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Functional Elements of the Steroid Hormone-Responsive Promoter of Mouse Mammary Tumor Virus

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Transcription from the promoter of mouse mammary tumor virus is subject to induction by several classes of steroid hormones as well as to repression by a negative regulatory element present in the long terminal repeats of proviral DNA. In order to characterize the functional elements of the promoter that in some way must respond to these regulatory signals, a number of promoter mutations were constructed, including a set of linker-scanning mutations across the entire promoter region. Analysis of these mutated promoters with a transient-transfection assay defined at least three mutation-sensitive promoter elements that are required for both basal and hormone-induced transcription. One mutation-sensitive region contains a TATA element located at approximately position −30 with respect to the start of transcription. A second mutation-sensitive region contains two 10-base-pair direct repeats located between positions −60 and −38, within which are embedded three copies of octamer-related sequences; complete disruption of this region of the promoter leads to a more severe decrease in transcription than do any of the linker-scanning mutations, suggesting that the repeated sequences may be at least partially functionally redundant. Gel electrophoresis mobility shift assays were used to demonstrate specific binding of a nuclear protein to this region of the promoter. A third mutation-sensitive region contains a binding site for nuclear factor 1 (NF-1) located between positions −77 and −63. Site-directed mutations in the NF-1-binding site which increase the apparent affinity of NF-1 for the promoter in vitro do not decrease the hormone dependence of transcription, suggesting that transcriptional activation mediated by steroid hormone-receptor complexes cannot be explained by facilitation or stabilization of the interaction of promoter sequences with NF-1 and consistent with the idea that binding of NF-1 is not rate determining in transcription from the mouse mammary tumor virus promoter. None of the promoter mutations functionally separates basal from glucocorticoid-induced transcription, suggesting that hormone induction does not make the promoter independent of any of the DNA-binding factors required for its basal activity.

Transcription of the proviral DNA of mouse mammary tumor virus (MMTV) is positively regulated by several classes of steroid hormones, including glucocorticoids (58, 70), progestins (9, 10), and androgens (9). The sequences that confer this hormone inducibility on the MMTV promoter have been extensively characterized and have been collectively called the hormone response element (HRE). This element, which has the properties of a steroid-dependent transcriptional enhancer, consists of sequences within the proviral long terminal repeat (LTR) approximately between positions −200 and −80 with respect to the start of transcription (27, 34, 37, 57). Analysis of MMTV proviral chromatin in isolated nuclei has revealed a hormone-inducible site of DNase I hypersensitivity within the HRE; the induction of this hypersensitive site parallels the transcriptional activation of the MMTV promoter and presumably is the direct result of a receptor interacting with the HRE (55, 72). Further studies have shown that the HRE contains multiple binding sites for purified hormone receptor complexes in vitro (21, 22, 54, 61, 62, 71). The MMTV LTR also contains a negative regulatory element (NRE) localized to 91 base pairs (bp) of DNA between bp −454 and −364, whose role appears to be to repress the inherent basal activity of the MMTV promoter; the ratio of gene expression in the presence of hormone to gene expression in the absence of hormone is thus increased when the element is present (44). A second negative element has also been identified within HRE sequences (33), but this element has not been demonstrated to affect transcription in vivo unless it is present in multiple copies.

In addition to the receptor-binding sites, sequences that bind the cellular transcription factor nuclear factor 1 (NF-1) have been identified within the MMTV LTR between about positions −80 and −60 (14, 15, 50). Several studies have suggested that this cellular protein acts as a necessary transcription factor for hormone-induced transcription from the MMTV promoter (5, 14, 15, 43), including the isolation of a naturally occurring point mutation in the NF-1-binding site of a transcriptionally defective endogenous MMTV provirus (32).

Beyond the receptor and NF-1-binding sites, little is known of which, if any, other DNA sequence elements are necessary for accurate and efficient transcription from the MMTV promoter, although several studies have suggested that additional factors are required. In one study employing deletion and linker-scanning (LS) analysis, the binding site for NF-1, as well as a sequence located around a TATA element near position −30, was implicated in hormone-mediated transcription (5); however, the extensive sequence modifications introduced in these experiments did not allow detailed delineation of specific sequence requirements. In another study, exonuclease III protection experiments in isolated nuclei were used to demonstrate binding of cellular factors between positions −82 and +12; the exonuclease III boundary at position −82 was shown to be the result of NF-1 binding, while the downstream boundary implicated at least one other factor (15). Further work in vitro has demon-

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strated a DNA-binding factor in nuclear extracts that binds to MMTV DNA and results in exonuclease III protection of sequences between positions −42 and −4 (14), a region that includes the TATA box.

The resolution of the questions of which factors are involved in transcription from the MMTV promoter and how these factors interact with the promoter and with each other to result in hormone-regulated transcription awaits a more detailed functional characterization of the MMTV promoter itself. To this end, we report here the construction and analysis of a number of mutated MMTV promoters, including a set of LS mutations across the entire promoter region. These experiments define at least three regions of the MMTV promoter, in addition to the hormone receptor-binding sites, which appear to be necessary for normal levels of transcription. One region includes the TATA element, a second consists of two 10-bp direct repeats within which are embedded three sequences related to the octamer element (17, 52), and a third consists of sequences that are recognized by NF-1. The mutation-sensitive region containing octamer-related sequences is specifically recognized by a nuclear protein. In addition, we show that promoter mutations resulting in apparent increased in vitro affinity for NF-1 do not decrease the extent of hormone receptor-mediated transcriptional activation, suggesting that the interaction of NF-1 with the promoter is not a primary target for such activation and consistent with the idea that NF-1 binding is not rate determining in transcription from the MMTV promoter.

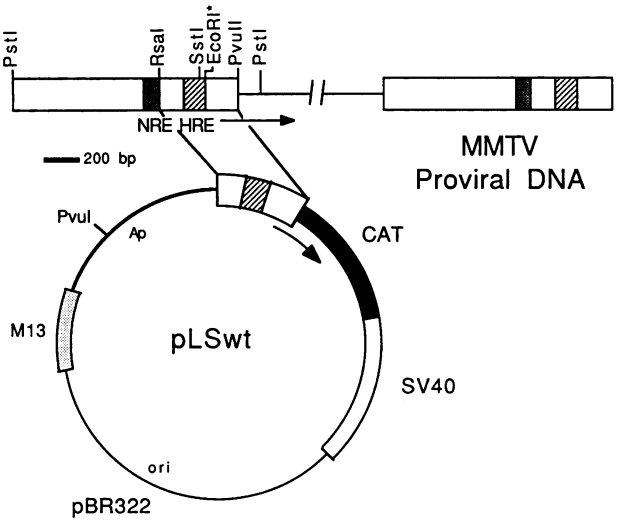

FIG. 1. Structure of MMTV LTR and pLSwt. Plasmid pLSwt (5.5 kilobases) contains MMTV LTR sequences from position −363 (*Rsa*I) to +133 (*Pvu*II) with respect to the transcription initiation site. The positions of the HRE (▨), NRE (▩), coding sequences for CAT (■), and simian virus 40 sequences that specify poly(A) addition (▭) are indicated. Vector sequences are identical to those in pLC1 and pGTC1, plasmids which we have described in detail (44, 69).

MATERIALS AND METHODS

Plasmid constructions. All restriction and modification enzymes were used according to the recommendations of the supplier (New England BioLabs or Bethesda Research Laboratories).

BAL 31 exonuclease was used to introduce 5′ and 3′ deletions into the MMTV promoter. Ends of the deleted fragments were made blunt by filling in with the Klenow fragment of DNA polymerase I, synthetic *Bgl*II linkers (AAGATCTT) were added at the deletion endpoints, and the deleted promoters were recloned. Individual members of the deletion sets were then sequenced on supercoiled plasmid DNA to determine the precise deletion endpoints. Approximately 300 deletion mutants were sequenced in order to obtain the desired deletion sets. The LS mutations were assembled by replacing a fragment of the appropriate 5′ deletion with the corresponding fragment of the appropriate 3′ deletion. The resulting LS mutants were then sequenced to verify their identity. The LS mutants, which contain the wild-type sequence configuration of the MMTV promoter between positions −363 (*Rsa*I) and +133 (*Pvu*II), are named such that the numbers in parentheses indicate the endpoints of the bases in the wild-type promoter that are replaced by the synthetic linker. The LS plasmids also contain the chloramphenicol acetyltransferase (CAT) reporter gene and simian virus 40 sequences containing the small-t intron and early poly(A) addition signal (Fig. 1). The LS plasmids do not contain pBR322 sequences that have been shown to exert an adverse effect on transient expression (56).

LS(−59/−38) was assembled by recombining the 5′ portion of LS(−59/−52) with the 3′ portion of LS(−45/−38) to construct a deleted promoter in which 22 bp of MMTV sequences were removed and replaced by the 8-bp *Bgl*II linker; this deleted promoter was designated LS(−59/−38Δ14). To restore the wild-type spacing of other MMTV promoter elements, a synthetic linker was inserted into the unique *Bgl*II site to generate LS(−59/−38), in which 20 out of 22 bp in this region of the promoter are altered (see Fig. 4).

Supercoiled monomer DNA was purified by alkaline extraction (3) and CsCl-ethidium bromide equilibrium density centrifugation followed by exhaustive dialysis against a buffer containing 250 mM NaCl, 10 mM Tris hydrochloride (pH 7.6), and 1 mM EDTA and ethanol precipitation. Inclusion of the dialysis step after the centrifugation resulted in a significant increase in gene expression from the transfected DNA and increased reproducibility between different preparations of the same plasmid. Plasmid concentrations were determined by A_{260} and verified by visualization after agarose gel electrophoresis and ethidium bromide staining. All large-scale plasmid preparations were analyzed by sequencing of the promoter region before transfection.

Plasmids were propagated in *Escherichia coli* HB101. Plasmid DNAs were sequenced by the chain termination method (59) with supercoiled templates (12) and [α-^{35}S] dATP (Dupont, NEN Research Products).

Site-directed mutagenesis. Site-directed mutagenesis was performed as described by Kunkel et al. (31) by using pGTL1, a plasmid identical to pLSwt (Fig. 1) except for changes in several restriction sites outside the MMTV-CAT transcription unit. Single-stranded pGTL1 DNA was isolated from a *dut ung E. coli* host, taking advantage of the M13 replication origin on the plasmid, and second-strand synthesis was primed with synthetic 32-base oligonucleotides (see Fig. 7A) synthesized on an Applied Biosystems DNA synthesizer (Oligonucleotide Synthesis Service, Department of Biochemistry and Biophysics, Texas A&M University). Altered DNA sequences of the mutant promoters were verified by dideoxy-DNA sequencing on plasmid DNA as described above. Mutated MMTV promoter sequences were subcloned subsequent to site-directed mutagenesis and DNA sequencing to ensure that the desired mutations were the only sequence alterations in the plasmids.

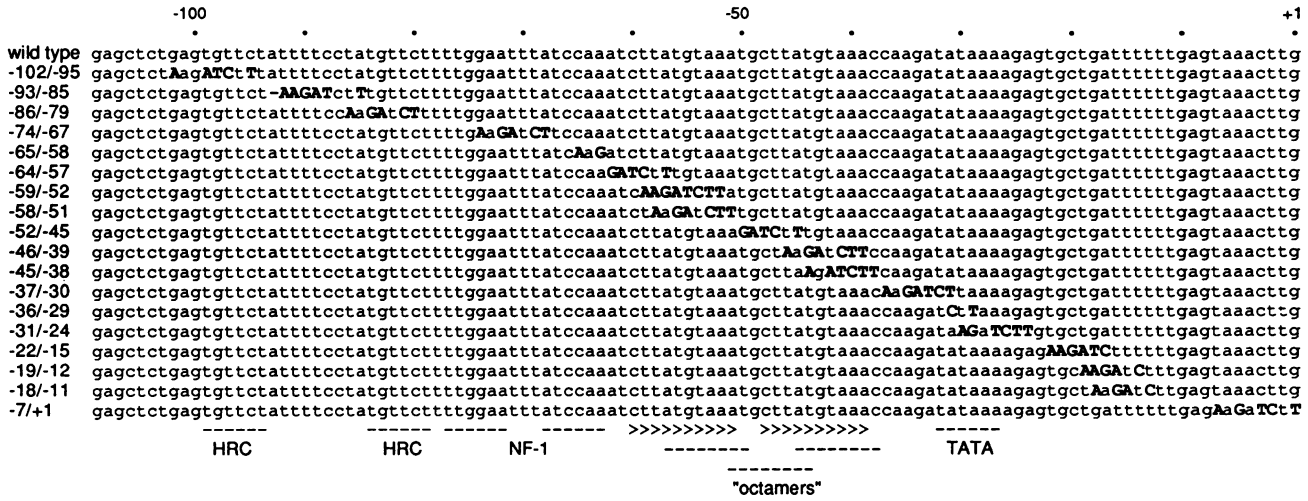

FIG. 2. Structure of LS mutations of the MMTV promoter. Wild-type sequences are in lowercase letters, while mutations introduced by the *Bgl*II linker are in boldface capital letters. The name of each mutant refers to the positions of sequences replaced by the linker. The locations of the TATA box, the 10-bp direct repeats containing sequences related to the octamer consensus, and recognition sequences for NF-1 and the glucocorticoid hormone-receptor complex (HRC) are indicated.

Gel electrophoresis mobility shift assay. Nuclear extracts of Ltk$^-$ cells were made according to the method of Dignam et al. (16) with previously described modifications (32). Protein concentration of the extract was determined by the method of Bradford (4) with bovine serum albumin as a standard. Gel electrophoresis mobility shift assays for NF-1 were performed by a method based on the procedure of Schneider et al. (64) with a probe consisting of complementary 32-base oligonucleotides (see Fig. 7A) containing the MMTV NF-1 site (extending from position −84 to position −53) as previously described (32).

Protein binding to the MMTV octamer-related sequences was assessed with a 41-bp probe containing MMTV sequences from position −67 to −33 flanked by *Bam*HI and *Bgl*II restriction sites and labeled with polynucleotide kinase and [γ-^{32}P]ATP (3,000 Ci/mmol; Dupont, NEN). The binding reaction mixtures contained 9 fmol of labeled oligonucleotide, 9 μg of nuclear extract protein, 1 μg of poly(dI-dC), and various amounts of competitor DNA in a buffer composed of 50 mM Tris hydrochloride (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol, 12.5 mM $MgCl_2$, 100 mM KCl, and 20% glycerol. These mixtures were incubated for 15 min on ice and then loaded on a 4% polyacrylamide gel (acrylamide-bisacrylamide [29:1]) containing 0.1 M Tris borate (pH 8.3), 2 mM EDTA, and 10% glycerol and electrophoresed at 4°C at 20 V/cm in the same buffer without glycerol. The gel was dried and exposed for 18 h to XAR-5 film (Eastman Kodak Co.) with an intensifying screen.

Competitor DNAs derived by polymerase chain reaction were prepared by amplification of 25 ng of plasmid DNA. The primers were 5′-CTGAGTGTTC TAGATTCCTA TG TTCT-3′ and 5′-GTGAATGTTA GGATCCTTGC AAGT TT-3′. These primers resulted in an amplified product consisting of MMTV sequences from position −104 to +20; the primers introduced base changes at positions −92, −91, +5, +6, and +7 which constructed restriction sites in the amplified DNA. Amplification was performed in a final volume of 100 μl at a total primer concentration of 1 μM with 2.5 U of *Taq* polymerase (Cetus Corp.) according to the recommendations of the supplier. Forty amplification cycles were performed at 91°C for 1 min, 45°C for 1 min, and 72°C for 1 min. Following amplification, samples were extracted with chloroform, phenol-chloroform (1:1), and chloroform and were then ethanol precipitated. DNA concentrations were then determined by A_{260}, and the integrity of the amplified product was verified by gel electrophoresis.

Transfections and CAT assays. Mouse Ltk$^-$ cells (30) were transfected by the DEAE-dextran procedure coupled with a dimethyl sulfoxide shock (36) as previously described in detail (69). Plasmid DNA was present at a concentration of 1.2 nM in the transfections unless otherwise indicated in the figure legends. Five micrograms of extract protein was assayed for 30 min for extracts from cells treated with dexamethasone, while 35 to 40 μg of protein was assayed for 2 h for extracts from cells grown in the absence of hormone. In both cases, the measured activity was within the linear range of the assay.

Quantitative RNase mapping by RNase T_2 protection was performed as previously described for RNase T_1 (69). MMTV-CAT expression was measured relative to expression from the endogenous cellular β-tubulin gene.

RESULTS

Construction of LS mutations in the MMTV promoter. In order to elucidate the molecular mechanisms whereby MMTV transcription is modulated by the HRE and NRE, it is necessary to understand what determines the basal level of transcription from the MMTV promoter. For our purposes, we have defined the MMTV promoter as the DNA sequence elements which are sufficient for basal levels of transcription but which do not specify hormone responsiveness. By this definition, the MMTV promoter has previously been shown to extend no more than 109 bp 5′ of the transcription start to an *Sst*I site within the LTR (Fig. 1) (34, 44). This 109-bp region contains a number of sequences of potential importance in MMTV transcription (Fig. 2). Two binding sites for the glucocorticoid hormone-receptor complex are present (54, 61, 62); both of these sites contain a copy of the consensus hexamer present in most DNA sequences recognized by the receptor (TGTTCT) (for a review, see reference 2) as well as the binding site for NF-1 (14, 15, 50). This region

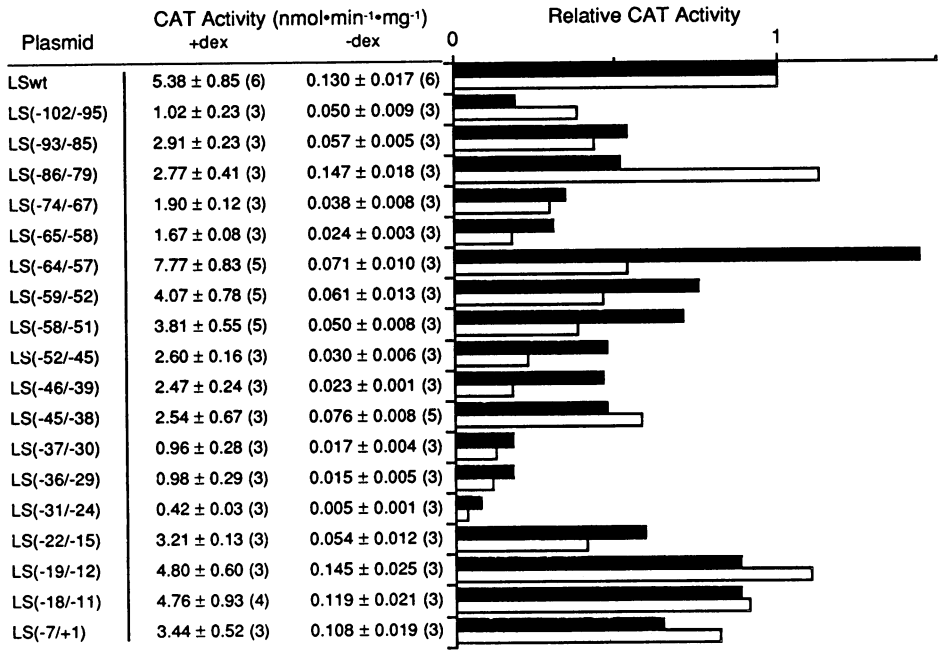

FIG. 3. CAT activity directed by promoters containing LS mutations. Plasmids containing LS mutations were transfected into Ltk$^-$ cells at a DNA concentration of 1.2 nM. Numerical values represent the means of absolute CAT specific activities of extracts of transfected cells ± the standard errors of the means with the number of independent transfections performed shown in parentheses. To compare the relative effects of the LS mutations on transcription in the presence (■) and the absence (□) of dexamethasone (dex), the data are shown graphically as CAT expression which has been normalized to that obtained from LSwt under the same transfection conditions.

of the LTR also contains a TATA element. Between the NF-1 site and TATA box are two direct repeats of the 10-bp sequence CTTATGTAAA; within the region containing these repeats are three sequences related to the promoter element known as the octamer (ATGCAAAT) (17, 52).

In order to investigate the functional significance of sequences in the MMTV promoter, LS mutations (40) across the MMTV LTR from position −102 to +1 relative to the site of transcription initiation were constructed. The mutations were introduced within the context of LTR sequences between positions −363 (*Rsa*I) and +133 (*Pvu*II) (Fig. 1). This region includes all of the sequences composing the HRE (27, 34, 37, 57) but not those of the NRE (44); this sequence context therefore allows determination of both basal and hormone-induced transcriptional activity without promoter repression due to the NRE. Downstream of the MMTV promoter in each construct was the bacterial CAT gene followed by the simian virus 40 small-t intron and sequences necessary for poly(A) addition similar to other plasmids we have described (44, 69) (Fig. 1).

The oligonucleotide linker chosen for mutagenesis was 5′-d(AAGATCTT)-3′. This linker, which contains a *Bgl*II restriction site, possesses the same G+C content as the MMTV promoter region (25%) and should, therefore, minimize any nonspecific sequence effects of the linker insertions on transcription. The LS mutations are named such that the numbers correspond to the nucleotides replaced by the linker. With one exception, LS(−93/−85), each of the mutant promoters analyzed contained an exact replacement of the wild-type promoter sequences by those of the synthetic linker and maintained, therefore, the relative positioning of the unaltered MMTV sequences (Fig. 2). Some LS mutations introduced only slight alterations in MMTV sequences [for example, only 2 bp were altered in LS(−65/−58)], while in others up to 8 bp were altered [LS(−59/−52)].

Transcriptional activity of mutant promoters. Plasmids containing each of the LS mutants were transfected into mouse Ltk$^-$ cells by the DEAE-dextran dimethyl sulfoxide shock procedure (36) as previously described in detail (69). In some experiments, the synthetic glucocorticoid dexamethasone was added 40 h after transfection to a concentration of 10^{-7} M, and 18 h later, cells were harvested and crude extracts were assayed for CAT activity (69) and total protein (4). We have previously demonstrated, with a wide variety of MMTV plasmid constructions similar to those containing the LS mutations, that CAT activity is linearly related to the abundance of RNA transcripts correctly initiated within the MMTV LTR (44, 69); this observation was confirmed in the present study (see below). CAT expression from MMTV promoters containing the LS mutations is shown in Fig. 3, in which the absolute levels of CAT expression are shown numerically and the expression relative to the wild-type promoter is shown graphically. For ease in comparing the effects of the LS mutations in the presence and absence of glucocorticoids, the values in the bar graph have been separately normalized to the corresponding wild-type levels for transfections performed in the presence or absence of dexamethasone. A number of LS mutations affected basal or hormone-induced transcription or both, and these results are discussed in detail below (see Discussion).

In order to verify that the altered levels of CAT expression obtained with the promoters containing the LS mutations were reflecting alterations in MMTV transcription, an RNase T_2 protection experiment was performed. Selected LS mutants were transfected into mouse Ltk$^-$ cells, and MMTV-CAT transcripts were quantitated relative to an internal standard of mRNA from the endogenous mouse β-tubulin gene (44, 69). Transcription from mutant MMTV promoters determined by this method was in good agreement with that assessed by CAT assay (data not shown), a

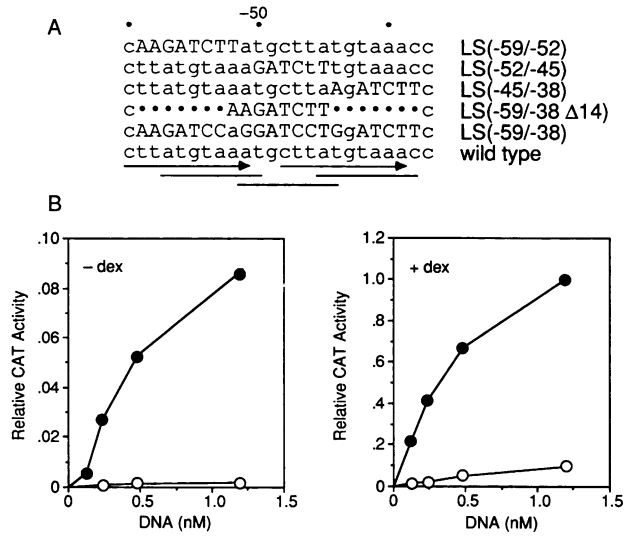

FIG. 4. Transcription from MMTV promoters with mutations in all three octamer-related sequences. (A) Sequences of promoter mutations. MMTV sequences from position −60 to −37 are shown, with the wild-type sequence at the bottom. The three octamer-related sequences and two 10-bp repeats are indicated. LS(−59/−38) was constructed by insertion of an oligonucleotide into LS(−59/−38Δ14) and contains sequences similar to the combination of mutations present in LS(−59/−52), LS(−52/−45), and LS(−45/−38). (B) CAT activity directed by LS(−59/−38). LSwt (●) or LS(−59/−38) (○) DNA was transfected into Ltk⁻ cells. Relative CAT activity (with expression from LSwt at 1.2 nM in the presence of hormone defined as 1.0) is shown as a function of DNA concentration in the transfections. Cells were propagated either in the absence (left) or presence (right) of 10^{-7} M dexamethasone (dex).

result we have repeatedly verified in transfection experiments with a number of related plasmids (44, 69).

Promoter mutations in all three octamer-related sequences. Of particular interest are the LS mutations in the 10-bp repeats containing octamer-related sequences [LS(−59/−52), LS(−58/−51), LS(−52/−45), LS(−46/−39), and LS(−45/−38)]. Basal promoter activity from LS(−52/−45) and LS(−46/−39), which alter the proximal 10-bp repeat and the two most proximal octamer-related elements, was impaired to an extent roughly equivalent to that of promoters with mutations in the NF-1-binding site, indicating the importance of this region of the promoter in MMTV transcription. However, glucocorticoid-induced transcription from these promoters was decreased by a factor of only about two. The potential role of sequences in the distal 10-bp repeat or the most distal octamerlike element is not clearly revealed by the LS mutations; LS(−59/−52) and LS(−58/−51) caused a reduction by a factor of two to three in basal promoter activity, but their effect on hormone-induced transcription was a reduction by a factor of only about 1.3. It is possible that these more distal sequences are not critical for MMTV promoter activity. However, it is also possible that relatively small effects of mutations in this region of the promoter are the result of the repeated nature of the sequences, which may be, at least to some extent, functionally redundant. To directly test this idea, a mutated promoter [LS(−59/−38)] in which this region was more extensively altered was constructed (Fig. 4). This promoter was created by insertion of an oligonucleotide into the deleted promoter present in LS(−59/−38Δ14) (see Materials and Methods). The ends of the mutated segment of LS(−59/−38) have a DNA sequence identical to those in LS(−59/−52) and LS(−45/−38), mutations which, when present alone, decreased hormone-induced transcription by a factor of two or less. The mutated promoter in LS(−59/−38) is very similar to the promoter that would result from the combination of the base substitutions in LS(−59/−52), LS(−52/−45), and LS(−45/−38). Transcriptional activity from LS(−59/−38) was decreased by a factor of at least 10 relative to the wild-type promoter over a range of DNA concentrations in both the presence and absence of glucocorticoids, confirming the importance of this entire region of the promoter for both basal and hormone-induced transcription (Fig. 4).

The DNase I footprint for NF-1 on the MMTV promoter extends from position −80 to −58 (50), and NF-1 binding to the promoter leads to an exonuclease III boundary at position −56 (14). The MMTV sequences modified in LS(−59/−38) therefore included a few nucleotides of these protected regions. Although a number of studies have indicated that the sequences modified in LS(−59/−38) are too far removed from the core recognition sequence to affect NF-1 recognition (see Discussion), we employed a gel electrophoresis mobility shift assay (19, 20) to address directly whether NF-1 binding to the promoter had been altered in LS(−59/−38). A synthetic 32-bp oligonucleotide containing MMTV sequences from position −84 to −53 was 5′-end labeled and used as a probe. Nuclear extracts from Ltk⁻ cells served as a source of NF-1, and a band of retarded mobility was observed, indicative of a protein-DNA complex (Fig. 5A, lane 1). Competition experiments have verified that the retarded band is due to specific recognition of the NF-1 site (reference 32 and data not shown). To assess NF-1 binding to mutated promoter sequences, we prepared competitor DNAs composed of MMTV sequences from position −104 to +20 by the polymerase chain reaction technique (45). Efficient competition was observed when the competitor was derived from the wild-type MMTV promoter (Fig. 5A, lanes 2 to 4), while a competitor prepared from LS(−74/−67), which alters the NF-1 core recognition sequence and has a relatively severe effect on MMTV promoter activity, showed little or no competition (Fig. 5A, lanes 5 to 7). A competitor DNA derived from LS(−59/−38) was as effective a competitor as that derived from the wild-type promoter (Fig. 5B, lanes 5 to 7). It therefore appears that impaired promoter recognition by NF-1 is not the source of the altered transcriptional activity of LS(−59/−38). This point is addressed further in Discussion.

A nuclear protein recognizes octamer-related sequences in the MMTV promoter. To determine whether a nuclear protein recognizes the promoter region containing the 10-bp repeats and the octamer-related sequences, we took advantage of a gel electrophoresis mobility shift assay similar to that described above. Nuclear extracts of Ltk⁻ cells were incubated with a synthetic oligonucleotide containing MMTV sequences from position −67 to −33. Two bands of retarded mobility indicative of protein-DNA complexes were observed; both of these bands were subject to competition by sequences from this region of the MMTV promoter but not by unrelated sequences (Fig. 6). One or more proteins in the nuclear extract, therefore, specifically recognize this region of the MMTV promoter.

Mutations that increase affinity of NF-1 for the MMTV promoter. Exonuclease III protection experiments in isolated nuclei have detected binding of NF-1 to the MMTV promoter only when the nuclei are isolated from glucocorticoid-treated cells (15). This observation has suggested a model in which one role of the activated glucocorticoid

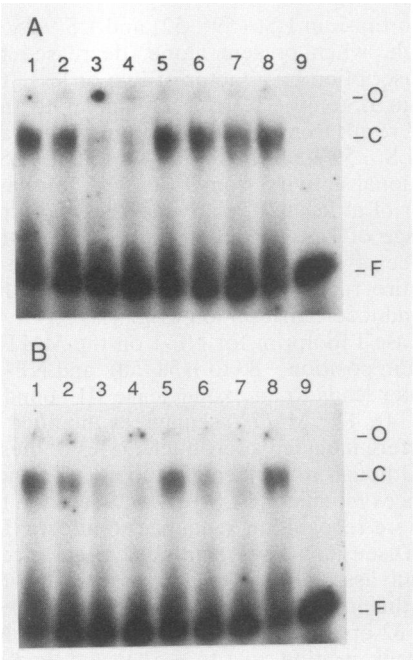

FIG. 5. NF-1 binding to mutated MMTV promoters assessed by gel electrophoresis mobility shift assay. A 5'-end-labeled, double-stranded oligonucleotide corresponding to the wild-type MMTV sequence from position −84 to −53 (30 fmol) was incubated with 4 μg of nuclear extract protein from Ltk$^-$ cells in the presence of poly (dI-dC) and various competitor DNAs corresponding to MMTV sequences from position −104 to +20. The products are displayed as autoradiograms after fractionation by polyacrylamide gel electrophoresis. The origins of the gels (O) and the positions of bands corresponding to free DNA (F) and the retarded band indicative of NF-1 binding (C) are indicated. (A) NF-1 binding to LS(−74/−67). Lanes 1 and 8, No competitor; lanes 2 to 4, competitor derived from LSwt in 10-, 50-, and 100-fold molar excess, respectively; lanes 5 to 7, competitor derived from LS(−74/−67) in 10-, 50-, and 100-fold molar excess, respectively; lane 9, no nuclear extract. (B) NF-1 binding to LS(−59/−38). Lanes 1 and 8, No competitor; lanes 2 to 4, competitor derived from LSwt; lanes 5 to 7, competitor derived from LS(−59/−38); lane 9, no nuclear extract. Amounts of competitor are identical to those in the corresponding lanes in panel A.

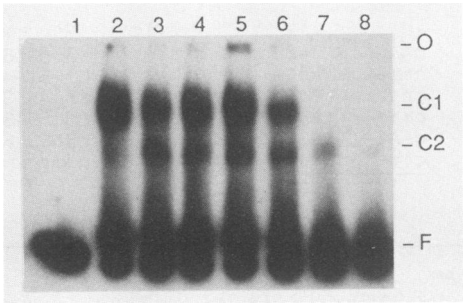

FIG. 6. Protein binding to MMTV octamer-related sequences assessed by gel electrophoresis mobility shift assay. A 5'-end-labeled, double-stranded oligonucleotide containing MMTV sequences from position −67 to −33 (9 fmol) was incubated with 9 μg of nuclear extract protein from Ltk$^-$ cells in the presence of poly (dI-dC). The products are displayed as an autoradiogram after fractionation by polyacrylamide gel electrophoresis. The origin of the gel (O) and the positions of bands corresponding to free DNA (F) and retarded bands indicative of protein-DNA complexes (C1 and C2) are shown. Unlabeled, double-stranded competitor oligonucleotides were used to assess the specificity of observed complexes for the labeled sequence. Lane 1, No extract added; lane 2, no competitor; lanes 3 and 4, NF-1(wt/wt) competitor (Fig. 7A) in 50- and 100-fold molar excess, respectively; lanes 5 and 6, a competitor consisting of a 32-bp oligonucleotide containing no sequences related to the MMTV promoter in 50- and 100-fold molar excess, respectively; lanes 7 and 8, a competitor consisting of an oligonucleotide containing MMTV sequences from position −67 to −33 (the same oligonucleotide used as a probe) in 50- and 100-fold molar excess, respectively.

receptor protein is to facilitate or stabilize binding of NF-1 to the promoter. A prediction of this model is that promoter mutations that lead to increased inherent affinity for NF-1 should increase the basal promoter activity and render it less responsive to hormonal activation. To explore this idea, we constructed mutations in the MMTV NF-1 site by oligonucleotide-directed, site-specific mutagenesis. Two mutant promoters based on pGTL1, a plasmid identical to pLSwt (Fig. 1) except for changes in several restriction sites outside the MMTV-CAT transcription unit, were constructed. The mutations created NF-1-binding sites which more closely match the consensus NF-1 site (Fig. 7A) and which were therefore expected to have higher affinity for NF-1. One of the mutated NF-1 sites contains a transversion (T to G) at position −67 to introduce a consensus sequence into the proximal half of the symmetrical NF-1 site (pGTL2), while the second mutated promoter contains a complete consensus NF-1 site obtained by introducing two additional transversions (AA to CT at positions −73 and −72) (pGTL4). While different studies have reported different NF-1 consensus sequences (23, 28, 35, 64), the consensus shown in Fig. 7A (derived from reference 28) maintains perfect symmetry in the two halves of the site.

The binding affinity of mutated MMTV NF-1 sites relative to the wild-type site was estimated with a gel electrophoresis mobility shift assay with the synthetic 32-bp oligonucleotide containing the wild-type MMTV NF-1 site as described above. To assess the relative affinity of NF-1 binding to the wild-type and mutant promoters, a competition experiment (Fig. 7B) in which competitor DNAs consisted of unlabeled 32-bp oligonucleotides containing the wild-type and mutated NF-1 sites was performed. This experiment revealed significant differences in the apparent affinity of NF-1 for the three binding sites in the order predicted on the basis of the relationship with the consensus NF-1 site: the wild-type NF-1 site [NF-1(wt/wt)] was significantly lower in affinity than the site containing the consensus sequence in the proximal symmetry element [NF-1(wt/c)], which was significantly lower in affinity than the complete consensus NF-1 site [NF-1(c/c)]. Determining the exact quantitative differences in binding affinity among the three sites is difficult because of the crude nature of the protein fraction. However, on the basis of densitometric scanning of the autoradiogram shown in Fig. 7B, the NF-1(wt/c) and NF-1(c/c) sites were estimated to have binding affinities 10 and 50 times higher than that of the wild-type site, respectively. These estimates are consistent with the results of Meisterernst et al. (42), who quantitated affinities of purified porcine liver NF-1 with a number of mutated NF-1 sites. Their results showed that mutations in an NF-1 site of the type TTGGCN$_5$GCCAA (similar to what we have defined as the consensus site) to TTGGAN$_5$GCCAA (with a distal half-site similar to that found in MMTV) reduces affinity for NF-1 by a factor of 5 to 10. Similarly, a mutation of G to T in the first base of the GCCAA element (similar to the proximal half-site found in MMTV) reduces affinity by a factor of five. We therefore believe that our estimates based on crude extracts are realistic.

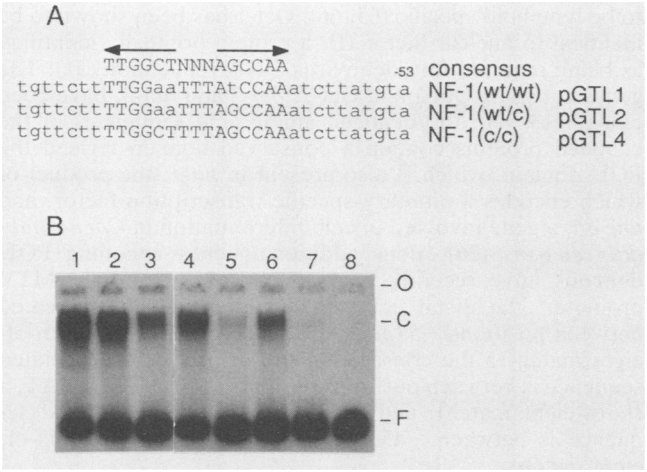

FIG. 7. Sequences of mutated promoters containing altered NF-1-binding sites. MMTV sequences between positions −84 and −53 with respect to the transcription initiation site are shown. The consensus NF-1-binding site derived from the analysis of multiple high-affinity sites (28) is shown at the top with the region of dyad symmetry indicated by arrows. These sequences correspond to the primers used in site-directed mutagenesis. (B) NF-1 binding to mutated MMTV promoters assessed by gel electrophoresis mobility shift assay. A 5′-end-labeled, double-stranded oligonucleotide corresponding to the wild-type [NF-1(wt/wt)] 32-bp sequence shown in panel A (20 fmol) was incubated with 7 μg of nuclear extract protein from Ltk⁻ cells in the presence of poly(dI-dC). The products are displayed as an autoradiogram after fractionation by polyacrylamide gel electrophoresis. The origin of the gel (O) and the positions of bands corresponding to free DNA (F) and the retarded band indicative of NF-1 binding (C) are indicated. Unlabeled, double-stranded competitor DNAs corresponding to the sequences shown in panel A were used to assess the relative affinity of NF-1 for wild-type and mutant MMTV promoters. Lane 1, No competitor DNA; lanes 2 and 3, NF-1(wt/wt) competitor in 8- and 38-fold molar excess, respectively; lanes 4 and 5, NF-1(wt/c) competitor in 8- and 38-fold molar excess, respectively; lanes 6 and 7, NF-1(c/c) competitor in 8- and 38-fold molar excess, respectively; lane 8, no extract added.

Transcriptional activity of promoters with increased NF-1 affinity. A comparison of the transcriptional activities of promoters containing wild-type (pGTL1) and complete consensus (pGTL4) NF-1-binding sites is shown in Fig. 8. At low concentrations of DNA, transcriptional activity of the promoter with the consensus binding site was increased about twofold in both the presence and the absence of hormone; however, at 1.2 nM DNA (the concentration used in the experiments shown in Fig. 3), the difference between the two promoters was only about 20%. The increased apparent affinity for NF-1 did not affect the extent of receptor activation of promoter activity; the hormone induction ratio of wild-type and mutant promoters was essentially constant (15- to 20-fold hormone induction in these experiments) and did not vary with DNA concentration. Transfection experiments with the promoter containing a consensus sequence only in the proximal NF-1 half-site (pGTL2) gave CAT expression intermediate to that given by the two constructions containing the wild-type or complete consensus NF-1 sites (data not shown).

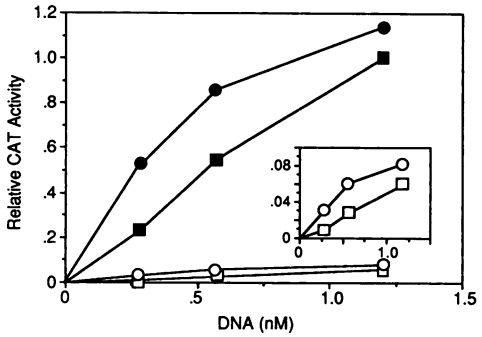

FIG. 8. Transcriptional activity of MMTV promoters with altered NF-1 sites. Ltk⁻ cells were transfected with pGTL1 (squares) or pGTL4 (circles) at various DNA concentrations. Filled symbols indicate CAT expression in the presence of 10^{-7} M dexamethasone, while open symbols indicate expression in the absence of this hormone. The CAT activities shown are the averages of three to five independent transfections and are expressed as relative activities. The insert shows CAT expression in the absence of hormone with an expanded scale.

DISCUSSION

Mutations in steroid hormone-receptor binding sites. LS mutations LS(−102/−95), LS(−93/−85), and LS(−86/−79) overlap two DNase I footprints of glucocorticoid hormone-receptor complexes (54, 62), each of which contains a hexameric TGTTCT consensus binding site. LS(−102/−95) disrupts the more distal of these hexamer consensus binding sequences (located between positions −99 and −94) in four of the six hexamer consensus bases, including the two G residues that have been previously shown to be protected from methylation in the presence of the hormone receptor in vitro (61). The glucocorticoid-induced transcriptional activity of this mutant was reduced by a factor of five. In a similar manner, the mutations introduced by LS(−86/−79) alter four nucleotides within the more proximal consensus hexamer for glucocorticoid receptor binding between positions −84 and −79, but this mutation resulted in a decrease in glucocorticoid-induced CAT expression by a factor of only about two. It is unclear whether the difference in sensitivity of these two regions, both of which bind the receptor in vitro, reflects specific differences in the mutations with respect to the consensus binding site sequence or the inherent differences in the necessity of the two sites for hormone regulation of MMTV transcription. Our results are generally consistent with the work of Cato et al. (11), who found that overall steroid responsiveness was dependent on the two proximal receptor-binding sites mutated in our study in addition to more distal receptor-binding sites also present in the HRE. This finding is in contrast to an earlier report in which the proximal sites were found to be relatively unimportant (5).

The mutations introduced by LS(−93/−85), which alters the sequence between the two glucocorticoid receptor-binding consensus sequences, resulted in a 50% decrease in hormone-induced CAT activity, suggesting, as has been previously reported (5), that the sequences outside of the hexamer consensus sites can affect the levels of hormone-mediated transcription. However, since LS(−93/−85) contains a 1-bp deletion relative to the normal MMTV sequence organization, it is also possible that this positional alteration is responsible for the decreased activity.

In the absence of hormone, transcription from the MMTV promoter became, not surprisingly, less dependent on the receptor-binding sites. This was particularly apparent with LS(−86/−79), in which transcriptional activity was not at all affected in the absence of hormone. It is not clear, however, why LS(−102/−95) and LS(−93/−85) should result in a

decrease of a factor of two in CAT expression in the absence of glucocorticoids. One possibility is that the media in which the transfected cells were propagated contained nontrivial amounts of glucocorticoid hormones and that the levels of gene expression we have defined as hormone independent in reality reflect some amount of hormone induction. We believe this is unlikely, however, since transcription from LS(−86/−79) was affected only in the presence of hormone (Fig. 3). Alternatively, it is possible that an additional basal promoter element overlaps the receptor-binding site that is altered in LS(−102/−95), and we are currently investigating this possibility.

One mutation-sensitive region of the MMTV promoter contains a TATA element. Our LS mutations have defined at least three mutation-sensitive regions of the MMTV promoter. One such region is defined by LS(−37/−30), LS(−36/−29), and LS(−31/−24) and contains a TATA element. LS(−37/−30) alters six bases in this region, at least two of which are in the TATA element, while LS(−36/−29) alters only two bases (−31 and −29), both of which are in the TATA sequence. The activity of both of these mutant promoters is decreased by a factor of six to eight relative to the wild type. LS(−36/−29) changes only two bases with respect to the wild-type sequence (ATCTTAA versus ATATAAA), suggesting that at least one of these bases may be intimately involved with the binding of the mouse homolog of transcription factor TFIID (48). LS(−31/−24), which also alters sequences within the TATA element, directed the lowest transcriptional activity of any of the LS mutants. The lower activity of this mutant, compared with those of the other TATA mutants, is possibly due to the more complete disruption of the TATA-like sequences in this promoter (Fig. 2).

While the importance of the TATA element in MMTV transcription cannot be considered surprising, previous mutational analysis had not convincingly established its role. The LS mutations of Buetti and Kühnel (5) in this region of the promoter contained a deletion between positions −37 and −11, altering spacing between other promoter elements and the transcription start site. In addition, the transcriptional activity of this mutated promoter was not determined in the absence of glucocorticoid induction, and therefore no conclusion regarding the significance of the mutated sequences on basal transcription could be made.

The transcriptional activity of mutations 3′ of the TATA element increased to essentially wild-type levels, indicating that sequences altered in LS(−22/−15), LS(−19/−12), LS(−18/−11), and LS(−7/+1) are not important for basal or hormone-induced transcription.

A second mutation-sensitive region contains two 10-bp direct repeats and three octamer-related sequences. MMTV sequences between positions −60 and −38 contain two direct repeats of the 10-bp sequence CTTATGTAAA, and embedded within these direct repeats are three homologies to the promoter element known as the octamer (ATGCAAAT) (17, 52). The octamer element is present in, among others, the promoters of the immunoglobulin heavy- and light-chain genes (17, 24, 39, 52), the herpes simplex virus thymidine kinase gene (53), small nuclear RNA genes (1, 8, 38, 46), the histone H2B gene (18), and simian virus 40 (67), in each of which it has been shown to be required for optimal levels of transcription. The octamer sequence has been shown to be the binding site for at least two proteins: one, termed Oct-1 (68), OTF-1 (18), OBP100 (67), or NF-A1 (66), which apparently is present in all cell types, and another, termed Oct-2 (13), OTF-2 (63), or NF-A2 (66), which appears to be lymphoid specific (65, 66). Oct-1 has been shown to be identical to nuclear factor III, a protein originally identified as being required for adenovirus DNA replication (51). The genes encoding Oct-1 and Oct-2, *oct-1* and *oct-2*, have been cloned (13, 68); the deduced amino acid sequences of the encoded proteins contain a conserved domain termed the POU domain, which is also present in *pit-1*, the product of which encodes a pituitary-specific transcription factor, and *unc-86*, a gene involved in cell differentiation in *Caenorhabditis elegans* (26). Four additional genes encoding POU domains have recently been cloned (25). In the MMTV promoter, the distal octamer-related sequence is located between positions −57 and −50 (ATGTAAAT, a seven-of-eight match to the consensus), the central octamer-related sequence is between positions −51 and −44 (ATGCTTAT, a six-of-eight match), and the proximal octamer-related sequence is between −45 and −38 (ATGTAAAC, a six-of-eight match).

The most severe effect of mutations in this region of the promoter was observed on basal transcription, where LS(−52/−45) and LS(−46/−39) decreased CAT expression by a factor of four to six, a decrease comparable to that in the mutations in the NF-1-binding site. These two LS mutations alter the proximal 10-bp element and the central and proximal octamer-related sequences. Mutations in the distal 10-bp element or the distal octamer-related sequence [LS(−59/−52) and LS(−58/−51)] decreased basal transcription by a factor of two to three. None of the LS mutations in this region of the promoter reduced hormone-induced transcription by a factor of more than two. These data indicated that at least the proximal portion of the 10-bp elements or octamer-related sequences plays a functional role in the MMTV promoter but left unclear the role of the repeated nature of these elements. The importance of this repeated nature was defined by the dramatically decreased transcriptional activity of LS(−59/−38) (Fig. 4), in which all of the repeated segments in this region of the promoter are altered. It is possible that the two 10-bp repeats or the three octamer-related sequences are functionally redundant in a manner similar to that previously observed with two G-C boxes in the promoter of the herpes simplex virus thymidine kinase gene (41). That is, mutations in the distal 10-bp repeat or the distal octamer-related sequence have relatively little effect on transcription in LS(−59/−52) or LS(−58/−51), but similar sequence alterations within the context of additional mutations in the proximal 10-bp repeat or the central and proximal octamer-related sequences [LS(−59/−38)] decrease transcription significantly (Fig. 4).

Alternatively, it could be argued that the observed effect of mutations in LS(−59/−38), which we ascribe to alterations in the 10-bp repeat or octamer-related sequences, may be an artifact of modifying sequences necessary for NF-1 binding on the 5′ side and TFIID binding at the TATA element on the 3′ side. A number of considerations make this possibility unlikely. First, if the observed effect of LS(−59/−38) were simply to due a combination of sequence alterations at the NF-1 site and TATA element, then the predicted magnitude of the effect would be expected to be the product of the effects caused by LS(−59/−52) and LS(−45/−38), since other sequences altered in LS(−59/−38) do not overlap with the defined binding sites for NF-1 or the putative TATA-binding factor (see below). However, the observed transcription level from LS(−59/−38) was one-fourth to one-third of the level predicted on the basis of this premise in the presence of glucocorticoids and less than 1/10 of the predicted level in the absence of hormone, indicating

that sequences altered in LS(−59/−38) but not altered in LS(−59/−52) or LS(−45/−38) play a role in MMTV transcription. Moreover, sequences altered in LS(−59/−38) do not affect NF-1 binding, as determined by competition in a gel electrophoresis mobility shift experiment (Fig. 5), nor do they alter sequences required for NF-1 recognition, as determined by Nilsson et al. (49) and Leegwater et al. (35). Finally, with regard to the possible effect of LS(−59/−38) on TFIID binding at the MMTV TATA element, several relevant observations can be made. The first T of the TATA element in the MMTV promoter is at position −32. LS(−59/−38) thus introduces sequence alterations beginning 6 bp upstream of TATA. DNase I footprints of the most highly purified preparations of mammalian TFIID on the adenovirus major late promoter have been shown to extend 6 and 9 bp upstream of TATA on the nontemplate and template strands of DNA, respectively, while DNase I footprints on the *hsp70* promoter extend 4 and 6 bp upstream of TATA on the nontemplate and template strands, respectively (48). Footprinting with methidiumpropyl-EDTA · iron(II), a chemical reagent that is not subject to the steric constraints of an enzymatic probe such as DNase I, has defined a TFIID binding site on the adenovirus major late promoter extending 1 and 3 bp upstream of TATA on the nontemplate and template strands, respectively (60). Purified TFIID from *Saccharomyces cerevisiae*, which has been shown to function in vitro in a mammalian transcription system, generates a DNase I footprint on the adenovirus major late promoter that extends 4 and 6 bp upstream of TATA on the nontemplate and template strands, respectively (7). On the basis of these experiments, it appears that mutations in LS(−59/−38) are unlikely to affect sequences that are involved in TFIID recognition. The binding studies described above did not define the minimal sequence required for TFIID recognition, however. At present, the best indication of the size of such a minimal sequence comes from functional studies of promoter activity. LS mutations of the herpes simplex virus thymidine kinase promoter indicate that DNA 7 bp upstream of the TATA sequence has no effect on promoter activity (40), while a detailed set of point mutations in the β-globin promoter indicates that DNA within 2 bp of the TATA element can be mutated without affecting promoter activity (47); functional analysis thus limits the sequences important for TFIID recognition to those very near the TATA sequence itself. These observations, coupled with the relatively minor effect of LS(−45/−38) on MMTV promoter activity (a reduction by a factor of two), lead us to conclude that sequences this far removed from TATA are not likely to have a significant effect on TFIID binding, and we believe that the effect of LS(−59/−38) on MMTV transcription cannot be ascribed to defects in recognition of the TATA element.

The importance of the 10-bp repeat or octamer-related sequences for MMTV transcription is further strengthened by the identification of a protein that recognizes this region of the promoter (Fig. 6). In addition, we have demonstrated that an oligonucleotide containing MMTV octamer-related sequences can specifically inhibit transcription from the MMTV promoter in vitro, suggesting that the added oligonucleotide titrates out a DNA-binding protein required for transcription (J. Pierce and D. O. Peterson, unpublished observation). Several lines of evidence suggest that this protein may be Oct-1, the ubiquitously expressed octamer-binding protein. We have shown that an oligonucleotide containing a consensus octamer can compete for binding with MMTV sequences, and we have also demonstrated that authentic Oct-1, expressed either in vitro or in *E. coli* from the cloned cDNA (68), can specifically recognize this region of the MMTV promoter. However, it is not completely clear that Oct-1 is the factor involved in MMTV transcription. We have not yet demonstrated an effect of authentic Oct-1 on in vitro transcription from the MMTV promoter, which would confirm its functional role. In addition, we have purified a protein that recognizes this region of the MMTV promoter from mammalian nuclear extracts by DNA affinity chromatography, and this protein is significantly smaller than Oct-1 (M. Huang and D. O. Peterson, unpublished observation). Finally, site-directed mutations in the MMTV promoter that convert either the distal and proximal or all three octamer-related sequences to consensus octamers result in a significant decrease in promoter activity (J. W. Lee and D. O. Peterson, unpublished observation). More work will be necessary to sort out these observations and definitively characterize the factor that mediates the transcriptional effect of the MMTV 10-bp repeats or octamer-related sequences.

A third mutation-sensitive region of the MMTV promoter contains sequences bound by NF-1. A third region of the MMTV promoter that is sensitive to the LS mutations is defined by LS(−74/−67) and LS(−65/−58), both of which direct transcription at approximately one-fifth to one-third of the wild-type level in both the presence and the absence of hormone. This region is resolved from mutations affecting receptor-binding sites by LS(−86/−79), which has no effect on basal transcription, and from promoter sequences more proximal to the transcription start by LS(−64/−57). The MMTV promoter in LS(−74/−67) contains base changes at five positions, of which two (−74 and −68) are conserved positions in the distal half of the NF-1 recognition site (28), while LS(−65/−58) replaces bases only at positions −65 and −63, with only the replacement at −65 altering a conserved sequence in the proximal half of the NF-1 recognition site. LS(−74/−67) was demonstrated to alter NF-1 recognition in vitro (Fig. 5).

On the basis of some previous reports, a larger reduction in promoter activity might have been expected from these mutations; the insertion of as few as three bases between the two NF-1 half-sites was reported to result in a decrease in hormone-mediated transcription from the MMTV promoter of greater than two orders of magnitude (43). Our results correspond more closely to those obtained in the nucleotide replacement mutagenesis experiments of Cato et al. (11), in which complete alteration of the distal NF-1 half-site resulted in a reduction by a factor of six to eight in glucocorticoid-induced transcription. As in that study, both mutants tested here maintained the wild-type spacing of sequences 5′ and 3′ of the two NF-1 half-sites, and only a portion of the sequences thought to be critical for recognition by NF-1 was mutated. Therefore, one explanation for the difference between our results (as well as those of Cato et al. [11]) and those of others is that promoters with one intact half-site retain the ability, albeit impaired, to bind NF-1 (28). Such weakened interactions may also be stabilized by the presence of additional proteins bound to this region of the promoter. It is interesting to note that a boundary for exonuclease III digestion in vitro has been observed with purified NF-1 at position −68 (14), which is between the two NF-1 half-sites, suggesting that both NF-1 half-sites need not be occupied simultaneously in the MMTV promoter.

The decreased transcription from mutant promoters with altered NF-1-binding sites indicates that this transcription factor is required for basal as well as hormone-induced

transcription (Fig. 3). Earlier studies have not been definitive on the role of NF-1 in basal transcription from the MMTV promoter. For example, oligonucleotide substitution mutagenesis of the distal NF-1 half-site results in reduction by a factor of about two in basal transcription (11), but a naturally occurring G-to-A transition at position −75 in endogenous MMTV proviral DNA at genetic locus *Mtv-17* significantly decreases the affinity of NF-1 for the promoter and dramatically affects glucocorticoid-induced transcription without an apparent effect on promoter activity in the absence of hormone (32). In addition, one study has included the MMTV NF-1 site in the HRE sequences required to confer maximum glucocorticoid inducibility to the herpes simplex virus thymidine kinase promoter (57). However, the thymidine kinase promoter employed in this study contained a deletion of the endogenous NF-1 site present in distal element 2 (28, 29, 40), and the effect of the MMTV NF-1 site may have been to partially restore the deleted element. The variable effects of mutations in the MMTV NF-1-binding site on basal transcription can be interpreted in a consistent manner if NF-1 binding, while being required for normal levels of transcription from the wild-type MMTV promoter, is not rate determining. Thus, if the step that is rate determining for basal transcription is sufficiently slow, significant sequence alterations in the NF-1 site, such as those present in the LS or insertion mutations, would be required in order to decrease NF-1 affinity to an extent sufficient to make NF-1 binding become the limiting factor in transcription. In the presence of hormone, where the rate-determining step in transcription is significantly faster, the decrease in NF-1 affinity caused by even the less extensive mutations (such as the single point mutation in the endogenous provirus at *Mtv-17* [32]) may be sufficient to make NF-1 binding become rate determining. The observation that mutant MMTV promoters with increased apparent affinity for NF-1 do not substantially increase basal transcription (Fig. 7 and 8) is consistent with the idea that NF-1 binding is not rate-determining.

The requirement for NF-1 in basal transcription from the MMTV promoter is supported by a recent study of Buetti et al. (6) and by our own in vitro transcription studies, in which we have demonstrated a requirement for NF-1 (Pierce and Peterson, unpublished observation).

It is interesting to note that an octamer-related sequence (ATCCAAAT, a seven-out-of-eight match to the consensus) is present from position −68 to −61 within the NF-1-binding site. This octamer-related sequence is apparently not recognized by the same protein that binds MMTV DNA within the 10-bp repeats, since an oligonucleotide containing the NF-1 site (−84 to −53) does not compete for this protein (Fig. 6).

The MMTV promoter and hormone-induced transcription. While a few of the LS mutants differentially affected transcription depending on whether glucocorticoids were present, the hormone induction ratio for all of the mutant promoters varied by a factor of only about two up or down relative to that seen with the wild-type promoter (Fig. 3 and 8). No promoter mutation functionally separated basal from hormone-induced transcription, suggesting that all of the *trans*-acting, DNA-binding transcription factors required for basal transcription are also required for hormone-induced transcription; stated in another way, hormone induction does not render the MMTV promoter independent of any of the DNA-binding transcription factors necessary for basal transcription. Furthermore, since promoters with substantially increased apparent affinity for NF-1 remained subject to the same extent of hormone induction as the wild-type promoter, facilitation or stabilization of NF-1 binding does not appear to be a potential target for transcriptional activation of the MMTV promoter by steroid receptor proteins; such a model has been suggested on the basis of detection of NF-1 binding to the MMTV promoter in isolated nuclei only when such nuclei are prepared from hormone-treated cells (15).

Structure of the MMTV promoter. Promoters recognized by RNA polymerase II generally have a modular structure composed of a TATA element near position −30 and one or more upstream elements whose position relative to the start of transcription is not as rigidly constrained. The MMTV promoter follows this basic pattern. Three distinct mutation-sensitive regions (in addition to the sequences composing the binding sites for the steroid hormone-receptor complexes) were identified by our LS analysis and define the MMTV promoter. One region contains a TATA element at the expected position, a second contains as many as three binding sites for proteins that recognize octamer-related sequences, and a third contains a binding site for NF-1. It will be of interest to determine how these basal promoter elements, which by themselves direct a relatively low level of transcription, are organized to allow the high degree of modulation of transcriptional activity imposed by the HRE and NRE.

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