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Functional analysis of the steroid hormone control region of mouse mammary tumor virus

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ABSTRACT

Gene fusions between the mouse mammary tumor virus long terminal repeat and the *E. coli lacZ* gene have been shown to exhibit hormone dependent expression of β-galactosidase activity. These constructions were used in transient expression experiments to assess the effects of specific modifications introduced into the region upstream of the transcription initiation site. 5' deletions demonstrate that sequences sufficient for wild-type promoter function are contained downstream of residue -64 relative to the initiation site. Other deletions define a region of approximately 80 base pairs between -220 and -140 which contains sequences essential for hormonal control. Between this control region and the promoter lie sequences dispensable for both functions.

INTRODUCTION

Mouse mammary tumor virus (MMTV) provides a unique opportunity for understanding the regulation of eukaryotic gene expression at the molecular level. A retrovirus, MMTV integrates a DNA copy of its own genome within that of its host, and transcription of the provirus is positively regulated by glucocorticoid hormones (1). While there are many examples now of specific gene regulation in eukaryotes, there are relatively few cases for which there exists any information on the cellular components which mediate the response. For steroid regulation, however, there is considerable genetic and biochemical evidence that cytoplasmic receptor proteins perform this function first by binding the hormone and subsequenly by binding to target DNA sequences associated with specific genes (2,3). The general mechanism for steroid receptor mediated regulation of cellular genes also applies for MMTV.

Recent studies on MMTV have provided information on several components of the regulatory system. Characterization of fusions between the long terminal repeat (LTR) of the virus and other genes clearly demonstrate that the LTR, which contains the single known viral promoter, also contains sufficient information to establish hormonal regulation (4,6). In addition, several groups have shown that purified receptor protein bound with steroid can bind *in vitro*

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to specific sites on the MMTV genome, including one or more sites within the LTR (7-12). These results are consistent with the postulated interaction between the steroid receptor complex and a specific recognition site(s) in the MMTV LTR.

In the studies described here, we have used several *in vitro* mutational approaches in combination with DNA transfection experiments to define sequences within the LTR which are involved in hormonal regulation of the MMTV promoter. We find that the region approximately 220 to 140 base pairs upstream of the transcription initiation site contains sequences essential for hormonal regulation. These sequences presumably constitute all or part of the target sequences which are recognized by and interact with the steroid-receptor complex. In addition, we wished to determine whether there exists physical or functional overlap between sequences involved in receptor interaction and sequences involved in promoter function. Our results suggest that these two control elements are separable.

MATERIALS AND METHODS

Plasmid constructions.

Construction of (Δ-64). pSVM-*gpt* (13) was cleaved with *Cla*I and *Sst*I. Following treatment with nuclease S-1 to produce blunt ends, the larger *Cla*I-*Sst*I fragment was purified by agarose gel electrophoresis, and recircularized using T4 DNA ligase. The ligation mix was used to transform *E.coli* strain HB101 and ampicillin resistant transformants were selected. Plasmids were screened initially by restriction analysis to estimate the length of the deletion. One deletion was analyzed by DNA sequencing.

Construction of Bal 31 deletions, Δ24, Δ28, and Δ94. pCH110 containing the MMTV LTR (Figure 1) was first digested with *Sst*I which cuts once in the MMTV LTR. Bal 31 exonuclease was added and samples of DNA were removed at various times to terminate the reaction. These samples were treated with T4 DNA polymerase and the four deoxynucleotide triphosphates to produce flush ends on the DNA. A synthetic *Xho*I linker (CCTCGAGG, Collaborative Research) was then ligated to each end. The DNA molecules were recircularized with T4 DNA ligase and transformed into HB101 selecting for ampicillin-resistant colonies. In a parallel experiment, the *Sst*I site was converted to an *Xho*I site without Bal 31 treatment creating a minor modification of the wild-type sequence around -102. A *Cla*I-*Xho*I fragment (from -858 to -102) from this plasmid was excised and replaced with the corresponding *Cla*I-*Xho*I fragments from the Bal 31 deletions. In this manner deletions removing only sequences upstream of the *Xho*I site were

produced. The approximate sizes of the deletions were determined by restriction analysis of plasmids isolated from small cultures. Larger cultures of candidate deletion plasmids were prepared for DNA sequence analysis and DNA transfections.

Construction of Δ-221 and Δ-174. The HaeIII-221/+124 fragment from the LTR was isolated by acrylamide gel electrophoresis and HindIII linkers ligated to each end. The fragment was then ligated into the unique HindIII site on pCH110 to construct Δ-221. The orientation was confirmed by DNA sequence analysis. Δ-174 was constructed by first linearizing Δ-221 with BglI, followed by Bal 31 digestion of both ends. HindIII linkers were ligated to each end and subsequent digestion with HindIII yielded shortened fragments with one end point at +124 and the other end variable due to the Bal 31 treatment. The heterogenous fragments were sized by gel electrophoresis and a shortened subpopulation isolated and ligated into the HindIII site on pCH110. One of these was selected for sequence analysis; the endpoint of the Bal 31 generated deletion was determined to be -174.

Construction of insertion mutants IS-1 and IS-2. pMT11 (H. Huang and K. Moore, unpublished) was digested with SmaI and HindIII and the SmaI/HindIII 57 base pair fragment isolated by gel electrophoresis. The HindIII sticky ends were filled in as described. Δ28 was linearized by digestion with XhoI and the sticky ends filled in as described. The 57 base pair fragment from pMT11 was blunt-end ligated to the linearized Δ28 under conditions favoring recircularization. Individual clones were screened for the presence of new unique linker restriction sites within the plasmid and the orientation subsequently determined by DNA sequence analysis.

IS-2 was constructed by digesting IS-1 with BglII and SalI, filling in the resulting sticky ends as described and recircularizing the shortened plasmid. The deleted sequences were confirmed by DNA sequence analysis.

Construction of Δ48.1, Δ48.2, and Δ56. IS-1 was linearized with XbaI and the ends digested with exonuclease III under conditions favoring removal of less than 50 base pairs (14). The exonuclease III was removed by phenol treatment followed by ethanol precipitation. The exonuclease treated plasmid was digested with XhoI to remove any of the remaining 57 base pair linker, the sticky ends filled in as described and the plasmids recircularized. Individual plasmids were digested with HaeIII to determine the approximate deletion size; three were sequenced.

DNA transfections.

Stable transformants of mouse 3T6 cells were isolated using pSVM-gpt DNA

and the $CaPO_4$ transfection procedure as described previously (4). The selection for gpt^+ transformants was performed using mycophenolic acid (25 μg/ml) according to the procedure of (5). Pools of clones were grown for further analysis as described in the legend to Figure 2.

Transient expression of β-galactosidase activity directed by MMTV-lacZ fusion plasmids was determined as described previously (15). β-galactosidase assays and protein determinations (16) were analyzed in 96 well microtiter dishes with the aid of an ELIZA reader (Dynatech).

S-1 nuclease mapping.

Total cellular RNA was isolated from cells grown in the presence or absence of 10^{-6}M dexamethasone according to the method of Chirgwin, et al (17). The probe used for hybridizations was an EcoRI-BglI fragment labelled at the BglI end (18). RNA-DNA hybridizations and S-1 treatment were performed according to Berk and Sharp (19), and hybrids were analyzed on polyacrylamide gels as described previously (18).

DNA sequencing.

DNA sequencing was performed using the chemical modification methods of Maxam and Gilbert (20). Fragments were end labelled with γ^{32}P-ATP and T4 polynucleotide kinase (21). Internal deletions and insertions were sequenced starting at a HaeIII site in the MMTV LTR (position -221). 5' deletions were sequenced starting at the HindIII linker (Δ -221, Δ -174), or in the case of Δ -64 at an HpaII site at +106 in the LTR.

RESULTS

Defining the limits of the MMTV promoter.

We have previously constructed and characterized plasmids which contain fusions between the MMTV regulatory region and the bacterial genes Eco-gpt and lacZ, and the mouse dihydrofolate reductase cDNA (4,13,15). In particular, fusions between the MMTV LTR and Eco-gpt exhibit hormonally inducible production of Eco-gpt mRNA in stable transformants of mouse 3T6 cells. S-1 nuclease mapping of the MMTV transcripts demonstrates that the initiation site and the extent of hormonal regulation corresponds to that observed for the intact viral genome (14).

Figure 1 shows the approximate location of the transcription start site within the 1.4 kilobase Hind III fragment carrying the LTR fused to either Eco-gpt (13) or lacZ (15). This fragment was originally cloned as a PstI-PstI fragment and contains almost an entire LTR (22). In order to locate the approximate boundary of the promoter sequences within the MMTV LTR, we isolated

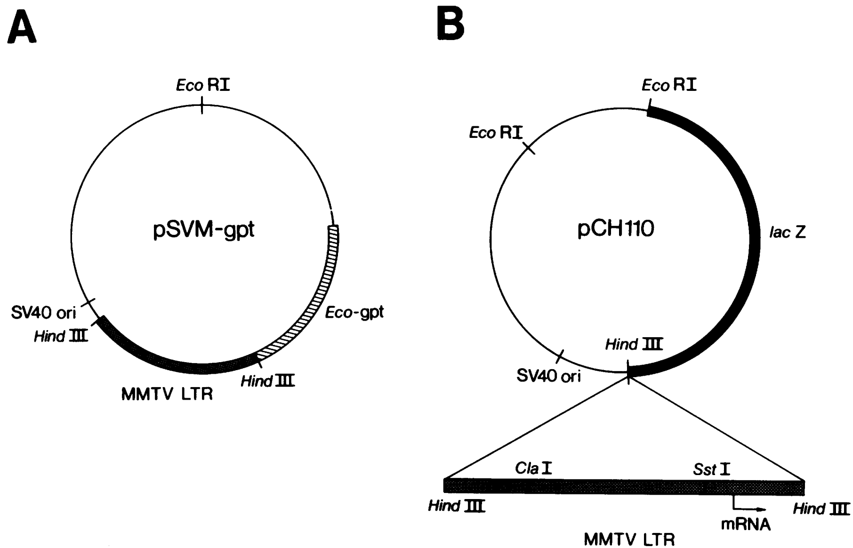

Fig. 1 A) Plasmid pSVM-gpt (18). The 1.4 kilobase MMTV LTR fragment is shown stippled; the Eco-gpt coding region is hatched. B) Plasmid pCH110. The MMTV LTR fragment is inserted into the unique HindIII site of pCH110. The ClaI and SstI restriction sites occur at positions -858 and -102, respectively, relative to the MMTV transcription start site indicated by the arrow (29). The lacZ containing fragment is shaded in black.

a deletion of the regulatory region by cleavage at the unique ClaI and SstI sites located 858 and 102 base pairs, respectively, upstream of the transcription start site (position +1) followed by recirculization of the shortened pSVM-gpt plasmid. During this cloning procedure, several unexpected plasmids were isolated which had deleted additional sequences closer to the initiation site. One of these was sequenced and found to be lacking sequences from -891 to -64. Since this deletion was initially isolated in pSVM-gpt, the Eco-gpt marker was used to introduce the plasmid into 3T6 cells, selecting for stable transformants. The deletion did not reduce the efficiency of transformation compared to the wild type. When the RNA from pools of transformants was analyzed by S-1 nuclease digestion, the results showed that the initiation site was the same as for wild-type pSVM-gpt, although production of the RNA was not stimulated by the addition of dexamethasone (Figure 2). These results demonstrate that removal of sequences upstream of -64 does not remove sequences essential for promoter function because the synthesis of MMTV RNA is unimpaired both in quantity and specificity of initiation. Since hormonal regulation is abolished by removal of the upstream sequences, the

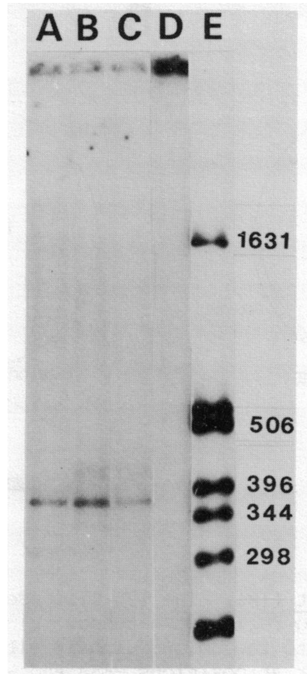

Fig. 2 S-1 nuclease mapping of MMTV transcripts. Hybridizations were performed between 5' end-labelled probe (EcoRI-BglII fragment, see Material and Methods) and 25 μg of total cellular RNA isolated from stable transformants. RNA was isolated from the following transformed mouse 3T6 cells: A) pSVM-gpt transformed, pool of 9 clones.; B) transformed with MMTV mutant Δ-64, pool of 19 clones, minus dexamethasone; C) same pool as (B), grown in 10^{-6}M dexamethasone; D) untransformed 3T6 cells; E) radiolabelled DNA molecular weight markers, sizes given in base pairs. For both pSVM-gpt and Δ-64, the number of transformants analyzed was 25% of the total obtained from transforming 10^6 initial cells.

region required for hormonal control must be located upstream of -64.

5' deletions into the MMTV regulatory region.

We have shown previously that the synthesis of β-galactosidase directed by MMTV-lacZ fusions in transient expression experiments provides a convenient and quantitative assay for function of the MMTV regulatory region (15). We have employed this assay to characterize a set of deletion mutants in the regulatory region.

We began by analyzing the function of truncated LTR DNA fragments to determine the extent of upstream sequences required for regulation of the MMTV promoter. A HaeIII fragment with endpoints at -221 and +124 was isolated from the wild-type MMTV LTR, and was inserted using HindIII linkers in the correct orientation into the unique HindIII site of pCH110, fusing it to lacZ (Δ-221).

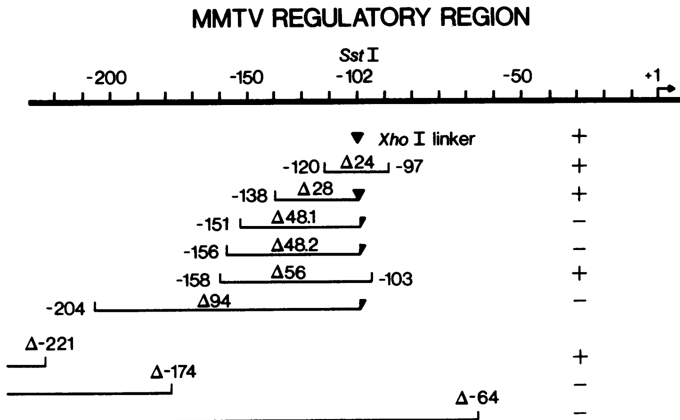

Fig. 3 Map of the MMTV regulatory region. The relative positions of 5' and internal deletions described in the text are indicated by horizontal lines. 5' deletions (those entering the regulatory region from the left) indicate the first base pair not removed by the deletion. Internal deletions are designated by the number of base pairs missing relative to the wild-type sequence. The numbers at the termini of internal deletions indicate the last residue missing in the deletion. The inverted triangles denote the presence of a synthetic XhoI oligonucleotide linker; half triangles indicate the presence of a portion of the linker oligonucleotide. The (+) or (-) symbols refer to the hormonal induction properties of each mutant.

In addition, a truncated HaeIII fragment was isolated using the exonuclease Bal 31 as described in the Methods. Nucleotide sequence analysis of this fragment showed that the deletion removed 5' sequences up to residue -174. This HaeIII fragment from -174 to +124 was also inserted into the HindIII site of pCH110 with HindIII linkers (Δ-174). Finally, the HindIII fragment carrying the Δ-64 deletion described above was also fused to lacZ in pCH110 (Figure 3).

The three MMTV-lacZ fusions (Δ-221, Δ-174, and Δ-64) were analyzed by transfection into mouse L-cells in the presence or absence of dexamethasone. As shown in Table 1, only Δ-221, which contains all the upstream sequences to base pair -221, is inducible by hormones. Δ-174 which has sequences extending only to -174 is not inducible. Δ-64 is also non-inducible as measured in these transient expression experiments, a result consistent with the analysis of RNA isolated from stable transformants. From these results and those in the previous section, we conclude first that the sequences within 221 base pairs of the transcription start site are sufficient for proper induction by steroid hormones and second, that basal promoter function is independent of steroid control.

TABLE 1.
5' Truncation of MMTV Regulatory Region
Expression of β-galactosidase from MMTV-lacZ fusions

β-galactosidase Levels (units/mg protein)				
Plasmid	n	- Hormone	+ Hormone	Induction Ratio
Mock transfection	6	0.5 ± 0.2	1.0 ± 0.4	--
pCH110/SV40-lacZ	7	30.0 ± 4.2	30.7 ± 4.2	--
MMTV-lacZ/wild-type	9	1.3 ± 0.4	12.7 ± 2.0	10
MMTV-lacZ/Δ-221	2	1.2 ± 0	7.0 ± 0.7	6
MMTV-lacZ/Δ-174	2	1.4 ± 0.2	2.0 ± 0.5	--
MMTV-lacZ/Δ-64	4	1.2 ± 0.4	1.1 ± 0.3	--

For transient expression experiments presented in this table and Tables 2 and 3, transfections into L-cells and β-galactosidase assays were performed as described previously (15). Cells were grown with or without 10^{-6}M dexamethasone. β-galactosidase specific activity is nmoles o-nitrophenyl-β-D-galactopyranoside cleaved per minute per mg of cellular protein. The activity for each DNA is the average determined for the number of individual determinations entered under the heading, n. The induction ratio is determined by the ratio of the induced relative to uninduced levels of β-galactosidase. Induction ratios less than or equal to that obtained with mock transfected cells (i.e., 2-fold) are not considered significant and are denoted by a dash.

Internal deletions of the MMTV regulatory region.

Having shown that the regulatory sequences can be functionally dissociated from the promoter, internal deletions were constructed to define the promoter proximal boundary of the regulatory region. The deletions described below have both endpoints between the transcription start site and position -221, which as shown above, contains sequences sufficient for hormonal regulation. Since the promoter sequences appear to be contained within 64 base pairs of the start site for transcription, we took advantage of a unique SstI site at residue -102 in the MMTV LTR and isolated Bal 31 deletions extending in both directions from this restriction site. The deleted plasmids were recircularized using XhoI linkers. In a parallel experiment, we employed the same procedure omitting the Bal 31 digestion in order to modify the SstI site to an XhoI site. A ClaI/XhoI fragment (-858 to -102) excised from this plasmid was replaced with the corresponding fragments from each Bal 31 deletion. This substitution made it

TABLE 2
Internal Deletions of MMTV Regulatory Region
Expression of β-galactosidase from MMTV-lacZ fusions

Plasmid	n	β-galactosidase Levels (units/mg protein) - Hormone	+ Hormone	Induction Ratio
MMTV-lacZ/XhoI linker	5	0.8 ± 0.2	9.0 ± 2.4	11
MMTV-lacZ/Δ24	4	1.1 ± 0.2	6.5 ± 1.0	5.5
MMTV-lacZ/Δ28	4	1.3 ± 0.3	13.4 ± 2.5	10
MMTV-lacZ/Δ48.1	5	0.5 ± 0.2	0.8 ± 0.4	—
MMTV-lacZ/Δ48.2	6	0.4 ± 0.1	0.75 ± 0.3	—
MMTV-lacZ/Δ56	6	0.75 ± 0.1	10.5 ± 1.9	14
MMTV-lacZ/Δ94	8	4.8 ± 1.5	3.8 ± 1.0	—

possible to isolate deletions primarily upstream of residue -102.

Three of the Bal 31 derived deletions, selected following preliminary restriction analysis, were sequenced to determine the exact deletion end points (Figures 3,4). These deletion plasmids, Δ24, Δ28, and Δ94, along with the XhoI linker modified mutant, were introduced into L-cells and the transient levels of β-galactosidase were determined with or without dexamethasone treatment. The results of the transfection analysis are summarized in Table 2. These results show that conversion of the SstI site to an XhoI site with the removal of 4 base pairs from the LTR and insertion of an 8 base pair linker does not interfere with hormonal induction. One Bal 31 deletion was isolated which removed sequences from -120 to -97 (Δ24) but which lacked an XhoI linker was also included in this analysis. This deletion is also wild-type in hormonal response as is Δ28 which removes upstream sequences as far as -138. These results suggest that the sequences between -97 and -138 are dispensable for hormonal induction. The longest Bal 31 deletion characterized was Δ94 whose left-hand endpoint is -204. This deletion cannot be induced by the addition of dexamethasome, although the constitutive levels of β-galactosidase appear to be somewhat elevated above the basal wild-type levels.

Consistent with the properties of the 5' deletions, the results with the Bal 31 deletions suggest a critical role for sequences upstream of -138 in the hormonal response. The longest internal deletion with wild-type hormonal

Fig. 4 The nucleotide sequence of linker modifications and deletion fusion points for internal deletions of the MMTV regulatory region. Bold-face residues represent base pairs originating from the synthetic XhoI linker oligonucleotide. Vertical dashed lines indicate the fusion point of the deletion. Residues are numbered relative to position +1 defined as the putative site of transcription initiation (30).

induction is Δ28. Using this deletion as the starting plasmid, an alternative approach to generating unidirectional deletions employing a synthetic linker DNA fragment and exonuclease III (see Materials and Methods) produced several deletions extending farther upstream of -138 but stopping short of -204 (Figure 2). The exact left-hand endpoints determined for three deletions are -158 (Δ56), -156 (Δ48.1) and -151 (Δ48.2). These deletions have somewhat different right-hand endpoints due to loss of part of the XhoI linker, but all retain residue -102 (Figure 4). Δ48.1 and Δ48.2 are both completely non-responsive to hormone, suggesting that at least some sequences essential for hormonal regulation are located between residues -138 and -156. Only Δ56 is an exception to this rule. Although this deletion removes an additional two base pairs of the wild sequence compared to Δ48.1, it remains fully inducible by hormone treatment (Table 2). It is possible that the new fusion sequence created by Δ56 produces a site recognized by the hormone receptor. Alternative explanations for the properties of this deletion will be discussed.

INSERTION MUTATIONS

WILD TYPE: -150 -140 -130 -120 -110 -100 -90 -80 -70

Δ28: -160 -150 -139 -102 -100 -90 -80 -70; XhoI linker

IS-1(+33): -139, 57 base pair insertion, -102; XbaI BglII SalI XhoI

IS-2(+3): -139, -102; BglII/SalI

Fig. 5 Insertions into the MMTV LTR. Top line is a map of the MMTV LTR. Second line indicates the region deleted by Δ28 with the inserted base pair XhoI linkers (thick black line). Third and fourth lines show the size and position of the foreign DNA sequences (thick black line) inserted into the XhoI site of Δ28.

Insertions between the promoter and regulatory region.

Results with deletion mutants Δ24 and Δ28 suggest that there are sequences upstream of the transcription start site which are dispensable for both promoter function and hormonal response. In order to investigate the relationship between the regulatory region and the promoter, we focused first on one question. Can the region deleted by Δ28, for example, be replaced by foreign DNA sequences and remain functional?

To attempt to answer this question, we used a fragment of synthetic DNA containing multiple restriction enzyme sites derived from the plasmid pMT11 (H. Huang and K. Moore, unpublished). This 57 base pair fragment extending from a SmaI site to a HindIII site was inserted into the unique XhoI site of Δ28 (IS-1 Figure 5). The nucleotide sequence of the inserted fragment shares no homology with the wild-type MMTV sequences absent in Δ28. The expression of β-galactosidase encoded by the plasmid carrying this insertion is essentially the same as in mock transfected cells with and without hormone treatment (Table 3). Thus, a deletion of 28 base pairs which does not affect hormonal regulation (Δ28, Table 2), is rendered poorly inducible when 57 base pairs of foreign DNA are substituted at the deletion site. A plasmid having two copies of the synthetic fragment inserted in tandem at the XhoI site is also poorly inducible (data not shown).

IS-1 replaces the deleted sequences of Δ28 with foreign sequences but, because of the length of the inserted fragment, increases the spacing between

TABLE 3
INSERTION MUTATIONS IN MMTV LTR

	β-galactosidase levels (units/mg protein)		
Plasmid	- Hormone	+ Hormone	Induction Ratio
Mock transfection	0.6	2.2	3.6
MMTV IS-1 (+33)-lacZ			
50 μg DNA	0.6	1.7	3
100 μg DNA	0.5	1.8	3.6
MMTV IS-1 (+33)-lacZ			
50 μg DNA	0.9	6.8	7.5
100 μg DNA	0.95	7.3	7.7

In this experiment, IS-1 and IS-2 were analyzed by transfection into L-cells with or without hormone treatment. Two concentrations of each plasmid DNA were used to assure that DNA saturation was achieved, and the levels of β-galactosidase at each concentration are shown.

sequences on either side of the XhoI site compared to wild-type. To investigate the possible effects of spacing or regulation, we constructed a derivative of IS-1 using restriction sites contained in the synthetic linker fragment to reduce the size of the inserted DNA. This derivative, IS-2, has a deletion of some of the inserted sequences producing a mutant having only 3 additional base pairs compared to the wild-type length. As shown in Table 3, this construction exhibits substantial although less than wild-type levels of hormonal induction (7X). These results with IS-2 suggest that substitution of unrelated DNA sequences into the region from -138 to -102 reduces but does not abolish regulation. However, enlarging this region by 30 base pairs compared to wild-type eliminates detectable hormonal response.

DISCUSSION

In these studies we have used expression of the E.coli lacZ gene under the control of the MMTV promoter to characterize the effect of specific alterations in the regulatory region of MMTV. The deletions of the LTR we have examined define the boundaries of the steroid hormone control region. The region between residues -138 and -220 contains sequences essential for hormonal regulation.

With the exception of one deletion which we discuss below, removal of sequences in this region abolishes induction by hormone. These 80 base pairs must contain all or part of the region which is recognized by and presumably interacts with the steroid receptor complex. There is good agreement between the location of these sequences and fragments of the LTR which have recently been reported to bind to purified receptor protein in vitro (7-12).

A second important conclusion we can draw from our results is that the sequences essential for the hormone response and promoter sequences are functionally non-overlapping. Transcription from the 5' deletion mutant, Δ-64, initiates properly in the presence or absence of hormone. Thus, sequences upstream of -64 are not essential for promoter function. The 64 base pairs upstream of the transcription initiation site contain sequences which resemble the TATA box of other eukaryotic polymenase II promoters, as well as a sequence between -64 and -61 which resembles the so-called CAT box, an upstream promoter element shared by some polymerase II promoters. However, the basal level of transcription encoded by this deletion mutant cannot be stimulated by the addition of hormone. These results are somewhat different than those presented by Hynes, et al (31), who find that a deletion retaining 50 base pairs upstream of the MMTV cap site still exhibits a residual level of hormone sensitivity. The presence of the SV40 enhancer on the vectors potentially complicates the analysis, but, because it is present in the presence or absence of hormone, the S-1 analysis (Figure 2) should reflect the inducibility of the -64 deletion. Our results suggest that it is possible to remove sequences responsible for hormonal induction without affecting the promoter. Furthermore, neither the deletion of sequences between -97 and -138 (Δ24, Δ28), nor the replacement of sequences from -102 to -138 (IS-2) with foreign DNA eliminates the response to hormone. These results suggest that the promoter and the hormone control region may be separated by as much as 75 base pairs, the region between -64 and -138.

The hormone-receptor complex acts as a positive regulator of transcription at the MMTV promoter. The exact mechanism by which the receptor protein acts is still unclear. One suggestion which has been made is that the hormone regulatory sequences may function as a hormonally activated "enhancer element" analogous to the SV40 enhancer (23-25). One property characteristic of enhancers is that they appear to be capable of activating transcription from promoters independent of the distance to the promoter up to several kilobases, although the precise nature of the intervening sequences can influence function of the enhancer. (26,27). The isolation of internal deletion mutations which still exhibit hormonal induction is consistent with the properties of an

enhancer element because the functional sites are moved closer to the transcription start site without affecting function. However, our results show that an insertion of 57 base pairs into a functional deletion mutant can substantially reduce hormonal responsiveness. Thus, either a relatively minor change in the spacing can affect hormone dependent activation of transcription or possibly sequences within this 57 base pairs preclude hormonal activation. Although this result suggests to us that the hormone receptor may require specific and relatively short range interactions with the promoter and/or RNA polymerase to exert its positive effects, it is too early for us to propose a precise mechanism. Consistent with either of these interpretations, removal of a portion of this insert restores near wild-type inducibility. Models that involve specific protein-protein interaction between the receptor and RNA polymerase as well as local or more long-range effects on DNA and chromatin structure must be considered.

Whatever the mechanism of activation is, our results allow us to propose a preliminary structure for the MMTV promoter-regulatory region. The promoter is contained downstream of base pair -64 relative to the transcription initiation site. The hormonal control region is contained either partially or entirely in the segment from -138 to -220. Intervening between these two elements is a region of unknown function, apparently dispensable for promoter function, and at least part of which is dispensable for hormonal response. One prediction we can make based on this structure of the regulatory region is that it might be functional when separated from the MMTV promoter and fused to a second heterologous promoter. We have recently constructed hybrids between the MMTV regulatory region and the adenovirus 2 late promoter or the SV40 early promoter (J. Culpepper and F. Lee, manuscript in prep; M. Costello and G. Ringold, manuscript in prep). When fused at the correct distance from the corresponding transcription initiation site, the MMTV sequences are capable of regulating expression from these heterologous promoters. Similar results have been obtained with fusions between MMTV and the herpes thymidine kinase promoter (23), as well as the Rous sarcoma virus promoter (28).

Several groups have recently reported that one or more steroid-receptor complexes are capable of binding to fragments of the MMTV LTR _in vitro_ (7-12). The results we have presented here do not distinguish between a single or multiple binding sites for receptor. They simply implicate a specific region upstream of the promoter in hormonal regulation. The properties of one deletion, however, may be consistent with the existence of multiple sites for receptor interaction. Although Δ56 removes sequences within the region we

believe is essential for hormonal response, this mutant retains full hormonal sensitivity. One simple explanation is that sequences on either side of the deletion are fused in such a manner that the resulting region is now recognized by the receptor. Alternatively, if multiple binding sites do exist, Δ56 may excise one site completely, bringing an upstream site more promoter proximal where it is functional. The Dnase I footprinting analyses conducted by Payvar, et al (10) and Scheidereit, et al (7) provide data consistent with this latter explanation. Results from both studies suggest that there are at least 2 receptor binding sites in the region from -100 to -200, which is the region affected by Δ56. In addition both studies find binding sites in the region from -70 to -100. Based on our findings that deletions removing sequences upstream of -140 can abolish hormonal regulation, we suggest that the presence of these promoter proximal binding sites alone is insufficient for regulation. Further studies in vitro with purified receptor as well as functional studies with additional mutatants, especially point mutants, will be needed to clarify the nature of the receptor-DNA interaction and the mechanism of receptor mediated induction of transcription.

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