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A small region of the mouse mammary tumor virus long terminal repeat confers glucocorticoid hormone regulation on a linked heterologous gene

(thymidine kinase/deletions/Rous sarcoma virus/long terminal repeat/promoters)

JOHN MAJORS AND HAROLD E. VARMUS

Department of Microbiology and Immunology, University of California, San Francisco, CA 94143

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ABSTRACT **Expression of mouse mammary tumor virus (MMTV) proviruses is transcriptionally regulated by glucocorticoid hormones. We have linked the MMTV long terminal repeat (LTR) to the coding region of the herpes simplex virus thymidine kinase gene and used this construction to characterize sequences within the LTR that are involved in glucocorticoid regulation. Our results show that 290 base pairs (bp) of the MMTV LTR, including 190 bp upstream from the start site for transcription, are sufficient to confer regulation on the downstream gene. Deletion of an additional 50 bp, leaving sequences from position −140 to +100, eliminates the response. However, the constitutive level of expression is maintained even after deletion of sequences upstream from position −80, indicating that sequences required for the hormone response can be distinguished from those required for basal expression. We also have shown, by making a 4-bp insertion or a 20-bp deletion around position −107, that the distance between the putative signal for hormone response and the start site of transcription can be varied without affecting regulation. Furthermore, replacement of MMTV sequences from position −59 to +100 with analogous sequences from the Rous sarcoma virus LTR does not change the regulation.**

Mouse mammary tumor virus (MMTV) gene expression is regulated at the transcriptional level by glucocorticoid hormones (1, 2). This control has been demonstrated after virus infection (3, 4) and after the introduction of molecularly cloned MMTV DNA into several different cell types (5–8). Two lines of evidence suggest that sequences within the MMTV long terminal repeat (LTR) are involved in the hormone response. First, subgenomic fragments containing the LTR confer glucocorticoid regulation on linked heterologous genes (7–9); second, in two assays glucocorticoid receptor protein binds preferentially to LTR DNA fragments (10–12). We have joined the LTR to sequences coding for the herpes simplex virus (HSV) thymidine kinase (TK) in studies which were designed, first, to identify viral sequences that are sufficient to confer glucocorticoid regulation on the linked gene and, second, to explore the relationship between those sequences and other promoter elements. We find that sequences located between 140 and 190 base pairs (bp) upstream from the start site of MMTV transcription (positions −140 to −190) are involved in the response and that the spatial relationship between these sequences and the transcriptional start site can be varied without affecting regulation.

MATERIALS AND METHODS

Plasmid Constructions. All restriction digests were carried out in standard buffers (10 mM Tris, pH 7.5/5 mM $MgCl_2$/0.5 mM dithiothreitol containing 0, 10, 50, or 150 mM NaCl). 5′ ends were dephosphorylated by treatment with bacterial alkaline phosphatase for 1 hr at 68°C in a buffered solution containing 10% dimethyl sulfoxide. Fragments for cloning were isolated on Sea-Plaque agarose (Marine Colloids, Rockland, ME) gels and extracted by melting the agarose at 68°C in two volumes of 0.3 M NaCl/20 mM Tris·HCl, pH 7.5/1 mM EDTA, followed by three extractions with phenol and precipitation with ethanol. DNAs to be ligated were treated with T4 DNA ligase overnight at 4°C. Individual constructions are described in the text. *Bal*-31 digestions were done in 1 M NaCl/12 mM $CaCl_2$/12 mM $MgCl_2$/20 mM Tris chloride, pH 8.0/1 mM EDTA for appropriate time intervals at room temperature. After extraction with phenol and precipitation with ethanol, fragments were treated with T4 polymerase (13) for 15 min at 37°C to provide blunt ends for ligation. The LTR *Bam*HI fragments used in p303 and p311 were made by cleaving the 1.4-kilobase (kb) *Pst* I fragment with *Hpa* II and either *Ava* I, which cuts 40 bp from the left end of the LTR, or *Hae* III, which cuts ≈1,000 bp from the left end of the LTR. The ends of each of these fragments were then filled in with T4 polymerase, and the fragments were cloned into *Bam*HI-cleaved pBR322, the ends of which also had been made blunt with T4 polymerase. Thereby *Bam*HI sites were regenerated. The *Bam*HI site at the end of the Rous sarcoma virus (RSV) LTR was generated by P. Luciw, who fused a *Bam*HI linker to the blunt end created by cleavage with *Bst*EII, filling in with T4 polymerase.

Plasmid Screening and Preparation. Plasmids were screened for appropriate inserts and deletions by the method of Birnboim and Doly (14). Plasmid DNAs were isolated on a large scale by a Triton X-100 lysis procedure. After the chromosomal DNA was pelleted, the plasmid-containing supernatant was treated with RNase A, extracted one time with phenol/chloroform, 1:1 (vol/vol), and then precipitated with ethanol. RNA fragments were removed by chromatography over a Bio-Gel A-50 column. After ethanol precipitation, the DNA was used directly in transfection experiments.

Transfection. DNA was introduced into mouse LTK^- cells by the calcium phosphate coprecipitation technique as described by Graham and van der Eb (15). Between 2 and 5 μg of the precipitated plasmid DNAs were applied to plates containing 5×10^5 cells. After 2 days hypoxanthine/aminopterin/thymidine (HAT) medium (16) was applied. HAT-resistant colonies (generally about 50 per 60-mm plate) were allowed to form, and the uncloned population was expanded; 2 days prior to har-

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Abbreviations: MMTV, mouse mammary tumor virus; HSV, herpes simplex virus; TK, thymidine kinase; LTR, long terminal repeat; HAT, a selective medium; RSV, Rous sarcoma virus; Dex, dexamethasone; bp, base pair(s); kb, kilobase(s).

vest, dexamethasone (Dex; 0.5 μM) was applied to half of the plates.

RNA Isolation and Analysis. RNA was isolated by the oligo(dT)-cellulose batch elution procedure described by Varmus *et al.* (17).

Blot Hybridization. Poly(A)$^+$ RNA, 5 μg per lane, was electrophoresed through agarose in the presence of formaldehyde as described by Nusse and Varmus (18); the RNA was then transferred to nitrocellulose membranes (19) and hybridized with ^{32}P-labeled DNA probes, either to the 1.8-kb *Pst* I MMTV *env* gene fragment (20) or to the 3.4-kb *Bam*HI HSV TK DNA fragment (16), prepared by using reverse transcriptase to copy denatured template DNA in the presence of calf thymus DNA primers.

S1 Nuclease Analysis. S1 nuclease analysis was performed as described in Hackett *et al.* (21). A 600-bp *Sac* I fragment was isolated from p311, cleaved with *Bst*NI, treated with bacterial alkaline phosphatase (22), and then labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase. The 300-bp *Sac* I–*Bst*NI fragment was then isolated by electrophoresis through polyacrylamide and elution by the "crush and soak method" (22). Hybridizations were done at 54°C for 2 hr in the presence of 15 μg of yeast RNA. The hybrids were then diluted into S1 buffer (21), digested with S1 for 30 min in the presence of 5 μg of single-stranded calf thymus DNA, precipitated with ethanol, and electrophoresed through an 8% polyacrylamide gel.

RESULTS

A Proviral Clone Directs the Synthesis of a Glucocorticoid Responsive *env* mRNA. Molecularly cloned MMTV DNA is responsive to glucocorticoids after introduction into mammalian tissue culture cells (5–10). The MMTV reagents for our studies were derived from an unusual provirus molecularly cloned from the DNA of rat XC cells that had been infected with the C3H strain of MMTV (23). We found that the provirus was probably generated by reverse transcription of an MMTV *env* mRNA (24). Fig. 1 shows the structure of that provirus and an analysis of RNA from LTK$^-$ cells that received the cloned proviral DNA by transfection. We found a 3.6-kb RNA that hybridizes to an MMTV probe and has the same mobility as MMTV *env* mRNA; its concentration was increased 3- to 5-fold by Dex, a synthetic glucocorticoid, indicating that the cloned DNA retains (after transfer to mouse L cells) the hormonal responsiveness observed with the provirus in its original setting.

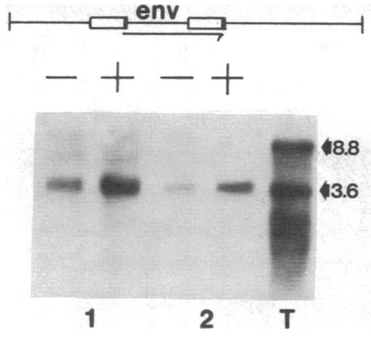

FIG. 1. A truncated provirus makes glucocorticoid-responsive *env* mRNA. Poly(A)$^+$ RNA, 5 μg per lane, from cells that received cloned proviral DNA (23) during cotransfection with the HSV TK gene was analyzed by electrophoresis through a formaldehyde/agarose gel, transferred to nitrocellulose filters, and hybridized with MMTV-specific [^{32}P]DNA as described. Samples 1 and 2 show RNAs from two different populations of cells, both of which were treated (lanes +) or untreated (lanes −) with Dex. Lane T shows mRNA from an MMTV-induced mammary tumor; the large (8.8 kb) species is MMTV genomic RNA; the arrow points to the 3.6-kb MMTV *env* mRNA. The line drawing at the top depicts the *Eco*RI fragment containing the truncated MMTV provirus with LTRs (boxes) and the *env* gene: arrow, transcribed domain; open boxes, LTR sequences upstream from the transcriptional start site (U3); closed boxes, downstream sequences (R-U5) (25).

The LTR Is Sufficient for the Response. To show that the LTR alone contains sufficient nucleotide sequences to generate the response, we took the LTR from the left end of the truncated provirus and linked it to a fragment containing the HSV TK gene (16) (Fig. 2). A 1.3-kb LTR fragment was joined to HSV TK gene sequences at a *Bgl* II site that lies within the TK mRNA leader to form the plasmid p303. The *Bgl* II site in the TK mRNA leader lies 50 bp downstream from the TK transcription initiation site, and the fused construction should result in a hybrid mRNA containing 100 bases from the MMTV LTR downstream from the transcriptional start site (positions +1 to +100), 50 bases of TK mRNA leader, and about 1,250 bases of TK mRNA coding and 3′ untranslated sequences (26).

We tested for glucocorticoid inducibility of RNA transcribed from p303 after calcium phosphate-mediated transfection of plasmid DNA into mouse LTK$^-$ cells. Mouse LTK$^-$ cells are growth-inhibited by Dex (27); therefore, we were unable to assess inducibility by a simple measurement of the efficiency of HAT-resistant colony formation. Instead we measured steady-state RNA levels after short-term (48 hr) incubation with the hormone. Because the uninduced MMTV promoter within plasmids such as p303 worked extremely poorly (see below), efficient colony formation in the absence of Dex demanded that we introduce large amounts of plasmid DNA (up to 5 μg per 60-mm plate) to the LTK$^-$ cells. After selection in HAT medium, populations of colonies (generally pools of 50 or more) were grown to confluence and analyzed for HSV TK poly(A)$^+$ RNA, with or without the addition of Dex. Fig. 2 shows that p303 serves as a template for an inducible 1.4-kb RNA species (arrow) that hybridizes to an HSV TK-specific DNA probe. Also apparent is a transcript of 900 bp, whose origin will be discussed later.

Deletions Define a Sequence Involved in the Hormone Response. Plasmid p303 contains all but 40 bp from the 5′ portion of the MMTV LTR; in plasmid p311, the LTR sequences terminate at a *Hae* III site 210 bp upstream from the transcription initiation site, thereby deleting approximately 1,000 bp from the 5′ end of the LTR. Included within the deleted region is a large open reading frame sufficient to encode a protein of M_r 36,000, whose function is as yet unknown (28–31). Fig. 2 shows the results of a test for the inducibility of MMTV–HSV TK RNA in cells transfected with a set of deletion mutants derived from p311 by progressive removal of sequences from the 5′ side. (Details of the constructions are described in the figure legend.) pdel(1), which retains MMTV sequences from position −190 to +100, still made the same amount of the 1.4-kb RNA in response to glucocorticoids as did p303. Removal of an additional 50 bp to generate pdel(2) (which retains sequences from position −140 to +100) and deletions extending further into the LTR [to position −80 in pdel(3) and to position −5 in pdel(4)] eliminated the capacity of the hybrid DNAs to synthesize the 1.4-kb RNA at high levels in the presence of hormone.

To ask whether the deletions affect only the glucocorticoid response of the MMTV promoter, we had to measure the uninduced (constitutive) MMTV expression. To focus more closely on correctly initiated transcripts, we used S1 nuclease protection experiments to analyze the RNA samples described in Fig. 2A. A 300-bp *Sac* I–*Bst*NI fragment, 5′-labeled at the *Bst*NI end, was denatured and hybridized with the RNAs prior to digestion with S1 nuclease. RNA initiated at the appropriate site within the MMTV LTR should protect exactly 200 bases of

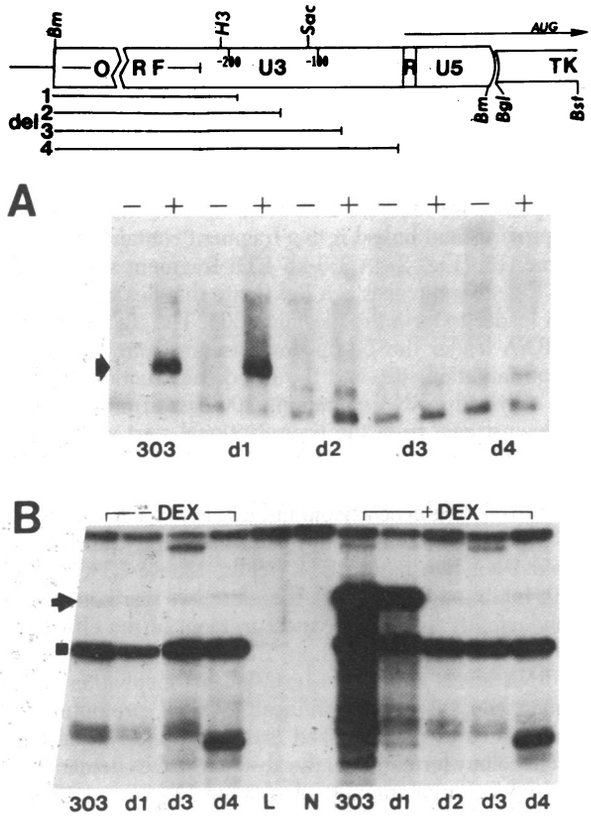

FIG. 2. MMTV-HSV TK gene fusions define sequences involved in glucocorticoid regulation. Shown schematically at the top of the figure is the structure of the control region of p303. The 1.3-kb LTR *Bam*HI fragment was cloned between the *Bam*HI (*Bm*) site of pBR322 and the *Bgl* II (*Bgl*) site in the HSV TK gene leader. p311 (not shown) deletes the open reading frame (ORF) sequences and terminates at the *Hae* III (*H3*) site, which was converted to a *Bam*HI site. The deletions (del 1–4) were constructed by cleaving p311 with *Bam*HI, followed by digestion with *Bal*-31, cleavage in adjacent plasmid DNA with *Hin*dIII, and religation. Thus, in each of the deletion plasmids, the MMTV sequences are joined to the *Hin*dIII site of pBR322. Deletion end points were determined by Maxam–Gilbert sequence determination procedures (22). Also shown are sites for *Sac* I (*Sac*) and *Bst*N1 (*Bst*), and the approximate location of the HSV TK gene initiation codon (AUG). Positions within the LTR are numbered with respect to the start site of transcription. (*A*) Blot hybridization analysis of RNAs from cells that received plasmid DNAs containing the hybrid MMTV–HSV TK gene fusions. Poly(A)$^+$ RNA, 5 μg per lane, from cells that received the indicated plasmid constructions is displayed after formaldehyde/agarose gel electrophoresis and hybridization with HSV TK-specific [^{32}P]DNA. The 1.4-kb hybrid MMTV TK RNA is indicated by the arrow. The shorter 0.9-kb RNA present in all lanes is discussed in the text. Lanes: −, cells received no hormone; +, cells were in Dex-containing medium for 2 days prior to harvest. (*B*) S1 nuclease analysis. Displayed are ^{32}P end-labeled DNA fragments that were protected from digestion with S1 nuclease by hybridization with the RNA samples analyzed in *A*. The arrow points to the 200-base fragment that extends from the MMTV cap site to the labeled *Bst*N1 site in the HSV TK gene. The fragment at the top of the gel is undigested probe. Lanes L and N show results of hybridizing the probe with RNA from L cells that received no plasmid and with only carrier RNA, respectively. The prominent band present in each of the lanes with RNA from transfected cells (■) is the result of contamination of the probe used in this particular experiment with a small amount of downstream sequences. (This band is not observed in similar experiments with other preparations of the same probe; see Fig. 3*B*).

the labeled DNA strand from digestion. Hybridization of the fragment to RNA from uninduced cells that received p303, the intact LTR fusion, protected a small amount of the 200-base fragment; RNA from induced cells protected significantly more (Fig. 2*B*). The constitutive level of expression was retained in cells receiving plasmids pdel(1) and pdel(3), indicating that basal expression does not require sequences upstream from position −80. RNA from cells that received pdel(4), deleted to within 5 bp of the initiation site, produced none of the 200-base fragment after S1 digestion. (The slightly larger fragment observed on analysis of this RNA probably resulted from read-through from upstream start sites and reflects the deletion end-point.) The addition of Dex had no effect on the constitutive level of RNA made in cells that received plasmids pdel(2) and pdel(3). Therefore, the deletions have allowed us to dissociate sequences responsible for the basal level of synthesis, located downstream from position −80, from those responsible for glucocorticoid regulation.

These experiments with deletion mutants show that sequences lying upstream from position −190 are not required for glucocorticoid induction, and that sequences residing between positions −190 and −140 are involved in the response. To better characterize the possible relationship of these sequences to other promoter signals, we analyzed two further sets of altered promoters.

Alterations at Position −107. We introduced a unique *Eco*RI site at position −107 in the LTR to produce plasmid p559 (Fig. 3). (The *Eco*RI site was generated by cleavage at the *Sac* I site in the LTR, trimming of the *Sac* I ends with T4 polymerase,

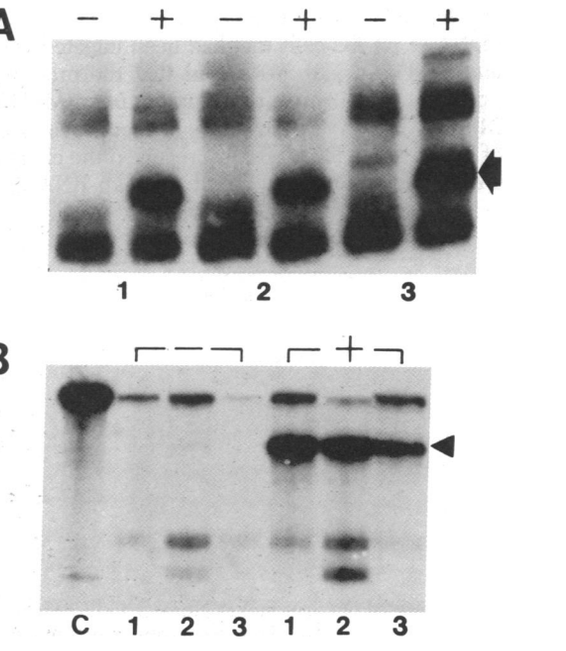

FIG. 3. Glucocorticoid-regulated expression from three MMTV promoters altered near position −107. The nature of the alterations in three plasmids derived from p303 is shown above *A*. (*A*) Autoradiogram of a blot hybridization of RNAs, 5 μg per lane, from Dex-induced (+) and uninduced (−) cells that received each of the three constructions by transfection. The filter was probed with HSV TK-specific [^{32}P]DNA. The 1.4-kb RNA is indicated with an arrow. (*B*) S1 nuclease protection analysis of the same RNA samples; the probe was a different preparation of the *Sac* I–*Bst*N1 fragment (Fig. 2). The filled triangle denotes the position of the 200-base fragment protected by transcripts initiated at the normal site within the MMTV LTR. Lane C, undenatured probe.

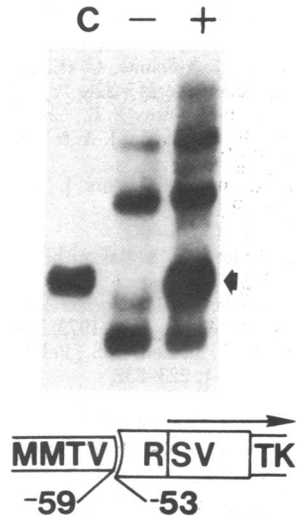

FIG. 4. Glucocorticoid-regulated expression from a hybrid MMTV–RSV promoter. (*Lower*) Structure of the hybrid promoter; the numbers are with respect to the MMTV (−59) and RSV (−53) transcription start sites. (*Upper*) Lanes: c, control lane showing RNA produced by a plasmid construction in which an intact RSV LTR was fused to the HSV TK gene; − and +, RNA from noninduced (−) and induced (+) cells that received the MMTV–RSV hybrid. In each case the RNA, 5 μg per lane, was analyzed by gel electrophoresis and hybridization with HSV TK [^{32}P]DNA. The arrow indicates the position of the 1.4-kb RNA.

and ligation of the resulting ends to *Eco*RI sites, whose ends also had been made flush with T4 polymerase.) p559 was then cleaved with *Eco*RI and treated either with T4 polymerase (to generate a 4-bp insertion, A-A-T-T, [p559-1]) or with *Bal*-31 (to generate a 19-bp deletion [p559-d19]) and religated. Fig. 3*A* shows that each plasmid served as a template for the inducible 1.4-kb RNA. To demonstrate that we did not affect the transcription initiation site, we subjected those RNAs to S1 nuclease analysis. Fig. 3*B* shows that the fidelity of initiation was unaffected by the deletion.

A Hybrid MMTV–RSV Promoter. Fig. 4 describes a different sort of promoter rearrangement involving hybrids between the MMTV and RSV LTRs (32). The RSV LTR encodes an efficient promoter (33), which is not affected by glucocorticoids (unpublished data). The construction shown in Fig. 4 has RSV sequences, extending leftward to position −53, fused to MMTV sequences that begin at the 5′ end of the MMTV LTR and extend rightward to −58. In turn, the RSV sequences were joined to HSV TK gene sequences approximately 100 bp downstream from the RSV cap site and again at the *Bgl* II site in the HSV TK leader. This hybrid promoted synthesis of a hormone-inducible 1.4-kb RNA detectable by the gel transfer method (Fig. 4); S1 nuclease analysis showed that this message is a hybrid RSV TK RNA initiated at the normal RSV initiation site (data not shown).

DISCUSSION

We have utilized fused MMTV–HSV TK gene constructions to show that a small region of the MMTV LTR is sufficient to express a linked gene under glucocorticoid hormone control. Our deletion analysis suggests that at least part of the hormone regulatory signal lies between 140 and 190 bp upstream from the start site of MMTV transcription and that there is probably no fixed relationship between that signal and the rest of the promoter. In addition, large parts of the MMTV promoter can be exchanged with equivalent parts of the RSV promoter with no effect on the regulation. With these deletions and substitutions, we have narrowed the region sufficient for hormone regulation to sequences between positions −190 and −120 and possibly those between −98 and −59. Although our work cannot rule out involvement of the latter sequences, the minimal region that is sufficient for glucocorticoid induction may be quite small. A better definition of these sequences will require a detailed mutational analysis.

What can we conclude about the structure and function of the MMTV promoter? In the least complicated scheme, the MMTV promoter would resemble a regulated prokaryotic promoter, with a recognition site for the regulatory (steroid receptor) protein that affects the efficiency of a nearby but functionally distinct binding site for RNA polymerase (34). Our results fit this picture in that they present evidence for a regulatory site that can be dissociated from a weak but functional promoter, located downstream from position −80. Studies showing that receptor protein binds to naked DNA from the same region of the LTR implicated here are also consistent with this simple scheme (10–12). Our results with p559-d19, however, suggest that the resemblance is superficial, as bacterial promoters appear to have rigidly fixed spatial relationships between binding sites for polymerases and regulatory proteins (34); in at least some cases, these probably reflect specific protein–protein contacts (35).

Our results suggest that the steroid response function of the MMTV promoter may replace an augmentation function present normally in promoters that are constitutively expressed at moderate or high levels. Clearly, within the context of our constructions, the uninduced MMTV promoter is extremely weak. [A comparison of the relative amounts of the 1.4-kb and the 0.9-kb transcripts made from the RSV LTR (Fig. 4, lane C) and from the uninduced MTV LTR (Figs. 2 and 3) gives some indication of the relative "strengths" of the two promoters.] Many eukaryotic promoters have sequences located more than 70 bp upstream from the transcription start site that are required for efficient expression *in vivo* (36, 37). Because the substitution of MMTV sequences for RSV sequences in this region greatly reduces the strength of the RSV promoter (Fig. 4, lane designated −; unpublished results), we suggest that this signal(s) is lacking in the MMTV promoter and that the hormone regulatory site is in some sense substituted for it. Two obvious possibilities for the receptor's mechanism of action are (*i*) that it induces a local change in chromatin conformation or (*ii*) that it provides a target or entry site either for RNA polymerase or for its accessory factors.

Further complexity is suggested by the work of Lee *et al.* (7) and Huang *et al.* (8), who have shown that the MMTV LTR promoter can be "strengthened" by the addition of "enhancer" elements either from simian virus 40 or from the murine sarcoma virus LTR. In each case the glucocorticoid function acts independently of the enhancer function, suggesting that the two elements provide additive functions. We note that a much higher level of constitutive RNA synthesis is seen with the intact provirus than with the LTR–HSV TK gene fusion. The resulting hormone induction is only 3- to 5-fold, much less than that seen with the fused template [or with most clones of rat hepatoma cells infected with single or multiple MMTV proviruses (38)]. We don't know yet whether there is an "enhancer" element outside the LTR—either in flanking rat sequences or in the internal MMTV sequences that act in the unique context of the truncated provirus—that is responsible for this difference.

Although we have placed the HSV TK gene, a selectable marker, under hormone control, LTK$^-$ cells are not easily used in a simple colony assay because their growth is affected by glucocorticoids (27). In experiments not described here we have used rat-2 cells (39), which are not sensitive to hormone, to

demonstrate that formation of HAT-resistant colonies by hybrid MMTV–HSV TK plasmids is hormone dependent. Although the colony assay is simple and convenient, it provides no information about RNA structure. Use of LTK$^-$ cells, in which expression of the TK$^+$ phenotype in the absence of hormone demanded that the cells incorporate relatively large amounts of DNA, increased the signal strength and facilitated RNA analysis. However, the specific manner by which the HSV TK gene is expressed during selection is obscure. The 0.9-kb transcript, present constitutively in all cells transformed with MMTV–HSV TK gene fusions (Figs. 2–4), may be responsible for the synthesis of the TK gene product. Roberts and Axel (40) described a similar transcript present in many cells that received HSV TK DNA cleaved at the *Bgl* II site and fused to plasmid sequences lacking a promoter. Subsequent selection in HAT medium elicited overexpression of the RNA as a consequence of amplification of the resident HSV TK gene-containing DNA. They argued that the transcript is initiated within the TK gene coding sequences and translated into a truncated polypeptide with partial TK activity. We believe that this truncated polypeptide may be the source of selectable TK activity in our experiments. Alternatively a small number of the incorporated fragments may integrate next to active promoters, which would drive the synthesis of HSV TK mRNA. Because our analysis of uncloned cell populations would obscure these transcripts, validation of either hypothesis will require further experimentation.

Note Added in Proof. Results related to those presented here have recently been reported by other laboratories (41, 42).

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