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Multiple Hormone-Inducible Enhancers as Mediators of Differential Transcription

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Sets of genes under a common regulatory control in a given cell type are often differentially transcribed. The possibility that this differential transcription can be modulated by the number or strength of *cis*-acting regulatory sequences associated with a given gene was tested by using the glucocorticoid-responsive enhancer element associated with the mouse mammary tumor virus promoter. Results indicate that differential levels of hormone-inducible gene expression can be modulated in an additive way by the number of glucocorticoid-responsive enhancers associated with this promoter. Realization of these effects shows little preference for position of the additional elements with respect to the promoter. When sequences that bind the glucocorticoid receptor in vitro with somewhat lower affinity than the enhancer were tested, these additive effects were not detected. The results support the view that differential transcription of genes subject to a common regulatory control can be mediated, at least in part, by the number or strength of their associated *cis*-acting regulatory sequences.

The control of gene expression can, in principle, occur at a number of levels, the most basic of which is transcription initiation. For eucaryotic genes transcribed by RNA polymerase II, the DNA sequences required in *cis* for this kind of control have been extensively studied in several systems (for a review, see reference 12). One class of these sequences, the enhancer elements (for a review, see reference 22), has been shown to play a role in the determination of cell specificity of viral gene transcription (5, 17) as well as in the tissue-specific expression of endogenous cellular genes (10, 63). These elements appear to act as binding sites for *trans*-acting factors (35, 47, 51) that are required for the enhancer-directed stimulation of transcription from a linked promoter (57, 65). An emerging view of enhancer action is that different enhancers may act independently of one another to confer different controls on a gene by two or more distinct biochemical pathways (66). An extension of this view is that differential regulation of transcription for a set of genes under a common control could be conferred by the strength or number of enhancer elements associated with each gene (13, 33, 52).

The regulated transcription of mouse mammary tumor virus (MMTV) provides a relevant biological system in which to test the proposal that differential levels of transcription can be mediated by multiple functionally related enhancer elements of different strength or number. The glucocorticoid-regulated transcription of the proviral genes of MMTV (Fig. 1A), a retrovirus, is mediated by *cis*-acting sequences that have been termed the glucocorticoid response element (GRE). These sequences are specifically bound by a partially purified hormone-receptor complex in vitro (9, 16, 40–42, 48, 49) and serve to increase the rate of transcription initiation from the MMTV promoter (60) in a manner reminiscent of enhancer elements (6, 43). Functional GRE sequences have been identified within the proviral long terminal repeats (LTRs) (6, 19, 25, 30, 43) and thus are present twice, once at each end, in the integrated provirus. Other sequences that bind the hormone receptor complex in vitro have been identified within the proviral genome (40, 41), but the function of these sequences as GREs with a defined promoter has not yet been investigated.

In this study, we used a transient-expression assay to show that the presence of multiple GRE sequences can differentially activate the MMTV promoter in an additive fashion and that this increased activity is relatively independent of the position of the GREs with respect to the promoter. We show that such additive effects are not obtained when sequences internal to the MMTV genome to which hormone receptor complex binds with lower affinity in vitro are placed in their normal position and orientation with respect to the LTR.

MATERIALS AND METHODS

Plasmid constructions. pMT1 is a derivative of pSVOd (34), a plasmid that is missing the pBR322 "poison" (29) sequences. The simian virus 40 (SV40) origin and early promoter signals of pSVOd were removed by linearizing with *Eco*RI, filling in the ends with the Klenow fragment of *Escherichia coli* DNA polymerase I (Bethesda Research Laboratories), ligating 10-base-pair (bp) *Hin*dIII linkers (Boehringer Mannheim Biochemicals) to these blunt ends, digesting with *Hin*dIII, and then recircularizing the plasmid with T4 DNA ligase (Bethesda Research Laboratories Inc.). A 455-bp bacteriophage M13 origin of replication was added to this construction by replacing the *Aha*III fragment of pBR322 (positions 3232 to 3943) with the corresponding fragment of pZ152 (67) and screening the ampicillin-resistant colonies for the M13 origin fragment. To construct pLC1, the 1.6-kilobase (kb) *Hin*dIII-*Bam*HI fragment of pSV2cat (15), which contains the chloramphenicol acetyltransferase (CAT) coding sequences followed by the SV40 small t intron and early poly(A) signal, was inserted between the *Hin*dIII and *Bam*HI sites (pBR322 positions 29 to 375) of pMT1. Next, a 1.5-kb *Hin*dIII fragment, modified from a 1.5-kb *Pst*I

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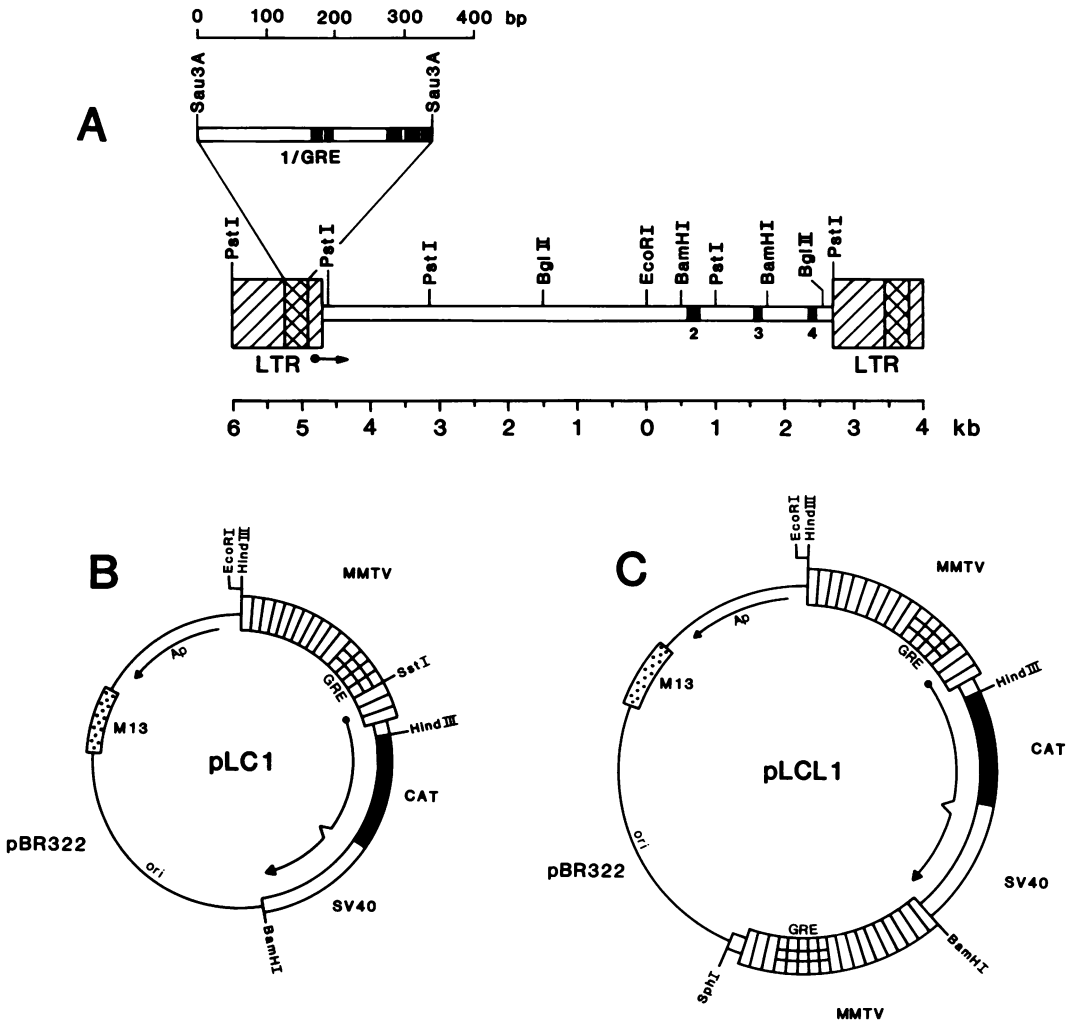

FIG. 1. Structure of the MMTV provirus and MMTV-CAT plasmids. (A) Structure of MMTV proviral DNA. The glucocorticoid response elements (cross-hatched boxes) are contained within the LTRs (striped boxes) at each end of the proviral genome. Sites of in vitro binding for purified glucocorticoid receptor (numbered 1 to 4) are indicated by filled boxes. The 344-bp *Sau*3A fragment used in these studies is enlarged above the provirus. The 1.3-kb *Bam*HI restriction fragment containing internal glucocorticoid binding sites 2 and 3 (40) is also shown. Transcription of viral RNA begins in the left LTR (arrow). (B) Structure of plasmid pLC1. The 1.5-kb *Pst*I fragment containing the MMTV LTR (striped box) is inserted upstream of the coding sequences for the CAT gene (filled box) and the SV40 small t intron and early poly(A) signals (open box). The vector sequences are derived from pSVOd (34), a "poison-less" (29) derivative of pBR322, into which we have inserted a bacteriophage M13 origin of replication (stippled box) (67) after deletion of the SV40 origin-early promoter region. Inclusion of the M13-derived sequences does not affect hormone regulation (data not shown) and is not relevant to the present study. The expected major transcript from this plasmid is indicated (arrow). (C) Structure of plasmid pLCL1. The 191 bp of pBR322-derived sequences between the *Bam*HI and *Sph*I sites (not shown) of pLC1 were replaced with an additional copy of the MMTV LTR in the orientation depicted. The scale of pLCL1 (8.0 kb) is the same as that of pLC1 (6.5 kb) in panel B.

fragment that contains the C3H MMTV LTR (31), was inserted upstream of the CAT gene. This *Hin*dIII fragment was constructed such that a *Bam*HI site was inserted 12 bp upstream of the MMTV LTR. In this construction, the CAT initiation codon is 308 bp downstream from the transcription initiation site within the MMTV LTR (58), and there are no other initiation codons 5′ of the CAT open reading frame. Plasmid pLC2 was constructed by replacing the 135-bp *Pvu*II-*Pst*I fragment (positions +134 to +269) of MMTV DNA from pLC1 with a small region of pUC18 polylinker, thus generating an MMTV-CAT RNA that is 139 bp shorter than that directed by pLC1. Plasmid pLCL1 contains a second copy of the MMTV LTR *Pst*I fragment, after modification of the ends, between the *Bam*HI and *Sph*I sites of pBR322-derived sequences in pLC1.

Plasmids containing multiple GREs were constructed by inserting the 344-bp *Sau*3A fragment of the C3H MMTV LTR (positions −112 to −455 with respect to the transcription initiation point) into pLC1 that had been partially digested with *Bam*HI to create linear molecules which were then isolated from an agarose gel with DEAE-membrane (Schleicher & Schuell, Inc.) and treated with calf intestinal phosphatase (Boehringer Mannheim). Plasmid p23LC1, which contains internal binding sites 2 and 3, was created by inserting the 1.3-kb *Bam*HI fragment of pMTV1 (59) into the *Bam*HI site downstream of the CAT gene in pLC1. The constructions used for RNase T1 mapping probes are described below. All plasmids were transformed into *E. coli* HB101 and screened for insertion site and orientation by restriction enzyme analysis. Supercoiled monomer plasmid

DNA was purified by alkaline extraction (2) followed by CsCl-ethidium bromide equilibrium density centrifugation. Plasmid concentrations were determined by A_{260} and verified by visualization after agarose gel electrophoresis-ethidium bromide staining. Restriction enzymes (Bethesda Research Laboratories or New England BioLabs, Inc.) were used according to the recommendations of the supplier.

Transfections and CAT assays. Mouse Ltk$^-$ cells (23) were grown at 37°C in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% fetal bovine serum (KC Biologicals) in a 5% CO_2, water-saturated atmosphere. Transfections were by the DEAE-dextran-dimethylsulfoxide (DMSO) shock procedure (28). The day before transfection, approximately 1.5×10^5 cells were seeded onto 100-mm tissue culture dishes (Corning Glass Works). The next day, the medium was replaced with 3.0 ml of Dulbecco modified Eagle medium containing 200 μg of DEAE-dextran (Pharmacia, Inc.) per ml, penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively; GIBCO), and DNA (1.2 nM). After 4 h (37°C, 5% CO_2) this mixture was replaced with 2.5 ml of 137 mM NaCl–5 mM KCl–0.7 mM Na_2HPO_4–6 mM dextrose–21 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.1] containing 10% DMSO (Sigma Chemical Co.). After a 2-min incubation at room temperature, the cells were washed once with phosphate-buffered saline (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 15 mM Na_2HPO_4), and 10.0 ml of Dulbecco modified Eagle medium containing 10% fetal bovine serum, penicillin, and streptomycin was added. 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, crude extracts were made by three freeze-thaw cycles, and the protein concentrations of the extracts were determined (3) with bovine serum albumin as the 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 [^{14}C]chloramphenicol (50 mCi/mmol; New England Nuclear), and 15 to 40 μg of cellular protein, depending upon the experiment. Reactions proceeded for 30 min at 37°C and were stopped by extraction of chloramphenicol and its acetylated derivatives with 1.0 ml of ethyl acetate (J. T. Baker Chemical Co.). The acetylated products were separated from the chloramphenicol substrate by ascending thin-layer chromatography in chloroform-methanol (95:5) and were viewed by overnight exposure to XAR-5 film (Eastman Kodak Co.). For quantitation, the spots were cut from the chromatography plates, and radioactivity was determined by liquid scintillation counting. Under these conditions, extracts of cells transfected with pLC1 resulted in a CAT specific activity of 0.65 nmol acetylated per min per mg of protein.

Probe constructions and quantitative RNase T1 mapping. The MMTV LTR probe was derived from a 1.5-kb *Pst*I restriction fragment which contains most of the MMTV LTR (31) cloned into the *Pst*I site of pUC8 (61). This LTR fragment was then removed from the pUC8 vector by digestion with *Hin*dIII and *Eco*RI and cloned into the *Hin*dIII and *Eco*RI sites of the SP6/T7 expression vector pGEM1 (50) to generate pGLTR1.2. For the dexamethasone induction time course experiment, pGLTR1.2 was linearized with *Cla*I, and a 1,170-nucleotide RNA probe (complementary to positions −861 to +274 of the MMTV LTR) was synthesized in vitro with T7 RNA polymerase. For RNase T1 mapping experiments, pGLTR1.2 was linearized with *Sst*I, the ends were made blunt with Klenow DNA polymerase (5 U/μg for 20 min at 25°C in 10 mM Tris hydrochloride [pH 8.0]–100 mM NaCl–10 mM $MgCl_2$) (New England BioLabs), and the 414-nucleotide probe (complementary to positions −105 to +274 of the MMTV LTR) was synthesized with T7 RNA polymerase. The mouse β-tubulin cDNA (kindly provided by D. Cleveland), consisting of sequences corresponding to codons 1 to 406 (27), was cloned into the *Eco*RI site of pGEM1 and screened for orientation by restriction enzyme mapping. This clone was digested with *Bam*HI, and a 212-nucleotide probe (complementary to 194 nucleotides of β-tubulin RNA) was synthesized with T7 RNA polymerase.

The RNA probe synthesis reactions were performed in a volume of 20 μl containing 20 ng of template DNA, 15 U of T7 RNA polymerase (Bethesda Research Laboratories), 40 U of RNasin (Promega Biotech), transcription buffer (40 mM Tris hydrochloride [pH 7.9], 10 mM NaCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine), 500 μM each ATP, GTP, and CTP (Sigma), 100 μCi of [α-^{32}P]UTP (>800 Ci/mmol; New England Nuclear), and either 50 μM (MMTV probes) or 500 μM (tubulin probe) cold UTP. The synthesis reactions were for 20 min at 37°C, after which the reaction mixtures were extracted with phenol in the presence of 100 μg of yeast RNA (Boehringer Mannheim). Unincorporated nucleotides were removed by P-10 (Bio-Rad Laboratories) "spin column" (37) gel exclusion chromatography. The probes were stored under ethanol until needed. This protocol generated 200 to 300 ng of RNA, of which greater than 95% was full length as judged by gel electrophoresis. MMTV probes had a specific activity of 3×10^8 to 5×10^8 cpm/μg.

For RNase T1 protection experiments (a modification of the procedure of Zinn et al. [69]), enough probe for 5×10^5 cpm of MMTV probe and 5×10^4 cpm of β-tubulin probe per reaction was precipitated in the same tube and then resuspended in hybridization buffer (80% deionized formamide, 400 mM NaCl, 40 mM PIPES [piperazine-*N*-*N'*-bis {2-ethane sulfonic acid}] [pH 6.4], 1 mM EDTA). Total RNA from transfected cells was prepared by dissolving cells in 6 M guanidinium thiocyanate (7) and pelleting the RNA by centrifugation through CsCl (11). RNA concentrations were determined by A_{260}. Ethanol-precipitated samples consisting of 25 μg (see Fig. 2A and 5C) or 15 μg (see Fig. 7) of RNA from transfected cells and yeast RNA to a total of 50 μg were then resuspended in 20 μl of the probe-hybridization buffer mix. The RNAs were denatured at 85°C for 10 min and allowed to hybridize overnight at 55°C. A 300-μl volume of RNase T1 (Bethesda Research Laboratories) at a concentration of 330 U/ml in RNase digestion buffer (10 mM Tris hydrochloride [pH 7.6], 300 mM NaCl, 1 mM EDTA) was added, and the samples were incubated for 1 h at 30°C. The RNase reaction was stopped by the addition of 20 μl of 10% sodium dodecyl sulfate and 5 μl of a 10-mg/ml proteinase K (Bethesda Research Laboratories) solution followed by incubation for 15 min at 37°C. After phenol extraction and ethanol precipitation, the RNAs were suspended in 5 μl of gel loading buffer (80% deionized formamide, 10 mM NaOH, 1 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) and denatured for 10 min at 85°C, and the protected fragments were separated on a 7% polyacrylamide gel containing 7 M urea. For quantitation of RNA, the gels were dried and exposed for various times to Kodak XAR-5 film without an intensifying screen. The resulting autoradiograms were scanned by using a Zeineh soft laser scanning densitometer (Biomed Instruments, Inc., Fullerton, Calif.), and the intensity of the protected fragment corresponding to transcripts

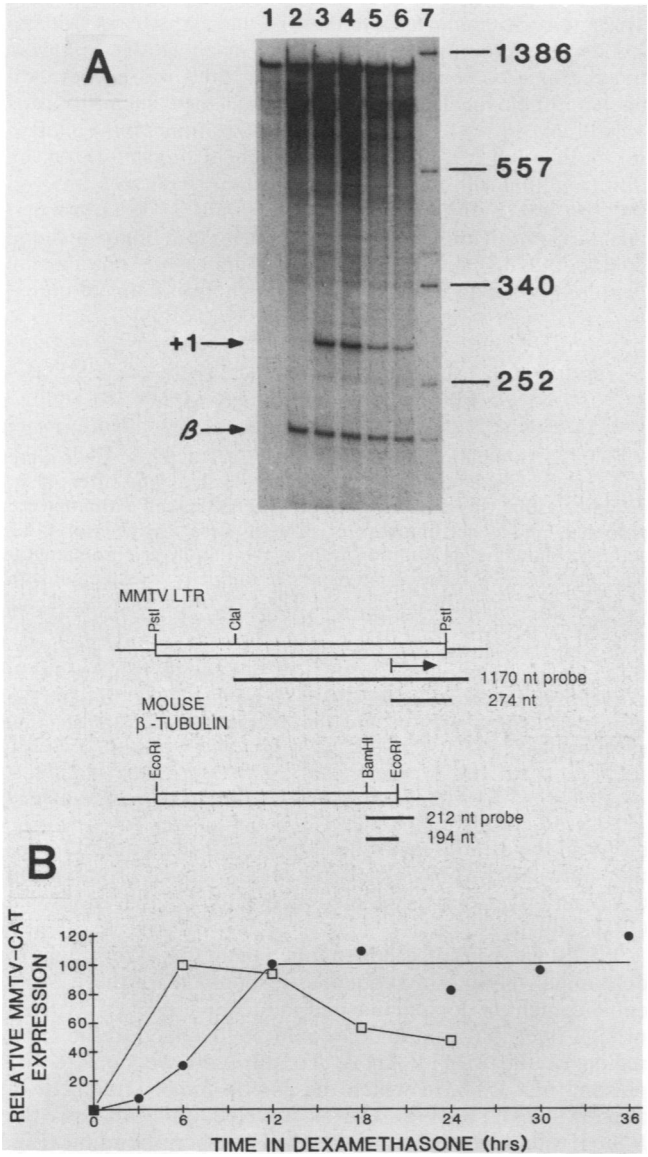

FIG. 2. Hormone inducibility of MMTV-CAT gene expression directed by pLC1. Ltk$^-$ cells were transfected with pLC1, and dexamethasone (10^{-7} M) was added 40 h later. At various times subsequent to hormone addition, either total cellular RNA or crude cell extracts were prepared. (A) Analysis of MMTV-CAT transcripts by nuclease protection. Uniformly labeled RNA probes of 1,170 (MMTV) and 212 (β-tubulin) nucleotides (nt) were synthesized in vitro with T7 RNA polymerase; plasmids containing the 1.5-kb *Pst*I fragment from the MMTV LTR or the 1.2-kb *Eco*RI fragment of mouse β-tubulin cDNA were linearized with *Cla*I or *Bam*HI, respectively, before probe synthesis (see diagram). RNA samples were hybridized to a mixture of these probes and then treated with RNase T1. Protected fragments are displayed as an autoradiogram after fractionation by polyacrylamide-urea gel electrophoresis. RNA samples consisted of 50 μg of yeast RNA (lane 1) or 25 μg of yeast RNA mixed with 25 μg of total cellular RNA from transfected cells incubated in the presence of dexamethasone for 0 h (lane 2), 6 h (lane 3), 12 h (lane 4), 18 h (lane 5) or 24 h (lane 6). The positions of protected fragments of 274 and 194 nucleotides expected from MMTV promoter-specific (+1) and mouse β-tubulin (β) RNAs, respectively, are indicated. In-vitro-generated RNA markers are also included (lane 7). Independent experiments with the separate probes verified the origins of the protected fragments (data not shown). (B) Kinetics of induction of MMTV-CAT RNA and CAT enzyme activity. The relative concentration of MMTV-CAT RNA (□) was determined by densitometic scanning of the autoradiogram shown in panel A as described in the text. CAT enzyme activities (●) were determined in a parallel experiment and are based on assays containing 40 μg of protein from crude extracts of transfected cells. The CAT activities are reported relative to the average of that obtained between 12 and 36 h, from which there is less than 20% variation for any given time point.

initiated at the +1 site in the MMTV LTR relative to the protected fragment from the mouse β-tubulin probe was determined.

RESULTS

Transient-expression assay for glucocorticoid-inducible gene expression. To study glucocorticoid hormone-regulated gene expression in vivo, we utilized a transient-expression assay system in which the activity of the glucocorticoid-responsive MMTV promoter could be monitored by way of expression of the CAT gene. As there is no enzymatic activity comparable to CAT in mammalian cells, this system is convenient for analyzing gene expression from various plasmid constructs in a rapid and sensitive way (15).

All of our experiments were performed with mouse Ltk$^-$ cells (23); this cell line is easily transfected and has been widely used to study hormone-regulated MMTV gene expression (19, 30, 41, 43, 68). In preliminary transfection experiments with a plasmid in which the CAT gene was under the direction of the non-hormone-responsive SV40 early promoter, CAT enzyme activity was not hormone inducible in the presence of 10^{-7} M dexamethasone, a synthetic glucocorticoid (data not shown). Thus, glucocorticoids do not induce increased gene expression from transfected DNA in general, and furthermore, the stability of both the CAT protein and this mRNA are insensitive to hormone.

Before attempting to utilize CAT activity as a measure of glucocorticoid-inducible gene expression, we first investigated the kinetics of hormone induction for both CAT enzyme activity and CAT RNA synthesized from the MMTV promoter. Plasmid pLC1 was constructed for these initial studies (Fig. 1B). In contrast to plasmid constructions in other studies in which transient-expression assays have been used to analyze hormone inducibility (25, 38), no other known eucaryotic promoter or enhancer sequences are located on pLC1.

To investigate the hormone-mediated induction of CAT activity, pLC1 was transfected into mouse Ltk$^-$ cells by the DEAE-dextran-DMSO shock procedure (28). The plasmid concentration during this and all subsequent transfections was 1.2 nM, corresponding to 5 μg of pLC1 per ml; this concentration was reported by Lopata et al. (28) to be optimal for transfection of L cells, a result which we have independently confirmed (data not shown). At 40 h after transfection, dexamethasone was added to a final concentration of 10^{-7} M, and crude cell extracts were prepared and assayed for CAT activity (15) and total protein (3) at various times after hormone addition (Fig. 2B). Under these conditions, CAT enzyme activity reached a maximal level within 12 h after hormone addition, and this level was maintained between 12 and at least 36 h. CAT activity from pLC1 was induced approximately 350-fold by the addition of dexamethasone.

The kinetics of MMTV-CAT RNA induction were determined by using a quantitative RNase T1 protection assay. At

40 h after transfection with pLC1, dexamethasone was added to 10^{-7} M, and total cellular RNA was isolated at various later times. Transcripts initiated at the expected position in the MMTV LTR (58) protected a 274-nucleotide fragment of the MMTV probe (indicated by +1), whereas β-tubulin mRNA protected a 194-nucleotide fragment of the β-tubulin probe (indicated by β) (Fig. 2A). The results of this experiment show that the amount of correctly initiated RNA from the MMTV promoter relative to the amount of β-tubulin RNA (Fig. 2B) reached a maximum 6 to 12 h after hormone treatment, after which the RNA levels decreased to 56% maximal at 18 h and 47% maximal at 24 h. This decrease was expected because of the continued growth of the cells throughout the time course of the assay and the lack of replication of pLC1 (see below). In addition to quantitating the relative concentration of MMTV-CAT RNA, this RNase protection experiment, along with S1 nuclease protection experiments (1) with a 5′-end-labeled probe (data not shown), confirmed that the hormone-inducible transcripts directed by plasmid pLC1 initiate at the correct site within the MMTV LTR. In this experiment an additional fragment from the MMTV probe of approximately 800 nucleotides was also protected; this fragment was observed with RNA from untreated as well as dexamethasone-treated cells and therefore does not represent a functional CAT mRNA.

Taken together, the kinetics of induction of CAT enzyme activity and MMTV-CAT RNA (Fig. 2B) indicate that, under the conditions employed, the CAT activity measured 12 h or later after the addition of dexamethasone reflects a steady-state level of hormone-inducible gene expression, provides a measure of the maximal amount of MMTV-CAT RNA, and is not a sensitive function of the time of cell harvest. Unless otherwise noted, cells were harvested 18 h after the addition of dexamethasone in all transfection experiments described in the remainder of this paper.

Increased gene expression when LTRs flank a regulated transcription unit. As a consequence of the retroviral replication strategy, the GRE sequences sufficient for hormone inducibility of MMTV are located at both ends of the proviral genome within the LTRs (Fig. 1A). We initially investigated whether a plasmid construct with a similar gene structure could give rise to a different level of gene expression than that from a plasmid which contains GRE sequences only 5′ of the promoter. Plasmid pLCL1 (Fig. 1C) was constructed for this purpose. This plasmid is identical to pLC1 except that 191 bp of pBR322-derived sequences from *Bam*HI to *Sph*I were replaced with a second copy of the MMTV LTR identical to that placed 5′ of the gene. In the MMTV provirus, the GRE sequences in the 3′ LTR are upstream of the MMTV poly(A) signal and are thus encoded in the 3′ end of the viral RNAs. However, to avoid complicating effects of GRE sequences on parameters other than transcription initiation that are potentially under hormone control (4, 32), the SV40 early poly(A) signal was retained upstream of the 3′ LTR in pLCL1.

When transfected into Ltk$^-$ cells, the hormone-inducible CAT activity directed by pLCL1 was 1.7 times that of pLC1 (Fig. 3), while in the absence of added hormone, the CAT activity of pLCL1 was essentially the same as that of pLC1 (data not shown). Therefore, this increase in CAT expression is due not to a general increase in the transcriptional activity of one plasmid construction over the other, but rather is specifically mediated by glucocorticoid hormones. A complicating factor in interpreting these results is that, while having additional GRE sequences downstream of the CAT gene, pLCL1 also contains an additional MMTV promoter in the 3′ LTR, and this experiment does not differentiate between the possible effects of this second promoter on CAT activity from those mediated only by the additional GRE sequences.

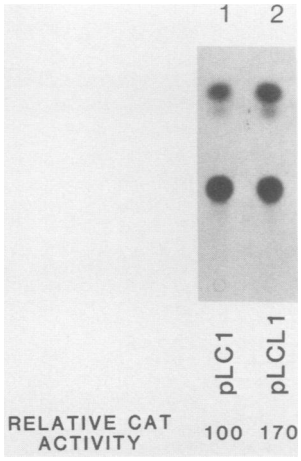

FIG. 3. An additional LTR increases CAT activity. Cells were transfected with equimolar amounts of plasmid, and after 40 h, dexamethasone was added to 10^{-7} M. After 18 h, cell extracts were prepared, and equal amounts of protein were assayed for CAT activity. Shown is an autoradiogram of the thin-layer chromatography plate from a representative assay for cells transfected with pLC1 (lane 1) and pLCL1 (lane 2). The two faster-migrating spots correspond to the 3-acetyl and 1-acetyl derivatives of chloramphenicol, while the slower-migrating spot is the unmodified substrate. CAT activity is expressed relative to the specific activity of an extract from cells transfected with pLC1 (arbitrarily set to 100). The relative activities reported are the average of four transfection experiments.

3′ LTR can be replaced by a functional GRE. We sought to determine whether the sequences responsible for the 1.7-fold enhancement of dexamethasone-inducible CAT activty from pLCL1 over that from pLC1 could be localized to the GRE region of the MMTV LTR. To this end we constructed plasmid pLC1.3A, in which the 344-bp *Sau*3A fragment of the MMTV LTR (Fig. 1) was inserted into pLC1 at the *Bam*HI site downstream of the CAT transcription unit (Fig. 4A). This *Sau*3A fragment has been shown to contain a functional GRE (6), even though it is missing approximately 30 bp of DNA that has been implicated in some studies as involved in both hormone induction in vivo (43) and receptor binding in vitro (48, 49). In pLC1.3A the GRE sequences are in the same orientation with respect to the transcription unit as those in the 3′ LTR of pLCL1, and these additional sequences were again inserted outside the transcription unit. Hormone-inducible CAT activity directed by pLC1.3A in transfected cells was 1.5-fold higher than that directed by pLC1 (Fig. 4B), confirming that GRE sequences were, in large part, sufficient to substitute for the 3′ LTR in regulated transcription units flanked by LTRs.

To investigate the position and orientation specificity of this additional GRE on hormone-inducible transcription, we constructed plasmids pLC1.5A, pLC1.5B, and pLC1.3B (Fig. 4A). These plasmids, together with pLC1.3A, contain the 344-bp *Sau*3A fragment of the MMTV LTR inserted into the *Bam*HI sites of pLC1 at either the 5′ (pLC1.5) or 3′ (pLC1.3) end of the transcription unit in each of the two possible orientations (A or B). Transfection of pLC1.5A resulted in a hormone-inducible CAT activity 1.8-fold

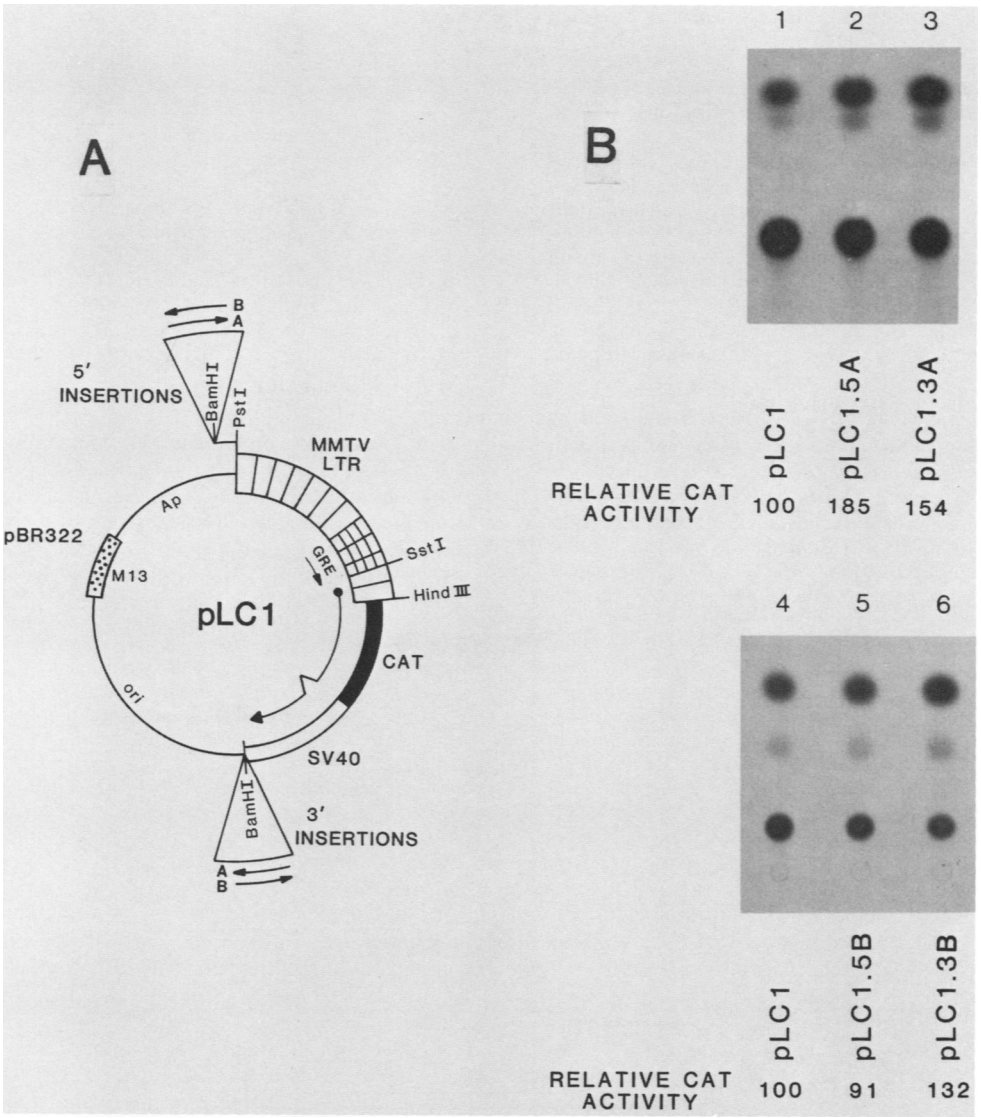

FIG. 4. Position and orientation dependence of additional GREs. (A) Structure of pLC1.5/3. The 344-bp *Sau*3A fragment from the MMTV LTR (Fig. 1) was inserted into the *Bam*HI sites of pLC1 at either the 5′ (pLC1.5 plasmids) or the 3′ (pLC1.3 plasmids) end of the MMTV-CAT transcription unit. Insertions in both orientations (A and B) were obtained. (B) Representative autoradiogram of CAT assays performed and reported as in the legend to Fig. 3. Extracts were prepared from cells transfected with equimolar amounts of pLC1 (lanes 1 and 4), pLC1.5A (lane 2), pLC1.3A (lane 3), pLC1.5B (lane 5), or pLC1.3B (lane 6). The relative CAT activities reported are the average of at least three transfection experiments.

greater than that of pLC1 and thus roughly comparable to the CAT activity of both pLCL1 and pLC1.3A; this result suggests that the effect of an additional GRE is relatively independent of position with respect to the promoter to which it is linked. Similar results have been observed by others in comparing gene constructs containing a single GRE with those containing no GRE sequences (43). In contrast to the results with pLC1.3A and pLC1.5A, transfection of pLC1.5B failed to show any increase in hormone-inducible CAT activity relative to pLC1, and transfection of pLC1.3B also led to less hormone-inducible CAT activity than the comparable construction with the *Sau*3A fragment in the normal orientation (Fig. 4B). These results were consistently obtained in multiple transfections with independent plasmid DNA preparations.

Taken together, the CAT activities derived from the pLC1.5 and pLC1.3 plasmids indicate that the increases in transcription due to the presence of additional GRE sequences is independent of the position but, in these sequence contexts, not the orientation of the added *Sau*3A fragment with respect to the gene (see below).

Effects of multiple GREs are additive. To address the possibility that further increases in the number of GREs might lead to additional activity from the MMTV promoter and, if so, to quantitate the nature of this increase, we constructed plasmid pLC1.5AA, in which two 344-bp *Sau*3A fragments were inserted in tandem into the *Bam*HI site located 5′ of the MMTV LTR in pLC1 (Fig. 4A). In pLC1.5AA both *Sau*3A fragments were inserted in the same orientation as that in the intact LTR. The hormone-inducible CAT activity directed by pLC1.5AA was 2.9 times that directed by pLC1 (Fig. 5A), which confirmed our earlier

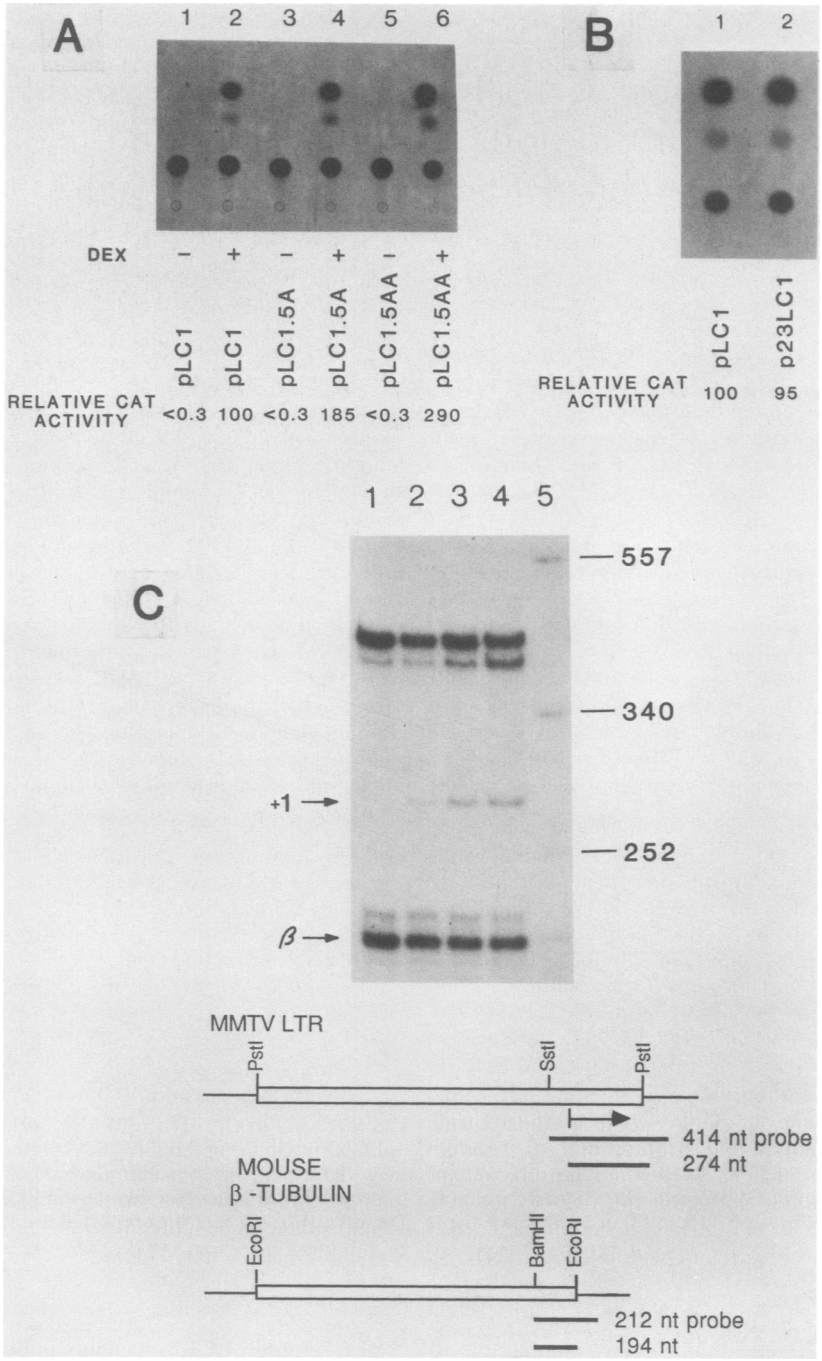

FIG. 5. Effect of multiple glucocorticoid receptor binding sites on the MMTV promoter. Plasmids containig either tandemly duplicated *Sau*3A fragments inserted 5′ of the MMTV-CAT transcription unit (pLC1.5AA) or a 1.3-kb *Bam*HI fragment containing internal binding sites 2 and 3 (Fig. 1) inserted 3′ of the MMTV-CAT transcription unit (p23LC1) were tested for their ability to affect transcription from the MMTV promoter. In both plasmids the insertions were into the *Bam*HI sites of pLC1 in the A (correct) orientation with respect to the promoter (Fig. 4A). (A) Effect of multiple GREs. A representative autoradiogram of CAT enzyme assays of extracts prepared from cells transfected with equimolar amounts of pLC1 (lanes 1 and 2), pLC1.5A (lanes 3 and 4), and pLC1.5AA (lanes 5 and 6) is shown. Assays were performed and reported as in the legend to Fig. 3 except that some cells were maintained in the absence of dexamethasone (DEX) (lanes 1, 3, and 5), while others were treated with hormone as described (lanes 2, 4, and 6). The relative CAT activities are the average of at least five determinations. (B) Effect of internal receptor binding sites 2 and 3. A representative autoradiogram of CAT enzyme assays performed and reported as in the legend to Fig. 3 is shown. Extracts were prepared from cells transfected with pLC1 (lane 1) or p23LC1 (lane 2) and treated with dexamethasone. The relative CAT activities are the average of three determinations. (C) RNase T1 protection analysis of MMTV-CAT transcripts from plasmids containing multiple GREs. Uniformly labeled RNA probes of 414 (MMTV) and 212 (β-tubulin) nucleotides (nt) were synthesized in vitro with T7 RNA polymerase and were utilized in an RNase T1 protection experiment as described in the legend to Fig. 2; in this experiment the MMTV probe was synthesized from a plasmid linearized at the *Sst*I site in the LTR (see diagram). RNA samples consisted of 25 μg of total RNA from cells transfected with pLC1 (lanes 1 and 2), pLC1.5A (lane 3), or pLC1.5AA (lane 4). At 40 h after transfection, cells were incubated for an additional 18 h in the absence (lane 1) or presence (lanes 2, 3, and 4) of 10^{-7} M dexamethasone. The positions of protected fragments of 274 and 194 nucleotides expected from MMTV promoter-specific (+1) and β-tubulin (β) RNAs, respectively, are indicated. In-vitro-generated RNA markers (lane 5) are also shown.

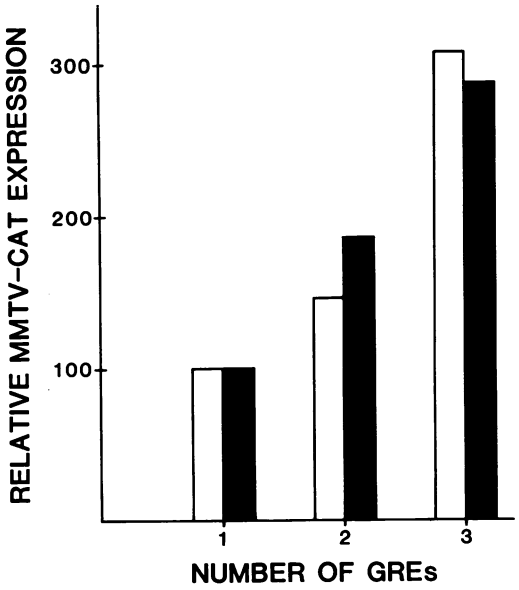

FIG. 6. Additive effects of multiple GREs. The relative concentration of MMTV-CAT RNA, as determined by densitometric scanning of the autoradiogram in Fig. 5C (open bars), and the relative CAT enzyme activity (filled bars) in extracts of transfected cells is shown as a function of the number of GRE-containing *Sau*3A fragments present at the 5' end of the MMTV-CAT transcription unit. Plasmids pLC1, pLC1.5A, and pLC1.5AA correspond to one, two, and three GREs, respectively.

observation that the MMTV promoter is not fully induced by the single GRE normally present in the LTR (pLC1) and further indicated that the promoter is also not maximally induced when associated with two GREs (pLC1.5A).

To verify that the increased CAT activity seen in cells transfected with the various plasmids containing multiple GREs was due to increased levels of correctly initiated MMTV-CAT RNA, we performed an RNase T1 protection experiment (Fig. 5C). As expected, a fragment of 274 nucleotides, corresponding to transcripts initiated at the MMTV promoter, was protected in RNA from hormone-treated cells but not in RNA from untreated cells. Densitometric scanning of the autoradiogram in Fig. 5C indicated that the relative levels of MMTV promoter-specific RNA from cells transfected with constructs containing multiple GREs correlated very well with the relative CAT activities derived from these plasmids; furthermore, the increases in transcription from the MMTV promoter were additive with respect to the number of GREs present on the plasmid (Fig. 6). A second protected fragment from the MMTV probe of 390 nucleotides was also detected (Fig. 5C). The origin of this fragment may be the same as that of the protected fragment of about 800 nucleotides observed in Fig. 2A, as again it is present in RNA from cells not treated with dexamethasone. These results show that the relative levels of CAT activity in cells transfected with plasmids containing multiple GREs can be completely accounted for by the relative abundance of transcripts initiated at the MMTV promoter.

Having shown that multiple GREs have the potential to increase transcription from the MMTV promoter, we investigated whether sequences internal to the MMTV genome that have been defined as lower-affinity glucocorticoid receptor binding sites in vitro were able to act in a similar fashion. A 1.3-kb *Bam*HI fragment (Fig. 1A) of the MMTV genome containing internal binding sites 2 and 3 (40) was inserted into the 3' *Bam*HI site in pLC1 to form plasmid p23LC1; the orientation of these additional sequences is the same with respect to the MMTV LTR as those in the intact provirus. Upon transfection into Ltk$^-$ cells, p23LC1 directed levels of CAT activity comparable to pLC1 both in the presence (Fig. 5B) and in the absence (data not shown) of dexamethasone.

Multiple GRE effects are not due to differential transfection efficiencies. Neither the CAT assays nor the RNase T1 protection experiments described above address the possibility that the differences in activity derived from plasmids containing multiple GREs were due to variability in transfection efficiency. We therefore performed a second RNase T1 protection experiment in which we incorporated a cotransfected reference plasmid whose transcriptional activity could be used to monitor this parameter (Fig. 7). Plasmid pLC2, which contains the *Pst*I-*Pvu*II fragment of the MMTV LTR upstream of the CAT coding sequences, was constructed for this purpose. The level of correctly initiated transcripts (indicated by +1) from pLC1.5AA (Fig. 7, lane 4) relative to those from pLC2 (indicated by +1*) was clearly greater than that of pLC1 (Fig. 7, lane 3).

In this experiment a protected fragment from a mouse β-tubulin probe (indicated by β) again served as an internal standard. Densitometric scanning of the autoradiogram in Fig. 7 indicated that, while expression from pLC2 was identical in each transfection, the level of steady-state RNA directed by pLC1.5AA was 2.7 times that of pLC1. These results confirm that differences in transfection efficiency were not responsible for the differences in activity directed by these plasmids. Furthermore, competition between the promoters in pLC1 and pLC2 was not evident in this experiment (compare Fig. 7, lanes 2 and 3), suggesting that the availability of *trans*-acting transcription factors was not limiting under these conditions.

Two protected fragments, in addition to those expected, were also evident in this experiment. The fragment appearing just above the β-tubulin protected band is derived from pLC2 and most likely corresponds to the fragments of 800 nucleotides in Fig. 2A and 390 nucleotides in Fig. 5C. The other fragment, appearing below the β-tubulin control, was also detected in S1 nuclease protection experiments by using a 5'-end-labeled probe (data not shown) and is derived from a hormone-responsive transcriptional start site downstream of the normal site within the MMTV LTR.

DISCUSSION

Characterization of transient-expression assay. The expression of CAT has been used previously in transient-expression assay systems to measure both promoter (14) and enhancer (24, 46, 51) activities. Although levels of CAT enzyme activity and RNA have been correlated (14, 54, 63), a characterization of this assay with a hormone-inducible promoter has not been reported. We have analyzed the kinetics of induction of both RNA and enzyme activity and have compared the relative amounts of RNA and CAT activity in cells transfected with plasmids that direct different levels of gene expression.

Kinetics of induction (Fig. 2) revealed that relative CAT enzyme activity reaches a constant level 12 h after hormone addition, whereas the relative concentration of MMTV-CAT RNA reaches a maximal level between 6 and 12 h after hormone addition and then declines. These kinetics are predicted if the transfected DNA is not replicated. We have

not obtained any evidence for replication of our plasmids in Ltk$^-$ cells; sites of adenine methylation introduced by the *dam*$^+$ *E. coli* strain in which the plasmids were amplified are not lost upon transfection (data not shown). Thus, even if the transfected DNA is not degraded and the rate of RNA synthesis from each transfected DNA template is not altered, the relative concentrations of RNA and protein gene products synthesized from the transfected plasmid compared with those synthesized from endogenous cellular genes would eventually be expected to decrease with time. These time course data show that CAT activity measured 18 h after the addition of hormone reflects the maximum CAT activity transiently expressed by the transfected cells and that this activity is not a sensitive function of time.

Our results indicate that relative CAT enzyme activity is a linear function of the relative concentration of MMTV-CAT RNA at a specific time in the transient-expression assay (Fig. 6). In addition to the results in Fig. 6, we have also observed a linear correlation between CAT activity and RNA levels from four other MMTV-CAT constructs, each with a different relative activity (data not shown).

We have investigated the copy number of plasmid DNA in the nuclei of transfected cells. Quantitative Southern blots (55) in which the hybridization of plasmid sequences was compared with that of endogenous MMTV DNA in Ltk$^-$ cells indicated that the average number of plasmid molecules per nucleus is approximately 30. This value is reproducible between independent transfections and is unchanged in experiments with plasmids that direct different levels of CAT gene expression (data not shown). The differential levels of hormone-inducible CAT activity reported here are thus unlikely to be the result of copy number effects of the different plasmid constructions.

It has been reported that transient-expression assay of CAT enzyme activity is not highly reproducible (17), most likely as a result of differences in transfection efficiency for different plates. We have not found this to be the case under the conditions we use for transient-expression assays. With a single exception, the CAT activity measured under our experimental conditions and over a number of trials is always within 20%, and in many cases within 10%, of the reported mean values. This high level of reproducibility is most likely due to a combination of pooling the cells before plating for transfection, performing transfections at a cell density low enough to ensure subconfluent monolayers at harvest (i.e., avoiding growth arrest), and normalizing CAT activity to total protein (i.e., correcting for variable cell growth and recovery of protein during extract preparation).

Contrary to reports elsewhere (18, 25, 26, 38), our results clearly demonstrate that the MMTV promoter-enhancer does not require additional heterologous enhancer elements for use in a transient-expression assay system. The differences between our results and those of others appear to be related to the absence in our plasmids of specific, *cis*-acting, pBR322 sequences that decrease transient expression from the MMTV and other promoters (D. Peterson, K. Beifuss, and K. Morley, submitted for publication).

Role of multiple GRE sequences in MMTV. MMTV DNA contains functional GRE sequences in the LTRs at each end of the proviral genome, and our initial studies on multiple enhancer effects sought to determine the significance of this sequence organization on transcription initiation from the upstream MMTV promoter. CAT gene expression in a transient-expression assay with the plasmids pLCL1 and pLC1 (Fig. 3) indicated that the effect of a second LTR located 3′ of the regulated transcription unit is to increase

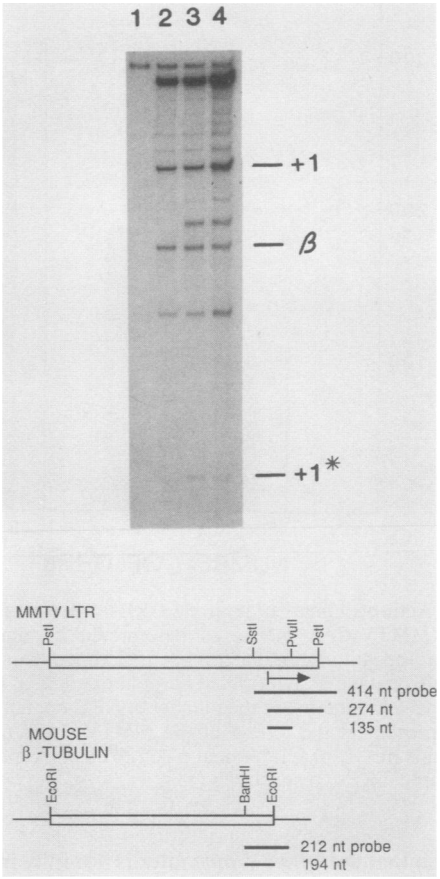

FIG. 7. Increased MMTV-CAT activity is not due to differential transfection efficiencies. Cells were transfected with pLC1 (1.2 nM), pLC1 (1.2 nM) and pLC2 (0.60 nM), or pLC1.5AA (1.2 nM) and pLC2 (0.60 nM). The total DNA concentration in each transfection was brought to 11.0 μg/ml with plasmid pMT1, which is identical to pLC1 except that the MMTV LTR and CAT coding sequences have been removed. Total RNA was prepared 12 h after the addition of dexamethasone (10^{-7} M). The probes used are identical to those used in Fig. 5. RNA samples consisted of 50 μg of yeast RNA (lane 1) or 35 μg of yeast RNA and 15 μg of total RNA from cells transfected with pLC1 (lane 2), pLC1 and pLC2 (lane 3), and pLC1.5AA and pLC2 (lane 4) and were treated with RNase T1 as described in the text. The position of the 274-nucleotide (nt) fragment corresponding to correct initation from pLC1 and pLC1.5AA is indicated (+1) as is the position of the 135-nucleotide fragment derived from correctly initiated transcripts from the reference plasmid, pLC2 (+1*), and the 194-nucleotide fragment derived from mouse β-tubulin mRNA (β).

transcription from the upstream MMTV promoter. It thus appears that the absolute levels of transcriptional enhancement of the intact MMTV provirus in the presence of glucocorticoid hormones may be due in some part to the repeated sequences flanking the provirus. This experiment suggested that the extent of hormone-inducible transcription initiation could be quantitatively modulated by the number of GRE sequences linked to a regulated transcription unit. As we had hypothesized, GRE sequences alone are sufficient for the enhancement of transcription observed with a complete LTR in pLCL1 provided they are in the correct orientation with respect to the promoter (Fig. 4). Furthermore, this increased activation of the MMTV promoter is

not the result of differential transfection efficiencies of the various plasmids (Fig. 7).

The results presented here clearly indicate that the MMTV promoter is not at maximal activity when under the influence of either one or two GRE sequences (Fig. 5 to 7). These results are consistent with the suggestion (40, 41) that the glucocorticoid receptor binding sites internal to the MMTV genome may serve as additional GREs that act to enhance transcription from the MMTV promoter. However, in a direct test of this proposal, a 1.3-kb *Bam*HI restriction fragment containing two of these internal binding sites (40) was shown to have no detectable effect on the transcription from a proviral promoter contained within an intact LTR (Fig. 5B). This fragment was located 3′ of the promoter in a sequence organization that in some respects mimics its position in a complete MMTV provirus. We have also been unable to demonstrate GRE activity on this fragment when linked in four different sequence contexts to a truncated MMTV LTR (position −110 to +274) which shows only a small hormone response in comparison with that of the intact LTR (unpublished data). The glucocorticoid receptor binding sites located on this 1.3-kb *Bam*HI fragment have been reported to be of lower affinity than those in the LTR (40), and such differences in affinity may correlate with the ability of the sequences to function as GREs (53). It is possible, however, that the combined action of a larger number of receptor binding sites that function as very weak GREs may have a significant effect on the MMTV promoter which was not detected in our assays of two isolated sites or that the internal receptor binding sites are functioning by a biochemical mechanism distinct from that of the GRE that is directly related to their normal position within the transcribed portion of the provirus.

MMTV GRE as a transcriptional enhancer. In light of studies by others (6, 43), the orientation dependence we observed in multiple GRE constructions was unexpected and, since based on a small of number plasmid constructions, must be interpreted with caution. These conflicting results, however, may have arisen because our system significantly differs from others in which orientation-independent function was reported for the MMTV GRE. In one of these systems (6), the plasmid sequences for testing the reverse orientation of the GRE had two *Sau*3A fragments linked to the herpes simplex virus thymidine kinase promoter and were stably integrated into the genome; the sequence organization of the transformed DNA was not characterized. If, as is often the case, these sequences were inserted in tandem linear arrays, each internal transcription unit was then flanked by a total of four GREs, two of which were 3′ of the promoter in a position and orientation in which we also detect transcriptional enhancement. In the other system (43), the sequences that were used contained a more complete GRE receptor-binding domain than that contained on the *Sau*3A fragment used in the present study (49). It is possible that the sequences necessary for maximal hormone activation from the reversed orientation of the GRE resides on two separable sequence elements. Also, in contrast to our transient-expression assay system, the plasmid constructions used in the latter experiments contained not only those constituting the previously defined hormone response element, but also sequences containing internal binding site 4 (Fig. 1), as well as an SV40 enhancer region, the presence of which may have independently affected glucocorticoid-responsive transcription (41). Finally, we have localized a negative transcriptional control element to the same 344-bp *Sau*3A fragment that contains the GRE sequences used in this study (K. Morley, M. Toohey, and D. Peterson, submitted for publication); the reverse orientation of this fragment appears to magnify the negative effect on the MMTV promoter, especially at an insertion site 5′ of the promoter. Thus, the absolute level of transcriptional enhancement observed here may be affected by the presence of both positive and negative elements on the inserted *Sau*3A fragment and may be determined in part by the relative positions of the two elements with respect to the promoter.

It has been suggested that the functional differences between enhancer elements and other RNA polymerase II control elements may be only one of degree (52, 64). In support of this view, both the SV40 21-bp repeats (36) and the herpes simplex virus thymidine kinase upstream elements (33) show some enhancerlike activity in that they can stimulate transcription when placed in the opposite orientation. Furthermore, the enhancers of SV40 (8) and polyoma (17) which are generally considered to be bidirectional regulatory elements, show a marked directional preference in at least some sequence contexts. It is not yet clear where in the functional spectrum of these regulatory sequences the MMTV GRE resides, although our data suggest that the GRE sequences contained on the 344-bp *Sau*3A fragment show relatively little distance-position dependence but do, in at least some sequence contexts, show an orientation dependence.

Multiple enhancers and differential transcription. Many of the RNA polymerase II promoter sequences defined to date consist of either large direct repeats or smaller regions containing well-conserved, multiply repeated regions (for a review, see reference 12). In the best characterized of these elements (6, 20, 35, 39, 51, 52), these repeated regions have been shown to require *trans*-acting factors capable of stimulating transcription from a linked promoter. The *cis*-acting DNA sequences for several systems which are under the regulatory control of more than one pathway have been defined. These regions generally adhere to the above generalization of repeating elements. In these cases, however, the repeated elements are clustered into groups, either physically separate from one another (21) or overlapping (44, 62), each of which carries the sequences responsible for the transcriptional response due to a particular biochemical signal. One view of this type of regulatory region is that different enhancers can act independently to stimulate transcription in response to different biochemical states within the cell (66). An extension of this view is that differential regulation of separate genes under the same type of biochemical control can be conferred by different numbers of a specific type of enhancer element associated with each gene (13, 33, 52). We tested this latter possibility in our experimental system.

The insertion of GRE sequences upstream of the intact MMTV LTR resulted in additive increases in the glucocorticoid-inducible activity of the MMTV promoter (Fig. 6). Whether this effect is mediated by multiple hormone receptor binding events or by the increased probability of a single binding event on plasmids with multiple GREs is unclear. Either mechanism is possible, not only for multiple transcription templates introduced by transfection, but also for single-copy genes, and the two mechanisms are not mutually exclusive. The lack of detectable competition between plasmids containing identical hormone-responsive promoters (Fig. 7) suggests that we have not titrated the *trans*-acting transcription factors required for a multiple binding event mechanism.

Experiments from other laboratories support the idea that

our results may be indicative of a general regulatory strategy. After the completion of our work, a series of plasmid constructs with increasing numbers of sequence elements conferring a metal response to the mouse metallothionein II gene was reported to result in increasing amounts of metal-inducible RNA from the herpes simplex virus thymidine kinase promoter (56). The repeat units used in these experiments were much smaller than those in the present study, suggesting that the additive response described here may work at the levels of both single repeat units or groups of these elements. It also should be noted that the human growth hormone gene has recently been shown to have a functional GRE located within its first intron (53) in addition to the previously defined GRE located 5′ of the transcription initiation site (45). Although it has not been investigated, it is tempting to speculate that glucocorticoid regulation of this gene is under the control of multiple hormone-responsive enhancer elements.

In conclusion, our results give direct support for the proposal that differential levels of transcription may be controlled in part by the number of functional enhancer elements associated with particular transcription units. In the specific case of glucocorticoid-responsive transcription described here, further increases in the number of GREs associated with the MMTV promoter would eventually be expected to saturate the level of transcription as some parameter, other than that which is supplied by the GRE, becomes rate limiting. Determination of the number of GREs necessary to reach, and the transcriptional activity at, this saturation point will help define the range of transcriptional modulation afforded by the multiple enhancer mechanism.

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