teristics (such as malignant potential and drug resistance) may depend on differences in gene expression that can be revealed by in situ hybridization with appropriate probes. The method of using biotin-substituted probes (*6, 7, 13, 14*) is particularly elegant since, once synthesized, such probes are stable and detection does not require lengthy exposure times. The usefulness of such probes will increase as methods are developed for improved sensitivity of detection and more accurate quantitation of the signal.

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Correlation of Glucocorticoid Receptor Binding Sites on MMTV Proviral DNA with Hormone Inducible Transcription

Abstract. *Steroid hormones, when complexed to their receptors, recognize and bind specific DNA sequences and subsequently induce increased levels of transcription. The mechanisms of steroid hormone action were analyzed by constructing chimeric DNA molecules from portions of mouse mammary tumor virus envelope and long terminal repeat (LTR) regions ligated to the thymidine kinase (tk) gene of herpes simplex virus. This construction allowed the tk gene to be expressed in a hormone-responsive fashion upon transfection into Ltk$^-$ cells. Comparison of transcription data with in vitro binding data showed that hormone-responsive transcription can be directly correlated to the presence of steroid hormone receptor binding sites on the DNA. There are at least two such receptor binding sites in the LTR region, one between −202 and −137 and another between −137 and −50 base pairs from the RNA cap site, as well as a site near the 5′ end of the envelope region. These results strengthen the hypothesis that steroid-receptor complexes regulate genes primarily by binding to DNA sites near the promoter region and thereby modulate transcription.*

Steroid hormones bind specific cytoplasmic receptor molecules which then undergo an activation process and accumulate in the cell nucleus. The activated steroid-receptor complex, which is thought to act as a modulator of gene expression, is capable of recognizing specific DNA sequences near the promoter region of genes that are under steroid hormone control (*1–6*). This suggests that such specific binding sites on the DNA have a primary role in the modulation of gene expression by the steroid-receptor complex. The mechanism of gene regulation by steroid hormones in eukaryotic cells might therefore be similar to prokaryotic gene regulation wherein regulatory proteins bind to a specific DNA sequence in the promoter region of genes under their control, thereby changing the rate of transcription.

Mouse mammary tumor virus (MMTV) serves as a convenient system in which to analyze the site or sites necessary for steroid hormone regulation. This system has been used to study both in vitro binding (*1, 4–6*), involving DNA restriction enzyme fragments and the glucocorticoid-receptor complex, and in vivo transcription, in which cell cultures are transfected with the desired DNA fragments (*7–9*).

In the experiments described here we attempted to correlate previously obtained transcription data (*9*) with DNA binding data to define the sequence requirements for gene expression induced by the glucocorticoid receptor. For this purpose we analyzed a set of chimeric DNA molecules in vitro for the presence of glucocorticoid receptor binding sites. The chimeric DNA molecules contain proviral MMTV sequences, including part of the envelope (env) gene and long terminal repeat (LTR), as well as the thymidine kinase (tk) gene of herpes simplex virus (HSV) (see Table 1). These chimeric genes, which lacked increasing length of sequences from the LTR, had previously been introduced into cultured L cells, and analysis of their transcripts had enabled investigators to define the LTR regions necessary for hormone-responsive transcription. Hormone-sensitive transcripts initiating correctly in the tk gene were also observed (Table 1) (*9*).

In Table 1 we show the DNA sequences present in the chimeric molecules and point out the DNA fragments that were assayed. Eco RI restriction fragments from the six different plasmids, containing various amounts of DNA from the MMTV env and LTR regions and 222 base pairs (bp) of mouse genomic DNA, were purified in microgram quantities and used in the DNA-cellulose competition assay (*4*). In this assay, ^{3}H-labeled triamcinolone acetonide (^{3}H-TA)–receptor complex is mixed with a DNA-cellulose suspension, where the cellulose-bound DNA is nonspecific (calf thymus) DNA. The amount of hormone-receptor complex bound to DNA-cellulose can be easily determined by pelleting the DNA-cellulose and counting the radioactivity associated with it. If free DNA is mixed with the DNA cellulose, the free DNA competes with the DNA cellulose for labeled steroid-receptor complex. DNA fragments containing specific binding sites for the glucocorticoid-receptor complex compete more strongly than nonspecific DNA fragments (*4*). Typical competition curves from the DNA-cellulose binding assay are shown in Fig. 1. Eco RI fragments from plasmids −236, −202, and −137 (Fig. 1, A and B) compete approximately five times better than nonspecific DNA [in this case, a 750-bp (Fig. 1D)

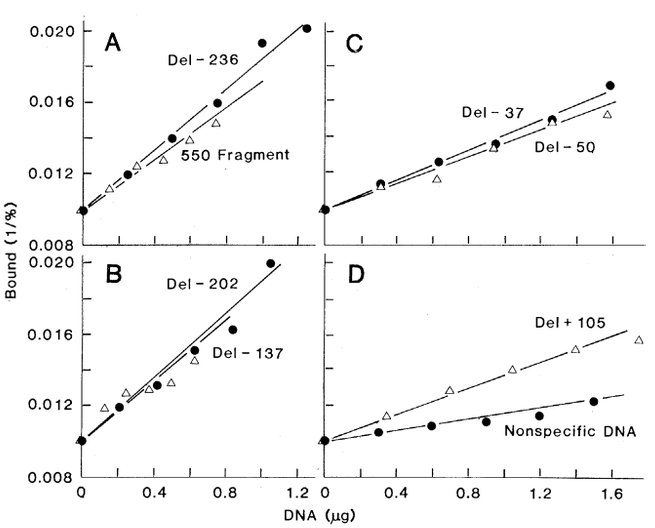
fragment from pBR322] and compete approximately to the same extent as a 550-bp Pst I fragment which had previously shown specific binding (*4*). These results are thus in good agreement since the 550-bp Pst I fragment contains LTR sequences 262 bp 5′ of the RNA cap site. The Eco RI fragments from plasmids −37, −50, and +105 (Fig. 1, C and D) compete considerably less well than the 550-bp fragment but still clearly better than the nonspecific DNA.

These results were reproducible and suggest that a major binding site or sites for the glucocorticoid receptor is contained on the Eco RI fragments from plasmids −236, −202, and −137; they also suggest that the Eco RI fragments from the other three plasmids also contain one or more sites with increased affinity for glucocorticoid receptor. The latter was somewhat surprising, especially in the case of plasmid +105 which had seemed unlikely to contain a binding site, since all LTR sequences are deleted except for 29 bp at the 3′ end of the LTR region. This suggested that the DNA fragments tested contained a receptor binding site outside the LTR region. To investigate this possibility, we isolated Bam HI–Eco RI subfragments from plasmids −236 and +105. In both cases, the Bam HI site separates the env region from the LTR region. Competition experiments with the Eco RI–Bam HI subfragments are shown in Fig. 2. The LTR fragment from plasmid −236 competes approximately four times better than nonspecific DNA, and the env fragments from plasmids −236 and +105 compete 2.5 to 3 times better than nonspecific DNA. The LTR fragment from plasmid +105 behaves like nonspecific DNA and, therefore, does not contain a receptor binding site. These results so far allow the conclusion that one glucocorticoid receptor binding site is located near the 5′ end of the env region and another site (or sites) is located in the LTR region. A binding site near the 5′ end of the env region has also been reported by Geisse *et al.* (*6*).

Table 1. LTR-tk DNA molecules and their characteristics. The plasmids and their response to hormonal induction have been described (*9*). In these constructs deletions extend from the env gene into the 3′ LTR such that the 5′ proximal region of the env region is fused to the 3′ proximal region of the LTR. The MMTV DNA originates from mouse genomic DNA and is therefore at the 3′ end flanked by mouse DNA (*12*). The plasmid names refer to the number of base pairs remaining upstream from the RNA cap site (vertical arrow) in the LTR. The LTR and env regions are drawn approximately on scale; broken lines represents deleted LTR and env regions; single line represents mouse DNA or vector DNA. Note that the hormonal induction of two transcripts (horizontal arrows), each with their own promoter, is measured [*results of S1 nuclease analysis to map the correctly initiated RNA (*9*)] and that the LTR region is upstream of the tk region (both are downstream of the steroid receptor binding sites). Eco RI (*R*) fragments were used in the DNA-cellulose competition assay. In the cases of plasmid −236 and +105, Eco RI-Bam HI (*B*) fragments were also analyzed.

Plasmid	Deletion of LTR (distance from cap site)	env (bp)	LTR (bp)	Mouse DNA (bp)	Eco RI fragment (bp)	Dex sensitive transcription from RNA initiation site*	
						tk	LTR
R B R env LTR TK PBR	-236	450†	370	222‡	1042	Yes	Yes
R R	-202	~389	336	222	~950	Yes	Yes
R R	-137	~121	271	222	~615	Yes	Yes
R R	-50	~153	184	222	~560	No	Yes
R R	-37	~160	171	222	~540	No	No
R B R	+105	450	29	222	701	No	No

*Dexamethasone (*Dex*)-sensitive transcription from RNA initiation site was measured by S1 analysis to map the correctly initiated RNA (*9*). †The sequence of the env region is described in (*13*). ‡See (*14*).



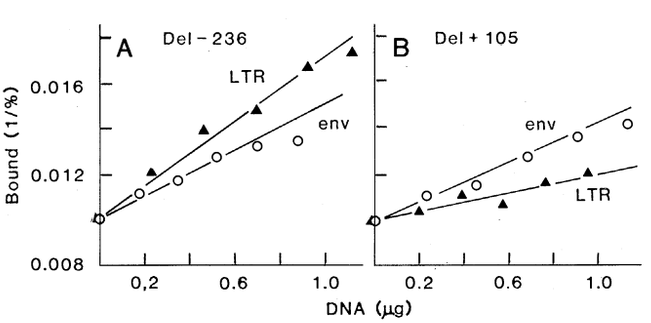


Fig. 1 (left). Receptor binding to Eco RI fragments. The DNA-cellulose competition assay and the preparation of activated receptor extract has been described (*4*). ^{3}H-Labeled triamcinolone acetonide (TA)–receptor complex from rat liver cytosol was incubated with a constant amount of DNA-cellulose and increasing amounts of purified Eco RI restriction enzyme fragments. The percentage bound represents specifically bound counts of ^{3}H-TA–receptor complex in the presence of competing DNA divided by the total competible bound counts in the absence of competing DNA. The 550-bp fragment is a Pst I fragment from the 5′ MMTV LTR and serves as a positive control (*4*). The nonspecific DNA (negative control) is a purified 750-bp Pst I–Eco RI DNA fragment from pBR322. For descriptions of the other DNA fragments, see Table 1. The Eco RI fragments from plasmids −236, −202, −137, and the positive control (A and B) compete four to five times as well as the nonspecific DNA (D). The Eco RI fragments from plasmids −50, −37, and +105 (C and D) compete about twice as well as the nonspecific DNA. All competition curves were obtained at the same time. Fig. 2 (right). Receptor binding to DNA subfragments. Competition experiments were carried out as described in Fig. 1 and as previously reported (*4*). (A) Eco RI–Bam HI subfragments from plasmid −236 (Table *1*); (B) Eco RI–Bam HI subfragments from plasmid +105. The LTR subfragment from plasmid 105 behaves like nonspecific DNA (data not shown). The env subfragments from plasmids −236 and +105 compete 2.5 to 3 times as well as nonspecific DNA. The LTR subfragment from plasmid −236 competes four times as well as nonspecific DNA.

A more detailed analysis of binding data is given in Table 2. Data from several individual competition experiments are combined. The increase in affinity observed for one restriction fragment over nonspecific DNA allows us to calculate the increase in affinity for a single hypothetical specific binding site on that DNA fragment. For these calculations a size of 10 to 50 bp for a specific binding site is assumed with the understanding that each base pair of a DNA fragment can start a new receptor binding site [for a discussion of these calculations, see (*4*)]. The binding site in the env region is approximately 750 times stronger than an average 10- to 50-bp DNA sequence (Table 2). The binding site in the LTR region is three to four times stronger than the env site and may be composed of several subsites. Support for existence of several subsites comes from the clearly decreased affinity observed for the −137 env-LTR fragment compared to the −236 and −202 fragments. This would place one receptor binding site in the LTR region between −202 and −137 of the cap site and a second receptor binding site between −137 and −50. The notion of subsites and their placement by our data finds support in the work of Payvar *et al.* (*10*).

The results obtained with the in vitro binding assay agree well with the previously reported transcription results (*9*), with the exception of plasmid −50 for which hormonal induction of the LTR transcript was observed (see Table 1) but which does not appear to have a binding site in the LTR region. In vitro, the −50 Eco RI fragment shows no higher specificity for the steroid-receptor complex than the −37 and +105 Eco RI fragments. Since the env region contained on the +105 fragment could be shown to contain a receptor binding site (results with Bam HI–Eco RI subfragments), it is possible that the increase in affinity observed for the −50 and −37 Eco RI fragments over nonspecific DNA is also, in these two cases, due to the binding site in the env region.

It therefore appears that the steroid-receptor complex bound to the site in the env region can promote downstream transcription. This would explain the hormone inducible LTR transcript obtained with the −50 plasmid (*9*). Lack of hormonal induction of LTR transcripts in plasmids −37 and +105 probably results from essential elements of the LTR promoter having been deleted. With the −50 plasmid weak steroid induction of downstream transcription of LTR sequences is readily observable because, in the absence of hormone, no LTR transcripts are found. However, in the case of the tk promoter transcripts, the basal level in the absence of hormones was much higher and therefore might have obscured detection of any weak hormonally induced downstream transcription. A strong decrease in transcription levels was observed with the −137 plasmid when compared to the plasmid −236 and −202. This decrease in transcription may be due to the deletion of a GTGGTTT (where G is guanine and T is thymine) sequence at −148; this sequence has been described as a consensus sequence for promoter enhancers (*11*).

To our knowledge this is the first time in which a rigorous comparison of transcription data with in vitro binding data has been carried out in a hormone responsive system. The DNA sequences of the MMTV LTR region which appeared necessary in the transcription assay for hormonal induction could be shown by the in vitro binding assay to contain glucocorticoid receptor binding sites. There appear to be at least two sites in the LTR region, one between −202 and −137 and the other between −137 and −50. One additional site was found near the 5′ end of the env region. In the absence of the LTR receptor binding sites, the env site, when placed near the LTR promoter, apparently can hormonally control LTR promoter transcripts. While data from the transcription assay helped locate the regions necessary for hormonal induction, the in vitro binding assay has shown that the steroid receptor complex has a marked increased affinity for sites in these regions.

The data thus show that specific DNA binding sites for the steroid receptor complex near the promoter region of a gene are necessary for hormonal regulation of transcription. Steroid hormone receptors thus appear to function as does the *Escherichia coli* cyclic AMP receptor protein. In the presence of cyclic AMP, this receptor protein recognizes specific DNA sequences in the promoter regions of responsive genes and thereby modulates the rates of their transcription.

Table 2. Receptor binding characteristics of deletion fragments.

DNA fragment tested (bp)	DNA for 1/2 maximum competition (μg)*	Increase in affinity†	
		For total fragment	For one specific site per fragment
−236 env-LTR (1050)	1.19 ± 0.10 (N = 5)	3.62	3800
−202 env-LTR (950)	1.18 ± 0.15 (N = 3)	3.66	3480
−137 env-LTR (615)	1.11 ± 0.10 (N = 3)	3.95	2430
−50 env-LTR (560)	2.7 ± 0.20 (N = 3)	1.04	580
−37 env-LTR (540)	2.4 ± 0.20 (N = 3)	1.29	700
+105 env-LTR (750)	2.7 ± 0.20 (N = 3)	1.03	770
550 LTR (550)	1.4 ± 0.10 (N = 5)	2.93	1610
−236 LTR- (595)	1.37 ± 0.10 (N = 4)	3.02	1740
−236 env (475)	1.92 ± 0.20 (N = 4)	1.86	880
+105 LTR (275)	4.6 ± 0.30 (N = 2)	0	0
+105 env (475)	2.2 ± 0.20 (N = 3)	1.5	710
Nonspecific (750)	5.5 ± 0.20 (N = 5)	0	0

*The amount of DNA needed for 1/2 maximum competition was determined from competition curves such as shown in Figs. 1 and 2. †The increase in affinity for a fragment is the ratio of the amounts needed for 1/2 maximum competition of nonspecific DNA over tested fragment minus one. For the calculation of the increase in affinity for one specific site per fragment, the assumption has been made that each base pair of a DNA fragment can start a new binding site (neglecting end effects). The increase in affinity for one specific site per fragment is therefore the observed increase for the total fragment times the number of base pairs per fragment (*4*).

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