A Transcriptionally Defective Long Terminal Repeat within an Endogenous Copy of Mouse Mammary Tumor Virus Proviral DNA

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Mouse mammary tumor virus proviral DNA is endogenous to most inbred strains of mice but in many strains is not transcriptionally active. This inactivity may be due to defects in the proviruses themselves or to position effects mediated by DNA sequences flanking the proviral units. The transcriptional competence of long terminal repeats (LTRs) derived from endogenous proviral DNA at genetic loci *Mtv-8*, *Mtv-9*, and *Mtv-17* of the C57BL/6 mouse strain was examined with a transient transfection assay in which gene expression was monitored by expression of chloramphenicol acetyltransferase. LTRs from *Mtv-8* and *Mtv-9* were able to direct glucocorticoid-induced chloramphenicol acetyltransferase expression in this assay, while the LTR from *Mtv-17* was only about 5% as effective. Analysis of chimeric LTRs indicated that the glucocorticoid-inducible transcriptional enhancer element within the *Mtv-17* LTR is active when linked to a functional promoter from *Mtv-8*, whereas the promoter from *Mtv-17* is defective in directing hormone-induced gene expression, even when linked to the *Mtv-8* glucocorticoid-responsive enhancer. The DNA sequence of transcriptional control regions of the LTRs of all three endogenous proviral units was determined; this analysis revealed that the source of the defect in *Mtv-17* is a single G-to-A transition at position −75 with respect to the site of transcription initiation that resides within the previously defined binding site for the transcription factor nuclear factor 1. Competition experiments with a gel electrophoresis mobility shift assay indicated that the affinity of nuclear factor 1 for DNA derived from *Mtv-17* is significantly less than for comparable sequences derived from *Mtv-8*.

Endogenous copies of the proviral DNA of mouse mammary tumor virus (MMTV) are present as stable genetic elements in the germ line DNA of most common laboratory strains of mice (28). Although most of this endogenous MMTV DNA is apparently organized into complete proviral units, the proviruses appear in most cases to be transcriptionally inactive (35, 64). Notable exceptions to this rule are the endogenous proviruses at genetic loci *Mtv-1* and *Mtv-2*, which have been shown to encode infectious virions and to contribute to the onset of mammary tumorigenesis in several inbred strains (39, 62, 63, 65). Other endogenous proviral units have been reported as transcriptionally active in some tissues or in specific cultured cell lines (31, 35, 46, 51), indicating that at least some endogenous proviruses are transcriptionally competent.

The molecular basis for the transcriptional inactivity of endogenous MMTV proviral DNA and the mechanisms by which it can become activated are not known. In some cases transcriptional inactivity may be maintained by position effects of the sequences flanking the provirus; it has been shown that the site of chromosomal insertion can play a major role in the transcription of an exogenously acquired MMTV provirus (12, 60), and transcription from the normally inactive *Mtv-8* provirus has been demonstrated when its chromosomal context is altered by integration at new genomic sites by stable transformation of cloned *Mtv-8* DNA (8, 23). It is also possible that transcriptional inactivity of some endogenous proviral units is due to defective MMTV sequence elements necessary for initiation of RNA synthesis. These elements include the transcriptional promoter; a glucocorticoid-responsive enhancer termed the glucocorticoid response element (GRE), which mediates the hormone inducibility of viral transcription (for a review, see reference

66) and serves as a binding site for the purified glucocorticoid receptor protein in vitro (15, 17, 47-49, 56, 57); and a recently characterized negative transcriptional regulatory element (NRE) which mediates repression of the basal activity of the MMTV promoter and increases the ratio of gene expression observed in the presence and absence of hormone (42).

In this study we have examined the transcriptional activity of four long terminal repeats (LTRs) derived from three endogenous copies of MMTV proviral DNA endogenous to C57BL/6 mice. Using a transient transfection assay, we have shown that LTRs derived from the endogenous proviruses at genetic loci *Mtv-8* and *Mtv-9* can support glucocorticoid-induced transcription but that the provirus from locus *Mtv-17* cannot; the defect in this provirus is due to a single G-to-A transition in the DNA sequence element that serves as a binding site for the transcription factor nuclear factor 1 (NF-1), and the affinity of NF-1 for the altered sequences is significantly diminished by this transition.

MATERIALS AND METHODS

Plasmid constructions. Restriction endonucleases and modifying enzymes (New England Biolabs or Bethesda Research Laboratories) were used according to the supplier's recommendations.

Plasmids pLV8.5 and pLV8.3 contain the 5' and 3' LTRs, respectively, from the provirus at *Mtv-8*, while the 3' LTRs from proviruses at *Mtv-9* and *Mtv-17* are in pLV9.3 and pLV17.3. These plasmids were constructed as follows. The 3' LTRs of MMTV DNA at genetic loci *Mtv-8*, *Mtv-9*, and *Mtv-17* and the 5' LTR from *Mtv-8*, previously cloned from the C57BL/6 cell line T1M1 (53), were subcloned as *Ava*I-to-*Bst*EII restriction fragments (see Fig. 1). These isolated fragments were treated with the Klenow fragment of *Escherichia coli* DNA polymerase I (Bethesda Research Laboratories) in the presence of deoxynucleotide triphosphates to

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fill in protruding single-stranded termini. Synthetic HindIII linkers were then ligated to the blunt-ended fragments by using T4 DNA ligase (Bethesda Research Laboratories); the added linker sequences were 10 base pairs (bp) in length for the LTR from Mtv-9 and the 5' LTR of Mtv-8 and 17 and 19 bp for the 3' LTR from Mtv-17 and 3' LTR from Mtv-8, respectively. These modified LTR sequences were inserted in the appropriate orientation into plasmid vector pMT2 (42), which has a unique HindIII restriction site immediately upstream of the chloramphenicol acetyltransferase (CAT) gene and simian virus 40-derived sequences encoding the small-t intron and poly(A) addition (16).

Plasmids pLV.G8.P8, pLV.G8.P17, and pLV.G17.P8 contained chimeric LTR sequences and were made as follows. Sau3A-HindIII restriction fragments containing the MMTV promoters (see Fig. 2) from LTRs in pLV8.5 and pLV17.3 were isolated and ligated into the BglII-HindIII sites of a CAT vector analogous to pMT2 (42) but containing both BglII and HindIII sites immediately 5' of the CAT coding sequences. The MMTV promoters, minus most GRE sequences, were thus inserted in a position to drive synthesis of CAT; these insertions resulted in the retention of the unique BglII site immediately 5' of the MMTV promoter. The 344-bp Sau3A fragment containing most of the GRE (Fig. 1) from Mtv-8 was then ligated into the unique BglII site of plasmids containing both promoters to generate pLV.G8.P8, in which both GRE and promoter were derived from Mtv-8, and pLV.G8.P17, in which the GRE-containing fragment was derived from Mtv-8 and the promoter-containing fragment was derived from Mtv-17. In similar fashion, the 344-bp GRE fragment from Mtv-17 was inserted into the BglII site of the plasmid containing the Mtv-8 promoter to generate pLV.G17.P8. These chimeric LTRs were then sequenced on double-stranded plasmid DNA to verify the origin of the GRE and promoter sequences and the orientation of each insert.

Preparation of DNA. Plasmid DNA was purified from chloramphenicol-treated E. coli HB101 by alkaline extraction (1) followed by banding on a CsCl-ethidium bromide density gradient. DNA for transfection was then dialyzed against 10 mM Tris hydrochloride (pH 7.6)–1 mM EDTA–0.25 M NaCl and precipitated with ethanol. Recovered DNA was dissolved in 10 mM Tris hydrochloride (pH 7.6)–1 mM EDTA. DNA concentrations were determined by absorbance at 260 nm and verified by agarose gel electrophoresis-ethidium bromide staining.

Transient expression assay. Mouse Ltk⁻ cells (27) were grown as monolayers in Dulbecco modified Eagle medium (GIBCO Laboratories) containing 10% fetal bovine serum (GIBCO Laboratories) at 37°C in an atmosphere of 5% CO_2 saturated with water. Transfections were by the DEAE-dextran–dimethyl sulfoxide shock procedure (32) as described in detail (59), except that cells were plated at a density of 5×10^5 cells per plate the day before transfection. Crude cellular extracts were prepared, and CAT assays were performed as described (59). Protein concentrations of the extracts were measured by the method of Bradford (2) with bovine serum albumin as the standard. Acetylated products were separated from the labeled chloramphenicol substrate by thin-layer chromatography (16), and radioactive spots were identified by autoradiography and quantitated by liquid scintillation counting. Multiple transfections with two independent plasmid preparations were performed for each plasmid construction; standard errors of the reported means were 10% or less for transfections performed in the presence

of dexamethasone and 30% or less for transfections performed in the absence of hormone.

DNA sequencing. Sau3A-Sau3A or Sau3A-HindIII fragments of the MMTV LTRs were subcloned into M13mp18 and M13mp19 vectors (38) and sequenced by the dideoxy chain termination method (55). All reported sequences were determined on both DNA strands, with the exception of a few bases just downstream from the 5' Sau3A site which were sequenced on multiple independent clones but only on one strand. Dideoxy sequencing to verify the construction of chimeric LTRs was performed on double-stranded plasmid templates (5) prepared by alkaline extraction (1).

Gel electrophoresis mobility shift assay. Nuclear extracts of Ltk⁻ cells were made by the method of Dignam et al. (9) with the following modifications. Cells (10^8 to 10^9) were grown on plates, and the synthetic glucocorticoid dexamethasone was added to a final concentration of 10^{-7} M 18 h before harvest. Cells were washed twice with phosphate-buffered saline (2.7 mM KCl, 1.5 mM KH_2PO_4, 15 mM Na_2HPO_4, 137 mM NaCl) containing 1 mM EDTA, and 0.1% Triton X-100 was added to the buffer described by Dignam et al. (9) for cell homogenization. The salt-extracted nuclear proteins were dialyzed against 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)–20% (vol/vol) glycerol–0.1 M KCl–0.2 mM EDTA–0.5 mM dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride, and the precipitated material was removed by centrifugation for 10 min at $1,000 \times g$ at 4°C. Protein concentration of the extract was determined by the method of Bradford (2) with bovine serum albumin as a standard.

Complementary 32-base oligonucleotides containing the NF-1 site (extending from position −84 to −53) were synthesized on an Applied Biosystems DNA Synthesizer and purified by denaturing polyacrylamide gel electrophoresis. The sequence was derived from the transcriptionally active C3H strain of virus and had the sequence 5'-TGTTCTT TTGGAATTTATCCAAATCTTATGTA-3'. Equal molar amounts of the complementary strands were mixed and annealed by heating for 2 min at 100°C and slowly cooling to room temperature. The resulting double-stranded fragment was purified by nondenaturing polyacrylamide gel electrophoresis and 5'-end labeled to a specific activity of 100 Ci/mmol with T4 polynucleotide kinase (Bethesda Research Laboratories) and [γ-³²P]ATP (New England Nuclear Corp.). Binding reactions were performed by a modification of the procedure of Schneider et al. (58). Labeled DNA fragment (10 fmol) and various amounts of competing DNA as indicated in the legend to Fig. 6 were incubated with 4 μg of nuclear extract protein for 15 min at room temperature in 25 mM HEPES (pH 7.9)–1 mM EDTA–5 mM dithiothreitol–10% glycerol–150 mM NaCl–67 μg of poly(dI-dC) (Pharmacia) per ml in a total volume of 15 μl. The reaction mixture was then loaded on a 4% polyacrylamide gel (29:1, acrylamide-bisacrylamide) containing 0.04 M Tris acetate (pH 7.8)–2 mM EDTA–10% glycerol and electrophoresed at 15 V/cm for 0.75 to 1 h in the same buffer (without glycerol). After electrophoresis the gel was dried and exposed for 16 h to XAR-5 film (Eastman Kodak Co.) with a Dupont Cronex Lightning-Plus intensifying screen.

RESULTS

Transcriptional potential of MMTV LTRs endogenous to C57BL/6 mice. We have previously reported the molecular cloning and characterization of MMTV DNA endogenous to C57BL/6 mice (53). This inbred strain contains three appar-
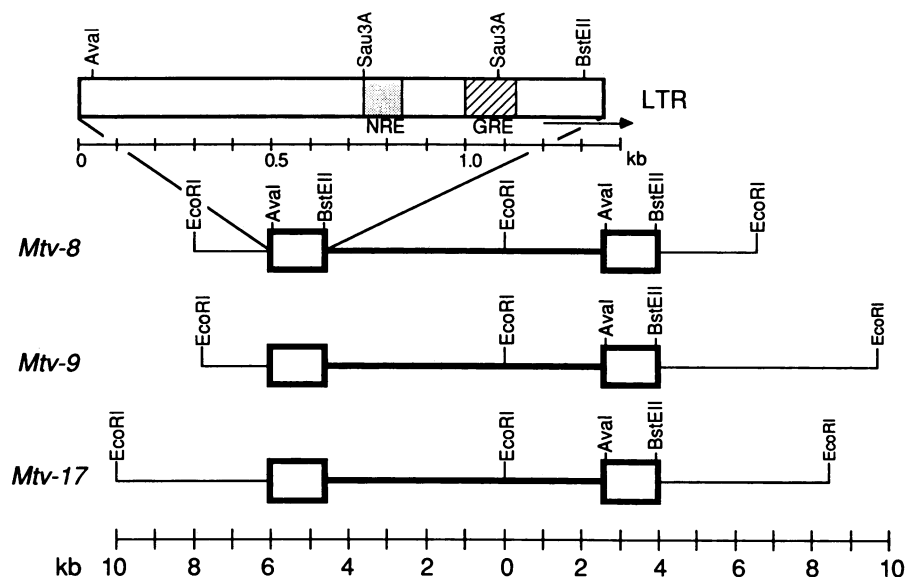
FIG. 1. MMTV proviral DNA endogenous to C57BL/6 mice. MMTV proviruses at genetic loci *Mtv-8*, *Mtv-9*, and *Mtv-17* are depicted as heavy lines with some relevant restriction sites indicated. The molecular cloning of the entire *Mtv-8* locus, as well as the 3' portions of *Mtv-9* and *Mtv-17*, has been previously described (53). An enlargement of the LTR with approximate positions of the GRE, as defined functionally (3, 4, 22, 29, 34, 54) and by sites of glucocorticoid receptor binding in vitro (47, 56, 57), and the NRE (42) are indicated. The arrow shows the start site and direction of MMTV transcription. The presence of *AvaI* and *BstEII* sites in the 5' LTRs of proviruses at *Mtv-9* and *Mtv-17* has not been verified.

ently intact copies of proviral DNA at genetic loci *Mtv-8*, *Mtv-9*, and *Mtv-17* (Fig. 1) (50, 53).

To determine the transcriptional potential of the LTRs of these endogenous proviruses, analogous *Ava*I-to-*Bst*EII restriction fragments from both LTRs of *Mtv-8*, as well as from the 3' LTRs of *Mtv-9* and *Mtv-17*, were cloned into plasmid pMT2 (42) after modification of the ends to *Hind*III sites with synthetic linkers (see Materials and Methods for details of constructions). Each resulting plasmid was constructed such that the MMTV promoter within the LTRs could drive synthesis of the CAT gene product when the plasmids were introduced into appropriate cultured cells (Fig. 2). The plasmids have been designated pLV8.5 (containing the 5' LTR from *Mtv-8*), pLV8.3 (containing the 3' LTR from *Mtv-8*), pLV9.3 (containing the 3' LTR from *Mtv-9*), and pLV17.3 (containing the 3' LTR from *Mtv-17*).

We have described an extensive characterization of a transient transfection assay with plasmids similar to these in which we defined highly reproducible transfection conditions (59). We demonstrated that under these conditions CAT specific activity measured in crude extracts of transfected cells is linearly related to the quantity of MMTV-CAT RNA transcripts (59); furthermore, we have shown that four different plasmid constructions, all of which are related to those described here, result in an essentially identical average plasmid copy number of 15 to 30 molecules per nucleus in a population of transfected Ltk⁻ cells (42, 52).

Each LTR-containing plasmid was transfected into mouse Ltk⁻ cells by the DEAE-dextran–dimethyl sulfoxide shock procedure (32) as previously described (59) (Fig. 3). CAT activity directed by the plasmid containing the 5' LTR from *Mtv-8* (pLV8.5) was more than 100-fold induced by the synthetic glucocorticoid dexamethasone (Fig. 3, lanes 1 and 2). CAT activity from plasmids containing the 3' LTRs of *Mtv-8* (pLV8.3) and *Mtv-9* (pLV9.3) was similarly inducible (Fig. 3, lanes 3 to 6). In dramatic contrast, CAT activity in extracts of cells transfected with the plasmid containing the

3' LTR of *Mtv-17* (pLV17.3) (Fig. 3, lanes 7 and 8) was significantly less in the absence of glucocorticoid induction and was inducible to a level only about 5% of that observed in extracts of cells transfected with the other three plasmids.

DNA sequence analysis of endogenous MMTV LTRs. The failure of the 3' LTR from *Mtv-17* to direct CAT gene expression at levels comparable to the other tested LTRs
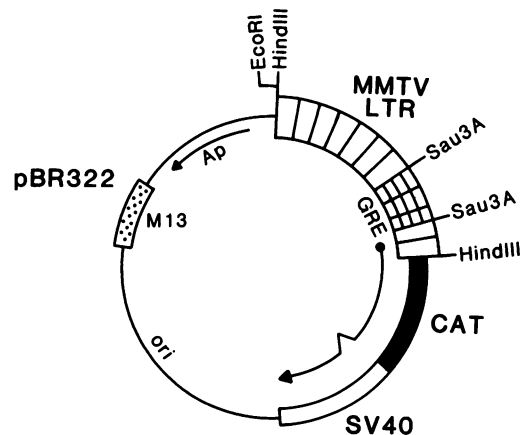
FIG. 2. Structure of MMTV LTR-CAT plasmids. Comparable *Ava*I-*Bst*EII restriction fragments of endogenous MMTV LTRs (striped box) were inserted into plasmid pMT2 (42) after converting the ends to *Hind*III sites. The resulting plasmids contain the LTRs as well as the coding sequences for CAT (filled box) and the simian virus 40 small-t intron and early poly(A) signals (open box). The vector sequences are derived from pSVOd (37), a "poisonless" (33) derivative of pBR322, into which we have inserted a bacteriophage M13 origin of replication (stippled box) (67) after deletion of the simian virus 40 origin region. The expected major transcript is indicated by the arrow. Plasmids pLV8.5 and pLV8.3 contain the 5' and 3' LTRs from *Mtv-8*, respectively, pLV9.3 contains the 3' LTR from *Mtv-9*, and pLV17.3 contains the 3' LTR from *Mtv-17*.

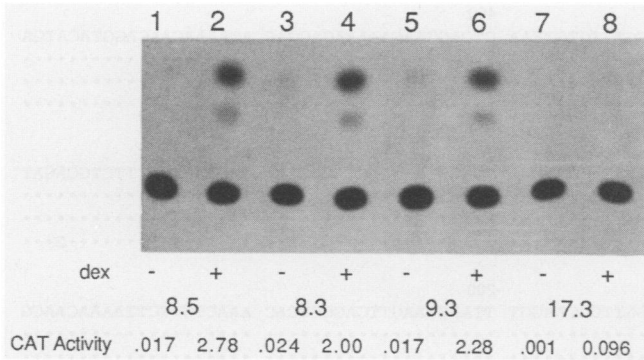FIG. 3. CAT expression from plasmids containing LTRs from endogenous MMTV proviral DNA. Ltk⁻ cells were transfected with the indicated plasmids, and after 58 h, cell extracts were prepared and assayed for CAT activity. Shown is an autoradiogram of the thin-layer chromatography separation of the unacetylated [^{14}C] chloramphenicol substrate (lower spot) from the 3-acetyl and 1-acetyl derivatives (upper spots) in a representative assay. Numerical values of CAT activity represent the averages of three to five independent transfections with two independent plasmid preparations and are reported as nanomoles of chloramphenicol acetylated per minute per milligram of extract protein. Cells were transfected with pLV8.5 (lanes 1 and 2), pLV8.3 (lanes 3 and 4), pLV9.3 (lanes 5 and 6), and pLV17.3 (lanes 7 and 8). Dexamethasone (10⁻⁷ M) was added to some transfections (lanes 2, 4, 6, and 8) 18 h before harvest. The CAT assays depicted in the autoradiogram proceeded for 1 h at 37°C with 40 μg (extracts of cells not treated with dexamethasone) or 5 μg (extracts of cells treated with dexamethasone) of total protein.

could be due to defective transcription, to mRNA instability, or to inefficient translation of the MMTV-CAT mRNA. To begin to investigate these possibilities, portions of the endogenous MMTV LTRs were subcloned into M13mp18 and M13mp19 (38) vectors and subjected to DNA sequence analysis by the dideoxy chain termination method (55). The regions sequenced were from approximately −450 to +100 with respect to the transcription start within the MMTV LTR and encompassed all known transcriptional regulatory elements within the MMTV LTR, including the NRE (42), GRE (3, 4, 22, 29, 34, 54), and promoter (3, 34, 42, 54).

A comparison of the derived DNA sequences of the four endogenous LTRs is presented in Fig. 4. All of the LTR sequences are highly homologous to each other and to previously published MMTV LTR sequences (10, 11, 26, 41). The DNA sequences from the 5′ and 3′ LTRs of *Mtv-8* are nearly identical, with only two base differences at positions −260 and +87. The sequence of the 3′ LTR of *Mtv-8* contains four base differences as compared with a previously published sequence (10), all of which are downstream of the transcription start. The 3′ LTR of *Mtv-9* represents a combination of the sequences of the two *Mtv-8* LTRs with the *Mtv-8* 5′ LTR sequence at −260 and the *Mtv-8* 3′ LTR sequence at +87. The 3′ LTR from *Mtv-17* differs from the *Mtv-8* 5′ LTR at 28 sites. However, the sequence of the *Mtv-17* LTR from +1 to the *Bst*EII site at +105 is identical to that of the 3′ LTRs of *Mtv-8* and *Mtv-9*, indicating that the expected major transcripts from the CAT plasmids containing these LTRs should be identical with the exception of slight differences in the linker DNAs used to modify the *Bst*EII end to a *Hind*III site, none of which introduced potential translation initiation sites into the mRNA. Moreover, a series of related plasmids containing variable polylinker sequences at an analogous position

within the MMTV-CAT transcript have been constructed, and alterations in the polylinker sequence are without effect on transiently expressed CAT activity (data not shown). It therefore appears very unlikely that differences in mRNA stability, processing, or translational efficiency (61) could explain the lack of CAT expression in cells transfected with pLV17.3.

Comparison of the DNA sequences, however, suggests that the lack of CAT expression from pLV17.3 may be due to altered transcriptional activity of the *Mtv-17* LTR. For example, a G-to-A difference between the LTRs of *Mtv-8* and *Mtv-17* at position −75 is within the footprint for the transcription factor NF-1, which has been implicated in hormone-inducible gene expression from MMTV (3, 7, 40, 45); in addition, there are 26 other base differences between the two LTRs in the sequenced 5′ flanking DNA, some of which reside in the glucocorticoid receptor footprints defined in vitro (47, 56, 57) and within sequences containing the NRE (42).

Functional analysis of chimeric transcription control regions. To analyze separately the potential effects of sequence differences between the various LTRs, we took advantage of the conserved *Sau*3A restriction site at position −114 (Fig. 4). A CAT expression plasmid was constructed which contained a chimeric transcription control region composed of *Mtv-8* sequences from −457 to −115 and *Mtv-17* sequences from −114 through the rest of the LTR to the *Bst*EII site at +105 (Fig. 5A). Because the *Sau*3A site at −114 roughly divides the LTR between the promoter and the GRE, we have called this plasmid pLV.G8.P17, indicating that the origin of the GRE is *Mtv-8* and the origin of the promoter is *Mtv-17*; the only difference in 5′ flanking sequences between this plasmid and a similarly constructed plasmid composed entirely of *Mtv-8* DNA (pLV.G8.P8) is the single G-to-A difference in the NF-1 footprint at position −75. A plasmid containing the converse chimeric transcription control region was also constructed with sequences from −457 to −115 from *Mtv-17* and sequences from −114 to +105 from *Mtv-8* (pLV.G17.P8).

These plasmids were transfected into Ltk⁻ cells to assess their ability to direct glucocorticoid-inducible expression of CAT (Fig. 5B). Transfection of pLV.G17.P8 (Fig. 5B, lanes 1 and 2) and pLV.G8.P8 (lanes 5 and 6) resulted in glucocorticoid-induced CAT activity comparable to that observed with the complete *Mtv-8* LTR (Fig. 3). In contrast, hormone-induced CAT expression in cells transfected with pLV.G8.P17 was approximately 10-fold lower (Fig. 5B, lanes 3 and 4). It therefore appears that the *Mtv-17* GRE is functional when linked to a functional promoter but that the single-base change in the NF-1 footprint of *Mtv-17* significantly affects the ability of the LTR to respond to the GRE. Interestingly, uninduced levels of CAT expression from pLV.G8.P17 were significantly higher than those observed when the *Mtv-17* promoter was in the context of the entire *Mtv-17* LTR (pLV17.3, Fig. 3, lane 7) and were comparable to those observed with pLV.G17.P8 and pLV.G8.P8, which contain the *Mtv-8* promoter (see Discussion).

NF-1 binding to endogenous LTR sequences. To assess whether the altered NF-1 site in *Mtv-17* DNA had, indeed, affected NF-1 binding, we took advantage of a gel electrophoresis mobility shift assay (13, 14) similar to that employed by Schneider et al. (58) to detect NF-1 binding in crude nuclear extracts. The basis of this assay is that protein-DNA complexes migrate more slowly during electrophoresis than does free DNA. Complementary 32-base oligonucleotides containing the NF-1 site (extending from

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              Sau3A                                                                    -400
Mtv-8 (5')    GATCCCTCCCCTTTCGT GAAAGACTCGCCAGAGCTAG ACCTCCTTGGTGTGTGTTAA CTCAGGAAGAAAAAGACGAC ATAAAACAACAGGTACATGA
Mtv-8 (3')    ................. .................... .................... .................... ....................
Mtv-9 (3')    ................. .................... .................... .................... ....................
Mtv-17 (3')   ................. .....G.............. .................... ....AA.............. ...G................

                                                                                       -300
Mtv-8 (5')    TTATATTTATCTAGGAACTG GAATGAACGTTTGGGGAAAG ATTTTTCATTATACCAAGGA GGGGGCAGTGGCTAGACAAT TAGAACACATTTCTGCAGAT
Mtv-8 (3')    ................... .................... .................... .................... ....................
Mtv-9 (3')    ................... .................... .................... .................... ....................
Mtv-17 (3')   ...........A....AA ....A.........AA.... .......G..G..T...... .................... ...............G...

                                                                                       -200
Mtv-8 (5')    ACTTTTGGCATGAGCTATAA TGGATAACCTTTATGAGCCC AACCTTGCGGTTCCCAAGGT TTAAGTAAGTTCAGGGTCAC AAACTGTTCTTAAAACAAGG
Mtv-8 (3')    G.................. .................... .................... .................... ....................
Mtv-9 (3')    ................... .................... .................... .................... ....................
Mtv-17 (3')   ..C....A...A..G..... .AA................. ...............G... .C..A........TT..... ..................A.

                                                               Sau3A          -100
Mtv-8 (5')    ATGTGAGACAAGTGGTTTCC TGACTTGGTTTGGTATCAAA TGTTTTGATCTAAGCTCTGA GTGTTCTATTCTCCTATGTT CTTTTGGAACTTATCCAAGT
Mtv-8 (3')    ................... .................... .................... .................... ....................
Mtv-9 (3')    ................... .................... .................... .................... ....................
Mtv-17 (3')   ................... .................... .................... .................... .....A..............

                                                                                       +1
Mtv-8 (5')    CTTATGTAAATGCTTATGTA AACCATGATATAAAAGAGTG CTGATTTTTGAGTAAACTT GCAACAGTCCTAACATTCAC CTCTCGTGTGTTTGTGTCTG
Mtv-8 (3')    ................... .................... ................... .................... ....................
Mtv-9 (3')    ................... .................... ................... .................... ....................
Mtv-17 (3')   ................... .................... ................... .................... ....................

                                                                                       BstEII
Mtv-8 (5')    TTCGCCATCCCGTCTCCGGT CGTCACTTATCCTTCACTTT CCAGAGAGTCCCCCCGCAGA CCCCGGTGACC
Mtv-8 (3')    ................... .................... .......G............ ............
Mtv-9 (3')    ................... .................... .......G............ ............
Mtv-17 (3')   ................... .................... .......G............ ............
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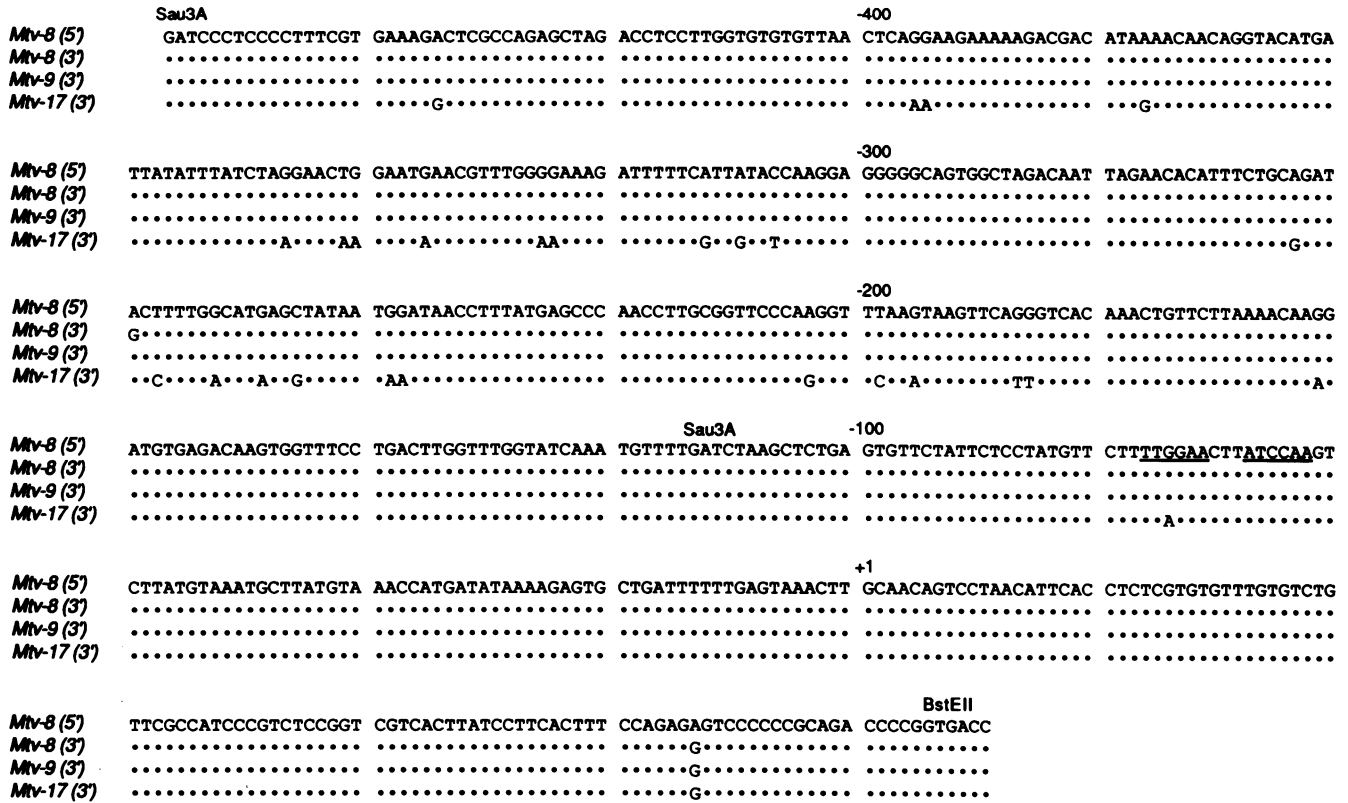
FIG. 4. Sequence of transcription control regions of endogenous MMTV LTRs. Sequences were determined by the dideoxy chain termination method and are numbered relative to the site of transcription initiation (+1). The positions of the *Sau*3A restriction sites (Fig. 1) are indicated. The underlined bases represent the sequences related to the consensus NF-1 binding site (TTGGCTNNNAGCCAA) (24); the previously defined NF-1 footprint extends from −79 to −59 (45). Functional analysis of the GRE has localized it to the region between approximately −200 and −80 (3, 4, 22, 29, 34, 54), with glucocorticoid receptor footprints located at approximately positions −198 to −164, −124 to −107, −101 to −88, and −85 to −73 (47, 56, 57). The NRE has been localized to positions −457 to −366 (42).

position −84 to −53) from the transcriptionally active C3H strain of virus were synthesized, allowed to anneal, and 5'-end labeled with T4 polynucleotide kinase and [γ-³²P]ATP. The sequence of the oligonucleotides differs from the *Mtv-8* sequence at two positions, both of which are outside the bases related to the NF-1 consensus (see Materials and Methods). Incubation of this labeled oligonucleo-

tide with a crude nuclear extract of Ltk⁻ cells resulted in the appearance of a labeled band of reduced electrophoretic mobility (Fig. 6). The specificity of this retarded band was verified by competition studies in which unlabeled 32-bp oligonucleotide was able to compete effectively with the labeled oligonucleotide for binding, while comparable amounts of oligonucleotides of similar size that did not
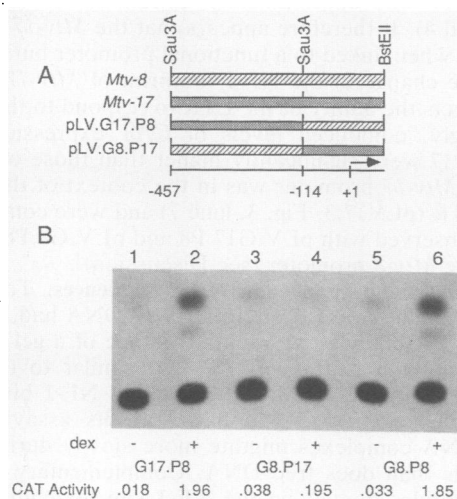

FIG. 5. CAT expression from plasmids containing chimeric transcription control regions. (A) Structures of chimeric transcription control regions. Sequences derived from *Mtv-8* are shown as a striped box, while those derived from *Mtv-17* are shown as a stippled box. The start site and direction of MMTV transcription are indicated by the arrow. Plasmid pLV.G17.P8 contains sequences from *Mtv-17* from −457 to −115 and sequences from *Mtv-8* from −114 to +105, while pLV.G8.P17 has the converse structure. Plasmid pLV.G8.P8 is composed entirely of sequences from *Mtv-8*. (B) CAT expression. A representative autoradiogram of CAT assays performed and reported as described in the legend to Fig. 3 is shown. Numerical values of CAT activity represent the averages of three to five independent transfections with two independent plasmid preparations. Extracts were prepared from cells transfected with pLV.G17.P8 (lanes 1 and 2), pLV.G8.P17 (lanes 3 and 4), and pLV.G8.P8 (lanes 5 and 6). Dexamethasone (10⁻⁷ M) was added to some transfected cells (lanes 2, 4, and 6) 18 h before harvest. The CAT assays depicted in the autoradiogram proceeded for 1 h at 37°C with 40 μg (extracts of cells not treated with dexamethasone) or 5 μg (extracts of cells treated with dexamethasone) of total protein.
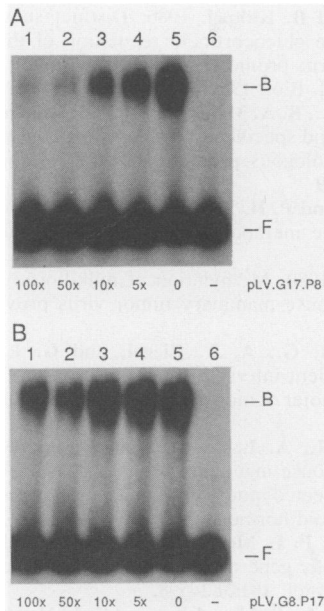
FIG. 6. NF-1 binding to endogenous MMTV LTRs, assessed by gel electrophoresis mobility shift assay. A 5′-end-labeled, double-stranded oligonucleotide containing MMTV sequences from −84 to −53 (10 fmol) was incubated with 4 μg of nuclear extract protein from Ltk⁻ cells, and the products are displayed as an autoradiogram after fractionation by polyacrylamide gel electrophoresis. The positions corresponding to the electrophoretic mobilities of free DNA (F) and the DNA–NF-1 complex (B) are shown. (A) Competition for NF-1 binding by pLV.G17.P8. Incubations were performed in the presence of unlabeled pLV.G17.P8, which contains *Mtv-17* sequences from −457 to −115 and *Mtv-8* sequences from −114 to +105, at molar ratios of 100 (lane 1), 50 (lane 2), 10 (lane 3), 5 (lane 4), and 0 (lane 5) relative to the labeled fragment. Lane 6 contains DNA with no added nuclear extract. (B) Competition for NF-1 binding by pLV.G8.P17. Incubations were performed in the presence of unlabeled pLV.G8.P17, which contains *Mtv-8* sequences from −457 to −115 and *Mtv-17* sequences from −114 to +105. Relative amounts of competing DNA are the same as in the corresponding lanes in panel A.

contain an NF-1 site were not able to compete (data not shown). In addition, plasmids pLV8.3 (data not shown) or pLV.G17.P8 (Fig. 6A), which contain functional NF-1 sites from *Mtv-8*, were able to compete, while pMT1, a plasmid that contains only the pBR322-derived sequences of the MMTV-CAT constructions, was not (data not shown). Little or no competition was observed with pLV.G8.P17 (Fig. 6B) or pLV17.3 (data not shown), indicating that the affinity of NF-1 for *Mtv-17* DNA containing the G-to-A transition is substantially reduced. Quantitative interpretation of these experiments is difficult due to the crude nature of the extract and unknown parameters relating to the kinetics of dissociation of NF-1–DNA complexes during electrophoresis, but the results suggest that NF-1 affinity for the altered site is reduced by at least an order of magnitude.

DISCUSSION

Transcriptional potential of endogenous MMTV LTRs. The transcriptional inactivity of endogenous MMTV proviral DNA has been ascribed to position effects of the chromosomal context of the integration sites of the proviral units. Mechanisms related to DNA hypermethylation (6, 19, 21) or specific patterns of chromatin packaging (12, 51) have been

postulated to explain these effects at a molecular level. These models predict that functional transcriptional regulatory signals within the endogenous proviruses should be revealed in transfection experiments in which molecular clones of proviral DNA are freed of their usual chromosomal context. Our transfection results indicate that the LTRs from endogenous proviruses at *Mtv-8* and *Mtv-9* are, indeed, capable of specifying hormone-induced transcription, and these results support the idea that position effects play a major role in determining the transcriptional inactivity of these proviral units. Similar results have been obtained in transfection experiments performed with a clone of *Mtv-8* DNA isolated from GR mice (8, 23).

In contrast, at least one endogenous provirus with fairly broad distribution among laboratory strains of mice (28) contains a transcriptionally defective LTR. We have not been able to show explicitly that both LTRs of *Mtv-17* are defective, owing to the difficulty in obtaining molecular clones of the 5′ portion of the provirus (53). However, our results suggest that the lack of transcription of the provirus at *Mtv-17* appears to be due to an inherent transcriptional defect that affects the DNA sequence element normally involved in binding NF-1. The DNA sequence alterations in the *Mtv-17* LTR within the GRE and NRE apparently do not significantly alter the function of these regulatory elements, as evidenced by their ability to mediate appropriate transcriptional activity when linked to the *Mtv-8* promoter (Fig. 5B). Thus, even if the chromosomal context of the *Mtv-17* provirus allowed its functional GRE to be recognized by the glucocorticoid receptor protein, transcription would not be induced. Indeed, indirect evidence provided by glucocorticoid-inducible sites of nuclease hypersensitivity (51, 68) that map to the GREs within both LTRs of the *Mtv-17* provirus suggests that these GREs are recognized by the receptor, even in cell lines in which no MMTV transcription can be detected (J. Marich and D. Peterson, unpublished observation).

We have determined that the G-to-A transition in the NF-1 site in *Mtv-17* is not specific to the clonal isolate analyzed here; the identical sequence is also present in an independently isolated clone of *Mtv-17* DNA (kindly provided by Jaqueline Dudley).

The portion of the *Mtv-17* LTR that we have sequenced includes a region spanning the C-terminal 73 amino acids of the 320-amino-acid open reading frame present in the MMTV LTR (10, 11, 26). Relative to the LTRs from *Mtv-8* and *Mtv-9*, the sequenced portion of the *Mtv-17* LTR contains 20 base alterations affecting 18 codons and 13 amino acids of this reading frame; however, none of these alterations would lead to premature termination of synthesis of the polypeptide encoded by this region.

NF-1 and MMTV transcription. NF-1 was originally characterized as a cellular protein required for adenovirus replication in vitro (43), but it has now been shown to interact with a number of eucaryotic RNA polymerase II promoters as well, including that of MMTV (45). Recently, construction and analysis of mutant MMTV promoters has allowed some transcriptional relevance to be ascribed to this binding. Buetti and Kühnel (3) constructed a mutant in which sequences between −90 and −70 were deleted and replaced with a 10-bp synthetic linker, resulting in a decrease in hormone induction of fivefold, but not affecting basal levels of transcription. More recently, Miksicek et al. (40) constructed a series of insertion mutations that altered the spacing between the elements of dyad symmetry of the NF-1 site without changing any wild-type nucleotides. These

mutations decreased hormone-induced transcription as well as NF-1 binding in vitro; however, their effect could not be definitively ascribed to changes in NF-1 binding because of simultaneous alterations in the relative spacing of other transcriptional regulatory sequences. Our analysis of the naturally occurring defect within the LTR of *Mtv-17* establishes the importance of NF-1 binding for hormone-induced transcription from the MMTV promoter, and the interpretation of our results is not complicated by deletion or insertion of nucleotides into the NF-1 site. Furthermore, the effect of the point mutation in the *Mtv-17* NF-1 site indicates the importance of this base in NF-1 recognition and transcriptional control. The G residue altered in the *Mtv-17* LTR is highly conserved in both half-sites of symmetrical NF-1 binding sites (18, 20, 24, 30, 44, 45).

It is difficult to reach any definitive conclusions regarding the role of the NF-1 site in basal promoter activity. The complete *Mtv-17* LTR, which contains sequence alterations in the NRE and GRE in addition to the altered NF-1 site, directs a level of CAT activity 10- to 20-fold less than the LTR from *Mtv-8* both in the presence and absence of dexamethasone (Fig. 3); when the single-base difference in the NF-1 site is isolated from the other divergent sequences, only hormone-induced CAT expression is affected (Fig. 5B). These observations by themselves suggest that the NF-1 site may be required only for hormone-induced transcription from the MMTV promoter. This idea is consistent with the results of one study in which the NF-1 site was included in the sequences required to confer maximal glucocorticoid inducibility to the herpes simplex virus thymidine kinase promoter (54). However, the thymidine kinase promoter employed in this study contained a deletion of distal sequence 2 (36), which contains binding sites for the transcription factors SP1 and NF-1 (24, 25), and the requirement for the MMTV NF-1 site in these experiments may simply have been to partially restore the deleted promoter element. Interpreting the role of NF-1 in basal MMTV transcription is further complicated by our observation that a number of mutations introduced into the MMTV NF-1 site lead to decreased transcription both in the presence and absence of hormone (M.G. Toohey and D. O. Peterson, manuscript in preparation), suggesting that NF-1 binding is required for basal, as well as glucocorticoid-induced, promoter activity. A final determination of the role of NF-1 in basal transcription from the MMTV promoter must await a more detailed analysis of a number of mutant promoters containing altered NF-1 sites.

ACKNOWLEDGMENTS

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