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Biochemical and genetic studies of enhancement of transcription by the glucocorticoid receptor

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Biochemical and Genetic Studies of Enhancement of Transcription by the Glucocorticoid Receptor

by

Crafford Arrington Harris

DISSERTATION

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GRADUATE DIVISION

of the

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San Francisco

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Crafford A. Harris

Dedication

I dedicate this dissertation to Rose Vera deBrosche Harris and Frederick Holladay Harris, Jr., my parents, who, recognizing that my intellectual curiosity and enthusiasm were being stifled, sacrificed the joy of watching me grow up to send me to Andover.

I also dedicate this dissertation to Tara Camille Stephenson, my wife, who has been wonderfully supportive and tolerant of the intensity of my specialization, and who has helped me to maintain and expand my broader awareness at the same time.

Crafford A. Harris

Acknowledgments

All of my colleagues in the Yamamoto laboratory have contributed ideas and/or materials that were critical to the development and completion of this work. In addition, I received invaluable assistance from other laboratories within and outside of the Biochemistry and Biophysics Department at UCSF. One of the very special things about UCSF is the openess to exchange of ideas, information, and materials among the various laboratories.

I wish to thank Leroy Liu for his generous gift of DNA topoisomerase II, Don Rio for his gift of HeLa whole cell extract, Kathy Jones for her assistance in the preparation of cell extracts, and the many members of the Tjian laboratory for their advice. I also wish to thank Greg Shackleford and Harold Varmus for their gift of C3H strain MTV.

As members of my thesis committee, Bruce Alberts and Peter Walter offered many helpful suggestions, and graciously suffered with me through the agonies of technical obstacles and biological surprises.

As advisor and instructor, Keith has taught me a great deal. Probably, much more of my knowledge and thinking than I realize is a direct product of my interaction with Keith.

Table of Contents

Figures

Crafford A. Harris

Biochemical and Genetic Studies of Enhancement of Transcription by the Glucocorticoid Receptor

Crafford Arrington Harris

Experiments presented in this dissertation attempted to identify nonreceptor proteins that function in glucocorticoid-dependent enhancement of transcription from promoters linked to Glucocorticoid Response Elements (GREs) from mouse mammary tumor virus (MTV), using biochemical and genetic approaches.

In some, but not in all, experiments, addition of partially purified glucocorticoid receptor and a major contaminating protein of apparent molecular weight 72 kD to in vitro transcription reactions stimulated transcription both from a promoter linked to MTV GREs and from an enhancerless promoter. Possible reasons for the irreproducibility of these experiments are discussed. Addition of partially purified DNA topoisomerase II further stimulated transcription from both promoters.

The variant cell line CR1 was previously selected from a wild type parent cell line containing approximately 15 MTV proviruses by using anti-MTV antibodies and complement to kill wild type cells that upon hormone induction express high levels of MTV

membrane proteins. The hormone induced level of transcription from the resident MTV proviruses in CR1 is only one-tenth that in wild type cells, yet glucocorticoid receptors in CR1 appear normal. Surprisingly, newly introduced MTV proviruses are induced normally in CR1. Therefore, CR1 either carries cis-acting defects affecting one or more of its resident MTV proviruses, or it is defective in a trans-acting factor that acts at non-MTV sequences close to its proviral integration sites. A simple and rapid transient assay was developed that can be used to determine whether other complement-resistant cell lines carry defects in trans-acting factors that act directly on MTV.

Considering the results of the in vitro transcription experiments, and earlier results in our laboratory showing that de-induction of transcription upon removal of hormone is rapid, and that receptor-dependent enhancement does not require any previously identified promoter elements that bind specific transcription factors, one possible hypothesis is that the glucocorticoid receptor enhances transcription by recruiting and/or activating the transiently-acting general transcription factor TFIIE, and that the 72 kd protein that copurifies with the glucocorticoid receptor is TFIIE. Experiments that could test this hypothesis are described.

I. Introduction

DNA-dependent RNA polymerases have evolved to initiate transcription preferentially at specific signals, promoters, in the DNA sequence. Variation in promoters for different genes has allowed individual genes to be transcribed at particular intrinsic rates, and regulatory mechanisms have arisen which allow transcription rates to be modulated. This specificity, variability, and regulability of transcription initiation has facilitated the evolution of ordered, dependent, and responsive patterns of expression of cell characteristics supporting efficient adaptation, growth, development, and replication.

Transcription initiation at promoters is a multi-step process. First the RNA polymerase must locate the promoter. Subsequently, multiple conformational changes of the polymerase and template are required before productive polymerization can be established.[22,269] All of the steps in the initiation process are potential sites for regulation.

Promoters for eukaryotic nuclear genes are composed of multiple sequence elements, each of which binds one or more specific transcription factors that contribute to promoter efficiency.[19,123,135,145] For example, the Herpes simplex virus *thymidine kinase* (HSV-*tk*) gene promoter, which is transcribed by RNA polymerase II, consists of at least five specific elements upstream of the major transcription initiation site.[145,167] The sequence from -26 to -21 bp from the major initiation site, 5'-TATTAA-3', is required for maximal promoter efficiency,[144,145] and is homologous to sequences in other promoters

that have been shown to specifically bind a "TATA" transcription factor,[33,63,165,201] also known as TFIID,[140,201] and as fraction [DB].[197] In addition to increasing the efficiency of promoter utilization, the DNA-bound TFIID factor also restricts the positions at which polymerase can start transcription to a small region about 25-30 bp downstream of the "TATA" sequence.[18,144] Transcription factor Sp1 binds to two *tk* promoter elements, each of which includes the Sp1 consensus core sequence 5'-GGGCGG-3' -- at -49 to -54, and at -96 to -101.[41,107,144,145] A fourth *tk* promoter element binds the "CCAAT transcription factor", or CTF, at the sequence 5'-GCCAAT-3', at -80 to -85, which is homologous to one half of the palindromic CTF consensus sequence.[41,106,107,144,145] Finally, an additional sequence element, at - 138 to -131, 5'-ATTTGCAT-3', which is identical with the "octanucleotide" element originally discovered in immunoglobulin gene promoters, has recently been shown in our laboratory to contribute to the activity of the *tk* promoter.[12,41,105,139,167,168] Factors that bind to this element have also been identified.[121,50,223,204,221] A similar multi-element structure has been found in many other promoters, in many cases including some of these same elements.[18,24,38,135,145,201] The positions of the *tk* upstream promoter elements can be moved to some extent relative to the position of the TFIID binding site without decreasing transcription, but do not seem to be able to act over the very large distances from which enhancers, described below, can function.[145]

Additional transcription factors have been identified that do not appear to bind to specific DNA sequences. TFIIA appears to facilitate sequence-specific binding of

TFIID,[187] and TFIIB and TFIIE bind to each other and to RNA polymerase II.[20,21,188,267]

Many of the eukaryotic transcription factors can bind quite stably, forming "stable complexes" that commit transcription to the promoter and are resistant to challenge by a second promoter,[19,33,48,85] and that can be purified by gel filtration.[195,231] It has conclusively been shown that such stable complexes remain assembled through multiple rounds of initiation on RNA polymerase I promoters,[270,271] and on RNA polymerase III promoters,[14,19] and on an unusual RNA polymerase II promoter.[141] For more typical polymerase II promoters the evidence is inconclusive and conflicting.[85,195]

In addition to promoter elements, other sequences, enhancers, stimulate transcription at linked promoters even from very large and variable distances -- from up to several kbp.[11,35,135,155,175,235,241] Enhancers appear also to consist of one to several binding sites for one or more transcription factors.[4,15,29,34,43,63,76,86,89,90,98,103,124,125,127,132,150,161,166,171,181,184,194,202,208,214,215,233,240,248,251,257,259,266,268] In yeast, in addition to TATA elements, upstream activation sequences, UASs, have been found.[26,224] These appear to share characteristics with both upstream promoter elements and enhancers of higher eukaryotes.[224]

The activities of many enhancers are regulated.[4,10,13,32,34,35,37,40,52,53,57,58,

62,66,67,69,96,97,99,108,110,111,113,125,127,135,150,162,175,176,181,206,213,216,241,234,244,262 For example, the glucocorticoid response elements (GREs) of mouse mammary tumor virus (MTV) are enhancers whose activities are induced by glucocorticoid hormones.[262] Glucocorticoids bind to a soluble intracellular receptor and activate its nuclear localization and possibly additional activities as well,[177] allowing the receptor to bind GRE DNA sequences and regulate gene activity. Partially purified glucocorticoid receptor was shown in our laboratory and others to bind specifically to MTV GRE DNA in vitro.[105,170,203] When these GREs were fused at various distances from promoters, transcripts initiated at the linked promoters became inducible by hormone.[25]

Factors that stimulate or regulate transcription must affect the rate or extent of polymerase binding to the promoter or of subsequent structural changes of polymerase and template on the pathway to productive initiation. These functions could be mediated by direct interactions with the polymerase or the DNA template, or by action on other transcription factors that in turn directly interact with the polymerase or the template.

That there are multiple possible mechanisms of stimulation and regulation of transcription is supported by studies in prokaryotes. The bacteriophage lambda C_I protein binds to a DNA sequence adjacent to the RNA polymerase binding site at a promoter for the C_I gene itself and stimulates transcription by increasing k_f, the rate of isomerization of the "closed complex" of the RNA polymerase bound to double stranded promoter DNA,[310] to the "open complex" of the polymerase bound to melted promoter DNA and poised for rapid

initiation.[83] The C_I protein probably directly contacts the RNA polymerase.[92] The NR_I protein of enteric bacteria acts at an enhancer upstream of the *glnAp2* promoter, also increasing k_f.[160] The E. coli cyclic AMP receptor protein binds upstream of the *lac* promoter and stimulates its transcription primarily by increasing the binding constant, K_B, for formation of the polymerase-promoter closed complex.[133] In eukaryotes, the adenovirus E1A protein appears to increase the activity of a promoter-specific transcription factor for the E2 gene promoter, which in turn stimulates transcription rate by an unknown mechanism.[264] In no case are the mechanisms of transcriptional stimulation or regulation understood in complete detail.

In eukaryotes, the existence of chromatin structures that can repress initiation of transcription allows for additional possible mechanisms of regulation.[75,115,172] Thus, for example, a transcription factor or regulatory protein might disrupt, open, or prevent formation of a condensed chromatin structure, thereby making a promoter more accessible and more active. Such a mechanism has been proposed as one function of the TFIIIA transcription factor for the RNA polymerase III-transcribed 5S RNA gene.[272] In addition, a number of biochemical features of chromatin are associated with transcriptionally active gene domains, including histone acetylation,[273] binding of proteins HMG 14 and 17,[274] and DNA demethylation.[275,276,277] These might also be used for regulation.

Induction of transcription by the glucocorticoid receptor acting at MTV GREs has been an especially attractive system for studying transcriptional enhancement, as the enhancer

has been well characterized,[25,105,171,278] the regulatory protein has been purified,[255,256] and, more recently, the receptor gene has been cloned.[148,149] Furthermore, genetic variants defective in glucocorticoid induction of MTV transcription have been isolated.[49]

The mechanism by which the glucocorticoid receptor enhances transcription is not known. The receptor might interact directly with RNA polymerase II or might affect the activities of other transcription factors, either factors involved in uninduced transcription or additional factors that are required only for induced transcription. As different genes are induced by glucocorticoids in different cell types, it is possible that the cell specificity of the hormone response might be achieved by interactions of the hormone binding receptor with other factors that modulate its function.[158,262]

Two results in our laboratory suggested that such additional factors affecting receptor function might exist. First, from the M1.54 cell line, which contains approximately 15 MTV proviruses, variant cell lines were selected that had a great reduction in the hormone-induced level of MTV transcription, yet appeared by several criteria to have unaltered glucocorticoid receptors.[49] Furthermore, in the CR1 variant, the glucocorticoid induction of transcription of the gene encoding tyrosine aminotransferase, a liver-specific enzyme, was reduced. One interpretation of these results was that the CR1 variant carries a defect in a non-receptor trans-acting factor that is required for induction of MTV and TAT. Since

several other hormone responses were normal, it seemed possible that the defect might be in a factor involved in determining whether one subset of potentially hormone-responsive genes is induced in a given cell type.49 Second, purification of the glucocorticoid receptor yielded a 72 kd protein that was co-purified with the receptor over four columns, and which did not appear to be a proteolytic fragment of the receptor.[255,256] Furthermore, this protein was reported to stimulate specific DNA binding by the purified receptor.[279]

Objectives

Inspired by the results discussed above, the objectives of this thesis were to develop biochemical and genetic methods that could identify nonreceptor proteins involved in glucocorticoid receptor-dependent transcriptional enhancement. Specifically, my objectives were:

1) To reconstitute receptor-dependent enhancement in vitro to provide a biochemical assay for nonreceptor proteins required for enhancement, and to allow dissection of the mechanism of receptor-dependent enhancement in detail.

and

2) To characterize the defect in the CR1 variant, and to develop efficient methods for characterization of other variants that might genetically identify nonreceptor trans-acting factors that modulate receptor-dependent enhancement.

II. An In Vitro Assay for Enhancement

1. Introduction

As a biochemical approach to identifying nonreceptor proteins that participate in glucocorticoid-dependent enhancement, and to begin dissecting the mechanism of enhancement, efforts were made to develop an in vitro assay for enhancement. Transcription in a cell-free extract of a GRE-containing gene and of a control gene were compared. Both genes were transcribed in the extract, and the transcripts were initiated faithfully at the sites used in vivo. Although in some experiments indications of receptor effects on transcription were seen, GRE-dependent effects were not reproducibly obtained. Experimental difficulties that plagued these experiments will be discussed here and in Methods, and suggestions for improved design of the experiments will be presented.

2. The HSV *tk* Gene Is Faithfully Transcribed in Vitro.

Transcription in a HeLa whole cell extract was programmed with a construct that fused an MTV GRE to the herpes simplex virus thymidine kinase gene (GRE-*tk*), and with a control *tk* gene that contained no enhancer. The gene containing the GRE was a pseudo-wild type gene carrying a 10 bp internal deletion, so that its transcripts are slightly smaller than the control, wild-type transcripts (Figure 1). GRE-*tk* constructs similar to the one used in these experiments have been shown by others to be induced in vivo by glucocorticoids by about six fold, both in stably transfected rat XC cells,[25] and in

Figure 1

Constructions Used For In Vitro Transcription Experiments

Plasmid paTk (*tk*) contains wild type HSV *tk* DNA sequence from 109 bp upstream of the major transcription initiation site to 293 bp downstream of the site of polyA addition.[143] The approximate regions of DNA protected from DNAase I digestion by transcription factors Sp1,[107] CTF,[107] and TFIID are shown as hatched boxes. The DNA covered by TFIID has not been reported for the *tk* gene itself, and is taken from determinations for the adenovirus major late promoter.[33] This construction does not include HSV sequences upstream of -109, and therefore does not contain the octanucleotide promoter element that was recently identified.[105,167] Plasmid pΨaAA'1 contains a fusion of MTV GRE sequences between the Sau3A sites at - 452 and -109 relative to the MTV transcription initiation site, fused to the promoter fragment from paTk at -109 in the *tk* sequence. The MTV GRE DNA fragment contains five receptor binding regions, whose positions and extents, as determined by DNAase I footprinting, are shown as hatched boxes labelled R 1.1 through R 1.5.[171] Only part of receptor binding region 1.5 is included in pΨaAA'1; since no footprints have been determined directly on pΨaAA'1, the extent of DNA covered by receptor at R 1.5 in pΨaAA'1 is not known. RNA was measured by primer extension. A radioactively labelled synthetic oligonucleotide primer complementary to nucleotides +82 to +58 of wild type *tk* mRNA was annealed to transcripts and extended with reverse transcriptase as described in Methods. Plasmid pΨaAA'1 contains a 10 bp deletion in the transcribed region of the *tk* gene, and therefore primer extension of pΨaAA'1 transcripts yields products that are 10 nucleotides shorter than those obtained with wild type transcripts.

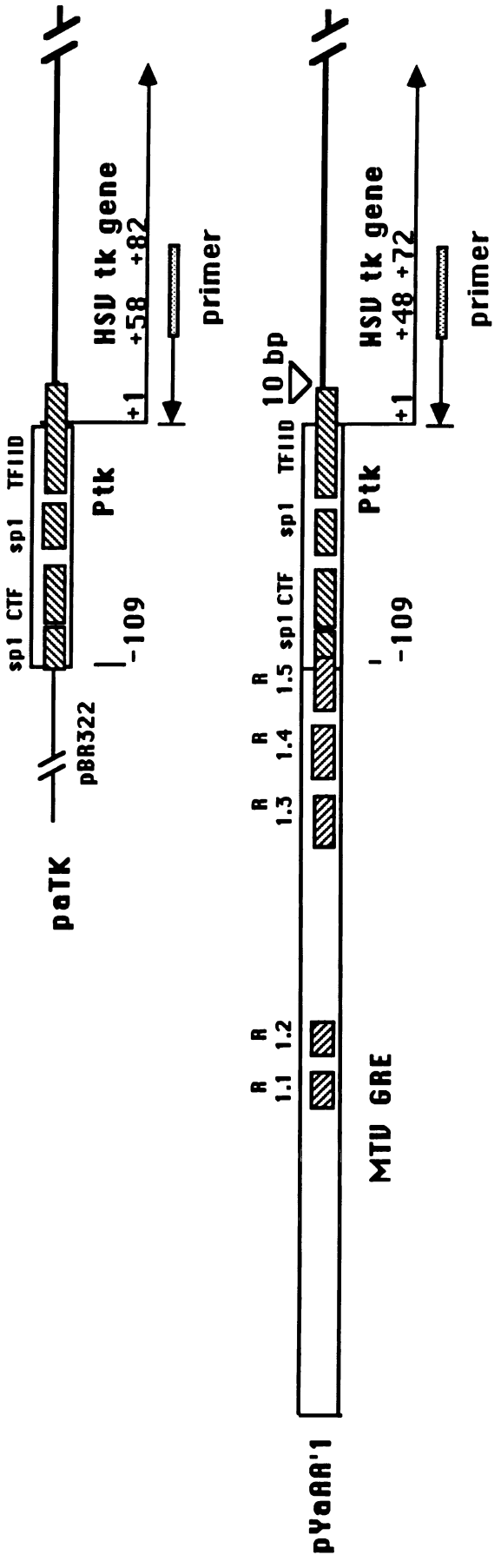

paTK
pBR322
sp1 CTF sp1 TFIID
Ptk
-109
+1
+58 +82
HSV tk gene
primer
pYaAA'1
MTV GRE
R R R R R
1.1 1.2 1.3 1.4 1.5
sp1 CTF sp1 TFIID
10 bp
Ptk
-109
+1
+48 +72
HSV tk gene
primer

Figure 1

transiently transfected rat HTC cells.[196] The whole cell extract was expected to contain the HeLa cell glucocorticoid receptor. However, as the preparation included ammonium sulfate precipitations, which reportedly inactivates steroid receptors,[169] it was not expected that receptor in the extract would be active. Transcription of the GRE-*tk* template was not observed to be significantly greater than transcription from the control *tk* template when reactions contained hormone but no added purified receptor. Transcription in the reactions was measured by primer extension, using a synthetic oligonucleotide as the primer for reverse transcriptase, as diagrammed in Figure 1.

Figure 2 shows that the *tk* gene is transcribed in the cell-free extract. Comparison of the primer extension products of the in vitro transcribed RNA, for example in lane B, with those of RNA transcribed in vivo, in lane A, shows that they are initiated at the same sites.

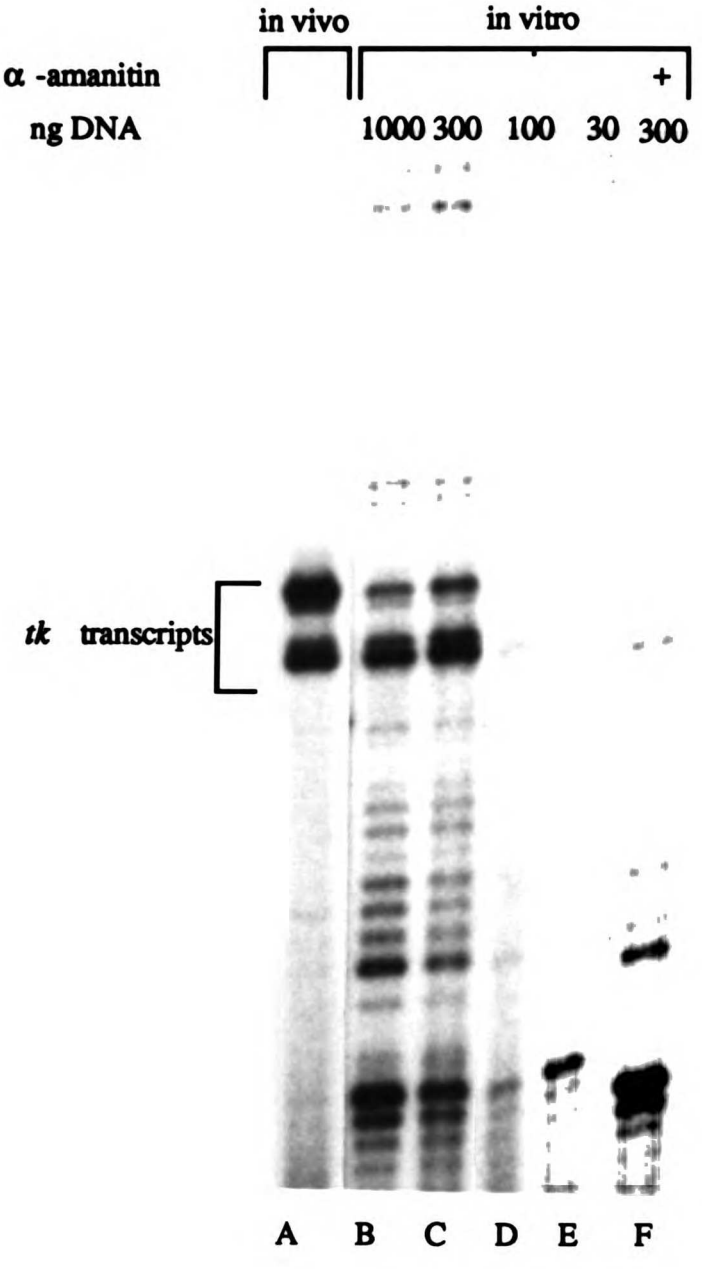
A number of bands at different positions on the gel were also seen, and these threatened to obscure the authentic starts. Some of these artefactual bands were the result of primer extension on contaminants in the plasmid template stock, likely bacterial RNA, or on the plasmid DNA itself, since these bands were seen even when the template DNA was assayed by primer extension directly, without incubating it in a transcription reaction. Other bands seemed dependent on transcription in the HeLa extract, and were α-amanitin-sensitive; these may have been derived from non-specific transcription initiating at DNA nicks. The amounts of both types of artefactual bands varied as a function of the batch of plasmid, and both were more conspicuous when plasmid stocks included a higher

Figure 2

Transcription of the *tk* Gene In Vitro

Plasmid paTK was used as template for in vitro transcription reactions in a total volume of 25 µl, containing 10 µl of HeLa whole cell extract, as described in detail in Methods. RNA transcribed in vitro or in vivo was measured by primer extension as described in Figure 1 and in Methods. The in vivo transcribed RNA analyzed in lane A was 24 ug of total RNA isolated from cell line XaPS1.13 after growth in dexamethasone overnight.[25] XaPS1.13 is a rat XC cell line stably transfected with plasmid paPS1, which contains MTV DNA sequences from -1183 bp to -106 bp (relative to the MTV transcription initiation site) fused to position -109 of the *tk* gene. In vitro transcription reactions contained 30 to 1000 ng paTK, as indicated. In lane F, RNA transcribed in vitro in the presence of 0.1 µg/ml α-amanitin, which is sufficient to inhibit transcription by RNA polymerase II, but not transcription by RNA polymerases I or III,[94,192,210] is measured. The positions of the major initiation sites for *tk* transcripts are marked.

Figure 2


in vivo
in vitro
α -amanitin
+
ng DNA
1000 300 100 30 300
tk transcripts
A B C D E F

proportion of nicked DNA. Both of these types of artefactual bands contributed only negligible background relative to authentic transcripts when a strong promoter such as the SV40 early promoter was used in the reactions, but the background was substantial when the *tk* promoter, which is only of moderate strength, was used, and often obscured the results.

Figure 2 also shows a titration of DNA added to the extract. The amount of transcription decreased abruptly when less than 300 ng plasmid (final concentration 3 nM) was added to a 25 µl reaction containing 10 µl of extract. In order to determine whether it was reasonable to attempt to detect receptor-dependent enhancement using these conditions, it was important to determine the amount of purified receptor necessary to bind this amount of DNA.

3. A large molar excess of purified receptor is required to saturate the MTV GRE binding sites in vitro.

It was assumed that detection of enhancement in vitro would require near saturation of the MTV GRE by the receptor protein. Therefore the amount of receptor required for saturation was determined by a filter binding assay and by a methylation footprinting assay.

Figure 3 shows the results of a filter binding assay. A mixture of DNA fragments from MTV and from pBR322 that had been labelled with ^{32}P was incubated with varying amounts of purified glucocorticoid receptor from rat liver.[255,256] After one hour at 4 °C, the mixture was passed through a nitrocellulose filter under conditions that retained protein and any DNA bound to protein, but allowed unbound DNA to flow through. Bound DNA was recovered, separated on an agarose gel, and visualized by autoradiography.

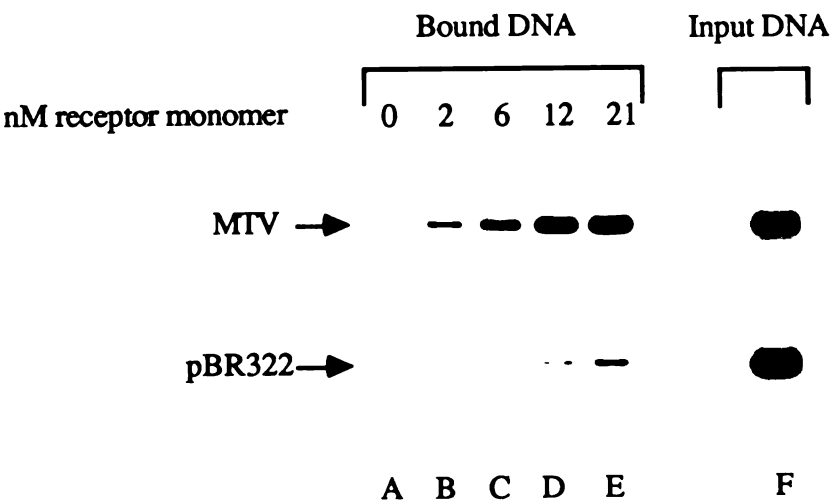
Densitometry of the autoradiogram and counting of filters before and after elution showed that about 8 nM receptor monomer was required to retain 50% of the MTV DNA fragments. The concentration of DNA in the reaction was 60 pM, so when 50% of the fragments were bound by receptor, 30 pM DNA was bound. This MTV DNA fragment contains 13 binding sites for receptor.[105,171] Assuming that binding of receptor to a single site is sufficient for retention of the DNA on the filter, that there is no cooperativity of binding, and that all the binding sites are of equal affinity, then the binding could be modeled by a binomial distribution, which revealed that when 30 pM of the DNA fragments had at least one site filled, the total concentration of filled sites was 41 pM.[252] This suggests that, since 8 nM receptor was required to bind 41 pM binding sites, nearly a two hundred fold molar excess of receptor would be required for saturation. This is a minimum estimate, since if some of the sites have a lower affinity for receptor, as has been suggested,[171] then the concentration of sites bound at 30 pM DNA fragment bound was less than 41 pM. If receptor binds as a tetramer, which is not incompatible with the appearance of receptor-DNA complexes in electron micrographs,[171] approximately a fifty

Figure 3

Filter Binding Assay of Glucocorticoid Receptor Binding to MTV DNA

Plasmid pA2, a pBR322 derivative which contains as an Eco RI fragment a single MTV LTR and all other MTV sequences except those between 1.3 kbp and 4.4 kbp from the left end of the left LTR, was cut with Eco RI. The resulting 4.5 kbp MTV fragment and 3 kbp pBR322 fragment were labelled with ^{32}P and incubated together with varying amounts of purified glucocorticoid receptor for 1 hour at 4 ^{o}C. The mixture was then passed through a nitrocellulose filter which bound protein and any DNA that was bound to protein. Bound DNA was eluted from the filter and displayed on an agarose gel. The positions of the MTV and pBR322 DNA fragments are indicated. In lane F is displayed an aliquot of input DNA before filtration.

Figure 3


Bound DNA
Input DNA
nM receptor monomer
0 2 6 12 21
MTV
pBR322
A B C D E
F

fold molar excess of receptor to DNA binding sites was required to achieve saturation of the DNA by receptor.

In Figure 4 is shown a methylation footprinting assay of receptor binding to the MTV LTR GRE. A DNA fragment including MTV sequences from -1089 bp to -106 bp relative to the transcription initiation site was labelled with ^{32}P at the - 106 end, and incubated at 75 pM with varying amounts of receptor. The DNA was methylated at guanines with dimethyl sulfate (DMS), cleaved with piperidine at the methylated positions, and displayed on an acrylamide gel. Guanines contacted by receptor were expected either to be protected from methylation, or to be made more reactive, as had been seen previously for other proteins.[104]

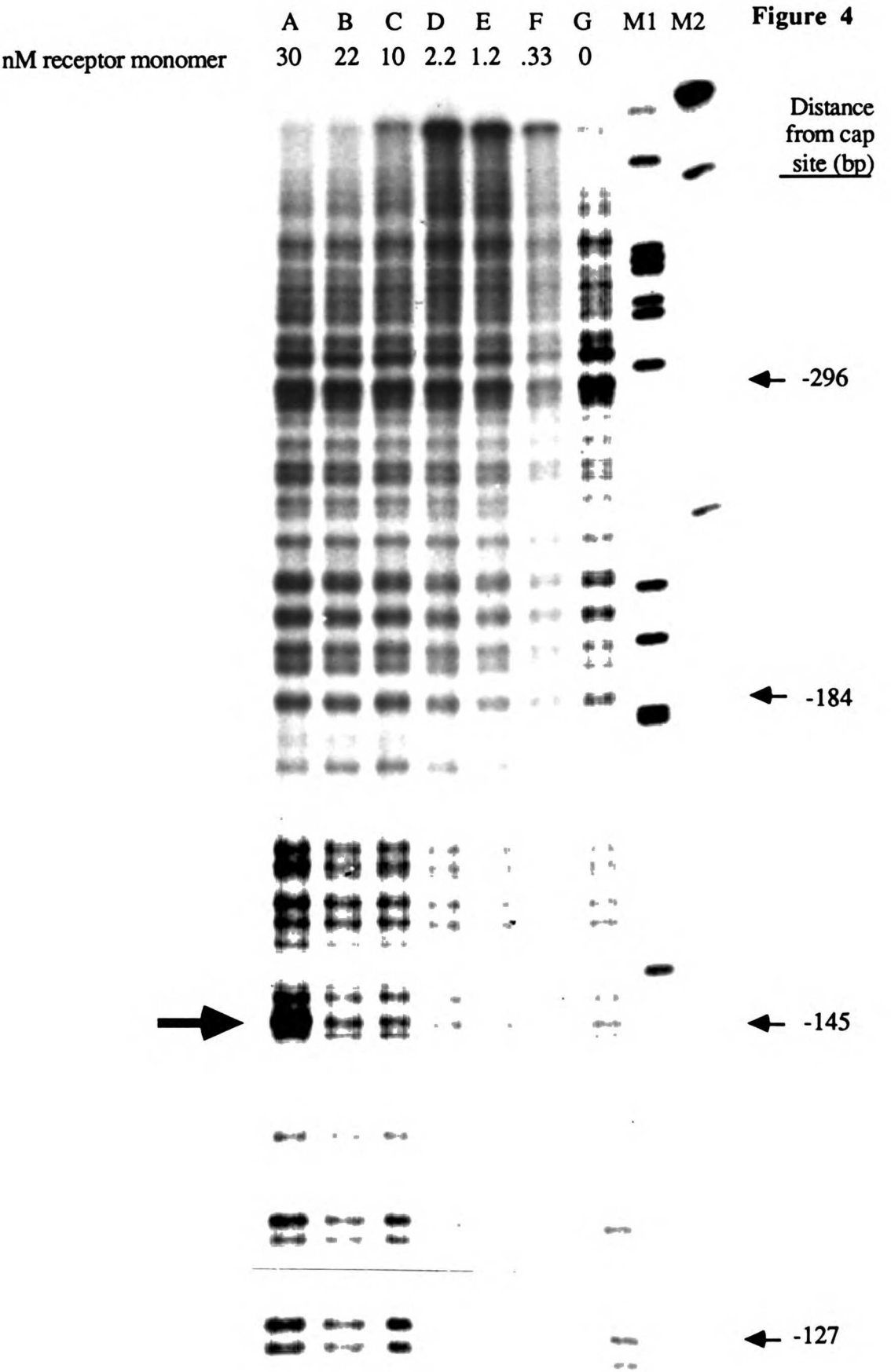
As can be seen in Figure 4, enhancement of the reactivity of the guanine at -145 bp occurred at 30 nM receptor; however, no protection from methylation was seen. The absence of protection was not simply caused by technical difficulties, as experiments done in parallel showed complete protection footprints of bacteriophage lambda repressor C_I on lambda operator DNA, at concentrations of each consistent with the binding parameters previously determined by others (not shown).[102] The absence of protection by the glucocorticoid receptor may have been caused in part by the extreme reactivity of DMS with thiols. The glucocorticoid receptor contains 9 cysteines in the DNA binding domain, and replacement of any one of these cysteines by glycine decreases enhancer-activating activity of the receptor by 100-fold.[44] Cysteines have also been shown to be involved in hormone

Figure 4

Methylation Footprinting Analysis of Glucocorticoid Receptor Binding to MTV LTR DNA

A DNA fragment containing MTV sequences from - 1089 bp to -106 bp relative to the mRNA initiation site, labelled with ^{32}P at the 3' end of the mRNA strand, was incubated with 0 to 30 nM receptor monomer for one hour at 4 ^{o}C, and then methylated with dimethyl sulfate. The DNA was cleaved at methylated positions with piperidine and displayed on an acrylamide gel. Distances relative to the MTV mRNA initiation site are indicated on the right side of the figure. Molecular weight markers in lane M1 are Sau 3A fragments of pBR322 DNA, and in lane M2 are Hind III fragments of bacteriophage lambda DNA. The large arrow points to the guanine at -145 bp whose reactivity with DMS is enhanced by the receptor.

Figure 4


A B C D E F G M1 M2
nM receptor monomer
30 22 10 2.2 1.2 .33 0
Distance from cap site (bp)
-296
-184
-145
-127

binding by the receptor.[156,280,281] If receptor were inactivated by methylation at one of these cysteines, and as a result dissociated from its binding site, the sequences that were previously protected would then become substrates for the reaction, and the protected "footprint" would be obscured; however, any enhancements of reactivity would remain detectable. Scheidereit and Beato did see receptor-dependent protection from methylation of GRE DNA, using slightly different conditions, higher concentrations of receptor, and a different strain of MTV that has slightly different sequences at many of the receptor binding sites.[203]

The concentration of DNA in the methylation reaction shown in Figure 4 was 75 pM, and, since there are five receptor binding sites on this fragment, the concentration of binding sites was 375 pM. Since binding of receptor was not detectable until 30 nM receptor was added, at least an eighty fold excess of receptor would have been required to saturate the GRE binding sites. Even assuming that receptor binds as a tetramer, at least a twenty fold excess of receptor to DNA binding sites would have been required to achieve saturation.

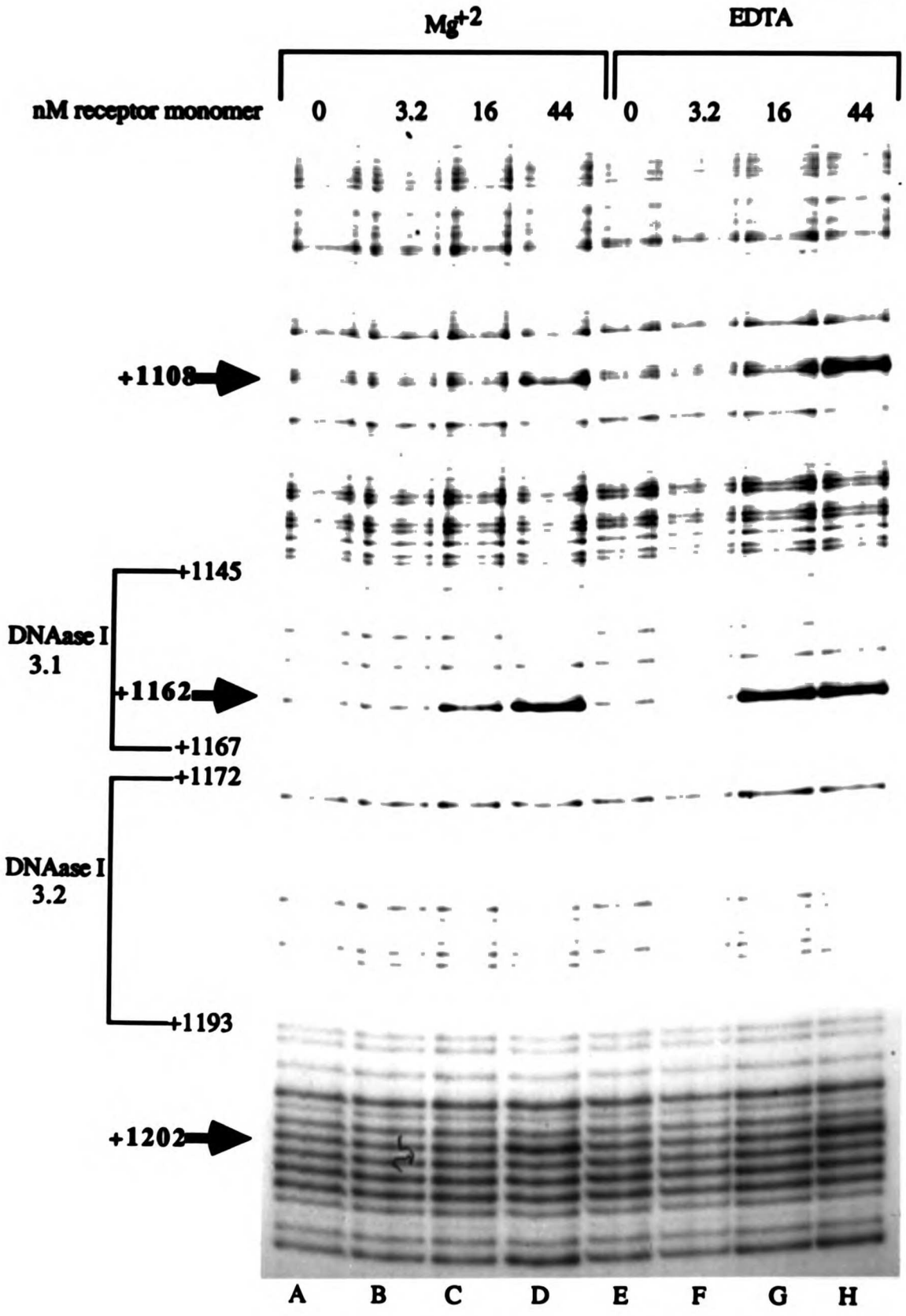
In Figure 5 is shown another methylation experiment, this time using a GRE-containing fragment from the transcribed region of MTV. Again, no strong protections against methylation were detected, but enhancements of reactivity were seen at several guanines. Interestingly, an enhancement of reactivity was seen at a guanine that lies quite far from the

Figure 5

Methylation Footprinting Analysis of Glucocorticoid Receptor Binding to GREs within Transcribed MTV DNA

An MTV DNA fragment from +484 bp to +1281 bp relative to the Bam HI site approximately 4.3 kbp downstream of the mRNA start site was labelled with ^{32}P at the 5' end of the antisense strand. The labelled fragment was incubated with purified glucocorticoid receptor, methylated, cleaved, and analyzed as described in Figure 4. Reactions analyzed in lanes A through D contained 1 mM Mg^{+2} during binding; reactions analyzed in lanes E through H contained 1 mM EDTA. The two DNAase I footprints of the receptor that were previously determined are indicated by brackets.[171] The large arrows point to guanines whose reactivity with DMS is enhanced by the glucocorticoid receptor.

Figure 5


Mg+2
EDTA
nM receptor monomer
0
3.2
16
44
0
3.2
16
44
+1108
+1145
DNAase I
3.1
+1162
+1167
+1172
DNAase I
3.2
+1193
+1202
A
B
C
D
E
F
G
H

independently-determined DNAase I footprints of receptor on this fragment,[171] at position +1108 bp, suggesting that receptor may alter DNA structure at a distance from its binding sites, or that receptor may make weak secondary contacts with DNA at positions separate from the primary binding sites.

Receptor at 16 nM was sufficient to enhance reactivity of DNA at 20 pM. Since there are two binding sites on this fragment,[171] the concentration of binding sites was 40 pM, and, again assuming that receptor binds as a tetramer, this was a 100 fold molar excess of receptor to DNA binding sites.

Thus, results of both filter-binding and methylation protection experiments suggested that, given the binding conditions as optimized at that time, a greater than twenty-fold molar excess of receptor to binding sites was required to approach saturation. Analysis of DNase I footprinting experiments also supported this conclusion.[171] These results were then used in designing appropriate receptor/DNA ratios for experiments designed to detect in vitro enhancement.

(The high molar ratio of receptor to DNA required for saturation was one of the most serious obstacles to in vitro transcription experiments. Concentrating the receptor after purification was not a satisfactory solution, as the absolute amount of purified receptor was also quite limiting. Subsequently, by adding 2% polyvinyl alcohol and 0.1% NP-40 to the binding reaction, conditions that had augmented binding of other DNA binding

proteins,[282] J. LaBaer has reduced the amount of receptor necessary to attain saturation by four-fold, to approximately a five-fold molar excess, again assuming binding as a tetramer.[117] Addition of polyvinyl alcohol and similar volume-excluding compounds may make in vitro conditions more closely approximate the nearly crystalline state of protein assemblies in vivo,[55] and has greatly facilitated in vitro reconstitution of initiation of DNA replication,[54] and of mu phage transposition.[154] Recently, the cloning of the receptor gene and the construction of cell lines that overproduce the receptor has provided new sources of greater amounts of receptor.[149,134,283] These advances should facilitate more rapid progress in the further development of the in vitro assay in the future.)

The purified rat liver receptor supplied by our collaborators typically was at a concentration of 3 ug/ml, or 30 nM monomer.[255,256] Therefore, if receptor were included at one-third of the volume of a transcription reaction, the concentration in the reaction would be 10 nM monomer, or 2.5 nM putative tetramer. This was sufficient to saturate only approximately 125 pM binding sites, or 25 pM pΨaAA'1 plasmid, which corresponds to about 2 ng per 25 µl reaction. The need to decrease the amount of DNA in a transcription reaction to approximately 2 ng per 25 µl reaction required surmounting the problem of the large decrease in transcription below 300 ng per 25 µl.

4. Polynucleotides Stimulate Transcription of Small Amounts of Template in the In Vitro Reaction

Other investigators had previously observed a sharp decrease in transcription in cell extracts below a certain level of template, and had found that addition of poly(dI-dC) abolished this sharp drop, stimulating transcription at low template concentration.[78] It is presumed that this effect is caused by titration by the polynucleotide of nonspecific DNA binding proteins, which otherwise would coat the small amount of template nonspecifically and render it inaccessible to the transcription machinery. Therefore, varying amounts of poly(dI-dC) were tested for their ability to stimulate transcription from low concentrations of the *tk* gene in our extracts. The addition of 900 ng of poly(dI-dC) allowed detection of transcription, though weak, from 9 ng of template per 75 µl reaction, conditions expected to allow near saturation of the GRE by receptor (not shown).

5. Purified Receptor In Some Experiments Affects Transcription In Vitro, but Specific Stimulation of Transcription from GRE-containing Genes Is Not Yet Consistently Reproducible.

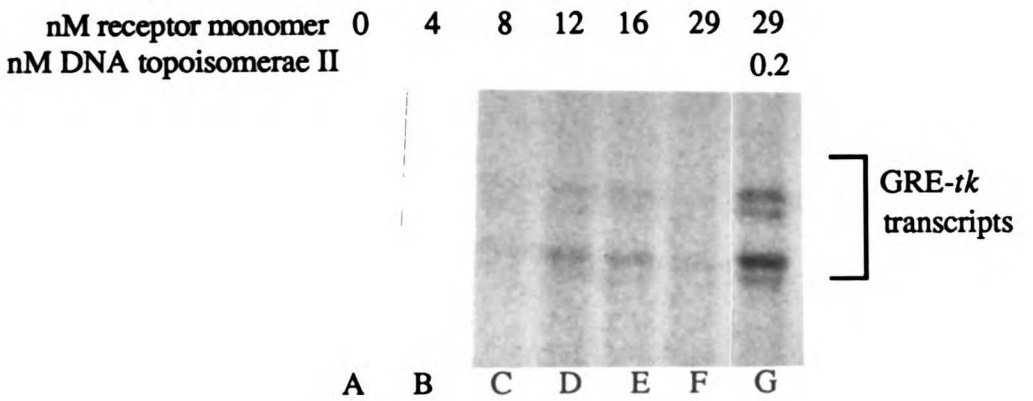
Once conditions had been established that allowed assay of transcription with the receptor near saturation, it became possible to ask whether receptor would stimulate transcription in vitro. In Figure 6 is shown an experiment in which receptor does appear to stimulate transcription of the GRE-*tk* fusion. As described in detail in Methods, the

Figure 6

Stimulation of Transcription From A GRE-*tk* Gene In Vitro By Purified Glucocorticoid Receptor

Plasmid pΨaAA'1 was pre-incubated with varying amounts of glucocorticoid receptor and DNA topoisomerase II, and then mixed with transcription extract that had been pre-incubated with poly(dI-dC), as described in detail in Methods. After transcription for 30 min at 30 ^{o}C, RNA was isolated and analyzed by primer extension. DNA concentration was 38 pM. The positions of products corresponding to GRE-*tk* transcripts are indicated.

Figure 6


nM receptor monomer 0 4 8 12 16 29 29
nM DNA topoisomerae II 0.2
GRE-tk transcripts
A B C D E F G

pΨaAA'1 DNA template was pre-incubated with receptor, while the transcription extract was pre-incubated with poly(dI-dC); the two were then mixed and the reaction was allowed to proceed. With the DNA template at 9 ng per 75 µl reaction (36 pM), 16 nM receptor monomer maximally stimulated transcription, while 29 nM receptor decreased transcription relative to that at 16 nM. This inhibition at the highest concentration of receptor, which proved to be specific for the GRE-*tk* template (see below), is not understood, but could be caused by receptor binding to a recently discovered weak binding site within the *tk* promoter,[117] which overlaps the binding site for transcription factor CTF.[107] A second possibility is that receptor bound to its 3'-most binding region in the MTV GRE fragment, region 1.5, interferes with the binding of transcription factor Sp1 to its 5'-most binding site in the *tk* promoter (Figure 1). Unfortunately, in this particular experiment the control template lacking a GRE had been omitted in order to decrease the background of artefactual bands that had been obscuring the results of recent experiments, as described above.

One potential mechanism of transcriptional enhancement could involve alterations in DNA topology, perhaps by recruitment of topoisomerase or gyrase activities by enhancer binding proteins. To explore this possibility, some transcription reactions were supplemented with additional DNA topoisomerase II. In lane G of Figure 6 it can be seen that the addition of partially purified topoisomerase II (a generous gift of Leroy Liu[151]) to approximately 30 ng/ml (0.2 nM if 50% pure) further stimulated transcription in the presence of receptor.

In Figure 7, a similar experiment again showed stimulatory effects of partially purified topoisomerase II and of receptor on transcription in vitro. Topoisomerase II at 0.2 nM plus receptor at 10 nM stimulated transcription from both the GRE-containing template and from the control template lacking the enhancer. Increasing receptor to 29 nM further stimulated transcription from the enhancerless *tk* promoter, but slightly inhibited transcription from the GRE-*tk* construct, as in the previous experiment. Increasing the concentration of topoisomerase II to 1 nM, or decreasing it to 0.04 nM, yielded the same amount of transcription as 0.2 nM. In lane F, novobiocin, a drug that inhibits topoisomerase II,[95] inhibited transcription at 1000 μg/ml. In subsequent experiments (not shown), novobiocin inhibited transcription in vitro even at 100 μg/ml, and novobiocin inhibited hormone induced transcription of MTV in vivo at 1000 μg/ml. It should be noted, however, novobiocin is a quite non-specific inhibitor at these concentrations, inhibiting other ATP-requiring processes, interfering with some protein-protein interactions, and precipitating some proteins.[56,238,211,71] Therefore, results of experiments using high concentrations of novobiocin must be interpreted with extreme caution.

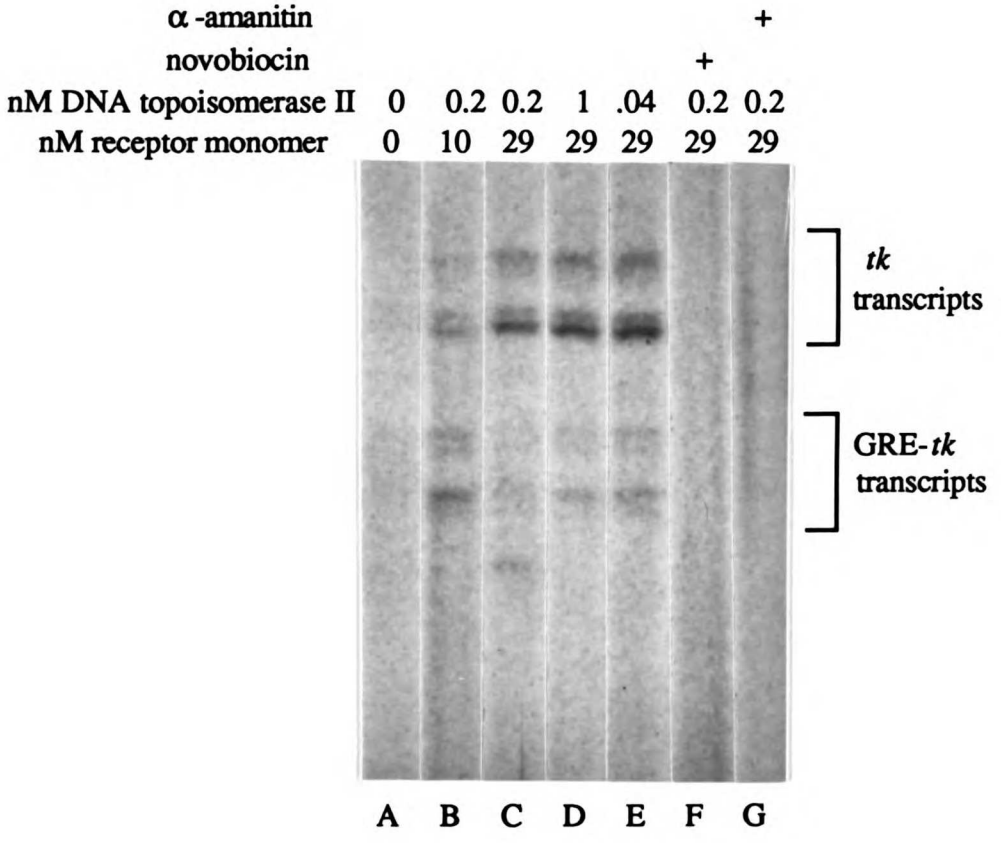
One interpretation of these results is that at moderate concentrations, up to 16 nM, receptor stimulated transcription in vitro of the GRE-containing template, while at 29 nM, receptor began to inhibit transcription of the GRE-containing plasmid, perhaps by interfering with the binding of another transcription factor, as described above. At higher

Figure 7

Stimulation of Transcription from Both a GRE-*tk* Template and a Wild Type *tk* Template by Purified Receptor In Vitro

A mixture of plasmids paTK and pΨaAA'1 was pre-incubated with varying amounts of purified glucocorticoid receptor and DNA topoisomerase II, and then added to transcription extract that had been pre-incubated with poly(dI-dC). After transcription for 30 min at 30 ^{o}C, RNA was analyzed by primer extension. DNA was at 38 pM. In lane F novobiocin was present during transcription at 1 mg/ml. In lane G α-amanitin was present during transcription at 0.1 μg/ml. The positions of primer extension products corresponding to GRE-*tk* transcripts and control *tk* transcripts are indicated.

Figure 7


α -amanitin
novobiocin
nM DNA topoisomerase II
nM receptor monomer
0 0.2 0.2 1 .04 0.2 0.2
0 10 29 29 29 29 29
+
+
tk transcripts
GRE-tk transcripts
A B C D E F G

concentrations than required to stimulate transcription from the GRE-containing plasmid, transcription from the control plasmid that did not contain a GRE was stimulated.

These reports are reminiscent of those reported recently in the case of the bacterial protein NR_I, which acts at an enhancer upstream of the *glnAp2* promoter.[160] In this case, 7 to 14 nM NR_I maximally enhanced transcription in vitro from 5 nM of a template containing the *glnAp2* promoter linked to the five upstream NR_I binding sites, while at 56 nM NR_I transcription from a deleted template containing no NR_I binding sites was also strongly stimulated.

The mechanism of the stimulatory effect of topoisomerase II is not known, and a number of questions need to be answered. First, is the effect dependent on the activity of the glucocorticoid receptor? Second, does topoisomerase II itself cause the stimulation, or is it caused by some other factor in the preparation? Third, is the lack of dependence of the stimulatory effect on the concentration of topoiosmerase II over the range tested, 0.04 nM to 1 nM, indicative of enzyme excess, or is the stimulatory effect caused not by a factor in the topoisomerase II preparation, but rather by a component of the topoisomerase dilution buffer (see Methods)?

Unfortunately, in further experiments, efforts to reproduce the stimulation of transcription by receptor without the addition of topoisomerase II were unsuccessful. In one experiment, although inhibition of transcription from the GRE-containing template at

29 nM receptor was again seen, stimulation of transcription at 16 nM receptor could not be reproduced (not shown). Conceivably, this reflected a failure to maintain transcription factors below saturation, as suggested by other experiments (see Methods). Transcription factor excess would not eliminate inhibition of transcription by high concentrations of receptor if it occurred by the mechanisms proposed above, and inhibition of transcription from the GRE-*tk* template was seen at 29 nM receptor in all experiments.

Further attempts to reproduce the enhancement of transcription by receptor were thwarted by difficulties in detecting the low levels of transcription. Therefore, although the DNA binding experiments had suggested that a large molar excess of receptor would be necessary to saturate the DNA and produce detectable enhancement, an attempt was made to boost the basal transcription signal to more easily detectable levels by using a higher concentration of DNA in the transcription reaction, and therefore a lower molar ratio of receptor to DNA. When 100 ng DNA was transcribed in a 25 µl reaction, specific enhancement was not observed, whether receptor was pre-incubated with the DNA, or pre-incubated with the transcription extract, and whether the input DNA was linear or supercoiled (data not shown).

Efforts were then re-directed towards developing more sensitive assays for transcription, on which some progress has been made by others.[239] In vitro enhancement has not yet been achieved using these assays.[239]

6. Suggestions for Improved Experimental Design

The limiting concentration of purified receptor was the greatest impedance to these experiments. Recently constructed cell lines that overproduce receptor at several fold the level of wild type cells should provide a valuable source.[134] Use of the improved DNA binding conditions recently adopted should also facilitate the experiments.[117] The combination of these two advances should allow the experiments to be repeated at approximately a ten fold higher concentration of template in the transcription reactions, which should both raise the transcription signal to consistently detectable levels, and help ensure that transcription factors can be kept limiting. Recently developed assays for post-translational receptor modifications may lead to an understanding of factors that affect receptor activity, and to improved preparations of receptor.[59] Since the hormone-binding activity of purified receptor decays rapidly and irreversibly,[285] it may be useful to use a receptor derivative, such as N556, whose hormone-binding domain has been deleted, and which is a constitutive activator of transcription.[283] Modification of the receptor to reduce its tendency to proteolysis, or the use of more highly purified, protease-free, transcription factors may also be beneficial. Freshly purified receptor might be more active than frozen receptor. Characterization of the putatively inactive HeLa receptor in the whole cell transcription extract-- its concentration by Western blot analysis, its hormone-binding activity, and its DNA-binding activity, and a determination of any inhibitory effects it might have on added purified receptor would also be useful.

The ideal template for these studies would be strongly transcribed in the absence of receptor and strongly induced in its presence in cells from which transcription extracts and receptor can be prepared. As extracts with high activity can be prepared from rat HTC cells,[239] and as HTC cell lines overproducing receptor are now available,[134] a number of potential plasmid templates for the in vitro assay should be screened for their levels of expression and inducibility by transient transfection assays in the overproducing HTC cell lines. The recently constructed synthetic GRE, GRE_{46},[163] is more active than the MTV LTR GRE, and GRE_{46} has a higher affinity for the receptor.[196] Therefore, plasmids containing strong promoters such as the SV40 early promoter, adenovirus major late promoter, or B-globin promoter and one or more copies of the GRE_{46} are good candidates as test templates. Darbre and King have shown that an MTV GRE can confer hormone-inducibility of about three fold on an SV40 promoter that lies several kbp away,[31] and similar results have been obtained in our laboratory.[196,286] It will be interesting to determine whether such a construction will be even more inducible when receptor levels are increased. Plasmids should be constructed so that the GRE is not immediately adjacent to the promoter, in order to avoid artefactual effects of receptor binding on transcription factor binding, which may have occurred on the GRE-*tk* plasmid used in the experiments described here, as discussed above.

More rapid assays for transcription in vitro have been developed that would facilitate much more rapid progress in these experiments.[137,287] These assays have not been

sufficiently specific when we have used crude extracts and/or weak promoters,[79,239] but might be usable if suitable strongly-inducible templates containing strong promoters were identified, and if their required transcription factors were purified.

It is possible that the mechanism of enhancement of transcription by the glucocorticoid receptor involves alterations in the chromatin structure of the template. As the type of extract used in these experiments may not efficiently assemble template DNA into chromatin, especially in the presence of large amounts of poly(dI-dC), use of a minichromosome template might increase the extent or reproducibility of the enhancement observed. Recent success in achieving high levels of replication in Cos cells of a plasmid containing a construct that is strongly induced by glucocorticoids suggests that it might be possible to prepare appropriate minichromosomes.[228] Chromatin can also be assembled on plasmids in vitro, using Xenopus oocyte extracts.[122] Minichromosomes prepared in this way have recently been used to study transcription complex assembly in vitro.[253]

A crude extract was used in these initial experiments because it seemed possible that factors not required for general transcription might be essential for receptor-dependent enhancement of transcription. However, the use of a crude extract does not allow independent variation of the concentrations of individual transcription factors. Thus, if enhancement of transcription by receptor requires that a specific factor be limiting, then it might not be possible to reconstitute enhancement in vitro with extracts containing factors at

inappropriate relative concentrations. Furthermore, some enhancer and promoter factors are members of families of related factors that bind to similar DNA sequences yet may have different activities in the context of other linked promoter and enhancer elements.[50,73,204] It seems likely therefore that incubation of templates in crude extracts may generate improper transcription complexes that contain non-functional combinations of transcription factors and prevent normal transcription or its regulation. Thus, it would seem worthwhile to explore some experiments using purified transcription factors. In addition, whole cell extracts may contain active or inactive, and perhaps interfering, glucocorticoid receptor. Nuclear extracts prepared from cells grown in the absence of hormone will be depleted of receptor.

Some of these suggestions might improve the in vitro assay. Initially, it might be useful to repeat the stimulatory effects seen in the experiments presented here using the same extract, templates, and conditions. The apparent stimulatory effect of toposiomerase II should be used initially simply as a means to increase the transcription signal to easily detectable levels, but understanding the mechanism of this phenomenon would be interesting in its own right.

7. Conclusions

In two experiments reported here, receptor stimulated transcription from a plasmid

containing a GRE fused to the HSV-*tk* promoter. These results were complicated by several other observations. Transcription from the GRE-*tk* template was inhibited by higher concentrations of receptor, perhaps because of interference between the binding of receptor and binding of an adjacent transcription factor. Receptor-dependent stimulation of transcription from the control template, which did not contain a GRE, was also seen, though this seemed to require higher concentrations of receptor than those required to stimulate transcription from the GRE-*tk* template. Stimulation of transcription from the GRE-*tk* template was not readily reproducible, perhaps because transcription factors were not consistently kept limiting in the reactions. No attempts were made to demonstrate that the stimulation of transcription could occur when the GRE was at widely different positions relative to the transcription initiation site, which is a defining characteristic of an enhancer. Because of these complications, the reconstitution of receptor-dependent enhancement must be considered tentative at this time.

Recently several other laboratories have detected stimulation of transcription by eukaryotic enhancers or enhancer binding proteins in vitro, using crude extracts or partially purified factors.[8,124,152,198,199,208,217,250] In most cases the enhancer effects have been less than 10% of their in vivo levels. Stimulation has not been detected when an enhancer was separated from a promoter by more than a few hundred base pairs; thus, the essential feature of an enhancer has not been observed. The results are further complicated by the presence in the enhancers of binding sites for proteins that also bind to sequences that have been considered promoters.[12,124,135,152,168] Thus, it seems possible that

these enhancers have activities that are promoter-like in addition to some fundamentally different type of activity that confers the ability to stimulate transcription from a large distance. That is, the in vitro stimulatory effects of enhancers that have been observed to date may have occurred via promoter-like activities, and may not be measuring the unique activities of enhancers. Alternatively, the inability of enhancers to stimulate transcription from as large distances in vitro as in vivo may reflect the greater distance between two DNA sites in vitro on DNA not packaged in chromatin relative to in vivo DNA that is compacted by nucleosomes and higher order chromatin structures. The use of minichromosome templates in in vitro transcription reactions may resolve this question.[253,272]

Progress in this investigation was greatly impeded by the low concentration, amount, and activity of the purified glucocorticoid receptor. The recent success in constructing cell lines that overexpress cloned receptor sequences should provide a more concentrated source of receptor.[134] This, along with the recently developed conditions that increase the efficiency of DNA binding by the receptor,[117] should facilitate the further development of the in vitro enhancement assay.

If the observation of glucocorticoid receptor-dependent transcriptional enhancement in vitro is confirmed, it should be possible to fractionate the transcription extract to identify the factors specifically required for receptor-dependent enhancement. It will also be

possible to probe the mechanism of enhancement by the receptor in detail, for example by isolating individual steps in the initiation reaction, as recently done by others,[85,195] and determining at which step the receptor acts.

III. Transcription in Injected Xenopus Oocytes

The purified glucocorticoid receptor retains at least some specific DNA binding activity, as shown, for example, in Figures 3 through 5. As one approach to testing whether the purified receptor was also competent for transcriptional enhancement, receptor was co-injected with the GRE-*tk* template and the control *tk* template into stage 5 or 6 Xenopus laevis oocytes. In a series of experiments done in collaboration with David Standring, one of which is shown in Figure 8, no effects of receptor on transcription from either template were seen, whether receptor and DNA were injected into the cytoplasm and into the nucleus, respectively, or coinjected into the nucleus. In these experiments the maximum molar ratio of receptor to DNA was 70 fold.

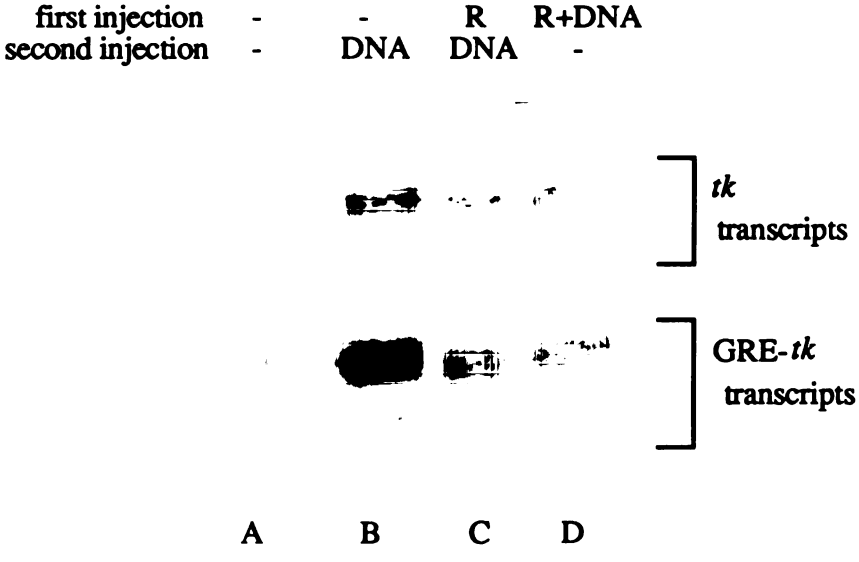
David Standring is currently beginning experiments to determine whether the glucocorticoid receptor can be translated in oocytes injected with receptor RNA transcribed in vitro from the cloned receptor gene. If the receptor is produced, then the coinjection experiments can be repeated. If receptor function can be measured, then it should eventually be possible to assess material purified from cells engineered to produce the receptor at high levels.

Figure 8

Transcription in Xenopus Oocytes Injected with Purified Glucocorticoid Receptor and *tk* and GRE-*tk* DNAs

Stage 5 or 6 Xenopus oocytes were not injected (lane A), injected intranuclearly with a mixture of GRE-*tk* and control *tk* DNAs (0.05 fmol each) 27 h before harvest (lane B), injected extranuclearly with 3.5 fmol glucocorticoid receptor monomer at 46 h before harvest and then with 0.05 fmol each DNA at 27 h before harvest (lane C), or injected intranuclearly with a mixture of 0.03 fmol each DNA and 0.3 fmol receptor at 31 h before harvest (lane D). RNA was isolated and analyzed by primer extension. The positions of the products of primer extension on *tk* and GRE-*tk* RNAs are indicated.

Figure 8


first injection - - R R+DNA
second injection - DNA DNA -
tk transcripts
GRE-tk transcripts
A B C D

IV. Characterization of the CR1 Variant

1. Introduction

One of the initial reasons for attempting to use an in vitro assay to identify additional proteins required for hormone dependent enhancement was the phenotype of the CR1 variant cell line. CR1 and other complement resistant variants were selected from the M1.54 cell line, which contains approximately 15 MTV proviruses, by using anti-MTV antibodies and complement to kill wild type cells that upon hormone induction express high levels of MTV proteins associated with the plasma membrane.[49] As described in Chapter 1, the hormone-induced level of MTV transcription is greatly reduced in CR1. Furthermore, the glucocorticoid induction of the tyrosine aminotransferase gene is also reduced in CR1. However, some other hormone responses are unaffected, and the CR1 glucocorticoid receptor appears normal by multiple assays.[49] CR1 was most simply interpreted as carrying a defect in a nonreceptor trans-acting factor required for hormone-dependent enhancement of transcription from the multiple MTV proviruses and the TAT gene.[49]

Somewhat surprisingly, experiments described in this chapter show that newly infected or transfected MTV genes are expressed and regulated equally well in CR1 and in M1.54. Thus, CR1 appears wild type for factors that act on MTV. Therefore, either the CR1 variant carries cis-acting defects affecting one or more of its MTV proviruses, or it is defective in a trans-acting factor that acts at non-MTV sequences close to the integration

sites of the proviruses. Experiments that might distinguish these possibilities are discussed. A simple and rapid transient assay was developed that can be used to determine whether other complement-resistant cell lines carry defects in trans-acting factors that act directly on MTV.

2. The Glucocorticoid Receptor is Not Detectably Altered in the CR1 Variant, and Expression of Additional Receptor Does Not Rescue the CR1 Defect.

Experiments were done to test whether the CR1 defect might be a subtle alteration in the glucocorticoid receptor. Initial experiments, in which CR1 cells were infected with an MuLV retroviral vector expressing the cloned receptor gene, showed some indication that increasing the amount of receptor significantly increased the hormone-induced transcription of MTV. However, the discovery that MuLV is itself induced by glucocorticoids,[64,79,288] coupled with the auto-repressing effects of receptor on its own expression,[289] meant that the level of receptor expression was changing during hormone induction, and could not be determined accurately. Various assays of receptor structure and function failed to reveal any alterations in the CR1 receptor; indeed no point mutations in CR1 receptor mRNA were detected by RNAase protection. Finally, CR1 cells were transfected with a non-hormone-inducible plasmid expressing receptor, and stably transfected clones were examined for levels of receptor expression and levels of hormone-induced MTV RNA. Clones that expressed several fold wild type levels of receptor had only slightly increased hormone-

induced MTV transcription (data not shown), as had been seen previously when receptor levels were increased in wild type cells.[284] Thus, expression was not rescued to wild type levels, and the slight increase in transcription simply reflected the previously demonstrated limiting amount of receptor in HTC cells.[284] Therefore, all evidence suggested that the glucocorticoid receptor in the CR1 variant is unaltered.

3. A stably transfected marked MTV provirus is induced equally in variant CR1 cells and wild type M1.54 cells.

To test whether CR1 had defects in any trans-acting factors required for expression or induction of MTV, a construction in which the central region of MTV was replaced with a *tk* structural gene, pLTL-1,[278] was transfected into CR1 and M1.54. Stably transfected cell lines containing single copies of pLTL-1 were isolated and the hormone induction of the LTL construct and of the originally infected MTV proviruses were compared in the variant and wild type cell lines. The pLTL-1 construction contained MTV sequences from the C3H strain of virus, whereas the originally infected virus was the GR strain; thus, it was possible to distinguish between the transcripts of each by use of an RNAase protection assay, as described in Figure 9 and in detail in Methods.

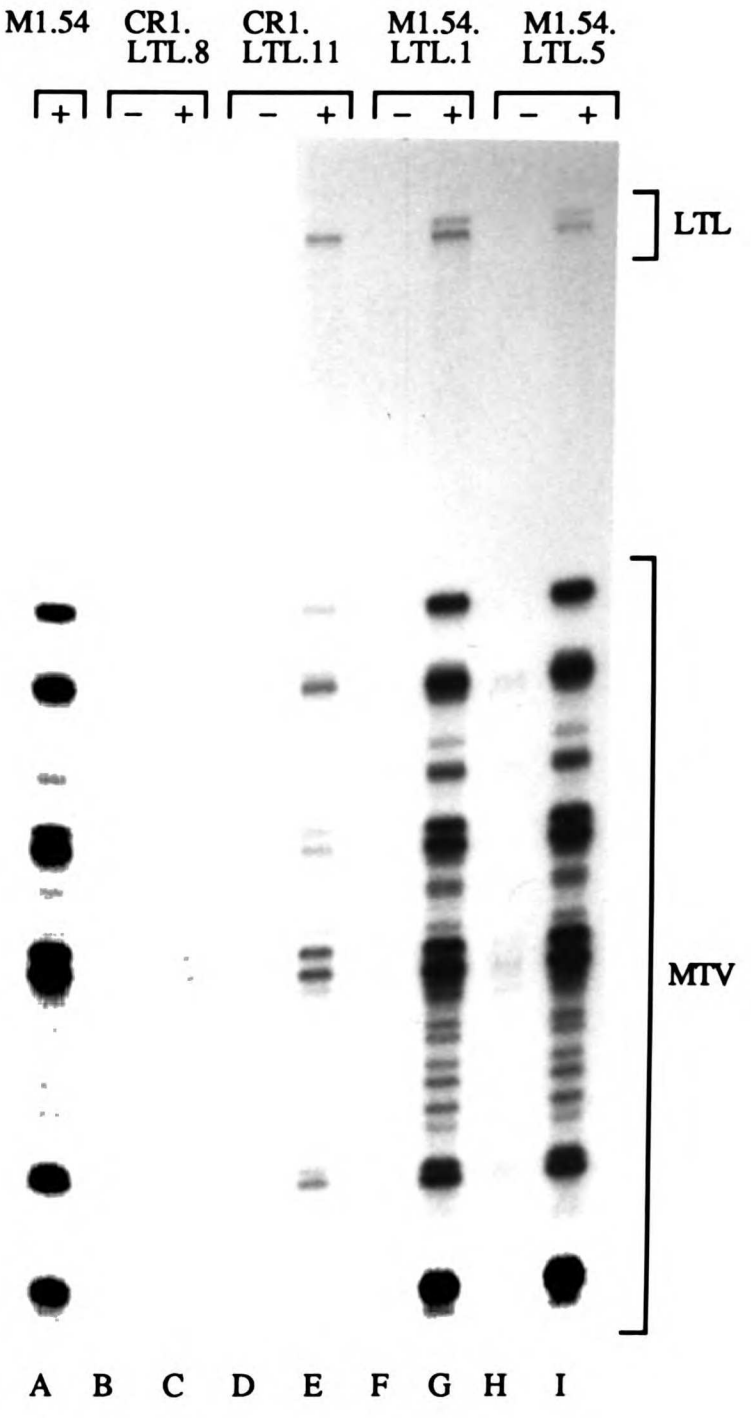
In Figure 9 it can be seen that hormone-induced transcription of the originally infected MTV proviruses is reduced approximately ten fold in the CR1 clones. In contrast, the

Figure 9

Expression of pLTL-1 and MTV in Stably Transfected CR1 and M1.54 Cell Lines

Single copy transfectants were grown in the presence (+) or absence (-) of 10^{-7} M dexamethasone overnight. RNA was isolated and measured by an RNAase protection assay, as described in detail in Methods. RNA was annealed to a radioactively labelled antisense RNA probe that included sequences from -106 bp to ~+557 bp (relative to the mRNA start site) of pLTL-1. The annealed RNA was then digested with 40 μg/ml RNAase A for 30 min at 30 °C, and the digested products were displayed on an acrylamide gel. RNA from pLTL-1 gives a band at ~557 bp, and when RNA is from M1.54 an additional slightly larger band. The larger M1.54 band is probably produced by annealing of the probe to two RNAs-- one is a transcript from pLTL-1, and the second, which extends the region protected from RNAase to sequences 5' of the start site, is the 3' end of endogenous MTV RNA, which extends past the sequence of the MTV start site in the 3' long terminal repeat.[236] MTV RNA gives a series of smaller protected bands, resulting from RNAase cleavage at sequence differences between the probe, which contains C3H MTV sequences, and the endogenous GR strain MTV RNA. It was necessary to use conditions that resulted in only partial digestion of the MTV GR strain RNA hybrids to prevent digestion of the fully complementary pLTL-1 RNA hybrids due to hybrid breathing (see Methods). In lane A is displayed the products resulting from analysis of RNA from nontransfected M1.54 cells; no pLTL-1 transcripts are detected in nontransfected cells. The positions of pLTL-1 RNA and MTV RNA bands are marked. Additional MTV RNA products were run off the gel.

Figure 9


M1.54
CR1. LTL.8
CR1. LTL.11
M1.54. LTL.1
M1.54. LTL.5
+
– +
– +
– +
– +
LTL
MTV
A B C D E F G H I

hormone-induced transcription of pLTL-1 is approximately equal in CR1 and in M1.54. Thus, somewhat surprisingly, trans-acting factors that act directly on the MTV provirus and are required for hormone induced transcription of MTV appear to be fully functional in CR1.

4. Superinfected wild type MTV proviruses are induced equally in variant CR1 cells and wild type M1.54 cells.

It could be argued that transcription of pLTL-1 does not accurately reflect MTV transcription because a large region of MTV has been deleted and replaced with the *tk* gene. Also, because only a few pLTL-1 transfectants were obtained, the analysis of the few lines might be skewed by position effects [290,291] on the expression of the LTL genes integrated at different chomosomal locations. Furthermore, it was possible that the enhancer on the co-transfected plasmid, pSV40neo, that was used to select the stably transfected clones, might have altered LTL expression. Therefore, wild type C3H strain virus was superinfected into the variant and wild type cells, using conditions that resulted in infection of nearly all the cells. The C3H transcripts and proviral DNA could be distinguished from those of the resident GR strain proviruses using RNAase protection assays, and restriction fragment length polymorphisms, respectively. Several days after infection, populations of infected CR1 and M1.54 were assayed for MTV transcription in the presence or absence of hormone, and expression was normalized to proviral copy number. As shown in Figure 10, induction of the newly infected C3H strain MTV was

Figure 10

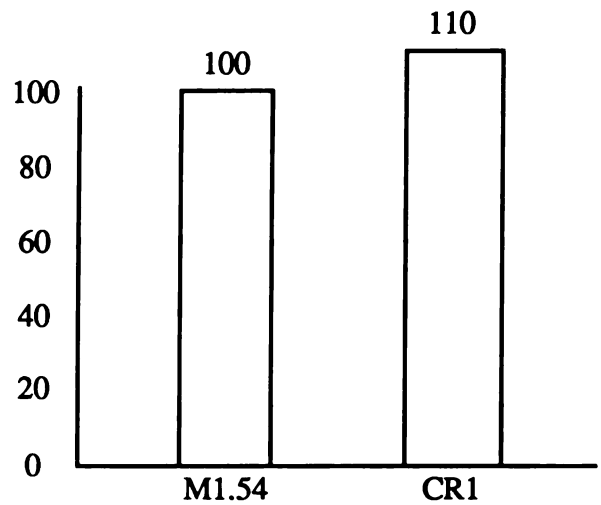
Expression of Superinfected MTV in M1.54 and CR1

CR1 and M1.54 cells were superinfected with C3H strain MTV, as described in Methods. C3H virus transcripts in populations of infected cells were measured by the RNAase protection assay described in the legend to Figure 9. To correct for different efficiencies of infection, C3H RNA levels were normalized to C3H proviral copy number as determined by Southern blotting. The two sets of results shown are from two independent infections. In both experiments the level of C3H transcripts in M1.54 cells was arbitrarily set to 100.

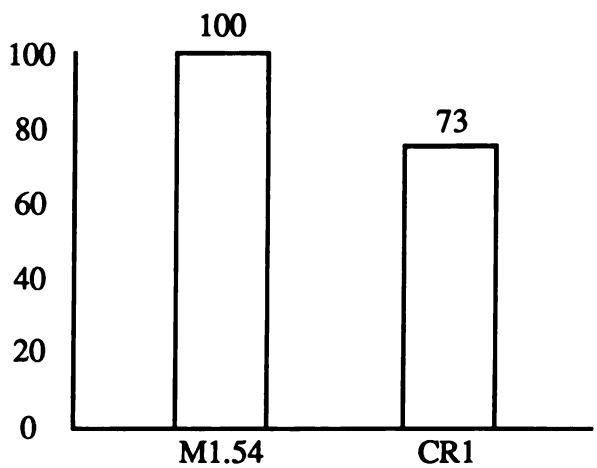
Figure 10

Relative Accumulation of RNA Transcribed from Newly Infected MTV

Experiment One



Experiment Two



indistinguishable in CR1 and M1.54, consistent with the interpretation of previous experiments.

5. The MTV GRE and Promoter Are Expressed and Regulated Equally in CR1 and M1.54 In Transient Transfections-- A Rapid Cis-Trans Test for CR Variants.

Although the results of the stable transfection of CR1 with pLTL and the super-infection with MTV clearly showed that the defect affecting the expression of the proviruses from the initial infection was not in a trans-acting factor that acts on MTV DNA, a more rapid and straightforward test was needed for efficient screening of additional variants. Therefore, a transient transfection assay was developed. Because HTC cells are difficult to transfect, development of the assay required considerable effort, as described in detail in Methods.

CR1 and M1.54 were co-transfected with pGMCO-gr/ch, a fusion of the GR strain MTV LTR to the chloramphenicol acetyltransferase (CAT) structural gene, in a vector especially favorable for expression,[174] and with pRSVβgal, a fusion of the Rous sarcoma virus LTR to the structural gene for beta-galactosidase.[292] Expression from the MTV LTR in the presence and absence of hormone was measured as relative CAT activity after controlling for differences in transfection efficiencies by normalization to beta-galactosidase

Figure 11

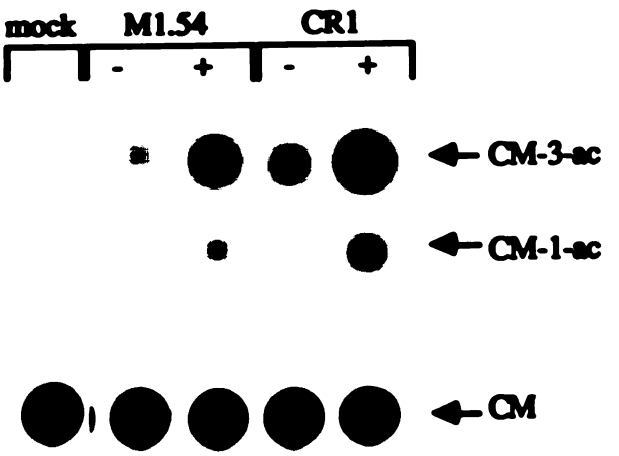
Transient Transfections of M1.54 and CR1 Cells

M1.54 or CR1 cells were co-transfected with pGMCO-gr/ch and pRSVβ-gal. After growth in the presence or absence of dexamethasone, cell extracts were prepared and chloramphenicol acetyltransferase and β-galactosidase acitivities were determined, as described in Methods. Panel A shows an assay of CAT activity. The spot marked CM is unreacted ^{14}C chloramphenicol. The spots marked CM-1-ac and CM-3-ac are the two isomers of mono-acetylated chloramphenicol. The first lane shows the activity of an extract of mock-transfected CR1 cells. Lanes marked - and + are assays of extracts from cells grown in the absence or presence of dexamethasone, respectively.

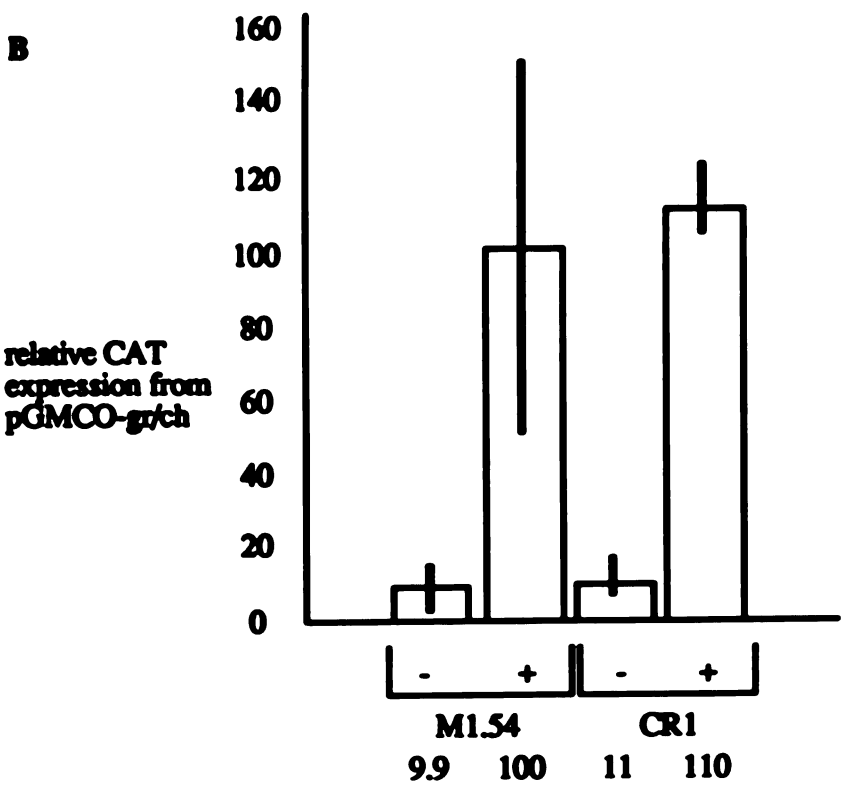
Panel B shows relative CAT expression from pGMCO-gr/ch, normalized to β-galactosidase expression to correct for differences in transfection efficiencies. Each value is the mean of results obtained in two or three experiments. Error bars show the range of values. Expression in hormone-treated M1.54 cells is arbitrarily set to 100.

Figure 11

A


mock
M1.54
CR1
-
+
-
+
CM-3-ac
CM-1-ac
CM

B


160
140
120
100
80
60
40
20
0
relative CAT expression from pGMCO-gr/ch
-
+
-
+
M1.54
CR1
9.9
100
11
110

expression from the uninduced internal control RSV-βgal gene. As shown in Figure 11, CR1 and M1.54 cells expressed and induced pGMCO-gr/ch equally, again demonstrating that the defect in CR1 does not operate in trans.

As the MTV LTR contains an open reading frame, it might be suggested that the MTV LTR encodes its own trans-acting activator, and that this function is defective in CR1. However, transient basal and induced expression from the plasmid pUC-G_{46}TCO,[196] a fusion of a synthetic GRE to the *tk* promoter and CAT gene which lacks the LTR open reading frame, is also identical in CR1 and M1.54 cells. Furthermore, the superinfections of CR1 populations with new MTV proviruses failed to rescue expression or induction of the resident proviruses. Thus, one obvious possible interpretation is that a cis-acting defect in CR1 decreases hormone-induced transcription of MTV; however, alternative explanations are possible.

6. What is the Nature of the Defect in CR1?

Although the M1.54 cell line contains approximately 15 MTV proviruses, it is likely that not all of the proviruses are active. Retroviral replication is extremely error-prone, with mutation frequencies as high as 10^{-3} per base pair.[293] This means that a fraction of any infecting population of virus carries mutations that block proper viral expression. Furthermore, some chromosomal sites may be non-permissive for proviral expression.[290]

Therefore, a significant fraction of proviral integrates may be non-expressing. This is supported by examination of MTV RNA expression in MTV-infected HTC cell lines that integrated only one or a few proviruses.[189]

However, if only a fraction of the proviruses in M1.54 are expressing, then the level of expression of MTV RNA in M1.54 in the presence of hormone is unusually high-- 1800 copies/cell,[49] compared to 70-100 copies/cell in strongly expressing lines containing a single provirus.[189] Furthermore, MTV expression in CR1, 175 copies/cell,[49] is about the level that might be expected from two or three strongly expressing proviruses. Thus one interpretation of the M1.54 and CR1 cell lines is that they each contain about three typical strongly expressing proviruses, and that M1.54 contains one provirus that is expressed at unusually high levels, whose expression has been greatly decreased, in cis, by the defect in the CR1 variant.

Certainly, if only a single MTV provirus in M1.54 is responsible for most of the MTV RNA expression, a classic spontaneous mutation could explain the low level of MTV expression in CR1. The mutation could lie in the MTV proviral DNA, in a linked cis-acting cellular transcriptional stimulatory element, perhaps a cellular enhancer, or in a gene encoding a trans-acting factor that acts at such a linked cis-acting element. No deletions or rearrangements could be detected in any of the MTV proviruses in CR1 relative to those in the wild type M1.54 parent (not shown). However, small deletions, point mutations, and distant rearrangements would not have been detected.

A second possible explanation for the CR1 defect is that more than one provirus is more weakly transcribed in CR1 than in M1.54. How could multiple cis-acting defects have arisen at a detectable frequency? Perhaps the defects are in fact not mutations, but rather epigenetic gene inactivations that occur at a much higher frequency. Many variants have been found that appear to have such epigenetic gene inactivations, and these high frequency epigenetic defects explain many of the cases in which recessive "mutations" have been isolated at high frequency in cultured cells.[219,254] In many instances, recessive phenotypes arise in two steps which occur at very different frequencies-- a low frequency step, occurring at about 10^{-5} (after mutagenesis), which is a classic mutation, and a high frequency epigenetic gene inactivation step which occurs at a frequency of up to 10^{-2} to 10^{-1},[16,219,220,254] or even higher.[258] Epigenetic gene inactivations have been shown in some cases to affect a large chromosomal region, as nearby genes are also affected.[17,193] In several cases, the inactivated DNA is hypermethylated, and/or the phenotypes can be reverted by treating the cells with azacytidine, which causes de-methylation, suggesting that one mechanism of gene inactivation may involve DNA methylation.[17,80,81,157,227,100] For most genes, only one allele can be inactivated by such a mechanism, probably because at most loci the large chromosomal region inactivated includes a lethal. Notably, however, MTV proviruses are like single allele genes, since each provirus is integrated at a different chromosomal locus. Thus, every provirus in a cell like M1.54 could in principle be inactivated by such a high frequency epigenetic mechanism as long as none is integrated near haplolethal genes. Darbre and King have reported that

mouse mammary tumor S115 cells grown for long periods in the absence of steroid hormones lose the ability to induce transcription from endogenous MTV genes, but retain the ability to induce transcription from newly transfected MTV genes, and that contemporaneously endogenous MTV DNA becomes hypermethylated.[30,31]

Further investigation of the CR1 defect is hampered by the multiple proviruses, and by not knowing which of the proviruses is strongly expressed in the wild type parent. In principle, however, since a fraction of MTV transcripts proceed through the poly-A addition site and beyond the end of the 3' LTR,[236] it would be possible to use cloned cellular DNA probes flanking each provirus to identify those proviruses expressed strongly in M1.54 and those which are defective in CR1.

CR1 also differs from M1.54 in that glucocorticoid induction of the tyrosine aminotransferase gene is not detectable in CR1. Whether the TAT defect is related to the MTV defect is not known. Some other lines of HTC cells have been reported to yield subclones with widely differing levels of TAT induction at high frequency, in the absence of any selection,[9] but this was not observed with HTC lines used in this laboratory.[261] It seems unlikely that the TAT and MTV defects are related, since the MTV defect does not act in trans on newly introduced MTV genes. It is possible, however, that there is only a single defect, and that it affects a trans-acting factor that acts at a cellular regulatory site adjacent to one unusually strongly expressing MTV provirus and also at a site at the TAT

gene. In principle, this model could be tested by selecting for revertants of CR1 that express MTV at the wild type level, and then asking whether these revertants are also revertants for TAT induction. It should also be possible to ask directly if the defect affecting TAT expression is trans-acting by testing the expression of a transfected cloned TAT gene.[294]

7. Conclusions

These experiments clearly show that newly introduced MTV genes are transcribed and hormonally regulated indistinguishably in CR1 and M1.54 cells. Therefore, the defect in CR1 that affects MTV expression is not in a trans-acting factor that directly acts on MTV sequences. The exact nature of the defect in CR1 will be difficult to establish because of the large number of MTV proviruses, although the relevant proviruses can in principle be identified. A repeat of the selection on a cell line containing a single MTV provirus, or MTV-driven reporter gene, while it would make the identification of defects in trans-acting factors that act directly on MTV even more unlikely, would facilitate the analysis of cis-acting defects like the one apparently identified here. As GRE function can now be studied in S. cerevisiae,[205] it should also be possible to efficiently select and study both trans-acting and cis-acting defects in yeast. There may be a class of cis-acting regulatory elements, and trans-acting regulators, that are involved in differentiation in higher eukaryotes, and perhaps altered in the CR1 variant, that are not present in yeast, however.

The altered TAT induciblity in CR1 is not understoood. However, if the defect in CR1 that affects TAT expression is in a trans-acting factor, and if a transient transfection assay can be developed that detects this defect, then it should be possible to map the DNA sequences in the TAT gene at which this trans-acting factor acts. If the factor binds directly to DNA, then in principle it should be possible to use this interaction to purify it by DNA affinity chromatography,[295] or by using specific DNA binding as an assay for conventional purification. It should also be possible to ask whether the defect decreasing TAT induction is the same as that affecting MTV expression by rescuing or reverting the MTV defect, and asking if the TAT defect is simultaneously corrected.

Finally, the transient screen described here is rapid and straightforward, and can be efficiently used in the future to screen other variants in search of those defective in previously unknown trans-acting factors that directly act on MTV DNA sequences and are required for hormone-dependent enhancement of MTV transcription. If such lines are identified it may be possible to use them to clone the genes for trans-acting factors by rescuing the variant lines by DNA transfection, or, once an in vitro assay for enhancement is realized, to use extracts from the variant cell lines to assay for purification of these factors by in vitro complementation. Similarly, if the defect affecting TAT induction in CR1 proves to be trans-acting, then similar assays could be developed to allow the purification of this factor and/or the cloning of the gene that encodes it.

V. Summary and Discussion

1. Introduction

Experiments described in this dissertation attempted to identify nonreceptor proteins that function in glucocorticoid-dependent enhancement from promoters linked to MTV GREs, using genetic and biochemical approaches. Some progress was made in developing an in vitro assay for receptor effects on transcription. Recent advances, particularly the cloning of the receptor gene,[149] and high level expression of the receptor,[51,134] may facilitate further development of the in vitro assay. The genetic variant CR1, which transcribes its resident MTV proviruses at only about one-tenth the rate of its wild type parent, was shown not to be defective for trans-acting factors required for expression of newly introduced MTV genes, but rather to harbor defects affecting one or more of the resident proviruses in cis. A rapid and simple transient cis-trans test was developed to classify such variants.

2. Transcription in vitro

Addition of purified glucocorticoid receptor to in vitro transcription reactions stimulated transcription both from a promoter linked to an MTV GRE and from an enhancerless promoter. A number of technical problems plagued these experiments. These are described in Chapter 2, where suggestions for improvement of in vitro reconstitution

experiments are also presented. Addition of partially purified DNA topoisomerase II further stimulated transcription in vitro from both promoters. This phenomenon will be useful for increasing the sensitivity of in vitro transcription assays, and its mechanism is worth investigating in its own right.

Rates of transcription initiation in vitro are much slower than in vivo rates.[85,137] Investigation of the causes of this discrepancy might lead to conditions that would favor efficient enhancer function in vitro. Careful attention to rates of reactions and identification of factors contributing to in vivo rates has resulted in great progress in identifying and understanding the molecular machinery and mechanisms of other biological processes.[2] Perhaps an efficient in vitro transcription and enhancement assay could be developed by a stepwise progression from transcription in an intact cell through transcription in a minimally perturbed, gently permeabilized cell, and so on until eventually a cell-free, soluble system is attained. Transcription from a gene rapidly and strongly induced by glucocorticoids could be assayed by primer extension after a one hour treatment with hormone. In M1.54 cells MTV RNA is induced 10 fold after treatment with hormone for only four hours.[79] A gene with a higher basal level of expression that is even more inducible would be even more favorable. The ability to measure transcription by primer extension, without needing to label the RNA directly and without having to use hybrid selection to measure labelled RNA, would greatly accelerate the experiments, and greatly reduce their cost.

Initially, transcription could be compared between cells induced in normal medium plus serum to transcription in cells centrifuged free of medium, washed extensively, and resuspended in a buffer of choice. When the resuspension buffer composition and other aspects of the protocol were refined sufficiently so that transcription after induction was at the same rate in the washed cells as in the cells maintained in growth medium, then the next step could be attempted. The initial step could prove to be very important, as it might reveal components of medium or serum that are essential for the maintenance of normal transcription rates. Such factors might, for example, affect the concentrations of general transcriptional regulatory molecules or the extent of protein phosphorylations that are critical for transcription and/or induction. Identification of extracellular factors could lead to further investigations of the intracellular regulatory cascades they initiate, and to critical understandings of the constitutive and regulatory transcriptional machinery.

Once optimal conditions for cell harvest and resuspension were identified, it would be possible to compare transcription in washed, resuspended and detergent-permeabilized cells. Permeabilization with digitonin might be ideal, as it solubilizes the plasma membrane without permeabilizing lysosomes, and therefore does not release lysosomal nucleases and proteases that could interfere with the assay.[131] Then, when transcription in permeabilized cells was optimized to in vivo levels, transcription in purified nuclei could be attempted, and subsequently transcription in nuclear extracts. Such a stepwise procedure might identify previously unknown molecules and aspects of cell physiology that strongly affect

transcription. One cell character that might affect transcription is position in the cell cycle. Some genes are not transcribed in certain cell cycle phases. In fact, it has been reported that glucocorticoid induction of TAT is decreased during a large fraction of the cell cycle surrounding mitosis, based on experiments using HTC cells synchronized by mitotic arrest with colcemid.[138] Thus, comparison of transcription in extracts of synchronized cells might be revealing. Of special interest would be whether extracts from non-inducing cells contain dominant diffusible factors that could inactivate otherwise active extracts or templates.

At some point in the development of this assay, exogenous template would have to be added. Comparison of transcription of exogenous templates to that of endogenous chromosomal templates could reveal proteins and features of chromatin structure critical for proper transcriptional rates.

3. Transcription in Xenopus oocytes

Experiments described briefly here attempted to measure receptor-dependent GRE-specific stimulation of transcription in injected Xenopus oocytes. Although there may be other difficulties with such experiments, here again a major obstacle was the low concentration and amount of purified receptor. If such a system can be developed, it will be useful for determining the activity of preparations of purified receptor, and perhaps also as an alternative system for developing assays with permeabilized cells and cell extracts.

David Standring is currently trying to determine whether Xenopus oocytes will translate receptor mRNA and, if so, whether this receptor will be functional.

4. Characterization of the CR1 variant

The analysis of the CR1 variant cell line reported here clearly shows that CR1 does not carry defects in trans-acting factors that are required for expression of MTV and act directly on MTV DNA, since newly introduced MTV genes were expressed equivalently in CR1 cells and wild type cells. However, it is quite possible that other complement-resistant variants from the same selection do in fact carry defects affecting such trans-acting factors. The transient cis-trans test described here can be used to characterize the remaining CR variants.

Analysis of the CR1 defect has pointed out several potential pitfalls in such selection schemes for defects in trans-actaing factors in somatic cells, and improvements can be suggested. First, selection against high level expression from multiple genes does not necessarily favor non cis-acting mutations, since the possibility exists that just one gene accounts for most of the expression in the parent cell line. Second, selection against exogenously introduced genes, such as proviruses, that are genetically haploid at each chromosomal locus, may increase the frequency of undesired variants surviving selection due to inactivation of the introduced genes by epigenetic mechanisms that affect large chromosomal domains. Cells in which both alleles of a diploid gene have been inactivated

by such a mechanism probably would not survive in most cases because of the presence of linked lethals,[219] and therefore would not clutter the search for desired mutants. Fourth, many mutants may be difficult to select in HTC cells, since these cells are pseudo-tetraploid, having four copies of most genes.[302]

Some of these potential difficulties could be minimized by improved selection schemes. To decrease the likelihood of generation of the variant phenotype by a single cis-acting mutation, multiple differently marked selectable markers could be inserted into the genome and selected against or screened for. Thus, for example, lines could be infected or transfected with a GRE-*tk* fusion, a GRE-*aprt* fusion, a GRE-VSV-G fusion, etc.. The different selectable genes should be transfected independently, because co-transfected DNAs often become linked, and integrate together,[301] and the entire block of transfected DNA is often inactivated or even deleted at high frequency.[193,300] As much as possible, the different GRE-regulated transfected genes should be driven by different promoters that utlilize different promoter factors, to reduce the likelihood of selecting for defects in these promoter factors, and to favor the identification of previously unknown factors.

The high frequency of epigenetic inactivation of transfected selectable markers may be difficult to avoid, so the selection must be made to favor the desired mutations by selecting against multiple transfected markers following mutagenesis. Where possible, selection against endogenous diploid genes would be more desirable.

Selections in pseudotetraploid lines should be avoided, and instead selections should be attempted in pseudodiploid lines. Some cell lines have been found to be functionally hemizygous at many loci; these might be especially advantageous for selections, since the frequency of mutant isolation for hemizygous genes is of course much higher than the frequency of isolation of simultaneous mutations in both alleles of a homozygous diploid gene.[72] Since such cell lines are not hemizygous for all loci, it would be advantageous to perform the selection on multiple different partially hemizygous lines to maximize the likelihood of isolating the mutants of interest.

Even in ideal systems, somatic cell genetics is still quite cumbersome. The recent discovery of receptor-dependent stimulation of GRE-containing genes in yeast will allow genetic selections in this much more powerful system.[205] The availability of haploid strains and rapid gene mapping techniques, and the ease of handling large numbers of cells make yeast particularly useful for the isolation and genetic characterization of both cis-acting and trans-acting mutations. There may be factors affecting the tissue-specific pattern of receptor function in mammalian organisms which cannot be found in yeast.

5. Two models for the mechanism of receptor-dependent transcriptional enhancement

At this time, after the further analysis of the phenotype of the CR1 variant described

here, there is no strong genetic evidence suggesting the existence of nonreceptor factors specifically required for receptor-dependent enhancement. There remains, however, the biochemical observation of the 72 kd protein that co-purifies with the rat liver glucocorticoid receptor; I shall denote this protein GAP72 (glucocorticoid receptor-associated protein, 72kD).

Intriguingly, a protein of similar apparent molecular weight-- 74 kd in humans and 72 kd in mouse, RAP74, has independently been purified by Greenblatt and collaborators using an RNA polymerase II affinity column, and has been shown to be required for transcription from several promoters in vitro.[20,21] This factor appears to be identical to the TFIIE transcription factor that has been purified by Roeder and collaborators. RAP74 appears also to bind to another protein, RAP30, which also binds to polymerase II, and RAP30 appears to be identical to TFIIB.[21,188] TFIIE and TFIIB appear to act transiently on assembled transcription complexes (containing upstream promoter factors, TFIID, TFIIA, and RNA polymerase II) to trigger initiation.[21,188]

It is intriguing to speculate that GAP72 and TFIIE are identical. The rapid decrease in transcription from GRE-linked genes after removal of hormone suggests that enhancement of transcription by receptor might involve an interaction between receptor and just such a transiently acting transcription factor.[298] Furthermore, the lack of an effect of purified receptor on the DNA-binding of transcription factors Sp1 and CTF in vitro,[117] and the demonstration that the deletion of any of the known transcription factor binding sites in the

tk promoter, those for Sp1, CTF, TFIID, and the octamer factor, although decreasing the absolute level of transcription from the promoter, had no effect on its inducibility by glucocorticoids when it was linked to a GRE,[105] suggested that receptor function might involve an interaction with a different, non-DNA-binding transcription factor. Finally, when purified receptor containing GAP72 was added to in vitro transcription reactions, stimulation of transcription from promoters not linked to GREs as well as from promoters linked to GREs might be expected.

Two initial tests of the relatedness of GAP72 and TFIIE are in principle immediately possible. First, purified receptor containing GAP72 could be applied to an affinity column containing RNA polymerase II and TFIIB. Specific binding of GAP72 would suggest that GAP72 is in fact the same protein as TFIIE. Second, TFIIE-depleted extracts could be supplemented with purified receptor and GAP72. If the addition of GAP72 rescues the activity of the depleted extract, GAP72 is likely to be the same protein as TFIIE. In vitro transcription experiments could then be designed to ask if receptor enhances transcription by tethering TFIIE in the vicinity of promoters linked to GREs. The magnitude of such an effect in vitro should be amplified in transcription extracts depleted of excess TFIIE.

A second model for the mechanism of receptor-dependent transcriptional enhancement involves opening of chromatin structure by receptor, perhaps by removal of a nucleosome or by unfolding of a higher order structure. The presence of a glucocorticoid-induced

DNAse I hypersensitive site,[278] whose intensity is dependent on the receptor concentration,[284] suggests that the receptor may increase DNA accessibility in chromatin around GREs. The apparent independence of receptor-dependent enhancement of the presence of transcription factors Sp1, CTF, TFIID, and the octamer factor is also consistent with this model.[105] In yeast, some enhancer-like elements are poly(dA-dT) sequences.[225] Such sequences inhibit nucleosome formation in vitro,[116,183] and it has been argued that this might be the mechanism of their enhancer-like activity in vivo.[225] In support of this model, it was shown that the *ded1* poly(dA-dT) element increased transcription from a linked bacteriophage T7 promoter when the T7 RNA polymerase was produced in yeast cells.[26] The T7 RNA polymerase was selected because it has little homology with eukaryotic nuclear RNA polymerases, and therefore would be less likely to be stimulated by mechanisms requiring specific protein contacts. Similar experiments might test the chromatin accessibility model for glucocorticoid-dependent transcriptional enhancement, and are currently in progress.[196]

Other models for receptor-dependent transcriptional enhancement also remain consistent with current knowledge. These include direct interactions between receptor and the polymerase, receptor-induced DNA gyration that alters template structure, and receptor-dependent gene localization to a subnuclear compartment favoring transcription. Identification of regions of the receptor protein that are required for enhancement activity may facilitate distinguishing between these models. Some yeast UAS binding proteins contain discrete regions apparently required for transcriptional stimulatory activity.[93,130]

It has been argued that one general property of transcriptional activation regions is acidity.[224] Regions of the glucocorticoid receptor required for enhancement activity are currently being determined.[64,299] Their identifcation should factilitate analysis of receptor mechanism. Thus, affinity columns containing the activation region(s) of receptor and control columns containing mutant activation region(s) can be used to search for transcription factors or other proteins specifically interacting with the receptor, and receptor genes containing mutant activation regions can be used to search for genes encoding such factors by selecting for allele-specific second-locus suppressor mutations, most efficiently in yeast.

6. Future Prospects: Tissue-specific Hormonal Regulation of Gene Expression

Some genes are regulated by glucocorticoids in a tissue-specific manner. For example, the single gene encoding phosphoenolpyruvate carboxykinase is induced by glucocorticoids in liver and kidney, but repressed in adipose cells.[158] The mechanism by which such tissue-specific regulation occurs is unknown but potentially could involve combinatorial interactions of multiple different or differently modified transcription factors, with the total set of factors determining the basal activity and extent and direction of regulation of a gene.[262] According to this view, the effect of a regulatory protein on transcription from a gene would depend on the context of the interaction, that is on the

collection of other factors assembled at the gene in the particular cell type, at the particular developmental stage, and in the particular milieu of other contemporaneous regulatory inputs.

Such context dependencies of enhancer and promoter factors have been seen in this laboratory and elsewhere. For example, when the Moloney murine leukemia virus lymphoid-specific enhancer was combined with part of the kidney and salivary gland-specific polyomavirus enhancer a pancreas specificity was obtained.[191] As another example, two distinct octamer-binding transcription factors, OTF-1, which is a ubiquitous factor, and OTF-2, which is found in lymphoid cells but not in HeLa cells, have recently been purified.[50,204] OTF-1 and OTF-2 bind with similar or identical affinities to identical sequences containing the octamer motif in the immunoglobulin κ light chain gene promoter. However, OTF-1 stimulates transcription in vitro from the histone H2b promoter but not from the κ promoter, while OTF-2 stimulates transcription only from the κ promoter. It has been proposed that this selectivity might result from other transcription factors that are different at the two promoters or from different spacings between the octamer and binding sites for other factors.[50,204]

Hopefully, future biochemical and genetic analyses will eventually reveal which transcription factors act at each promoter in each cell type, at each developmental stage, and in each physiological state, how they interact, and at what steps they regulate transcription initiation. A complete explanation of development, however, will require understanding

many other processes, including how cell identities are asymmetrically distributed. This seems to involve the assymetric localization of specific proteins, a process which may be determined in each cell in part by pre-existing asymmetric positional information that is not encoded in the DNA genome.

Appendix 1: Methods

1. Solutions and Common Procedures

All solutions were made with doubly distilled water, and were either autoclaved or filter-sterilized. Common recombinant DNA methods were performed essentially as described in Maniatis et al.[305]

2. Transcription Extracts

Whole cell extracts of human HeLa cells were made by the method of Manley et al..[137] The extract used in the experiment shown in Figure 2 was a generous gift of Don Rio and Robert Tjian. The extract used in the experiment shown in Figures 6 and 7, and in most experiments, was prepared in collaboration with Kathy Jones and Robert Tjian. Extracts were prepared from cells actively growing (5-7 x 10^5 cell/ml) in suspension in F-12 medium supplemented with 5% calf serum. Cell pellets were stored at -20 ^oC until used. Storage at -70 ^oC might be preferable to provide greater protection against accidental thawing.

Nuclear extracts were prepared, according to Dignam and Roeder.[306] Such extracts prepared from cells treated with hormone should contain very little receptor, while those prepared from hormone-treated cells should contain receptor that was translocated to the nucleus upon binding hormone. Nuclear extracts prepared from HeLa cells grown as monolayers seemed especially active. Subsequent experiments by others using such extracts have so far failed to detect transcriptional enhancement by receptor.[239]

3. Receptor

Purified rat liver glucocorticoid receptor was generously supplied by our collaborators in the Gustafsson laboratory. Unless otherwise indicated, cytosolic receptor was purified by phosphocellulose chromatography, DNA-cellulose chromatography on nonspecific DNA, before and after heat-activation of DNA-binding activity, and DEAE-Sepharose chromatography, as described in detail elsewhere.[255,256] Receptor was eluted from the final DEAE-Sepharose column with a NaCl gradient, eluting at approximately 110 to 125 mM NaCl, in 20 mM Tris pH7.9 (adjusted at 2 to 4 °C), 1 mM EDTA, 20% glycerol, and 1 uM triamcinolone acetonide. Insulin and DTT were then added, to 250 μg/ml and 20 mM, respectively. Receptor was shipped to our laboratory on dry ice, and was subsequently stored at -70 °C. Fresh DTT was added to 20 mM and $MgCl_2$ to 2.8 mM upon thawing, unless otherwise noted.

The nature of the insulin added to the receptor may be an important issue. Many preparations of insulin contain Zn^{+2} in millimolar amounts. RNA polymerase II requires bound Zn^{+2}.[297] The DNA binding domain of the glucocorticoid receptor appears from primary structure to contain two "zinc fingers",[307,308] and recent EXAFS (extended X-ray absorption fine structure) studies show two atoms of zinc bound to each molecule of the receptor.[309] Recent experiments suggest that the specific DNA binding activity of transcription factor Sp1, another zinc finger protein, has a sharp optimum for Zn^{+2}, binding strongly at 400 μM Zn^{+2}, while having no activity at 800 μM Zn^{+2}.[312,313] Thus, Zn^{+2} added to receptor with insulin may possibly have inhibited the DNA binding or

transcriptional acitiviation activities of receptor in the experiments described here.

The receptor preparations were not homogeneous. The principle contaminating protein, which was consistently observed, was GAP72 (see Chapter 5).

4. In Vitro Transcription Reactions

The reaction procedure was generally similar from experiment to experiment, with minor differences as noted. In the experiment shown in Figure 2, 10 µl of extract was mixed with 2.5 µl of 10x rNTPs (2.5 mM each, final concentration 250 µM), and 1.6 µl of 15.6x creatine phosphate mix (78 mM Tris-HCl pH 7.9, 156 mM KCl, 19.53 mM $MgCl_2$, 7.2 mM Na_2EDTA, 1 mM DTT, 50% glycerol, 78 mM creatine phosphate, and 3.1 mg/ml creatine phosphokinase) at 4 °C. This mixture was then added to the DNA and the reaction was incubated for 30 min at 30 °C. The final concentrations in the reaction were 25 mM Tris pH7.9, 50 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM Na_2EDTA, 0.5 mM DTT, 10% glycerol, 5 mM creatine phosphate, 0.2 mg/ml creatine phophokinase, 250 µM each rNTP, including 10 µl extract. The creatine phosphate and creatine phosphokinase ATP regeneration system was included because of a report that it stimulated in vitro transcription.[77] Subsequent experiments showed that the ATP regeneration system did not stimulate transcription with the extracts used; therefore, it was not added to most later experiments. A broad optimum was observed for Mg^{+2} between 2 mM and 10 mM, in agreement with previous reports.[296] DTT between 0.5 and 10 mM, and glycerol between 10 % and 20 % had little effect.

DNA was added as supercoiled plasmids, unless otherwise noted. Analysis of DNA

after a 30 min reaction showed that approximately 70% of the plasmid was nicked or relaxed, and approximately 30% was linear. A small amount of plasmid (approximately 0.1% to 1%) continued to migrate in agarose gels at the position of supercoiled plasmid (data not shown).

For the experiments shown in Figures 6 and 7, conditions were modified to allow the addition of receptor. All components were aliquoted at 4 ^{o}C . For each 75 µl reaction, to 15 µl rNTPs (2.5 mM each) were added, in order: water to volume, 3 µl plasmid (9 ng pΨaAA'1 in Figure 6, 9 ng each pΨaAA'1 and paTK in Figure 7), and 27 µl of receptor buffer (20 mM Tris pH 7.9 (at 4 ^{o}C), 125 mM NaCl, 1 mM EDTA, 20% glycerol, 20 mM DTT, 250 µg/ml insulin, 1µM dexamethasone, and 2.8 mM $MgCl_2$), containing varying amounts of receptor. This was incubated at 30 ^{o}C for 15 min. Poly(dI-dC) (900 ng, from a 500 ng/µl stock) was added to a separate tube. Extract (22.5 µl) was then added to the poly(dI-dC) tube, which was then incubated at 30 ^{o}C for 2 min, at which time the extract plus poly(dI-dC) was transferred to the tube containing DNA and receptor, and the reaction was incubated at 30 ^{o}C for an additional 30 min. The final concentrations in the reaction were 25 mM Tris pH7.9, 75 mM NaCl, 4.8 mM $MgCl_2$, 0.44 mM EDTA, 12.3% glycerol, 7.8 mM DTT, 90 µg/ml insulin, 0.36 µM dexamethasone, 500 µM each rNTP, 22.5 µl extract and 9 ng plasmid per 75 µl reaction, and the indicated amount of poly(dI-dC).

The concentration of transcription factors in the reaction may be a critical variable, as the experiments were done just at the crossover point between template excess and factor

excess. Titration of DNA in a 25 µl reaction mix containing 12.5 µl of the extract used for all of the in vitro enhancement experiments, showed that at optimal concentrations of poly(dI-dC) transcription was constant between 9 ng and 100 ng of template, suggesting transcription factor excess. Experiments designed to detect enhancement used 75 µl reactions containing 9 ng of template, 27.5 µl of the extract, and receptor. Thus, these conditions may have been just on the edge of factor excess. This might explain why enhancement was seen in some cases but not in others, since, if receptor enhances transcription by stimulating the binding of one or more transcription factors, then in cases where the extract was in excess no enhancement would be seen. The inhibition of transcription seen at the highest concentration of receptor is not inconsistent with this possibility if the inhibition reflects steric interference with the binding of transcription factor Sp1 (see Chapter 2).

Nucleic acids were isolated after phenol/chloroform extraction and addition of 60 µg tRNA by precipitation on dry ice after the addition of 2.5 volumes of 0.45 M ammonium acetate dissolved in 100% ethanol, resuspended in 25 µl of distilled water, and quantitated by primer extension.

5. Primer Extension

A synthetic oligonucleotide primer complementary to wild type *tk* mRNA from +82 bp to +58bp relative to the major initiation sites was labelled at the 5' end with ^{32}P using T4 polynucleotide kinase. The oligonucleotide was a generous gift of Robert Tjian. After phenol/chloroform extraction and addition of tRNA carrier, labelled primer was purified by

multiple rounds of precipitation by the addition of 2.5 volumes of 0.45 M ammonium acetate in 100% ethanol, and was then resuspended in 6x hybridization salts (1x is 250 mM KCl, 100 mM Tris pH8.0, 10 mM EDTA). Primer (~100 fmol in 5 µl) and RNA (25 µl) were mixed and incubated at 62 °C for 1 hr, at which time hybridization was complete. The tubes were then cooled briefly on ice, and then extension was initiated by the addition of 70 µl of reverse transcriptase mix (10 mM $MgCl_2$, 10 mM Tris pH7.5, 5 mM DTT, 100 µg/ml actinomycin D, 100 units/ml reverse transcriptase obtained from Life Sciences). Extension was for 1 hr at 37 °C. Nucleic acids were precipitated by the addition of 2.5 volumes of ethanol, and subsequently analyzed on an 8% denaturing acrylamide gel.

6. Filter Binding

Plasmid pA2, which contains the Eco R1 MTV insert from pMTV2 cloned into the Eco R1 site of pML2,[311] was provided by V. Chandler. The plasmid was digested with Eco R1 and labelled with α^{32}PdATP using T4 DNA polymerase. Labelled fragments (60 pM) were incubated with varying concentrations of receptor in 20 mM Tris pH 7.8 (at 25 °C), 180 mM NaCl, 1 mM EDTA, 10% glycerol, 2 mM β-mercaptoethanol, 100 µg/ml insulin, and 1µM dexamethasone for 1 hr at 4 °C in a total volume of 250 µl. Receptor was from the 82/3/10 batch.

Nitrocellulose filters (BA85, 27 mm; Schleicher and Schuell) were presoaked for 1 hr in wash buffer (10 mM Hepes pH7.4, 20 mM KCl, 20 mM DTT). Reactions were filtered at 250 µl per 1-2 min, and then allowed to dry for 1-3 min before washing. Filters were then washed twice with 0.5 ml of wash buffer, and allowed to dry for another 90 sec.

Filter bound radioactivity was determined by Cerenkov counting. DNA was then eluted with 300 µl elution buffer (10 mM Tris pH7.4, 20 mM NaCl, 0.1 % SDS, 0.1 mM EDTA) for 30 min at 25 ^{0}C, with occasional vortexing. After addition of 10 µg yeast total RNA carrier and NaAc to 0.3 M, nucleic acids were precipitated with ethanol, and DNA was analyzed by electrophoresis on a 1% agarose gel followed by autoradiography.

7. Methylation Footprinting

Methylation reactions were performed essentially as described by Maxam and Gilbert.[142]

The DNA fragment used in the experiment shown in Figure 4 was a Hha I to Bam H1 fragment containing MTV sequences from -1089 bp to -106 bp relative to the MTV mRNA start site. The fragment is from pPS1, which contains the Pst I to Sst I fragment from the MTV LTR inserted into pBR322 via Bam H1 linkers.[25] After digestion with Bam H1 and labelling with α^{32}PdGTP using T4 DNA polymerase, the DNA was digested with Hha I, and the desired fragment was purified by acrylamide gel electophoresis, electroelution, and ethanol precipitation. DNA and receptor were incubated in 20 mM Tris pH 7.8 (at 25 ^{o}C), 180 mM NaCl, 1 mM EDTA, 10% glycerol, 2 mM β-mercaptoethanol, 100 µg/ml insulin, and 1µM dexamethasone for one hour at 4 ^{o}C.

The DNA fragment used in the experiment shown in Figure 5 was a Pst I to Bam HI fragment from pMTV5, containing sequences from +484 to +1281 relative to the Bam H1 site approximately 4.3 kbp downstream of the mRNA initiation site. After digestion of

pMTV5 with Bam H1 and labelling with ^{32}P using T4 polynucleotide kinase, the labelled DNA was digested with Pst I, and the desired fragment was purified by electrophoresis on an acrylamide gel followed by electroelution. Receptor used in the experiment shown in Figure 5 was from the 11/24/82 batch. DNA and receptor were incubated in 20 mM Tris pH8.0, 20% glycerol, 1 mM EDTA or 1 mM $MgCl_2$, 55 mM NaCl, 250 μg/ml insulin, and 1 μM dexamethasone for 15 min at 25 °C.

After binding, dimethyl sulfate was added to the reactions to 0.5%. After 5-15 min on ice, 300 mM NaAc pH7.0, 1.0 M mercaptoethanol (to quench the DMS), and tRNA carrier were added, and the DNA was precipitated twice with ethanol. DNA was then resuspended in 100 μl of 20 mM NH_4Ac pH 8.0, 0.1 mM EDTA and incubated for 15 min at 90 °C. Piperidine was then added to 1 M, and incubation at 90 °C was continued for another 30 min. After lyophilization and resuspension in loading solution containing 80% formamide, DNA was analyzed by electrophoresis on 10% or 12% acrylamide gels followed by autoradiography.

8. Growth of HTC Cells.

Rat hepatoma HTC cells were grown in monolayer culture at 37 °C, and were passaged after detachment using either PBS-TE (Dulbecco's Phosphate-Buffered Saline plus 10 mM Tris-HCl pH7.4 and 2 mM Na_2EDTA) or trypsin. In early experiments cells were grown in DME-H16 (Dulbecco's Modified Eagle's medium with glutamine and glucose) plus 10 % calf serum supplemented with iron. In later experiments cells were grown in medium supplemented with 10 % fetal calf serum, as it had been found that some

component of supplemented calf serum activated the glucocorticoid receptor and therefore slightly induced MTV transcription even in the absence of added dexamethasone.[64,79,196] DME, PBS, and STV were obtained from the UCSF Cell Culture Facility. Sera were obtained from HyClone Laboratories.

9. DNA topoisomerase II

Calf thymus DNA topoisomerase II, batch P 16, was a gift of Leroy Liu.

10. Transcription in Xenopus oocytes

Stage 5 or 6 Xenopus laevis oocytes were isolated and injected as previously described.[222] For co-injection of receptor and DNA the two solutions were mixed on the injection slide. Nucleic acids were isolated, and *tk* RNA was measured by primer extension.

11. RNAase Protections

RNAase protection assays were modifications of the methods described by Myers et al.,[303] and by Winter et al..[304] Antisense probes complementary to C3H RNA were transcribed in vitro using SP6 RNA polymerase and α-^{32}P rCTP. An aliquot of the probe was examined by acrylamide gel electrophoresis, followed by autoradiography. If contamination with smaller or larger labelled RNAs was substantial the entire probe preparation was purified by electrophoresis and electroelution. The probe was synthesized from pLT64 cleaved with Eco RI.

Total RNA (10 μg) was precipiated with ethanol, and then resuspended in 30 μl

hybridization buffer (80% formamide, 40 mM Pipes pH6.7, 0.4 M NaCl, 1 mM EDTA). To this was added 0.1 to 1 pmol probe in 1 µl hybridization buffer. The mixture was heated to 85 °C for 10 min, and then incubated overnight at 37 °C. For digestion of unhybridized RNA and cleavage at mismatches, 40 µg/ml to 80 µg/ml RNAase A was added in 300 µl of digestion buffer (10 mM Tris pH7.5 at 25 °C, 5 mM EDTA, 300 mM NaCl), and the reaction was incubated at 30 °C for 30 min. Digestion was stopped by the addition of 20 µl 10% SDS, 50 µg proteinase K, and incubation at 37 °C for 15 min. After addition of tRNA carrier and phenol/chloroform extraction, nucleic acids were precipitated with ethanol, and then analysed by acrylamide gel electrophoresis followed by autoradiography.

A preliminary titration showed that 40 µg/ml RNAase A provided sufficient cleavage of mismatched hybrids of endogenous MTV GR strain RNA and C3H strain probe to allow unobscured measurement of RNA transcribed from newly infected or transfected C3H strain MTV genes. At 120 µg/ml RNAase A or higher, degradation of the C3H-C3H hybrids began to be observed (Figure 12). Therefore 40 to 80 µg/ml RNAaseA was used for subsequent experiments.

12. Plasmids

Plasmid pLTL-1 was previously described.[278] Plasmid pLT64 was constructed by inserting a fragment from the Acc I site at ~+557 to the Sst I site at -106 into pSP64.

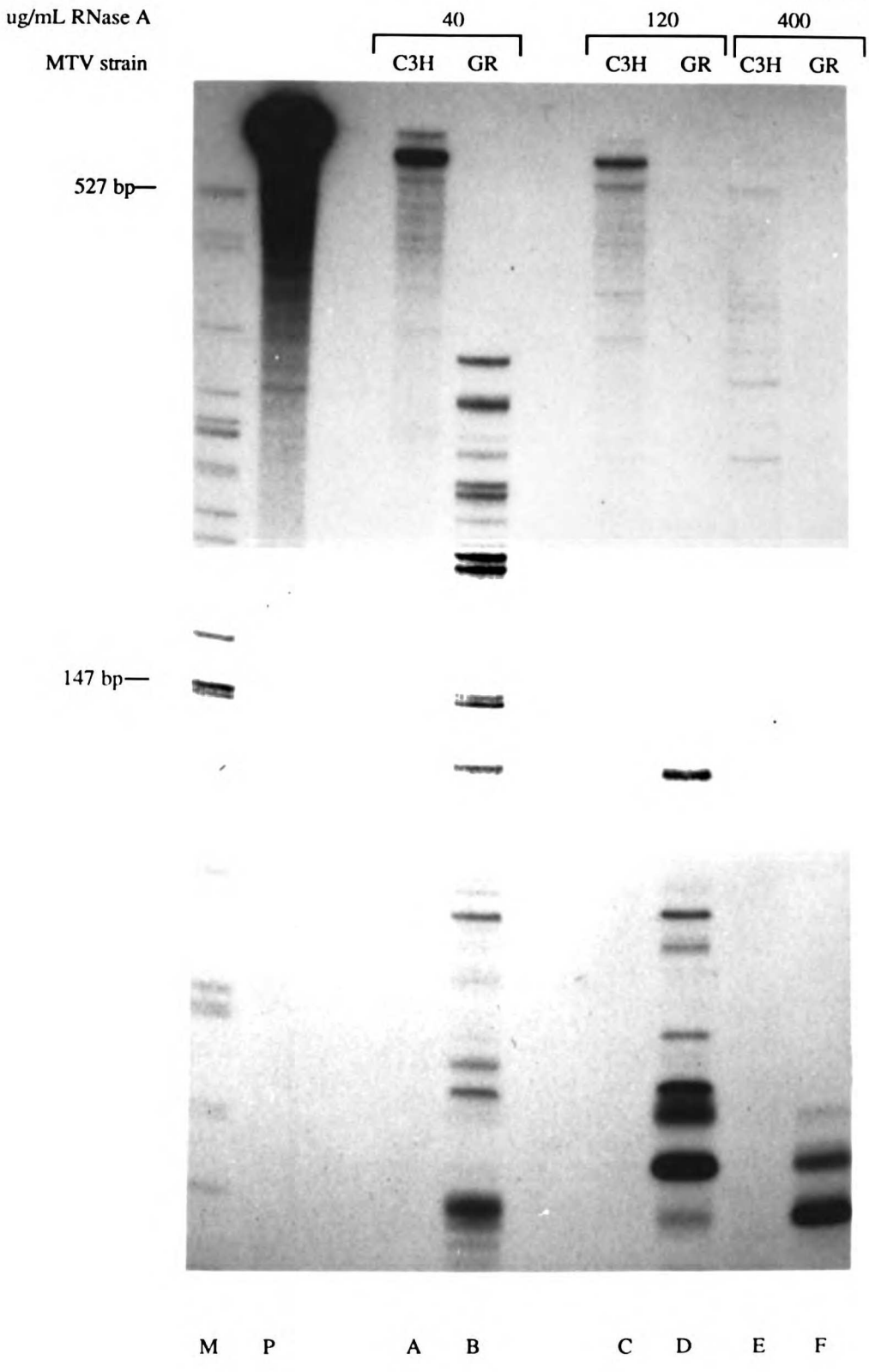
Plasmid pGMCO-gr/ch was constructed from pLC1 [174] by the following procedure:

Figure 12

Determination of RNAase A Concentrations for Distinguishing C3H from GR Strain MTV RNA by RNAase Protection

Total hormone-induced RNA from L1.2.2, an L cell line stably transfected with the C3H strain based plasmid pLTL-1, or from M1.54, a line infected with GR strain MTV, was hybridized with a radioactively labelled antisense C3H RNA probe, as described in the text, and then digested with varying concentrations of RNAase A, as indicated. The digested products were measured by electorphoresis on an acrylamide gel, followed by autoradiography.

Figure 12


ug/mL RNase A
40
120
400
MTV strain
C3H
GR
C3H
GR
C3H
GR
527 bp—
147 bp—
M
P
A
B
C
D
E
F

The C3H strain MTV LTR of pLC1 was removed by digestion with Hind III, and replaced with a Hind III-linked GR strain MTV LTR. The GR strain LTR was isolated as a 1.45 kbp Pst I fragment.[46] To convert the Pst I fragment to a Hind III fragment, an intermediate vector was constructed with Hind III sites flanking a Pst I site: the "Bluescript" plasmid Eco RI to Acc I fragment containing a Hind III site was inserted into the large Eco RI to Acc I fragment of pSP64. The Pst I GR LTR fragment was then cloned into this intermediate vector, and excised as a Hind III fragment for the construction of pGMCO-gr/ch.

13. Stable transfections

Cells were transfected using the calcium-phosphate coprecipitation method described by Wigler et al. with the following modifications.[249] Cells were plated at 5 x 10^5 cells per 100 mm dish 24 hours prior to transfection, and were re-fed with fresh medium one hour prior to transfection. Cells were transfected with 5 μg pLTL-1 and 1 μg pSV40neo (pKOneo; D. Hanahan, personal communication) per 100 mm dish. Upon addition of the DNA:calcium phosphate coprecipitate to the cells, 0.25 ml of 4 mM chloroquine in PBS was added to the cells for a final concentration of 100 μM chloroquine.[129] After 4 hours, the medium was removed by aspiration, and the cells were subjected to glycerol shock for 2 min in 2 ml of growth medium containing 20% glycerol.[164] The glycerol was then diluted with 8 ml of PBS and this was aspirated. After a wash with another 8 ml of PBS, cells were fed with growth medium. After 2 days, selection was applied by changing to growth medium containing 300 μg/ml G418 (GIBCO). When large surviving colonies

became visible colonies were picked by scraping with plastic pipet tips.

14. Retroviral infections

Cells were infected by a modification of the method previously described.[190] Concentrated strain C3H MTV from Frederick Cancer Research Center was a generous gift of Greg Shackleford and Harold Varmus. Cells were plated at 4 x 10^5 per 100 mm dish on the day prior to infection. One hour prior to infection cells were re-fed with medium plus 20 µg/ml polybrene without serum. This was then replaced with 100 µl virus plus 2 ml medium plus serum, and the plates were rocked every ten minutes for 2hr to prevent drying. Additonal medium plus serum (10 ml) was then added, and 24 hr later the medium was replaced with fresh medium plus serum. Determination of viral titer by quantitation of viral RNA purified after addition of tRNA carrier, using known amounts of M1.54 RNA as standards, showed that the infection procedure applied approximately 10^6 viral particles per cell. Such a high multiplicity of infection was previously shown necessary for efficient infection of these rat HTC cells.[190]

15. Transient transfections

Transient expression assays used calcium-phosphate transfections as described above, but with the essential modifications described below. Initial attempts using standard protocols were unsuccessful. M1.54 cells were particularly difficult to transfect, and CAT

activity expressed from pGMCS was only marginally detectable above the activity of extracts from mock-transfected cells, even when the transfected cells were induced with hormone. Co-transfection with pRSV-βgal was essential to allow correction for varying transfection efficiencies. Cells were plated at 6 x 10^5 per 60 mm dish on the day prior to transfection, and were transfected with 10 μg pGMCO-gr/ch and 4 μg pRSVβ-gal per dish.

Three modifications gave consistently measurable expression. First, the 2x Hepes-phosphate solution was adjusted to pH 7.00 with dilute (200 mM) NaOH after mixing the Hepes and sodium phosphate solutions. Relative to the expression after transfection with pH 7.00 Hepes-phosphate, use of pH 7.02 gave 50% expression, pH 7.10 gave 10% expression, pH 6.92 gave 50% expression. Second, CAT expression from the pGMCO/gr-ch plasmid was significantly higher than expression from pGMCS, even though the latter plasmid contains an MSV enhancer downstream of the CAT gene. The higher expression from the pGMC0/gr-ch plasmid is probably a result of the deletion from the vector of pBR322 sequences that inhibit transcription in eukaryotes.[174] Third, decreasing the concentration of ^{14}C chloramphenicol in the CAT assay as described below was essential to lower the background. In addition, the use of fresh $CaCl_2$ stock solution seemed to improve transfections.

Other modifications of the transfection protocol decreased expression. Decreasing the chloroquine concentration to 33 μg/ml decreased expression slightly. Increasing chloroquine to 300 μg/ml, or eliminating choloroquine treatment, decreased expression

about three fold. Omission of the glycerol shock decreased expression, while increasing the glycerol to 25% had no effect. Attempts to adapt and optimize the DEAE-dextran transfection protocol [128] resulted in high yields of CAT activity on a per cell basis, but a lower total activity due to extensive cell killing. Transfection by electroporation,[28] by lipofection,[47] or by scrape-loading,[146] were less successful. Transfection of cells at various positions in the cell cycle after synchronization by thymidine block, in hopes of facilitating nuclear incorporation of transfected DNA at the time of nuclear membrane breakdown, was also unsuccessful. Growth of cells in the presence of 2-aminopurine, which is thought to stimulate translation of mRNA transcribed from transfected plasmids by inhibiting a kinase that is stimulated by double stranded RNA (as might be formed from antisense RNA transcribed from adventitious promoters encoded in plasmid sequences) and that locally inhibits eIF-2 activity,[112] had no effect on CAT expression.

16. Preparation of cell extracts

Cells were rinsed with TBS (150 mM NaCl, 10 mM Tris pH 7.5), and then scraped off the plates in TBS. After centrifugation at 300 xg for 5 min, cells were resuspended in 250 mM Tris pH7.5 (150 µl), and lysed by sonication with a microtip for 5 sec. After centrifugation for 10 min at 13,000 xg, the supernatant was transferred to a new tube and its protein concentration was determined. An aliquot of the supernatant was used to determine β-galactosidase activity. Extracts were not frozen before determination of β-galactosidase activity. The remainder of the supernatant was incubated at 65 °C for 10

min to inactivate an activity that interferes with the CAT assay. This step was essential for efficient detection of CAT activity from HTC cells. The heated extract was then centrifuged for 10 min at 13,000 xg, and the supernatant was transferred to a new tube. After determination of protein concentration, CAT activity was measured.

17. CAT assays and β-galactosidase assays

CAT assays were performed essentially as described in Gorman et al.[68] However, the amount of ^{14}C-chloramphenicol added was 0.1 μCi. Reactions proceeded overnight.

β-galactosidase assays were performed as described by An et al.[3] The extent of the reaction was determined by measurement of the absorbance at 420 nm.

CAT activities were normalized to β-galactosidase activities.

References

1. Aguilera, R. J., Hope, T. J., and Sakano, H. (1985). Characterization of immunoglobulin enhancer deletions in murine plasmacytomas. EMBO J. 4, 3689-3693.

2. Alberts, B. M. (1985). The DNA enzymology of protein machines. Cold Spring Harbor Symp. Quant. Biol. 49, 1-12.

3. An, G., Hikada, K., and Siminovitch, L. (1982). Expression of bacterial β-galactosidase in animal cells. Mol. Cell. Biol. 2, 1628-1632.

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

5. Ares, M., Jr., Chung, J.-S., Giglio, L., and Weiner, A. M. (1987). Distincit factors with Sp1 and NF-A specificities bind to adjacent functional elements of the human U2 snRNA gene enhancer. Genes and Devel. 1, 808-817.

6. Atchison, M. L., and Perry, R. P. (1986). Tandem kappa immunoglobulin promoters are equally active in the presence of the kappa enhancer: implications for models of enhancer function. Cell 46, 253-262.

7. Atchison, M. L., and Perry, R. P. (1987). The role of the κ enhancer and its binding factor NF-κB in the developmental regulation of κ gene transcription. Cell 48, 121-128.

8. Augereau, P., and Chambon, P. (1986). The mouse immunoglobulin heavy-chain enhancer: effect on transcription in vitro and binding of proteins present in HeLa and lymphoid B cell extracts. EMBO J. 5, 1791-1797.

9. Aviv, D., and Thompson, E. B. (1972). Variation in tyrosine aminotransferase induction in HTC cell clones. Science 177, 1201-1203.

10. Banerji, J., Olson, L., and Schaffner, W. (1983). A lymphocyte-specific cellular enhancer is located

downstream of the joining region in immunoglobulin heavy chain genes. Cell 33, 729-740.

11. Banerji, J., Rusconi, S., and Schaffner, W. (1981). Expression of a β-globin gene is enhanced by remote SV40 sequences. Cell 27, 299-308.

12. Bergman, Y., Rice, D., Grosschedl, R., and Baltimore, D. (1984). Two regulatory elements for immunoglobulin κ light chain gene expression. Proc. Natl. Acad. Sci. USA 81, 7041-7045.

13. Bienz, M., and Pelham, H. R. B. (1986). Heat shock regulatory elements function as an inducible enhancer in the Xenopus *hsp70* gene and when linked to a heterologous promoter. Cell 45, 753-760.

14. Bogenhagen, D. F., Wormington, W. M., and Brown, D. D. (1982). Stable transcription complexes of Xenopus 5S RNA genes: a means to maintain the differentiated state. Cell 28, 413-421.

15. Bohmann, D., Keller, W., Dale, T., Scholer, H. R., Tebb, G., and Mattaj, I. W. (1987). A transcription factor which binds to the enhancer of SV40, immunoglobulin heavy chain and U2 snRNA genes. Nature 325, 268-272.

16. Bradley, W. E. C., and Letovanec, D. (1982). High-frequency nonrandom mutational event at the adenine phosphoribosyltrasnferase (*aprt*) locus of sib-selected CHO variants heterozygous for *aprt*. Somat. Cell Gen. 8, 51-66.

17. Bradley, W. E. C. (1983). Mutation at autosomal loci of Chinese hamster ovary cells: involvement of a high-frequency event silencing two linked alleles. Mol. Cell. Biol. 3, 1172-1181.

18. Breathnach, R., and Chambon, P. (1981). Organization and expression of eucaryotic split genes coding for proteins. Ann. Rev. Biochem. 50, 349-384.

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

20. Burton, Z. F., Ortolan, L. G., and Greenblatt, J. (1986). Proteins that bind to RNA polymerase II are

required for accurate initiation of transcription at the adenovirus 2 major late promoter. EMBO J. 5, 2923-2930.

21. Burton, Z. F., Killeen, M., Sopta, M., Ortolan, L. G., and Greenblatt, J. (submitted). RAP30/74: A general initiation factor that binds to RNA polymerase II.

22. Cai, H., and Luse, D. S. (1987). Variations in template production by the RNA polymerase II transcription complex during the initiation process. Mol. Cell. Biol. 7, 3371-3379.

23. Calame, K. (1985). Mechanisms that regulate immunoglobulin gene expression. Ann. Rev. Immunol. 3, 159-195.

24. Carthew, R. W., Chodosh, L. A., and Sharp, P. A. (1985). An RNA polymerase II transcription factor binds to an upstream element in the adenovirus major late promoter. Cell 43, 439-448.

25. Chandler, V. L., Maler, B. A., and Yamamoto, K. R. (1983). DNA sequences bound specifically by glucocorticoid receptor in vitro render a heterologous promoter hormone responsive in vivo. Cell 33, 489-499.

26. Chen, W., Tabor, S., and Struhl, K. (1987). Distinguishing between mechanisms of eukaryotic transcriptional activation with bacteriophage T7 RNA polymerase. Cell 50, 1047-1055.

27. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18, 5294-5299.

28. Chu, G., Hayakawa, H., and Berg, P. (1987). Electroporation for the efficient transfection of mammalian cells with DNA. Nucl. Acids Res. 15, 1311-1326.

29. Church, G. M., Ephrussi, A., Gilbert, W., and Tonegawa, S. (1985). Cell type specific contancts to immunoglobulin enhancer in nuclei. Nature 313, 798-801.

30. Darbre, P. D., and King, R. J. B. (1984). Progression to steroid autonomy in S115 mouse mammary tumor cells: role of DNA methylation. J. Cell Biol. 99, 1410-1415.

31. Darbre, P. D., and King, R. J. B. (1987). Progression to steroid insensitivity can occur irrespective of the presence of functional steroid receptors. Cell 51, 521-528.

32. Davidson, I., Fromental, C., Augereau, P., Wildeman, A., Zenke, M., and Chambon, P. (1986). Cell-type specific binding to the enhancer of simian virus 40 in nuclear extracts. Nature 323, 544-548.

33. Davison, B. L., Egly, J. M., Mulvihill, E. R., and Chambon, P. (1983). Formation of stable preinitiation complexes between eukaryotic class B transcription factors and promoter sequences. Nature 301, 680-686.

34. DeFranco, D., and Yamamoto, K. R. (1986). Two different factors act separately or together to specify functionally distinct activities at a single transcriptional enhancer. Mol. Cell. Biol. 6, 993-1001.

35. deVilliers, J. and Schaffner, W. (1981). A small segment of polyoma virus DNA enhances the expression of a cloned rabbit β-globin gene over a distance of at least 1400 base pairs. Nucl. Acids Res. 9, 6251-6264.

36. Dorn, A., Bollekens, J., Staub, A., Benoist, C., and Mathis, D. (1987). A multiplicity of CCAAT box-binding proteins. Cell 50, 863-872..

37. Dudler, R., and Travers, A. A. (1984). Upstream elements necessary for optimal function of the hsp 70 promoter in transformed flies. Cell 38, 391-398.

38. Dynan, W., and Tjian, R. (1985). Control of eukaryotic mRNA synthesis by sequence-specific DNA binding proteins. Nature 316, 774-778.

39. Eckhardt, L. A., and Birshtein, B. K. (1985). Independent immunoglobulin class-switch events occurring in a single myeloma line. Mol. Cell. Biol. 5, 856-868.

40. Edlund, T., Walker, M. D., Barr, P. J., and Rutter, W. J. (1985). Cell-specific expression of the rat1 insulin gene: evidence for role of two distinct 5'-flanking elements. Science 230, 912-916.

41. Eisenberg, S. P., Coen, D. M., and McKnight, S. L. (1985). Promoter domains required for expression of plasmid-borne copies of the herpes simplex virus *tk* gene in virus-infected mouse fibroblasts and microinjected frog oocytes. Mol. Cell. Biol. 5, 1940-1947.

42. Elion, E. A., and Warner, J. R. (1986). An RNA polymerase I enhancer in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 6, 2089-2097

43. Ephrussi, A., Church, G. M., Tonegawa, S., and Gilbert, W. (1985). B lineage-specific interaction of an immunoglobulin enhancer with cellular factors in vivo. Science 227, 134-140.

44. Evans, R. M.; personal communication.

45. Falkner, F. G., and Zachau, H. G. (1984). Correct transcription of an immunoglobulin κ gene requires an upstream fragment containing conserved sequence elements. Nature 310, 71-74.

46. Fasel, N., Pearson, K., Buetti, E., and Diggelman, H. (1982). The region of mouse mammary tumor virus DNA containing the long terminal repeat includes a long coding sequence and signals for hormonally regulated transcription. EMBO J. 1, 3-7.

47. Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielson, M. (1987). Lipofection: A novel, highly efficient lipid mediated DNA transfection procedure. Proc. Natl. Acad. Sci. USA, in press.

48. Fire, A., Samuels, M., and Sharp, P. A. (1984). Interactions between RNA polymerase II, factors, and template leading to accurate transcription. J. Biol. Chem. 259, 2509-2516.

49. Firestone, G. L. and Yamamoto, K. R. (1983). Two classes of mutant mammary tumor virus-infected HTC cell with defects in glucocorticoid-regulated gene expression. Mol. Cell. Biol. 3, 149-160.

50. Fletcher, C., Heintz, N., and Roeder, R. G. (1987). Purification and characterization of OTF-1, a transcription factor regulating cell cycle expression of a human histone H2b gene. Cell 51, 773-781.

51. Freedman, L., unpublished results.

52. Friedman, R., and Stark, G. (1985). α-interferon induced transcription of HLA and metallothionein genes containing homologous upstream sequences. Nature 314, 637-639.

53. Fujita, T., Ohno, S., Yasumitsu, H., and Taniguchi, T. (1985). Delimitation and properties of DNA sequences required for the regulated expression of human interferon-β gene. Cell 41, 489-496.

54. Fuller,R. S., Kaguni, J. M., and Kornberg, A. (1981). Enzymatic replication of the origin of the Escherichia coli chromosome. Proc. Natl. Acad. Sci. USA 78, 7370-7374.

55. Fulton,A.B. (1982). How crowded is the cytoplasm? Cell 30, 345-347.

56. Gallagher, M., Weinberg, R., and Simpson, M. V. (1986). Effect of the bacterial DNA gyrase inhibitors, novobiocin, nalidixic acid, and oxolinic acid, on oxidative phosphorylation. J. Biol Chem. 261, 8604-8607.

57. Garabedian, M. J., Shepherd, B. M., and Wensink, P. C. (1986). A tissue-specific transcription enhancer from the Drosophila yolk protein 1 gene. Cell 45, 859-867.

58. Garabaeian, M. J., Hung, M.-C., and Wenskink, P. C. (1985). Independent control elements that determine yolk protein gene expression in alternative Drosophila tissues. Proc. Natl. Acad. Sci. USA 82, 1396-1400.

59. Garabedian, M., unpublished results.

60. Gerster, T., Matthias, P., Thali, M., Jirichy, J., and Schaffner, W. (1987). Cell type-specificity elements of the immunoglobulin heavy chain gene enhancer. EMBO J. 6, 1323-1330.

61. Geyer, P. K., and Corces, V. G. (1987). Separate regulatory elements are responsible for the complex pattern of tissue-specific and developmental transcription of the *yellow* locus in Drosophila melanogaster. Genes and Devel. 1, 996-1004.

62. Gillies, S. D., Morrison, S. L., Oi, V. T., and Tonegawa, S. (1983). A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. Cell 33, 717-728.

63. Gilman, M. Z., Wilson, R. N., and Weinberg, R. A. (1986). Multiple protein-binding sites in the 5'-flanking region regulate c-fos expression. Mol. Cell. Biol. 6, 4305-4316.

64. Godowski, P., unpublished results.

65. Goldberg, M. (1979). Sequence analysis of Drosophila histone genes. Ph. D. Thesis, Stanford University.

66. Goodbourn, S., Burnstein, H., and Maniatis, T. (1986). The human β-interferon gene enhancer is under negative control. Cell 45, 601-610.

67. Goodbourn, S., Zinn, K., and Maniatis, T. (1985). Human β-interferon gene expression is regulated by an inducible enhancer element. Cell 41, 509-520.

68. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. 2, 1044-1051.

69. Gorman, C. M., Rigby, P. W. J., and Lane, D. P (1985). Negative regulation of viral enhancers in undifferentiated embryonic stem cells. Cell 42, 519-526.

70. Gorski, K., Carneiro, M., and Schibler, U. (1986). Tissue-specific in vitro transcription from the mouse albumin promoter. Cell 47, 767-776.

71. Gottesfeld, J. (1986). Novobiocin inhibits RNA polymerase III transcription in vitro by a mechanism distinct from DNA topoisomerase II. Nucl. Acids Res. 14, 2075-2088.

72. Gottesman, M. M., ed. (1985). Molecular Cell Genetics. John Wiley & Sons, New York.

73. Graves, B. J., Johnson, P. F., McKnight, S. L. (1986). Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. Cell 44, 565-576.

74. Greene, J., Larin, Z., Taylor, I. C., Prentice, H., Gwinn, K. A., and Kingston, R. E. (1987). Multiple basal elements of a human hsp70 promoter function differently in human and rodent cell lines.

75. Gross, D. S., and Garrard, W. T. (1987). Poising chromatin for transcription. TIBS 12, 293-297.

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

77. Handa, H., Kaufman, R. J., Manley, J., Gefter, M., and Sharp, P. A. (1981). Transcription of simian virus 40 DNA in a HeLa whole cell extract. J. Biol. Chem. 256, 478-482.

78. Hansen, U., Tenen, D. G., Livingston, D. M., and Sharp, P. A. (1981). T antigen repression of SV40 early transcription from two promoters. Cell 27, 603-612.

79. Harris, C., unpublished observations.

80. Harris, M. (1982). Induction of thymidine kinase in enzyme-deficient Chinese hamster cells. Cell 29, 483-492.

81. Harris, M. (1984). High-frequency induction by 5-azacytidine of proline independence in CHO-K1 cells. Somat. Cell Mol. Gen. 10, 615-624,

82. Haslinger, A., and Karin, M. (1985). Upstream promter element of the human metallothionein-II_A gene can act like an enhancer element. Proc. Natl. Acad. Sci. USA 82, 8572-8576.

83. Hawley, D. K., and McClure, W. R. (1982). Mechanism of activation of transcription initiation from the λ P_{RM} promoter. J. Mol. Biol. 157, 493-525.

84. Hawley, D. K., and Roeder, R. G. (1985). Separation and partial characterization of three functional steps in transcription initiation by human RNA polymerase II. J. Biol. Chem. 260, 8163-8172.

85. Hawley, D. K., and Roeder, R. G. (1987). Functional steps in transcription initiation and reinitiation from the major late promoter in a HeLa nuclear extract. J. Biol. Chem. 262, 3452-3461.

86. Hayes, T. E., Kitchen, A. M., and Cochran, B. H. (1987). Inducible binding of a factor to the c-fos regulatory region. Proc. Natl. Acad. Sci. USA 84, 1272-1276.

87. Hearing, P., and Shenk, T. (1986). The adenovirus type 5 E1A enhancer contains two functionally distinct domains : one is specific for E1A and the other modulates all early units in cis. Cell 45, 229-236.

88. Hearing, P., and Shenk, T. (1983). The adenvirus type 5 E1A transcriptional control region contains a duplicated enhancer element. Cell 33, 695-703.

89. Herbomel, P., Bourachot, B., and Yaniv, M. (1984). Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell 39, 653-662.

90. Herr, W., and Clarke, J. (1986). The SV40 enhancer is composed of muliple functional elements that can compensate for one another. Cell 45, 461-470.

91. Hiromi, Y., Kuroiwa, A., and Gehring, W. J. (1985). Control elements of the Drosophila segmentation gene *fushi tarazu*. Cell 43, 603-613.

92. Hochschild, A., Irwin, N., and Ptashne, M. (1983). Repressor structure and the mechanism of positive control. Cell 32, 319-325.

93. Hope, J. A., and Struhl, K. (1986). Functional dissection of a eukaryotic transcriptional activator

protein, GCN4 in yeast. Cell 46, 885-894.

94. Hossenlopp, P., Wells, D., and Chambon, P. (1975). Animal DNA-dependent RNA polymerases--Partial purification and properties of three classes of RNA polymerases from uninfected and adenovirus-infected HeLa cells. Eur. J. Biochem. 58, 237-251.

95. Hshieh, T.-S., and Brutlag, D. (1980). ATP-dependent DNA topoisomerase from D. melanogaster reversibly catenates duplex DNA rings. Cell 21, 115-125.

96. Imbra, R. J., and Karin, M. (1986). Phorbol ester induces the transcriptional stimulatory activity of the SV40 enhancer. Nature 323, 555-558.

97. Imbra, R. J., and Karin, M. (1987). Metallothionein gene expression is regulated by serum factors and activators of protein kinase C. Mol. Cell. Biol. 7, 1358-1363.

98. Israel, A., Kimura, A., Kieran, M., Yano, O., Kanellopoulos, J., Le Bail, O., and Kourilsky, P. (1987). A common positive trans-acting factor binds to enhancer sequecnes in the promoters of mouse H-2 and β_2-microglobulin genes. Proc. Natl. Acad. Sci. USA 84, 2653-2657.

99. Israel, A., Kimura, A., Fournier, A., Fellous, M., and Kourilsky, P. (1986). Interferon response sequence potentiates activity of an enhancer in the promoter region of a mouse H-2 gene. Nature 322, 743-746.

100. Ivarie, R. D., and Morris, J. A. (1982). Induction of prolactin-deficient variants of GH_3 rat pituitary tumor cells by ethyl methanesulfonate: Reversion by 5-azacytidine, a DNA methylation inhibitor. Proc. Natl. Acad. Sci. USA 79, 2967-2970.

101. Jantzen, H.-M., Strahle, U., Gloss, B., Stewart, F., Schmid, W., Boshart, M., Miksicek, R., and Schutz, G. (1987). Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. Cell 49, 29-38.

102. Johnson, A., Pabo, C., O., Sauer, R. T. (1980). Bacteriophage λ Repressor and cro protein: Interactions with Operator DNA. Meth. Enzymol. 65, 839-856.

103. Johnson, P. F., Landschulz, W. H., Graves, B. J., and McKnight, S. L. (1987). Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes and Devel. 1, 133-146.

104. Johnsrud, L. (1978). Contacts between Escherichia coli RNA polymerase and a *lac* operon promoter. Proc. Natl. Acad. Sci. USA 75, 5314-5318.

105. Jones, S. D. (1986). Sequence elements of enhancers and promoters that determine enhancer activity. Ph. D. thesis, University of California, San Francisco.

106. Jones, K. A., Kadonaga, J. T., Rosenfeld, P. J., Kelly, T. J., and Tjian, R. (1987). A Cellular DNA-binding protein that activates eukaryotic transcription and DNA replication. Cell 48, 79-89.

107. Jones, K. A., Yamamoto, K. R., and Tjian, R. (1985). Two distinct transcription factors bind to the HSV thymidine kinase promoter in vitro. Cell 42, 559-572.

108. Karin, M., Haslinger, A., Heguy, A., Dietlin, T., and Cooke, T. (1987). Metal-responsive elements act as positive modulators of human metallothionein-II_A enhancer activity. Mol. Cell. Biol. 7, 606-613.

109. Karin, M., Haslinger, A., Holtgreve, H., Richards, R. I., Krauter, P., Westphal, H. M., and Beato, M. (1984). Characterization of a DNA sequence through which cadmium and glucocorticoid hormones induce human metallothionein-II_A gene. Nature 308, 513-519.

110. Karin, M., Haslinger, A., Holtgreve, H., Cathala, G., Slater, E., and Baxter, J. D. (1984). Activation of a heterologous promoter in response to dexamethasone and cadmium by metallothionein gene 5'-flanking DNA. Cell 36, 371-379.

111. Katinka, M., Vasseur, M., Montreau, N., Yaniv, M., and Blangy, P. (1981). Polyoma DNA sequences involved in control of viral gene expression in murine embryonal carcinoma cells. Nature 290, 720-722.

112. Kaufman, R. J., and Murtha, P. (1987). Translational control mediated by eucaryotic initiation factor-2 is restricted to specific mRNAs in transfected cells. Mol. Cell. Biol. 7, 1568-1571.

113. Kenney, S., Natarajan, V. Strike, R., Khoury,G., and Salzman, N. P. (1984). JC Virus enhancer-promoter active in human brain cells. Science 226, 1337-1339.

114. Klein, S., Sablitzky, F., and Radbruch, A. (1984). Deletion of the IgH enhancer does not reduce immunoglobulin heavy chain production of a hybridoma IgD class switch variant. EMBO J. 3, 2473-2476.

115. Knezetic, J. A., and Luse, D. S. (1986). The presence of nucleosomes on a DNA template prevents initiation by RNA polymerase II in vitro. Cell 45, 95-104.

116. Kunkel, G. R., and Martinson, H. G. (1981). Nucleosomes will not form on double stranded RNA or over poly(dA):(dT) tracts in recombinant DNA. Nucl. Acids Res. 12, 7057-7070.

117. LaBaer, J.; personal communication.

118. Labhart, P. and Reeder, R. H. (1984). Enhancer-like propeties of the 60/81 bp elements in the ribosomal gene spacer of Xenopus laevis. Cell 37, 285-289.

119. Laimins, L. A., Khoury, G., Gorman, C., Howard, B., and Gruss, P. (1982). Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA 79, 6453-6457.

120. Laimins, L., Tsichlis, P., and Khoury, G. (1984). Multiple enhancer domains in the 3' terminus of the Prague strain of Rous sarcoma virus. Nucl. Acids Res. 12, 6427-6442.

121. Landolfi, N. F., Capra, J. D., and Tucker, P. W. (1986). Interaction of cell-type-specific nuclear proteins with immunoglobulin V_H promoter region sequences. Nature 323, 548-551.

122. Laskey, R. A., Mills, A. D., and Morris, N. R. (1977). Assembly of SV40 chromatin in a cell-free

system from Xenopus eggs. Cell 10, 237-243.

123. Learned, R. M., Learned, T. K., Haltiner, M. M., and Tjian, R. T. (1986). Human rRNA transcription is modulated by the coordinate binding of two factors to an upstream control element. Cell 45, 847-857.

124. Lee, W., Haslinger, A., Karin, M., and Tjian, R. (1987). Two factors that bind and activate the human metallothionein II_A gene in vitro also interact with the SV40 promoter and enhancer regions. Nature 325, 368-372.

125. Lee, W., Mitchell, P., and Tjian, R. (1987). Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell 49, 741-752.

126. Lenardo, M., Pierce, J. W., and Baltimore, D. (1987). Protein-binding sites in immunoglobulin gene enhancers determine transcriptional activity and inducibility. Science 236, 1573-1577.

127. Levis, R., Hazelrigg, T., and Rubin, G. M. (1985). Separable cis-acting control elements for expression of the *white* gene of Drosophila EMBO J. 4, 3489-3499.

128. Lopata, M. A., Cleveland, D. W., and Sollner-Webb, B. (1984). High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. Nucl. Acids Res. 12, 5707-5717.

129. Luthman, H., and Magnusson, G. (1983). High efficiency polyoma DNA transfection of chloroquine treated cells. Nucl. Acids Res. 11, 1295-1308.

130. Ma, J., and Ptashne, M. (1987). Deletion analysis of GAL4 defines two transcriptional activating segments. Cell 48, 847-853.

131. Mackall, J., Meredith, M., and Lane, M. D. (1979). A mild procedure for the rapid release of cytoplasmic enzymes from cultured animal cells. Anal. Bioc. 95, 270-274.

132. Maeda, H., Kitamura, A., Kudo, A., Araki, K., and Watanabe, T. (1986). Trans-acting nuclear protein responsible for induction of rearranged human immunoglobulin heavy chain gene. Cell 45, 25-33.

133. Malan, P., Kolb, A., Buc, H., and McClure, W. R. (1984). Mechanism of CRP-cAMP activation of *lac* operon transcription initiation: Activation of the P1 promoter. J. Mol. Biol. 180, 881-909.

134. Maler, B., unpublished data.

135. Maniatis, T., Goodbourn, J., and Fischer, J. A. (1987). Regulation of Inducible and Tissue-Specific Gene Expression. Science 236, 1237-1245.

136. Mangin, M., Ares, M., Jr., and Weiner, A. M. (1986). Human U2 small nuclear RNA genes contain an upstream enhancer. EMBO J. 5, 987-995.

137. Manley, J. L., Fire, A., Samuels, M., and Sharp, P. A. (1983). In Vitro transcription: Whole-cell extract. Meth. Enzym. 101, 568-582.

138. Martin, D., Tomkins, G. M., and Granner, D. (1969). Synthesis and induction of tyosine aminotransferase in synchronized hepatoma cells in culture. Proc. Natl. Acad. Sci. USA 62, 248-255.

139. Mason, J. O., Williams, G. T., and Neuberger, M. S. (1985). Transcription cell type specificity is conferred by an immunoglobulin V_h gene promoter that includes a functional consensus sequence. Cell 41, 479-487.

140. Matsui, T., Segall, J., Weil, P. A., and Roder, R. G. (1980). Multiple factors required for accurate initiation of transcription by purified RNA polymerase II. J. Biol. Chem. 255, 11,992-11,996.

141. Mattaj, I. W., Lienhard, S., Jiricny, J., and De Robertis, E. M. (1985). An enhancer-like sequence within the Xenopus U2 gene promoter facilitates the formation of stable transcription complexes. Nature 316, 163-167.

142. Maxam, A. M., and Gilbert, W. (1980). Sequencing end-labeled DNA with base-specific chemical cleavages. Meth. Enzymol. 65, 499-560.

143. McKnight, S. L., Gavis, E. R., Kingsbury, R., and Axel, R. (1981). Analysis of transcriptional regulatory signals of the HSV thymidine kinase gene: identification of an upstream control region. Cell 25, 385-398.

144. McKnight, S. L. and Kingsbury, R. C. (1982). Transcriptional control signals of a eukaryotic protein-coding gene. Science 217, 316-324.

145. McKnight, S. and Tjian, R. (1986). Transcriptional selectivity of viral genes in mammalian cells. Cell 46, 795-805.

146. McNeil, P. L., Murphy, R. F., Lanni, F., and Taylor, D. L. (1984). A method for incorporating macromolecules into adherent cells. J. Cell Biol. 98, 1556-1564.

147. Mercola, M., Governman, J., Mirell, C., and Calame, K. (1985). Immunoglobulin heavy chain enhancer requires one or more tissue specific factors. Science 227, 266-270.

148. Miesfeld, R., Okret, S., Wikstrom, A.-C., Wrange, O., Gustafsson, J.-A., and Yamamoto, K. R. (1984). Characterization of a steroid hormone receptor gene and mRNA in wild-type and mutant cells. Nature, 312, 779-781.

149. Miesfeld, R., Rusconi, S., Godowski, P. J., Maler, B., Okret, S., Wikstrom, A.-C., Gustafsson, J.-A., and Yamamoto, K. R. (1986). Genetic complementation of a glucocorticoid receptor deficiency by expression of cloned receptor cDNA. Cell 46, 389-399.

150. Miksicek, R., Heber, A., Schmid, W., Danesch, U., Posseckert, G., Beato, M., and Schutz, G. (1986). Glucocorticoid responsiveness of the transcriptional enhancer of the Moloney murine sarcoma virus. Cell 46, 283-290.

151. Miller, K. G., Liu, L. F., and Eglund, P. T. (1981). A homogeneous type II DNA topoisomerase

from HeLa cell nuclei. J. Biol. Chem. 256, 9334-9339.

152. Mitchell, P. J., Wang, C., and Tjian, R. (1987). Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. Cell 50, 847-861.

153. Mizushima-Sugano, J., and Roeder, R. G. (1986). Cell-type-specific transcription of an immunoglobulin κ light chain gene in vitro. Proc. Natl. Acad. Sci. USA 83, 8511-8515.

154. Mizuuchi, K. (1983). In vitro transposition of bacteriophage Mu: A biochemical approach to a novel replication reaction. Cell 35, 785-794.

155. Moreau, P., Hen, R., Wasylyk, B., Everett, R., Gaub, M. P., and Chambon, P. (1981). The SV40 72 base pair repeat has a striking effect on gene expression both in SV40 and in other chimeric recombinants. Nucl. Acids Res. 9, 6047-6068.

156. Bodwell, J. E., Holbrook, N. J., and Munck,A. (1984). Sulfhydryl-modifying reagents reversibly inhibit binding of glucocorticoid-receptor complexes to DNA-cellulose. Biochemistry 23, 1392-1398.

157. Nakamura, N., and Okada, S. (1983). Mutations resistant to bromodeoxyuridine in mouse lymphoma cells selected by repeated exposure to EMS; characteristics of phenotypic instability and reversion to HAT resistance by 5-azacytidine. Mut. Res. 111, 353-364.

158. Nechushtan, H., Benvenisty, N., Brandeis, R., and Rashef, L. (1987). Glucocorticoids control phosphoenolpyruvate carboxykinase gene expression in a tissue specific manner. Nucl. Acids. Res. 15, 6405-6417.

159. Neuberger, M. S. (1983). Expression and regulation of immunoglobulin heavy chain gene transfected into lymphoid cells. EMBO J. 2, 1373-1378.

160. Ninfa, A. J., Reitzer, L. J., and Magasanik, B. (1987). Initiation of transcription at the bacterial *glnAp2* promoter by purified E. coli components is factilitated by enhancers. Cell 50, 1039-1046.

161. Ohlsson, H., and Edlund, T. (1986). Sequence-specific interactions of nuclear factors with the insulin gene enhancer. Cell 45, 35-44.

162. Ondek, B., Shepard, A., and Herr, W. (1987). Discrete elements within the SV40 enhancer region display different cell-specific enhancer activities. EMBO J. 6, 1017-1025.

163. Palmiter, R., unpublished results.

164. Parker, B. A., and Stark, G. R. (1979). Regulation of simian virus 40 transcription: sensitive analysis of the RNA species present early in infection by virus or viral DNA. J. Virol. 31, 360-369.

165. Parker, C. S., and Topol, J. (1984). A Drosophila RNA polymerase II transcription factor contains a promoter-region-specific DNA binding activity. Cell 36, 357-369.

166. Parker, C. S., and Topol, J. (1984). A Drosophila RNA polymerase II transcription factor binds to the regulatory site of an hsp 70 gene. Cell 37, 273-283.

167. Parslow, T. G., Jones, S. D., Bond, B., and Yamamoto, K. R. (1987). The immunoglobulin octanucleotide: independent activity and selective interaction with enhancers. Science 235, 1498-1501.

168. Parslow, T. G., Murphy, W., Blair, D., and Granner, D. K. (1984). Structure of the 5' ends of immunoglobulin genes: a novel conserved sequence. Proc. Natl. Acad. Sci. USA 81, 2650-2654.

169. Payvar, F., unpublished results.

170. Payvar, F., Wrange, O., Carlstedt-Duke, J., Okret, S., Gustafsson, J.-A., and Yamamoto, K. R. (1981). Purified glucocorticoid receptors bind selectively in vitro to a cloned DNA fragment whose transcription is regulated by glucocorticoids in vivo. Proc. Natl. Acad. Sci. USA 78, 6628-6632.

171. Payvar, F., DeFranco, D., Firestone, G. L., Edgar, B., Wrange, O., Okret, S., Gustafsson, J. -A. and

Yamamoto, K. R. (1983). Sequence-specific binding of glucocorticoid receptor to MTV DNA at sites within and upstream of the transcribed region. Cell 35, 381-392.

172. Pederson, D. S., Thomas, F., and Simpson, R. T. (1986). Core particle, fiber, and transcriptionally active chromatin structure. Ann. Rev. Cell Biol. 2, 117-147.

173. Peterson, C. L., Orth, K., and Calame, K. L. (1986). Binding in vitro of multiple cellular proteins to Ig-heavy-chain enhancer DNA. Mol. Cell. Biol. 6, 4168-4178.

174. Peterson, D. O., Beifuss, K. K., and Morley, K. L. (1987). Context-dependent gene expression: cis-acting negative effects of specific procaryotic plasmid sequences on eucaryotic genes. Mol. Cell. Biol. 7, 1563-1567.

175. Picard, D. (1985). Viral and cellular transcription enhancers. In "Oxford Surveys on Eukaryotic Genes", Vol. 2 (M. Maclean, ed.). Oxford University Press, pp. 24-48.

176. Picard, D., and Schaffner, W. (1984). A lymphocyte-specific enhancer in the mouse immunoglobulin κ gene. Nature 307, 80-82.

177. Picard, D., and Yamamoto, K. R. (1987). Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. EMBO J. 6, 3333-3340.

178. Piette, J., and Yaniv, M. (1986). Molecular analysis of the interaction between an enhancer binding factor and its DNA target. Nucl. Acids Res.14, 9595-9611.

179. Piette, J., Kryszke, M. H., and Yaniv, M. (1985). Specific interaction of cellular factors with the B enhancer of polyoma virus. EMBO J. 4, 2675-2685.

180. Piette, J., and Yaniv, M. (1987). Two different factors bind to the a-domain of the polyoma virus enhancer, one of which also inteacts with the SV40 and c-fos enhancers. EMBO J. 6, 1331-1337.

181. Pirotta, V., Steller, H., and Bozzetti, M. D. (1985). Multiple upstream regulatory elements control

the expression of the Drosophila *white* gene. EMBO J. 4, 3501-3508.

182. Plon, S. E., and Wang, J. C. (1986). Transcription of the human β-globin gene is stimulated by an SV40 enhancer to which it is physically linked but topologically uncoupled. Cell 45, 575-580.

183. Prunell, A. (1982). Nucleosome reconstitution on plasmid inserted poly(dA:dT). EMBO J. 1, 173-179.

184. Prywes, R., and Roeder, R. G. (1986). Inducible binding of a factor to the c-fos enhancer. Cell 47, 777-784.

185. Queen, C., and Baltimore, D. (1983). Immunoglobulin gene transcription is activated by dowstream sequence elements. Cell 33, 741-748.

186. Ringold, G. M., Yamamoto, K. R., Bishop, J. M., and Varmus, H. E. (1977). Glucocorticoid-stimulated accumulation of mouse mammary tumor virus RNA: increase rate of synthesis of viral RNA. Proc. Natl. Acad.Sci. USA 74, 2879-2883.

187. Reinberg, D., Horikoshi, M., and Roeder, R. G. (1987). Factors involved in specific transcription by mammalian RNA polymerase II: functional analysis of initiation factors IIA and IID and identification of a new factor operating downstream of the initiation site. J. Biol. Chem. 262, 3322-3330.

188. Reinberg, D., and Roeder, R. G. (1987). Factors involved in specific transcription by mammalian RNA polymerase II: Purification and functional analysis of initiation factors IIB and IIE. J. Biol. Chem. 262, 3310-3321.

189. Ringold, G. M., Shank, P. R., Varmus, H. E., Ring, J., and Yamamoto, K. R. (1979). Integration and transcription of mouse mammary tumor virus DNA in rat hepatoma cells. Proc. Natl. Acad. Sci. USA 76, 665-669.

190. Ringold, G. M., Cardiff, R. D., Varmus, H. E., and Yamamoto, K. R. (1977). Infection of cultured rat hepatoma cells by mouse mammary tumor virus. Cell 10, 11-18.

191. Rochford, R., Campbell, B. A., and Villareal, L. P. (1987). A pancreas specificity results from the combination of polyomavirus and Moloney murine leukemia virus enhancers. Proc. Natl. Acad. Sci. USA 84, 449-453.

192. Roeder, R. G. (1976). Eukaryotic nuclear RNA polymerases. In RNA Polymerase, Losick, R., and Chamberlin, M., eds. (Cold Spring Harbor Laboratory), pp. 285-329.

193. Roginski, R. S., Skoultchi, A. I., Henthorn, P., Smithies, O., Hsiung, N., and Kucherlapati, R. (1983). Coordinate modulation of transfected HSV thymidine kinase and human globin genes. Cell 35, 149-155.

194. Rosales, R., Vigneron, M., Macchi, M., Davidson, I., Xiao, J. H., and Chambon, P. (1987). In vitro binding of cell-specific and ubiquitous nuclear proteins to the octamer motif of the SV40 enhancer and related motifs present in other promoters and enhancers. EMBO J. 6, 3015-3025.

195. Safer, B., Yang, L., Tolunay, H. E., Anderson, W. F. (1985). Isolation of stable preinitiation, initiation, and elongation complexes from RNA polymerase II-directed transcription. Pro. Natl. Acad. Sci. USA 82, 2632-2636.

196. Sakai, D., unpublished results.

197. Samuels, M., Fire, A., and Sharp, P.A. (1982). Separation and characterization of factors mediating accurate transcription initiation by RNA polymerase II. J. Biol. Chem. 257, 14419-14427.

198. Sassone-Corsi, P., Wildeman, A., and Chambon, P. (1985). A trans-acting factor is responsible for the simian virus 40 enhancer activity in vitro. Nature 313, 458-463.

199. Sassone-Corsi, P., Dougherty, T. P., Wasylyk, B., and Chambon, P. (1984). Stimulation of in vitro transcription from heterologous promoters by SV40 enhancer. Proc. Natl. Acad. Sci. USA 81, 308-312.

200. Sassone-Corsi, P., Hen, R., Borrelli, E., Leff, T., and Chambon, P. (1983). Far upstream sequences are required for efficient transcription from the adenovirus-2 E1A transcription unit. Nucl. Acids. Res. 11, 8735-8746.

201. Sawadago, M., and Roeder, R. G. (1985). Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell 43, 165-175.

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

203. Scheidereit, C., and Beato, M. (1984). Contacts between hormone receptor and DNA double helix within a glucocorticoid regulatory element of mouse mammary tumor virus. Proc. Natl. Acad. Sci. USA 81, 3029-3033.

204. Scheidereit, C., Heguy, A., and Roeder, R. G. (1987). Identification and purification of a human lymphoid-specific octamer binding protein (OTF-2) that activates transcription of an immunoglobulin promoter in vitro. Cell 51, 783-793.

205. Schena, M., Silvera, P., and Yamamoto, K. R., manuscript in preparation .

206. Schirm, S., Jirieny, J., and Schaffner, W. (1987). The SV40 enhancer can be dissected into multiple segments, each with a different cell type specificity. Genes and Devel. 1, 65-74.

207. Schlokat, U., Bohmann, D., Scholer, H., and Gruss, P. (1986). Nuclear factors binding specific sequences within the immunoglobulin enhancer interact differentially with other enhancer elements. EMBO J. 5, 3251-3258.

208. Scholer, H. R., and Gruss, P. (1985). Cell type-specific transcriptional enhancement in vitro requires the presence of trans-acting factors. EMBO J. 4, 3005-3013.

209. Scholer, H. R., and Gruss, P. (1984). Specific interaction between enhancer-containing molecules and cellular components. Cell 36, 403-411.

210. Schwartz, L. B., Sklar, V. E. F., Jaehning, J., Weinmann, R., and Roeder, R. G. (1974). Isolation and partial characterization of the multiple forms of deoxyribonucleic acid-dependent ribonucleic acid polymerase in mouse myeloma, MOPC 315. J. Biol. Chem. 249, 5889-.

211. Sealy, L., Cotten, M., and Chalkley, R. (1986). Novobiocin inhibits passive chromatin assembly in vitro. EMBO J. 5, 3305-3311.

212. Searle, P. F., Stuart, G. W., and Palmiter, R. D. (1985). Building a metal-responsive promoter with synthetic regulatory elements. Mol. Cell. Biol. 5, 1480-1489.

213. Sekikawa, K., and Levine, A. (1981). Isolation and characterization of polyoma host-range mutants that replicate in nullipotential embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA 78, 1100-1104.

214. Sen, R., and Baltimore, D. (1986). Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell 46, 705-716.

215. Sen, R., and Baltimore, D. (1986). Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a postranslational mechanism. Cell 47, 921-928.

216. Serfling, E., Lubbe, A., Dorsch-Hasler, K., and Schaffner, W. (1985). Metal-dependent SV40 viruses containing inducible enhancers from the upstream region of metallothionein genes. EMBO J. 4, 3851-3859.

217. Sergeant, A., Bohmann, D., Zentgraf, H., Weiher, H., and Keller, W. (1984). A transcription enhancer acts in vitro over distances of hundreds of base-pairs on both circular and linear templates but not on chromatin-reconstituted DNA. J. Mol. Biol. 180, 577-600.

218. Shuey, D. J., and Parker, C. S. (1986). Binding of Drosophila Heat Shock Gene Transcription Factor to the hsp 70 promoter. J. Biol. Chem. 261, 7934-7940.

219. Siminovitch, L. (1985). Mechanisms of genetic variation in Chinese hamster ovary cells. In Molecular Cell Genetics, M. M. Gottesman, ed. (John Wiley & Sons, New York), pp. 869-879.

220. Simon, A. E., Taylor, M. W., Bradley, W. E. C., and Thompson, L. H. (1982). Model involving gene inactivation in the generation of autosomal recessive mutants in mammalian cells in culture. Mol. Cell. Biol. 2, 1126-1133.

221. Singh, H., Sen, R., Baltimore, D., and Sharp, P. A. (1986). A nuclear factor that binds to a conserved sequence motif in transcriptional control elements of immunoglobulin genes. Nature 319, 154-158.

222. Standring, D. N., Ou, J.-H., and Rutter, W. J. (1986). Assembly of viral particles in Xenopus oocytes: Pre-surface-antigens regulate secretion of the hepatitis B viral surfacr envelope particle. Proc. Natl. Acad. Sci. USA 83, 9338-9342.

223. Staudt, L. M., Singh, H., Sen, R., Wirth, T., Sharp, P. A., and Baltimore, D. (1986). A lymphoid-specific protein binding to the octamer motif of immunoglobulin genes. Nature 323, 640-643.

224. Struhl, K. (1987). Promoters, activator proteins, and the mechanism of transcriptional activation in yeast. Cell 49, 295-297.

225. Struhl, K. (1985). Naturally occurring poly dA:dT sequences are upstream elements for constitutive transcription in yeast. Proc. Natl. Acad. Sci. USA 82, 8419-8423.

226. Stuart, G. W., Searle, P. F., Chen, H. Y., Brinster, R. I., and Palmiter, R. D. (1984). A 12-base-pair motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. Proc. Natl. Acad. Sci. USA 81, 7318-7322.

227. Sugiyama, R. H., Arfin, S. M., and Harris, M. (1983). Properties of asparagine synthetase in asparagine-independent variants of Jensen rat sarcoma cells induced by 5-azacytidine. Mol. Cell. Biol. 3, 1937-1942.

228. Thomas, J., unpublished results.

229. Thomas, K. R., Folger, K. R. and Capecchi, M. R. (1986). High frequency targeting of genes to specific sites in the mammalian genome. Cell 44, 419-428.

230. Thompson, E. B., Granner, D. K., Gelehrter, T., Erikson, J., and Hager, G. (1979). Unlinked multiple glucocorticoid-induced processes in HTC cells. Mol. Cell. Endo. 15, 135-150.

231. Tolunay, H. E., Yang, L., Anderson, W. F., and Safer, B. (1984). Isolation of an active transcription initiation complex from HeLa cell-free extract. Proc. Natl. Acad. Sci. USA 81, 5916-5920.

232. Treisman, R. and Maniatis, T. (1985). SV40 enhancer increases number of RNA polymerase II molecules on linked DNA. Nature 315, 72-75.

233. Treisman, R. (1986). Identification of a protein-binding site that mediates transcriptional response of the *c-fos* gene to serum factors. Cell 46, 567-574.

234. Treisman, R. (1985). Transient accumulation of c-fos RNA following serum stimulation requires a conserved 5' element and c-fos 3' sequences. Cell 42, 889-902.

235. Tyndall, C., LaMantia, G., Thacker, C. M., Favaloro, J., and Kamen, R. (1981). A region of the polyoma virus genome between the replication origin and late protein coding sequences is required in cis for both early gene expression and viral DNA replication. Nucl. Acids Res. 9, 6321-6250.

236. Ucker, D. S., Firestone, G. L., and Yamamoto, K. R. (1983). Glucocorticoids and chromosomal position both modulate MTV transcription by affecting the efficiency of promoter utilization. Mol. Cell. Biol. 3, 551-561.

237. Ucker, D. A., and Yamamoto, K. R. (1984). Early events in the stimulation of mammary tumor virus RNA synthesis by glucocorticoids: novel assays of transcription rates. J. Biol. Chem. 259, 7416-7420.

238. Van Dyke, W., and Roeder, R. G. (1987). Novobiocin interferes with the binding of transcription factors TFIIIA and TFIIIC to the promoters of class III genes. Nucl. Acids Res. 15, 4365-4374.

239. Vanderbilt, J., unpublished results.

240. Veldman, G. M., Lupton, S., and Kamen, R. (1985). Polyoma enhancer contains multiple redundant sequence elements that activate both DNA replication and gene expression. Mol. Cell. Biol. 5, 649-658.

241. Voss, S. D., Schlokat, U., and Gross, P. (1986). The role of enhancers in the regulation of cell-type-specific transcriptional control. Trends in Biochem. Sci. 11, 287-289.

242. Wabl, M. R., and Burrows, P. D. (1984). Expression of immunoglobulin heavy chain at a high level in the absence of a proposed immunoglobulin enhancer element in cis. Proc. Natl. Acad. Sci. USA 81, 2452-2455.

243. Wang, X.-F., and Calame, K. (1986). SV40 enhancer-binding factors are required at the establishment but not the maintenance step of enhancer-dependent transcriptional activation. Cell 47, 241-247.

244. Wasylyk, C., Imler, J. L., Perez-Mutul, J., and Wasylyk, B. (1987). The c-Ha-ras oncogene and a tumor promoter activate the polyoma virus enhancer. Cell 48, 525-534.

245. Wasylyk, C., and Wasylyk, B. (1986). The immunoglobulin heavy-chain B-lymphocyte enhancer efficiently stimulates transcription in non-lymphoid cells. EMBO J. 5, 555-560.

246. Weber, F., and Schaffner, W. (1985). SV40 enhancer increases RNA polymerase density within the linked gene. Nature 315, 75-77.

247. Weinberger, C., Hollenberg, S. M., Ong, E. S., Harmon, J. M., Brower, S. T., Cidlowski, J., Thompson, E. B., Rosenfeld, M. G., and Evans, R. M. (1985). Identification of human glucocorticoid receptor complementary DNA clones by epitope selection. Science 228, 740-742.

248. Wiederrecht, G., Shuey, D. J., Kibbe, W. A., and Parker, C. S. (1987). The Saccharomyces and Drosophila heat shock transcription factors are identical in size and DNA binding properties. Cell 48, 507-515.

249. Wigler, M., Silverstein, S., Lee, L.-S., Pellicer, A., Chang, Y.-C., and Axel, R. (1977). Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell 11, 223-232.

250. Wildeman, A. G., Sassone-Corsi, P., Grundstrom, T., Zenke, M., and Chambon, P. (1984). Stimulation of in vitro transcription from the SV40 early promoter by the enhancer involves a specific trans acting factor. EMBO J. 3, 3129-3133.

251. Wildeman, A. G., Zenke, M., Schatz, C., Wintzerith, M., Grundstrom, T., Matthes, H., Takahashi, K., and Chambon, P. (1986). Specific protein binding to the simian virus 40 enhancer in vitro. Mol. Cell. Biol. 6, 2098-2105.

252. Wonnacott, T. H. and Wonnacott, R. J. (1984), Introductory Statistics for Business and Economics, 3rd ed., Wiley, New York.

253. Workman, J. L., and Roeder, R. G. (1987). Binding of transcription factor TFIID to the major late promoter during in vitro nucleosome assembly potentiates subsequent initiation by RNA polymerase II. Cell 51, 613-622.

254. Worton, R. G., and Grant, Stephen, G. (1985). Segregation-like events in Chinese hamster cells. In Molecular Cell Genetics, M. M. Gottesman, ed. (John Wiley & Sons, New York), pp. 831-866.

255. Wrange, O., Carlstedt-Duke, J., and Gustafsson, J. -A. (1979). Purification of the glucocorticoid receptor from rat liver cytosol. J. Biol. Chem. 254, 9284-9290.

256. Wrange, O., Okret, S., Radojcic, M., Carlsedt-Duke, J., and Gustafsson, J.-A. (1984). Characterization of the purified glucocorticoid receptor from rat liver cytosol. J. Biol. Chem. 259, 4534-4541.

257. Wu, C. (1984). Two protein binding sites in chromatin implicated in the activation of heat shock

genes. Nature 309, 229-234.

258. Wyke, J. A., Beamand, J. A., and Varmus, H. E. (1980). Factors affecting phenotypic reversion of rat cells transformed by avian sarcoma virus. Cold Spring Harbor Symp. Quant. Biol. 44, 1065-1075.

259. Xiao, J. H., Davidson, I., Ferrandon, D., Rosales, R., Vigneron, M., Macchi, M., Ruffenach, F., and Chambon, P. (1987). One cell-specific and three ubiquitous nuclear proteins bind in vitro to overlapping motifs in the domain B1 of the SV40 enhancer. EMBO J. 6, 3005-3013.

260. Xiao, J.-H., Davidson, I., Macchi, M., Rosales, R., Vigneron, M., Staub, A., and Chambon, P. (1987). In vitro binding of several cell-specific and ubiquitous nuclear proteins to the GT-1 motif of the SV40 enhancer. Genes and Devel. 1, 794-807.

261. Yamamoto, K. R., personal communication.

262. Yamamoto, K. R. (1985). Steroid receptor regulated transcription of specific genes and gene networks. Ann. Rev. Genet. 19, 209-252.

263. Yamamoto, K. R., Stallcup, M. R., Ring, J., and Ringold, G. M. (1978). Mammary tumor virus DNA: a glucocorticoid-responsive transposable element. Cold Spring Harbor Symp. on Quant. Biol. XLII, 625-638.

264. Yee, A. S., Reichel, R., Kovesdi, I., and Nevins, J. R. (1987). Promoter interaction of the E1A-inducible factor E2F and its potential role in the formation of a multi-component complex. EMBO J. 6, 2061-2068.

265. Zaller, D. M., and Eckhardt, L. A. (1985). Deletion of a B-cell-specific enhancer affects transfected, but not endogenous, immunoglobulin heavy-chain gene expression. Proc. Natl. Acad. Sci. USA 82, 5088-5092.

266. Zenke, M., Grundstrom, T., Matthes, H., Wintzerith, M., Schatz, C., Wildeman, A., and Chambon, P. (1986). Multiple sequence motifs are involved in SV40 enhancer function. EMBO J. 5, 387-

397.

267. Zheng, X.-M., Moncollin, V., Egly, J.-M., and Chambon, P. (1987). A general transcription factor forms a stable complex with RNA polymerase B (II). Cell 50, 361-368.

268. Zinn, K., and Maniatis, T. (1986). Detection of factors that interact with the human β-interferon regulatory region by DNAase I footprinting. Cell 45, 611-618.

269. McClure, W. R. (1985). Mechanism and control of transcription initiation in prokaryotes. Ann. Rev. Biochem. 54, 171-204.

270. Wandelt, C., and Grummt, I. (1983). Formation of stable initiation complexes is a prerequisite for ribosomal DNA transcription in vitro. Nucl. Acids Res. 11, 3795-3809.

271. Cizewski, V., and Sollner-Webb, B. (1983). A stable transcription complex directs mouse ribosomal RNA synthesis by RNA polymerase I. Nucl. Acids Res. 11, 7043-7056.

272. Kmiec, E. B., Ryoji, M., and Worcel, A. (1986). Gyration is required for 5S RNA transcription from a chromatin template. Proc. Natl. Acad. Sci. USA 83, 1305-1309.

273. Allegra, P., Sterner, R., Clayton, D. F., and Allfrey, V. G. (1987). Affinity chromatographic purification of nucleosomes containing transcriptionally active DNA sequences. J. Mol. Biol. 196, 379-388.

274. Dorbic, T., and Wittig, B. (1987). Chromatin from transcribed genes contains HMG17 only downstream from the starting point of transcription. EMBO J. 6, 2393-2399.

275. Keshet, I., Leiman-Hurwitz, J., and Cedar, H. (1986). DNA methylation affects the formation of active chromatin. Cell 44, 535-543.

276. Bird, A. P. (1984). DNA methylation-- how important in gene control? Nature 307, 503-504.

277. Kelley, D. E., Pollok, B. A., Atchison, M. L., and Perry, R. P. (1987). The coupling between

enhancer activity and hypomethylation of κ immunoglobulin genes is developmentally regulated. Mol. Cell. Bio., in press.

278. Zaret, K. S., and Yamamoto, K. R. (1984). Reversible and persistent changes in chromatin structure accompany activation of a glucocorticoid-dependent enhancer element. Cell 38, 29-38.

279. Payvar, F., and Wrange, O. (1984). Relative selectivities and efficiencies of DNA binding by purified intact and protease-cleaved glucocorticoid receptor. In Steroid Hormone Receptors: Structure and Function. Eriksson, H., and Gustafsson, J.-A., eds., Amsterdam, Elsevier/North Holland, pp. 267-282.

280. Carlstedt-Duke, J., Stromstedt, P.-E., Persson, B., Cederlund, E., Gustafsson, J.-A., and Jornvall, H. (1987). Identification of hormone-interacting amino acid residues within the steroid-binding domain of the glucocorticoid receptor in relation to other steroid hormone receptors. Manuscript submitted.

281. Simons, S. S., Pumphrey, J. G., Rudikoff, S., and Eisen, H. J. (1987). J. Cell. Biochem. suppl. 11A, 111.

282. Briggs, M. R., Kadonga, J. T., Bell, S. P., and Tjian, R. (1986). Purification and biochemical characterization of the promoter-specific transcription factor Sp1. Science 234, 47-52.

283. Godowski, P. J., Rusconi, S., Miesfeld, R., and Yamamoto, K. R. (1987). Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. Nature 325, 365-368.

284. Vanderbilt, J. N., Miesfeld, R., Maler, B. A., and Yamamoto, K. R. (1987). Intracellular receptor concentration limits glucocorticoid-dependent enhancer activity. Molec. Endo. 1, 68-74.

285. Wrange, O., personal communication.

286. DeFranco, D., unpublished results.

287. Sawadogo, M. and Roeder, R. G. (1985). Factors involved in specific transcription by human RNA

polymerase II: analysis by a rapid and quantitative in vitro assay. Proc. Natl. Acad. Sci. USA 82, 4394-4398.

288. Celander, D., and Haseltine, W. A. (1987). Glucocorticoid regulation of murine leukemia virus transcription elements is specified by determinants within the viral enhancer region. J. Virol. 61, 269-275.

289. Kalinyak, J. E., Dorin, P. I., Hoffman, A. R., and Perlman, A. J. (1987). Tissue-specific regulation of glucocorticoid receptor mRNA by dexamethasone. J. Biol. Chem. 262, 10441-10444.

290. Feinstein, S., C., Ross, S. R., and Yamamoto, K. R. (1982). Chromosomal position effects determine transcriptional potential of integrated mammary tumor DNA. J. Mol. Biol. 156, 549-565.

291. Baker, W. K. (1968). Position-effect variegation. Adv. Genet. 14, 133-169.

292. Walker, M. D., Edlund, T., Boulet, A. M. and Rutter, W. J. (1983). Cell-specific expression controlled by the 5'-flanking region of insulin and chymotrypsin genes. Nature 306, 557-561.

293. Coffin, J. M., Tsichlis, P. N., Barker, C. S., and Voynow, S. (1980). Variation in avian retrovirus genomes. Ann. N.Y. Acad. Sci. 354, 410-425.

294. Shinomiya, T., Scherer, G., Schmid, W., Zentgraf, H., and Schutz, G. (1984). Isolation and characterization of the rat tyrosine aminotransferase gene. Proc. Natl. Acad. Sci. USA 81, 1346-1350.

295. Kadonaga, J. T., and Tjian, R. (1986). Affinity purification of sequence-specific DNA binding proteins. Proc. Natl. Acad. Sci. USA 83, 5889-5893.

296. Weil, P. A., Luse, D. S., Segall, J., and Roeder, R. G. (1979). Selective and accurate initiation of transcription at the Ad2 major late promotor in a soluble system dependent on purified RNA polymerase II and DNA. Cell 18, 469-484.

297. Lewis, M. K., and Burgess, R. R. (1982). Eukaryotic RNA Polymerases. In The Enzymes, Vol. 15,

Boyer, P. D., ed., Academic Press, New York.

298. Ringold, G. M., Shank, P. R., and Yamamoto, K. R. (1978). Production of unintegrated mouse mammary tumor virus DNA in infected rat hepatoma cells is a secondary action of dexamethasone. J. Virol. 26, 93-101.

299. Schena, M., unpublished results.

300. Schena, M., and Foster, B., unpublished results.

301. Perucho, M., Hanahan, D., and Wigler, M. (1980). Genetic and physical linkage of exogenous sequences in transformed cells. Cell 22, 309-317.

302. Thompson, E. B., Tomkins, G. M., and Curran, J. F. (1966). Induction of tyrosine α–ketoglutarate transaminase by steroid hormones in a newly established tissue culture cell line. Proc. Natl. Acad. Sci. USA 56, 296-303.

303. Myers, R. M., Zoia, L., and Maniatis, T. (1985). Detection of single base substitutions by ribonuclease cleavage at mismatches in RNA:DNA duplexes. Science 230, 1242-1246.

304. Winter, E., Yamamoto, F., Almoguera, C., and Perucho, M. (1985). A method to detect and characterize point mutations in transcribed genes: Amplification and overexpression of the mutant c-Ki-ras allele in human tumor cells. Proc. Natl. Acad. Sci. USA 82, 77575-7579.

305. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular cloning-- a laboratory manual. Cold Spring Harbor Laboratory.

306. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nuc. Acids Res. 11, 1475-1489.

307. Miller, J., McLachlan, A. D., and Klug, A. (1985). Repetitive zinc-binding domains in the protein

transcription factor IIIA from Xenopus oocytes. EMBO J. 4, 1609-1614.

308. Brown, R. S., Sander, C., and Argos, P. (1985). The primary structure of transcription factor TFIIIA has 12 consecutive repeats. FEBS Lett. 186, 271-274.

309. Luisi, B., Freedman, L., Sigler, P., and Yamamoto, K. R., unpublished results.

310. Chamberlin, M. J. (1976). RNA Polymerase-- An overview. In RNA Polymerase, Losick, R., and Chamberlin, M., eds., Cold Spring Harbor Laboratory, pp. 17-67.

311. Lusky, M., and Botchan, M. (1981). Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences. Nature 293, 79-.

312. Kadonaga, J. T., Carner, K. R., Masiarz, F. R., and Tjian, R. (1987). Isolation of cDNA encoding transcription factor Sp1 and funtional analysis of the DNA binding domain. Cell 51, 1079-1090.

313. Kadonaga, J. and Tjian, R., personal communication.

314. Herman, P. W., personal communication.