Transcriptional repression of a hormone-responsive promoter

Kimberlin L.Morley, Mark G.Toohy and David O.Peterson*

Department of Biochemistry and Biophysics, Texas A&M University and Texas Agricultural Experiment Station, College Station, TX 77843, USA

Received April 13, 1987; Revised and Accepted August 6, 1987

ABSTRACT

The activity of the mouse mammary tumor virus promoter was assessed in various sequence contexts with a transient transfection assay in which promoter activity was determined by way of expression of a linked gene encoding chloramphenicol acetyltransferase, as well as by direct analysis of RNA transcripts. The results indicate that the proviral long terminal repeat contains a negative transcriptional control element in addition to the glucocorticoid-responsive transcriptional enhancer that has been described previously. The negative element is able to function in both orientations and, at least to some extent, at multiple positions with respect to the regulated transcription unit. The effects on gene expression cannot be explained by alterations in transfection efficiency. The element has been localized to a 91 base pair fragment located immediately 5' of binding sites for the glucocorticoid receptor protein that have been defined *in vitro*. The role of the negative element may be to repress the inherent activity of the proviral promoter in the absence of glucocorticoids, resulting in an increased ratio of gene expression in the presence and absence of hormone.

INTRODUCTION

Multiple classes of *cis*-acting regulatory elements have been shown to be responsible for modulating the transcriptional activity of eukaryotic genes. Many viral and cellular genes require a class of DNA sequences known as transcriptional enhancers for normal expression (for a review, see reference 1); these elements appear to act as binding sites for *trans*-acting factors that are required for enhancer-directed stimulation of promoter activity (2-4). Negative regulation of gene expression is common in prokaryotes and yeast (for reviews, see references 5 and 6), and *cis*-acting negative regulatory elements in higher eukaryotes have been reported in association with several genes (7-14). The mechanisms by which these negative elements function is not well understood, but at least in some cases, direct (15) and indirect (8, 10-12) evidence suggests that negative elements also act as binding sites for *trans*-acting molecules.

Previous studies have shown that transcription of the proviral genes of mouse mammary tumor virus (MMTV), a retrovirus, is induced by glucocorticoid hormones (16-18). DNA sequences required in *cis* for hormone induction have been localized

to a region of the MMTV long terminal repeat (LTR) upstream from the transcription initiation site and have been termed the glucocorticoid response element (GRE) (19-23); these sequences have been shown to be bound specifically by purified glucocorticoid receptor protein in vitro (24-29) and to have the properties of a hormone-inducible transcriptional enhancer element (19, 23). In this study, we use a transient expression assay to show the presence of a negative regulatory element within the MMTV LTR. This element, which is contained within a 91 base pair (bp) restriction fragment located just 5' of the GRE, represses the inherent activity of the MMTV promoter in the absence of hormone. The overall glucocorticoid inducibility of MMTV transcription appears to be due to a combination of promoter activation by the GRE enhancer in the presence of hormone and to promoter repression by the negative element in the absence of hormone.

MATERIALS AND METHODS

Plasmid constructions

All plasmid constructions were based on pSV0d (30), a derivative of pBR322 that does not contain the pBR322 "poison" sequences (31); all of the constructions have deleted the simian virus 40 (SV40) origin fragment present in pSV0d. In all plasmids, the chloramphenicol acetyltransferase (CAT) gene, the SV40 small t intron, and sequences required for polyadenylation were derived from pSV2CAT (32). The M13 origin was derived from pZ152 (33) and is not relevant to the present studies. Plasmid pLC1 (Fig.1) contains a C3H MMTV LTR contained on a 1.5 kilobase (kb) *Pst*I restriction fragment (34), the ends of which were converted to *Hind*III sites; the construction of pLC1 has been described (35). As shown in Fig. 1, pLC2 contains an intact LTR within the *Pst*I-*Pvu*II restriction fragment, pGTC1 contains a 497 bp *Rsa*I-*Pvu*II LTR fragment which spans the GRE and transcription start, and pMC1 contains a 375 bp *Sst*I-*Pst*I LTR fragment which is missing most GRE sequences but which contains the MMTV promoter and transcription start. These three plasmids were constructed by converting the ends of the MMTV fragments to *Hind*III sites and replacing the LTR-containing *Hind*III fragment of pLC1. Plasmid pMT2 was constructed by digesting pLC1 with *Hind*III and religating to delete the LTR. Plasmids pGMC1.5A/B and pGMC1.3A/B were constructed by inserting (in both orientations) the 344 bp *Sau*3A restriction fragment containing most GRE sequences (Fig. 1) into a *Bam*HI site in pMC1 on either the 5' or 3' side of the MMTV-CAT transcription unit. Insertion on the 5' side generated pGMC1.5A and pGMC1.5B, while insertion on the 3' side generated pGMC1.3A and pGMC1.3B. Orientation A with respect to the MMTV promoter is that present in an intact LTR. Plasmid pNMC1 was constructed by inserting a 91 bp *Sau*3A-*Rsa*I fragment, in which the *Rsa*I site had been converted to

Figure 1. (A) Structure of MMTV-CAT plasmids. All plasmid constructions contain a portion of the MMTV LTR located upstream from the CAT gene (solid box) and the SV40 small t intron and sequences required for poly (A) addition (open box). Shown is plasmid pMC1, which contains a 375 bp *SstI*-*PstI* LTR fragment (striped box) which is missing most GRE sequences but which contains the MMTV promoter and transcription initiation site (see B). Other plasmids contain different LTR fragments as shown in B. Details of the constructions are provided in Materials and Methods. (B) Structure of MMTV LTR. LTR sequences are represented as a box, and relevant restriction sites are indicated. The GRE is identified by the hatched areas that represent regions of glucocorticoid receptor binding as defined by DNase I footprinting (26, 29). The arrow indicates the start site and direction of viral RNA transcription (49). The heavy lines indicate LTR fragments present in specific plasmid constructions; pLC1 contains the *PstI* restriction fragment with an essentially intact LTR, pLC2 contains the *PstI*-*PvuII* fragment also with an essentially intact LTR, pGTC1 contains a 497 bp *RsaI*-*PvuII* restriction fragment that spans the GRE and the transcription start, and pMC1 contains the 375 bp *SstI*-*PstI* fragment described in A. Plasmid pMT2 (not shown) is identical to the other plasmids but contains no MMTV sequences.

a *Bam*HI site via a synthetic linker, into the 5' *Bam*HI site of pMC1. Plasmids pNMC2 and pNMC3 contain similarly modified 231 bp *Sau*3A-*Hae*III and 269 bp *Sau*3A-*Mae*III fragments, respectively, inserted into the 5' *Bam*HI site of pMC1. The orientation of the pNMC insertions with respect to the MMTV promoter is the same as that in an intact LTR (orientation A). Supercoiled, monomer plasmid DNA was purified by alkaline extraction (36) and equilibrium centrifugation on a CsCl-ethidium bromide

density gradient. Concentrations were determined by absorbance at 260 nm and verified by ethidium bromide staining after agarose gel electrophoresis. In all MMTV-CAT constructions, the first AUG downstream of the expected transcription start site was the beginning of the CAT open reading frame.

Transfections and CAT assays

Mouse Ltk⁻ cells (37) were grown at 37°C in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% fetal bovine serum (KC Biologicals) in a 5% CO₂, water-saturated atmosphere. Transfections were performed by the DEAE-dextran/ DMSO shock procedure (38) at a plasmid concentration of 1.2 nM (equivalent to 5 µg/ml of plasmid pLC1) as previously described (35). For cells receiving hormone treatment, the synthetic glucocorticoid dexamethasone (Sigma) was added to a final concentration of 10⁻⁷ M 40 h after the DMSO shock. At 58 h after the DMSO shock the cells were harvested, and crude extracts were assayed for CAT activity (32) and for total protein (39) with bovine serum albumin as a standard. CAT enzyme activities were determined in a reaction containing 460 mM Tris hydrochloride (pH 7.8), 0.53 mM acetyl Coenzyme A (Pharmacia), 23 µM [¹⁴C] chloramphenicol (50 mCi/mmol; New England Nuclear), and 20-80 µg of extract protein, depending on the experiment. Reactions proceeded for 30 or 60 min at 37°C and were stopped by extraction of chloramphenicol and its acetylated derivatives with 1 ml of ethyl acetate (J. T. Baker, Co.). The acetylated products were separated from the chloramphenicol substrate by ascending thin layer chromatography in chloroform:methanol (9:1) and were visualized by overnight exposure to XAR-5 film (Eastman Kodak Co.). CAT activity was quantitated by excising the radioactive spots from the TLC plate and liquid scintillation counting. The specific activities of CAT were calculated and are reported as relative CAT activities normalized to the specific activity of an extract from hormone-treated cells transfected with pLC1 (defined as 100). This relative activity corresponds to an absolute specific activity of 3.0 nmol of chloramphenicol acetylated per min per mg of extract protein. Under these experimental conditions, transiently expressed CAT activity is highly reproducible (35); multiple independent transfection experiments resulted in errors of the mean of less than 20%.

Nuclear DNA dot blot

Nuclei were isolated from transfected cells as previously described (40) except that the buffer for Dounce homogenization contained 0.1% Nonidet P-40. Nuclear DNA was purified by phenol extraction and ethanol precipitation as described (40). The DNA was denatured in NaOH, neutralized with an equal volume of 2 M ammonium acetate, and serial 2-fold dilutions were prepared in 1 M ammonium acetate (41) and dotted onto nitrocellulose (Schleicher & Schuell) using a filter manifold. The filter was baked for 2 h at 80°C in a vacuum oven, and

prehybridization, hybridization, and wash conditions were as described (42), except that the hybridization with the probe of genomic Ltk⁻ cell DNA (Fig. 3B) was performed with only 1×10^6 cpm (instead of 5×10^6 cpm) of ^{32}P -labeled DNA in the absence of dextran sulfate. Probes were labeled with ^{32}P by nick translation (43) to a specific activity of $1\text{--}2 \times 10^8$ cpm/ μg . Filters were exposed to XAR-5 film (Eastman Kodak Co.) at -70°C without an intensifying screen. Sequential hybridization with multiple probes was performed as described (42). Autoradiograms were scanned with a Zeineh soft laser scanning densitometer (Biomed Instruments, Inc., Fullerton, CA).

Quantitative RNase T1 mapping

Total RNA was prepared (35) from transfected cells by extraction with guanidinium thiocyanate (44) and centrifugation through CsCl (45). A 414 nucleotide, uniformly labeled RNA probe that spanned the MMTV transcription initiation site was synthesized in vitro with T7 RNA polymerase utilizing a template of MMTV LTR DNA in pGLTR1.2 (35). In addition, a 212 nucleotide RNA probe complementary to mouse

Figure 2. CAT gene expression from plasmids containing deleted LTRs. Plasmids pLC1, pGTC1, pMC1 and pMT2 were transfected into mouse Ltk⁻ cells. Crude cell extracts were assayed for CAT activity and total protein. Specific activities were calculated and are reported as relative CAT activities normalized to the specific activity of an extract from dexamethasone-treated cells transfected with pLC1 (defined as 100). Numerical values of relative CAT activity represent the mean of 3 to 5 transfections with independent plasmid preparations. The autoradiogram shows a TLC separation of acetylated chloramphenicol products (upper spots) from the [^{14}C]chloramphenicol substrate (lower spot) in a representative CAT enzyme assay from one of the transfection experiments. The assayed extracts were derived from cells transfected with pLC1 (lanes 1 and 2), pGTC1 (lanes 3 and 4), pMC1 (lanes 5 and 6) and pMT2 (lanes 7 and 8). Assays contained 20 μg of protein from extracts of dexamethasone-treated cells (lanes 2, 4, 6, and 8) and 40 μg from extracts of untreated cells (lanes 1, 3, 5 and 7); reactions were allowed to proceed for 30 min at 37°C . In this particular experiment, the assay shown in lane 4 had depleted the chloramphenicol substrate and had extended beyond the time period in which the enzyme reaction was linear; this extract was reassayed under linear conditions for quantitation of CAT activity (not shown).

β -tubulin mRNA was also synthesized (35) from a cDNA cloned into pGEM1 (46). Total RNA (50 μ g) from transfected cells was simultaneously hybridized to the MMTV LTR probe and the mouse β -tubulin probe, and the RNase T1 protection experiment was performed as previously described (35). Protected fragments were fractionated by electrophoresis on an 8% polyacrylamide gel containing 8 M urea and were visualized by autoradiography on XAR-5 film (Eastman Kodak Co.) at -70°C using an intensifying screen. Autoradiograms were scanned as described above.

RESULTS

Transient expression from genes containing LTR deletions

We have described an extensive characterization of a transient transfection assay for determining the activity of the MMTV promoter (35). Taking advantage of gene constructions in which the MMTV promoter was fused to the coding sequences for chloramphenicol acetyltransferase (CAT), we determined that transiently expressed CAT enzyme activity was linearly related to the abundance of MMTV-CAT transcripts in cells transfected under carefully defined conditions. Using this assay, we found the activity of the MMTV promoter in the absence of glucocorticoids to be extremely low, with a ratio of gene expression in the presence and absence of hormone of 200-300.

The transient transfection system we have described appears to provide a sensitive assay for accurately quantitating the relatively low levels of expression from the MMTV promoter in the absence of hormone. To begin to more systematically quantitate this basal activity and to assess whether basal activity could be determined, at least in part, by the sequence context of the MMTV promoter within the proviral LTR, two deleted LTRs were constructed and inserted into plasmid vectors in which transcription could be monitored by way of expression of the CAT gene product. The resulting plasmids, pGTC1 and pMC1, as well as a comparable plasmid containing an undeleted MMTV LTR, pLC1, were transfected (35, 38) into mouse Ltk⁻ cells, and cell extracts were assayed for CAT activity (32) and total protein (39). In the absence of hormone, the deletions resulted in increased CAT activity of 8.6- (pGTC1) and 6.4-fold (pMC1) relative to that directed by the intact LTR (pLC1), while hormone-induced CAT activity was increased by 1.6-fold (pGTC1) or decreased by 11-fold (pMC1) (Fig. 2). The 2- to 3-fold hormone inducibility of CAT activity in cells transfected with pMC1 is most likely due to a portion of the GRE that was not deleted in this construction; this region consists of 30 to 40 bp of DNA, at least part of which is within a glucocorticoid receptor binding domain defined *in vitro* (26, 28, 29) (Fig. 1 and Fig. 5A). A further deletion of 36 bp to the *EcoRI** site (Fig. 1) abolishes this residual activity while maintaining the relatively high basal activity of pMC1 (data not shown).

To determine whether increased basal CAT expression directed by the plasmids containing LTR deletions could have resulted by positioning cryptic transcription start

Figure 3. Relative plasmid concentration in nuclei of transfected cells. DNA was purified from nuclei isolated from cells transfected with either pLC1 or pGTC1. Serial 2-fold dilutions were prepared and dotted onto nitrocellulose (0.5 μ g of DNA in the most concentrated dot); an identical dilution series of total DNA from untransfected Ltk⁺ cells (L) served as a control. The filter was hybridized sequentially with nick translated probes of pMT2 (see legend to Fig. 1B) (A) and genomic DNA from Ltk⁺ cells (B).

sites in vector DNA nearer to the site of translation initiation for the CAT protein, pMT2 was tested in the transient expression assay. This plasmid is identical to those described above but contains no eukaryotic promoter sequences and thus indicates the maximum possible CAT activity obtained in the absence of promoter-directed transcription. As shown in Fig. 2, the relative CAT activity directed by pMT2 was 3-fold lower than that from pGTC1; therefore, potential transcription starts within vector DNA cannot account for the increased basal CAT activity observed with the deleted LTRs. This conclusion is strongly supported by the direct analysis of MMTV-CAT transcripts described below.

Transfection efficiency

One possible explanation for our results is that the observed effects on gene expression were artifactually caused by differential transfection efficiency of the various plasmid constructions. To directly test this idea, the relative amount of plasmid DNA in the nuclei of cells transfected with pLC1 or pGTC1 was measured. Total DNA was purified from nuclei isolated from transfected cells, and the relative amount of plasmid in nuclear DNA was determined by dot blot analysis of serial dilutions using a nick translated probe of pMT2 (Fig. 3A); the extent of homology of this plasmid with pLC1 and pGTC1 is identical, and it should therefore hybridize with equal efficiency to both transfected plasmids. The total amount of nuclear DNA in each dot was compared by rehybridizing the same filter with a probe of nick translated

genomic DNA from Ltk⁻ cells (Fig. 3B). Densitometric scanning of the dot blots indicated that the relative amounts of pLC1 and pGTC1 in the nuclei of transfected cells were similar, with the amount of pLC1 approximately 2-fold greater than the amount of pGTC1. Therefore, if anything, we have underestimated the increased basal gene expression in cells transfected with pGTC1 relative to those transfected with pLC1, and differential transfection efficiency cannot explain the observed effects on gene expression. To extend this result, we performed quantitative Southern blot (47) analysis of nuclear DNA derived from pLC1- and pGTC1-transfected cells in which the intensity of LTR-containing restriction fragments from the transfected plasmid was compared to that of fragments derived from MMTV sequences endogenous to Ltk⁻ cells [(48) and data not shown]; this analysis has allowed us to estimate that the average number of plasmid molecules in the nuclei of the transfected cell population is approximately 15-30 copies/nucleus. This copy number has been obtained with four different plasmid constructions over a total of seven independent nuclear isolations.

Direct measurement of MMTV-CAT RNA transcripts

Increased gene expression from plasmids containing deleted LTRs suggested that the activity of the MMTV promoter is increased when the promoter is removed from the context of the entire LTR. To directly test this hypothesis, RNA transcripts from transfected cells were analyzed by nuclease protection. S1 nuclease mapping using a 5'-end-labeled probe showed that transcripts initiate at the expected position (49) within the LTR of hormone-treated, pLC1-transfected cells (data not shown), but this technique did not prove sensitive enough to detect transcripts from uninduced cells.

To circumvent this problem, we took advantage of an RNase protection assay that utilizes a uniformly labeled probe (Fig. 4) (50). Total RNA was prepared from cells transfected with either pGTC1 or pLC2, a plasmid similar to pLC1 but containing LTR sequences between the *Pst*I and *Pvu*II restriction sites (Fig. 1). Uninduced CAT activity from cells transfected with pLC2 was comparable to that obtained from pLC1 (data not shown). Purified RNA was hybridized to a mixture of two uniformly labeled RNA probes; one spanned the MMTV transcription initiation site, while the other was homologous to mouse β -tubulin mRNA and served as an internal control for the quantity and quality of the RNA preparations. Samples were then treated with RNase T1, and protected fragments were displayed by electrophoresis on a denaturing polyacrylamide gel and subsequent autoradiography.

Correctly initiated MMTV transcripts (49) were expected to result in a 135 nucleotide protected fragment of the MMTV probe, and intense protected fragments of this size were observed with RNA from glucocorticoid-treated cells transfected with both pGTC1 (Fig. 4, lane 1) and pLC2 (data not shown). A protected fragment of the same size was also detected with RNA from cells transfected with pGTC1 and

Figure 4. Direct analysis of MMTV-CAT transcripts by nuclease protection. A 414 nucleotide, uniformly labeled RNA probe that spanned the MMTV transcription initiation site, as well as a 212 nucleotide RNA probe complementary to mouse β -tubulin mRNA were synthesized *in vitro* (see diagram). Total RNA was prepared from cells transfected with either pLC2 (lane 2) or pGTC1 (lanes 1 and 3); some pGTC1-transfected cells were treated with dexamethasone prior to RNA purification (lane 1). RNAs (50 μ g) were simultaneously hybridized to both probes, and the resulting hybrids were treated with RNase T1. Transcripts initiating at the expected position at the MMTV promoter in pLC2 or pGTC1 protect a probe fragment of 135 nucleotides, while β -tubulin mRNA protects a probe fragment of 194 nucleotides (see diagram). Protected fragments are displayed as an autoradiogram after fractionation by 8% polyacrylamide-urea gel electrophoresis. Positions of the expected fragments of 135 nucleotides from the MMTV probe and 194 nucleotides from the β -tubulin probe (β), are indicated. Independent experiments with the individual probes verified the origins of the protected fragments. Size markers consisted of labeled RNAs synthesized *in vitro*. The autoradiogram was exposed to film for 9 days to show the MMTV protected fragment, while a 24 h exposure of the β -tubulin fragment is shown.

maintained in the absence of hormone (lane 3), and a much less prominent band of this size was observed with RNA from uninduced cells transfected with pLC2 (lane 2). Comparable amounts of RNA were loaded in each lane of Fig. 4 as demonstrated by the protected fragments from the internal standard homologous to mouse β -tubulin mRNA (β). Densitometric scanning of the autoradiogram allowed us to estimate the ratios of the integrated intensities of the protected fragments; the glucocorticoid induction of MMTV-CAT transcripts directed by pGTC1 was about 30-fold (comparison of lanes 1 and 3), while the basal levels of MMTV-CAT RNA from pLC2 and pGTC1 differed by about 5-fold (comparison of lanes 2 and 3). These ratios are in reasonable agreement with the corresponding ratios of CAT activity of approximately 50 and 9, respectively (Fig. 2). The origin of the protected fragment that runs just above the 135

Figure 5. (A) Structure of pGMC1.5/3. The 344 bp *Sau3A* fragment containing most GRE sequences (see Fig. 1) was inserted into a *Bam*HI site in pMC1 on either the 5' or 3' side of the MMTV-CAT transcription unit in both orientations. Insertion on the 5' side generated pGMC1.5A and pGMC1.5B, while insertion on the 3' side generated pGMC1.3A and pGMC1.3B. Orientation A with respect to the MMTV promoter is that present in an intact LTR. The nucleotide sequence of a portion of the GRE is shown below. Nucleotides from -135 to -70 with respect to the transcription initiation site are indicated. Boxes represent glucocorticoid receptor footprints defined by Payvar, *et al.* (26) (single, narrow box) and Scheidereit, *et al.* (29) (three shorter boxes). Open triangles represent G residues (some of which are on the opposite strand) that are protected from methylation in the presence of bound receptor (28). Deletion of the T at position -111 and insertion of 10 nucleotides at this position represents the difference in GRE sequences between pLC1 and pGMC1.5A (see text). **(B)** CAT gene expression from pGMC1.5/3. Plasmids pMC1, pGMC1.5A, pGMC1.5B, pGMC1.3A, and pGMC1.3B were transfected into mouse Ltk⁻ cells. Crude cell extracts were assayed for CAT activity and for total protein, and results are reported in terms of relative CAT activity as in Fig. 2. Numerical values of relative CAT activity represent the mean of 3 to 5 transfections with independent plasmid preparations. The autoradiogram shows a representative CAT enzyme assay from one transfection experiment. The assayed extracts were derived from cells transfected with pMC1 (lanes 1 and 2), pGMC1.5A (lanes 3 and 4), pGMC1.5B (lanes 5 and 6), pGMC1.3A (lanes 7 and 8), and pGMC1.3B (lanes 9 and 10). Assays contained 40 µg of protein from extracts of dexamethasone-treated cells (lanes 2, 4, 6, 8 and 10) and 80 µg from extracts of untreated cells (lanes 1, 3, 5, 7, and 9); reactions were allowed to proceed for 60 min at 37°C.

nucleotide fragment is unknown, but it is present in all lanes including a control lane derived from hybridization of yeast tRNA to the labeled probes (data not shown). Densitometric scanning revealed that the integrated intensity of this fragment in all lanes was identical within a factor of 1.3; the fragment appears more intense in lane 2 due to the increased background in that lane. These results indicate that the increased basal CAT gene expression observed in transfections with plasmids containing deleted LTRs is mediated by a direct effect on the MMTV promoter. An identical result was obtained in a duplicate RNase protection experiment with RNA from independently transfected cells.

Localization and position/orientation dependence of the negative regulatory element

Our results have indicated that a negative regulatory element 5' of the promoter within the proviral LTR reduces the activity of the MMTV promoter. In order to more specifically localize the sequences required to mediate the negative effect, the 344 bp *Sau3A* fragment that contains most of the GRE (see Fig. 1) was inserted into the *Bam*HI site at the 5' end of the transcription unit in pMC1 in its normal orientation with respect to the promoter (orientation A). This insertion restored a sequence organization closely resembling that of an intact LTR; however, a single base pair of LTR DNA within the GRE was deleted and 10 bp of synthetic linker DNA inserted in its place (Fig. 5A). Thus, in this plasmid (pGMC1.5A), the exact sequences of the GRE, as well as the relative spacing of most of the GRE with respect to the MMTV promoter, have been altered. In the absence of glucocorticoids, CAT gene expression from pGMC1.5A was reduced over 50-fold relative to pMC1 (Fig. 5B, lane 3), indicating that the functional sequences required for the negative effect on the MMTV promoter are contained within the inserted 344 bp *Sau3A* fragment. Together with the observation that the LTR deletion in pGTC1 (Fig. 1), which extends 91 bp into the *Sau3A* fragment, inactivates the negative element (Fig. 2), these results indicate that at least the 5' end of the negative element lies within this 91 bp region in a portion of the LTR that has not been identified as part of the GRE (20-23) and that contains no receptor binding sites as defined by footprinting (26, 27) or methylation protection (28).

The observed decrease in CAT expression was highly dependent on the specific sequences inserted upstream of the MMTV promoter. For example, pMC1 derivatives comparable to pGMC1.5A but containing insertions of either a 2.3 kb *Pvu*II-*Kpn*I fragment from the 5' portion of MMTV DNA of the endogenous provirus at genetic locus *Mtv-8* (42) or a 1.0 kb *Kpn*I-*Bgl*II fragment from the 5' portion of the MMTV(C3H) provirus (34, 42) resulted in CAT activities comparable to those obtained with pMC1 (data not shown).

The orientation and position specificity of the negative regulatory element was investigated with three other constructions (Fig. 5A) in which the 344 bp *Sau3A* GRE fragment was inserted into pMC1 at either the 5' *Bam*HI site in the opposite

Figure 6. Localization of the negative regulatory element. Plasmids pNMC1, pNMC2 and pNMC3 contain a 91 bp *Sau3A-RsaI* fragment, a 231 bp *Sau3A-HaeIII* fragment and a 269 bp *Sau3A-MaeIII* fragment, respectively, cloned into the *Bam*HI site on the 5' side of the MMTV-CAT transcription unit in pMC1 in orientation A (see Fig.1). The plasmids were transfected into mouse Ltk⁻ cells, and crude cell extracts were assayed for CAT activity and total protein. Results are reported in terms of relative CAT activity, the values of which represent the mean of 3 to 5 transfections with independent plasmid preparations. An autoradiogram of representative CAT assays from one of the transfections is shown. The assayed extracts were derived from cells transfected with pMC1 (lane 1), pNMC1 (lane2), pNMC2 (lane 3), pNMC3 (lane 4) and maintained in the absence of glucocorticoids. Assays contained 80 µg of extract protein, and reactions proceeded for 60 min at 37°C.

orientation to that normally present in the LTR (pGMC1.5B) or at a *Bam*HI site 3' of the CAT transcription unit in both orientations (pGMC1.3A and pGMC1.3B). These insertions were outside the predicted MMTV-CAT transcription units and therefore were not expected to alter the structure of the RNAs encoded by the respective plasmids. In the absence of glucocorticoids, CAT gene expression from pGMC1.5B was approximately 50-fold lower than from pMC1 (Fig. 5B, lane 5), indicating that the negative element is able to function in both orientations with respect to the MMTV promoter. CAT expression in the absence of hormone from pGMC1.3A and pGMC1.3B was also reproducibly lower than from pMC1 in multiple transfections with independent plasmid preparations, although the effect was less pronounced than in pGMC1.5A or pGMC1.5B. In the presence of glucocorticoids, cells transfected with any of the pGMC plasmids resulted in lower CAT expression than obtained with the complete LTR (Fig. 5B, lanes 2, 4, 6, 8, and 10) (see Discussion).

In order to more precisely determine the location of the negative element, three plasmids were constructed by insertion of various LTR-derived restriction fragments into pMC1. Plasmid pNMC1 contains a 91 bp *Sau3A-RsaI* fragment, pNMC2 contains a 231 bp *Sau3A-HaeIII* fragment and pNMC3 contains a 269 bp *Sau3A-MaeIII* fragment (see Fig. 1). Each plasmid was transfected into Ltk⁻ cells, and crude extracts

were assayed for uninduced CAT activity (Fig. 6). Plasmid pNMC1 (lane 2) yielded an uninduced CAT activity comparable to that of cells transfected with pLC1 (Fig. 2). The uninduced levels of CAT activity from plasmids pNMC2 and pNMC3 (lanes 3 and 4, respectively) were slightly higher than for pNMC1 but were much lower than pMC1 (lane 1). This result shows that the 91 bp *Sau3A*-*RsaI* fragment contains a functionally intact negative element and that additional sequences, including those that are recognized by the glucocorticoid receptor, are not required for MMTV promoter repression.

DISCUSSION

A negative element within the MMTV LTR

Our results indicate that the MMTV LTR contains a negative transcriptional regulatory element that decreases the activity of the proviral promoter. The negative element is functionally contained within 91 bp of DNA between *Sau3A* and *RsaI* sites in a region that is not part of the GRE (20-23) and that is located between positions -455 and -364 with respect to the site of transcription initiation. At least within the larger context of the 344 bp *Sau3A* restriction fragment, the negative element is able to function in both orientations and, to some extent, at multiple positions with respect to the regulated promoter. Similar to the results described here, the 344 bp *Sau3A* fragment has been reported to reduce the activity of the Moloney murine leukemia virus promoter when inserted in both orientations at two different positions (51), suggesting that the negative element we have described is able to function on at least one heterologous promoter and in a sequence context significantly different from the one employed in the present study. The relative orientation and position independence observed with the MMTV negative element are properties associated with a number of regulatory elements (10, 12, 13, 15, 52) that have been termed transcriptional silencers (52). At least two kinds of mechanisms appear to mediate these kinds of effects; establishing transcriptional repression mediated by the silencer associated with unexpressed copies of yeast mating type information requires a round of DNA replication (53), while the negative effects mediated by the MMTV negative element are observed in the absence of detectable replication of the test genes (48).

Previous analysis of the MMTV LTR failed to reveal the presence of the negative regulatory element described here. This may be due at least in part to the numerous cell lines and various experimental strategies employed to assess MMTV promoter activity. For example, some studies have employed exogenous enhancer elements as components of the MMTV gene constructions (21, 54, 55) which, in our hands, decrease the hormone induction ratio by 20-30 fold, primarily by increasing the basal level of expression (unpublished observation). One such study (55) did ascribe a

regulatory function to the MMTV GRE by which it could negatively modulate the activity of an exogenous enhancer element; however, it is unclear whether the observed effects reflected negative regulation or whether, as has been observed in several systems, the activity of one enhancer element in a particular promoter and cellular context could be detected only when a second enhancer was activated (for a review, see reference 56). In other kinds of studies, gene constructions were employed in which the MMTV promoter was fused to the coding sequences for the thymidine kinase of herpes simplex virus (19, 20, 22, 23); stably transformed cells containing the test genes were selected on the basis of expression of thymidine kinase activity in the absence of glucocorticoids, introducing a potential bias into the cell populations subsequently chosen for analysis. Comparison of our results to these studies is further complicated because the S1 nuclease analysis used to determine transcriptional activity in many instances did not detect transcription in the absence of hormone (20, 22, 23). In contrast to some of these strategies, our constructions did not contain any known eukaryotic transcriptional regulatory elements other than those in the MMTV LTR, and our transient transfection assay circumvented potential problems associated with preselection for expression of the reporter gene linked to the MMTV promoter. In addition, our detection of basal promoter activity was facilitated by the sensitive CAT and RNase protection assays and by the absence in our constructions of specific pBR322 sequences that decrease expression from transcription units containing the MMTV, as well as other, eukaryotic promoters (48).

Interestingly, sequence rearrangements or deletions in and near the negative transcriptional regulatory element we have described appear to alter the tissue specificity of oncogenic transformation induced by integrated MMTV proviral DNA (57, 58). These deletions and rearrangements have been interpreted as altering the specificity of viral gene expression, suggesting that the negative element may play a role in determining this specificity.

Gene regulation by multiple classes of elements

One intriguing aspect of our results is that, although transfection with any of the pGMC plasmids resulted in lower glucocorticoid-inducible CAT activity than obtained with a construction containing the intact LTR (Fig. 5), the ratio of gene expression in the presence and absence of hormone was higher for all four pGMCs than the ratio of 3.8 obtained with pMC1. These induction ratios varied from 5.8 for pGMC1.3A to more than 220 for pGMC1.5A. However, in all cases the increased induction ratio was primarily due to a decrease in gene expression in the absence of hormone rather than an increase in expression in the presence of hormone. Thus, the addition of the 344 bp *Sau3A* fragment containing a functional negative regulatory element as well as a functional GRE increased the hormone induction ratio without greatly increasing the absolute level of hormone-induced CAT gene expression. The failure of the

inserted 344 bp *Sau3A* fragment to activate the MMTV promoter to the level observed with a complete LTR may be due to the specific sequence organization of the pGMC plasmids, all of which differ from pLC1 (see Fig. 5A). These results suggest that overall glucocorticoid inducibility of MMTV transcription may be due to a combination of promoter activation by the GRE enhancer in the presence of hormone and to promoter repression by the negative element in the absence of hormone. Although the inherent activity of the MMTV promoter in the absence of the glucocorticoid receptor-activated GRE is fairly low, repressing this activity leads to a greatly increased ratio of gene expression in the presence and absence of hormone. Interestingly, transfection with pGMC1.5B, in which the negative element is positioned between the MMTV promoter and most of the GRE, resulted in the lowest absolute level of glucocorticoid-induced CAT expression, although the hormone induction ratio was about 30. It is possible that this sequence organization does not allow the receptor activated GRE to efficiently modulate promoter activity. This observation is analogous to a result obtained with several yeast genes (59, 60) in which a negative transcriptional regulatory element was found to exert its greatest effect when positioned between the promoter and an associated upstream activation sequence.

The relationship between positive and negative control elements involved in gene regulation is not well understood. Negative elements may function alone or in conjunction with positive elements, such as transcriptional enhancers, to provide appropriately regulated gene expression. Differential expression could then depend on the number (35) and strength of positive and negative control elements associated with each gene, as well as on the intracellular concentrations of *trans*-acting molecules required to mediate the transcriptional effects (56) in response to appropriate temporal and tissue-specific signals.

ACKNOWLEDGMENTS

We thank Katherine Beifuss and Bob Hathorn for expert technical assistance and Don Cleveland for generously supplying us with the mouse β -tubulin cDNA clone. This work was supported by U.S. Public Health Service Grant CA32695 from the National Cancer Institute and grant A-904 from the Robert A. Welch Foundation. This work was also partially funded by the Texas Agricultural Experiment Station.

*To whom correspondence should be addressed

REFERENCES

1. Khoury, G., and Gruss, P. (1983) *Cell* **33**, 313-314.
2. Mercola, M., Goverman, J., Mirell, C., and Calame, K. (1985) *Science* **227**, 266-270.
3. Sassone-Corsi, P., Wildeman, A., and Chambon, P. (1985) *Nature* **313**, 458-463.

4. Schöler, H. R., and Gruss, P. (1984) *Cell* **36**, 403-411.
5. Brent, R. (1985) *Cell* **42**, 3-4.
6. Reznikoff, W. S., Siegele, D. A., Cowing, D. W., and Gross, C. A. (1985) *Ann. Rev. Genet.* **19**, 355-387.
7. Bienz-Tadmor, B., Zakut-Houri, R., Libresco, S., Givol, D., and Oren, M. (1985) *EMBO J.* **4**, 3209-3213.
8. Gonzalez, F. J., and Nebert, D. W. (1985) *Nucl. Acids Res.* **13**, 7269-7288.
9. Goodbourn, S., Burstein, H., and Maniatis, T. (1986) *Cell* **45**, 601-610.
10. Jones, P. B. C., Galeazzi, D. R., Fisher, J. M., and Whitlock, J. P., Jr. (1985) *Science* **227**, 1499-1502.
11. Larsen, P. R., Harney, J. W., and Moore, D. D. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8283-8287.
12. Muglia, L., and Rothman-Denes, L. B. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7653-7657.
13. Remmers, E. F., Yang, J.-Q., and Marcu, K. B. (1986) *EMBO J.* **5**, 899-904.
14. Rosen, C. A., Sodroski, J. G., and Haseltine, W. A. (1985) *Cell* **41**, 813-823.
15. Zinn, K., and Maniatis, T. (1986) *Cell* **45**, 611-618.
16. Ringold, G. M., Yamamoto, K. R., Tomkins, G. M., Bishop, J. M., and Varmus, H. E. (1975) *Cell* **6**, 299-305.
17. Ringold, G. M., Yamamoto, K. R., Bishop, J. M., and Varmus, H. E. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2879-2883.
18. Ucker, D. S., and Yamamoto, K. R. (1984) *J. Biol. Chem.* **259**, 7416-7420.
19. Chandler, V. L., Maler, B. A., and Yamamoto, K. R. (1983) *Cell* **33**, 489-499.
20. Hynes, N., van Ooyen, A. J. J., Kennedy, N., Herrlich, P., Ponta, H., and Groner, B. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3637-3641.
21. Lee, F., Hall, C. V., Ringold, G. M., Dobson, D. E., Luh, J., and Jacob, P. E. (1984) *Nucl. Acids Res.* **12**, 4191-4206.
22. Majors, J., and Varmus, H. E. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 5866-5870.
23. Ponta, H., Kennedy, N., Skroch, P., Hynes, N. E., and Groner, B. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1020-1024.
24. Geisse, S., Scheidereit, C., Westphal, H. M., Hynes, N. E., Groner, B., and Beato, M. (1982) *EMBO J.* **1**, 1613-1619.
25. Govindan, M. V., Spiess, E., and Majors, J. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5157-5161.
26. Payvar, F., DeFranco, D., Firestone, G. L., Edgar, B., Wrange, Ö., Okret, S., Gustafsson, J. Å., and Yamamoto, K. R. (1983) *Cell* **35**, 381-392.
27. Payvar, F., Wrange, Ö., Carlstedt-Duke, J., Okret, S., Gustafsson, J. Å., and Yamamoto, K. R. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6628-6632.
28. Scheidereit, C., and Beato, M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 3029-3033.
29. Scheidereit, C., Geisse, S., Westphal, H. M., and Beato, M. (1983) *Nature* **304**, 749-752.
30. Mellon, P., Parker, V., Gluzman, Y., and Maniatis, T. (1981) *Cell* **27**, 279-288.
31. Lusky, M., and Botchan, M. (1981) *Nature* **289**, 79-81.
32. Gorman, C. M., Moffatt, L. F., and Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044-1051.
33. Zagursky, R. J., and Berman, M. L. (1984) *Gene* **27**, 183-192.
34. Majors, J. E., and Varmus, H. E. (1981) *Nature* **289**, 253-258.
35. Toohey, M. G., Morley, K. L., and Peterson, D. O. (1986) *Mol. Cell. Biol.* **6**, 4526-4538.
36. Birnboim, H. C., and Doly, J. (1979) *Nucl. Acids Res.* **7**, 1513-1523.
37. Kit, S., Dubbs, D. R., Piekarski, L., and Hsu, T. C. (1963) *Exp. Cell Res.* **31**, 297-312.
38. Lopata, M. A., Cleveland, D. W., and Sollner-Webb, B. (1984) *Nucl. Acids Res.* **12**, 5707-5717.
39. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248-254.
40. Peterson, D. O. (1985) *Mol. Cell. Biol.* **5**, 1104-1110.

41. Kafatos, F. C., Jones, C. W., and Efstratiadis, A. (1979) *Nucl. Acids Res.* **7**, 1541-1552.
42. Peterson, D. O., Kriz, K. G., Marich, J. E., and Toohey, M. G. (1985) *J. Virol.* **54**, 525-531.
43. Feinstein, S. C., Ross, S. R., and Yamamoto, K. R. (1982) *J. Mol. Biol.* **156**, 549-566.
44. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., Cowan, M. J., Rutter, W. J., and Kirschner, M. W. (1979) *Biochemistry* **18**, 5294-5299.
45. Glisin, V., Cryvenjakov, J., and Byus, C. (1974) *Biochemistry* **3**, 2633-2637.
46. Schenborn, E. T., and Mierendorf, R. C. (1985) *Nucl. Acids Res.* **13**, 6223-6236.
47. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503-517.
48. Peterson, D. O., Beifuss, K. K., and Morley, K. L. (1987) *Mol. Cell. Biol.* **7**, 1563-1567.
49. Ucker, D. S., Firestone, G. L., and Yamamoto, K. R. (1983) *Mol. Cell. Biol.* **3**, 551-561.
50. Zinn, K., DiMaio, D., and Maniatis, T. (1983) *Cell* **34**, 865-879.
51. Overhauser, J., and Fan, H. (1985) *J. Virol.* **54**, 133-144.
52. Brand A. H., Breeden, L., Abraham, J., Sternglanz, R., and Nasmyth, K. (1985) *Cell* **41**, 41-48.
53. Miller, A. M., and Nasmyth, K. A. (1984) *Nature* **312**, 247-251.
54. Lee, F., Mulligan, R., Berg, P., and Ringold, G. (1981) *Nature* **294**, 228-232.
55. Ostrowski, M. C., Huang, A. L., Kessel, M., Wolford, R. G., and Hager, G. L. (1984) *EMBO J.* **3**, 1891-1899.
56. Yamamoto, K. (1985) *Ann. Rev. Genet.* **19**, 209-252.
57. Michalides, R., Wagenaar, E., and Weijers, P. (1985) *Mol. Cell. Biol.* **5**, 823-830.
58. Wellinger, R. J., Garcia, M., Vessaz, A., and Diggelmann, H. (1986) *J. Virol.* **60**, 1-11.
59. Brent, R., and Ptashne, M. (1984) *Nature* **312**, 612-615.
60. Johnson, A. D., and Herskowitz, I. (1985) *Cell* **42**, 237-247.