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Sequence elements of enhancers and promoters that determine enhancer activity



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# Sequence Elements of Enhancers and Promoters that Determine Enhancer Activity

by

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**Susan D. Jones**

## Dedication

I dedicate this thesis to my whole family. To Jill, Ronald, and Stephen Jones who have provided love and support through my life. To Jim, Louise, Ted, and Bob Arnold who have taken me as a family member and encouraged me in my pursuit of science. To my dear husband and friend, Ken Arnold, without whose love and support it would have been much more difficult. And to Shamus and Mačka, who never understand what I'm doing, but provide hours of comfort and support anyway.

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I am extremely grateful for the education that I have received in graduate school from my advisor, Keith Yamamoto. I would especially like to thank Keith for the continuing encouragement and support even when the results were less than encouraging.

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**Susan D. Jones**

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# Sequence Elements of Enhancers and Promoters that Determine Enhancer Activity

*Susan Dana Jones*

Enhancers are *cis*-acting DNA sequence elements that increase the rate of transcription from linked promoters. They are activated when bound by *trans*-acting proteins. Experiments presented in this thesis investigate the sequences within enhancers and promoters that affect enhancer activity. Two enhancers were examined: the glucocorticoid response element (GRE) from mouse mammary tumor virus (MTV) long terminal repeat (LTR) and the mouse immunoglobulin κ enhancer. The effects of mutations introduced either in the GRE or in the linked thymidine kinase (*tk*) promoter were examined.

*In vitro* experiments had revealed that five footprints of the glucocorticoid receptor protein were formed on the MTV LTR GRE; each footprint contained a conserved sequence element. Linker scanning mutagenesis of one conserved sequence abolished the underlying footprint and was sufficient to decrease enhancer activity. Additional receptor binding regions within the MTV genome also had GRE activity. These results suggest that binding of receptor at the GRE is the only sequence specific event necessary for enhancer function.

Mutations both within and outside of identified *tk* promoter elements, which are binding sites for transcription factors, were tested for effects on enhancer action. GRE activity was not altered when any identified *tk* promoter element was disrupted, but was slightly lowered when sequences near the cap site were mutated. GRE activity therefore affects a step in transcription initiation that either occurs after sequence-specific binding of initiation factors, or occurs at a previously unidentified promoter element near the cap site.

A new promoter element was discovered in the HSV *tk* gene: an octanucleotide sequence that is found in all vertebrate light chain immunoglobulin genes. The immunoglobulin κ enhancer functioned equally well on *tk* promoters either containing or lacking the octanucleotide: in fact, the κ enhancer also

increased transcription from a *tk* promoter that contained no known upstream promoter elements.

Both enhancers therefore act upon a transcription factor that does not bind to any known upstream promoter element. The GRE may activate a factor that acts near the cap site. The κ enhancer and the GRE may activate the same transcription factor or may activate different factors that were not distinguished by these experiments.

# I. Introduction

## 1. Positive control of transcription

Regulation of gene expression is a primary mechanism by which cells can respond to changes in their environment. In unicellular organisms, changes in nutritional sources can cause a rapid change in the gene products required for survival. The cells within a multicellular organism often need to change their pattern of expressed genes as they undergo differentiation from a pluripotential stem cell to a highly specialized terminal cell type. One level of gene expression that is highly regulated is transcription. Since transcription initiation is the first of many steps that lead to the final accumulation of a gene product, it is a reasonable step to regulate, although certainly not the only one. Energy is not wasted making transcripts for which the cell has no use, and the transcription factors can be used to transcribe only required genes.

Accurate and efficient initiation of transcription requires both defined *cis*-acting sequences, the promoter, and a set of *trans*-acting factors, the transcription machinery. In prokaryotes, the *cis*-acting elements include the Pribnow box, which is always located 10±1 bp from the site of initiation, and a second conserved sequence at −35. The spacing between these two elements is rigidly conserved, 17±1.[142] Additional sites that are required either for repression or activation of gene expression are often found interdigitated within the promoter sequences in prokaryotes. These include the binding site for CRP-cAMP, whose locations are not conserved between different promoters, but whose location is inflexible within a promoter.[23,24]

The three classes of eukaryotic promoters each have a distinct pattern of *cis*-acting elements. RNA polymerase III promoters require sequences within the transcribed unit to function.[76,128] This requirement can be broken down into two sequence motifs: box A and box B.[16,42,69] These two sequences are juxtaposed in the 5S RNA gene, but will function even if they are separated by a spacer.[17] Neither site alone is sufficient for accurate and efficient transcription. RNA polymerase I and II promoters usually have their sequence determinants located in the 5′ flanking region of the transcribed gene. Recent studies on the mouse ribosomal genes (pol I) have demonstrated that a core sequence that encompasses the initia-

tion site is required for any transcription to occur.[58, 79] A second sequence, the upstream control element, is necessary for maximal levels of transcription. This upstream region is a binding site for one or more transcription factors that are necessary for fully efficient transcription of this promoter *in vitro*.[79]

Eukaryotic RNA polymerase II promoters usually contain a TATA homology near the initiation site.[45, 89] In mammalian promoters, it is most often between −20 and −30; in yeast it is usually closer to −60.[56] The TATA box can be correlated with accurate initiation at the cap site, and is also necessary for maximal levels of transcription in some promoters.[89, 97] A variety of upstream sequence motifs have been found associated with pol II promoters.[6, 13, 18, 26, 36, 50, 51, 52, 55, 63, 95, 97] Each promoter can contain one or more of these sequences. The spacing between the various upstream elements and the TATA region in eukaryotes is much more flexible than in the prokaryotic counterparts. In addition, many different arrangements of upstream elements are transcriptionally active.

Eukaryotic promoters may also contain sites for binding repressor molecules, just as in their prokaryotic counterparts. Examples of both direct steric hindrance and repression from a distance have been seen.[9, 71, 106, 126] Lastly, they may contain binding sites for activator proteins that are considered distinct from the upstream elements. These enhancer sequences will be discussed in detail below.

Transcriptional initiation in prokaryotes has been separated *in vitro* into at least two functionally distinct events, each of which may be composed of multiple steps.[11] The first event is the binding of RNA polymerase to the promoter, represented by the equilibrium constant $K_B$, to form a closed complex. Binding is highly reversible, and can be regulated both negatively and positively. The second event of transcription initiation is an isomerization from a closed complex to a transcriptionally active open complex, that can be approximated by the forward rate constant, $k_f$. Increases in either $K_B$ or $k_f$ will cause an increase in the overall rate of transcription from a given promoter.

Positive control of initiation in E. coli can affect either $K_B$ or $k_f$. In the lactose and galactose operons, removal of a repressor precedes positive activation. Binding of the cyclic AMP receptor protein (CRP-cAMP) to the promoter region provides the positive activation.[23, 24] The *lac* operon consist of two

overlapping promoters, P1 and P2.[85] P2 is a weak promoter, but it serves to divert the polymerase away from the more active P1. When the repressor is bound, RNA polymerase binding to P1 is prevented; P2 can still be occupied, although it cannot initiate transcription. Upon removal of repressor, the P1 site is still blocked by the polymerase that is bound at P2. CRP-cAMP binding increases the binding constant, $K_B$, of RNA polymerase at P1 by a direct, although not understood, action. In addition, CRP-cAMP has a small, indirect effect on the binding of polymerase to P1, because by preventing RNA polymerase from binding to P2 it allows RNA polymerase to bind and transcribe from P1. Thus, the CRP-cAMP influences transcription from P1 both directly and indirectly. Other bacterial genes that are positively regulated by CRP-cAMP may use either direct or indirect methods of activation.

In contrast, the effect of λ repressor binding is to increase the rate of open complex formation, $k_f$, at $P_{RM}$ without changing the polymerase binding constant, $K_B$.[59] The λ repressor protein concomitantly represses transcription from $P_R$ and activates its own transcription from $P_{RM}$.[68, 70] This activation is thought to result from direct contact between the bound repressor protein and RNA polymerase. Mutations in the repressor that fail to activate transcription are localized to amino acids in the domain of the protein that would spatially overlap with the polymerase when both are bound at the right operator.[60, 67] Although this genetic evidence supports the conclusion that this contact is necessary for maximal transcription of the λ $P_{RM}$, the molecular mechanism by which contact with a second protein increases $k_f$ is unknown.

Activation from a distant sequence is another method of positive activation that has been found often in eukaryotic organisms, and recently in bacteria.[4, 105, 122] These sequences, termed upstream activator sequences in yeast[57] and enhancers in higher eukaryotes,[4] can function over large (>1 kb) distances, in either orientation, and, in the case of enhancers, from a position 3′ of a transcriptional unit. They are binding sites for proteins that activate transcription from linked genes by unknown mechanisms.

Modification of cellular transcription factors is used by some eukaryotic viruses to increase the specific initiation from their own promoters during an infection. The E1a protein of adenovirus has been

reported to increase the binding of a cellular transcription factor to the E2 promoter, which is stimulated by E1a.[75] Ela is itself a nuclear protein, but it does not bind to DNA. If cells are infected with adenovirus containing a deletion of the E1a gene, *dl312*, the cellular factor still binds to the E2 promoter, but does so much later after infection than with the wild-type virus: thus, Ela is thought to increase this binding during early stages of infection. It is not known if E1a increases the concentration of the cellular transcription factor or changes its capacity to bind its site of action. However, the pseudorabies virus IE protein is homologous in function to the E1a protein, and can even substitute for E1a in a *dl312* infection. This protein has been shown to modify an existing cellular factor rather than increase its concentration in the cell.[1] These proteins may be similar to enhancer binding proteins in their activity, without a requirement for DNA binding.

## 2. Transcription initiation: The site of much regulation

The repertoire of sequence-specific transcription factors isolated from both prokaryotes and eukaryotes is huge.[27, 28, 29, 32, 33, 72, 90, 104, 109, 110, 128, 129, 133] Much information has already been learned about the protein requirements and the changes in the template that accompany prokaryotic transcription initiation, and eukaryotic initiation is likely to be similar. Bacterial RNA polymerase holoenzyme recognizes the −10 and −35 regions of prokaryotic promoters, and is alone sufficient for accurate and efficient levels of transcription *in vitro*, except in the case of promoters that require positive activation. It consists of a core of 4 subunits plus a fifth subunit termed σ, which is required for initiation at promoters, but not for elongation. σ affects the specific DNA binding of the polymerase, although it is not known which subunits of the enzyme are directly involved in DNA-binding; the σ subunit alone has not been shown to bind specifically to DNA. After transcription of the first few nucleotides, σ dissociates from the elongating transcriptional apparatus, and cycles to another RNA polymerase to participate in the next round of transcription initiation. There is more than one type of σ factor in E. coli and in B. subtilis. Each σ factor is specific for a subset of promoters. The modulation of the activity of a given σ factor may be one way to regulate the level of transcription from its responsive promoters.[123]

Eukaryotic RNA polymerase II alone is unable to direct accurate and efficient transcription initiation *in vitro*. Upon supplementation with nuclear extracts, however, RNA polymerase II will transcribe a variety of templates.[87, 134, 145, 155] It appears, therefore, that eukaryotic transcription requires additional nuclear factors besides just the enzyme itself. These factors may include those that cycle through each round of initiation, analogous to the prokaryotic σ factors. Some *in vitro* studies suggest that such transient factors exist.[127] Experiments performed *in vitro* have shown that certain factors form a stable interaction with a promoter during a preincubation in the absence of nucleotides (*i.e.*, no initiation), such that a second promoter added later cannot be transcribed.[19, 39, 61, 127] This so-called stable complex may remain active through multiple rounds of transcription. The class of factors that recognize and bind stably to the previously described upstream sequence motifs are necessary for promoter recognition and utilization.[72, 104, 133] Some of these factors are specific to a small family of promoters.[110] Others are found that bind to several promoters, often in multiple species.[29]

How stable factors bound at sites upstream of the initiation site contribute to transcription initiation is not known. Consideration of this question is further complicated by the observation that many different factors exist, and that many combinations of upstream binding factors constitute transcriptionally active promoters. Some possibilities of upstream factor activity are that they form a complex with RNA polymerase after it has bound or they provide a recognition site for polymerase that helps it bind, that they allow more efficient entry or activity of the proposed σ- type, transient factors, or that they modify the template.

### 3. Enhancer action on eukaryotic initiation

Enhancers are *cis*-acting DNA elements that increase the rate of transcription from linked promoters, and are flexible in both the distance and orientation relative to the promoter. Enhancers were first described in viral genomes such as SV40, MSV, and others.[4, 8, 22, 78, 80, 81, 105, 148] They have since been found to be associated with many endogenous genes as well.[5, 43, 46, 48, 91, 121, 125, 152] Many enhancers are cell-type specific. It was suggested that these sequences are sites of action for proteins, and that the

activating protein for a given enhancer was present in only some cell types.[77, 141, 164] The discovery that a sequence motif that conferred responsiveness to glucocorticoid hormones upon linked genes was bound by purified glucocorticoid receptor protein *in vitro* and was a glucocorticoid dependent enhancer *in vivo* was consistent with the notion that enhancers are sites for binding of *trans*-acting proteins.[116, 163, 166] The hypothesis that enhancers are activated by enhancer-binding factors is becoming widely accepted as more enhancer sequences are shown to be binding sites for cellular proteins.[15, 35, 99, 107, 116, 119, 137]

Enhancers can be considered distinct from upstream promoter elements. Unlike promoter elements, enhancers can function from several kb upstream or downstream of the cap site.[4, 105] As more enhancers have been described, however, the functional distinction between upstream elements and enhancers has become more vague. Many enhancer elements overlap the upstream promoter elements and it is difficult to analyze either one separately.[48, 152] Nevertheless, it seems useful to consider these elements as regulatory sequences, distinct from the sequences that comprise promoters. An analogy can be made with the regulatory proteins CRP-cAMP and λ-repressor, which are not considered transcription factors but rather regulatory proteins. Thus, factors that interact with the upstream elements of eukaryotic promoters will be considered as functional subunits of RNA polymerase, while the factors that bind to enhancer elements will be regarded as regulatory proteins.

Enhancers increase the rate of transcription from linked promoters. Nuclear run-off assays have shown that enhancers increase the number of RNA polymerase molecules on the linked transcription unit.[146, 154] There are many steps in eukaryotic transcription initiation that might be positively regulated by enhancers, just as both $K_B$ and $k_f$ in prokaryotic initiation are regulated. Three possible steps that enhancers can regulate are: (1) enhancers might act to increase the occupancy of any or all of the factor binding sites upstream of the polymerase, (2) enhancers may perform a function that is also done by one or more of the upstream binding factors, but at a significantly faster rate and in a way that is less sensitive to the distance from the promoter, and (3) enhancers might increase the activity of the σ-type factors that are needed in eukaryotic initiation. Different steps in eukaryotic initiation can conceivably be regulated

by different enhancers. Some enhancer elements increase the transcription of a linked gene for a short time, perhaps by acting on a step that requires the σ-type, transient factors.[12, 41, 48, 73, 125, 147, 149] Other enhancers act as developmental switches, turning on tissue-specific expression at the appropriate time.[5, 44, 118, 152] These enhancers might regulate a step that is involved in the formation of a heritable, stable initiation complex.

Many molecular mechanisms have been suggested for enhancer action. Five possible models are: (1) Enhancers act as bidirectional entry sites for polymerase or other factors.[153] These factors could then reach the promoter either by traversing the intervening DNA sequences until a promoter is found, or by the intervening DNA bending such that the factor is brought directly to the promoter; (2) Enhancers have an effect on the local chromatin structure, such that the environment becomes more favorable to transcription in the region of the promoter. One related suggestion is that enhancer-binding proteins are site-specific gyrases; (3) Enhancer binding factors form a complex with the upstream binding factors upon being bound to the enhancer sequences. Such a complex would then be more efficient at directing rapid and efficient initiation than the upstream elements alone; (4) Enhancer binding proteins have a direct action on the RNA polymerase molecule; (5) Enhancers bring the transcriptional unit to a nuclear location that is enriched in transcription factors, nucleotides, and/or polymerase, such as the nuclear matrix.

Detection of enhancer activity has classically been accomplished by linking the test sequences to a eukaryotic promoter *in vitro* and reintroducing the construct back into cells to test for transcriptional activation *in vivo*. The success of such an assay depends on several context effects. The cell type into which the enhancer is introduced must contain the necessary enhancer activating protein. Presumably the cell type specific enhancers are such because only the active cell type contains the enhancer binding proteins. A report by Maeda et al. is consistent with this notion.[83] They demonstrated that a rearranged immunoglobulin heavy chain gene that was stably integrated into mouse fibroblast cells is transcribed upon microinjection of myeloma cell nuclear extract, and that this effect is dependent on the presence of the immunoglobulin heavy chain enhancer. Another striking example of this context effect are the results

of DeFranco and Yamamoto in which the MoMSV $S_a$ enhancer activity was not detectable in rat pancreas exocrine cells, despite the fact that these cells are permissive for activation of many other enhancers and the MoMSV $S_a$ activity is active in other cell types.[21]

Another context effect is imposed by the fact that enhancer activity is detected only through the promoter used in the test construction. Enhancers have no activity that can be measured independently of the promoter that they act upon. Based on the model suggested above in which different enhancers act on different steps in the transcription initiation pathway, it is likely that a given promoter will respond only to the set of enhancers that act at a rate limiting step, and will be immune to the effects of enhancers that act at alternate steps. It may be possible to classify enhancers by which step of initiation they affect, based on what set of promoters are responsive to each enhancer.

### 4. Two specific transcriptional enhancers

Glucocorticoid hormones have long been known to increase the transcription of several genetic loci.[144, 162] Like all steroid hormones, glucocorticoids act by binding to a soluble receptor protein in the cytoplasm of cells.[165, 166] Upon hormone binding, the receptor is then localized to the nucleus where it interacts with specific DNA sequences. One genetic locus that is stimulated by glucocorticoids is the mouse mammary tumor virus (MTV) promoter. Initiation of transcription from the unique promoter in the left hand long terminal repeat (LTR) is rapidly and specifically induced upon addition of glucocorticoids to cells harboring integrated copies of this virus.[124, 149] *In vitro* analysis of the MTV genome revealed several regions that were bound by purified glucocorticoid receptor protein.[115, 116, 135, 136] One such region is a 220 bp sequence located upstream of the promoter that is able to confer hormone responsiveness upon linked, heterologous promoters, including that of the herpes simplex virus thymidine kinase gene (HSV *tk*).[12, 84, 120, 163] This element, termed a glucocorticoid response element (GRE), is a glucocorticoid-dependent transcriptional enhancer. Activation of this enhancer is accompanied by the appearance of a DNAase I hypersensitive site, a phenomenon associated with many transcriptional enhancers, but only correlated directly with enhancer activation in this case.[117, 167] The appearance of

this site may be due directly to the binding of the receptor protein, or it may reflect some action of the receptor and other factors upon the chromatin. GRE activity requires the continuous presence of hormone-bound receptor. Upon removal of hormone from the environment, transcription decreases rapidly back to the basal levels.[149, 167]

The mouse immunoglobulin κ enhancer is functionally distinct from the GRE. Evidence for its existence was first presented by Parslow and Granner, who demonstrated that activation of the mouse κ gene in development was accompanied by the appearance of a nuclease hypersensitive site within the first intron of the gene.[111] This site was later shown by Picard and Schaffner to be a transcriptional enhancer.[118] It is required during B-cell development to turn on high levels of transcription of the κ gene. Since it is a developmental switch, evidence for its being turned off after it has been activated has never been shown. The κ promoter upon which this enhancer acts consists of a TATA box and a conserved sequence element located at −70.[37, 113] This enhancer has not worked well when linked to other promoters, which may be due to the context effects discussed above.

### 5. Objectives

The goal of my thesis work has been to use a genetic approach to test how an enhancer can increase initiation from a linked promoter. The working model is that enhancers act to facilitate the utilization of one or more transcription factors that are rate-limiting in the transcription reaction. The approach taken was to introduce mutations in either the enhancer or the promoter. The resulting activity of the promoter-enhancer combination was then analyzed in a transient expression system. By determining which, if any, *cis*-acting elements were necessary for enhancement to occur, we might then infer that the known *trans*-acting factor that bound at this site is directly involved in the enhancement reaction. The specific objectives that I have addressed are:

(1) What sequences in the enhancer are necessary for MTV GRE activity?

(2) Can the site of GRE action at the HSV *tk* promoter be determined by mutational analysis? What does this reveal about the enhancers' action in the initiation reaction?

(3) What is the relationship between two evolutionarily conserved elements in the mouse κ light chain gene, one of which is a transcriptional enhancer?

# II. Sequence Requirements for GRE Function

## 1. Introduction

A glucocorticoid response element (GRE) located within the 5′ LTR of mouse mammary tumor virus (MTV) is a glucocorticoid dependent transcriptional enhancer that contains five distinct footprints of its activator protein, the glucocorticoid receptor protein.[12, 116, 163, 165] It was of interest to determine what features of this GRE contributed to the functional activity of this element. This study describes a systematic analysis of the *cis*-acting sequences responsible for receptor binding and GRE function.

This GRE resides on a 340 bp Sau3a restriction fragment. The five distinct footprints of purified rat liver glucocorticoid receptor reside within the promoter-proximal 220 bp of the fragment (see Figure 1). Electron microscopic studies suggested that receptor bound as a tetramer, and that more than one tetramer could bind to a single DNA fragment.[116] Each footprint covered a sequence that contained at least one copy of a conserved, degenerate octanucleotide, of sequence 5′AGA$^{A}_{T}$CAG$^{A}_{T}$3′. No other sequences that were homologous between the five footprints were apparent in the 15-40 bp that were protected in each footprint. Conceivably, one binding site might be the active site, whereas the others maintain a high local concentration of the receptor. Alternately, a cooperative mechanism for sequential binding may exist. Lastly, the sites may all be functional.

Binding of receptor protein to MTV sequences was initially detected by a filter-binding assay in which restriction fragments from MTV were tested for their ability to be bound by the purified receptor.[115] In addition to the Sau3a fragment located upstream of the promoter, four fragments within the transcribed and translated sequences were also specifically bound by the receptor. The upstream sequence is termed receptor binding region 1, RB1. Additional regions, termed RB2-RB5, are depicted in the upper diagram in Figure 1.

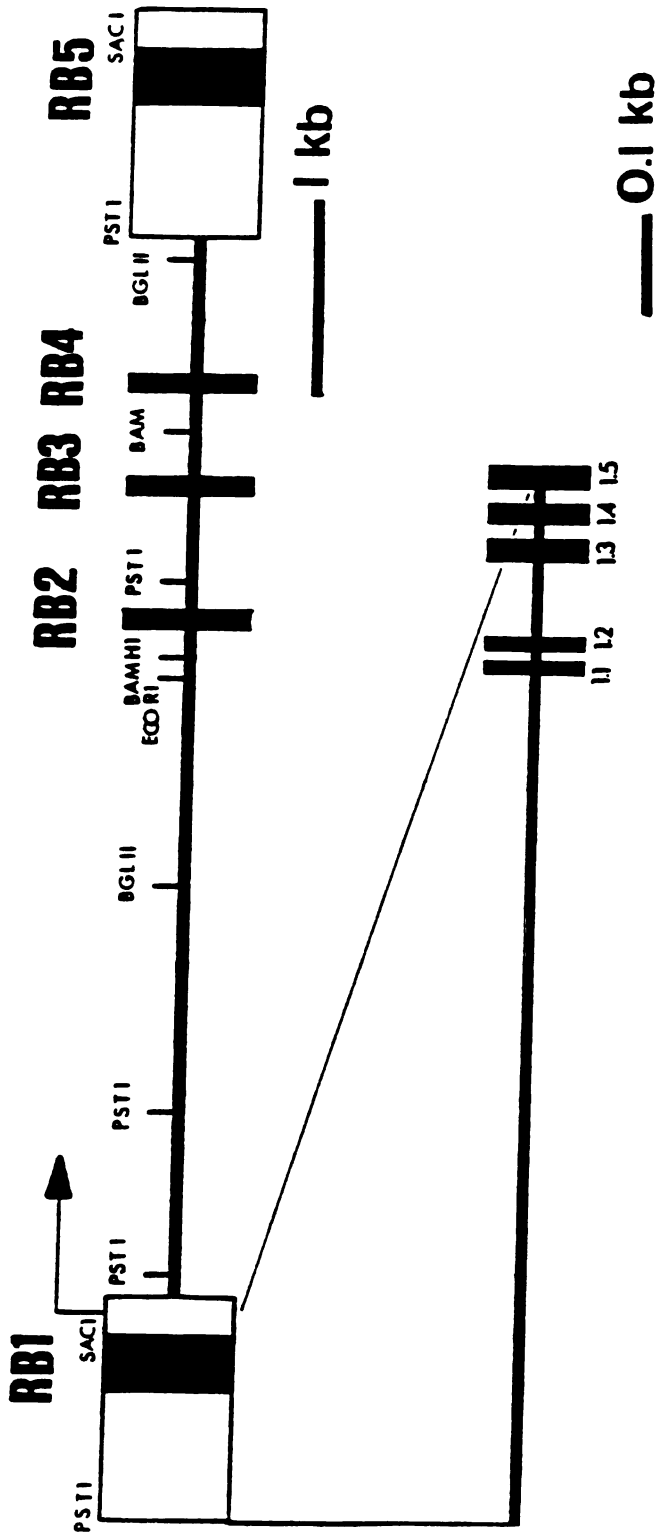
Two approaches were taken. A series of linker scanning mutations within RB1 was constructed and analyzed for GRE activity in a transient expression assay. These mutations were both within and outside of the defined glucocorticoid receptor footprints. Some mutations within footprints did not disrupt the integrity of the conserved octanucleotide, while others did. A second approach was to test the set

**Figure 1**
**Location of Glucocorticoid Receptor Binding Regions in MTV**

A restriction map of the mouse mammary tumor virus (MTV) provirus and the locations of glucocorticoid receptor binding regions within the provirus are represented in the upper diagram. Receptor binding region one (RB1) is located upstream of the transcription initiation site, while at least four additional receptor binding regions, RB2-RB5, lie within the transcribed sequences. These additional regions are located from 3 to 7 kb from the transcription initiation site. The receptor binding regions, termed RB1-RB5, are shown as dark boxes. The start site of the transcript that results from the utilization of the unique promoter in the 5' LTR is indicated with an arrow.

A representation of the footprints that are formed by purified, activated glucocorticoid receptor within RB1 is shown in the lower diagram. RB1 contains five distinct footprints termed 1.1-1.5. Footprint 1.1 is the furthest from the MTV promoter; footprint 1.5 is promoter proximal and is located 110 bp upstream of the transcription initiation site.

Fig. 1


RB1
RB2
RB3
RB4
RB5
SAC I
PST I
BGL II
BAM
BAMHI
ECO RI
1 kb
0.1 kb
L1
L2
L3
L4
L5

of additional receptor binding regions within MTV for GRE activity. In both cases, a footprinting analysis was done using purified rat liver receptor. The GRE activity of each fragment was assayed by transient transfection. Each fragment was fused to the promoter of the herpes simplex virus thymidine kinase (HSV *tk*) gene, which was used to drive the expression of the bacterial chloramphenicol resistance gene, chloramphenicol acetyl transferase (CAT).[2,49] A diagram of these constructions is presented in Figure 2. The plasmids used to test the linker scanning mutations also include the Moloney murine sarcoma virus (MoMSV) enhancer, which was intended to increase basal signal. After completion of this study, it was discovered that the MoMSV enhancer also carries a GRE.[21,103] This of course reduces the sensitivity of testing the MTV GRE mutations. Nevertheless, some interesting phenotypes were detected (see below).

**2. Receptor footprints are correlated with GRE activity of RB1**

The location of several linker scanning mutations that were analyzed is shown in Figure 3. When these were footprinted using the purified rat liver receptor, it was found that only mutations that interfered directly with the consensus octanucleotide disrupted the footprint.[20] Furthermore, while the footprint whose octanucleotide had been disrupted was affected, the other four footprints formed normally, indicating that receptor binding is not cooperative in this assay.

Figure 3 shows the phenotypes that were obtained in CAT assays using some of the linker scanning mutations. It can be seen that the mutations that affect footprints 1.3 and 1.4 decrease GRE activity. The results of several CAT assays suggest that a decrease to 20-50% of wild type enhancer activity is obtained. Similar results were obtained for mutations that disrupted the octanucleotide in footprint 1.2 (data not shown). Mutations in other sequences that do not alter the formation of footprints in RB1 also do not affect the GRE activity. Thus, only those sequences required for footprint formation appear necessary for GRE activity..

The ability of these mutated enhancer elements to direct and enhance transcription from the proper initiation site of the *tk* promoter was analyzed using a primer extension analysis of RNA isolated from

**Figure 2**
**CAT Construction for Testing Enhancer-Promoter Activity**

The general layout of the constructions used to analyze enhancer activity is diagramed. A fragment that contained both the enhancer of interest and the *tk* promoter was inserted into a BglII restriction site located at the start of the coding sequences of the bacterial chloramphenicol acetyl transferase (CAT) gene (see appendix A for details about the constructions). The SV40 intron and poly-adenylation signal were linked to the 3′ end of the bacterial gene to provide proper signals for RNA processing of this gene in mammalian cells. In some cases, a second enhancer was inserted downstream of the SV40 sequences, at a unique BamHI site as shown. Constructions that tested the enhancer activity of linker scanning mutations in the MTV GRE contained the MSV enhancer at this BamHI site, as did the constructions used in Chapter 3. Constructions described in Chapter 4 contain the origin and enhancer region of polyomavirus at this site. The experiments that were designed to test the GRE activity of the additional receptor binding regions in MTV did not include an additional enhancer.

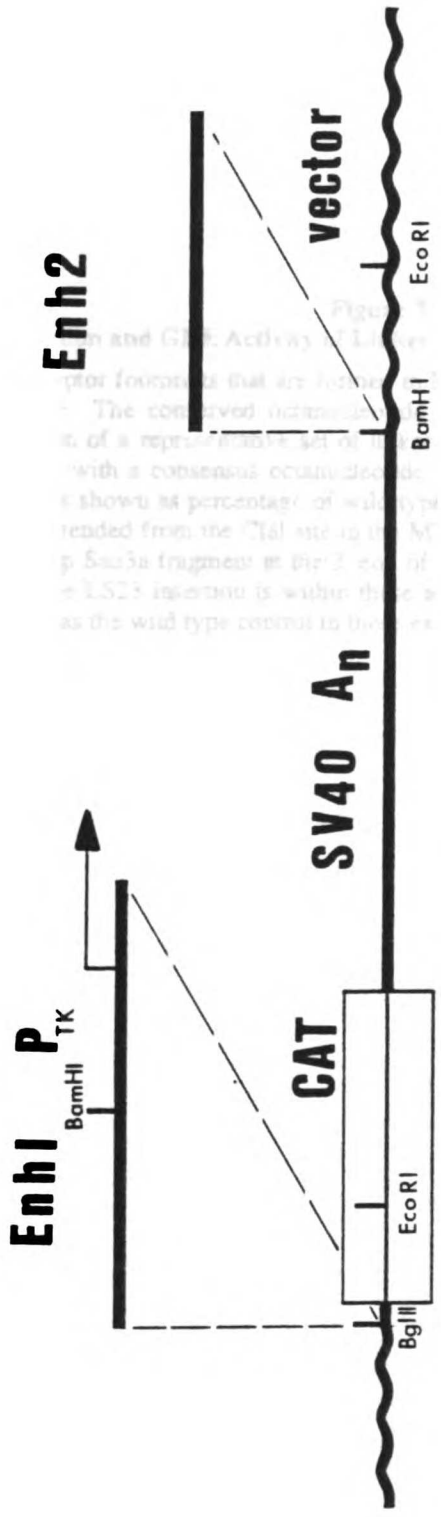

Enh2
vector
EcoRI
BamHI
SV40
$A_n$
CAT
EcoRI
BglII
EnhI
BamHI
$P_{TK}$

Fig. 2

**Figure 3**
**Location and GRE Activity of Linker Scanning Mutations in RB1**

The glucocorticoid receptor footprints that are formed in RB1 are indicated as open boxes in the diagram at the top of the figure. The conserved octanucleotide that is found within each footprint is shaded. Below RB1, the location of a representative set of linker scanning mutations is diagramed. Any part of the linker that overlaps with a consensus octanucleotide is shaded. The relative GRE activity as determined by CAT assays is shown as percentage of wild type induction. The actual fragment that was used in these experiments extended from the ClaI site in the MTV LTR to the SacI site, encompassing 753 bp. This includes the 340 bp Sau3a fragment at the 3' end of the fragment, and an additional 413 bp of LTR sequence upstream. The LS23 insertion is within these additional sequences, is a fully functional GRE, and was therefore used as the wild type control in these experiments.

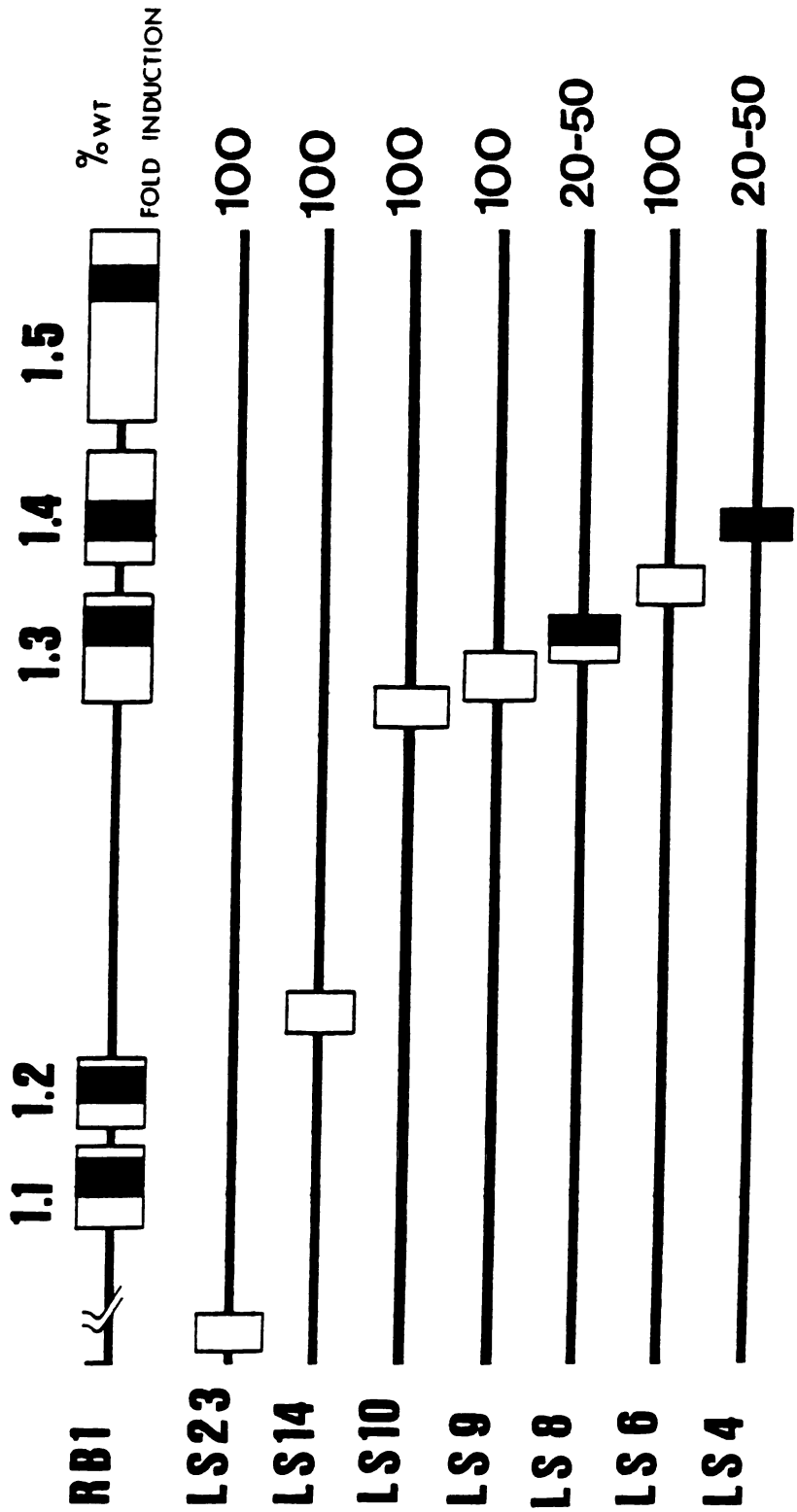

1.1
1.2
1.3
1.4
1.5
% WT
FOLD INDUCTION
RB1
LS23
100
LS14
100
LS10
100
LS9
100
LS8
20-50
LS6
100
LS4
20-50

Fig. 3

cells 12-24 hr after transfection. The CAT primer that was used in this assay corresponded to sequences from −5 to −31 from the translation initiation codon of the CAT gene. In the constructions under analysis, this primer gave an extension product of 86-90 bp. The internal control, RSV-CAT, gave an extension product of 68 bp. As shown in Figure 4, all of the LS mutants initiate and enhance transcription from the normal *tk* cap sites. LS8, a mutation that disrupts footprint 1.3 *in vitro* and the induction of CAT enzyme activity *in vivo*, yields a glucocorticoid response only 25% the level of the wild type control. In no case were aberrant start sites observed. Two of the mutations shown, LS9 and LS10, produce nucleotide substitutions within footprint 1.3 that leave intact the consensus octanucleotide; these alter neither the CAT activity nor the transcriptional enhancement. Thus, these sequences appear dispensable under the conditions tested. Surprisingly, LS4, which disrupts footprint 1.4 and yields lower induction levels by CAT assay (Figure 3), appears to respond fully to dexamethasone when examined in the primer extension assay; the reason for this discrepancy is not known. These experiments demonstrate that footprints in RB1 are correlated with its GRE activity, and that the effects of several mutations within single footprint sites are sufficiently strong to be detected even in the presence of the remaining receptor binding sites, and the intact MoMSV enhancer. Although cooperativity of receptor binding was not seen *in vitro*, these results suggest that some aspect of GRE activity is cooperative *in vivo*. This may be binding either of receptor or of an auxiliary factor that will not bind until contacts between bound receptor molecules have been made, or an activity that ensues upon formation of a receptor complex at the GRE.

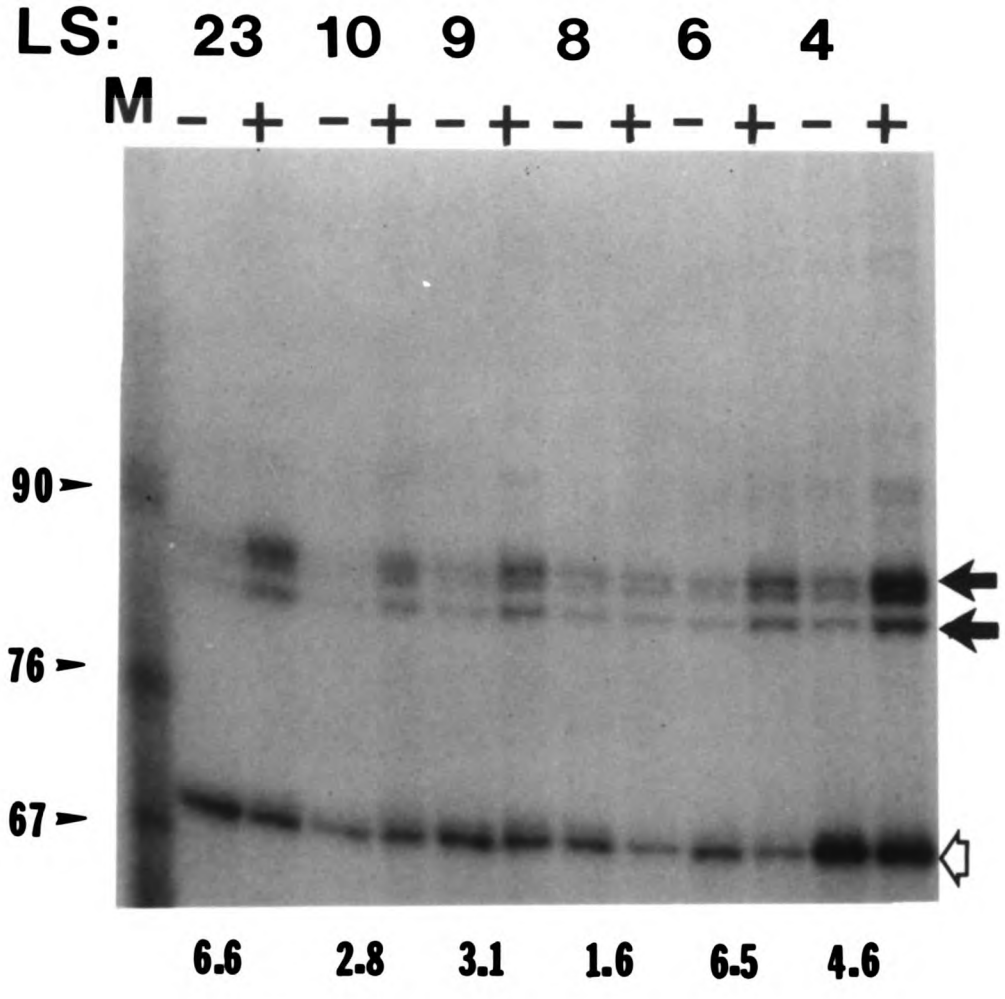
### 3. RB4 is a glucocorticoid dependent transcriptional enhancer

A 900 bp BamHI-BglII restriction fragment encompasses the receptor binding region RB4; the BamHI site is 5.5 kb downstream of the MTV transcription initiation site, and is within sequences that are transcribed and translated (Figure 1). The demonstration that RB1 can enhance the *tk* promoter when it is linked either to CAT coding sequences or to its own coding sequences indicates that downstream receptor binding regions are not essential for GRE activity. RB2-RB5 may act passively, increasing the local concentration of receptor protein relative to RB1. Alternatively, the four may contribute actively by them-

**Figure 4**
**Primer Extension Analysis of Transcripts from Linker Scanning Mutations**

Primer extension analysis of the unfractionated RNA isolated from transiently transfected XC cells is shown. Cells were cotransfected with CAT constructs containing a linker scanning mutation in the GRE fused to the *tk* promoter, and RSVCAT as an internal control. The *tk* promoter gives rise to primer extension products of 90, 89, and 87 nucleotides; these normal start sites are indicated by the dark arrows. These are separated from the RSVCAT control extension product, which is 68 nucleotides long, indicated by the open arrow. Lanes marked "+" represent analysis of RNA from cells that were treated with 0.1μM dexamethasone for 12 hr following transfection. The lanes marked "–" were not treated with hormone and represent the basal level of transcription from the construction under investigation. Each pair of lanes represents transcripts isolated from cells that were transfected with the linker scanning mutations in the GRE that are indicated above the figure. Lane M indicates the markers; their size (in nucleotides) is indicated at the left. The fold induction as determined by scanning densitometry of several primer extension analyses of these GRE mutations is shown under the Figure. Each was normalized to the relative level of transcript from the RSVCAT plasmid.

Fig.4



selves functioning as GREs. Lastly, these sites may have no activity.

Four footprints were detected within the 5′ half of RB4 (Figure 5); as in the RB1 footprints each site contained at least one conserved octanucleotide. The sequences found within each footprint are listed in Table 1. Although a direct comparison was not executed, it appeared that these footprints formed at close to same concentration of receptor as the footprints in RB1. Filter-binding experiments performed earlier had shown that RB4 was less effective at receptor binding than RB1.[116] Within each site, no other sequence homologies were found.

RB4 was fused in both orientations to the *tk* promoter and tested for its GRE activity in transient transfections. Figure 6 shows that RB4 displays GRE activity, although apparently at a lower level than RB1. Restriction fragments containing RB2 and RB3 were also active as GREs (D. DeFranco, unpublished results); each of these regions contains two footprints, each with the expected conserved octanucleotide.[116] Subfragments of RB4 that contained just two of the four footprints were examined; a subfragment containing no footprints was also tested. Consistent with similar studies of RB1, only those subfragments containing receptor binding sites were functional GREs.

---

**TABLE 1**
**Sequences of footprints in RB4**

| *Footprint* | *Sequence* |
|---|---|
| 4.1 | 5′CCAGGATTTCAAGAACATGAAATGATTCC3′ |
| 4.2 | 5′GAGAAAAGAGGATCTACTTTTCATATTTCCTGTTCTTCTTG3′ |
| 4.3 | 5′AGCCTTATCCGAACAAAGAATAATAGATTT3′ |
| 4.4 | 5′AGAAGAAGTAGTTTTAGAGTTGGGACAAGATGTGGCAAA CTTAAAGACCAGAATGTCCACTA3′ |

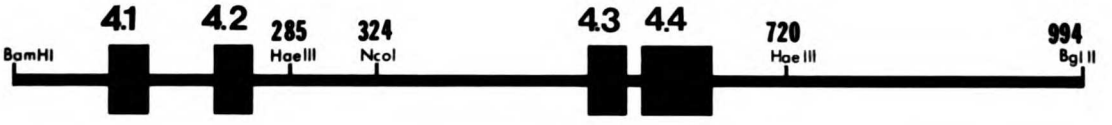
**Table 1**: Sequences protected by purified rat liver glucocorticoid receptor in RB4 are shown. The copy of the conserved octanucleotide that is found in each footprint is underlined. See methods for experimental details.

---

**Figure 5**
**Glucocorticoid Receptor Footprints in RB4**

The location of the footprints that were observed in RB4 is shown in the upper diagram. The shaded areas represent protected sequences. Restriction sites within this 900 bp fragment are also indicated, as is the distance of each restriction site from the BamHI site at the 5′ end. The lower diagram shows the actual footprints obtained by a DNAase protection assay. The location of each footprint is indicated at the left, in bp from the BamHI site. The fragment was end-labeled on either strand at the unique NcoI site. The BamHI-NcoI and the NcoI-BglII subfragments were gel-purified and analyzed as described in the methods section. The figure shows the G reaction of Maxam-Gilbert sequencing in the left lane of each panel. The unlabeled lanes in footprint 4.2 are the other sequencing reactions. Lanes **a-d** represent addition of 0 ng receptor, 200 ng receptor, 400 ng receptor, and 800 ng receptor respectively. Similar amounts of protein were required to generate footprints at RB4 and RB1.

Fig. 5


4.1
4.2
285
HaeIII
324
NcoI
4.3
4.4
720
HaeIII
994
BglII
BamHI

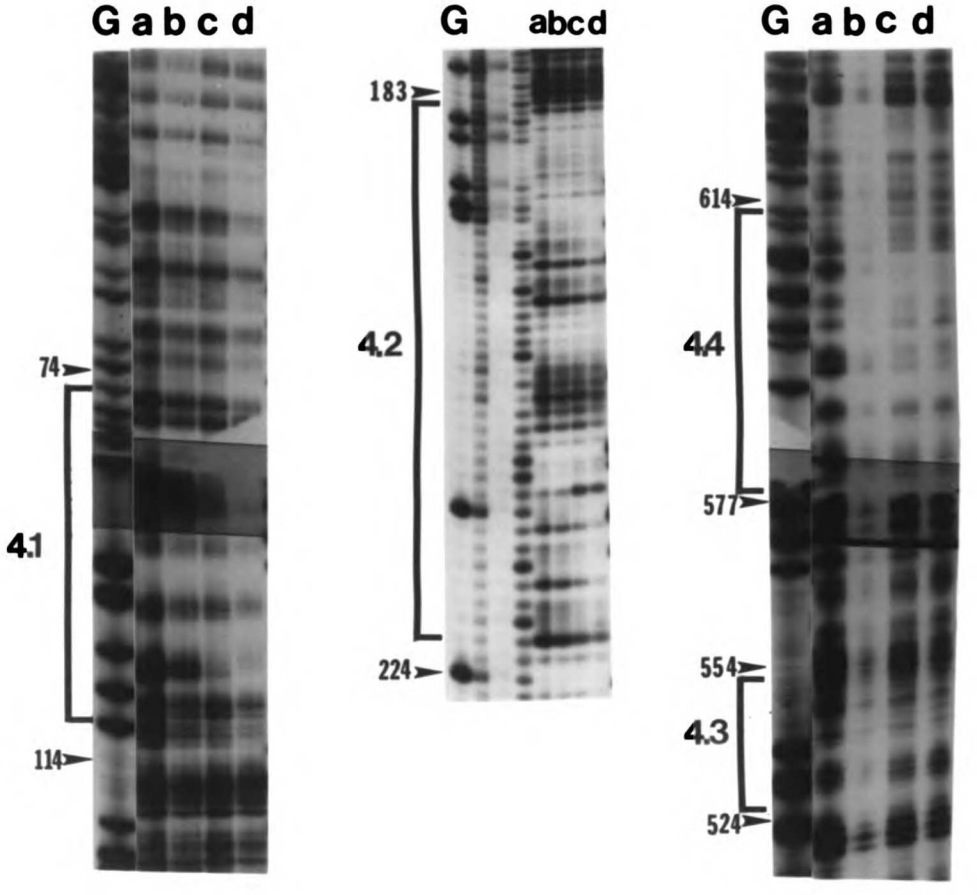

G a b c d
74
4.1
114
G abcd
183
4.2
224
G a b c d
614
4.4
577
554
4.3
524

**Figure 6**
**CAT Assay of RB4 Activity on $P_{tk}$**

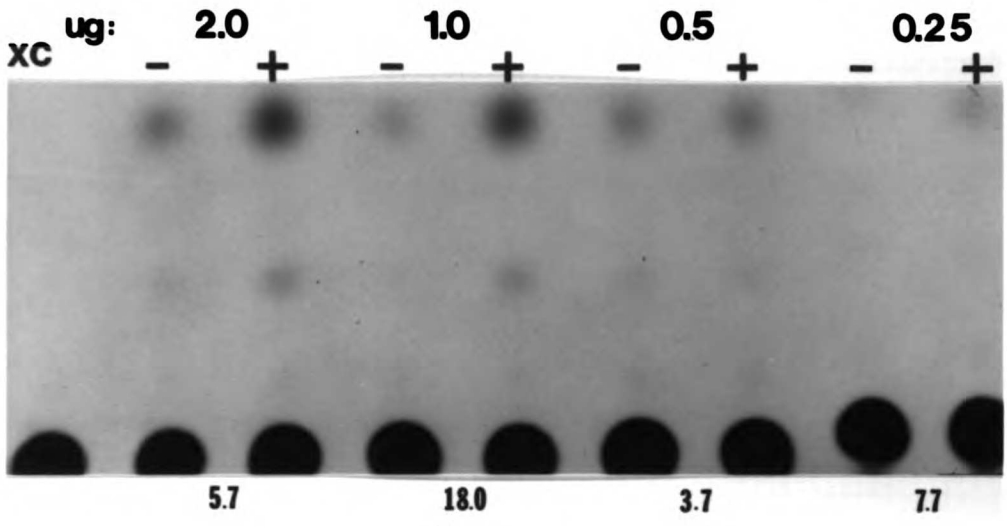
The upper panel shows a representative CAT assay of an analysis of RB4 GRE activity in XC cells. A DNA concentration curve is shown. The lowest spot is unreacted $^{14}C$ chloramphenicol; the upper two spots are the products, which are two isomers of mono-acetylated chloramphenicol.

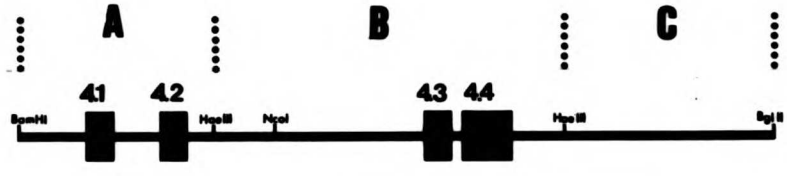
The construction analyzed is RB4 linked to the *tk* promoter such that footprint 4.1 is promoter-proximal; this is referred to as the 2 orientation. It was analyzed by transient transfection. The lane marked "XC" represents the background CAT enzyme activity that is present in the untransfected XC cells. Lanes marked "+" represent analysis from cells that were treated with 0.1μM dexamethasone for 20 hr following transfection. The lanes marked "–" were not treated with hormone and represent the basal level of transcription from the construction under investigation. The amount of test DNA added per 100mM plate of cells is shown above the autoradiogram, in μg, while the fold of induction that resulted from the treatment with dexamethasone is shown below each pair of assays.

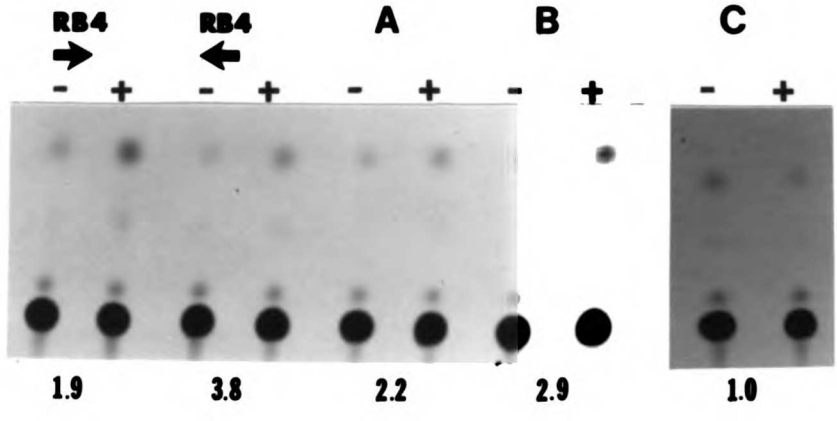
The center diagram shows the location of the three subfragments of RB4 that were tested for GRE activity in a transient expression assay. Fragments A and B each contain two receptor footprints, while fragment C contains no footprints. Each fragment was linked to the *tk* promoter.

The lower panel shows a representative CAT assay of the subfragments shown in panel B. Lanes marked "+" and "–" are as above. The test fragment analyzed in each pair of assays is shown above the autoradiogram, while the fold induction is shown below.

Fig.6


ug: 2.0 1.0 0.5 0.25
XC
- + - + - + - +
5.7 18.0 3.7 7.7


A
B
C
4.1
4.2
4.3
4.4


RB4
RB4
A
B
C
- + - + - + - + - +
1.9 3.8 2.2 2.9 1.0

The downstream receptor binding regions are capable of GRE activity when placed in a position 5′ of a heterologous promoter. It is likely that they are also active in MTV. Evidence to support this notion is that all of these regions exhibit hypersensitivity upon stimulation with glucocorticoids of cells harboring integrated proviruses:[117] hypersensitivity has been correlated with the activity of some enhancers. If the downstream receptor binding regions are contributing to the effect in the provirus, then transcription must be occurring across active enhancer elements that are bound by their activating protein. Transcription across templates that are bound by activator proteins has been seen in the case of the 5S RNA gene, which can be transcribed by a linked phage promoter and polymerase even when a complete transcription complex has formed on the gene.[159] Likewise, transcription occurs across many cellular enhancers such as those of the immunoglobulin genes.[5, 46, 118, 121] Hence, transcription of the MTV provirus probably occurs across active GREs.

## 4. Conclusions

The experiments described above suggest that receptor binding is necessary for activation of a GRE. There is no evidence that specific binding of any other protein to the enhancer sequences is required. The results also indicate that sequences outside of the conserved octanucleotide have no effect on activation. This is shown in the linker scanning mutations in which the sequences within a footprint but outside of the octanucleotide do not render the GRE less active. The results do not rule out a mechanism in which the receptor protein serves to localize another protein on nearby DNA. Moreover, it is conceivable that sequences between the octanucleotides are functional in other contexts. For example, a separate enhancer that is not detectable in some cell types overlaps the GRE in the MoMSV enhancer.[21]

Receptor binding regions that are responsible for the rapid and specific increase in MTV transcription stimulated by glucocorticoids may also be located within MTV transcribed sequences. The various receptor binding regions within the MTV genome can act independently in the assays described herein. Each region can confer glucocorticoid responsiveness on a linked promoter without the presence of additional GREs, although RB4 is not as effective an enhancer as RB1. One possibility is that the sequences

within the footprints that are between the octanucleotides modulate the strength of the binding site. It is likely that activity as a GRE is proportional to the strength of a receptor binding site; this would explain why the downstream sites, which have conserved octanucleotide sequences, appear to be less active than RB1. Differences in GRE activity between different regions should be abolished in a background where receptor is not limiting. This has not been examined. The observation that the downstream sites are active at all, however, supports the interpretation that receptor binding is the sole requirement for GRE activity in this system.

## III. Promoter Mutations Do Not Affect GRE Activity

## 1. Introduction

The purpose of the experiments described in this chapter was to use a genetic approach to investigate the interaction between enhancers and promoters. The effects of specific promoter mutations on enhancer activity was therefore examined. Since enhancer activity is measured only as a function of its action on a linked promoter, changes in the promoter might alter the enhancers relative activity. Enhancer activity can vary over large ranges depending on which promoter is used to assess the enhancer action. The relative activity of a given enhancer may depend on the presence of specific promoter elements, and therefore a mutational analysis of the linked promoter elements may reveal which elements a given enhancer is dependent on. Any sequence in the promoter in which a mutation had an effect on enhancer activity would be implicated as important for the enhancer action. If no sequence alteration in the promoter affected enhancer activity, then the step of initiation at which enhancement occurred would be a step that did not involve specific sequences. The particular system used was fusions of the MTV GRE to Herpes simplex virus thymidine kinase (HSV *tk*) promoters carrying mutations in one or a combination of its identified promoter elements, or in regions with no apparent promoter function.[97]

The *tk* gene promoter consists of at least four sequence motifs shown in Figure 7, panel A. The TATA box is located from −26 to −21, while a complex upstream region extends from −50 to −105.[95,96] An additional element, newly discovered, is located from −131 to −138 and will be described in Chapter 4.[114] This functionally defined promoter is also sufficient for the induction of *tk* expression by the *trans*-acting immediate early gene product ICP4 in herpes virus infections.[31]

The upstream region contains three elements that interact *in vitro* with two purified transcription factors, Sp1 and CTF (Figure 7).[51,72] Sp1, isolated on the basis of its activity on SV40 early promoter *in vitro*, binds at 6 sites in the SV40 promoter and at two sites in the *tk* promoter; all share a hexanucleotide, 5'GGCGGG3'.[28,72,98] This so-called G/C box is also found in several other promoters, including human metallothionein I, mouse hypoxanthine phosphoribosyl transferase, and rat type II procollagen.[29] Hence, Sp1 is a rather general transcription factor; incorporation of Sp1 binding sites in the promoters of several

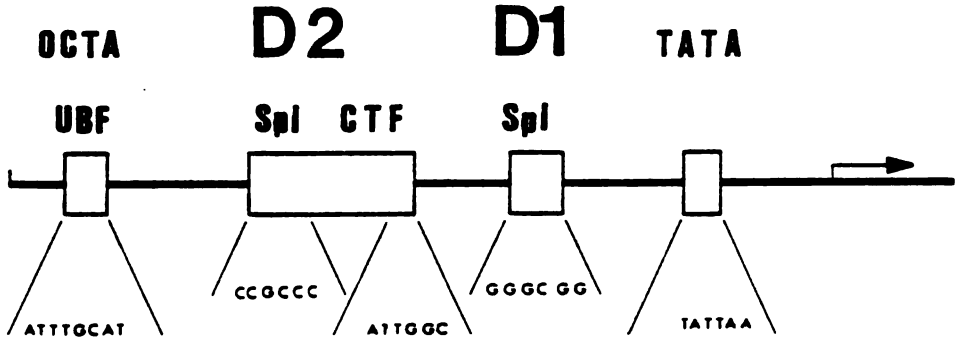
**Figure 7**
**Location of Identified Promoter Elements in $P_{tk}$**

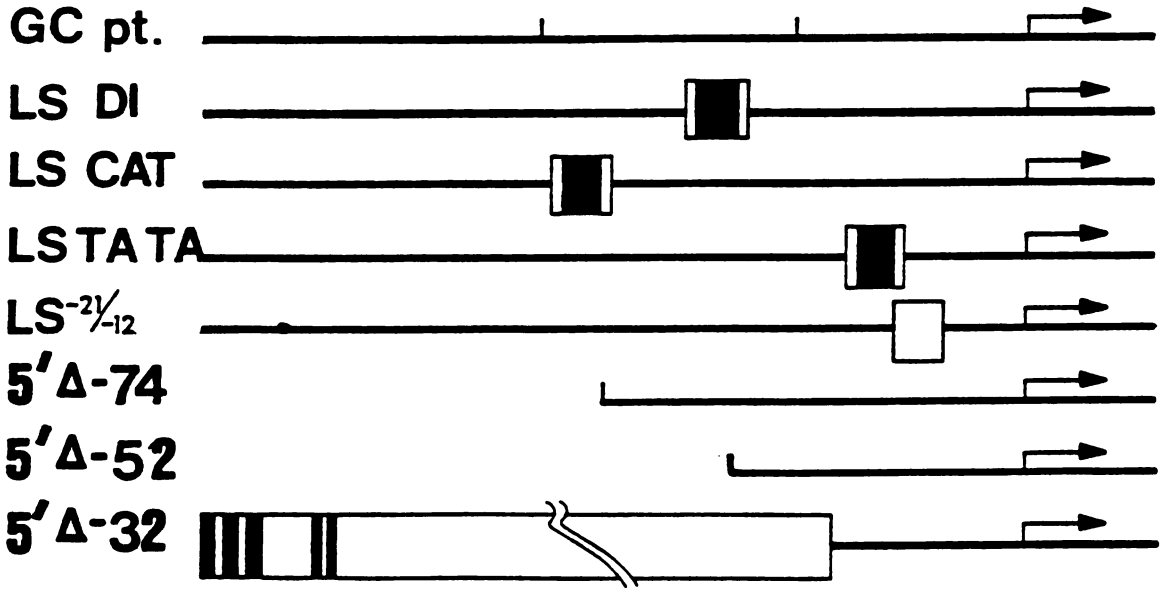
The upper diagram represents the known functional promoter elements of the HSV *tk* promoter as open boxes. The arrow represents the start site of transcription from this promoter. Above the promoter, factors that interact with each element *in vitro* are identified (see text for details). Below each element, the core sequences of each element that are required for full promoter activity *in vitro* and *in vivo* are shown. The lower diagrams represent the sites of mutations that were used to examine the activity of the MTV GRE upon *tk* promoters containing mutations in defined promoter elements. The location of the linker, when present, is shown as a open box. Any core sequences that are replaced by the linker is shaded within the box. Locations of point mutations and the endpoints of deletion mutations are also shown.

The mutations are located as follows: with the bases that are altered from the wild type sequence underlined. The G/C box point mutations are at position −102, creating the sequence 5′CGGCCC3′, and at position −52, creating the sequence 5′GGGCCG3′. These are complementary mutations in the two binding sites for the *trans*-acting factor Sp1. The linker scanning mutation LS D1 replaces the sequence from −59 to −49, 5′GTCGGGGCGG3′, with the sequence 5′CCGGATCCGG3′. LS CAT replaces the sequence from −84 to −74, 5′TGGCGAATTC3′, with the sequence 5′CCGGATCCGG3′. LS TATA replaces the sequence from −29 to −18, 5′GCATATTAAG3′, with the sequence 5′CCGGATCCGG3′. LS−21/−12 replaces the sequence from −21 to −12, 5′AGGTGACGC3′, with the sequence 5′C(C)GGATCCGG3′. Note that this linker scanning mutation also created a 1 bp insertion because the 10 bp linker replaces a 9bp wild type sequence. Deletion mutations are as described on the figure: they all contain a 10 bp Bam HI linker at the deletion endpoint.

The bottom diagram demonstrates the fusion of the PstI-SacI fragment of the MTV LTR to a deletion that extends to −32. Locations of the glucocorticoid receptor footprints are also shown; the distance from the deletion endpoint to the nearest footprint is 731 bp.

Fig. 7


OCTA
D2
D1
TATA
UBF
Spl
CTF
Spl
ATTTGCAT
CCGCCC
ATTGGC
GGGC GG
TATTAA


GC pt.
LS DI
LS CAT
LSTATA
LS-21/-12
5'Δ-74
5'Δ-52
5'Δ-32

viruses suggests that an Sp1 activity is found in a variety of cell types.

The role of these sites in the *tk* promoter has been demonstrated by linker scanning and point mutagenesis.[97,98] All mutations that disrupt the G/C motif have reduced transcriptional activity in microinjected oocytes, in transfected mammalian cells, and *in vitro*.[97] A point mutation in the upstream G/C box, which is within the region termed D2, had a more severe effect on transcription in microinjected oocytes than those in the promoter-proximal G/C box, D1.[98] If the fragment containing both the G/C boxes and all sequences between was inverted, then the mutations in the D1 G/C box had little or no effect. This suggests that sequences within D2 bind to the factor more strongly, or that additional factors were interacting with Sp1 at the D2 site but not at the D1 site.

Additional factors that bind to the *tk* upstream region within D2 have been identified by two laboratories.[51,72] These factors are not the same. One of these factors, termed CCAAT-binding transcription factor (CTF), is required for maximal *tk* promoter activity *in vitro*.[72] It binds to the CCAAT motif, a promoter element that is found in many eukaryotic pol II promoters and has been shown to be required for maximal activity of the mouse and rabbit β-globin promoters.[13,26,55] In transfected mammalian cells, this sequence motif is also required to direct maximal levels of transcription from the HSV *tk* promoter. The other factor, termed CCAAT-binding protein (CBP), binds to the CCAAT motif as well, but has not been tested for transcriptional activity *in vitro*.[51]

In my experiments, mutations in the two G/C boxes, the CCAAT motif, the TATA box, and in sequences not essential for maximal promoter function were fused to the GRE and tested for ability to respond to the enhancer. If GRE activity alters the activity of a sequence-specific transcription factor, then a mutation in the binding site for that particular factor should alter the relative enhancer activity. Therefore, a complete deletion of the binding site should render the GRE either inactive, since the factor that the GRE acts on has no site of action, or superactive, since the factor would otherwise be interacting with the promoter at a very low frequency. If GRE activity occurs through direct interaction with polymerase or non sequence-specific factors, or through an effect on the structure of the template, then muta-

tions in defined promoter elements might have no effect on enhancer function. Lastly, mutation of sequences that are not required for basal transcription may affect relative GRE activity, and therefore identify a sequence that is required for positive activation of the *tk* promoter.

**2. Mutations in the G/C boxes do not diminish GRE activity**

GRE action on the *tk* promoter derivatives was measured by transient transfection. The GRE was fused at a position 148 bp upstream of the cap site, with the exception of deletion mutations (Figure 7, panel B). These enhancer-promoter combinations were used to drive the expression of a reporter gene, bacterial chloramphenicol acetyl transferase (CAT).[2,49] The MoMSV enhancer was placed downstream of the CAT gene: this element carries both a "constitutive" enhancer activity, $S_a$, and a GRE activity, $S_g$.[21,103] The general construction is shown in Figure 2. In these constructs, only the $S_g$ activity was functional; the $S_a$ activity was blocked by additional plasmid sequences located on the enhancer fragment (see appendix for details). The additional GRE in these constructs increases the sensitivity of detecting the effects of the promoter mutations.

Experiments were performed as described in chapter 2. An additional cell line was used in some of these experiments: G10 is a rat hepatoma HTC cell derivative stably transfected with glucocorticoid receptor cDNA;. it carries three times the normal level of glucocorticoid receptor activity.[102]

Initial experiments examined GRE action on *tk* promoters containing point mutations in the G/C boxes and the TATA motif.[96,98] In XC cells, the mutations in the G/C boxes reduce basal CAT expression by as much as four-fold, whereas the relative induction upon hormone treatment was in each case similar to that seen for the wild type promoter, 20-25 fold (Table 2). As shown in Figure 8, the sites of initiation are not affected in these mutants and the accumulation of transcripts is proportional to the relative levels of CAT activity. The G/C point mutations tested therefore have no apparent effects on either the accuracy or the efficacy of the GRE.

**Figure 8**
**Primer Extension Analysis of GRE Activity on $P_{tk}$ Point Mutations**

Primer extension analysis of the unfractionated RNA isolated from transiently transfected XC cells is shown. Cells were cotransfected with CAT constructs containing mutations in the *tk* promoter linked to the GRE at position −148 relative to the cap site, and RSVCAT as an internal control. The *tk* promoter gives rise to primer extension products of 90, 89, and 87 bases. These are separated from the RSVCAT control extension product, which is 68 bases long. Lanes marked "+" represent analysis of transcripts from cells that were treated with 0.1μM dexamethasone for 12 hr following transfection. The lanes marked "−" were not treated with hormone and represent the basal level of transcription from the construction under investigation. The mutations that were tested in each pair are listed above the autoradiogram. The location of the normal *tk* start sites are marked by arrows, as is the location of the RSVCAT internal control.

Fig. 8

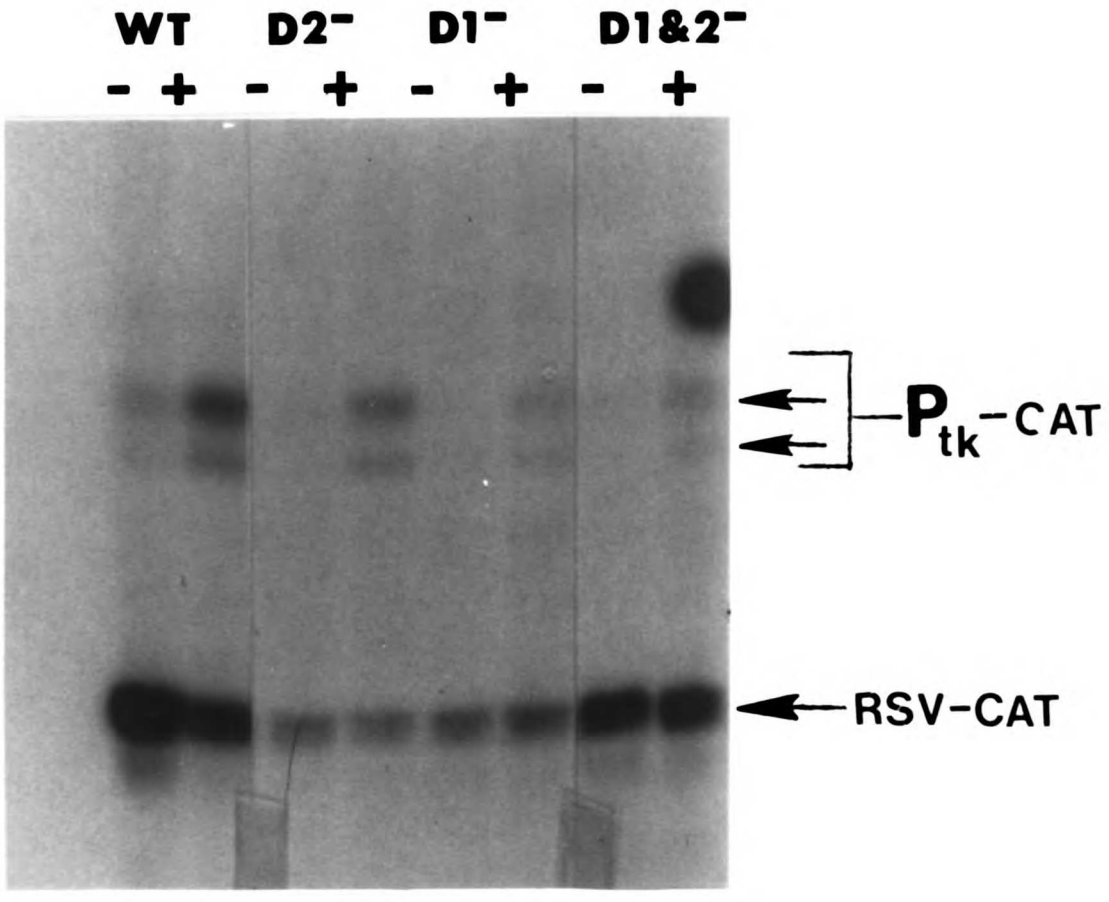


**TABLE 2**
**Effect of promoter point mutations on GRE activity in XC cells**

| *Promoter* | *Basal Transcription (% wild type)* | *Fold Induction* |
|---|---|---|
| wild type | 100 | 19 |
| −102 D2 G/C point | 47 | 19 |
| −51 D1 G/C point | 97 | 14 |
| −102,−51 D2, D1 G/C points | 20 | 15 |
| LS−21/−12 | 112 | 9 |
| LS−21/−12 & D2 point | 69 | 12 |
| LS−21/−12 & D1 point | 150 | 6 |
| LS−21/−12 & D1, D2 points | 41 | 8 |
| 5′Δ−32 + spacer | 6 | 7 |

**Table 2**: The effects of point mutations within the *tk* promoter on GRE activity upon the promoter as measured by CAT expression in transiently transfected XC cells is shown. Cells were transfected with plasmids that contained the promoters indicated, with the GRE fused to −148, and CAT activity was assayed 12-20 hr after transfection. All data were normalized to the levels of β-galactosidase activity that were produced from a cotransfected internal control, RSV β-galactosidase. This latter plasmid did not respond to dexamethasone, and so provided a measure of the transfection efficiency between two plates. The basal level of transcription of the various promoter mutations is presented as the percent of wild type promoter activity. Values given represent the mean of 2 to 7 determinations for a given promoter mutation. In all data presented here, the GRE was in the 1 orientation relative to the promoter; the results were not affected by the orientation. The exact location of the mutations is described in Figure 7.

A linker scanning mutation, LS−21/−12, extending from −21 to −12 bp, alters the last nucleotide of the TATA element from A to C; this mutant reduces transcription by 15-fold in the frog oocytes, but displayed no mutant phenotype in mammalian cells. A slight effect was observed when LS−21/−12 was combined with the G/C box mutations. GRE effects on these mutants were 30-80% of the induced levels achieved with the wild type promoter. These modest apparent alterations may indicate that the GRE affects an aspect of the formation or function of the initiation complex that involves sequences between −21 and −12. This mutation may in fact identify a binding site for a factor that is required for regulation of the *tk* promoter, but not for its basal activity.

A direct test of the function of the upstream factor binding sites in GRE responsiveness of the *tk* promoter was next performed. All sequences upstream of −32 in the *tk* promoter were removed. The

GRE was placed at −1 kb, with MTV LTR sequences between the GRE and the promoter (Figure 7, panel C). When this construct was analyzed for GRE action using the CAT assay, it was induced 8.5-fold by the addition of dexamethasone. Although no wild type control with the same spacing was constructed, this represents a significant level of transcriptional activation. A representative CAT assay of this construction is presented in Figure 9. The basal level of transcription arising from this promoter was just 3% of the wild type activity, suggesting that the spacer DNA was not contributing to the promoter activity or the response (see Table 2); consistent with this view, transcripts arising from the opposite strand of the MTV LTR have not been detected. Therefore, it seems most likely that the GRE acts upon factors that facilitate initiation complex formation or function, but that do not themselves bind specifically to DNA. Alternately, the GRE might act at sequences that are at or extremely near the cap site, since mutations at sequences between the TATA box and the cap site have a slight effect on GRE activity.

### 3. Promoters with mutations in functional elements retain GRE responsiveness

GRE action on *tk* promoters containing linker scanning mutations, deletions, or an inversion was tested in an additional series of constructions in which the $S_a$ activity of the MoMSV enhancer was used to increase basal promoter function sufficiently to allow analysis of both CAT activity and transcripts arising from severely altered promoters (Figure 7). Under these conditions, GRE activation of the wild type promoter in transfected XC cells was 5-6 fold.

The calculated activities of several repeats of the CAT assays are shown in Table 3. Linker scanning mutations that affect the CCAAT box, the D1 G/C box, or the TATA box reduce basal expression to 43%, 23%, and 16%, respectively, of wild type levels. In no case is GRE activity affected; relative induction ratios with the three mutants were 82-125% of that seen with the wild type promoter. The linker scanning mutations tested completely disrupt the binding sites for the corresponding transcription factors. Therefore, the simplest conclusion is that the GRE does not affect the action of factors that associate with these elements. The result with the TATA box mutation, LS−29/−18, implies that the effects

**Figure 9**
**CAT Assay of GRE Activity on $P_{tk}$ Mutations**

A representative CAT assay of *tk* promoter mutations under regulation by the MTV GRE analyzed in rat XC cells is shown. The basal activities of the promoter mutations are shown in the lanes marked "–", which represent CAT activity from transiently transfected cells that were not treated with dexamethasone. The lanes marked "+" were treated with 0.1μM dexamethasone for 12 hr following transfection. The promoter mutation examined in each pair of assays is indicated above the autoradiogram. The lane marked "XC" shows the trace amount of CAT activity that is obtained from mock-transfected XC cells.

Fig. 9

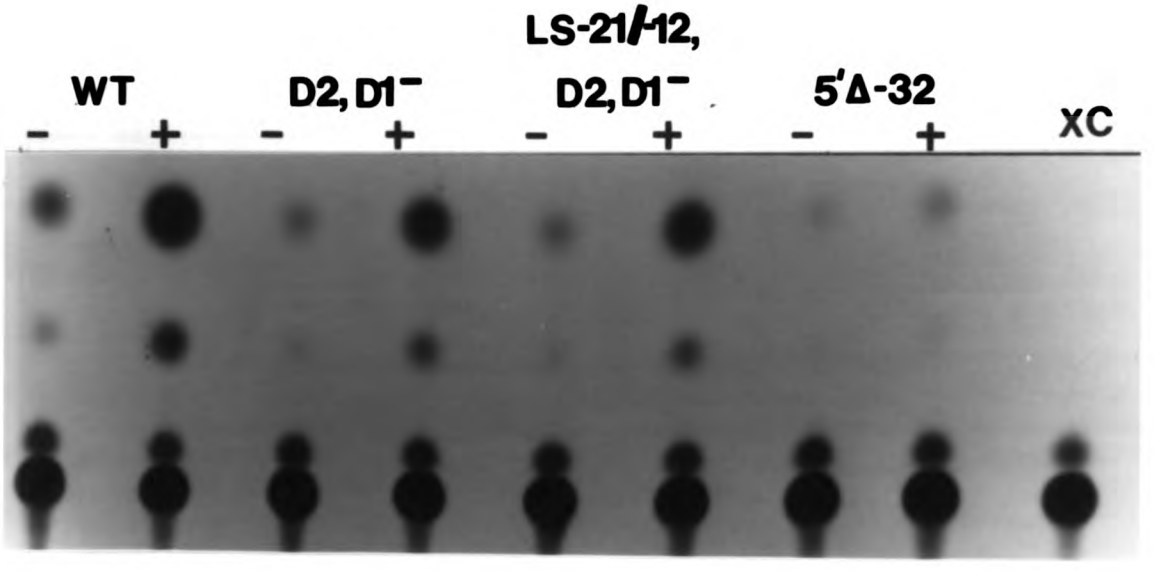


**TABLE 3**
**Effect of linker scanning and deletion mutations in the promoter on GRE activity in XC cells**

| *Promoter* | *Basal Transcription (% wild type)* | *Fold Induction* |
|---|---|---|
| wild type | 100 | 5.0 |
| LS–29/–18 (TATA) | 16 | 4.1 |
| LS–54/–49 (D1 G/C) | 23 | 5.2 |
| LS–84/–74 (CCAAT) | 43 | 6.1 |
| 5'Δ–74, GRE 1 | 3 | 10.4 |
| 5'Δ–74, GRE 2 | 2 | 7.2 |
| 5'Δ–54, GRE 1 | 4 | 4.2 |
| 5'Δ–54, GRE 2 | 1 | 5.1 |
| –195/+55 inversion | 69 | 4.0 |

**Table 3**: The effects of linker scanning and deletion mutations in the *tk* promoter on GRE activity upon these promoters as determined by CAT expression in transiently transfected XC cells is shown. Cells were transfected with plasmids containing the promoter mutation indicated, with the GRE linked either to –148 or to the deletion endpoint, and CAT activity was assayed 12-20 hr after transfection. Except where indicated, the GRE was in orientation 1; the orientation did not alter the results. All data were normalized to the levels of β-galactosidase activity that were produced from a cotransfected internal control, RSV β-galactosidase. This latter plasmid did not respond to dexamethasone, and so provided a measure of the transfection efficiency between two plates. The basal level of transcription of the various promoter mutations is presented as the percent of wild type promoter activity. Values given represent the mean of 2 to 7 determinations for a given promoter mutation.

of LS–21/–12 on GRE activity indeed reflect sequence alterations downstream of the TATA box.

One interpretation of the results is that the GRE acts on the factors that have formed stable interactions with the DNA and each other, regardless of whether any component is missing from the stable complex. To test this model, the GRE was linked to promoters containing 5' deletions that eliminated most or all of the known factor binding sites upstream of the TATA box. If all the upstream factor-binding sites are deleted from the promoter, then the probability that any stable complex will form upstream of the cap site region is lowered or eliminated. The GRE might either act on whatever stable complex forms on the remaining promoter, or it might require some upstream factor binding sites to act. The GRE was linked to the deletion endpoints in both orientations. Since the receptor binding sites are asymmetrically distributed on this fragment (see Figure 1), the inverse orientation (denoted "2") places a 120 bp spacer

between the enhancer and the deletion endpoint. Orientation 1 leaves no spacer DNA between the enhancer and the promoter, but the receptor binding sites are so close to the cap site that the possibility that the enhancer will substitute for the deleted promoter elements exists.

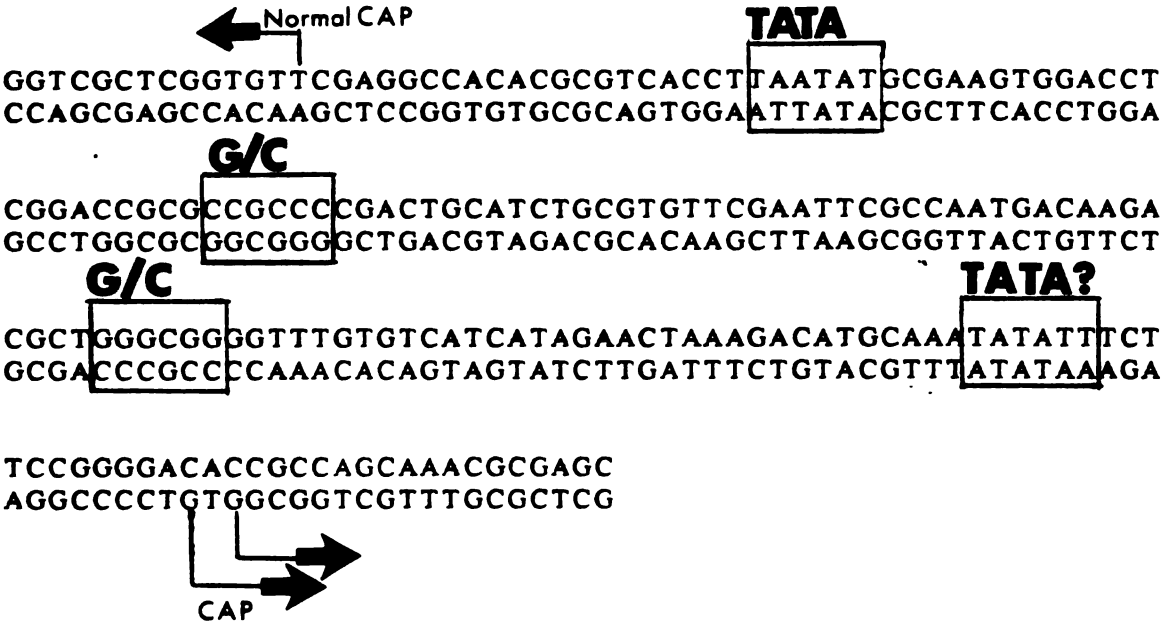
GRE activity on promoters that have deletions to −74 bp and −52 bp from the cap site was examined. The −74 deletion removes all upstream promoter elements except the D1 G/C box; the −52 deletion removes all upstream elements (Figure 7, panel C). Both mutants exhibit basal transcription at 1-4% the level of the wild type promoter (Table 3). Induction ranges from 144% to 207% that of the wild type for the −74 deletion, and from 84-100% for the −52 deletion. The increase over wild type induction ratios in the −74 mutant may be due to the change in spacing. Alternately, the GRE activity may affect binding of factors or other events at the cap site. The apparent increased induction of the −74 deletion relative to wild type may be due to the higher fluctuation in measuring the activity of a weak promoter. It appears that the GRE does not require the presence of any upstream factor binding site to function, and therefore that the GRE can act on whatever pre-formed complex exists at the promoter.

An interesting promoter that was examined for its responsiveness to the GRE is an inversion of the entire promoter fragment containing a linker scanning mutation in the CCAAT motif. This was inverted from −195 to +55, such that any detectable CAT activity would arise from transcription on the non-coding strand. It seemed possible that this would create a functional promoter for several reasons. First, McKnight et al. [98] showed that the sequences between −109 and −42 could be inverted with respect to the cap site and still direct accurate and efficient transcription from the correct site. Second, the G/C boxes of the *tk* promoter are in an inverse orientation relative to each other, suggesting that the polarity is not important. In fact, Sp1 binding sites are found in both orientations in a variety of promoters. Lastly, the CCAAT box motif of the *tk* promoter is in fact backwards relative to that of the MoMSV promoter and of the β-globin gene.[26,51] Hence, it may well be a bidirectional element as well. The inverted promoter is lacking a defined TATA box and cap site. There exists, however, a possible TATA motif at −139 that might serve to accurately position the polymerase on the DNA template as shown in Figure 10.

**Figure 10**
**Sequence and Features of Inverted $P_{tk}$**

The sequence of the fragment of the *tk* promoter that was inverted and tested for promoter activity and ability to respond to activation by the GRE is shown. The identified upstream elements of the *tk* promoter are indicated within the rectangles. In addition, a putative TATA motif located at −139 from the normal *tk* start site, and at −16 from the cap site used by the inverted promoter is indicated. The initiation sites that are used by this promoter are indicated by the arrows, and are denoted "cap"; the normal *tk* cap site is also indicated. A representative primer extension analysis of this promoter under the regulation of the MTV GRE is shown in Figure 11.

**Figure 10: Sequence and Features of Inverted $P_{st}$**



Analysis of the inversion in the transient expression system revealed that it was in fact transcriptionally active, was able to direct the synthesis of 69% of the wild type level of CAT activity, and was responsive to the GRE (Table 3). The high level of transcriptional activity arising from this construct was particularly striking since the inversion itself contained a mutation in the CCAAT box sequence.

**4. Transcription is correlated with CAT activity**

Primer extension analyses were performed to determine whether the effects of the GRE on CAT activity in the constructions containing such severe mutations reflect transcription from the normal *tk* cap site. Transcripts for most of these mutations were detected and are displayed in Figure 11. It can be seen that the LS mutation in D1 allows accurate initiation and enhancement. On the other hand, LS–29/–18, which disrupts the TATA box, leaves little initiation at the correct site, which is the usual effect of mutations in the TATA box of eukaryotic promoters.[89] There is instead a cluster of start sites ranging from –52 to +4. One especially strong start site is located at –4. This site is weakly visible in the wild type lanes, and may be an alternate cap site. The entire set of start sites in the TATA mutation is induced by the GRE, suggesting that the GRE affects equally the initiation efficiency of a family of initiation complexes differing slightly in their precise positions.

Analysis of the inversion reveals that it has 2 predominant transcription initiation sites located at –154 and –158 relative to the normal *tk* cap site (Figure 11 and see also Figure 10 for the sequence). These are the sites that are enhanced by the GRE, and they lie 16-20 bp downstream of the postulated TATA-like sequence. The inversion is therefore a valid eukaryotic promoter, and it is able to respond to the GRE.

Analysis of the transcripts from the deletion mutations is rendered more difficult by their low abundance and by the presence of multiple start sites (Figure 12). Lanes 5 and 6 show the transcripts initiating from the –74 deletion when the GRE is in orientation 1. Although transcription from the correct cap site is barely detectable, an induced start site is located at –4. Note that this same start site is seen in the LS TATA mutation (Figure 11, lanes 3 and 4),[97] and weakly in the wild type promoter. It is conceivable that

**Figure 11**

**Primer Extension Analysis of GRE Activity on $P_{tk}$ Linker Scanning and Inversion Mutations in XC Cells**

Primer extension analysis of the unfractionated RNA isolated from transiently transfected XC cells is shown. Cells were cotransfected with CAT constructs containing mutations in the *tk* promoter linked to the GRE either at position −148 relative to the cap site, or to the deletion endpoint, and RSVCAT as an internal control. The *tk* promoter gives rise to primer extension products of 90, 89, and 87 bases, as indicated by the arrows. Lanes marked "+" represent analysis of transcripts from cells that were treated with 0.1μM dexamethasone for 12 hr following transfection. The lanes marked "−" were not treated with hormone and represent the basal level of transcription from the construction under investigation. The GRE was in orientation 1 in all cases. M represents pBR325 HpaII markers; the sizes of the markers are shown to the left, and the distances from the *tk* cap site are indicated to the right of the figure (in nucleotides). Panel B shows the results of a primer extension analysis of the wild type promoter and the inversion promoter. Lanes marked "WT" are transcripts from cells transfected with the wild type *tk* promoter, while lanes marked "inv" arise from transcription from the inversion. The location of the start sites of the inversion are indicated on the sequence presented in Figure 10.

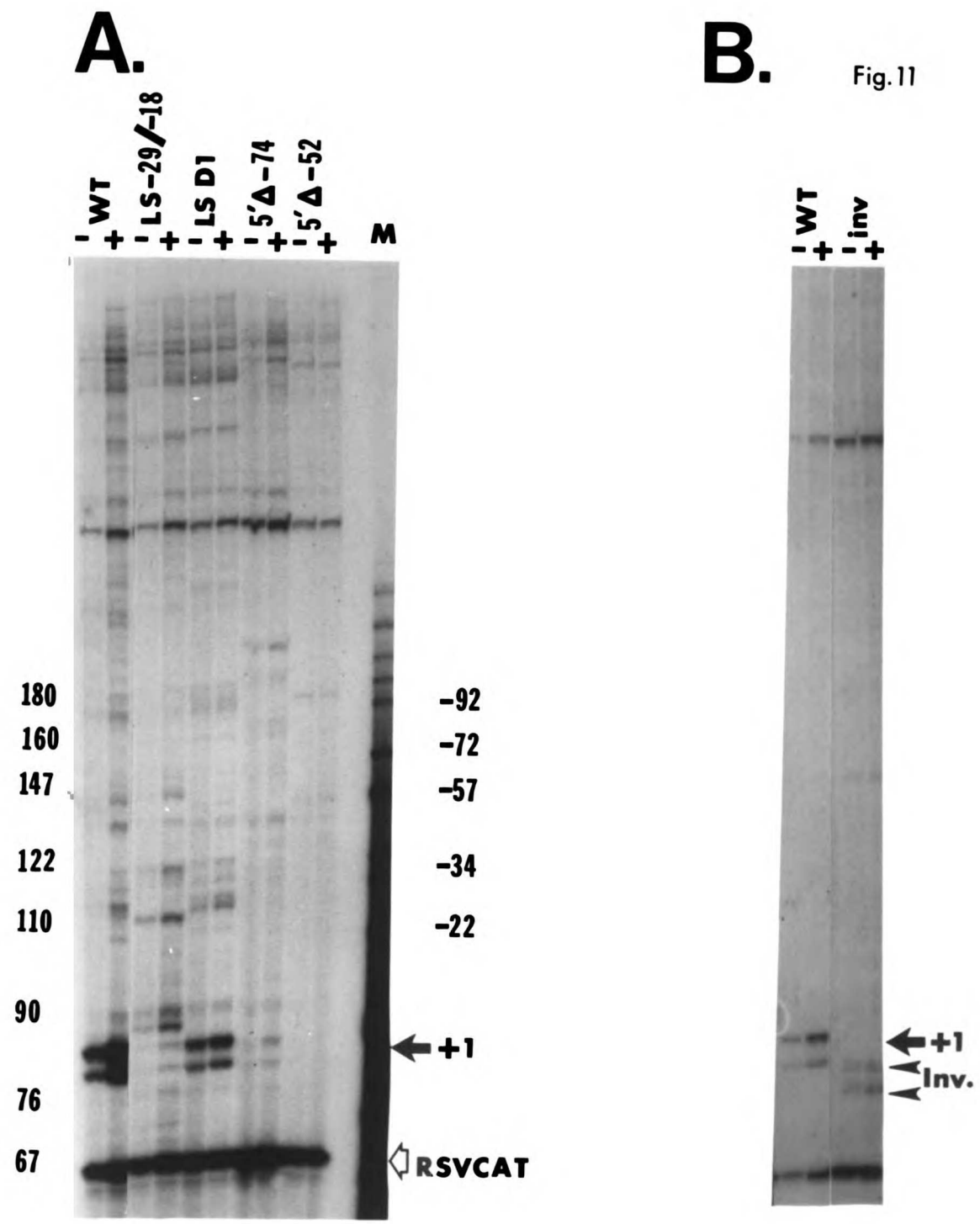

A.
B.
- WT +
- LS-29/-18 +
- LS D1 +
- 5'Δ-74 +
- 5'Δ-52 +
M
180
160
147
122
110
90
76
67
-92
-72
-57
-34
-22
+1
RSVCAT
- WT +
- inv +
+1
Inv.

Fig. 11

**Figure 12**
**Primer Extension Analysis of GRE Activity on $P_{tk}$ Deletion Mutations in XC Cells**

Primer extension analysis of the unfractionated RNA isolated from transiently transfected XC cells is shown. Cells were cotransfected with CAT constructs containing deletions of the *tk* promoter linked to the GRE, and RSVCAT as an internal control. The normal start sites from the *tk* promoter are indicated by the solid arrows. Lanes marked "+" represent analysis of transcripts from cells that were treated with 0.1μM dexamethasone for 12 hr following transfection. The lanes marked "–" were not treated with hormone and represent the basal level of transcription from the construction under investigation. The mutations that were analyzed in each pair of lanes is indicated above the autoradiogram. The numbers "1" and "2" denote the orientation of the GRE in each construction relative to the promoter. Markers are as described for Figure 11.

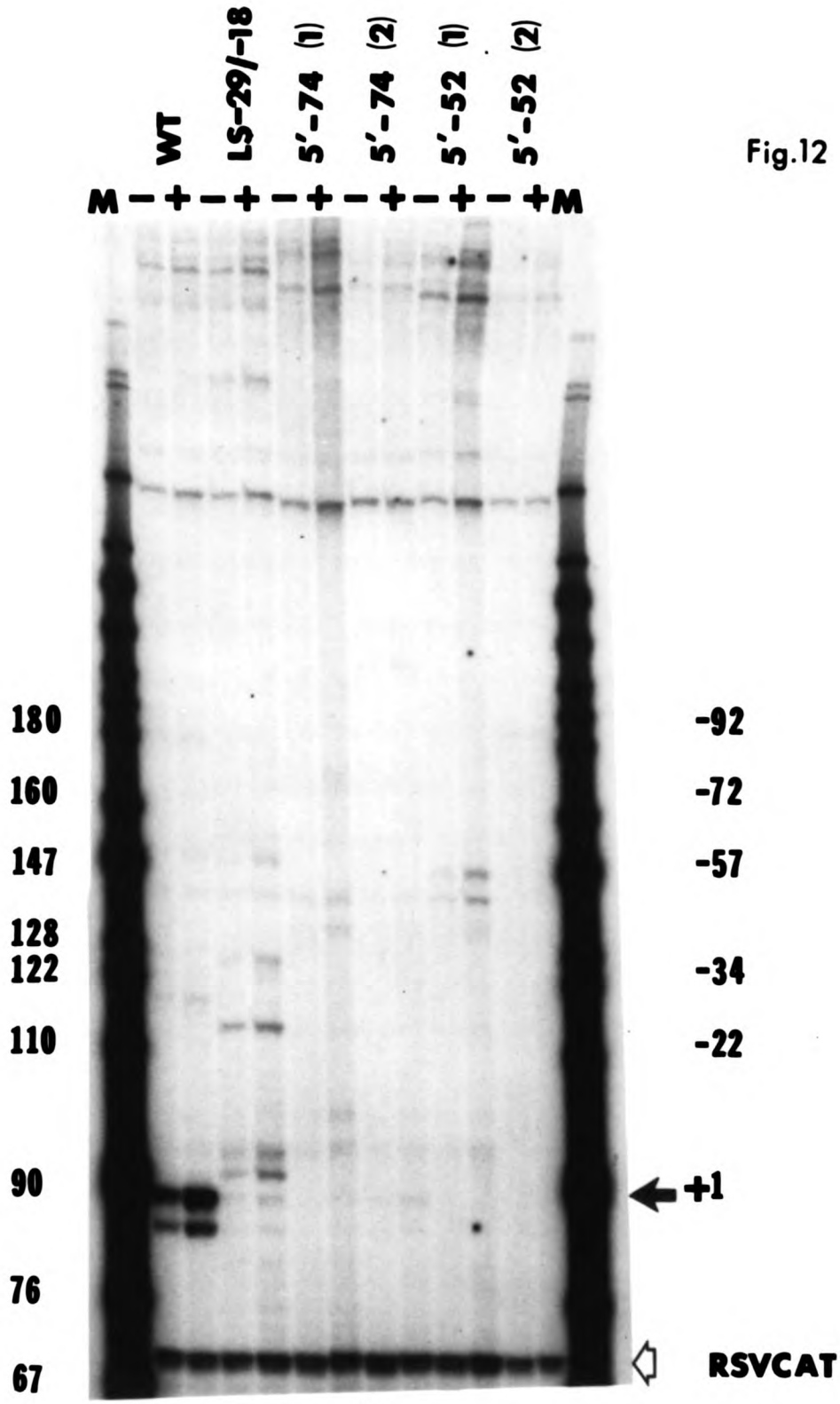

M
-
+
WT
-
+
LS-29/-18
-
+
5'-74 (1)
-
+
5'-74 (2)
-
+
5'-52 (1)
-
+
5'-52 (2)
M
180
160
147
128
122
110
90
76
67
-92
-72
-57
-34
-22
+1
RSVCAT

Fig.12

this altered site results from an alteration of the initiation complex by the receptor itself. That is, footprint 1.5 of the GRE overlaps the deletion endpoint and may impinge on part of the promoter, perhaps altering the orientation of RNA polymerase such that a different cap site is preferred. Consistent with this view, a different result is obtained when the GRE is in orientation 2, in which the nearest footprint, 1.1, is 120 bp from the deletion endpoint. Lanes 7 and 8 demonstrate that the initiation site utilized in these constructs is predominantly the correct *tk* cap site. In both cases, it can be seen that the addition of glucocorticoids to the cells transfected with these constructions causes an increase in the utilization of whatever cap sites are being used. Additional initiation sites are also observed well upstream of the the GRE; these are also enhanced by the GRE, providing evidence that the GRE is acting as an enhancer, rather than simply substituting for upstream promoter elements.

Analysis of the −52 deletion provides essentially the same results. When the GRE is in orientation 1, a weak but inducible start is located at −4. Again, this may be due to alterations in the configuration of sequences and factors around the actual cap site when the receptor is bound so close. In this case the receptor bound to footprint 1.5 overlaps sequences that are just 20 bp away from the TATA box. Analysis of the transcripts arising from the same deletion with the GRE in orientation 2 were not successful, probably due to the extremely low level of signal obtained from these constructs. As with the −74 fusions, enhanced transcripts originating upstream of the GRE are seen in orientation 1.

### 5. Analysis of *tk* promoter mutants in a cell line overexpressing glucocorticoid receptor

Recent efforts in our laboratory resulted in the successful cloning of the glucocorticoid receptor coding sequences.[100, 101, 102] Cell lines stably transfected with the receptor sequences have been isolated that expressed 1.5, 3, or 4 times the normal level of receptor as measured by a dexamethasone binding assay.[102] It was found that the magnitude of various hormone responses in these lines was proportional to the amount of receptor present, suggesting that receptor occupancy at GREs may limit the strength of many glucocorticoid effects. In any case, it seemed that such overproducer lines might increase the sensitivity of analysis of GRE effects on the *tk* promoter mutants.

Table 4 shows the data obtained for the linker scanning and inversion mutations in G10, which produces three-fold more receptor than the HTC parent line. The LS mutation in D1 was induced as well as wild type (18.5-fold), whereas LS–29/–18, which completely disrupts the TATA box, was induced only 30% as much as the wild type promoter; recall that it had been fully responsive to the GRE in XC cells. Note that the basal level of transcription of the LS–29/–18 mutant is increased in these cells relative to the wild type. Possibly, a second step that is not relieved by the GRE become rate limiting sooner than in the wild type promoter. The GRE can activate until this new step becomes rate limiting for transcription from this promoter. These results again imply that initiation events that occur close to the cap site may be involved in GRE activity. In particular, a previously unidentified transcription factor that is required for activation but not for basal expression from the *tk* promoter may act at sequences between the TATA box and the cap site.

**TABLE 4**
**GRE Activity on *tk* promoter mutations in G10 cells**

| *Promoter* | *Basal Transcription (% wild type)* | *Fold Induction* |
|---|---|---|
| wild type | 100 | 18.5 |
| LS–29/–18 (TATA) | 77 | 5.0 |
| LS–59/–49 (D1 G/C) | 42 | 12.2 |
| LS–84/–74 (CCAAT) | 34 | 27.0 |
| –195/+55 inversion | 94 | 12.4 |

Table 4: Cells were transfected with plasmids containing the promoter mutation indicated, with the GRE linked either to –148 or to the deletion endpoint, and CAT activity was assayed 12-24 hr after transfection. The GRE was in orientation 1; the orientation did not alter the results. All data were normalized to the levels of β-galactosidase activity that were produced from a cotransfected internal control, RSV β-galactosidase. This latter plasmid did not respond to dexamethasone, and so provided a measure of the transfection efficiency between two plates. The basal level of transcription of the various promoter mutations is presented as the percent of wild type promoter activity. Values given represent the mean of 2 to 5 determinations for a given promoter mutation.

### 6. Analysis of transcription in the G10 cells

Transcripts from the G10 cells were analyzed by primer extension as described for the XC cell system. Because this cell line transfected with lower efficiency than the XC line, it was necessary to transfect the cells with four times as much DNA to obtain detectable signals. The results of such an analysis are shown in Figure 13. Although there are several additional start sites in all lanes, the correct initiation site can clearly be seen in both the wild type and the −74 deletion mutation. In this case, the GRE was in orientation 2 relative to the deletion, which also led to proper initiation in the XC cells. Both the wild type and the mutant are induced by the GRE at the correct initiation site. A sample of RNA from XC cells transfected with the normal amount of DNA of the wild type construction is shown in lane 7; this demonstrates that the same cap site is used in both cell lines. In addition, some upstream starts are again present, and some of these are induced. Lanes 3 and 4 show the results of analysis of the LS−29/−18 TATA mutation; the disperse series of start sites that were used and induced in the XC cells are also seen in G10 cells.

### 7. Conclusions

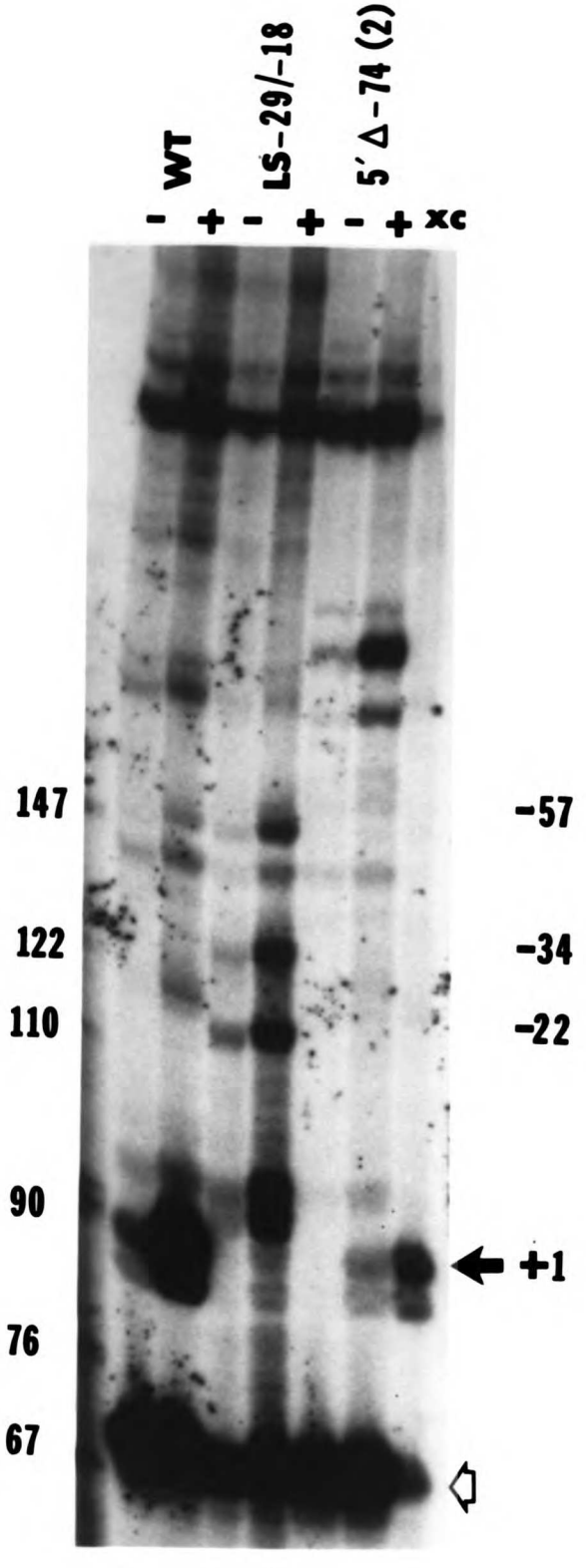
No evidence was found that any previously identified promoter element is the site that GRE activity affects. The simplest interpretation of the results is that the GRE facilitates the utilization of a transcription factor that is not directly involved in protein:DNA contacts. Such a factor might well be of the class of transcription factors that resemble the σ factors in E. coli, the so-called transient factors. Alternately, the GRE may activate more than one transcription factor, perhaps by directly interacting with a pre-formed stable complex.

Interestingly, mutations between the TATA box and the cap site seemed to reduce GRE activity slightly. This may define a new element in the *tk* promoter that is not required for constitutive activity but is necessary for regulation by the GRE. If this region is a binding site for factors that have yet to be identified, then the GRE may facilitate the utilization of these sequence-specific factors. Alternately, the GRE may influence some step other than factor binding that occurs at these mutation-sensitive sequences.

**Figure 13**
**Primer Extension Analysis of GRE Activity on $P_{tk}$ Mutations in G10 Cells**

Primer extension analysis of the unfractionated RNA isolated from transiently transfected G10 cells is shown. Cells were cotransfected with CAT constructions containing mutations in the *tk* promoter linked to the GRE, and RSVCAT as an internal control. The wild type *tk* promoter gives rise to primer extension products of 90, 89, and 87 bases as marked by the arrows. These are separated from the RSVCAT control extension product, which is 68 bases long. Lanes marked "+" represent analysis from cells that were treated with 0.1μM dexamethasone for 12 hr following transfection. The lanes marked "–" were not treated with hormone and represent the basal level of transcription from the construction under investigation. The lane marked "XC" is a positive control showing the extension products from the wild type promoter tested in XC cells, treated with hormone for 12 hr post-transfection. Markers are as described in Figure 11.

Fig.13



In the weakest promoters examined, the GRE activated some alternate start sites, sometimes without activating the correct one. This may reflect a less rigorous positioning of the initiation complex when the upstream factor binding sites are missing. Since these alternate sites are clearly less efficient than the correct *tk* cap site, this result indicates one role that the upstream elements may play transcription initiation: they may accurately position the initiation complex such that initiation occurs more frequently. The GRE can increase the transcription of any start site that is being utilized. This is especially noticeable in the LS–29/–18 mutation of the TATA box, in which several start sites situated near the normal cap site are all enhanced by the GRE. In these experiments, however, the GRE was never seen to create new cap sites. It only enhanced sites that were existent before GRE activation. Furthermore, it enhanced the transcription from sites that were both downstream and upstream from itself.

The simplest model of GRE activity is that the GRE acts at a preformed stable initiation complex. The LS–29/–18 result is consistent with this model. When the TATA box is disrupted, the stable complexes are less rigidly positioned at the normal cap site. The GRE activates any of these complexes, suggesting that it acts at a step of initiation after stable complex formation. The results do not eliminate the possibility that the GRE does increase the incorporation of a stable factor that binds equally well to the complex even when its target DNA sequence is missing. This might occur if the contacts that the factor makes with other proteins in the complex are sufficient to insure stability in the absence of the factor's DNA contact points. The severe deletions that were examined make this extremely unlikely, however, since sites for all upstream binding factors are removed. Furthermore, there is no precedent for incorporation of factors into the initiation complex without the DNA sequence present. It is more likely, then, that the GRE acts to increase the activity of an existing initiation complex. This activity may occur at or near the cap site, perhaps by altering the activity of a transcription factor that acts at this region.

# IV. A Functional Relationship Between Two Linked, Conserved Sequence Elements

**Susan D. Jones IV. A Functional Relationship Between Two Linked, Conserved Sequence Elements**

## 1. Introduction

The experiments presented in this chapter tested the relationship between two conserved sequence elements of the mouse immunoglobulin κ gene. One of these, occupying a 250 bp (bp) region of an intron adjacent to the constant region exon,[34, 112] is the κ enhancer.[118] This element is preferentially active in B lymphoid cells, undergoes a localized change in chromatin structure that correlates with the onset of κ transcription, and is required for maximal expression from the κ promoter.[7, 111, 112, 121] The immunoglobulin heavy chain genes contain a similarly located enhancer. The second conserved sequence element, an octanucleotide, 5'ATTTGCAT3', is found 70±10 bp upstream from the transcription initiation sites of all light chain genes, (and in inverted orientation (5'ATGCAAT3') at the same location in heavy chain gene promoters).[38, 113] The octanucleotide has been stringently conserved in vertebrate evolution, and deletions of DNA segments that encompass this element abolish κ promoter activity.[7, 38, 88] A recent study suggests that the octanucleotide may be included in the recognition site for a specific protein.[140]

During plasma cell differentiation, the immunoglobulin enhancers are brought into the proximity of promoters upstream of the variable regions by DNA rearrangement. In the case of the heavy chain genes, secondary rearrangements occur that alter the constant region that is being expressed, and hence alter the class of antibody that is produced. In certain myeloma cell lines, class switch variants have been detected that delete the enhancer sequence during rearrangement. Interestingly, expression from the heavy chain promoter was retained in these variants;[74, 151] one interpretation of these findings is that these enhancers facilitate the binding of stable transcription initiation factors at the immunoglobulin promoters, such that the enhancers become dispensable after their initial action. That is, unlike the GRE, the κ enhancer might trigger during development an irreversible activation of its promoter; such a "determinative enhancer" might may facilitate binding of a stable sequence specific factor, conceivably one that binds to the octanucleotide motif. Here, I describe experiments to determine if the octanucleotide is a promoter element, and to determine the relationship of this element to the κ enhancer.

## 2. The octanucleotide is a functional promoter element

The approach was to determine whether the octanucleotide element is necessary for maximal activity of the herpes simplex virus thymidine kinase (*tk*) promoter.[72, 97] A perfect copy of the κ octanucleotide resides 131 to 138 bp upstream of the start site in a region thought to be dispensable from previous studies.[31, 97, 98] The activities of two *tk* promoter fragments were therefore examined. The first, "promoter A", comprised sequences from 55 bp downstream to 109 bp upstream of the transcriptional start site, whereas "promoter B" extended to an additional 39 bp upstream to position −148, and so included the octanucleotide locus (Figure 14).

A series of recombinant plasmids was constructed by fusing each promoter fragment to the coding sequences of the bacterial chloramphenicol acetyltransferase (CAT) gene (see Figure 2).[49] The plasmids were constructed both with and without the polyoma viral early region and origin of replication, which enable plasmids to replicate in mouse cells and thereby greatly increase the sensitivity of the transient expression assay;[121] polyoma sequences had no systematic qualitative effect upon the results (see below). The plasmids were transfected into the mouse myeloma cell lines P3X63Ag8 and SP2/0, and relative *tk* promoter activity was inferred by comparing the levels of CAT enzyme activity in lysates of the transfected cells.

Table 5 and Figure 15 show the results of these analyses. It can be seen that, although the absolute levels of CAT activity were greatly enhanced by the polyoma origin region, the relative levels of expression between the various constructions remained constant. As shown in Table 5, plasmids containing promoter A yielded a relatively low basal level of enzyme expression, which has been assigned an arbitrary value of one in all cases, for the sake of direct comparisons between experiments. The plasmids that contained promoter B, and therefore the octanucleotide sequence, produced approximately 3- to 4-fold higher levels of CAT activity ($p < 0.01$).

The difference between expression of these two promoters was seen in both cell lines, in the presence or absence of the polyoma origin and enhancer region, and in multiple repeats of the experiments

**Figure 14**
**Promoter Fragments used to Study Octanucleotide Function**

The organization of the wild type HSV *tk* promoter is shown. Locations of the cap site (init.), TATA box, and octanucleotide element (OCTA) are indicated. The locations of the two previously described upstream control regions, D1 and D2, are shown. The promoter fragments used in this study extended from a BglII site at position +55 to BamHI linkers at either $-109(P_A)$, $-148(P_B)$, or $-52(P_C)$.

Fig. 14


Pb
Pa
-140
-120
-100
AAGAAAAT ATATTTGCAT GTCTTTAGTT CTATGATGAC ACAAACCCCG CCTAGCGTCT
OCTA
D2
-80
-60
-40
TGTCATTGGC GAATTCGAAC ACGCAGATGC AGTCGGGGCG GCGCGGTCCC AGGTCCACTT
D1
Init
-20
+20
CGCATATTAA GGTGACGCGT GTGGCCTCGA ACACCGAGCG ACCCTGCAGC GACCCGCTTA
TATA
+40
ACAGCGTCAA CAGCGTGCCG CA

**Figure 15**

**CAT Assay Analysis of Octanucleotide Element Function in the *tk* Promoter in Myeloma Cells**

All panels show representative CAT assays of analyses of promoters A and B in myeloma cells. These promoters were analyzed by transient transfection. The upper panel represents the results of transfection with constructions that do not contain the origin region of Polyomavirus. The CAT reactions used 160 μg of protein extract, and were done for 18 hr. The center panel shows the results of analysis of the same constructions with the Polyomavirus origin inserted downstream of the CAT gene. These reactions used 30μg of protein extract, and were done for 1 hr. The promoter and enhancer that are contained in each construction are indicated (P=promoter, E=enhancer). Lanes marked "–" had no enhancer present at the 5′ end of the gene; lanes marked "κ" had the κ enhancer; and lanes marked "S" had the MoMSV enhancer. The lower panel compares the level of CAT activity that arises from promoter A with or without the added octanucleotide sequence.

Fig. 15

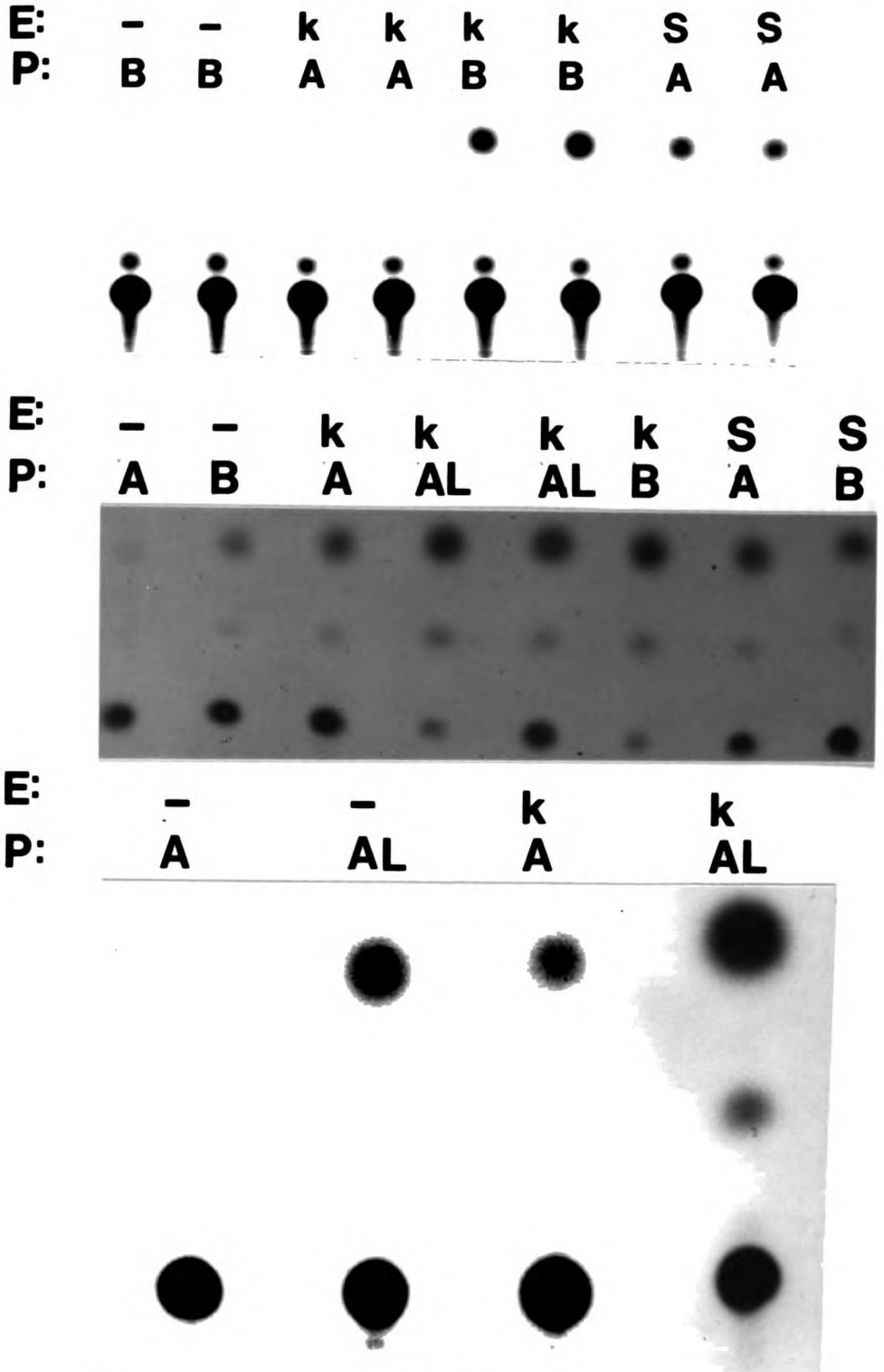


**TABLE 5**
**CAT assay of *tk* promoters with or with out the κ octanucleotide**

| *Promoter* | *Enhancer* | *Myeloma Cells* | *3T3 Cells* | *XC Cells* |
|---|---|---|---|---|
| A | None | 1.0 | 1.0 | |
| B | None | 3.4 | 3.9 | |
| AL | None | 3.4 | 3.6 | |
| A | κ | 4.0 | | 1.0 |
| B | κ | 15.4 | | 2.8 |
| AL | κ | 10.9 | | |
| A | MoMSV | 7.3 | 18.3 | |
| B | MoMSV | 3.8 | 12.1 | |

**Table 5**: Cells were transfected with plasmids that contained the promoters and enhancers indicated, and CAT activity was assayed 48 hr (myeloma cells) or 12 hr (fibroblasts) after transfection. All data were corrected for the trace level of CAT activity present in untransfected cells. The activities are normalized to one construct for each cell line, and therefore, are not comparable between different cell lines. Values given represent the average of 2 to 9 determinations; in most cases, more than one preparation of each plasmid was tested. Data shown for myeloma cells are a composite of results from two different cell lines using vectors that contained or lacked polyoma sequences; analysis of variance confirmed that neither of these variables significantly influenced the results obtained. The polyoma sequences do not support plasmid replication in XC cells (see Figure 17).

with different preparations of the plasmid DNA. To determine whether this difference resulted from the light chain octanucleotide present in promoter B, or from elsewhere within the 39 bp difference between the two promoters, we inserted immediately upstream of promoter A a synthetic 16 bp oligomer containing the octanucleotide sequence (Figure 16): this modification (promoter AL) increased CAT expression to a level equivalent to that from promoter B. However, when a single copy of this synthetic oligonucleotide was inserted in the opposite orientation, that found in the immunoglobulin heavy chain gene promoters, promoter activity was unaffected. Likewise, insertion of this element at +55 had no effect (see below). The octanucleotide therefore appears to be an orientation-dependent element of the *tk* promoter.

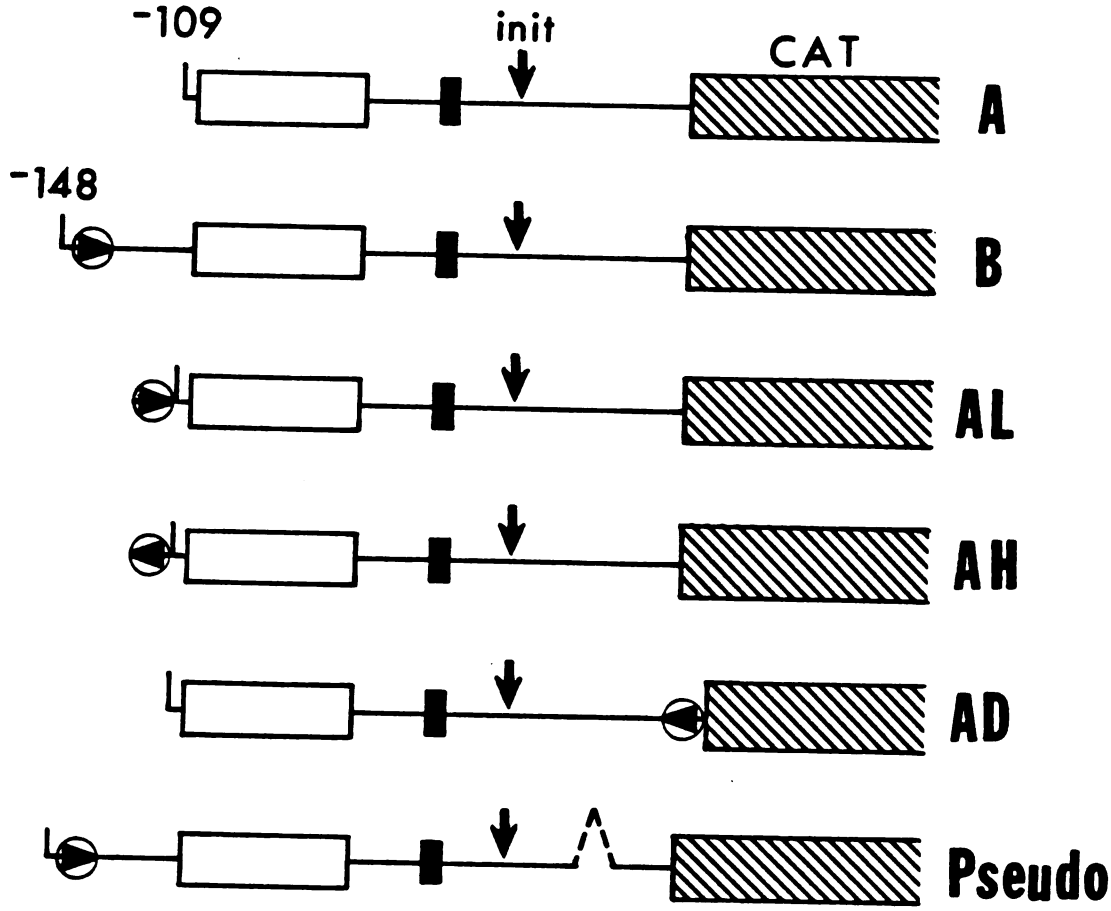
### 3. The κ enhancer acts independently from the octanucleotide

To test the possibility that the κ enhancer might aid in binding of an octanucleotide-specific transcription factor, the effect of the κ enhancer upon *tk* promoters containing or lacking the octanucleotide

**Figure 16**
**Sites of Insertion of Octanucleotide Oligonucleotide in $P_{tk}$**

Locations of the TATA box (closed rectangle) and the *tk* upstream elements (open rectangle) are shown. The light chain octanucleotide (circle) lies at position −138 in promoter B and in the ψ-wild type construct (Pseudo). A double-stranded synthetic oligonucleotide whose sequence (5'TATTTGCATGCA3') included the octanucleotide was modified by the addition of BglII-complimentary ends and ligated into the BamHI site at at the 5' end of promoter A; insertion of this element in either the light chain or heavy chain orientation gave rise to promoters AL and AH, respectively. Promoter AD was produced by inserting the same sequence in the heavy chain orientation into the BglII site at +55. The ψ-wild type gene contains a derivative of promoter fragment B in which residues +16 to +36 have been deleted and been replaced with a 10 bp BamHI linker.

Fig. 16



was examined. A 1.2 kb HindIII-HpaII fragment containing the κ enhancer near its midpoint was inserted upstream of the promoter such that the enhancer was at –0.6kb. When fused to promoter A, the κ enhancer increased CAT expression 3-5 fold above the basal level (Table 5). Fusion of the enhancer fragment to promoter B yielded CAT activity that also averaged 3-5 fold above the level from promoter B alone, which corresponds to 11-15 fold above the basal level of promoter A. Results similar to those with promoter B were obtained with promoter AL. The κ enhancer also increased transcription 3-5 fold from promoter AH (data not shown). Thus, the κ enhancer appears to augment *tk* promoter activity by a fixed proportion regardless of the presence of the octanucleotide, and the combined actions of both these sequence elements linked to a single promoter approximates the algebraic product of the individual effects. These results were duplicated in both cell lines, and in the presence or absence of the polyoma control sequences, implying that the κ enhancer acts at a step in transcription initiation other than binding of a transcription factor to the octanucleotide.

Strikingly different results were obtained when the κ enhancer was replaced by the MoMSV enhancer. In myeloma cells, this element increased CAT expression from promoter A more than 7-fold, but had little or no effect upon the activity of promoter B (Table 5). It appears therefore that the presence of the octanucleotide precludes the promoter's ability to respond to the MoMSV enhancer. There are several possible explanations for this result. One simple explanation is that the additional sequences in the longer promoter block enhancer activity. However, the MoMSV enhancer does work on this promoter when examined in fibroblast cells. In addition, the κ enhancer is active at promoter B, showing that the sequences between –109 and –148 do not block all enhancer activity in myeloma cells. Instead, the octanucleotide-binding factor and the MoMSV enhancer-binding factor may operate upon the activity of another transcription factor; the octanucleotide alone perhaps being sufficient to relieve a rate limitation at that step. Alternatively, the MoMSV enhancer may increase the utilization of the octanucleotide-binding factor. In the absence of the octanucleotide, this factor might still be incorporated inefficiently into the transcription complex, and its incorporation maybe increased when the MoMSV enhancer is linked. However, in the presence of the octanucleotide, this factor binds and is incorporated into a

transcription complex efficiently even in the absence of the MoMSV enhancer. Analysis of the effects of the MoMSV enhancer from downstream of *tk* promoters containing or lacking the octanucleotide are in progress, and will determine if either the sequences or a bound protein between −109 and −148 is causing the observed effect. Distinguishing which transcription factor the MoMSV enhancer activates awaits the development of an *in vitro* reaction in which various factors can be made limiting by lowering their concentration. The MoMSV enhancer can then be directly tested for increasing the activity of the octanucleotide binding factor.

**4. The octanucleotide is not cell type specific**

Others had demonstrated that the κ enhancer was not active in cells that were not of the lymphoid lineage.[118] This suggestion had initially been made for the conserved octanucleotide as well.[40,88] However, the suggestion that factors from other cell types might bind to this sequence led us to test whether this element might function in other cell types.[72,140] The same constructions were therefore introduced into mouse NIH 3T3 cells and rat XC fibroblast lines. As expected, the κ enhancer is not active in these cells (data not shown). However, the octanucleotide had a 3-4 fold stimulatory effect on transcription in the fibroblasts, as shown in Figure 17. Hence, a transcription factor that binds to the octanucleotide must be present in non-lymphoid cells. It is not known if this is the same factor that is detected in myeloma cells.

**5. Analysis of transcription**

The 5′ termini of the CAT transcripts produced by the *tk* promoters in these experiments were determined by primer extension assays of unfractionated RNA from the transfected cells. Figure 18, panel A, shows an autoradiogram of the elongation products from myeloma cell RNA after fractionation by gel electrophoresis. For each of the plasmids tested, the vast majority of transcripts initiated within a 4 bp region spanning residues −1 through +3, a pattern of initiation that is characteristic of the *tk* promoter in a variety of cell types and in cell-free transcription (e.g., see Figures 11 and 13).[30,31,72,97] Thus,

**Figure 17**
**CAT Assays Analysis of Octanucleotide Promoter Element Function in XC Cells**

The figure shows a representative CAT assay of an analysis of promoter A and promoter B activity in XC cells. Duplicate analyses of each construction are presented. The relative activity of these constructions is shown below the figure. All constructions contained the κ enhancer, which is not active in these cell (data not shown), and the promoter indicated above the figure. The first four lanes represent the CAT activity from constructions that do not contain the Polyomavirus origin of replication, while the next four lanes are analyses of constructions that contain this region.

Fig. 17

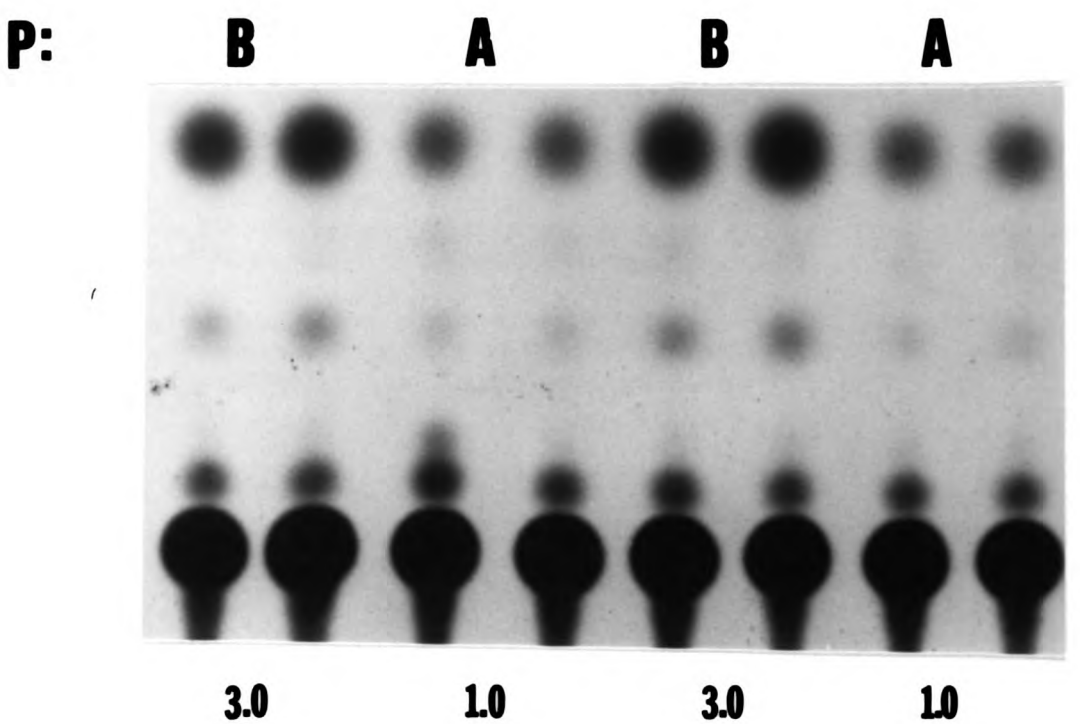


**Figure 18**

**Primer Extension Analysis of Octanucleotide Element Effect on the *tk* Promoter in Myeloma Cells**

Primer extension analysis of the unfractionated RNA isolated from transiently transfected myeloma cells is shown. Cells were transfected with CAT constructs containing the indicated promoters and enhancers. The *tk* promoter gives rise to primer extension products of 90, 89, and 87 bases. Panel **A** shows the accuracy of initiation from the *tk* promoter in these cells. In cells transfected with each of four plasmids, initiation is confined to a discrete 4 bp region ("*"). The constructions analyzed in this panel are the same as shown in lanes 1-4 of panel **B**. Panel **B** shows the relative transcript abundance in cells transfected with the same four plasmids. Lane 5 shows the background abundance of cross-hybridizing transcripts in myeloma cells that were mock-transfected. Lanes 7-10 show the relative abundance of transcripts from promoter A alone, with the κ enhancer, or with the enhancer and the synthetic oligonucleotide that contains a copy of the octanucleotide. Lanes 11 and 12 show cotransfections of the test plasmid with 0.25× of a second plasmid that contained the ψ-wild type promoter fragment and the κ enhancer; the latter plasmid produced slightly truncated transcripts which provide an internal standard for quantifications. Lengths (in nucleotides) of the molecular weight markers are indicated.

- 527
- 309
- 201
- 110
- 67
*
A
A+Kappa
B+Kappa
B+MSV
Sham
MW
-92 nt
- 76 nt
- 67 nt
1 2 3 4 5 6
7 8 9 10
11 12
+ Kappa
A
AL
AH
AL
A
A
AL
*
Pseudo

all of these promoter constructs directed accurate transcriptional initiation. In addition, the relative abundance of transcripts that initiated correctly from the various constructs, as determined by scintillation counting, paralleled closely the observed differences in CAT enzyme activity. Because identical sequences are transcribed from each plasmid, the relative levels of accumulated transcripts or of enzyme activity most probably reflect differences in the rate of initiation. We therefore conclude that promoters containing the light chain octanucleotide (promoter B and AL) were approximately 3-fold more active than promoter A. The activities of all three promoters increased approximately 4-fold when linked to the κ enhancer; in constructs containing both the κ enhancer and the light chain octanucleotide, promoter activity exceeded the basal value by more than an order of magnitude. Analysis of the transcripts also confirmed that the octanucleotide had no effect when placed in the inverted orientation immediately upstream of promoter A (promoter AH, lane 10). or when inserted 55 bp downstream from the initiation site (promoter AD).

**6. The octanucleotide increases transcription from a *tk* promoter devoid of all its upstream elements**

The octanucleotide is a functional promoter element of the HSV *tk* promoter. This promoter contains three additional identified upstream elements; the κ promoter is not known to contain any promoter elements other than the octanucleotide. The octanucleotide may therefore be able to function alone in the *tk* promoter as well, or its activity may be dependent on the other *tk* promoter elements in this context.

The sequences sufficient for optimal *tk* promoter activity had been reported previously to reside within 105 bp upstream of the start site. A 5′ deletion to −52, termed promoter C, displays very low activity. Signals from this promoter were barely detectable in either of the myeloma cell lines as shown in Figure 19. This promoter retains a TATA box, but lacks upstream promoter elements. A synthetic oligonucleotide that contained a copy of the octanucleotide was tested for restoration of competent transcriptional ability to this deletion mutation.

**Figure 19**
**CAT Assay of $P_{\alpha}$ Deletion and Derivatives in Myeloma Cells**

A representative CAT assay of promoter C activity in myeloma cells is shown. The promoter and enhancer that are present in each plasmid is indicated. The promoter and enhancer used in each assay is indicated (P=promoter, E=enhancer). Lanes marked "κ" contained the κ enhancer, while lanes marked "–" contained no enhancer at the 5′ end of the gene. All constructions tested contained the polyoma viral origin and enhancer region at the 3′ end of the gene.

Fig.19

The synthetic oligonucleotide was fused to the BamHI site at the –52 deletion endpoint in either orientation. The kappa enhancer was then linked to this set of promoters. The resulting distance between the conserved octanucleotide and the cap site of the *tk* promoter was 63 bp, which is within the normal range that it is found in the immunoglobulin promoters.[113] Relative to promoter C, 40-50 fold more CAT activity was observed with the octanucleotide in the light chain orientation, promoter CL (Table 6 and Figure 19); insertion of the element in the opposite orientation, promoter CH, yielded 7-14 fold more CAT activity than produced by promoter C. The kappa enhancer element is active on all of these promoters. Promoter C alone was enhanced 7 fold, while promoter CH was enhanced 13 fold; the significance of these apparent differences is questionable, however, since absolute activities were very low.

Analysis of the transcripts by the primer extension assay revealed a heterogeneous family of start sites rather than the strong, discrete sites expected. Utilization of some of these sites was increased when

**TABLE 6**
**Relative CAT Enzyme Activity of Promoter C Derivatives**

| *Promoter* | *Enhancer* | *Myeloma Cells* |
|---|---|---|
| C | None | 1 |
| C | κ | 7 |
| CL | None | 40 |
| CL | κ | 360 |
| CH | None | 7 |
| CH | κ | 90 |
| A | None | 150 |
| A | κ | 375 |

**Table 6**: Promoter C, a derivative of $P_{tk}$, lacks sequences upstream of –52, and therefore is lacking all known upstream promoter elements. The basal activity of this promoter has been assigned an arbitrary value of one that corresponds to an absolute activity of .26% conversion; the relative activity of promoter A is then 150. Cells were transfected with plasmids that contained the promoter and enhancer indicated, and CAT activity was assayed 48 hr after transfection. All data were corrected for the trace levels of CAT activity present in untransfected cells. The activity of these constructions was not tested in non-myeloma cell lines.

the octanucleotide was included in the promoter; see Figure 20, lanes 2 and 3 for example. Two sites that exhibit increased utilization when the octanucleotide is present are located downstream of the octanucleotide, at –4 and –50 relative to the *tk* cap site. These sites were also seen in experiments in which the GRE was fused to this same deletion, and the –4 site was induced by the GRE upon its activation (see Figure 12). In the case of the GRE, additional sites that were located upstream of the enhancer were also induced, and this was attributed to the fact that the GRE is an enhancer element, which can activate transcription from linked promoters both upstream and downstream. The octanucleotide had not functioned when placed in a downstream position of a fully functional *tk* promoter. However, when the octanucleotide was used to replace the upstream elements of the *tk* promoter, it was also able to increase the utilization of transcription initiation sites that were located upstream. There were many such sites, as shown in Figure 20. They were either in vector sequences or in the κ enhancer fragment if it was present. It should also be noted that the ψ-wild type gene is included as an internal control; the signal from this wild type promoter is much stronger than any of the weak start sites used in the reconstructed promoter although the total transcript abundance may be greater from the reconstructed promoters. The ψ-wild type promoter alone does not create a heterogeneous family of start sites (see Figure 18).

The octanucleotide is a functional promoter element when it is linked to the *tk* TATA box alone. In this context it functions as a bidirectional activator of transcription. Transcriptional activity is higher when the octanucleotide is placed in the light chain orientation than when placed in the heavy chain orientation, although the basis for this difference is not known.

## 7. Conclusions

These findings indicate that the immunoglobulin promoter octanucleotide is a functional promoter element of the HSV *tk* promoter. It can function from positions other than 70±10 bp upstream from the initiation site, as it is situated in the immunoglobulin genes. Unlike the action of an enhancer, this quantitative effect is lost when the octanucleotide is inverted (promoter AH) or placed downstream from the initiation site (promoter AD) of a wild type promoter. It retains promoter activity in the absence of the

**Figure 20**
**Primer Extension Analysis of $P_{tk}$ Deletions and Derivatives in Myeloma Cells**

Myeloma cells were transfected with CAT constructs containing the indicated promoters. The wild type *tk* promoter normally gives rise to primer extension products of 90, 89, and 87 bases: the severe deletion of promoter C renders transcription initiation much more inaccurate. All lanes exhibit extension products that arise from promoter C plus the κ enhancer. In addition, the plasmid analyzed in lane c contains the light chain octanucleotide element. Utilization of many initiation sites is increased in this promoter, promoter CL. Some of the predominant sites are indicated with arrows. In all cases, the ψ-wild type gene was cotransfected to provide an internal standard; the transcript arising from this promoter is the dark band at –76.

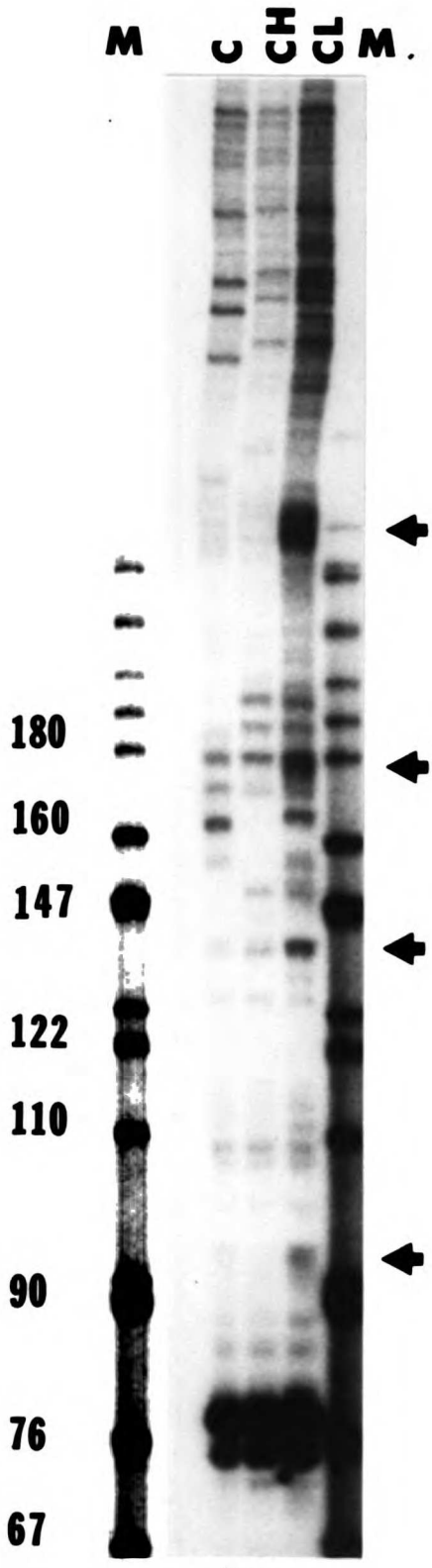



Fig. 20

other upstream elements of the *tk* promoter, although the start sites used in this promoter are disperse. The octanucleotide does not appear to play a primary role in specifying the exact site of initiation, as shifts in the position of this element (to position −138 in promoter B, −120 in promoter AL, or +55 in promoter AD) did not affect the fidelity of initiation.

The octanucleotide element had been proposed to account, in part, for the tissue-specificity of immunoglobulin gene expression.[37,40,53,88] The present results indicate, however, that the octanucleotide can also function in at least some non-lymphoid cell types. These results are compatible with the observation that proteins capable of binding the octanucleotide are present in both lymphoid and non-lymphoid cells, and with evidence that HeLa cell nuclear extracts contain factors that bind near this region of the *tk* promoter.[72,140] Indeed, the report that the octanucleotide may have a similar role in the promoters of Xenopus snRNA genes supports the notion that it is a phylogenetically ancient and highly-conserved genetic signal that may influence the activity of a variety of genes in diverse species and cell types.[91]

The results of this analysis closely parallel the results in the GRE study. Neither enhancer could be shown to act at any specific promoter element. Initially, it seemed likely that the κ enhancer might activate binding of a factor at the octanucleotide to aid formation of a stable complex, since the immunoglobulin heavy chain promoter in class-switch mutants that deleted the enhancer was still transcriptionally active. However, in recent experiments that allowed defined deletions from plasmids, the results indicate that the heavy chain enhancer is in fact not dispensable, suggesting that in earlier studies a second enhancer located downstream of the heavy chain gene may have been brought into proximity of the promoter by the class switch deletion (R. Grosschedl, personal communication). This recent result is consistent with the results presented in this chapter, in which the κ enhancer does not appear to act by formation of a stable complex at the octanucleotide.

In both myeloma cells and fibroblasts, a combination of the octanucleotide with the MoMSV enhancer is less efficacious than the enhancer alone (Table 5). The MoMSV enhancer activity is not at

all detectable upon a promoter containing the octanucleotide when analyzed in myeloma cells, and only a slight effect is seen in 3T3 cells. This implies that promoters containing the octanucleotide may be less responsive to certain types of enhancers than are promoters lacking the octanucleotide. Since there is also a spacing change in these constructions, a more telling experiment will be to examine the activity of the MoMSV enhancer upon these promoters from position downstream of the cap site. In marked contrast, the actions of the κ enhancer and the octanucleotide augment one another in myeloma cells, producing a multiplicative effect on promoter function. This favorable interaction between the κ enhancer and promoter octanucleotide may have special importance for the immunoglobulin genes, which are encoded as widely-separated gene segments in germline DNA. During the course of B-cell differentiation, sequences encompassing the promoter and 5′ exons of an immunoglobulin gene become fused, through a process of DNA rearrangement, to genomic loci containing the enhancer and downstream coding the regions of the gene. Conceivably, such rearrangements may serve not only to juxtapose the necessary coding sequences, but also to bring together two dissimilar and independent regulatory elements whose actions then magnify one another, and so contribute to the enormous transcriptional activity that is characteristic of this family of genes.

# V. Summary and Discussion

## 1. Summary of Results

The goal of the experiments presented in this thesis was to probe genetically the mechanisms of enhancer function. The results of a mutational analysis of one enhancer, the MTV GRE, showed that there are specific sequences within the enhancer that are necessary for the GRE to be fully functional. These sequences correspond to a conserved sequence element that is necessary for the binding of this enhancer's *trans*-acting activator, the glucocorticoid receptor protein. The analysis suggested that this protein is the only sequence-specific DNA binding protein that is required for enhancer activity, at least under the conditions tested.

A mutational analysis of the linked promoter revealed a different result. Mutations of specific sequence elements of the HSV *tk* promoter upstream of the TATA box region do not alter GRE activity. In my opinion, therefore, the GRE acts to increase the rate of a step in transcription initiation that is subsequent to the binding of upstream sequence-specific transcription factors, at least to any of the sequences tested. It may alter the activity of a transcription factor that either binds at or near the cap site, or does not bind directly to DNA, such as the transient transcription factors. The slight reduction in GRE activity that is seen when it is linked to promoters containing mutations near the cap site suggests that the GRE might act at a step of initiation that occurs in this region. This region may be a binding site for a sequence-specific transcription factor that is required only for positive activation. An alternate but unlikely possibility is that the GRE facilitates the utilization of a upstream sequence-specific factor that can still be incorporated into the initiation complex when the usual binding site has been removed.

An analysis of the two conserved sequence elements of the mouse κ gene revealed that the conserved octanucleotide that is located at $-70\pm10$ from the transcription initiation site of all vertebrate light chain immunoglobulin genes is a promoter element that is not cell-type specific. This sequence is found naturally in the promoter of the HSV *tk* gene, and contributes to the overall expression of this gene. The fact that this sequence motif in the HSV *tk* promoter had not been seen prior to this study may be due to the context in which the promoter mutations were analyzed. The κ enhancer can increase transcription

from the linked HSV *tk* promoter independently from this octanucleotide. In contrast, the MoMSV enhancer is not able to increase transcription from a linked *tk* promoter that contains the octanucleotide when analyzed in myeloma cells, suggesting either that the octanucleotide sequence or its bound protein block the activity of the MoMSV enhancer, or that this enhancer provides a function that is superseded by octanucleotide function in this cell type. These two functions are not necessarily identical. Experiments in progress will place the enhancer downstream of the promoter, thus eliminating the possibility that the observed activity results from the spacing change between the two promoters.

**2. Regulation of initiation**

When considering how an enhancer might increase the rate of transcription initiation from a linked promoter, it is useful to determine what steps in the initiation reaction are likely to be regulated. In prokaryotes, there are two experimentally distinguishable events that occur sequentially preceding transcriptional initiation. Each may be a composite of multiple steps. RNA polymerase first binds to the promoter in a reversible reaction: the promoter-polymerase complex then undergoes an isomerization to an open complex that is not readily reversible.[11,94] Initiation ensues immediately in the presence of ribonucleotide triphosphates. Regulators of transcription in prokaryotes have been shown to affect RNA polymerase binding, complex isomerization, or both of these reactions.

Regulation of the concentration or activity of σ factors provides another level of control.[123] σ is a subunit of RNA polymerase that is required for initiation, but leaves the RNA polymerase after elongation has begun.[10] E. coli transcription uses predominantly one σ factor, σ70. However, it has another σ factor that only transcribes the genes in the heat shock network. This factor is activated only during heat shock, and hence its promoters function only when needed.[54] B. subtilis has several different σ factors that are specific for the transcription of various sets of genes that are needed at different times in the life cycle of the organism.[47]

Eukaryotic promoters are more complex than prokaryotic, and the array of additional factors required for initiation suggests that there might be more steps at which regulation could occur. *In vitro*,

three events have been defined preceding initiation from the adenovirus major late promoter; each of these undoubtedly consists of multiple steps (Figure 21). Factors and RNA polymerase first interact with the promoter to form a stable, or template-committed complex.[39, 61, 127] This can be broken down into at least two steps: binding of factors followed by binding of RNA polymerase.[127] Formation of the template-committed complex is followed by formation of an activated, or rapid-start, complex. This requires the addition of other factors. This complex may be similar to the open complex of prokaryotic transcription, since addition of nucleotides to the rapid-start complex leads immediately to transcription initiation.[61]

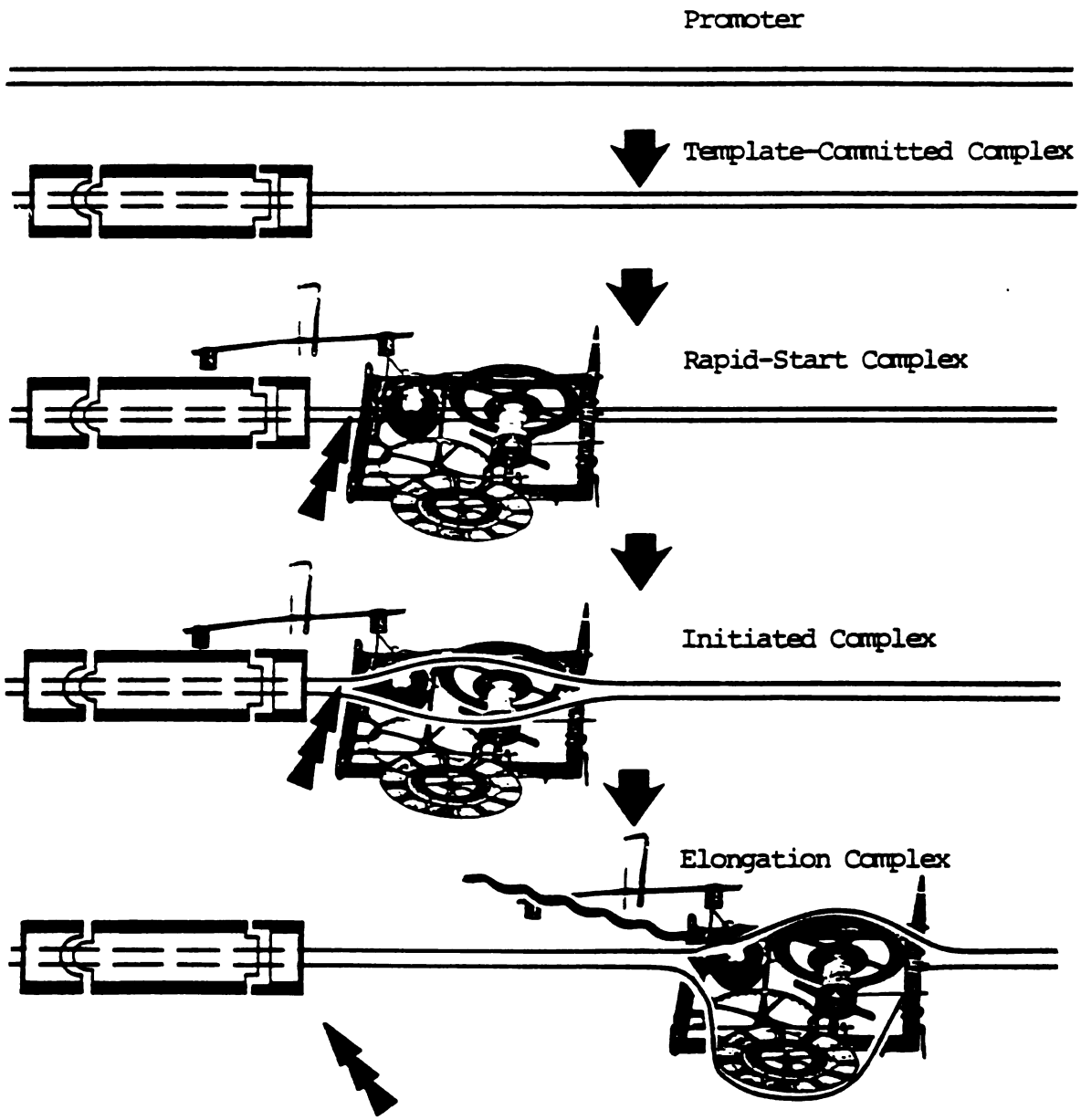
In addition to stable factors, the template-committed complex sequesters a factor that is limiting in an *in vitro* reaction, but can be released upon initiation to form a new complex and start a new round of initiation. Safer et al. have suggested one mechanism that might be used predominantly in the regulation of eukaryotic RNA polymerase II transcription is the regulation of the association of this transient transcription factor with the various promoters in a cell.[127] It is likely that there are several of these factors that have a specificity for given classes of promoters, just as in the case of the σ factors of B. subtilis. One might imagine stable complexes ready to transcribe at every promoter, waiting for the recycling factor to join the complex to complete the series of events which leads to initiation.

Enhancers, then, might increase the rate of formation of the template-committed complex, by increasing the rate at which either the stable factors needed for this complex, or RNA polymerase itself, is bound to the DNA. Alternately, they could increase the concentration or activity of transient factors. Lastly, they might stimulate formation of the rapid start complex, possibly by affecting the template. Clearly, our understanding of these events is primitive. For example, it is difficult to reconcile the apparent flexibility in the organization and combinations of the modular *cis*-acting promoter elements with the specificity of the initiation reaction. Thus, the HSV *tk* promoter contains at least five binding sites for four distinct factors,[72] whereas the SV40 promoter contains six binding sites for one of the factors used by the *tk* promoter, but no other apparent binding sites.[29] Are these factor/site combinations

**Figure 21**
**Events That Precede Transcription Initiation** *In Vitro*

A schematic representation of the formation of a template-committed transcription complex, a rapid start complex, and an elongation complex is shown. The open shapes represent stable transcription factors that bind to the upstream promoter elements; the interaction shown between the various factors are hypothetical. The triangular shapes represent transient factors; while RNA polymerase is represented as a large machine that contacts both the stable factors and the transient factors. Addition of nucleotides to the rapid-start complex leads to immediate transcription initiation. Hence, all steps that precede initiation must have been accomplished at this stage. This is indicated by the *open* configuration at the cap site; there is no evidence that the cap site region is really in this configuration prior to the actual initiation event. The nascent RNA transcript is represented in the elongation complex as a wavy line.

Figure 21


Promoter
Template-Committed Complex
Rapid-Start Complex
Initiated Complex
Elongation Complex

interchangable? Can functional promoters be created simply by combining various elements?[118] An important underlying question is whether the factors function additively, or whether different factors perform distinct functions. It is apparent that if they all perform different functions, then not all of these functions are required at all promoters. If they are additive, then the multitude and complexity of the upstream elements is difficult to rationalize.

Transcription initiation *in vitro* appears to depend on both transient and stable factors. Regulation of the concentration of active transient factor *in vivo* therefore might alter the rates of transcription from promoters that require different transient factors. Alternately, promoters may bind to the same transient factors with varying affinities, and this may be governed in part by what set of stable factors are present. Enhancers may act to increase the activity of either transient or stable factors.

### 3. Enhancers and initiation

The two enhancers that have been examined in this thesis did not exhibit a preferential site of action within the linked promoters that were examined. Assuming that formation of template-committed complexes involves sequence-specific binding of stable transcription factors to the promoter, it appears that both the MTV GRE and the mouse κ enhancer operate upon a subsequent event, such as the activity of the transient factors or the formation of the rapid-start complex. By necessity, the GRE must act upon a transient or reversible event, since its effects require the continuous presence of hormone; by extension, all inducible enhancers may act upon such a common step in initiation.

In principle, an enhancer could function as a developmental switch by facilitating the binding or activity of a stable transcription factor. There is certainly no reason to expect that it will affect the same step of initiation as an inducible enhancer, such as the activity of a transient factor. Interestingly, the mouse κ enhancer, which seems to effect a developmental switch, may be similar to the GRE in its actions. The only upstream element identified within the κ promoter is the octanucleotide motif,[113] and the κ enhancer stimulates the function of *tk* promoter derivatives with or without this octanucleotide. Consistent with this view that the κ enhancer does not require any defined upstream sequence element is

its activity upon a *tk* promoter mutation lacking all elements upstream of −52. Thus, we suggest that both enhancers affect initiation at some step after the formation of stable transcription complexes. While the results with the κ enhancer are contrary to our initial hypothesis, recent evidence in fact suggests that the immunoglobulin heavy chain enhancer is required continuously, consistent with our observations ( R. Grosschedl, personal communication).

The possibility that the GRE or the κ enhancer acts directly on the binding or activity of RNA polymerase is not ruled out by my experiments. *In vitro*, RNA polymerase has not been shown to bind in a sequence-specific manner, but might recognize some aspect of the stable complex. However, enhancer activity on RNA polymerase would not allow much flexibility in regulated networks. All promoters require RNA polymerase, and hence all promoters should respond to enhancers that affect polymerase action. Subtle changes in transcription could not be controlled if regulation occurred upon a global activity such as polymerase. We therefore suggest that the enhancers that were examined in this study either act upon transient transcription factors or increase the rate of formation of the rapid-start complex.

Enhancers other than those examined here might act at any step of initiation and might increase the activity of any transcription factor. Evidence that the MoMSV enhancer increases the activity of a different factor than the κ enhancer was presented in chapter 4. Unlike the κ enhancer, the MoMSV $S_a$ activity was not observed in myeloma cells on a promoter that contains the octanucleotide promoter element. It behaves as though these two transcriptional regulatory elements perform similar actions upon the initiation reaction, and if one is present, the other one does not work. Since the MoMSV $S_a$ enhancer was functional on this promoter in fibroblasts, the octanucleotide sequence alone does not intrinsically block non-immunoglobulin enhancer action. A difference in the rate-limiting step at this promoter between the two cell types might explain the results; alternately, the sequences or bound protein at the octanucleotide might block enhancer activity. These models are being tested by determining the activity of the MoMSV enhancer from a position downstream of this promoter. Preliminary results suggest that the enhancer does not work from a position downstream of the gene; if correct, then the activities of the

MoMSV enhancer and the octanucleotide are mutually exclusive.

**4. Enhancers vs. Upstream Elements**

Experiments presented in this thesis demonstrate that the κ octanucleotide is a promoter element, is somewhat distance flexible, and does not work from a position downstream of a fully functional promoter. However, when this same sequence element is linked to a very weak promoter, the –52 deletion of the *tk* promoter, then it is seen to increase the utilization of transcription initiation sites both upstream and downstream of itself. Might one then assume that this promoter element has become an enhancer? It is possible that promoter elements may play a role in transcriptional activation from larger distances if there is not a fully functional promoter nearby. It should be noted that the relative transcript abundance from any of these increased sites is still much lower than that from the wild type promoter.

Most of the sequence elements that have been described in eukaryotic promoters have some degree of freedom in orientation, which is often described as characteristic of enhancer elements. Perhaps they share other properties that have not been looked for yet. Promoter elements are binding sites for factors that are necessary for maximal transcription from the promoter. These factors presumably interact with one another to form the stable, template-committed complex. If a factor binds to a promoter element and does not find other stable factors nearby, might it not look up or down the DNA for them? Although this is not a traditional approach to describing promoter element function, the possibility that these sequences can exhibit some properties of enhancer elements when placed in the context of a sub-optimal promoter has not been investigated. The experiments presented herein are consistent with this model.

Many enhancers have been shown to contain more than one independent, functional sequence motif. For example, the immunoglobulin heavy chain enhancer contains a "core" enhancer consensus sequence, one copy of the octanucleotide that is also in its promoter, and a third sequence motif to which factors have been shown to be bound *in vivo*.[15,35] The MoMSV enhancer contains two separable enhancer activities, a GRE termed $S_g$ and a constitutive enhancer termed $S_a$.[21,103] The SV40 enhancer contains three separable enhancer functions, and the polyoma enhancer contains two.[62,64,66,150] Rever-

tants of enhancer mutations often are found to consist of simple changes in one of the sequence motifs followed by duplication of a fragment containing the motif.[65, 143] It is possible that duplications of promoter elements would also form functional enhancers, but this has not been directly tested. The inverse experiment has been done, however, and enhancer sequences have not been shown to function as promoter elements. This observation is complicated by the recent identification of many cellular enhancer elements that are interdigitated within the upstream control region such that one cannot be tested alone.

## 5. Conclusions

There may be an overlap in the factor/sequence element combinations that can act as enhancers and as promoter elements. However, I believe that each function is distinct. The experiments in this thesis asked how enhancers act to increase transcription initiation. We propose that the GRE activates a pre-existing stable initiation complex to increase the rate of transcription initiation. It may act on steps that occur at or near the cap site, such as open complex formation or sequestering transient transcription factors. A site in the *tk* promoter that may be required for positive activation is located downstream of the TATA box. The κ enhancer also appears to act at a step subsequent to stable complex formation. This enhancer may act at the same step as the GRE. The two enhancers could not be distinguished in these experiments with respect to the affected step of initiation.

Experiments are in progress to determine more precisely what transcription initiation step the GRE affects. *In vitro* glucocorticoid-regulated transcription initiation will help to determine at which step of initiation the GRE is acting. Other groups have been successful at breaking initiation into steps *in vitro*, but regulation by glucocorticoids has not yet been accomplished.[61, 127] Some advances in achieving enhancement *in vitro* have been reported recently.[131, 132, 138, 139, 158] These experiments have been done on constitutive enhancer elements, not inducible enhancers such as the GRE.

Another approach that may help to determine on which step in initiation the GRE is acting is to determine what cellular factors interact with the glucocorticoid receptor protein. This will be possible because the receptor has been recently cloned and expressed in both prokaryotic and eukaryotic

systems.[102, 156] In the future, the same approaches may be used for other enhancers as well. A continuation of the genetic approach will also be useful in continuing to understand enhancer action. Different enhancer and promoter combinations can be examined for activity to see if any classes of enhancers can be distinguished. Future experiments may reveal enhancers that are members of classes other than the GRE and the κ enhancer as determined by their differential ability to activate promoters. A class of enhancers that increases the rate of formation of the stable transcription complexes may be described in future studies, although it was not seen in this study.

# Appendix A: Methods

## 1. Cell lines and cell culture

Rat XC tk⁻ cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% defined calf serum. Cells were passed every two days, such that they were grown continuously in mid-log phase. Fresh cells were thawed from storage in liquid nitrogen every four weeks. Rat HTC 6.10.2 G10 cells were carried essentially the same way, except they were passed every three days. Mouse myeloma cell lines P3X63Ag8 and SP2/0 were grown in F12 medium supplemented with 10% fetal calf serum.

## 2. Transfections

XC cells (rat fibroblasts) and G10 cells (hepatoma cells expressing cloned glucocorticoid receptor) were transfected with 1-5μg of test DNA plus 0.5-2.5μg of an internal control DNA per $4\times10^6$ cells, using calcium-phosphate coprecipitation (see below). Cells were transfected in duplicate, and one plate was then treated with 0.1μM dexamethasone, a synthetic glucocorticoid hormone. After 10-24 hr, cells were harvested and either protein or RNA was isolated. CAT assays were performed on 50-100 μg of total protein, which corresponds to approximately $2\text{-}4\times10^6$ cells. The transfection efficiency on the duplicate plates was monitored by using an internal control plasmid, RSV-β-galactosidase. Analyses of β-galactosidase activity were performed in parallel and the results were used to normalize the CAT data. Primer extension analyses were performed on 50-100μg of unfractionated RNA, which corresponds to $5\text{-}10\times10^7$ cells. In this case, RSV-CAT was used as an internal control in the transfection. The transcript from this gene was recognized by the same primer as the test gene, but was 20 bases shorter. Both internal controls were not responsive to glucocorticoids.

The XC and G10 cells were transfected using the calcium-phosphate coprecipitation method described by Wigler et al.[157] Cells were plated at $10^6$ cells per 100mm dish 24 hr prior to transfection. They were fed with fresh medium 30-60 min prior to the transfection. Upon addition of the DNA:calcium phosphate coprecipitate to the cells, 0.25 ml of 4mM chloroquine in PBS was added to the cells for a final concentration of 100μM chloroquine.[82] Cells were incubated for 4 hr. The medium was

then removed by aspiration, the attached cells were rinsed twice with 10ml PBS, and subjected to glycerol shock for 2 min in 2.5ml of medium containing 20% glycerol.[108] The glycerol was diluted by the addition of 7.5ml of PBS. This was removed from the plates. They were then refed with media ± 0.1μM dexamethasone. Cells were harvested 12-24 hr later, and either protein cell extracts or total RNA was prepared.

Both myeloma cell lines used in this study were transfected using the DEAE-dextran method described by Banerji et al.[5] Cells were grown to 10-20% confluency on 100mM tissue culture plates. They were rinsed twice with Tris-buffered saline (TBS), and then exposed to 1μg plasmid DNA in 0.6 ml of TBS containing 0.5 mg/ml DEAE-dextran for 30 min at room temperature. After removal of the TBS/DNA/DEAE-dextran, the cells were treated with 100μM chloroquine in media for 3.5 hr. Cells were then refed with fresh media and harvested 48 hr later. The β-galactosidase assay was not performed in these assays. For the RNA analysis, an internal deletion of the non-coding leader sequence of the *tk* gene served as an internal control.

### 3. Preparation of cell extracts

Cells were removed from the plate using PBS that contained 10mM Tris (7.5) and 2mM EDTA. They were collected by centrifugation at 1500-2500rpm for 5 min and the pellets were frozen at −70°C until the day of the assay. Cells were lysed by adding 5 volumes of 0.25M Tris(7.5) and sonicating with a microtip for five seconds. Particulate material was then removed by a full-speed centrifugation in an Eppendorf centrifuge for 5 min. The supernatant was then transferred to a new tube containing 0.25 volumes 80% glycerol, for a final concentration of 16% glycerol (this stabilizes β-galactosidase during freezing and thawing, but is unnecessary for CAT stability). Aliquots of extracts were always assayed on the day that they were prepared; the remainder was stored at −70°C for re-assay if necessary.

**4. Isolation of RNA from transfected cells**

RNA was prepared by the guanidinium thiocyanate lysis procedure described by Chirgwin et al.[14] Cells were isolated by centrifugation, and were then lysed in 3ml of 5M guanidinium thiocyanate, 50mM β-mercaptoethanol, 0.5% Na-S-lauryl sarcosine, and 25mM sodium citrate (pH 7.0). This was then gently layered on 2ml of 5.7M CsCl, 0.1M $Na_2EDTA$, and was centrifuged at 36,000rpm in an SW50.1 rotor for 16 hr. The RNA pellet was resuspended in 300µl of sterile water to which 30µl of 3M NaOAc(pH 7.0) was added, phenol:chloroform extracted once, and precipitated with 3 volumes of ethanol. Concentration was determined by absorbance at 260nm.

**5. CAT assays and β-galactosidase assays**

CAT assays were performed essentially as described in Gorman et al.[49] However, the amount of $^{14}C$–chloramphenicol that was added to the reaction was 0.2µCi. Reactions proceeded for 1-18 hr, depending on the experiment, cell line, and previous experience with the constructs being tested. Reactions in which >50% of the reactant was converted to the acetylated product were considered out of the linear range and were repeated for shorter times.

β-galactosidase assays were performed as described by An et al.[3] using 75-100µg of protein in 3-24 hr reactions, which were terminated when a yellow color was visible; the extent of the reaction was determined by measurement of the absorbance at 420nm. CAT activities were normalized to the β-galactosidase activities.

**6. Primer extension analysis of RNA**

Primer extensions were performed on 50-100µg of unfractionated RNA. T4 polynucleotide kinase was used to end-label 40ng of a single-stranded synthetic primer. The primer was separated from the unincorporated label on a 5% denaturing TBE polyacrylamide gel. The 27 base primer co-migrated with the Bromphenol blue in this gel system. The primer, a kind gift from Daryl Granner, was homologous to sequences from −5 to −31 from the CAT translation initiation site; the *tk*-CAT fusion used in this thesis

yielded extension products of 86-90 bases. The RSVCAT internal control that was included in most experiments generated an extension product of 68 bases.

End-labeled primer (0.134 ng) was added to the RNA in a buffer that contained 250mM KCl, 10mM Tris(8.0), and 10mM EDTA, in a total volume of 30µl. Hybidization was then allowed to occur at 62°C for 1-6 hr. Time course analyses revealed that the hybridization was complete within 1 hr. The tubes were then cooled to room temperature, and the extension was initiated by the addition of 70µl of reverse transcriptase mix (10mM $MgCl_2$, 10mM Tris(7.5), 5mM DTT, 100µg/ml actinomycin D, 100 units/ml of reverse transcriptase obtained from Life Sciences). Extension proceeded at 37°C for 2-4 hr, and was terminated by the addition of 300µl of cold ethanol. The products were separated on a 5% denaturing acrylamide gel and detected by autoradiography. In some cases, the bands were cut out of the gel and counted; in others the relative levels of transcripts was determined by scanning densitometry.

### 7. Footprinting

Footprinting experiments were performed using purified, activated glucocorticoid receptor that was a generous gift from J.-A. Gustafsson and his colleagues.[160, 161] DNA was end-labeled using either T4 polynucleotide kinase or E. coli Klenow fragment. The end-labeled fragment was isolated by preparative gel electophoresis on either acrylamide or agarose gels. The purified fragment (1-10ng) was incubated with increasing amounts of the receptor protein in 20mM Tris(7.5), 1mM EDTA, 20% glycerol, 1µM dexamethasone, 1.5mM $MgCl_2$, 250 µg/ml insulin, and 20mM DTT. After the binding reaction had proceeded for 15 min at room temperature, DNAase I was added to a concentration of 70 µg/ml, or neocarzinostatin was added to 150 µg/ml. Mixtures were incubated at 25°C for 1 or 5 min respectively, and digestions were terminated in 10mM EDTA, 0.1% SDS, and 100 µg/ml Proteinase K. After incubation for 20 min at 37°C, the nucleic acids were extracted once with phenol:chloroform, brought to final concentration of 0.3M NaOAc(pH 7.0) and precipitated with three volumes of ethanol. Samples were analyzed by electrophoresis in 5-8% denaturing acrylamide gels followed by autoradiography. Footprinting reactions were co-electrophoresed with Maxam-Gilbert sequencing reactions to provide precise

alignment of protected sequences.

**8. Recombinant plasmids and plasmid constructions**

All procedures were performed essentially as described by Maniatis et al.[86] The starting plasmid for all of the CAT constructs is CAT3M.[78] A restriction map of this plasmid is shown in Figure 22, panel A. The XbaI site of CAT3M was converted to a SalI site by filling in the 5′ overhang with Klenow, and reclosing the vector onto a SalI linker, as shown in Figure 22. This manipulation created the acceptor vector, CAT3MS, into which all the *tk* promoter-enhancer fragments were introduced (Figure 22).

In some vectors, a second enhancer was inserted into the BamHI site of CAT3MS. This site is downstream of the CAT coding sequences and the SV40 transcription termination signals. Insertion of the MoMSV enhancer at this site created CAT3MSV.3 and CAT3MSV.9; the number at the end refers to the length of the enhancer fragment that was inserted, in kb, see Figure 23.[25] Insertion of the polyoma origin and enhancer region created the vector CAT3MSP.[121]

The *tk* promoter and gene was located between the BamHI and HindIII sites of the pBR322-based vector pRI-, in which the EcoRI site had been destroyed and the sequences between the NruI site and the PvuII site had been deleted. The BamHI site was at the 5′ end of the gene, either 109 or 148 bp upstream of the cap site, and was used as an insertion site for all the enhancers which were either BamHI-linked or BglII-linked. The resulting promoter-enhancer fragment was then excised using BglII and SalI, and inserted into the CAT3MS vector that had been cut with BglII and SalI. This scheme is diagramed in Figure 24.

Construction of the plasmids into which a synthetic oligonucleotide containing the κ light chain octanucleotide sequence was inserted within the *tk* promoter is shown in Figure 25. Basically, two complementary 16 bp oligonucleotides (5′GATCTATTTGCATGCA3′and5′GATCTGCATGCAAATA3′) that generated a BglII 5′ overhang were annealed in TE(7.5) at room temperature for 5-60 min. A 10-fold molar excess of this fragment was then ligated onto a BamHI deletion endpoint of the *tk* promoter, either −109 or −52 from the cap site. The reaction mix was then digested with an excess of BglII, which

**Figure 22**
**Construction of CAT Acceptor Vectors**

The starting vector, CAT 3M, is shown at the upper left. CAT3M was cleaved with XbaI, filled in using the Klenow fragment of DNA Polymerase I, and ligated to a SalI linker as shown in the lower diagram. The resulting vector, CAT3MS, is shown in the upper right. CAT3MS was then used as an acceptor vector for enhancer-promoter pairs (see Figure 24).

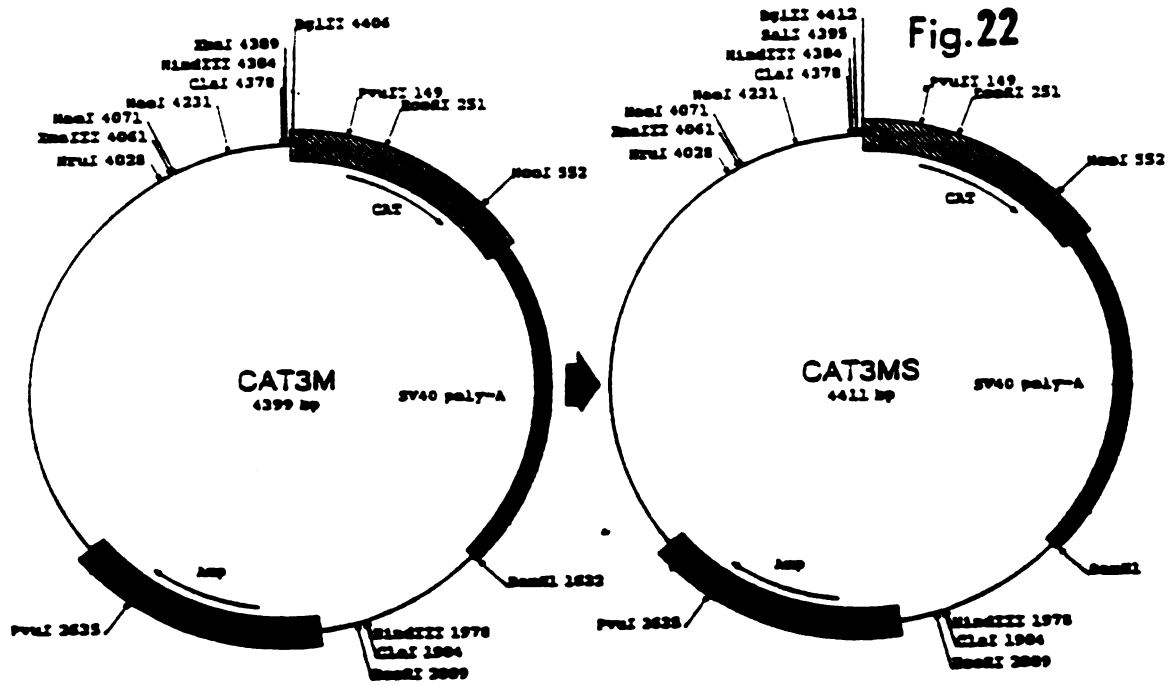

CAT3M
4399 bp
SV40 poly-A
CAT
Amp
CAT3MS
4411 bp
SV40 poly-A
CAT
Amp

Fig.22

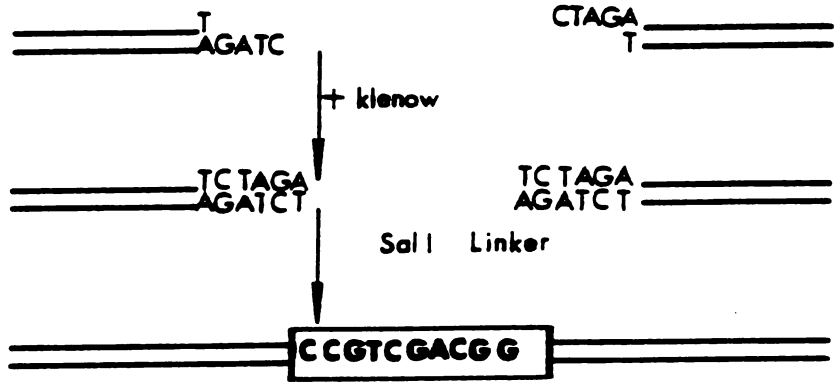
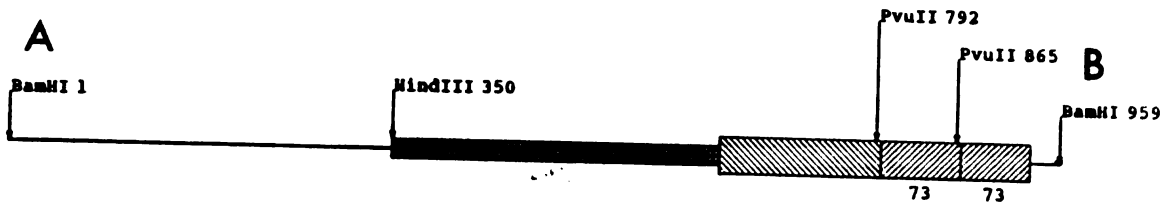

T
AGATC
CTAGA
T
+ klenow
TCTAGA
AGATCT
TCTAGA
AGATCT
Sal I Linker
CCGTCGACGG

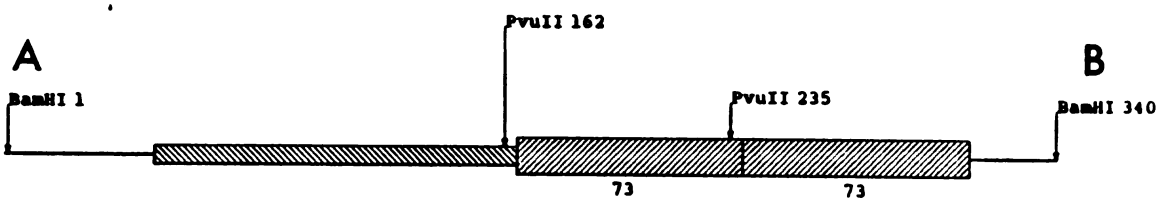
**Figure 23**
**Enhancer Fragments Inserted at 3′ end of CAT Gene**

Enhancer fragments that were inserted at the 3′ end of the CAT gene in CAT3MS are shown. Fragment 2 extends from an XbaI site located 147 bp upstream of the MoMSV transcription start site through the remaining LTR sequences and 47 bp of flanking mink cell DNA to an XhoII site. These sites were filled in and BamHI linked. Fragment 1 is a predecessor of fragment 2, with the same endpoint in the MoMSV LTR, but extending through 300 bp of flanking mink cell DNA and 350 bp of pBR322. Insertion of fragment 1 created the acceptor vector CAT3MSV.9, while insertion of fragment 2 created CAT3MSV.3. Fragment 3 is a 3.6 kb derivative of polyoma viral DNA. This fragment extends from a BglII site located at map position 631 through the origin (map position 0/5292) to a BamHI site at map position 2323. It therefore contains the origin, early promoter and T-antigen coding sequences, and the polyoma enhancer. Insertion of fragment 3 created CAT3MSP. The enhancer fragment was inserted such that the end marked "A" was proximal to the end of the CAT gene, as shown in the lower diagram. The arrow in the polyoma fragment indicates the cap site for transcription of T antigen. Note that this promoter resides between the polyoma enhancer and the CAT gene, thus preventing this enhancer from acting on the *tk* promoter that is upstream of the CAT gene.

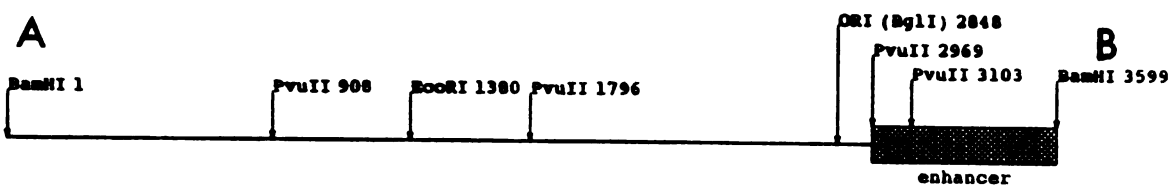
Fig. 23




MSV.9
959 bp




MSV.3
340 bp




Polyoma
3600 bp

**Figure 24**
**Construction and Isolation of *tk* Promoters Linked to Enhancers**

The *tk* promoter and its surrounding sequences are shown. The vector sequences are indicated as wavy lines. The indicated BamHI site is always at the junction of the promoter and the vector. This promoter endpoint was either at −148, −109, −74, or −52, as described in the text. All enhancer fragments were BamHI- or BglII-linked, and were inserted into this BamHI site. The orientation of the enhancer was determined by restriction analysis or sequencing. A SalI-BglII fragment containing both the enhancer and the promoter was isolated as shown, and was ligated to the CAT3MS vector or derivative that had been cleaved with SalI and BglII.

Fig 24

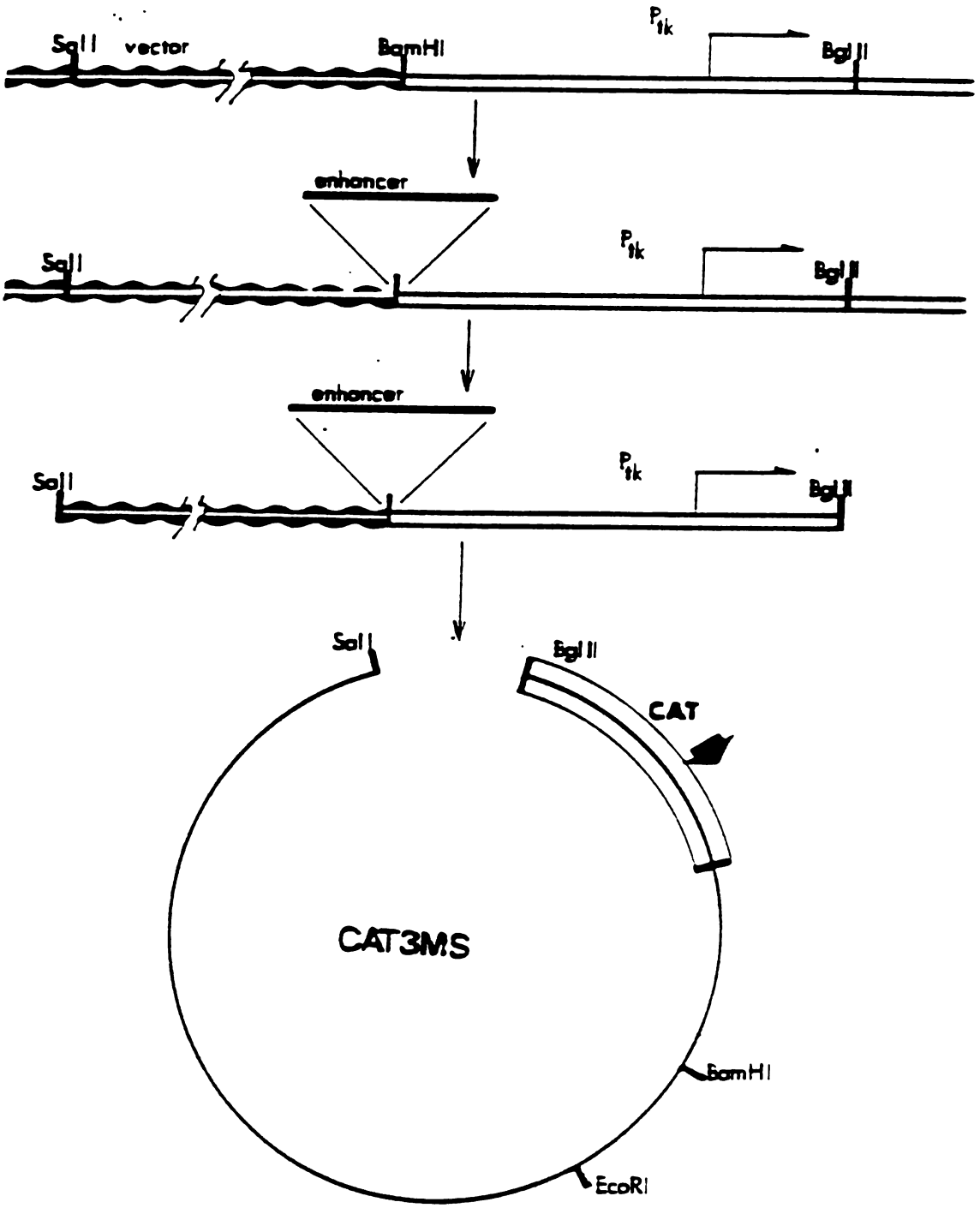
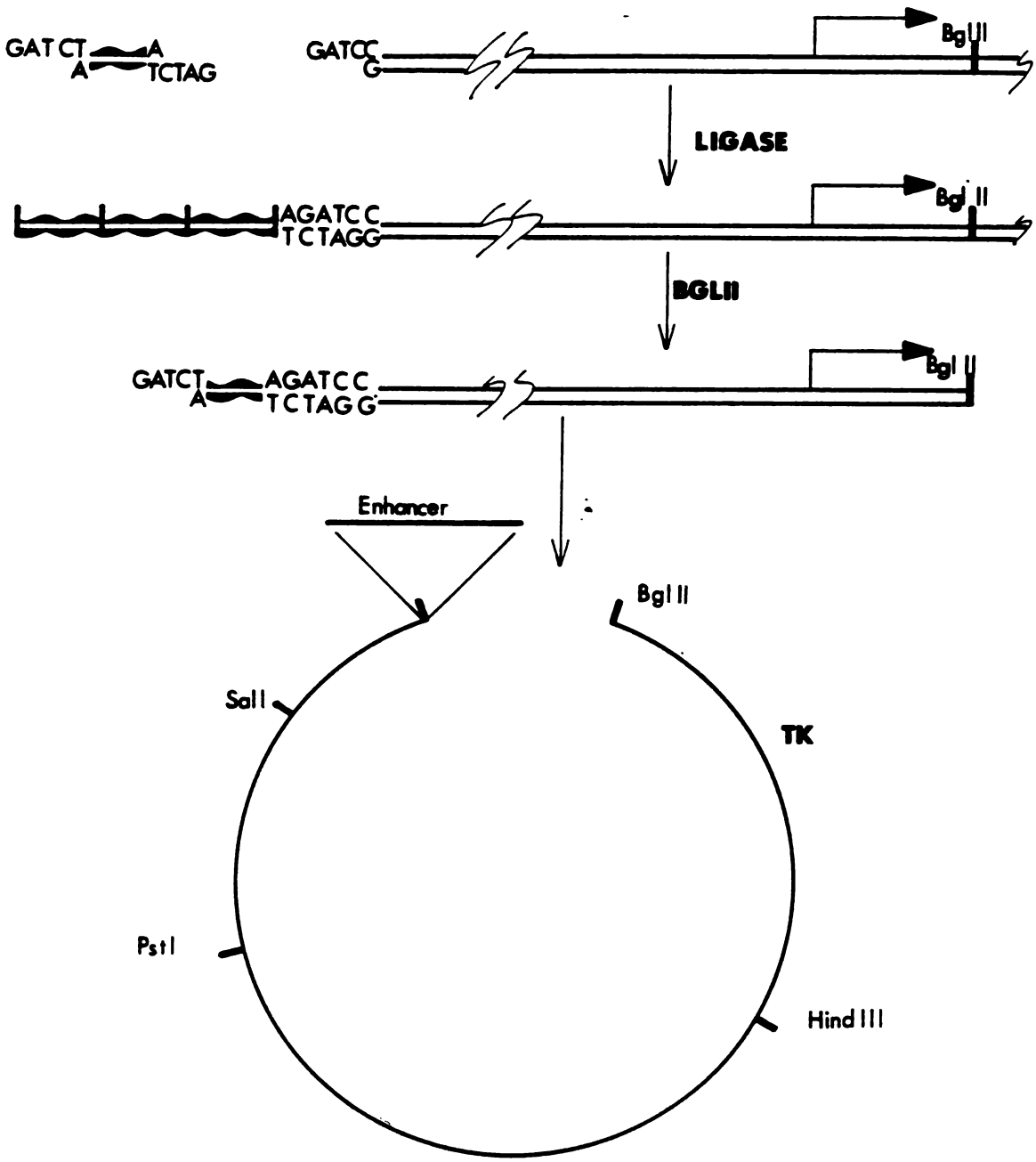

Sal I
vector
BamHI
P tk
Bgl II
enhancer
Sal I
P tk
Bgl II
enhancer
Sal I
P tk
Bgl II
Sal I
Bgl II
CAT
CAT3MS
BamHI
EcoRI

**Figure 25**
**Insertion of Synthetic Oligonucleotide into *tk* Promoter**

Insertion of a synthetic oligonucleotide that contained one copy of the κ octanucleotide onto the *tk* promoter is shown. The oligonucleotide, indicated as a wavy line, generated BglII sites when self-ligated. It was ligated to a promoter that had been cleaved with Bam HI at its endpoint (see Figure 24). After ligation, all but the promoter-proximal oligonucleotide was removed by a 12 hr incubation with BglII. The isolated promoter fragment was then reintroduced into the vector from which it was derived. Subsequent incubation of this intermediate construction with BglII and SalI released a promoter fragment, either containing or lacking an enhancer fragment, that was then inserted into the CAT3MS or CAT3MSP vector.

Fig 25


GATCT
A
A
TCTAG
GATCC
G
BglII
LIGASE
AGATCC
TCTAGG
BglII
BGLII
GATCT
A
AGATCC
TCTAGG
BglII
Enhancer
BglII
SalI
TK
PstI
HindIII

removed all but the terminal oligonucleotide fragment. BglII also cleaves at +55 bp; the promoter-containing fragment thus liberated was isolated and ligated back into the vector. In some cases, a 1.2kb HindIII-HpaI fragment from the mouse κ gene intron that contained the κ enhancer [92] was present in the acceptor vector, such that upon insertion of the promoter fragment, the enhancer would be upstream of the promoter (Figure 25). The promoter-enhancer combination was then inserted into the CAT3MS vector as described above. All constructions were confirmed by sequencing.[93, 130]

The series of *tk* promoter mutants that were examined were provided by Dr. Steve McKnight. pRSVβgal contains the Rous sarcoma virus (RSV) LTR upstream of the coding sequences for the bacterial β-galactosidase gene,[3] and was provided by Dr. Michael Walker. Likewise, pRSVCAT contained the RSV LTR upstream of the CAT gene coding sequences.[49]

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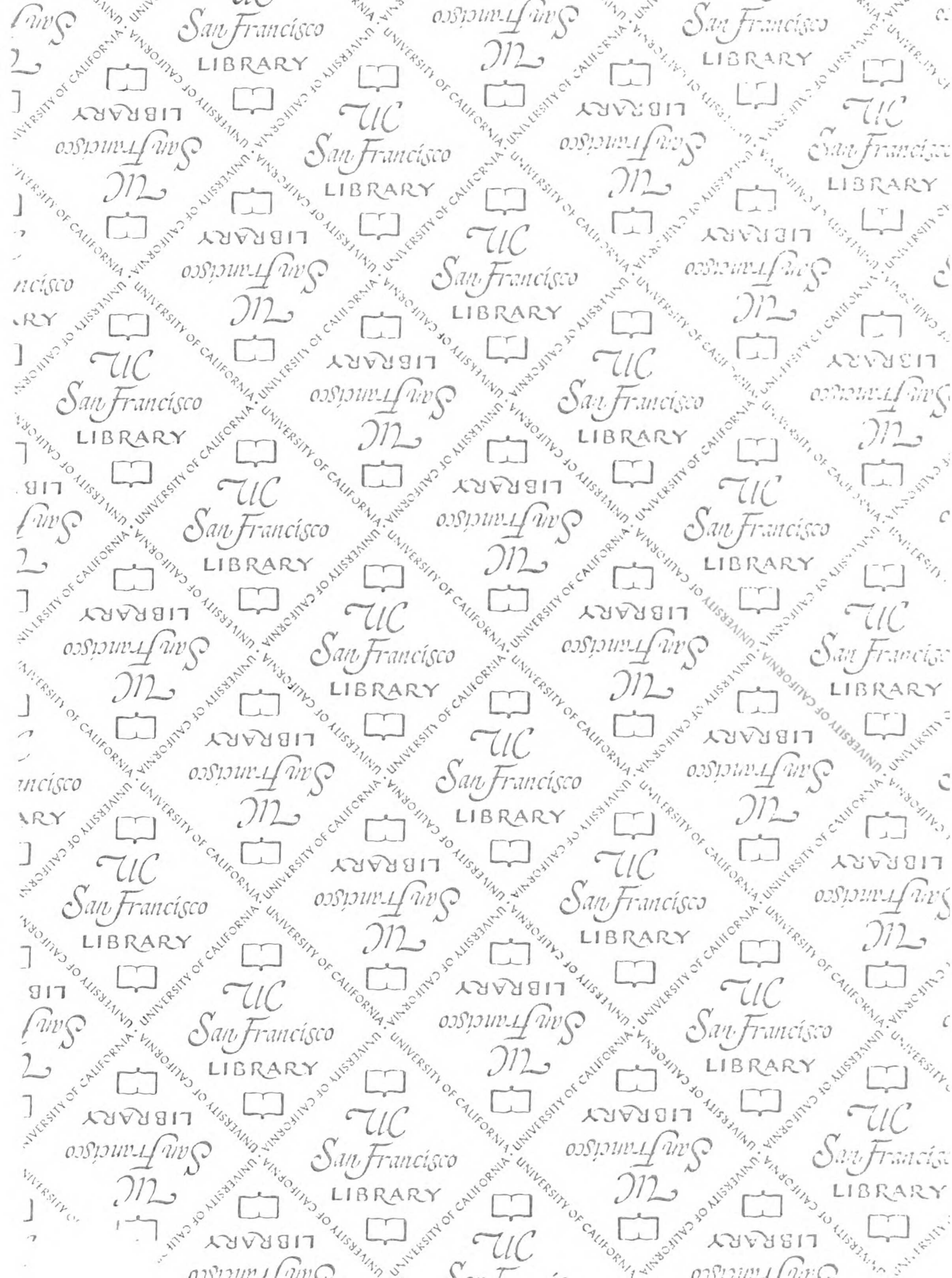
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