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A Redundant Nuclear Protein Binding Site Contributes to Negative Regulation of the Mouse Mammary Tumor Virus Long Terminal Repeat

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The tissue specificity of mouse mammary tumor virus (MMTV) expression is controlled by regulatory elements in the MMTV long terminal repeat (LTR). These regulatory elements include the hormone response element, located approximately between −200 and −75, as well as binding sites for NF-1, Oct-1 (OTF-1), and mammary gland enhancer factors. Naturally occurring MMTV deletion variants isolated from T-cell and kidney tumors, transgenic-mouse experiments with MMTV LTR deletions, and transient transfection assays with LTR constructs indicate that there are additional transcription regulatory elements, including a negative regulatory element (NRE), located upstream of the hormone response element. To further define this regulatory region, we have constructed a series of BAL 31 deletion mutants in the MMTV LTR for use in transient transfection assays. These assays indicated that deletion of two regions (referred to as promoter-distal and -proximal NREs) between −637 and −201 elevated basal MMTV promoter activity in the absence of glucocorticoids. The region between −637 and −264 was surveyed for the presence of nuclear protein binding sites by gel retardation assays. Only one type of protein complex (referred to as NRE-binding protein or NBP) bound exclusively to sites that mapped to the promoter-distal and -proximal NREs identified by BAL 31 mutations. The promoter-proximal binding site was mapped further by linker substitution mutations and transfection assays. Mutations that mapped to a region containing an inverted repeat beginning at −287 relative to the start of transcription elevated basal expression of a reporter gene driven by the MMTV LTR. A 59-bp DNA fragment from the distal NRE also bound the NBP complex. Gel retardation assays showed that mutations within both inverted repeats of the proximal NRE eliminated NBP binding and mutations within single repeats altered NBP binding. Intriguingly, the NBP complex was detected in extracts from T cells and lung cells but was absent from mammary gland cells. These results suggest that a factor contributing to high-level expression of MMTV in the mammary gland is the lack of negative regulation by NBP.

Mouse mammary tumor virus (MMTV) specifically causes breast carcinomas in mice (40). Much of this specificity is derived from high levels of viral transcription in the mammary gland. High-level mammary gland transcription can be conferred on various reporter genes by linkage to the MMTV long terminal repeat (LTR) (6, 46), and deletion analysis of the MMTV LTR has indicated that at least one region, called the mammary gland enhancer, is necessary for elevated MMTV transcription in the mammary gland (25, 31, 34, 58). Although the mammary gland enhancer is located near the 5′ end of the LTR, many additional regulatory elements near the transcription start site contribute to mammary gland expression, including the hormone response element (HRE), several negative regulatory elements (NREs), and NF-1, Oct-1, and TFIID binding sites (5, 8, 16, 35, 52).

The best known MMTV regulatory region is the well-characterized HRE (5, 18, 27, 42). The HRE is composed of multiple domains (3, 20) with the consensus sequence GGTAC AN_3TGTTCT and is recognized by a hormone-bound receptor (33). Binding of the hormone receptor complex to the MMTV HRE leads to a 10- to 50-fold increase in the levels of viral transcription (38). While deletion of any one of the binding domains results in a decrease in the levels of LTR-directed transcription, deletion of the distal HRE, located between −181 and −172, appears to have the greatest effect (3).

MMTV also is transcribed in a number of nonmammary tissues, including salivary gland, testis, thymus, spleen, kidney, lung and seminal vesicles but not brain, heart, skeletal muscle, or liver (14), despite the fact that glucocorticoid receptors are found in most tissues (13). Therefore, other factors in addition to the HRE must control the tissue specificity of MMTV expression.

The presence of additional regulatory elements for MMTV transcription first was suggested by the isolation of MMTV LTR variants from T-cell tumors (1, 16, 21, 24, 29, 30) as well as kidney, testicular, and pituitary tumors of mice (44, 56). In each case, the acquired MMTV proviruses in these tumors had sustained large (approximately 350- to 500-bp) deletions within the LTR (1, 16, 21, 24, 29). The MMTV LTR deletions in T-cell tumors all occupy a part of the U3 region extending from a maximum of −165 to −655 upstream of the MMTV transcription start site, and some deletions were accompanied by duplications of the LTR sequence flanking the deletion (1, 24, 29, 30). Virtually all of the MMTV proviruses acquired in the T-cell tumors had LTR deletions, and the deleted proviruses appeared to have a transcriptional advantage over the full-length endogenous MMTVs in these tumors (16, 29). Subsequent results from our laboratory (16) and others (32, 35) from transient transfection assays have localized at least one NRE upstream of the MMTV HRE. Furthermore, our experiments with transgenic mice have shown that deletion of MMTV LTR

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sequences between −201 and −344 as well as sequences upstream of −364 allows reporter gene expression in the brain, heart, and skeletal muscle, tissues that suppress expression from the full-length LTR (45).

To delineate the sequences responsible for tissue-specific negative regulation of the MMTV LTR and to determine if these sequences function through the binding of nuclear protein factors, we have constructed a series of deletion and substitution mutations within the LTR region known to be affected in MMTV T-cell variants. Results of these experiments indicated that there are two NRE regions (here called promoter distal and proximal) that bind to nuclear proteins from several cell types in which there is low-level MMTV expression. The NRE-binding activity was absent from mammary cells, suggesting that this tissue lacks negative regulation of the MMTV LTR.

MATERIALS AND METHODS

Cell culture. Nuclear extracts were prepared from D-6 mouse mammary epithelial cells from C57BL/6 mice (53), NMuMG mammary cells (37), and the T-cell lines ERLD and P4C, a subclone of EL4 (12). D-6 cells, a subclone of C57MG cells, were cultured in Dulbecco's modified Eagle medium containing 10% NuSerum IV (Collaborative Research, Bedford, Mass.), 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.4), penicillin-streptomycin, and 10 μg of insulin (Sigma) per ml. NMuMG cells were cultured in the same medium as D-6 cells except that 10% fetal bovine serum (GIBCO BRL, Bethesda, Md.) was used. D-6 cells were provided by A. Vaidya, and NMuMG cells were obtained from Susan Ross. ERLD cells were positive for the T-cell markers Thy-1, Lyt-1, and T-3 and negative for the markers MEL-14 and B220; P4C cells had the same phenotype except that they were positive for MEL-14 (28). CCL-64, P4C, and ERLD cells were cultured as described previously (12) except that 10% NuSerum IV was used.

Construction of plasmids and deletion mutants. The parental plasmid for the deletion mutations was pLC1 (kindly provided by D. Peterson), which contains the C3H exogenous MMTV LTR upstream of the chloramphenicol acetyltransferase (CAT) gene in the vector, pMT2 (35). pMT2 lacks pBR322 sequences that inhibit eukaryotic gene expression (41). pLC1 has been shown to correctly initiate CAT transcription from the MMTV promoter (35). The 5′-to-3′ deletion mutants (pS series) were constructed by digesting pLC1 DNA at the unique *Stu*I site (−637) and then for various lengths of time with BAL 31 nuclease (New England Biolabs, Beverly, Mass.). After the ends of the digestion products were filled by using the Klenow fragment of DNA polymerase I (New England Biolabs), the DNA was cleaved at the 3′ end of the LTR with *Eco*RI and the fragments were purified by agarose gel electrophoresis. The fragments then were cloned directionally into the pLC1 plasmid that had been cut with *Stu*I and partially digested with *Eco*RI. The sizes of the deletions obtained were determined with a kit for dideoxynucleotide sequencing (U.S. Biochemicals, Cleveland, Ohio). Plasmid DNAs were purified extensively, first by the alkaline lysis method (48) and subsequently by CsCl gradient purification, RNase digestion, proteinase K digestion, phenol-chloroform extraction, and dialysis. DNA concentrations were determined by A_{260} and confirmed by agarose gel electrophoresis and ethidium bromide staining.

Construction of substitution mutants. Substitution mutants were constructed in the pLC-LUC vector. This vector was prepared by digesting pLC1 with *Hin*dIII and inserting the 1.4-kb LTR fragment into the *Hin*dIII site of p19LUC (54) in the correct orientation. Substitution mutations in pLC-LUC were prepared essentially as described by Huguchi (17). In most cases a *Bgl*II site (absent from the wild-type pLC-LUC vector) was introduced to facilitate identification of mutants. In other instances, a purine was substituted for a pyrimidine residue or vice versa. Briefly, pLC-LUC was used as template in two PCRs in a Coy thermocycler (Coy Laboratory Products, Inc., Grasslake, Mich.). One reaction mixture contained a 5′ primer containing the C3H LTR *Stu*I site (5′ TGACCA CAGGCCTAGAAGTA 3′) and a 3′ primer containing a 6- to 8-bp mutation bearing the *Bgl*II site flanked on either side by 15 bp of the C3H LTR sequence. The 3-bp mutation in plasmid p909 did not contain the *Bgl*II site. The second reaction mixture contained a 5′ primer exactly complementary to the 3′ primer of the first reaction and a 3′ primer containing the C3H LTR *Afl*II site (5′ CTTACTTAAGCCCTGGGAAC 3′). Reactions were performed for 1.5 min at 94°C, 1.5 min at 50°C, and 3.0 min at 72°C for 30 cycles. Primers were removed on spun G-50 columns or by purification on acrylamide gels, the products of the two reactions were mixed at a 1:1 molar ratio, and PCR was repeated with the *Stu*I- and *Afl*II-containing primers. The product was subjected to chromatography on spun G-50 columns and then digested with *Afl*II and *Stu*I. Digested products were ligated into a pLUC-LUC vector that had been digested with the same enzymes and purified away from the resulting LTR fragment on SeaPlaque agarose (FMC BioProducts, Rockland, Maine). Transformed plasmids were screened for the presence of a *Bgl*II site, and the integrity of the region between the *Stu*I and *Afl*II sites was confirmed by sequencing. The pTBLV plasmid was constructed from a PCR product synthesized from the MMTV LTR primers 5′ TGGATCCTTGGCATAGCTCTGCTTTGC 3′ and 5′ CTCTAGAGGTGAAT GTTAGGAC 3′ and 1 μg of genomic DNA from type B leukemogenic retrovirus-infected T cells (1). This product was digested with *Cla*I and *Sst*I and substituted for the same region in pLC-LUC (−862 to −106). The substitution was verified by restriction enzyme digestions and sequencing.

Transfections. Transfections were performed by electroporation according to the method of Knutson and Yee (19). Each deletion mutant was electroporated up to five times, and each electroporation and CAT assay included both the full-length MMTV LTR construct (pLC1) and the promoterless plasmid vector (pMT2). Mink lung cells (CCL-64) were used because they are permissive for MMTV infection (15), because the high efficiency of DNA uptake allowed us to monitor MMTV LTR expression in the absence of hormones (16), and because basal expression from the MMTV LTR appears to correlate well with results obtained with transgenic mice (45). Under these conditions, DNA uptake of the different constructs was equivalent as measured by dot blot hybridization of nuclear DNA or by cotransfection of a second reporter plasmid, pBAG, carrying the *lacZ* gene (43) (data not shown).

CAT, luciferase, and β-galactosidase assays. CAT assays were performed by the procedure of Lopata et al. (26) as previously described (16). Assays were quantitated by excising acetylated and nonacetylated forms of chloramphenicol from the chromatography plate and counting in a Beckman liquid scintillation counter. Extracts used for luciferase assays were prepared as described above and assayed by the method of Brasier (2) using a Turner TD-20e luminometer. Typical luciferase values for pLC-LUC transfections using 40 μg of DNA and cells grown in the absence of hormone (basal pLC-LUC levels) were 1.6 U/100 μg of protein extract (average of 23 determinations). Cells transfected with the promoterless luciferase vector p19LUC had no detectable luciferase activity. The β-galactosidase assays were performed by standard procedures (48).

Gel retardation experiments. Nuclear lysates were prepared by the method of Dignam et al. (10). The clarified lysate was stored in aliquots at −70°C. In later experiments, 0.2 μg of pepstatin A (Sigma) per ml was added to all buffers.

Purified restriction enzyme fragments or PCR products were end labeled with Sequenase or T4 polynucleotide kinase for use in gel retardation assays. The primers for the 120-bp fragment from the proximal NRE were 5′ CGACATGA AACAACAGGT 3′ and 5′ TGGAGAATAATGTTCTA 3′, whereas the primers for the 59-bp fragment in the distal NRE were 5′ CCTTACCATATACAGGA 3′ and 5′ GCCATTGACTGTAACCCACCTATCCCAATTTAAGTC 3′. Primers with appropriate mutations in the proximal NRE were used to prepare the PCR products for Fig. 4 and 5. Reaction mixtures contained 1×10^4 to 5×10^4 cpm of an end-labeled fragment, 0.1 M KCl, 20 mM HEPES (pH 7.9), 0.5 mM dithiothreitol, 4% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 μg of pepstatin A per ml, and 2 μg of poly(dI-dC) in a volume of 10 μl. This mixture was incubated for 10 min at room temperature prior to electrophoresis on 4% nondenaturing polyacrylamide gels (acrylamide/bisacrylamide ratio, 19:1) containing 10% glycerol in a running buffer containing 12.5 mM Tris-HCl (pH 8.05), 5 mM sodium acetate, and 0.5 mM EDTA. Gels were run at 10 to 15 V/cm for 3 to 4 h at 4°C before they were dried and subjected to autoradiography.

Competition for NRE binding. The 100-bp fragment extended from the *Pst*I site at −262 to the *Rsa*I site at −364. The two subfragments of 90 and 180 bp were derived from the 270-bp fragment (−637 to −365) by subcloning at the *Dpn*I site (−454) into pUC9 (55). All fragments were purified on agarose gels. For the competition experiments involving 180- and 100-bp fragments, either gel-purified restriction fragments or PCR products were used. Competition reactions were performed as described above except that the protein lysate was incubated on ice for 10 min with poly(dI-dC). The unlabeled competitor DNA then was added and the samples were incubated on ice for 10 min; the incubations were followed by addition of the probe. In off-rate experiments, a wild-type probe or probe containing the 924 mutation was added to mink lung extract for 10 min and an aliquot was removed for the zero time point essentially as described elsewhere (4). Subsequently, a 100-fold molar excess of the homologous unlabeled probe was added, and aliquots were removed and quenched at intervals by loading onto a running 4% acrylamide gel.

RESULTS

Analysis of 5′ and 3′ MMTV LTR deletion mutants. Previous experiments indicated that the MMTV LTR contained one or more negative regulatory elements upstream of the HRE (16, 32, 35). To localize such regulatory elements in the MMTV U3 region, we constructed a series of 5′-to-3′ BAL 31 deletion mutants in the C3H LTR starting from the internal *Stu*I site at −637. These deletions were generated in the pLC1 vector that contains the MMTV LTR inserted upstream of the CAT reporter gene (35). Constructs containing these deletions were electroporated into mink lung cells in transient assays, and the level of CAT activity was quantitated relative to the full-length MMTV LTR construct (pLC1) in the presence and

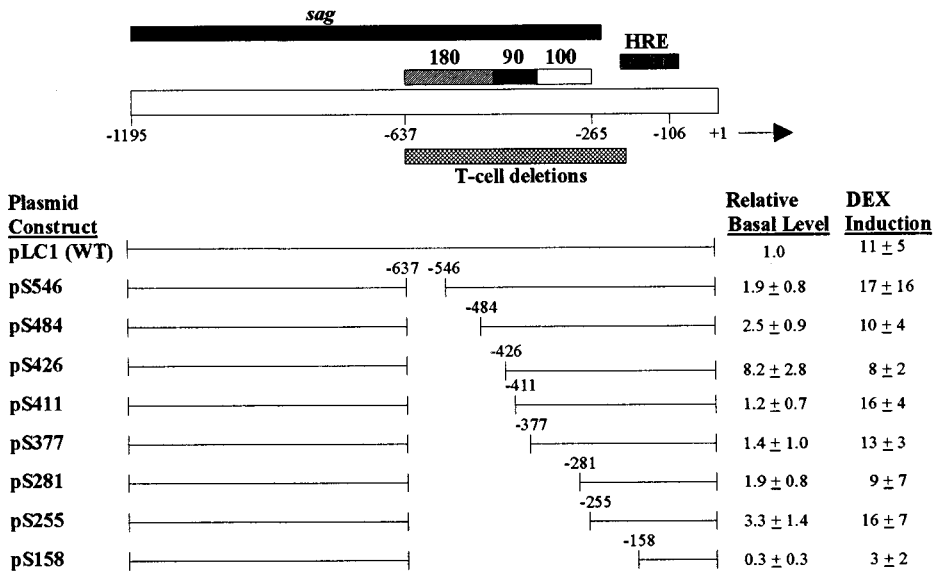

FIG. 1. CAT activity of mutants with 5′-to-3′ BAL 31 deletion mutations in the MMTV LTR. BAL 31 deletion mutants (designated pS since the deletions started from the internal *Stu*I site at −637) were constructed from the pLC1 plasmid (35). Deletions are shown by gaps, and the remaining sequence is represented by solid lines relative to the entire MMTV U3 region. The relative positions of the HRE and the superantigen (*sag*) open reading frame are shown above the LTR. The location of the largest LTR deletions in MMTV-induced T-cell tumors is shown below the U3 region (−165 to −655), whereas the minimum deletions observed occur between −195 and −575. Cells electroporated with each plasmid were plated, and half of each sample was treated with hormone (DEX) for 24 h at 37°C. After an additional 24-h incubation, cells were trypsinized, washed twice with phosphate-buffered saline, and resuspended in 100 μl of 0.25 M Tris-HCl, pH 7.8. Cells were disrupted by three cycles of freezing and thawing, and the cellular debris was removed by centrifugation for 2 min at 10,000 × *g* at 4°C. In a few cases, the CAT constructs (30 μg) and pSV2gpt (3 μg) were coelectroporated in parallel with cultures coelectroporated with wild-type pLC1 and pSV2gpt as previously described (16). No significant difference was obtained for the expression of mutants relative to the wild-type (WT) construct in transient versus stable transfections. Relative basal expression represents the value of each mutant in the absence of DEX relative to the wild-type construct. Values for DEX induction represent the increase in CAT expression of DEX-treated cultures compared with untreated cultures derived from the same electroporation. Standard deviations are given for each average value. The positions of the 180-, 90-, and 100-bp fragments used for gel retardation assays are shown relative to the start of transcription (+1).

absence of dexamethasone (DEX) (Fig. 1). Removal of LTR sequences between −637 and −546 (pS546) elevated basal CAT activity two- to threefold over that of pLC1, and further deletion to −426 (pS46) gave basal activity that was approximately eightfold higher than that of pLC1. Surprisingly, deletion of an additional 25 bp to −411 lowered basal CAT activity, suggesting that the sequences between −426 and −411 are important for maintaining basal promoter levels. Removal of sequences between −377 and −281 again raised basal CAT activity, as did deletions to −255. Deletion of sequences up to −158 again lowered basal activity and decreased DEX inducibility (mutant pS158), probably because of the removal of the distal HRE (3). Similar decreased responsiveness to DEX was noted for a naturally occurring MMTV deletion mutant (−655 to −165) isolated from a T-cell tumor (p1BMT) (16). However, the p1BMT mutant had high basal transcription (sevenfold over that of pLC1), whereas both pS158 and pS106 had low basal CAT activity similar to that of pLC1 (16). Examination of the results indicated that deletion of two regions between −484 and −426 and between −281 and −255 had the greatest effect on basal transcription from the MMTV LTR. We have designated these regions (promoter-) proximal and distal NREs.

Identification of nuclear factor binding sites in the NRE. Analysis of the deletion mutants in transient assays indicated that there were at least two NREs in the region between −637 and the HRE. Use of the labeled 100-bp fragment in gel shift assays resulted in the appearance of two high-molecular-weight complexes (Fig. 2). The more slowly migrating complex has been referred to as upper binding protein (UBP), whereas the faster migrating complex has been referred to as NRE-binding protein (NBP) (see below). Formation of these com-

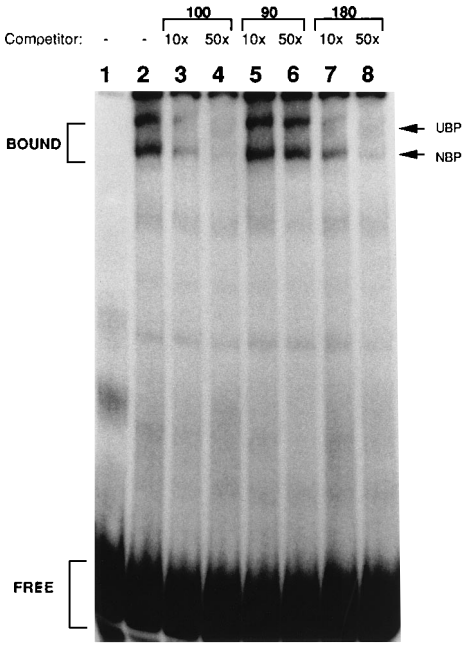

FIG. 2. Gel shift assays show similar nuclear protein binding sites in the 180- and 100-bp LTR fragments. Complex formation with the labeled 100-bp fragment was inhibited by the homologous fragment (lanes 3 and 4), the 90-bp fragment (lanes 5 and 6), and the 180-bp fragment (lanes 7 and 8). Lanes 1 and 2 had no nuclear extract and no competitor DNA, respectively. Lanes 3, 5, and 7 had 10-fold molar excess competitor, and lanes 4, 6, and 8 had 50-fold excess competitor.

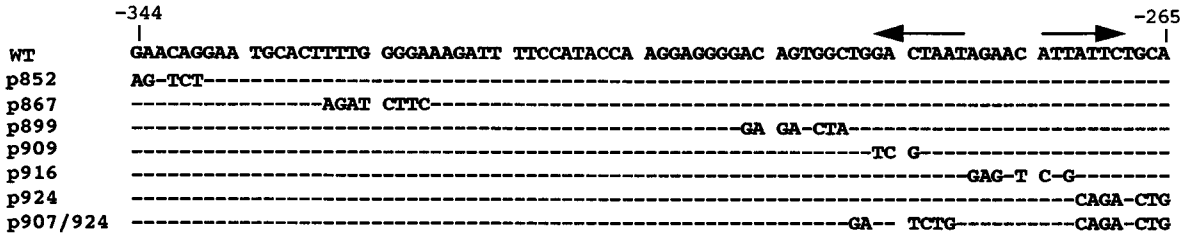

FIG. 3. Locations of substitution mutations in the proximal NRE. The top line shows the sequence of the wild-type (WT) C3H LTR in the region of the proximal NRE (−344 to −265). Each mutant sequence is indicated below, with dashes indicating identity with the wild-type sequence. The arrows indicate the position of the inverted repeat. Mutants were named for the first base of the substitution relative to the first base of the C3H LTR.

plexes was inhibited by the presence of unlabeled homologous 100-bp fragment and by the heterologous 180-bp LTR fragment but not by the intervening 90-bp fragment (Fig. 1). Moreover, a reciprocal result could be obtained when the 180-bp fragment was labeled, i.e., the 100- and 180-bp fragments, but not the 90-bp fragment, could compete for binding (not shown). Thus, these experiments indicated that the NBP and UBP binding sites were redundant within the NRE and that the high-molecular-weight complexes bound to these sites were specific as judged by their ability to be inhibited by the presence of excess homologous fragment. The relative location of these complexes was consistent with the position of deletion mutations that relieved negative regulation of the MMTV LTR (Fig. 1).

Mapping of the nuclear protein binding sites in the NRE. To further map the nuclear protein binding sites in the NRE, we focused on the sites contained within the smallest fragment (100 bp). Using PCR-generated fragments, we were able to localize binding sites for both complexes to the 85 bp at the 3′ end of the 100-bp fragment. Substitution mutations spanning the 85-bp region were introduced into a second vector containing the wild-type C3H MMTV LTR upstream of the luciferase reporter gene (pLC-LUC) (Fig. 3). Each of the mutants was transfected into mink lung cells in transient assays, and transfected cells grown in the presence and absence of DEX were assayed for luciferase activity relative to pLC-LUC (Table 1). A mutation near the 3′ end of the 85-bp fragment (p924) (starting at −272) showed a 2.6-fold elevation of basal LTR transcription over that of the wild-type plasmid, whereas the other mutations in plasmids p852, p867, p899, and p916 had wild-type or near-wild-type expression levels (Table 1). Each of these mutants showed glucocorticoid induction that was similar to that observed for the pLC-LUC wild-type plasmid (Table 1).

TABLE 1. Basal and hormone-induced luciferase expression of MMTV LTR substitution mutants in transient assays

Plasmid	Relative basal expression[a]	DEX induction[b]
pLC-LUC	1.0	16 ± 9
p852	1.4 ± 0.2	24 ± 5
p867	1.5 ± 0.6	20 ± 12
p899	0.7 ± 0.3	18 ± 15
p909	2.0 ± 0.4	12 ± 5
p916	1.4 ± 0.7	20 ± 5
p924	2.6 ± 0.7	24 ± 8
p907/924	2.1 ± 0.4	44 ± 16

[a] Luciferase activity relative to that of the wild-type LTR reporter construct in the same assay. The data are averages of 9 to 15 independent electroporations with standard deviations. Transfections were performed with at least two plasmid preparations.

[b] Fold increase in reporter gene expression following treatment of transfected cells with 10^{-6} M DEX for 24 h relative to untreated cells.

Examination of the MMTV sequences around the p924 mutation revealed an imperfect inverted repeat (Fig. 3) separated by 5 bp that spanned the sequence encompassing the p916 and p924 mutations. Because each of the mutants had a 5- to 8-bp sequence alteration, we introduced a 3-bp substitution mutation specifically into the 3′ end of the inverted repeat in the proximal NRE region of pLC-LUC. Transfection of this mutant (p909) into mink lung cells revealed a twofold increase in basal transcription relative to the wild-type plasmid (Table 1). Although the observed effect is small, this increase is three times our average standard deviation (30%). Because of the small increase in basal expression observed for proximal NRE mutants, we repeated these experiments with a number of the mutants under slightly different conditions for transient assays. A smaller amount (20 μg) of the luciferase plasmid was used in the presence of another plasmid, pRSV*lacZ* (57) (5 μg), to control more stringently for variations in DNA uptake. Results from these experiments showed that only plasmids p924 and p909 with mutations within the inverted repeats of the proximal NRE had elevated basal expression compared with the wild-type C3H plasmid (Table 2). Moreover, a naturally occurring LTR deletion variant (pTBLV) (1) also showed a 2.5-fold increase in basal luciferase activity. Thus, a mutation resulting in a 3-bp alteration within the inverted repeat of the proximal NRE, and lacking potential spacing effects caused by deletion mutations, had elevated basal MMTV transcription in the context of the entire LTR.

A mutant (p907/924) that had *Bgl*II substitution mutations in both inverted repeats of the proximal NRE also was constructed. Basal expression of this mutant in transient assays was similar to that observed for the p909 and p924 mutants (Table 1), suggesting that alteration of a single inverted repeat is sufficient for disruption of the proximal NRE.

Mapping of NBP binding activity. Substitution mutants in the proximal NRE were used to generate PCR products in the proximal NRE that were end labeled and incubated with nuclear extracts in gel shift assays. Gel assays showed that fragments having the same 5′ and 3′ ends but containing the 867, 899, 916, or 924 mutation all formed the UBP and NBP complexes. The formation of the NBP complex was detectable but appeared to be decreased in the presence of DNA probes containing the 909 mutation. However, PCR products containing mutations in both inverted repeats in the proximal NRE (907/924) formed only the UBP complex (Fig. 4 and data not shown).

Transient transfection experiments indicated that the 924 mutation had a greater effect on basal MMTV expression than did the 909 mutation, yet gel retardation assays showed little difference in binding of NBP to proximal NRE probes contain-

TABLE 2. Basal and hormone-induced reporter gene activities of MMTV LTR substitution mutants in the presence of pRSV*lacZ*

Expt and plasmid[a]	DEX added	β-Gal/100 μg[b]	LUC[c]/100 μg	Normalized LUC/100 μg[d]	Relative basal expression	DEX induction[e]
1						
pLC-LUC		0.075	0.11	0.11	1.0	
pLC-LUC	+		6.52			59
p924		0.090	0.38	0.32	2.9	
p924	+		24.90			65
pTBLV		0.035	0.13	0.28	2.5	
pTBLV	+		17.30			133
2						
pLC-LUC		0.069	0.17	0.17	1.0	
pLC-LUC	+		2.99			18
p867		0.069	0.14	0.14	0.8	
p867	+		2.37			17
p899		0.074	0.16	0.15	0.9	
p899	+		2.19			14
p909		0.070	0.27	0.27	1.6	
p909	+		4.94			18

[a] Each of the plasmids (20 μg) was transfected together with 5 μg of pRSV*lacZ* as a control for DNA uptake.
[b] The β-galactosidase (β-Gal) activity (A_{420}) of 100 μg of protein lysate was measured in cells grown in the absence of DEX. An A_{420} reading of 0.1 equals approximately 5×10^{-4} U of enzyme.
[c] LUC, luciferase activity.
[d] Luciferase activity (in relative light units) for each plasmid construct per 100 μg of cell lysate was normalized to pLC-LUC DNA uptake by dividing the β-galactosidase activity of pLC-LUC by the β-galactosidase activity of the mutant and multiplying this factor by the mutant luciferase activity. Approximately five times more protein was assayed from cells grown in the absence of DEX than from hormone-treated cells to obtain values in the linear range of the assay.
[e] For unknown reasons, we occasionally observe high levels of DEX induction. β-Galactosidase activity was not used to normalize for hormone induction since DEX-treated and untreated cultures were from the same transfection.

ing the 924 mutation (Fig. 4A, lanes 13 and 14) relative to assays using wild-type probes (Fig. 4A, lanes 2 to 4). Therefore, we compared the ability of NBP to be displaced from proximal NRE probes containing the 924 mutation in the presence of 100-fold excess homologous unlabeled DNA (off-rate) (Fig. 5). We observed that approximately 20 min was required to completely eliminate NBP from the wild-type probe (Fig. 5A), whereas NBP was completely eliminated from the 924 mutant probe within 30 s (Fig. 5B). Similar experiments using 852, 867, and 909 mutant probes had no effect on the off-rate for NBP binding, but the 909 probe showed poor initial binding to NBP (Fig. 4A and data not shown). Therefore, mutations that had

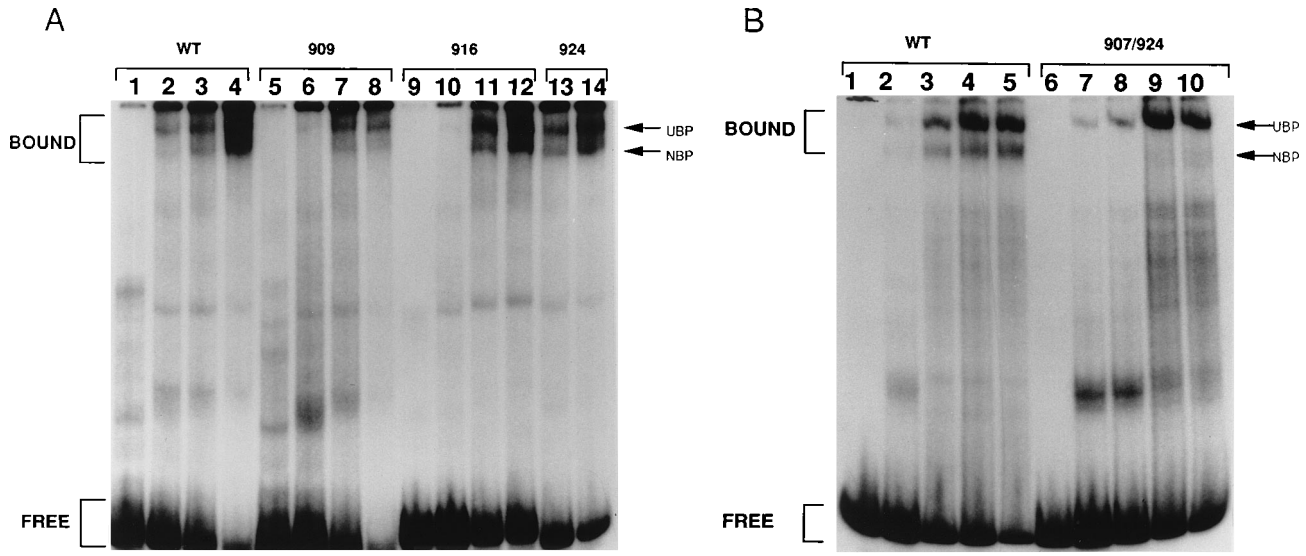

FIG. 4. Gel shift assays indicate that mutation of both halves of the proximal NRE prevents NBP complex formation. (A) A 120-bp end-labeled PCR product containing the wild-type or mutant sequence of the proximal NRE was incubated with no extract (lanes 1, 5, and 9) or 0.4 μg (lanes 2, 6, and 10), 0.8 μg (lanes 3, 7, 11, and 13), or 1.6 μg (lanes 4, 8, 12, and 14) of mink lung extract prior to nondenaturing gel electrophoresis. The following probes were used: wild-type (WT) probe (lanes 1 to 4), the p909 probe with a 3-bp mutation in the 5′ half of the proximal NRE (lanes 5 to 8), the p916 probe with a 6-bp mutation spanning the spacer and 2 bp in the 3′ half of the proximal NRE (lanes 9 to 12), and the p924 probe with a 7-bp mutation spanning the 3′ half of the proximal NRE and the flanking sequence (lanes 13 and 14). (B) An experiment similar to that in panel A, except that the wild-type probe is shown in lanes 1 to 5 and a mutant p907/924 probe is shown in lanes 6 to 10. The mutant probe contains mutations in both halves of the proximal NRE (Fig. 3). Reactions had no extract (lanes 1 and 6) or 0.4 μg (lanes 2 and 7), 0.8 μg (lanes 3 and 8), 1.6 μg (lanes 4 and 9), or 2.2 μg (lanes 5 and 10) of mink lung nuclear extract. The positions of the UBP and NBP complexes are indicated.

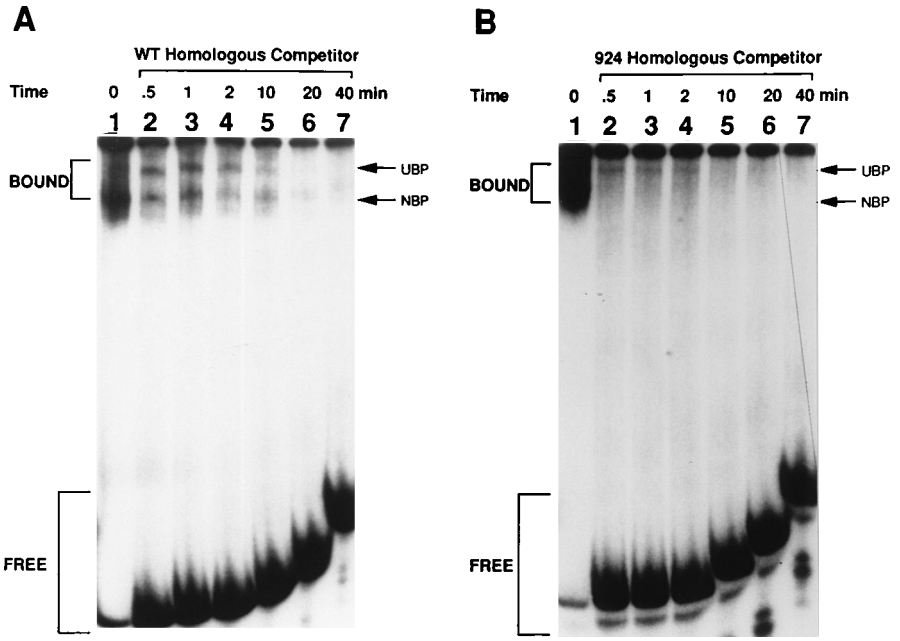

FIG. 5. Off-rate experiments using wild-type (WT) and 924 proximal NRE probes. The rate of dissociation between NBP and 120-bp proximal NRE probes lacking (A) or containing (B) the 924 mutation shown in Fig. 3 was measured after addition of 100-fold molar excess unlabeled DNA and electrophoresis on 4% polyacrylamide gels. Reaction mixtures contained 0.4 μg of mink lung nuclear extract per 10,000 cpm of probe. Lane 1 in each panel was overexposed to increase the sensitivity for NBP detection.

an effect on NBP binding (909 and 924) were located within the inverted repeats of the proximal NRE and elevated MMTV basal expression, whereas mutants that had no effect on NBP binding had little or no effect on basal expression. Because transfection assays indicated that mutations affecting the inverted repeat near −270 elevated basal transcription from the MMTV LTR, these results strongly suggest that the NBP complex participates in negative regulation of MMTV transcription.

Previous results also indicated that the 180-bp NRE fragment contained both UBP and NBP binding sites (Fig. 2), and deletion mutations between −484 and −426 (within the 3′ end of the 180-bp fragment in Fig. 1) gave a large increase in basal MMTV expression. To determine if the binding site within the 180 fragment was localized to the predicted region, we synthesized a 59-bp PCR product (−530 to −472) that contained several repeats similar to that in the proximal NRE, and this product was used in gel shift assays with lung cell extracts. As predicted, the 59-mer gave two bands that were specific since they could be inhibited in the presence of excess homologous fragment and a 120-bp fragment containing the proximal NRE (Fig. 6). Moreover, a 41-bp fragment (−512 to −472) gave a single shifted complex in gel retardation assays; the shifted band was shown to be NBP since it was not formed with mammary cell nuclear extracts (data not shown; see below).

Tissue specificity of NRE protein complexes. MMTV is known to cause mammary tumors as well as T-cell tumors, but acquired proviruses within T-cell tumors have deletions encompassing the NRE (1, 16, 21, 24, 29, 30) whereas MMTV proviruses from mammary tumors do not (40). Our previous experiments have shown that full-length MMTV LTR reporter gene constructs are expressed at much higher levels in the mammary gland than in other tissues (e.g., thymus and lung) of female transgenic mice (45). If NBP mediates negative regulation of the MMTV LTR, NBP activity may be differentially regulated in T cells and mammary gland cells. Therefore, nuclear extracts were prepared from mammary and T-cell lines and used for gel shift assays with the 100-bp NRE fragment (Fig. 7). The T-cell extract formed both the NBP complex and the UBP complex observed when lung cell extracts were used in gel assays (Fig. 7A, compare lanes 2 and 6). However, nuclear extracts from a mammary cell line lacked the NBP complex, suggesting that NBP activity is absent or inactive in mammary cells (Fig. 7B). The mammary extracts were not

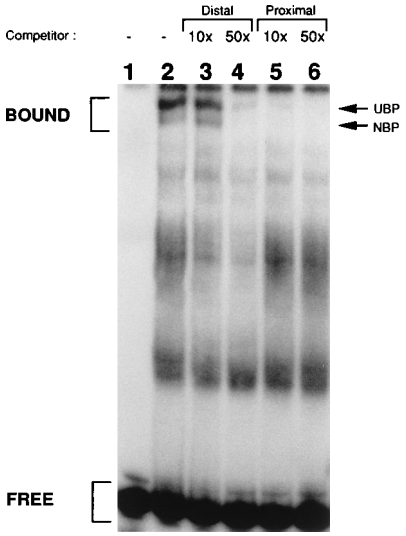

FIG. 6. Gel shift assays localize the distal NRE site within a 59-bp fragment. A 59-bp end-labeled PCR fragment was incubated with no nuclear extract (lane 1) or 1.6 μg of mink lung nuclear extract (lanes 2 to 6). Unlabeled homologous competitor DNA was added prior to the labeled probe in lanes 3 to 6 at a 10-fold (lanes 3 and 5) or 50-fold (lanes 4 and 6) molar excess. The competitor DNA was the 59-bp PCR product from the distal NRE (lanes 3 and 4) or the 120-bp PCR product from the proximal NRE (lanes 5 and 6).

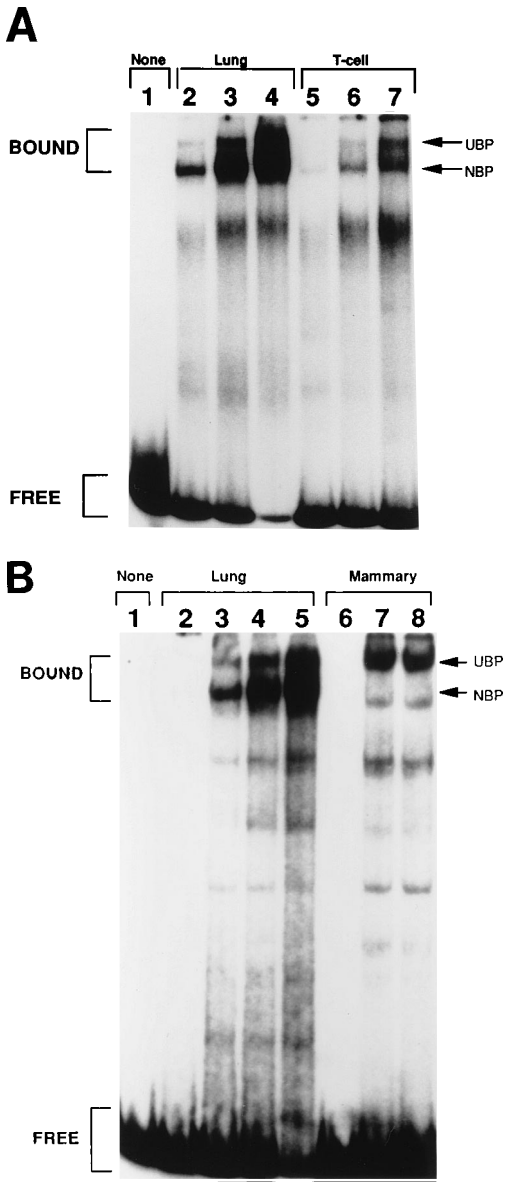

FIG. 7. Tissue-specific expression of the NBP complex in gel shift assays. (A) A 120-bp PCR product from the proximal NRE was end labeled and incubated with no extract (lane 1), mink lung nuclear extract (lanes 2 to 4), or ERLD mouse T-cell nuclear extract (lanes 5 to 7). The amount of extract used was 0.35 μg (lanes 2 and 5), 0.7 μg (lanes 3 and 6), or 1.4 μg (lanes 4 and 7). The positions of UBP and NBP complexes are indicated. (B) An end-labeled 100-bp probe from the proximal NRE was incubated with no extract (lane 1), mink lung cell nuclear extract (lanes 2 to 5), or D-6 normal mouse mammary cell nuclear extract (lanes 6 to 8). The amount of extract used was 0.2 μg (lanes 2 and 6), 0.4 μg (lane 3), 0.8 μg (lanes 4 and 7), or 1.6 μg (lanes 5 and 8). A nonspecific band (slightly faster mobility than NBP) observed with mouse mammary extracts was not detected in the presence of higher concentrations of poly(dI-dC).

degraded since the UBP complex easily was detected in these assays, and use of nuclear extracts from additional mammary and T-cell lines confirmed these results (data not shown). Thus, NBP activity appears to be regulated in a tissue-specific manner.

DISCUSSION

Negative regulation of the MMTV LTR by a redundant protein binding site. The following data support the role of NBP in negative regulation of MMTV transcription. (i) MMTV LTR deletion variants found in T-cell tumors invariably lacked the proximal and distal NBP binding sites (1, 16, 24, 29, 30) and showed increased basal transcription. (ii) Transient transfections of sequential LTR deletion mutants revealed that removal of two regions (promoter distal and proximal) located between −484 and −426 and between −281 and −255 produced the greatest elevation of basal MMTV expression (Fig. 1). (iii) Experiments with transgenic mice expressing MMTV LTR deletion mutants identified two regions (one upstream of −364 and one between −344 and −201) that elevated basal expression in tissues nonpermissive for MMTV expression (45). (iv) LTR substitution mutations within the inverted repeats of the proximal NRE elevated basal expression (Tables 1 and 2) and had a destabilizing effect on or abolished NBP binding (Fig. 4 and 5). Mutants outside the inverted repeats had no effect on expression or binding. (v) NBP activity was detectable in tissues that are nonpermissive or semipermissive for MMTV expression (Fig. 7) and was undetectable in the mammary gland, a tissue that shows the highest level of MMTV expression (45). (vi) Preliminary experiments with mice expressing an LTR transgene with the 924 mutation suggest that transcription is elevated in lymphoid tissues (12a). Thus, negative regulation of MMTV expression appears to be mediated in part by binding of NBP to redundant sites within the LTR.

Visual inspection of the proximal NRE revealed the presence of an imperfect inverted repeat 5′ ATTA(G/T)TC 3′ separated by 5 bp (Fig. 3). Transient assays showed small (two- to threefold) effects of mutations in this repeat, but these effects were reproducible, and all assays were performed in the context of the entire MMTV LTR that contains a second (distal) NRE (Fig. 6). PCR products from the proximal NRE with mutations in both halves of the inverted repeat (907/924) bound the UBP complex but did not bind the NBP complex (Fig. 4). Moreover, two mutations in a single repeat (909 and 924) were sufficient to destabilize NBP binding, and the same mutations elevated basal MMTV transcription. Together these data suggest that a single repeat can mediate DNA binding, whereas both repeats participate in negative regulation. Thus, NBP dimerization may be required for transcriptional repression.

Interaction of the NBP complex with other transcription factors. The high-molecular-weight nature of the NBP complex suggests that it is composed of a number of protein factors (Fig. 2 and 4 to 7), and we have preliminary evidence that the NBP complex contains one or more factors of 105 kDa (25a). There are no exact matches of the inverted repeats in the proximal NRE with other known transcription factor binding sites. Previous studies of glucocorticoid receptor binding sites have mapped one binding site to a region that overlaps with the promoter-proximal NRE (39).

Recent studies also indicate that the HRE can act as a negative regulator of transcription if the HRE is juxtaposed to other transcription factor binding sites such as AP-1 (9, 50; for a review see reference 7). One hypothesis suggests that the binding of two factors to neighboring sites holds the glucocorticoid receptor and the adjacent factor in conformations incompatible with transcriptional activation (9). However, participation of the glucocorticoid receptor in the NBP complex seems unlikely for several reasons. First, negative regulation of MMTV transcription is demonstrable in cells that have not been treated with glucocorticoids, whereas genes such as proliferin, prolactin, and osteocalcin are repressed in the presence of hormone (9, 47, 50). Second, deletion of the promoter-distal NRE around −500 appeared to increase basal MMTV tran-

scription, yet there is no known glucocorticoid receptor binding site in this region (39, 42, 49). Third, substitution mutations in the proximal NRE had little or no effect on glucocorticoid-induced reporter gene expression, with the possible exception of p907/924 (Table 1). Fourth, polyclonal glucocorticoid receptor antibody did not alter the NBP complex in gel shift assays (data not shown). Nevertheless, the 924 substitution mutation that had the greatest effect on MMTV basal transcription also maps to a putative glucocorticoid receptor binding site (39, 42, 49).

NREs and tissue-specific expression of the MMTV LTR. Transient transfection experiments by Morley et al. (35) indicated the presence of an NRE between −455 and −364 in the MMTV LTR, and Mink et al. (32) reported two NREs in the MMTV LTR between −631 and −560 and between −428 and −364. Other data have shown that MMTV LTR sequences between −452 and −117 downregulate expression from the murine sarcoma virus promoter (36). More recent results suggest that the element described by Morley et al. (35) and an element located between the proximal and distal binding sites of the glucocorticoid receptor (22) may cooperate to downregulate MMTV expression (23). Because the map position of the NRE elements that we have described is different from that initially reported by Morley et al. (35), it seems likely that there are at least two types of NRE in the MMTV LTR. One type of NRE appears to affect general transcription from the MMTV LTR, whereas the second type described here appears to influence the tissue-specific expression of viral genes.

Removal of one or more negative elements explains the high level of transcription from MMTV proviruses with LTR deletions in T-cell lymphomas relative to full-length proviruses (11, 16, 51). Indeed, we have shown that lung cells and T cells have NBP binding activity (Fig. 7A), and cells from these tissues expressed a reporter gene driven by the MMTV LTR at much lower levels than mammary gland cells in transgenic mice (45). However, MMTV proviruses from mammary tumors never contain deletions within the NRE region (40), a result suggesting that MMTV transcription is not suppressed in breast tissue. In support of this, NBP activity is undetectable in at least two mouse mammary cell lines that we have tested (Fig. 7B and data not shown). Thus, the highest levels of MMTV transcription may occur in mammary tissue because of active hormone receptors (13), the presence of mammary gland enhancer binding factors (25, 31, 34, 58), and the absence of tissue-specific negative factors such as NBP.

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