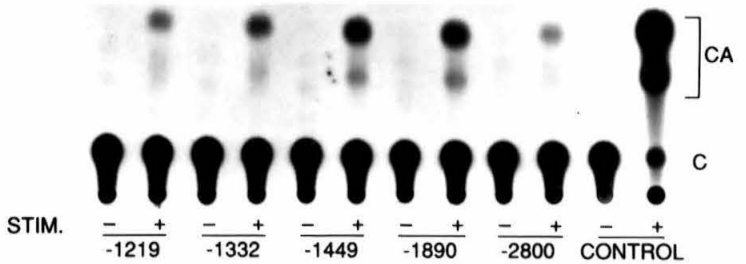

CA
C
STIM.
– + – + – + – + – + – +
-1219
-1332
-1449
-1890
-2800
CONTROL

Fig. 7. CAT assay data from transfected Jurkat (A) and EL4.E1 (B) cells. Relative CAT activity calculated as in Materials and Methods. Numbers in bars are the number of times that a given plasmid was tested in parallel with pIL2 (-321) CAT as a reference standard. Only results from 18 hr stimulated cells are shown. Results are presented as mean ± S.E.M.

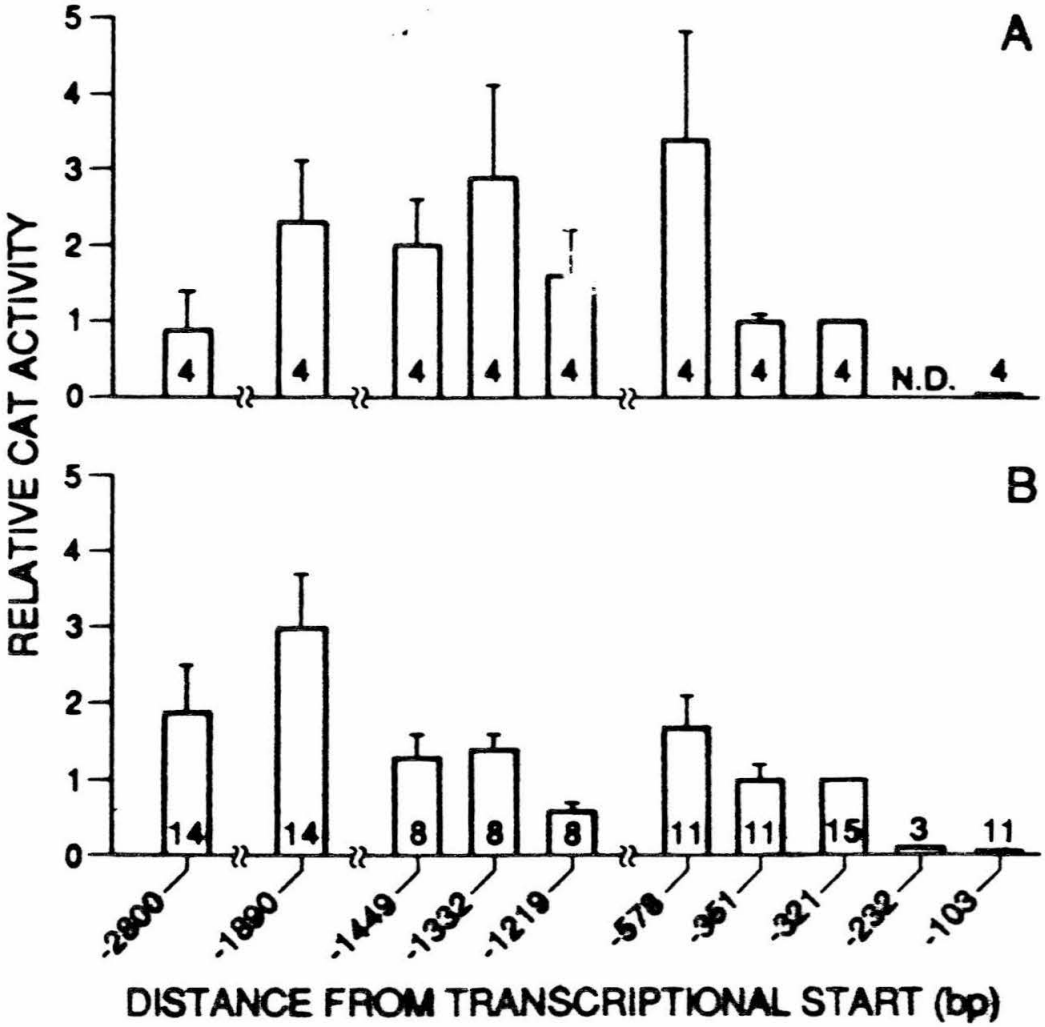

A
B
RELATIVE CAT ACTIVITY
0
1
2
3
4
5
4
4
4
4
4
4
4
4
N.D.
4
14
14
8
8
8
11
11
15
3
11
-2800
-1890
-1449
-1332
-1219
-578
-361
-321
-232
-103
DISTANCE FROM TRANSCRIPTIONAL START (bp)

Fig. 8. Gel blot analysis of cytoplasmic RNA extracted from induced and noninduced hematopoietic cell lines. Total cytoplasmic RNA was fractionated on denaturing agarose/formaldehyde gels, blotted, and hybridized with the indicated probes as described in Methods. Data presented are from sequential hybridizations of the different probes with the same filter. - = uninduced; + = induction with TPA + A23187. Cell lines are (1) EL4.E1, (2) BW5147, (3) HT-2, (4) P388D1, (5) WEH1-3B, (6) 32 D cl 5, (7) NS-1. Note that our subline of BW5147 is not inducible for IL2, in contrast to others in the literature (Hagiwara *et al.*, 1988).

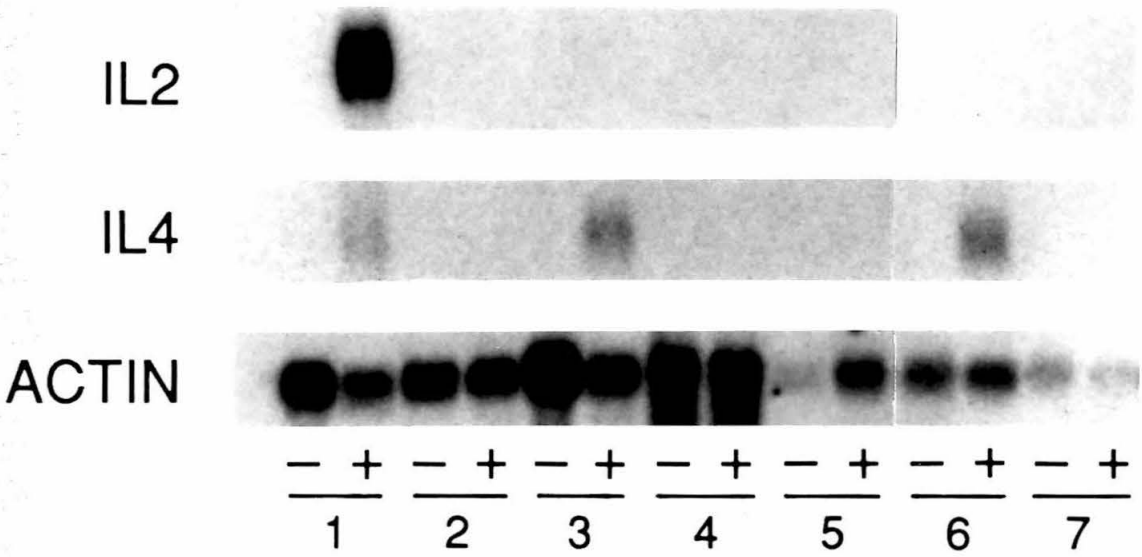

IL2
IL4
ACTIN
− + − + − + − + − + − + − +
1 2 3 4 5 6 7

Fig. 9. Both cyclosporin A (CsA) and increased intracellular cAMP depress expression of CAT from the IL2 promoter while increasing expression from the RSV LTR. (Left) EL4.E1 cells transfected with the indicated plasmids were stimulated with TPA + A23187 for 18 hr in the presence and absence of 10^{-5} M forskolin or 1 μg/ml CSA. For each plasmid results were calculated as [(CAT activity with drug)/(CAT activity without drug)] x 100%. Forskolin results are given as mean ± S.E.M. of 2-3 experiments. CSA results are from a single experiment. (Right) Effect of 10^{-6} M PGE_1 on IL2-CAT expression in Jurkat cells relative to drug-free controls. Results shown are from a single experiment.

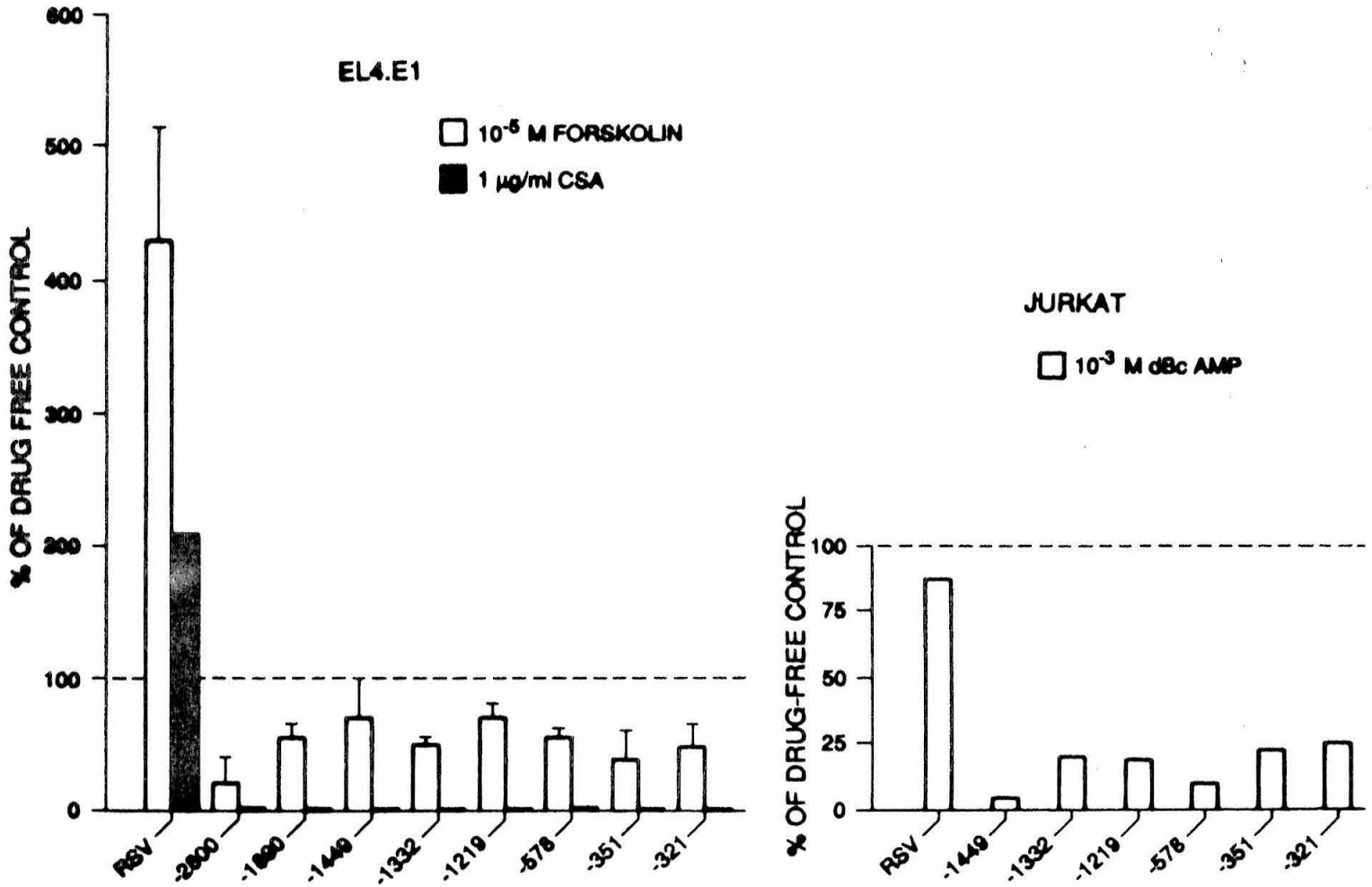
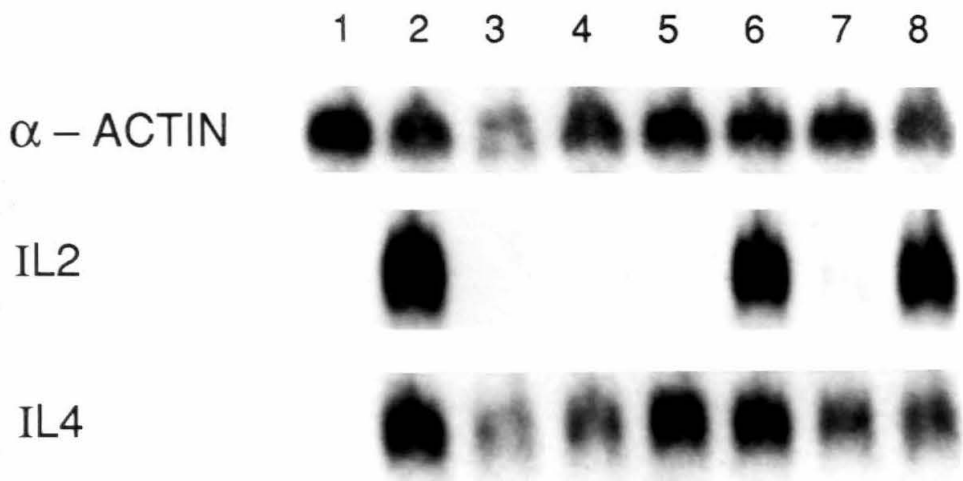
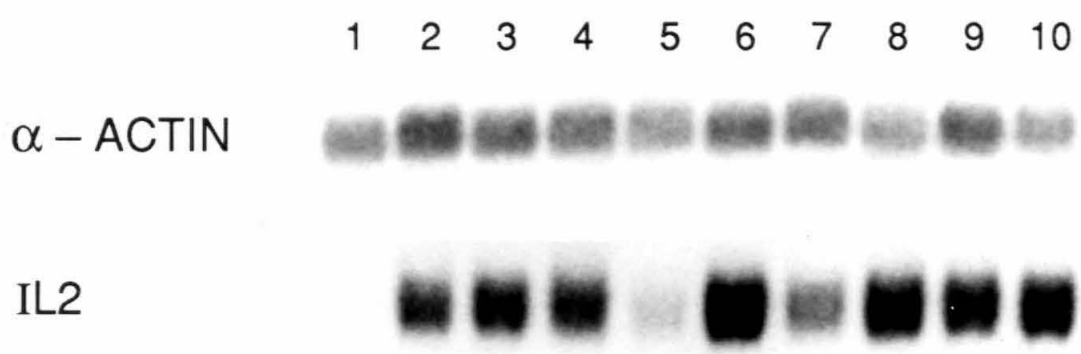

EL4.E1
10-5 M FORSKOLIN
1 µg/ml CSA
% OF DRUG FREE CONTROL
600
500
400
300
200
100
0
RSV
-2800
-1960
-1449
-1332
-1219
-578
-351
-321
JURKAT
10-3 M dBc AMP
% OF DRUG-FREE CONTROL
100
75
50
25
0
RSV
-1449
-1332
-1219
-578
-351
-321

Fig. 10. Effects of drugs on IL2 mRNA induction: Increased intracellular cAMP depresses IL2 expression but not IL4 expression. Gel blot showing the effects of increased intracellular cAMP on lymphokine mRNA levels. (A) EL4.E1 stimulated in the presence of various cAMP agonists. Lane 1, unstimulated cells; Lanes 2-8, cell stimulated 5 hr with TPA + A23187 in the presence of: Lane 2, no additives; Lane 3, 10^{-5} M PGE; Lane 4, 10^{-6} M PGE; Lane 5, 10^{-3} dBcAmP; Lane 6, 10^{-4} dBcAmP; Lane 7, 10^{-5} M forskolin; and Lane 8, 10^{-6} M forskolin. (B) Jurkat cells. Lanes 1-8 are as in (A). Lane 9, 10^{-5} M Dex; Lane 10, 10^{-6} M Dex.

A



B



CHAPTER 3

Prepared for Publication

Interleukin 2 Gene Expression in a Phorbol Ester and Calcium Ionophore - Inducible Murine Thymoma is Upregulated by Addition of Interleukin 1: Description of a Novel IL1-Responsive Phenotype

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Abstract

The macrophage-derived cytokine interleukin 1 (IL1) is known to have numerous effects on cells of the immune system, including a long-held belief that it functions as a necessary "second signal" with antigen to evoke production of interleukin 2 (IL2) by helper T cells. However, we have recently shown that IL1 has no effect on mature T cells stimulated to produce IL2 by the combination of phorbol ester and calcium ionophore. In contrast, immature, T cell-receptor-negative thymocytes do not secrete IL2 in response to phorbol ester + ionophore unless IL1 is present, suggesting that the effect of this cytokine is limited to a particular state in T-cell development. Here we show that EL4.E1, a murine thymoma that can be stimulated by high-level IL2 production by phorbol ester + ionophore alone, nevertheless responds to IL1 costimulation by increasing the steady-state level of IL2 mRNA. Thus, this cell line may represent a developmental intermediate between immature and mature T cells. Expression of a transfected reporter gene linked to a cloned fragment of the murine IL2 gene promoter is also increased by IL1 costimulation, thus suggesting that the observed increase in steady-state IL2 mRNA results from an IL1-induced increase in IL2 transcription rate and not just increased transcript stability. The disparate effects of IL1 and forskolin on IL2 gene expression suggest that, in these cells, IL1 does not mediate its effects via a cAMP intracellular second messenger as has been demonstrated in other cell types. Our data do not rule out a role for NF-κB in mediating the IL1 effects we describe.

Introduction

Interleukin 1 (IL1) is a potent mediator of cellular function that is produced primarily by activated macrophages (Koide and Steinman, 1987) and a role for IL-1 in T-cell activation has been demonstrated in a number of experimental systems

(reviewed in Dinarello, 1989; Durum *et al.*, 1985; Mizel, 1982). *In vitro*, IL1 synergizes with mitogenic lectins or anti-T-cell receptor (TcR) antibodies to induce expression of interleukin 2 (IL2) and IL2 receptor α chain (IL2Rα) genes (Gillis and Mizel, 1981; Smith *et al.*, 1980; Kaye *et al.*, 1984). In these roles IL-1 can be substituted for by tumor-promoting phorbol esters (e.g., 12-O-tetradecanoylphorbol 13-acetate; TPA) (Farrar *et al.*, 1980). As IL1 was known to provide a necessary, antigen-nonspecific signal for T-cell activation, this apparent equivalence with TPA suggested that its action might result from activation of protein kinase C (PKC). However, IL1 does not promote phosphoinositide hydrolysis nor cause PKC translocation to an active membrane-bound form (Abraham *et al.*, 1987). It therefore seems unlikely that its role is to augment the low levels of diacylglycerol (DAG) produced by TcR-mediated phosphoinositide breakdown. More recently, it has been demonstrated that IL1 treatment of the human T-leukemia line, Jurkat, causes increased production of two PKC co-factors—phosphatidylserine and DAG, the latter from hydrolysis of membrane phosphatidylcholine (Mary *et al.*, 1988; Rosoff *et al.*, 1988). However, because Jurkat lacks conventional IL1 receptors (Rosoff *et al.*, 1988) the significance of these results is unknown.

Recent work by Mizel and colleagues has shown that IL1 can utilize cAMP as an intracellular second messenger for the induction of κ immunoglobulin light chain synthesis in the mouse pre-B-cell line, 70Z/3 (Shirakawa *et al.*, 1988). In 70Z/3 cells, IL1-mediated induction coincides with the activation of a protein that can bind to the NF-κB consensus site in 5' flanking region of the κ chain genes (Shirakawa *et al.*, 1989a). IL1 can also induce NF-κB binding activity in a variety of T-cell lines (Osborn *et al.*, 1989). Hoyos *et al.* (1989) have utilized a transient transfection system to demonstrate a role for NF-κB in mitogen and HTLV-I Tax-mediated expression from the human IL2 gene promoter. Mutations that

prevented binding of this factor depressed promoter function, relative to wild-type, in optimally stimulated cells.

NF-κB is not the only transcription factor implicated in IL1 effects, however. IL1 can also induce transcription of the c -jun protooncogene, which encodes a component of AP-1, in the mouse IL1-dependent T-cell line LBRM-331A5 (Muegge *et al.*, 1989). In this cell line,expression of a linked reporter gene from the human IL2 promoter required an intact AP-1 site at -185, relative to the transcriptional start site. However, their deletions, which removed this site, also removed the NF-κB site centered at -200. Therefore, the resulting loss of promoter function can not be attributed unequivocally to loss of this AP-1 site. In fact, deletion analysis of the human IL2 promoter has not demonstrated an important role for this AP-1 consensus site, *in vivo* (Durand *et al.*, 1988). Footprint analysis of the mouse IL2 promoter has led to the same conclusion (Serfling *et al.*, 1989).

The studies that have reported a costimulatory role for IL1 have usually demonstrated this effect on cells suboptimally stimulated with mitogenic lectins (refs. cited above; Hackett *et al.*, 1988). Recently, however, we and others have obtained results that call into doubt the notion that IL1 is a necessary cofactor for the high-level production of IL2 (Lichtman *et al.*, 1988; Rothenberg *et al.*, 1990). We have shown that purified splenic T cells produced IL2 in response to TPA and the calcium ionophore A23187 and that this response was both independent of and insensitive to added IL1 (Rothenberg *et al.*, 1990). Lichtman *et al.* (1988) have obtained similar results with a panel of cloned antigen-dependent T_H1 cell lines stimulated with an anti-CD3 monoclonal antibody.

In addition to indicating a suboptimal stimulus a requirement for IL1 may also be reflective of a T cell's developmental status. Mature TcR^+ thymocytes resemble peripheral T cells in their insensitivity to IL1 when stimulated to

produce IL2 in response to TPA + A23187. In contrast, immature TcR^- double negative (DN) thymocytes did not respond to TPA and A23187 at all unless IL1 was present. Although these immature thymocytes secreted relatively high titers of IL2 over a 20 h period, *in situ* hybridization analysis showed that IL2 mRNA reached peak levels at 5 h and was largely gone by 20 h. The extreme transience of IL2 mRNA accumulation seen in these cells stood in contrast to the more prolonged appearance of IL2 transcripts in stimulated mature T cells (McGuire *et al.*, 1988). Thus, these immature thymocytes differ from their more mature predecessors in two important aspects of gene expression- inductive signal and kinetics.

Here we report a third response profile that is distinct from the two reported previously. Using the murine thymoma EL4.E1 as a homogeneous population of IL2 producers we demonstrate that it possesses characteristics of both immature and mature T cells. Like mature cells, it can respond to phorbol ester and ionophore alone. In addition, it possesses two traits normally associated with immature thymocytes, namely IL1 responsiveness when stimulated with TPA + A23187 and a peak of IL2 mRNA accumulation at 5 h. We have used these cells to explore the mechanism by which this unique synergy between IL1 and TPA + A23187 occurs.

Materials and Methods

Reagents—TPA and A23187 were from Sigma or Calbiochem. They were dissolved in DMSO to final concentrations of 10 μg/ml and 0.37 mg/ml, respectively, and stored in small aliquots at -20°C. Recombinant human and mouse IL1α (IL1) was purchased from Genzyme. The specific activity, as reported by the supplier, was 10^8 units per mg.

Cells—EL4.E1, a mouse IL2-producing thymoma cell line, and the human T-

cell leukemic line Jurkat (generously provided by G. Crabtree, Stanford University) were grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 50 μM 2-ME, and antibiotics.

Plasmids—The series of pIL2-CAT plasmids containing varying lengths of the mouse IL2 gene 5' DNA linked to the bacterial gene for chloramphenicol acetyltransferase (CAT) has been described in detail (Novak *et al.*, 1990). Briefly, cloned fragments of the IL2 gene 5' flanking region, all terminating 3' at +45 in the 5' untranslated region, were ligated to the CAT gene from pTK-CAT in the vector pSP65 (Promega). They are designated pIL2 (-X) where X is the 5' terminal nucleotide, relative to the transcriptional start site, that is present in the construct. All plasmids were purified in CsCl/EtBr density gradients before use.

Transfections and CAT Assays—Transfection of logarithmically growing cells was by DEAE-Dextran facilitation as described (Novak *et al.*, 1990). In short, cells were washed in serum-free DMEM and then resuspended at 10^7 per ml in a transfection cocktail containing 250 μg/ml DEAE-Dextran (2×10^6 M.W.), 0.1 mM chloroquine and 10 μg/ml supercoiled plasmid DNA all in DMEM. After incubation for 30-60 min at 37°C/7% CO_2 cells were pelleted, washed, and plated into four to twelve identical cultures. Approximately 20 h after plating, cells were stimulated with TPA and A23187 at final concentrations of 10 ng/ml and 37 ng/ml, respectively. Some of the cells also received IL1 to the indicated concentrations. Human rIL1α was found to be as effective as the mouse version on EL4.E1 cells and was used in most experiments.

Cells were harvested after various times of stimulation and extracts from equivalent numbers of cells were assayed for CAT activity in a 5 h assay as described (Novak and Rothenberg, 1986).

RNA Analysis—Cytoplasmic RNA was extracted by the method of Favaloro *et al.* (1980). The RNA from equivalent cell numbers was electrophoresed in

denaturing 1% agarose-formaldehyde gels, stained with acridine orange to visualize the rRNA, and then blotted onto nylon membranes (Nytran, Schleicher and Schuell). The RNA was fixed to the membrane by baking at 80°C for 60 min. Hybridization probes were generated by random priming and consisted of cDNAs for mouse and human IL2 (Yokota *et al.*, 1985; Holbrook *et al.*, 1984), mouse IL4 (Lee *et al.*, 1986), and mouse α skeletal actin (S. Sharp and N. Davidson, Caltech, unpublished). Hybridizations were carried out for 20 h at 42°C in 5 x SSPE, 50% formamide, 0.2% SDS, 5 x Denhardt's, and 10% dextran sulfate. Filters were washed three times at room temperature for 1 min each in 2 x SSC, 0.2% SDS and 0.05% $NaPP_i$, followed by two 30 min washes at 68°C in 0.2 x SSC, 0.1% SDS, 0.05% $NaPP_i$. Filters were exposed to film at -70°C with an intensifying screen and autoradiograms in the linear response range were densitometrically scanned. The amount of IL2 or IL4 mRNA was normalized with respect to the actin signal.

Results

Previous work in this lab has demonstrated a correlation between the developmental state of a T cell, as measured by surface TcR expression, and the inductive signals it requires to express the gene for IL2 (Rothenberg *et al.*, 1990). Cells that were able to transcribe IL2 in response to the combination of TPA and A23187 did not exhibit a need for IL1. These agonists of the universal phosphatidylinositol bisphosphate (PIP_2) hydrolysis pathway were also effective in eliciting transient high-level IL2 expression in purified, peripheral $CD8^+$ cells (McGuire *et al.*, 1988). These cells generally do not transcribe this gene in response to either antigen or mitogenic lectins. This result suggests that a T cell's functional lineage may, in part, be a reflection of the efficiency with which TcR perturbation is coupled to PIP_2 breakdown. TcR-negative T cells that do not respond to TPA + A23187 presumably have some other defect that can be

complemented by IL1.

IL1 is a Costimulus for IL2 Expression by Phorbol Ester and Ionophore-Treated EL4.E1 Cells

The EL4.E1 thymoma has been used as a model for T_H1 cells in studies of IL2 gene expression even though it is responsive to TPA alone. We have begun to study the response profile of this cell line because it is a useful recipient in DNA transfection experiments (Novak *et al.*, 1990). As shown below, unlike normal mature T cells, it shows synergy between IL1 and TPA/A23187.

An RNA gel blot of EL4.E1 cells stimulated for 5 h with various combinations of TPA, A23187, and IL1 is shown in Fig. 1. EL4.E1 cells can transcribe IL2 in response to TPA alone, but addition of calcium ionophore greatly increased the steady-state level of this message (Fig. 1, lanes 2 and 3). The addition of 20 units per ml of mouse rIL1α resulted in a further increase in IL2 mRNA (Fig. 1, lanes 3 and 4; see also Fig. 2). In three experiments the addition of IL1 increased the steady-state level of IL2 mRNA at 5 h almost fivefold over TPA + A23187 alone.

No IL2 mRNA was detected when EL4.E1 cells were stimulated 5 h with 20 u/ml IL1, alone or in combination with 37 ng/ml A23187 (not shown). This inability to respond to IL1 + ionophore is in contrast to results reported by others for several IL1-independent cell lines (Zlotnik and Daine, 1986; Simon, 1984). This difference may be attributable to the lower concentration of ionophore used here (37 ng/ml = 70 nM, compared to 250-500 nM).

On the other hand, the concentration of TPA used (10 ng/ml; ~17 nM) is sufficient to activate PKC in a number of cell lines (Nishizuka, 1984) and thereby to induce AP-1 and NF-κB (Angel *et al.*, 1987; Shirakawa *et al.*, 1989b). Therefore it is unlikely that the IL1-mediated enhancement of steady-state IL2 mRNA is due

solely to its ability to induce appearance of these DNA-binding activities (see also Osborn *et al.*, 1989).

The synergistic increases in IL2 mRNA induced by the addition of A23187 and IL1 to TPA-stimulated EL4.E1 cells appear to be specific for the IL2 gene, as IL4 mRNA showed almost no increase over TPA stimulation alone (Fig. 1). We have previously reported that increased intracellular cAMP suppressed IL2 but not IL4 expression in these cells (Novak *et al.*, 1990). The results in Fig. 1 extend this difference in regulation to positive mediators of IL2 as well.

Costimulatory Effects of IL1 are Mediated Through the IL2 Gene Promoter

In order to determine whether the IL2 promoter was responsible for mediating this positive effect we utilized a series of pIL2-CAT plasmids that contains varying lengths of IL2 5' flanking DNA, including the transcriptional start site, driving expression of CAT (Novak and Rothenberg, 1990). In this way we can assay transcriptional initiation without concern for mRNA stability because unlike authentic IL2 mRNA, the CAT message does not contain the AUUUA motifs that target lymphokine transcripts for rapid degradation (Shaw and Kamen, 1986; Reeves *et al.*, 1987). If IL1 mediates its effects through upstream sequences of the IL2 gene, it may be possible to dissect the IL1 regulating element away from more proximal general induction elements.

Several pIL2-CAT plasmids were transfected into EL4.E1 cells along with pRSV-CAT as a positive control. CAT activity was measured after 5 h, 12 h and 18 h of stimulation with TPA and A23187 in the presence and absence of IL1 (Figs. 2B and 3). In addition, cytoplasmic RNA was extracted from untransfected EL4.E1 cells that had been stimulated in parallel to monitor induction of endogenous IL2 mRNA. The relative steady-state levels of IL2 mRNA were determined by RNA gel blot analysis using message-specific probes for IL2 and

actin followed by densitometric scanning of the resultant autoradiograms. Note that the CAT assay measures the translation product of the induced RNA, not the RNA directly.

The accumulation of IL2 transcripts in EL4.E1 cells stimulated with TPA + A23187 alone occurred rapidly and was transient (Fig. 2A, lanes 2, 6, and 10). IL2 mRNA levels peaked around 5 h and declined during the next 13 h. This was in contrast to the kinetics of appearance of IL4 and IL2Rα mRNAs, which increased between 5 h and 18 h (not shown). Costimulation of EL4.E1 cells with either 2.5 or 20 units per ml IL1 resulted in a two- to sixfold increase in IL2 mRNA at 5 h (Fig. 2A, lanes 1-4). This effect was transient, however. At 12 h the enhancement due to IL1 was only 1.5-fold (Fig. 2A, lanes 6-9) and by 18 h IL1 ceased to augment the level of steady-state IL2 mRNA at all (Fig. 2A, lanes 9-12).

Transfection experiments using IL2-CAT plasmids suggest that in part, IL1 increases the rate of transcription from the IL2 promoter, even in cells optimally stimulated with TPA and ionophore. A CAT assay of EL4.E1 transfected with pIL2 (-2800) is shown in Fig. 2B. Expression of the transfected IL2-CAT gene requires an appropriate inductive signal because unstimulated cells contain no enzyme activity (Fig. 2B, lanes 1, 5, and 9). Thus, even when introduced as a naked DNA template, promiscuous transcription from the IL2 promoter does not occur in these cells. EL4.E1 transfectants stimulated with TPA + A23187 contained easily detectable CAT activity at 5 h (Fig. 2B, lane 2), which would be expected if the peak in endogenous IL2 mRNA seen at this time resulted from transcriptional activation of the IL2 promoter. CAT activity in these cells continued to rise over the next 13 h, reaching a maximum at 18 h, the latest time examined. This increase in CAT activity occurred even as the steady-state level of endogenous IL2 mRNA dropped (Fig. 2A).

The addition of IL1 to the stimulation regimen had two dramatic effects.

First, cells stimulated in the presence of IL1 contain up to 2.5x as much CAT activity per cell at 5 h as those stimulated with TPA and A23187 alone (Fig. 2B, left panel and Fig. 3A). This effect is IL1-dose-dependent because the enhancement is greater with 20 units per ml than with 2.5 units per ml.

The second point to be made concerns the 18 h time point. Although the steady-state level of IL2 mRNA at this time was equivalent for all stimulation conditions (Fig. 2A, lanes 10-12), the amount of CAT activity per cell in the presence of IL1 was now at least 60-70% lower than in cells stimulated without IL1. This disparity was due in part to a sustained increase in absolute CAT activity in the control stimulated cells (unlike the behavior of endogenous IL2 mRNA) and, in part, to a decrease in absolute CAT activity in IL1-costimulated cells (Fig. 3 and data not shown).

IL2 Promoter Sequences Upstream of -321 are not Required for IL1 Responsiveness

Our previous work on the upstream region of the mouse IL2 gene indicated the presence of several positive and negative regulatory elements between -321 and -2800, any of which might be involved in IL1-mediated effects. However, the data presented in Fig. 3A make it unlikely that far upstream sequence required.

The normalized curves for pIL2 (-321), pIL2 (-1890), and pIL2 (-2800) are virtually superimposable (Fig. 3A). Thus, the degree of both the 5 h enhancement and the 18 h suppression is unaffected by IL2 5' flanking sequences extending upstream of -321. It should be noted, however, that the absolute amount of CAT activity per cell at each time point was higher with pIL2 (-1890) and pIL2 (-2800) than with pIL2 (-321), in accord with our previously reported results (Novak *et al.*, 1990).

Expression of pRSV-CAT in stimulated EL4.E1 cells was not affected by

the presence of IL1 when examined after 3 h or 5 h of stimulation, but it was 31% lower at 18 h in cells costimulated with IL1 compared to cells stimulated with phorbol ester and ionophore alone (Table 1). This suggests that at later times IL1 causes a general decrease in CAT expression, possibly via reduced translational efficiency, which is distinct from its promoter-specific transcriptional enhancing effects seen at 5 h.

Possible Factors Contributing to the Effects of IL1 on EL4.E1 Cells

1. **cAMP.** It has been shown that IL1 can utilize cAMP as an intracellular second messenger in a number of non-T-cell lines (Shirakawa *et al.*, 1988). The results with pRSV-CAT argue against such a role for cAMP in EL4.E1 cells. We have previously demonstrated that after 18 h of stimulation, pRSV-CAT expression is increased fourfold in the presence of 10 μM forskolin—a diterpene activator of adenylate cyclase (Novak and Rothenberg, submitted), unlike its decreased expression in the presence of IL-1. Forskolin treatment had a strong suppressive effect on pIL2-CAT expression when examined at 5 h, in contrast to the transient stimulation by IL-1. These results, which are summarized in Table 1, do not support a role for cAMP in the IL1-mediated effects we have described.

2. **IL1 Receptor.** These effects require the expression of IL1R, as demonstrated by the results from identical transfection experiments using $IL1R^-$ Jurkat cells instead of EL4.E1. A similar time course experiment is shown in Fig. 3B. These cells showed much slower kinetics of expression of pIL2-CAT plasmids than EL4.E1. No activity was detected at 5 h even in the presence of 20 units per ml IL1. Cells had begun to express by 12 h and CAT activity was still rising by 18 h. However, no significant effect could be attributed to IL1. These results provide compelling evidence that surface IL1R expression is a prerequisite for the IL1 effects we have described. It is also unlikely that the effects seen in EL4.E1

cells are due to the type of phosphatidylcholine hydrolysis described by Rosoff *et al.* (1988) as this occurs within seconds of exposure to IL1.

3. **DNA-Binding Proteins.** The results presented in Fig. 3A are consistent with IL1 working through increased expression of NF-κB, NFAT-1, and/or AP-1, since binding sites for all three proteins are present in pIL2 (-321). Osborn *et al.* (1989) have shown that activation of NF-κB by tumor necrosis factor α(TNFα) is not sufficient to induce expression of IL2 in EL4 cells. Both TPA and TNFα can each activate this factor, as determined by a gel mobility shift assay. However, only TPA induces IL2 production and the combination of TPA and TNFα is not additive over TPA alone. A similar discrepancy between *in vitro* binding activity and *in vivo* transcriptional activation has been reported by Cross *et al.* (1989) in a comparison of the NF-κB sites upstream of the κ immunoglobulin gene and IL2Rα gene. Therefore, we evaluated a role for this protein directly by using an IL2-CAT construct, pIL2 (-232), that retains the NF-κB site at -200 but lacks the NFAT-1 site at -270 (Shaw *et al.*, 1988). In two experiments we have failed to detect significant expression of this construct in TPA + A23187 treated EL4.E1 cells even when IL1 was used as a costimulus (data not shown). Thus, it seems unlikely that NF-κB and AP-1 are sufficient to induce expression from the IL2 promoter in the absence of NFAT-1 or other upstream sequences. Muegge *et al.* (1989) reported expression of a construct containing 218 bp of the human IL2 promoter in a mouse IL1-dependent lymphoma, LBRM-331A5 stimulated with PHA or PHA + IL1. However, this construct differed significantly from ours in that it contained SV40 enhancer sequences to boost expression. Removal of these heterologous sequences resulted in a significant drop in expression consistent with our observations.

4. **Prostaglandin Synthesis.** IL1 induces expression of cyclooxygenase

(COX) in fibroblasts (Raz *et al.*, 1988). This enzyme is responsible for converting arachidonic acid to prostaglandin H, the first step in the synthesis of a variety of eicosanoids including prostaglandins E_1 and E_2. E-series prostaglandins can activate adenylate cyclase, thereby impairing expression of IL2. Because arachidonic acid-containing polyphosphoinositides are preferentially hydrolyzed in mitogen-stimulated lymphocytes (Sugiura and Waku, 1984), it remained a formal possibility that IL1-induced suppression of pIL2-CAT plasmids was a result of increased cAMP arising late in the stimulation. To address this we performed a time course experiment like those shown in Fig. 3 and included 5 uM indomethacin in all samples. Indomethacin inhibits the action of COX but its inclusion during the stimulation period failed to prevent the IL1-mediated decreased expression of pIL2 (-2800) at 18 h (data not shown).

5. **Cell Cycle.** It has been well demonstrated that proliferation and lymphokine secretion represent distinct responses to antigenic stimulation. For a given T cell the choice to do one precludes, for some time, the ability to do the other (Heckford *et al.*, 1986; Harris *et al.*, 1987; Nau *et al.*, 1988). Although EL4.E1 is a malignant line that grows without antigenic restimulation, it was possible that IL1 overcame the TPA-induced proliferation block seen with these cells. As a final control we stimulated EL4.E1 cells with TPA and A23187 in the presence and absence of 20 units per ml IL1. Cells harvested at 5 h and 18 h were stained with acridine orange, and cell cycle profile was determined by flow cytometry. As seen in Table II, IL1 caused no obvious perturbation in the cell cycle distribution at either time point. Therefore, it is likely that the early promoter-specific role we have described for IL1 does not result from decreased cell cycle progression. This enhancement is evident even in cells that have been stimulated with optimal concentrations of TPA and A23187 and it does not represent a "second signal" for T-cell activation as it is presently defined.

Discussion

Antigen- and mitogen-induced IL2 production by T-helper cells, *in vitro*, requires the presence of non-T accessory cells (Haber and Roff, 1977; Hünig *et al.*, 1983). This accessory cell requirement can be met with the macrophage-derived cytokine IL1 (Gillis and Mizel, 1981; Smith *et al.*, 1980). However, the increased understanding of T-cell physiology has been accompanied by contradictory reports of the role of IL1 in modulating T-cell function. Not all assays of T-cell response have demonstrated a need for IL1. Some of this discrepancy is due to the different types of T cells used to detect effects of IL1- primary peripheral T cells, lymphomas, and/or mature-type cloned T-cell lines (Lichtman *et al.*, 1988; Holsti and Raulet, 1989; Ho *et al.*, 1987; Greenbaum *et al.*, 1988; Gillis and Mizel, 1981). These cells represent distinct lineages or stages of T-cell development that differ in their physiological response to similar stimuli (Rothenberg *et al.*, 1990). In many cases a role for IL1 was demonstrated in conjunction with a deliberately suboptimal stimulus that gave rise to the original view of IL1 as an obligatory second signal in T-cell activation.

The EL4.E1 cell line is an IL2-producing, $IL1R^+$ thymoma of "mature" phenotype. It produces high levels of IL2 upon stimulation with TPA + A23187, in contrast to typical immature thymocytes (Rothenberg *et al.*, 1990). The requirement for IL1R in the effects we obtained was demonstrated by the failure of Jurkat cells, another mature, T lymphoma to respond to IL1 in an 18 h stimulation period and its slower kinetics of CAT expression (Fig. 3B). It should be noted that the peak of endogenous steady-state IL2 mRNA in these cells is also seen at 5 h (data not shown).

In this report we have examined the role of IL1 in mediating transcriptional enhancement from the IL2 promoter in the presence of both the phorbol ester,

TPA, and calcium ionophore, A23187. Thus, we have been able to assess the effects of IL1 in cells maximally stimulated by conventional criteria. By utilizing recombinant constructs containing only the IL2 gene promoter, it was possible to distinguish between effects on IL2 gene transcription and mRNA stability. In EL4.E1 cells, addition of as little as 2.5 units of IL1 per ml led to a significant rise in CAT activity expressed from the IL2 promoter early after stimulation. This effect was also manifest as an increase in the level of endogenous IL2 mRNA detected at 5 h. IL1 did not alter the transient nature of IL2 gene induction but rather it enhanced expression only when the cells were maximally activated; even in the absence of IL1, IL2 mRNA accumulation peaked at 5 h. This enhancement was promoter-specific as neither IL4 nor pRSV-CAT expression was increased at 5 h. By contrast the suppression of CAT activity seen at 18 h can be attributed to a more general pancellular decrease in translational efficiency because even constitutively expressed pRSV-CAT was affected (Table 1).

The effect of IL1 seen in EL4.E1 cells is not likely due to increased intracellular cAMP as has been proposed for NK cells and pre-B-cells (Shirakawa *et al.*, 1988). As shown in Table 1, the effects of 20 units per ml IL1 and 10 μM forskolin are clearly different. pRSV-CAT expression is enhanced at 18 h with forskolin but is inhibited with the concentration of IL1 used here. While expression of pIL2 (-321) is depressed at 18 h by both IL1 and forskolin, the 5 h results clearly demonstrate the dissimilarity of these two stimuli. Forskolin is even more suppressive at 5 h than at 18 h while IL1 is stimulatory at this time point (Table 1; see also Fig. 2B, left panel, and Fig. 3A). However, we can not rule out completely the possibility that the early stimulatory effects of IL1 are due to small rises in intracellular cAMP because in some experiments stimulation with 10^{-7}-10^{-8} M forskolin in conjunction with TPA and A23187 resulted in increased (less than twofold) expression from pIL2 (-2800) (data not shown).

Clearly, though, increases in cAMP of the magnitude seen by Shirakawa *et al.* (1988), are not responsible for mediating the IL1 enhancement that we have noted.

The possibility that IL1 mediates its effects through NF-κB is not ruled out by our data. In a number of cell lines, IL1 has been shown to induce expression of a protein whose DNA-binding specificity is similar to NF-κB (Osborn *et al.*, 1989; Shirakawa *et al.*, 1989a). We have confirmed the observation of Osborn *et al.* (1989) that TPA induce the appearance of this factor in EL4.E1 cells and have extended this to show that costimulation with IL1 increases NF-κB binding activity over TPA alone (D. Chen and E. V. Rothenberg, unpublished). The failure of IL1 to have an effect on Jurkat cells (Fig. 3B) is consistent with its working through NF-κB because in these cells IL1 does not induce appearance of this DNA-binding protein (Osborn *et al.*, 1989). Our results also suggest that NF-κB, if implicated, may be activated by a pathway distinct from cAMP or phorbol ester/PKC. In addition, the results with pIL2 (-232) indicate that in the absence of detectable IL2 promoter activity the activation of NF-κB has no effect, thus implying that this factor, by itself, is not sufficient to ensure IL2 gene expression.

Lastly, an extensive examination of the IL2 5' flanking region has revealed numerous positive and negative regulatory elements, all of which are subordinate to a more powerful activation signal (Novak *et al.*, submitted). Thus, in stimulated EL4.E1 cells transfected with pIL2-CAT plasmids a hierarchy of expression is seen that is dependent on the length of the upstream sequence included in a particular construct. Negative modulators of IL2 expression (e.g., cyclosporin A, cAMP) affect all expressible constructs to the same extent. Here we have shown that positive modulation of IL2 transcription by IL1 is mediated by sequences proximal to -321 of the IL2 gene promoter. Addition of upstream DNA to -2800 revealed no additional IL1-specific effects. This result, combined with our previous observations (Novak and Rothenberg, submitted) suggests that

modulation of IL2 expression is secondary to and subordinate to the ability to transcribe this gene in the first place. In this light, the results of Hoyos *et al.* (1989) are instructive. Using a human IL2 promoter-CAT construct they demonstrated that directed mutations that abrogate NF-κB binding also cause a decrease in CAT expression. However, none of their mutations had the result of completely abolishing expression. The ability of upstream sequences to compensate for the deletion of the NF-κB binding site has been noted previously (Durand *et al.*, 1988; Williams *et al.*, 1988). These results suggest that it is unlikely that this factor is necessary, let alone sufficient, for IL2 expression.

References

Abraham, R. T., S. N. Ho, T. J. Barna, and D. J. McKean. 1987. Transmembrane signaling during interleukin 1-dependent T-cell activation. J. Biol. Chem. **262**:2719-2728.

Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, M. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin. 1987. Phorbol ester-inducible genes contain a common Cis element recognized by a TPA-modulated trans-acting factor. Cell **49**:729-739.

Boyer, P. D., S. A. Diamond, and E. V. Rothenberg. 1989. Changes in inducibility of IL-2 receptor α-chain and T cell-receptor expression during thymocyte differentiation in the mouse. J. Immunol. **142**:4121-4130.

Cross, S. L., N. F. Halden, M. J. Lenardo, and W. J. Leonard. 1989. Functionally distinct NF-κB binding sites in the immunoglobulin κ and IL-2 receptor α chain genes. Science **244**:466-469.

Dinarello, C. A. 1989. Interleukin-1 and its biologically related cytokines. Adv. Immunol. **44**:156-205.

Durand, D. B., J.-P. Shaw, M. R. Bush, R. E. Reployle, R. Belagaje, and G. R. Crabtree. 1988. Characterizaiton of antigen receptor response elements within the interleukin-2 enhancer. Mol. Cell. Biol. **8**:1715-1724.

Durum, S. K., J. A. Schmidt, and J. J. Oppenheim. 1985. Interleukin 1: an immunological perspective. Ann. Rev. Immunol. **3**:263-287.

Farrar, J. J., S. B. Mizel, J. Fuller-Farrar, W. L. Farrar, and M. L. Hilfiker. 1980. Macrophage-independent activation of helper T cells. I. Production of interleukin 2. J. Immunol. **125**:793-798.

Favaloro, J., R. Treisman, and R. Kamen. 1980. Transcription maps of polyoma virus-specific RNA: Analysis by two-dimensional nuclease S1 gel mapping. Meth. Enzymol. **65**:718-749.

Hackett, R. J., L. S. Davis, and P. E. Lipsky. 1988. Comparative effects of tumor necrosis factor-α and IL-1β on mitogen-induced T cell activation. J. Immunol. **140**:2639-2644.

Harris, D. T., W. J. Kozumbo, P. Cerutti, and J.-C. Cerottini. 1987. Molecular mechanisms involved in T cell activation. I. Evidence for independent signal-transducing pathways in lymphokine production vs. proliferation in cloned cytotoxic T lymphocytes. J. Immunol. **138**:600-605.

Heckford, S. E., E. P. Gelmann, C. L. Agnor, S. Jacobson, S. Zinn, and L. A. Matis. 1986. Distinct signals are required for proliferation and lymphokine gene expression in murine T cell clones. J. Immunol. **137**:3652-3663.

Kaye, J., S. Gillis, S. B. Mizel, E. M. Shevach, T. R. Malek, C. A. Dinarello, L. B. Lachman, and C. A. Janeway, Jr. 1984. Growth of a cloned helper T cell line induced by a monoclonal antibody specific for the antigen receptor: interleukin 1 is required for the expression of receptors for interleukin 2. J. Immunol. **133**:1339-1345.

Koide, S. and R. M. Steinman. 1987. Induction of murine interleukin 1: Stimuli and responsive primary cells. Proc. Natl. Acad. Sci. USA **84**:3802-3806.

Lee, F., T. Yokota, T. Otsuka, P. Meyerson, D. Villaret, R. Coffman, T. Mosmann, D. Rennick, N. Roehm, G. Smith, A. Zlotnik, and K.-I. Arai. 1986. Isolation and characterization of a mouse interleukin cDNA that expresses B-cell stimulatory factor 1 activities and T-cell- and mast-cell-stimulating activities. Proc. Natl. Acad. Sci. USA **83**:2061-2065.

Lichtman, A. M., J. Chin, J. A. Schmidt, and A. K. Abbas. 1988. Role of interleukin 1 in the activation of T lymphocytes. Proc. Natl. Acad. Sci. USA **85**:9699-9703.

Mary, D., C. Aussel, C. Pelassy, and M. Fehlmann. 1988. IL-1 signaling for IL-2 production in T cells involves a rise in phosphatidylserine synthesis. J. Immunol. **141**:3078-3080.

McGuire, K. L., J. A. Yang, and E. V. Rothenberg. 1988. Influence of activating stimulus on functional phenotype: Interleukin-2 mRNA accumulation differentially induced by ionophore and receptor ligands in subsets of murine T cells. Proc. Natl. Acad. Sci. USA **85**:6503-6507.

Mizel, S. B. 1982. Interleukin 1 and T cell activation. Immunol. Rev. **63**:51-72.

Muegge, K., T. M. Williams, J. Kant, M. Karin, R. Chiu, A. Schmidt, U. Siebenlist, H. A. Young, and S. K. Durum. 1989. Interleukin-1 costimulatory activity on the interleukin-2 promoter via AP-1. Science **246**:249-251.

Nau, G. J., D.-K. Kim, and F. W. Fitch. 1988. Agents that mimic antigen receptor signaling inhibit proliferation of cloned murine T lymphocytes induced by IL-2. J. Immuno.. **141**:3557-3563.

Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transducted and tumour promotion. Nature **308**:693-698.

Novak, T. J. and E. V. Rothenberg. 1986. Differential transient and long-term expression of DNA sequences introduced into T lymphocyte lines. DNA **5**:439-451.

Novak, T. J., P. M. White, and E. V. Rothenberg. 1990. Regulatory anatomy of the murine interleukin-2 gene. Submitted for publication.

Novak, T. J. and E. V. Rothenberg. 1990. cAMP inhibits induction of IL2 but not of IL4 in T cells. Submitted for publication.

Osborn, L., S. Kunkel, and G. J. Nabel. 1989. Tumor necrosis factor α and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor NF-κB. Proc. Natl. Acad. Sci. USA **86**:2336-2340.

Raz, A., A. Wyche, N. Siegel, and P. Needleman. 1988. Regulation of fibroblast cyclooxygenase synthesis by interleukin-1. J. Biol. Chem. **263**:3022-3028.

Reeves, R., T. S. Elton, M. S. Nissen, D. Lehn, and K. R. Johnson. 1987. Posttranscriptional gene regulation and specific binding of the nonhistone protein HMG-I by the 3' untranslated region of bovine interleukin 2 cDNA. Proc. Natl. Acad. Sci. USA **84**:6531-6535.

Rosoff, P. M., N. Savage, and C. A. Dinarello. 1988. Interleukin-1 stimulates diacylglycerol production in T lymphocytes by a novel mechanism. Cell **54**:73-81.

Rothenberg, E. V., R. A. Diamond, K. A. Pepper, and J. A. Yang. 1990. Interleukin-2 gene inducibility in T cells prior to T-cell receptor expression: Changes in signalling pathways and gene expression requirements during intrathymic maturation. J. Immunol. **144**:1614-1624.

Serfling, E., R. Barthelmäs, I. Pfeuffer, B. Schenk, S. Zarius, R. Swoboda, F. Mercurio, and M. Karin. 1989. Ubiquitous and lymphocyte-specific factors are involved in the induction of the mouse interleukin 2 gene in T lymphocytes. EMBO J. **8**:465-473.

Shaw, G. and R. Kamen. 1986. A conserved AU sequence from the 3' untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell **46**:659-667.

Shaw, J.-P., P. J. Utz, D. B. Duncan, J. J. Toole, E. A. Emmel, and G. R. Crabtree. 1988. Identification of a putative regulator of early T cell activation genes. Science **241**:202-205.

Shirakawa, F., U. Yamashita, M. Chedid, and S. B. Mizel. 1988. Cyclic AMP—an intracellular second messenger for interleukin 1. Proc. Natl. Acad. Sci. USA **85**:8201-8205.

Shirakawa, F. M. Chedid, J. Suttles, B. A. Pollk, and S. B. Mizel. 1989a. Interleukin 1 and cyclic AMP induce κ immunoglobulin light chain expression via activation of an NF-κB-like DNA-binding protein. Mol. Cell. Biol. **9**:959-964.

Shirakawa, F. and S. B. Mizel. 1989b. *In vitro* activation and nuclear translocation of NF-κB catalyzed by cyclic AMP-dependent protein kinase and protein kinase C. Mol. Cell. Biol. **9**:2424-2430.

Simon, P. L. 1984. Calcium mediates one of the signals required for interleukin 1 and 2 production by murine cell lines. Cell. Immunol. **87**: 720-726.

Smith, K. A., K. J. Gilbride, and M. F. Favata. 1980. Lymphocyte activating factor promotes T cell growth factor production by cloned murine lymphoma cells. Nature **287**:853-855.

Sugiura, T. and K. Waku. 1984. Enhanced turnover of arachidonic acid-containing species of phosphatidylinositol and phosphatidic acid of concanavalin A-stimulated lymphocytes. Biochim. Biophys. Acta **796**:190-198.

Williams, T. M., L. Eisenberg, J. E. Burlein, C. A. Norris, S. Pancer, D. Yao, S. Burger, M. Kamoun, and J. A. Kant. 1988. Two regions within the human IL-2 gene promoter are important for induible IL-2 expression. J. Immunol. **141**:662-666.

Yokota, T., N. Arai, F. Lee, D. Rennick, T. Mosmann, and K.-I. Arai. 1985. Use of a cDNA expression vector for isolation of mouse interleukin 2 cDNA clones: Expression of T-cell growth-factor activity after transfection of monkey cells. Proc. Natl. Acad. Sci. USA **82**:68-72.

Zlotnik, A. and B. Daine. 1986. Activation of IL1-dependent and IL1-independent T cell lines by calcium ionophore and phorbol ester. J. Immunol. **136**:1033-1037.

Table I. IL1 and forskolin have distinct effects on expression of pRSV-CAT and pIL2(-321) in EL4.E1 cells.

Construct	Stimulation Time	CAT Activity (% of control)[a]	
		+IL1 (20 u/ml)	+10 μM forskolin[b]
pRSV-CAT	18h	68 ± 0.5	427 ± 99
pIL2(-321)	5h	169 ± 22	28 ± 4
	18h	43 ± 6	51 ± 17

a Cells were stimulated for the indicated lengths of time with 10 ng/ml TPA + 37 ng/ml A23187 in the presence or absence of IL1 or forskolin. CAT activity per 1 x 10^6 cell equivalents was determined and the value for the drug-treated samples was normalized with respect to the drug-free control. Results are presented as mean ± S.E.M. of 2-4 experiments each.

b These results have been presented in more detail previously (Novak and Rothenberg, submitted).

Table II. IL1 treatment does not affect the cell cycle distribution of phorbol ester and ionophore-treated EL4.E1 cells[a]

	% of Total Cells in					
	5h			18h		
	Unstim.	Stim.	Stim. + IL1	Unstim.	Stim.	Stim. + IL1
G_0	5.2	1.9	2.2	4.3	0.5	0.5
G_1	49.4	22.0	23.3	49.4	27.6	31.3
S	26.3	37.1	34.4	27.0	24.9	30.0
G_2 + M	18.4	37.6	38.9	18.9	45.3	36.4

[a] Cells were stimulated with 10 ug/ml TPA + 37 ng/ml A23187 with or without 20 u/ml rILα or they were left unstimulated. At the indicated times cells were harvested, fixed in 50% EtOH and stored at 4°C. Acridine orange analysis of cell cycle distribution was as described (Boyer *et al.*, 1989) using an Ortho cytofluorograf 50H.

Fig. 1 Gel blot analysis of cytoplasmic RNA extracted from unstimulated EL4.E1 cells (lane 1) or from cells stimulated for 5h with 10 ng/ml TPA (lane 2), TPA + 37 ng/ml A23187 (lane 3), and TPA + A23187 + 20 u/ml hrIL1α (lane 4). RNA from ~3 x 10^6 cells was electrophoresed on a denaturing 1% agarose-formaldehyde gel, transferred to a nylon filter, (Nytran, S & S), and hybridized sequentially with cDNA probes specific for IL4, IL2 and α skeletal actin.

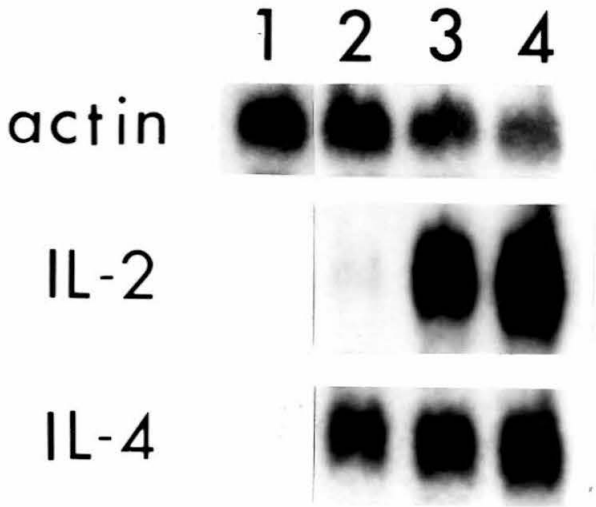

1 2 3 4
actin
IL-2
IL-4

Fig. 2 (A) Gel blot of cytoplasmic RNA extracted from unstimulated or stimulated EL4.E1 cells. Cells were stimulated as indicated in the figure and RNA from 1 x 10^6 cells was extracted, electrophoresed on a denaturing 1% agarose-formaldehyde gel, transferred to a nylon membrane (Nytran, S & S), and hybridized to cDNA probes for IL2 and α skeletal actin.

(B) CAT assay of extracts from EL4.E1 cells tranfected with pIL2(-2800). Cells were stimulated as in (A). Each lane represents the identical stimulation conditions of the RNA gel blot shown above it. Extracts were assayed at 37°C for 5h. Rightmost two lanes represent negative and positive assay controls, respectively. Positive control contains commercial CAT enzyme. [C] = unacetylated chloramphenicol; [CA] = monoacetylated chloramphenicol.

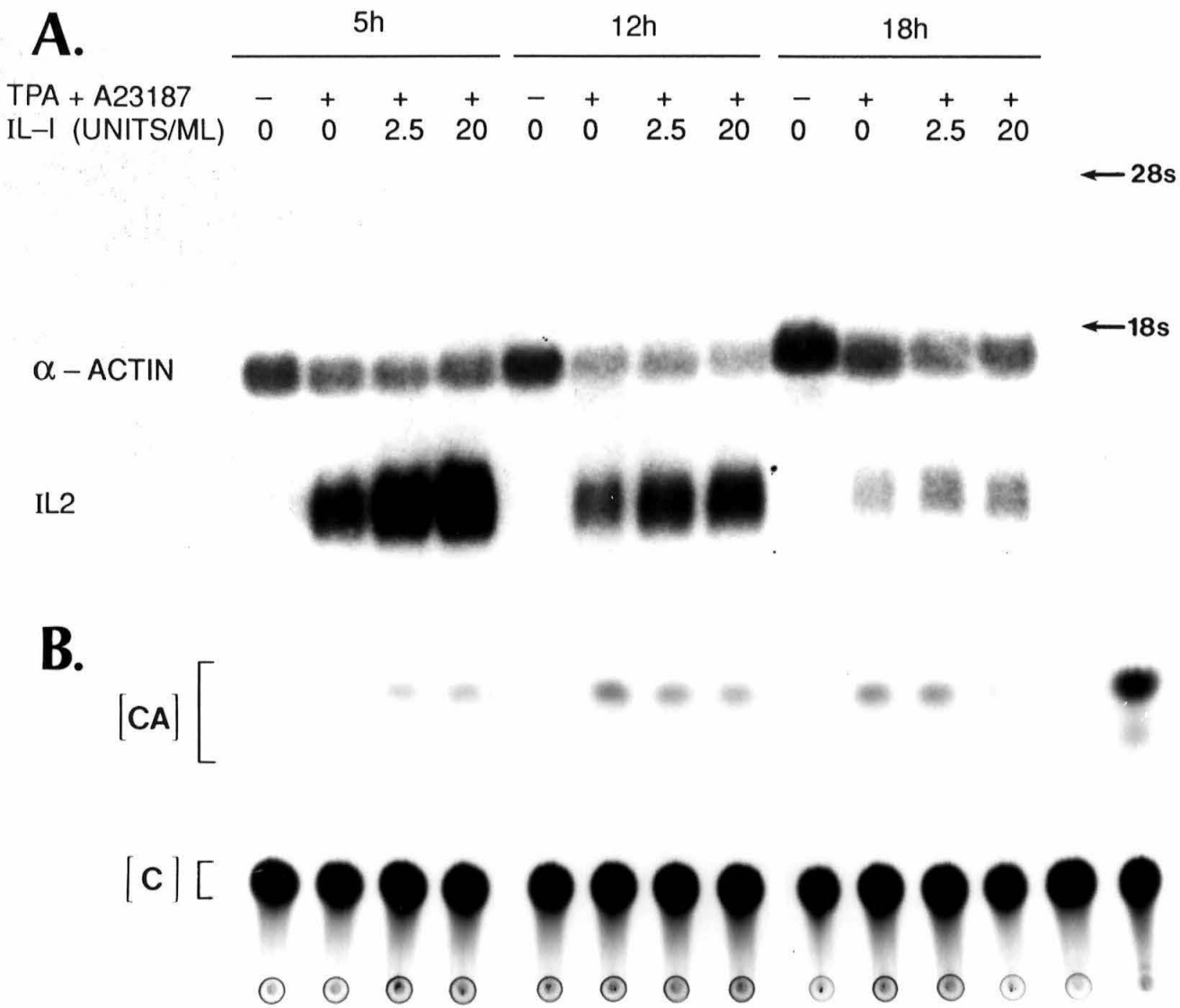
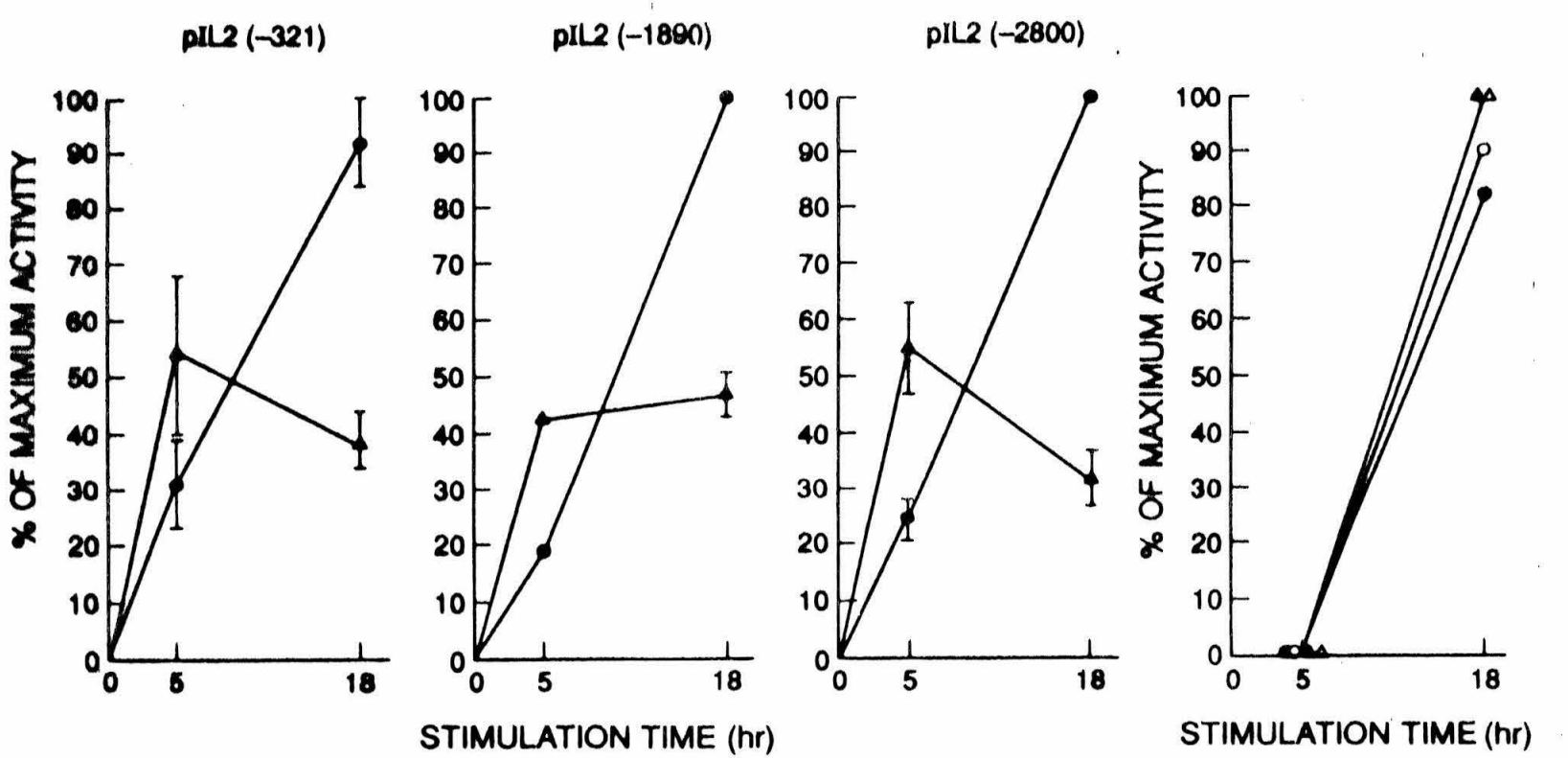

A.
5h
12h
18h
TPA + A23187 – + + + – + + + – + + +
IL–I (UNITS/ML) 0 0 2.5 20 0 0 2.5 20 0 0 2.5 20
28s
18s
α – ACTIN
IL2
B.
[CA]
[C]

Fig. 3 (A) Normalized CAT assay curves for pIL2(-321), pIL2(-1890), and pIL2(-2800). Calculations for each plasmid were done separately. Each experiment consisted of 4 data points: 5h stimulated ± 20 u/ml hrIL1α and 18h stimulated ± 20 u/ml hrIL1α. Cat activity per 10^6 cells was normalized with respect to the highest value in that experiment (in all but one case this was the 18h, no IL1 sample). Results are the mean ± S.E.M. of 2-5 separate experiments, except for the pIL2 (-1890) 5h values, which were determined once.

(B) Normalized CAT assay curve for transfected Jurkat cells. Experimental conditions and calculations were performed exactly as in (A). Open symbols are pIL2 (-321); closed symbols are pIL2 (-2800). Data is from one experiment.


A. EL4.EI
● TPA + A23187
▲ TPA + A23187 + 20 u/m/IL1
B. JURKAT
●,○ TPA + A23187
▲,△ TPA + A23187 + 20 u/m/IL1
pIL2 (-321)
pIL2 (-1890)
pIL2 (-2800)
% OF MAXIMUM ACTIVITY
STIMULATION TIME (hr)
0
5
18
10
20
30
40
50
60
70
80
90
100

CONCLUSION

Understanding the mechanisms controlling IL2 gene transcription is important for more than just the insight it provides into regulatory mechanisms of inducible gene expression. Because IL2 is a potent growth and differentiation factor for both T and B cells, the expression of this lymphokine is central to the control of immune responsiveness. Thus it could be argued that the ultimate stimulators of the immune response are those factors which directly result in transcription of this gene. The proximate stimulators of IL2 expression: antigen, accessory cells, other cytokines, signaling pathways, etc., may shed light on the related question of lineage commitment during T-cell development. One or more of these proximate factors may selectively trigger precommitted IL2 producers, or may cause a cell to select IL2 production from among a number of possible responses (Rothenberg *et al.*, 1990). The lineage relationship between IL2 producing Th_1 cells and IL4 producing Th_2 cells have been the focus of much current research. A simple model which affixes these different effector functions upon separate parallel lineages of T cells is almost certainly incorrect. Instead, what has emerged is a sense that Th_1 and Th_2 dominated responses reflect distinct types of immune responses. For example resting T cells produce abundant IL2, but little IL4 and IL5, upon stimulation with antigen or mitogenic lectin. However, if purified $CD4^+$ T cells are cultured for several days *in vitro* (priming) before being stimulated they tend to produce primarily IL4 and very little IL2 (Salmon *et al.*, 1989; Swain *et al.*, 1990). These same observations have been made for antigenic challenge, *in vivo*. The response to primary immunization is characterized by IL2 production, whereas the recall response to antigen is dominated by IL4 secretion (Bottomly, 1988). This progression from IL2 dominated responses to IL4 dominated responses could, in theory result from two different mechanisms. One possibility is that Th_2 cells require a longer priming period than Th_1 cells before responding and thus are only seen at later times in the response. Of course, it is

necessary for Th_1 cells to be short lived or in some way inactivated, otherwise one would still detect abundant IL2. This corollary suggests another possible explanation for the temporal separation of Th_1 and Th_2 responses, namely that Th_2 cells arise from activated Th_1 cells. The latter hypothesis is consistent with data demonstrating that memory T cells are usually of the Th_2 type. This developmental progression is also corollated with changes in the cell surface expression of several T-cell antigens. Primary responders are $CD44^-$, CD45R hi and $CD29^-$ while memory T cells are $CD44^+$, CD45 lo and $CD29^+$ (Sanders *et al.*, 1988). Such corollated changes in surface antigen expression and lymphokine production can be seen easily, *in vitro*, with lectin-stimulated human peripheral blood lymphocytes (Akbar *et al.*, 1988). The expression of CD29 by IL4-producing Th_2 cells is provocative in that it provides a mechanism by which this lymhokine is selectively secreted. An anti-CD29 antibody has been reported to increase cAMP in $CD29^+$ T cells (Groux *et al.*, 1989). As shown in Chapter 2, increased concentrations of this second messenger inhibit expression of IL2 but not of IL4, even in a cell line which can express both lymphokine genes. If T cells do in fact progress from IL2 producers to IL4 producers it is likely that this change is accompanied by changes in transcription factors, signaling pathways and/or other aspects of cellular physiology. By elucidating the factors which are responsible for the stringent cell-type and inducible control of IL2 gene expression it should become possible to identify candidate *trans*-acting factors which may identify a T cell as type 1 or type 2.

The factors responsible for high level production of IL2 in Th_1 cells are a combination of ubiquitous proteins (AP-1, NF-κB, Oct-2) and NFAT-1, an inducible transcription factor which has been reported to be restricted to IL2 producers. The data presented in Chapter 2, and results obtained by this lab more recently, are consistent with the notion that NFAT-1 is necessary but not sufficient for IL2

gene transcription. The evidence that NFAT-1 is required for expression comes from transient expression assays using mutant IL2 promoter constructs. The expression of a 5' deletion mutant, pIL2(-232), that lacks sequences upstream of -232, including the NFAT-1 binding site, is several orders of magnitude lower than a similar construct, pIL2(-321), that contains this binding site. In addition, we have since made an internal deletion in pIL2(-1890) by removing sequences between two XmnI restriction enzyme sites located at -326 and -266. This plasmid is not expressed in either EL4.E1 or Jurkat cells (T. J. Novak, P. M. White and E. V. Rothenberg, submitted). Thus, the NFAT-1 site, or sequences directly upstream that are also removed in this construct, or both, are required for high level expression from the IL2 promoter.

While these results are consistent with a requirement for NFAT-1, other data suggest that the presence of this factor is not sufficient for transcription of the IL2 gene. An analysis of nuclear protein extracts by gel retardation assays indicated that NFAT-1 is constitutively expressed in EL4.E1 cells (D. Chen and E. V. Rothenberg, unpublished observations). These cells, however, do not transcribe either their endogenous IL2 gene or transfected IL2-CAT constructs unless stimulated by TPA. Constitutive expression of NFAT-1 was also detected in a number of long-term murine T-cell lines, including CTL and Th_2 lines which do not express IL2 under any of the stimulation conditions examined.

Deletion analysis of the human IL2 promoter has indicated a role for AP-1, Oct 2, and NFκB in modulating expression of this gene. However, deletion of the binding sites for these factors did not prevent expression, suggesting they are not required for transcription. A construct, pIL2(-232), that contains binding sites for all three factors was expressed only slightly above background when transfected into EL4.E1 cells. Since gel shift experiments have demonstrated the presence of these factors in stimulated EL4.E1, (D. Chen and E. V. Rothenberg, unpublished),

their presence is insufficient to cause transcription of the IL2 gene.

The inhibition and enhancement of IL2 expression by cAMP and IL1, respectively, may result from changes in NFκB binding activity. Gel shift experiments indicate that co-stimulation with TPA/A23187 and IL1 results in an increase in NFκB activity, while increased cAMP caused the opposite result (D. Chen and E. V. Rothenberg, unpublished). These results support the prediction reached from transient transfection experiments (Chapter 3), that if IL1 upregulated NFκB activity in T cells, it did so via a mechanism that was distinct from the cAMP-dependent pathway operating in pre-B and NK cells.

The results presented here do not suggest that a single factor (*e.g.*, NFAT-1) plays the role of a "master switch" in the control of IL2 gene expression. There is strong evidence, however, that the presence of this factor is necessary for high level expression, and that other identified factors which bind to the IL2 promoter can modulate transcription. Far upstream DNA sequences also play a role in transcription regulation, but these regions, and the factors which interact with them, have not been characterized in detail.

REFERENCES

Akbar, A. N., L. Terry, A. Timms, P. C. L. Beverly, and G. Janossy. 1988. Unidirectional phenotypic changes within the T200 complex during activation of T cells. *J. Immunol.* **140**, 2171-2178.

Bottomly, K. 1988. A functional dichotomy in $CD4^+$ T lymphocytes. *Immunol. Today* **9**, 268-271.

Groux, H., S. Huet, H. Valentin, D. Pham, and A. Bernard. 1989. Suppressor effects and cyclic AMP accumulation by the CD29 molecule of $CD4^+$ lympohocytes. *Nature* **339**, 152-154.

Rothenberg, E. V., D. Chen, R. A. Diamond, M. Dohadwala, T. J. Novak, P. M. White, and J. A. Yang-Snyder. 1990. Acquisition of mature functional rsponsiveness: Programming for function via signaling. Manuscript in preparation.

Salmon, M., G. D. Kitas, and P. A. Bacon. 1989. Production of lymphokine mRNA by $CD45R^+$ and $CD45R^-$ helper T cells from human peripheral blood and by human $CD4^+$ T cell clones. *J. Immunol.* **143**, 907-912.

Sanders, M. E., M. W. Makgoba, S. O. Sharrow, D. Stephany, T. A. Springer, H. A. Young, and S. Shaw. 1988. Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, LFA-1) and three other molecules (UCLL1, CDw29 and Pgp-1) and have enhanced IFN-γ production. *J. Immunol.* **140**, 1401-1407.

Swain, S. L., Weinberg, A. D., and M. English. 1990. $CD4^+$ T cell subsets: Lymphokine secretion of memory cells and of effector cells that develop from precursors in vitro. *J. Immunol.* **144**, 1788-1799.

APPENDIX

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Developmental Biology

Inducible expression of a cloned heat shock fusion gene in sea urchin embryos

(microinjection/gene regulation)

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ABSTRACT **A fusion gene construct, in which the coding sequence for bacterial chloramphenicol acetyltransferase (CAT; acetyl-CoA: chloramphenicol 3-*O*-acetyltransferase, EC 2.3.1.28) was placed under the control of the regulatory region of the *Drosophila* gene encoding the 70-kilodalton heat shock protein (see Di Nocera, P. P. & Dawid, I. B. (1983) *Proc. Natl. Acad. Sci. USA* 80, 7095–7098), was microinjected into the cytoplasm of unfertilized sea urchin eggs. Pluteus-stage embryos developing from the injected eggs were exposed to high temperature conditions that we found would elicit an endogenous sea urchin heat shock response. These embryos express the gene for CAT and, after heat treatment, display 8–10 times more CAT enzyme activity than do extracts from control embryos cultured at normal temperatures. The injected DNA is present in high molecular weight concatenates and, during development, is amplified about 100-fold. Amplified sequences are responsible for all or most of the induced CAT enzyme activity.**

We recently developed procedures for the microinjection of cloned DNA into sea urchin eggs and determined the fate of the exogenous DNA during development (1, 2). In the present paper, we provide initial evidence that DNA sequences introduced into these eggs can be transcribed and productively expressed, in response to internal physiological signals. The DNA is injected into the cytoplasm before fertilization, sperm is then added, and development is allowed to proceed. We found earlier that when linear DNA molecules are injected, they are rapidly ligated into high molecular weight end-to-end concatenates, and that these undergo extensive replication during the first 24 hr of embryogenesis. The average net amplification of exogenous DNA mass during this period is 25-fold (1); in experiments reported below, an amplification of about 100-fold was observed.

The present experiments were conducted with a fusion gene assembled by Di Nocera and Dawid (3), in which the bacterial structural gene for chloramphenicol acetyltransferase (CAT, acetyl-CoA:chloramphenicol 3-*O*-acetyltransferase, EC 2.3.1.28) is placed under the control of the 5′ regulatory region of the gene coding for the *Drosophila* 70-kDa heat shock protein (hsp70). Di Nocera and Dawid showed that when this construct is transfected into *Drosophila* tissue culture cells, synthesis of the CAT enzyme is induced by heat treatment. Nucleotide sequences that are shared between the several different *Drosophila* heat shock genes have been demonstrated to be required for transcriptional induction under high temperature conditions (4–7). Most important for our present considerations is the finding that the *Drosophila* hsp70 regulatory region functions inductively in heterospecific systems, including the *Xenopus* oocyte nucleus (7, 8), where it is inactive at 20–27°C and induced at 34–37°C; in monkey cells (5, 6) and mouse cells (9), where it is inactive at 37°C and induced at 43°C and 45°C, respectively; and in yeast cells (10), where it is inactive at 24°C and induced at 40°C. In *Drosophila* and *Drosophila* tissue culture cells, the gene is inactive at 25°C and induced at 37°C (3, 11–13). Thus, in transfection experiments the gene seems to respond to whatever temperature is stressful for the cell in which it is placed.

MATERIALS AND METHODS

Microinjection of Plasmid hsp-cat1 into Unfertilized Eggs. Collection of gametes and microinjection of sea urchin eggs were as developed by McMahon *et al.* (1). About 2 pl of a solution (24 μg/ml) of the plasmid hsp-cat1 (3) dissolved in 40% glycerol was injected into the egg cytoplasm. The plasmid, which is about 5.7 kilobases (kb) in length, was first linearized by digestion at its unique *Bgl* I site, which is located in the pBR322 component of the construct. The amount nominally injected per egg represents about 7500 molecules. As described by Di Nocera and Dawid (3), hsp-cat1 contains a 1.2-kb 5′ regulatory sequence from the *Drosophila* gene for hsp70, cloned by them into pSVO-cat, a vector that contains the Tn*9* structural gene for CAT and simian virus 40 (SV40) early region intron splice and poly(A) addition sequences, but which lacks the early region promoter.

Protein Labeling of Pluteus-Stage Embryos. At 72 hr postfertilization, embryos were suspended at a concentration of 20,000 per ml in 1 ml of Millipore-filtered sea water (FSW). Heat-shocked embryos were incubated for 1, 2, and 4 hr at 25°C, and control embryos were incubated for 4 hr at 15°C in FSW. For protein labeling, the embryos were further incubated for 1 hr at 25°C (heat shocked) or 15°C (control) in the presence of 180 μCi of [^{35}S]methionine (Amersham, >600 Ci/mmol; 1 Ci = 37 GBq). The embryos were pelleted in a Microfuge, washed once in FSW, pelleted again, and suspended in 200 μl of electrophoresis sample buffer containing 0.1 M Cleland's reagent, 2.0% $NaDodSO_4$, 80 mM Tris (pH 6.8), 10% glycerol, and 0.025% bromophenol blue.

Protein Gel Electrophoresis. Aliquots of 20 μl of labeled embryo samples were heated to 80°C for 1 min, spun briefly in a Microfuge to remove debris, and loaded onto a 10% polyacrylamide/$NaDodSO_4$ slab gel. Electrophoresis was as described by Hubbard and Lazarides (14). Gels were soaked in EN^{3}HANCE (New England Nuclear), dried under vacuum, and exposed to Kodak XAR5 film at −70°C for 90 min.

CAT Assay. Heat-shocked or control plutei were pelleted in a Microfuge and suspended in 0.25 M Tris·HCl (pH 7.8). CAT assays were essentially as described by Gorman *et al.* (15) with the following minor alterations. Embryos were lysed by two freeze-thaw cycles in a 95% ethanol/dry ice bath and spun briefly to remove cellular debris. Final reac-

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Abbreviations: hsp, heat shock protein; hsp70, 70-kDa hsp; SV40, simian virus 40; CAT, bacterial chloramphenicol acetyltransferase; *cat*, gene for CAT; FSW, Millipore-filtered sea water; kb, kilobase(s).

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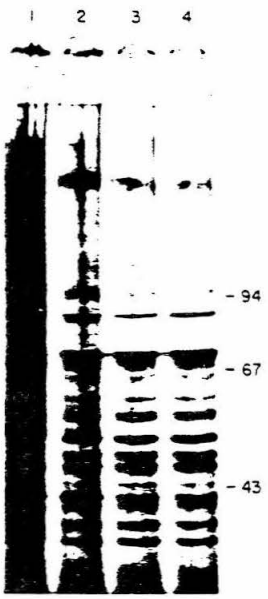

FIG. 1. Heat shock proteins in *S. purpuratus*. Proteins synthesized in pluteus-stage embryos were displayed by gel electrophoresis and autoradiographed. Embryos were maintained at a concentration of 2×10^4 per ml at the normal culture temperature of 15°C for 4 hr (lane 1) or at 25°C for 1 hr (lane 2), 2 hr (lane 3), and 4 hr (lane 4), followed in each case by a further 1 hr of incubation at 15°C or 25°C, respectively, in the presence of [^{35}S]methionine. Size markers are shown in kDa.

tion volumes were 150 μl. This contained 0.5 μCi of [^{14}C]chloramphenicol (45 mCi/mmol, Amersham) and 0.53 mM acetyl CoA (Sigma). Bacterial CAT (Pharmacia P-L Biochemicals) was used as a positive control in CAT assays and in calibrating the reaction. Assays were run for 2 hr at 37°C, and the acetylated products were separated on an Eastman Kodak silica gel TLC plate. TLCs were exposed to Kodak XAR5 film at −70°C.

RESULTS

Heat Shock Response in *Strongylocentrotus purpuratus*. Our initial problem was to determine the optimal heat shock conditions for *S. purpuratus*, which in the laboratory are raised routinely at 15°C. We chose 25°C as an appropriate temperature for studies of protein synthesis after heat shock, on the basis of preliminary survival experiments and unpublished protein synthesis data obtained by B. Brandhorst (personal communication) on the same species. Fig. 1 displays protein synthesis patterns in embryos exposed to 25°C for 2, 3, and 5 hr and in control embryos held at 15°C. Newly synthesized proteins were labeled in each sample by addition of [^{35}S]methionine for the final 1 hr of incubation. As observed in many previous studies, the autoradiograph of newly synthesized proteins of control embryos (15°C; Fig. 1, lane 1) reveals a nearly continuous size distribution. Exposure to 25°C results in a general reduction in protein synthesis and the appearance of several abundant new proteins (lanes 2–4). As in many other organisms (10), the major heat-induced species was about 70 kDa. In addition, a peptide of about 90 kDa and about five lower molecular weight bands that cannot be distinguished in the control sample also were labeled in heat-shocked *S. purpuratus* embryos. Several other fairly abundant proteins appeared to be synthesized in both control and heat-shocked *S. purpuratus* embryos. Fig. 1 shows that the accumulation of the heat shock proteins was already maximal within 2 hr (lane 2) and further incubation (lanes 3 and 4) had little effect. An incubation of 2 hr at 25°C was utilized for the subsequent experiments.

Ligation and Replication During Development of Injected hsp-cat1 DNA. Unfertilized eggs were injected from a continuously flowing microneedle with ≈7500 molecules of hsp-cat1 DNA as described in *Materials and Methods*. The injected hsp-cat1 DNA underwent the expected ligation and replication process (1, 2). This is demonstrated in the autoradiographs shown in Fig. 2. DNA was extracted from pluteus-stage (72 hr) embryos raised from injected eggs as described by McMahon *et al.* (1), run on a 0.8% agarose gel, and after transfer to nitrocellulose, hybridized to the ^{32}P-labeled hsp-cat1 probe. Lanes 1–4 of Fig. 2*a* contain serial dilutions of

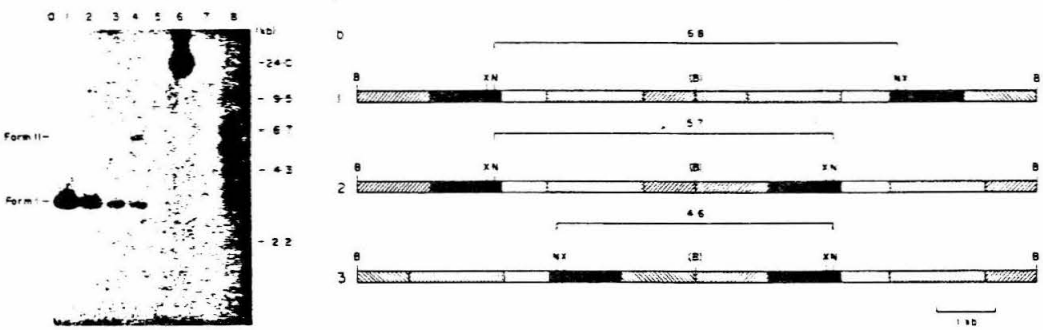

FIG. 2. Concatenation and amplification of injected hsp-cat1 DNA. (*a*) Autoradiograph of gel blot-hybridization between hsp-cat1 probe and DNA extracted from pluteus-stage embryos. Lanes: 1–4, DNA standards containing 1.80×10^7 (lane 1), 0.90×10^7 (lane 2), 0.45×10^7 (lane 3), and 0.22×10^7 (lane 4) molecules of hsp-cat1; 5–8, DNA samples from pluteus-stage embryos: DNA from embryos grown from uninjected eggs (lanes 5 and 7), DNA from embryos derived from eggs injected with hsp-cat1 (lanes 6 and 8), undigested DNA (lanes 5 and 6), and *Nru* I-digested DNA (lanes 7 and 8). DNA size markers (kb) are indicated. Exposure was for 3 hr with an intensifying screen. (*b*) Restriction maps of hsp-cat1 dimers that would be formed by ligation of linearized plasmid at *Bgl* I site. Solid bar, 5′ regulatory region from a *Drosophila* 87C hsp70 gene; open bar, gene for CAT; stippled bar, SV40 "t" intron and early region poly(A) addition sequences; hatched bar, pBR322 [from Di Nocera and Dawid (3)]. Only relevant restriction sites are shown: B, *Bgl* I; X, *Xho* I; N, *Nru* I. Maps: 1, "head to head" concatenate; 2, "head-to-tail" or "tail-to-head" concatenate; 3, "tail-to-tail" concatenate. Sizes of fragments that would be released on *Nru* I digestion are indicated by brackets.

Table 1. Amplification of hsp-cat1 and CAT enzyme activity in pluteus-stage embryos

Data	Exp. 1	Exp. 2
Amplification of hsp-cat1*	×107	×99
CAT enzyme activity		
Control embryos (15°C)		
Acetylation of [^{14}C]CA†	0.9%	0.8%
Units of CAT activity per sample‡	7.2×10^{-4}	6.9×10^{-4}
Heat-shocked embryos (25°C)		
Acetylation of [^{14}C]CA†	7.5%	8.0%
Units of CAT activity per sample‡	29.5×10^{-4}	31.2×10^{-4}
Estimated molecules of CAT subunit per embryo‡	0.91×10^{6}	0.90×10^{6}
Molec. CAT subunit§ / molec. hsp-cat1	0.86	0.85

*Amplification of injected sequences was estimated from densitometry or scintillation counting of pooled embryo DNA samples with reference to known standards as in Fig. 2. Each egg was assumed to have received 2 pl of a solution (24.0 μg/ml) of hsp-cat1, or 7.5×10^3 molecules.

†Percentage acetylation of chloramphenicol (CA) was determined by scintillation counting of substrate and product spots after TLC. Corresponding CAT enzyme activity was then determined by reference to the standard curve, using the equations given in the legend to Fig. 4.

‡Estimates of the number of CAT enzyme molecules were made by utilizing a nearly pure CAT enzyme preparation as a specific activity standard. The CAT enzyme preparation was produced in tissue culture using a baculovirus expression vector as described by Smith *et al.* (24). In $NaDodSO_4$ gels, the only protein visible in this preparation is the 25.6-kDa CAT subunit (15). The specific activity, S, measured as in Fig. 4, was about 90 units/μg of protein (this, of course, could be an underestimate because some of the enzyme molecules in the standard preparation could have been inactive). The number of enzyme subunit molecules per embryo is 2.35×10^{13} A/SN, where 2.35×10^{13} is the number of 25.6-kDa subunits per μg, A is the measured number of units of activity per sample, and N is the number of embryos in the sample, about 840, for Exp. 1 and 900 for Exp. 2. The estimates given here and in the bottom row assume that all embryos contain injected, amplified hsp-cat sequences. However, Flytzanis *et al.* (2) have shown that an average of only 60% of larvae actually contain amplified exogenous DNA after injection. Thus positive larvae could easily have expressed as much as 2-fold more molecules of CAT enzyme than shown.

§(‡ row)/(* row) (7.5×10^3), where 7.5×10^3 is the number of hsp-cat1 molecules injected per egg.

hsp-cat1 as reference standards. No hybridization of the probe was detected with DNA from uninjected embryos (lanes 5 and 7), whereas in the undigested DNA sample from injected eggs (lane 6), the probe hybridized to a high molecular weight band that occupied the same position as the total high molecular weight genomic DNA of the embryos. On digestion with *Nru* I, three reactive fragments of approximately 6.8, 5.7, and 4.6 kb were observed. These are exactly the fragments predicted if the injected plasmid forms a random concatenate by end-to-end ligation (1), as illustrated in Fig. 2*b*. Thus, with arbitrary reference to the *Bgl* I end closest to the SV40 sequences in linearized hsp-cat1 as the "head" and the other end as the "tail," digestion of a random end-to-end concatenate with *Nru* I would generate the reactive head-to-head (Fig. 2*b*, map 1), head-to-tail (Fig. 2*b*, map 2), and tail-to-tail (Fig. 2*b*, map 3) fragments of the lengths shown.

Reference to the DNA standards included in Fig. 2*a* indicates that the mass of the exogenous DNA was amplified greatly during development. Had no amplification occurred, with total recovery assumed, only about 3.4×10^5 molecules of hsp-cat1, or 2.2 pg, would have been present in the 45 embryos included in the experiment. This is less by a factor of 6.5 than the DNA content of the lowest standard, in lane 4 of Fig. 2*a*. The extent of amplification was estimated by densitometry or scintillation counting after hybridization to the hsp-cat1 probe. Data for the two experiments included in Table 1 indicate a net amplification in the sample as a whole of 99-fold and 107-fold, respectively. Thus, the average pluteus-stage embryo should include about 7×10^5 molecules of hsp-cat1 and, were this distributed equally to all cells, about 500 molecules per cell.

Heat-Induced Expression of CAT Enzyme in Pluteus-Stage Embryos. After exposure to 15°C or 25°C for 2 hr, pluteus-stage embryos bearing the amplified hsp-cat1 DNA were assayed for CAT activity as described. The results of a representative experiment are shown in Fig. 3. No background of CAT activity was detected in uninjected control plutei (lanes 2 and 4). Some CAT enzyme activity was present in 15°C embryos grown from injected eggs (lane 1) and the amount of active CAT enzyme was greatly augmented in the heat-treated embryos (lane 3). Lane 5 contains the reaction products of a bacterial CAT enzyme preparation to provide markers for the positions of the major acetylated forms of chloramphenicol.

It is almost certain that the amplified DNA is responsible for the inducible CAT activity. As noted earlier, supercoiled DNA molecules failed to amplify, and a relatively very low level of CAT enzyme activity was obtained from embryos injected with supercoiled hsp-cat1. Occasionally, we en-

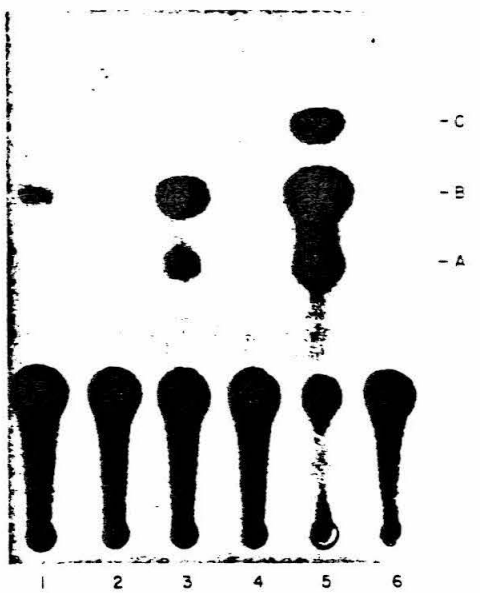

FIG. 3. CAT enzyme activity in injected embryos at 15°C and 25°C. Pluteus-stage embryos grown from eggs injected with hsp-cat1 and control plutei from uninjected eggs were incubated at the normal culture temperature of 15°C (lanes 1 and 2) or were exposed to 25°C for 2 hr (lanes 3 and 4) immediately prior to assay. Extracts for CAT assay were prepared from 837 and 836 injected plutei (lanes 1 and 3, respectively) and 1000 control plutei (lanes 2 and 4). Bacterial CAT enzyme (0.37 units) (lane 5) and the assay reaction mixture with no enzyme or extract added (lane 6) were assayed in parallel as described. Acetylated reaction products were separated from unacetylated [^{14}C]chloramphenicol substrate by ascending TLC. Reaction products (A, B, and C) are indicated. The primary and predominant product of the reaction is 3-acetyl chloramphenicol (B). Other products are a diacetylated form (C) and a product of an inefficient, nonenzymatic acyl migration (A) (16, 17). Autoradiography was for 24 hr.

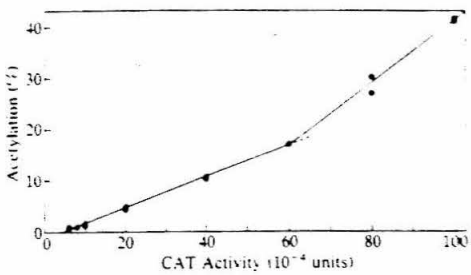

FIG. 4. Assay of CAT enzyme activity. Enzyme activity was measured in 2-hr reactions, as described by Gorman *et al.* (16) and in *Materials and Methods*. Commercial bacterial CAT enzyme was utilized in the presence of L cell extracts. The specific activity (0.37 units/μl) of the starting preparation was calibrated by the manufacturer. A unit is defined as the amount sufficient to acetylate 1 μmol of chloramphenicol per min (18). The abscissa is calibrated in 10^{-4} units, termed "U" in the following data. At U < 60, by linear least squares, A = 0.297U − 1.25, where A is % acetylation. At U = 60, the second acetylation product first becomes visible in autoradiographs of CAT assay carried out as in Fig. 3. At this point the slope doubles. Where U = 60–100, the solution is A = 0.619U − 20.5. The small abscissa intercept in the initial phase of the curve shown (A = 0 at U = 4.2) indicates that there is a slight inhibition of CAT activity caused by unknown substances in the cellular extracts. At the specific activity of the [^{14}C]chloramphenicol utilized, the sensitivity of the assay is about 5000 cpm per % acetylated products formed, though of course this value varies according to the length of time the reaction is allowed to proceed, the radioactivity of precursor utilized, and other such factors.

counter batches of eggs that fail to ligate and amplify injected linear DNA, although the DNA remains intact (1). This occurred in an experiment carried out with hsp-cat1 DNA (data not shown). Exogenous DNA could just be detected in the pluteus-stage embryos of this experiment, and the amount of CAT activity was less than 1% of that measured in the experiment shown in Fig. 3. Were the induced expression of CAT the product of a small subfraction of the hsp-cat1 molecules that do not undergo amplification, the amount of enzyme activity recovered would be expected to be independent of the net DNA amplification, contrary to the result obtained.

Approximate Quantities of CAT Enzyme Synthesized in Heat-Shocked Embryos. An easily detectable signal was obtained from about 840 injected, heat-treated embryos (Fig. 3). The sample chromatographed in lane 3 contained about 4×10^4 cpm of [^{14}C]acetylated chloramphenicol. To prepare the number of pluteus-stage embryos included in the heat-treated and control samples together (i.e., lanes 1 and 3) required 3 days of microinjection. Thus, the extremely high sensitivity of the CAT assay (16) offers for these purposes an important practical advantage. In control studies carried out with cultures of L cells transformed with a construct bearing the same CAT gene, we compared directly the signal obtained by S1 nuclease measurement of CAT mRNA and by CAT enzyme assay. Using a high-specific-activity single-stranded probe generated from an M13 primer, we recovered a protected fragment of the appropriate size containing about 1 cpm from a number of L cells (10^6) that yielded sufficient CAT activity to produce about 10^5 cpm of [^{14}C]acetylated chloramphenicol products. This result indicated that, for monitoring CAT gene expression in practical numbers of injected embryos, the enzyme assay would be greatly preferable.

The fraction of chloramphenicol acetylated in the assay was linear with enzyme activity over the range of these experiments (Fig. 4). The molecular size of the purified enzyme is known (15), and from its specific activity, the number of molecules synthesized in the heat-induced sea urchin embryos can be calculated. Data for two experiments are given in Table 1. It can be seen that the extracts from heat-induced embryos produced 8- to 10-fold more acetylated chloramphenicol than did the extracts from the 15°C embryos, which (because of the abscissa intercept of the standard curve) translates to 4–5 times more units of enzyme activity. The calculated number of molecules of CAT enzyme subunit after induction is about 10^6 per embryo. Table 1 also shows that, were all of the amplified hsp-cat1 DNA sequences active, the average (steady state) yield in heat-treated embryos would be on the order of 1 enzyme subunit molecule per gene copy. Since all may not be active and since all embryos may not contain amplified hsp-cat1 DNA (see the legend to Table 1), these are to be regarded as minimal estimates.

DISCUSSION

These results show that exogenous DNA sequences injected into the cytoplasm of the unfertilized sea urchin egg can be productively expressed during embryological development. The injected DNA also undergoes massive net amplification during development, as noted for a number of other plasmid sequences (1). Though there is as yet no direct cytological evidence, the observations that at least some of the exogenous sequences participate in both transcription and replication imply strongly that they have been sequestered into the embryo nuclei. Furthermore, the amplified hsp-cat1 sequences in the embryos are at least to some extent inducible. Several consequences follow from the observation that the *Drosophila* hsp70 control sequences can be activated in heat-stressed sea urchin embryos. First, new evidence is provided to the effect that the heat shock gene functions at the distress temperature of the host cells, here only 25°C, though in the species of origin 25°C is the standard environmental culture condition at which the gene is silent. Second, these results imply that at least some of the exogenous DNA present in the pluteus-stage embryos exists in a physical conformation such that sequence-specific regulatory interactions (4, 5, 7) may take place. Third, induction occurs in the presence of a number of hsp-cat1 molecules that is probably, on the average, 50–100 times the number of endogenous heat shock gene sequences. This implies a sufficient excess of diffusible regulatory molecules so that at least some of the hsp70 control sequences are engaged at least some of the time. It would be interesting to determine whether the presence of the injected hsp70 sequences affects the transcription of the endogenous heat shock genes.

The great sensitivity of the CAT assay (see the legend to Fig. 4) means that the signal obtained even after heat induction could be the product of fairly rare mRNAs. This is indeed suggested by calculation of the number of mRNA molecules required to produce the estimated number of CAT enzyme subunits in 2 hr. On the assumption that the rate of CAT mRNA translation at 25°C is equal to the rate of translation of sea urchin histone mRNA at 15°C, which has been measured at 0.7 codons per sec (19), to accumulate 10^6 CAT subunit molecules in 2 hr would require about 5000 RNAs per embryo, or four molecules per average cell. Of course, were translation of this heterologous sequence several times less efficient, as observed for the *Drosophila* hsp70 gene in *Xenopus* oocytes (7), proportionately more mRNAs would be needed. Nonetheless, this result implies that the induced CAT mRNAs are far less prevalent than those evidently required for the synthesis of the prominent endogenous heat shock proteins in the same embryos (see Fig. 1). The probable low ratio of CAT mRNAs per total hsp-cat1 sequence (Table 1) means either that only a small fraction of the exogenous genes participate at any one time in the induction or

that the CAT mRNA itself is unstable, or perhaps both. It may be relevant that only 0.1–0.2% of L cells receiving nuclear microinjections of pBR322 containing a thymidine kinase gene stably express this gene after a few rounds of DNA replication (20).

There are many possible reasons why the observed ratio of induced-to-uninduced CAT activities is not higher than 8–10 (Table 1). In *Drosophila* tissue culture cells transformed with hsp-cat1, a ratio of about 30 in CAT activity was obtained (3). A larger fraction of the amplified hsp-cat1 molecules in sea urchin embryos may be competent to express than to express inductively, or the concentration of positive regulators may be insufficient. Furthermore, the *Drosophila* mRNA hsp70 leader sequence in fact may not be recognized in sea urchin cells in such a way as to permit efficient translation at high temperature (7). A major factor may be the extent of expression in uninduced (15°C) embryos. Higher uninduced levels of expression of the gene for hsp70 than expected have been reported in several other transfection experiments (3, 21, 22). We could possibly have stimulated a mild heat shock response in the control embryos by maltreating them during the collection and centrifugation process. Finally, it is possible that the rate of expression at 15°C is indeed very low, but that the enzyme is stable, and thus that we are comparing the induced enzyme synthesized in 2 hr at 25°C to the uninduced enzyme accumulated over the preceding 3 days. Whatever the explanation, it remains that expression of some of the exogenous genes for CAT is clearly activated under the correct physiological conditions.

We do not yet know whether sea urchin genes that are ontogenically regulated and that normally function only in certain embryonic cell lineages will perform with fidelity when introduced into unfertilized eggs in the same manner. We have so far demonstrated merely that environmentally modulated expression can occur from some fraction of the exogenous genes. However, it is important to explore the possibility that ontogenic regulation can be studied by these methods as well. The sea urchin embryo offers a unique and important advantage for the study of embryological development—the availability of many cloned genes that operate in specific early cell lineages (23). The nature of the *cis* acting genomic information required for the embryonic regulation of these genes may be accessible by the simple and practical experimental route described in this initial study.

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1. McMahon, A. P., Flytzanis, C. N., Hough-Evans, B. R., Katula, K. S., Britten, R. J. & Davidson, E. H. (1984) *Develop. Biol.*, in press.
2. Flytzanis, C. N., McMahon, A. P., Hough-Evans, B. R., Katula, K. S., Britten, R. J. & Davidson, E. H. (1984) *Develop. Biol.*, in press.
3. Di Nocera, P. P. & Dawid, I. B. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 7095–7099.
4. Pelham, H. R. B. (1982) *Cell* **30**, 517–528.
5. Mirault, M.-E., Southern, E. M. & Delwart, E. (1982) *EMBO J.* **1**, 1279–1285.
6. Pelham, H. R. B. & Bienz, M. (1982) *EMBO J.* **1**, 1473–1477.
7. Bienz, M. & Pelham, H. R. B. (1982) *EMBO J.* **1**, 1583–1588.
8. Voellmy, R. & Rungger, D. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1776–1780.
9. Corces, V., Pellicer, A., Axel, R. & Meselson, M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7038–7042.
10. Lis, J., Costlow, N., de Banzie, J., Knipple, D., O'Connor, D. & Sinclair, L. (1982) in *Heat Shock: From Bacteria to Man*, eds. Schlesinger, M. J., Ashburner, M. & Tissières, A. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 57–62.
11. McKenzie, S. L., Henikoff, S. & Meselson, M. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1117–1121.
12. Tissières, A., Mitchell, H. K. & Tracy, U. M. (1974) *J. Mol. Biol.* **84**, 389–398.
13. Lewis, M., Helmsing, P. J. & Ashburner, M. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3604–3608.
14. Hubbard, B. D. & Lazarides, E. (1979) *J. Cell Biol.* **80**, 166–182.
15. Shaw, W. V., Packman, L. C., Burleigh, B. D., Dell, A., Morris, H. R. & Hartley, B. S. (1979) *Nature (London)* **282**, 870–872.
16. Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051.
17. Shaw, W. V. (1975) in *Methods in Enzymology*, ed. Hash, J. H. (Academic, New York), pp. 737–756.
18. Shaw, W. V. & Brodsky, R. F. (1968) *J. Bacteriol.* **95**, 28–36.
19. Goustin, A. S. & Wilt, F. H. (1982) *Biochim. Biophys. Acta* **699**, 22–27.
20. Capecchi, M. R. (1980) *Cell* **22**, 479–488.
21. Findly, R. C. & Peterson, T. (1981) *J. Cell Biol.* **88**, 323–328.
22. Kloetzel, P. M. & Sautz, E. K. F. (1983) *EMBO J.* **2**, 705–710.
23. Angerer, R. C. & Davidson, E. H. (1984) *Science*, in press.
24. Smith, G. E., Summers, M. D. & Fraser, M. J. (1983) *Mol. Cell. Biol.* **3**, 2156–2165.