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The Matrix Attachment Region-Binding Protein SATB1 Participates in Negative Regulation of Tissue-Specific Gene Expression

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The nuclear matrix has been implicated in several cellular processes, including DNA replication, transcription, and RNA processing. In particular, transcriptional regulation is believed to be accomplished by binding of chromatin loops to the nuclear matrix and by the concentration of specific transcription factors near these matrix attachment regions (MARs). A number of MAR-binding proteins have been identified, but few have been directly linked to tissue-specific transcription. Recently, we have identified two cellular protein complexes (NBP and UBP) that bind to a region of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) previously shown to contain at least two negative regulatory elements (NREs) termed the promoter-proximal and promoter-distal NREs. These NREs are absent from MMTV strains that cause T-cell lymphomas instead of mammary carcinomas. We show here that NBP binds to a 22-bp sequence containing an imperfect inverted repeat in the promoter-proximal NRE. Previous data showed that a mutation (p924) within the inverted repeat elevated basal transcription from the MMTV promoter and destabilized the binding of NBP, but not UBP, to the proximal NRE. By using conventional and affinity methods to purify NBP from rat thymic nuclear extracts, we obtained a single major protein of 115 kDa that was identified by protease digestion and partial sequencing analysis as the nuclear matrix-binding protein special AT-rich sequence-binding protein 1 (SATB1). Antibody ablation, distamycin inhibition of binding, renaturation and competition experiments, and tissue distribution data all confirmed that the NBP complex contained SATB1. Similar types of experiments were used to show that the UBP complex contained the homeodomain protein Cux/CDP that binds the MAR of the intronic heavy-chain immunoglobulin enhancer. By using the p924 mutation within the MMTV LTR upstream of the chloramphenicol acetyltransferase gene, we generated two strains of transgenic mice that had a dramatic elevation of reporter gene expression in lymphoid tissues compared with reporter gene expression in mice expressing wild-type LTR constructs. Thus, the 924 mutation in the SATB1-binding site dramatically elevated MMTV transcription in lymphoid tissues. These results and the ability of the proximal NRE in the MMTV LTR to bind to the nuclear matrix clearly demonstrate the role of MAR-binding proteins in tissue-specific gene regulation and in MMTV-induced oncogenesis.

The nuclear matrix is a network of RNA and nonhistone proteins that serves as a scaffold for loops of chromatin (10, 11, 64). This matrix has been associated with the regulation of DNA replication, transcription, and RNA processing (for a review, see reference 64). The DNA regions anchoring chromatin to the matrix (called matrix-associated regions [MARs] or scaffold-associated regions) (18) are composed of AT-rich sequences that have a unique structure characterized by high unwinding potential and the formation of a narrow minor groove (1, 52). MARs have been shown to colocalize with transcriptional enhancers (18, 49), and they also may provide insulation between transcription units, similar to locus control regions (26, 33).

A variety of proteins are associated with the nuclear matrix. Some of these proteins (e.g., matrins [9], lamins [37], nuclear mitotic apparatus protein [56], attachment region-binding protein [90], and scaffold proteins [76]) are candidates for structural components of this fibrous network, similar to the cytoskeleton, whereas others, such as high-mobility group proteins 1 and 2 (85), topoisomerase II (12), and a variety of kinases (23), may play regulatory roles in matrix function. Another group of proteins, such as special AT-rich sequence binding protein 1 (SATB1) (26, 62), MAR-binding protein 1 (99), and nucleolin (27), have been identified by their ability to bind to MARs. These proteins have properties similar to those of transcription factors, and indeed, many transcription factors have been shown to associate with the nuclear matrix (31, 88).

A number of experiments support a role for MARs in the regulation of gene transcription. MARs have been shown to stimulate the expression of some reporter constructs introduced stably into the chromosome of tissue culture cells (34, 68, 92). Gene transcription that is both dependent on copy number and independent of chromosomal location can be conferred by the presence of MARs (34, 83). Furthermore, actively transcribed genes appear to be associated with the nuclear matrix whereas transcriptionally inactive genes are not (32, 38). In the case of the immunoglobulin heavy-chain locus, MARs flank the enhancer located between the joining and constant regions (19), and the intronic enhancer and flanking MARs are required for B-cell-specific expression of μ heavy chains (81). The presence of these MARs enhances the expression of heavy-chain mRNA by 35- to 1,000-fold in spleens or in B-cell lines derived from transgenic mice carrying rearranged μ heavy-chain genomic sequences (34). In addition, the MARs were responsible for an extended DNase I-hypersensitive re-

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gion in the vicinity of the heavy-chain variable region promoter (34).

MARs participate in transcriptional suppression, as well as transcriptional enhancement, and transcriptional suppression mediated by the nuclear matrix is thought to have a key role in tissue-specific gene expression (22, 34). For example, binding of the transcription factor NF-μNR to the MARs flanking the μ intronic enhancer has been correlated with the lack of μ gene expression in non-B cells (77). Other proteins involved in negative regulation of transcription, such as YY-1 (also known as NMP-1) (42) and the glucocorticoid receptor (24, 84), have been shown to bind to the nuclear matrix.

Mouse mammary tumor virus (MMTV) has been studied extensively as a model hormone-inducible promoter (71, 95) that is regulated through changes in chromatin structure (70, 98). The activity of the TATA box element in the MMTV long terminal repeat (LTR) is high in in vitro systems (53); however, the basal level of this promoter in the context of the integrated provirus is very low, presumably due to general repression by chromatin proteins (3). Ample evidence suggests that the MMTV LTR is occupied by an array of six nucleosomes (16, 70) that have compositions typical of eukaryotic chromatin (two each of histones H2A, H2B, H3, and H4) (72). Chromatin structure has been shown to change in the presence of glucocorticoids (69, 70, 86, 98), and it is believed that this change allows promoter activation through the binding of transcriptional activators such as NF-1 and Oct-1 and the basal transcription apparatus (20, 48). In agreement with this idea is the recent observation that the RNA polymerase II holoenzyme contains SWI/SNF, a protein complex that can disrupt nucleosomes (94).

MMTV induces mammary carcinomas, as well as T-cell lymphomas, in mice (29, 60). Integrated MMTV proviruses acquired in T-cell tumors invariably have a large deletion within the U3 region of the LTR that removes at least two negative regulatory elements (NREs) and the 3′ one-third of the *superantigen* (*sag*) gene (47, 54, 59, 61). A naturally occurring MMTV variant, DMBA-LV or TBLV, with deleted LTRs induces T-cell lymphomas (4–6), and substitution of the full-length LTRs of a mammotropic MMTV with the deleted LTRs of a thymotropic MMTV results in the production of thymotropic virus (96). Therefore, determinants of MMTV disease tropism reside in the LTRs.

We previously have shown that T-cell lymphomas with acquired proviruses preferentially transcribe RNA from deleted proviruses in certain cell types compared to proviruses with full-length LTRs, suggesting that loss of this LTR region conferred a transcriptional advantage for MMTV proviruses in T cells (47). In support of this, we constructed deleted-LTR–reporter gene constructs that displayed higher basal transcription in transient transfection assays than did wild-type LTR constructs (47). Subsequent experiments with transgenic mice and transient transfection assays showed that deletion of two regions (termed the promoter-proximal and -distal NREs) could elevate transcription from the MMTV promoter (15, 75). Furthermore, binding sites for two different nuclear protein complexes called UBP and NBP mapped to the promoter-proximal and -distal NREs (15). Mutations in the proximal NBP-binding site elevated basal transcription from the MMTV promoter and destabilized binding to NBP (15).

To further study the negative regulation of MMTV transcription, we have used a 22-bp multimerized sequence from the MMTV NRE to purify NBP from nuclear extracts of rat thymus. By a variety of criteria, NBP appears to be special AT-rich sequence-binding protein 1 (SATB1) and mutation of SATB1 binding site 924 dramatically altered tissue-specific MMTV expression in transgenic mice.

MATERIALS AND METHODS

Probes and antibodies. The oligonucleotides 5′ gggGACTAATAGAACATT ATTC 3′ and 5′ cccGAATAATGTTCTATTAGTC 3′, representing the imperfect inverted repeat in the promoter-proximal MMTV NRE (pNRE), were annealed and end labeled with Sequenase (U.S. Biochemicals). Nucleotides in lowercase letters on the primer sequences were added to facilitate labeling and the formation of concatemers. Alternatively, the annealed oligonucleotides were ligated with T4 DNA ligase and cloned into pUC9 (89). Individual clones containing two (pNRE2) or four (pNRE4) repeats of pNRE were identified by restriction enzyme digestion and electrophoresis and confirmed by sequencing. Clones pNRE2 and pNRE4 were removed from the plasmid vector by digestion with *Eco*RI and *Hin*dIII, and the fragments were purified on polyacrylamide gels as described by Maxam and Gilbert (58) and end labeled with Sequenase for gel shift assays. The 120-bp fragment spanning the promoter-proximal inverted repeat sequence was obtained by PCR from the C3H MMTV LTR as described in Bramblett et al. (15); this fragment was cloned into pCRII (Invitrogen, San Diego, Calif.) and purified for gel shift assays as described above.

Polyclonal rabbit antisera against recombinant SATB1 were provided kindly by T. Kohwi-Shigematsu (Burnham Institute, La Jolla, Calif.) (26) or Paul Gottlieb (University of Texas at Austin) (7). Polyclonal rabbit antisera against human CDP were generated by inoculation of two New Zealand White rabbits with a glutathione *S*-transferase fusion protein containing the C-terminal two-thirds of the CDP protein purified as previously described (80). Immunizations were performed in accordance with the standard procedures of Cocalico Biologicals (Reamstown, Pa.). The fusion protein construct and guinea pig anti-CDP were provided generously by E. Neufeld (Harvard University). Anti-p50 and anti-p65 sera (Santa Cruz Biotechnology, Inc.) were provided by Henry Bose (University of Texas at Austin).

Preparation of nuclear extracts. Cell lines CCL-64, NMuMG, and D-6 were grown as described by Bramblett et al. (15). Jurkat cells were grown in RPMI medium containing 5% fetal calf serum (FCS) and 5% NuSerum IV (Collaborative Research, Bedford, Mass.), penicillin-streptomycin, and 50 μg of gentamicin per ml. LBB.A cells (65) (kindly provided by Brigitte Huber, Tufts University) were grown in RPMI medium containing 10% FCS, 10 mM HEPES (pH 7.4), and 5×10^{-5} M 2-mercaptoethanol. Crude nuclear lysates were prepared by the method of Dignam et al. (28) or by the method of Olnes and Kurl (67) and stored in aliquots at −70°C.

Crude nuclear extracts from mouse and rat tissues were prepared by the method of Dignam et al. (28) with modifications. All purification steps were performed at 4°C. Tissues were rinsed in phosphate-buffered saline, resuspended in 3 volumes of buffer A (10 mM HEPES [pH 7.9], 10% glycerol, 1.5 mM $MgCl_2$, 10 mM KCl, 250 mM sucrose, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride [PMSF], 0.32 μg of pepstatin A per ml, 10 μg of leupeptin per ml, 2 μg of aprotinin per ml), and allowed to incubate on ice for 10 min. Tissues were homogenized subsequently with a Dounce B pestle, and the crude nuclear lysate was passed through four layers of cheesecloth to remove particulate material and connective tissue. Nuclei were collected by centrifugation at 5,000 rpm (3,000 × *g*) for 10 min in a Sorvall SS-34 rotor. The nuclear pellet was resuspended in 6 volumes of buffer A and then pelleted at 14,500 rpm (25,000 × *g*) in the same rotor. The pellet was resuspended in buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM PMSF, 0.32 μg of pepstatin A per ml, 10 μg of leupeptin per ml, 2 μg of aprotinin per ml), and the suspension was stirred for 30 min. The supernatant was dialyzed against buffer D (20 mM HEPES [pH 7.9], 20% glycerol, 0.1 M KCl, 2 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM PMSF, 0.32 μg of pepstatin A per ml) for 3 h. The dialysate was centrifuged at 25,000 × *g* for 20 min at 4°C, and the supernatant was frozen in aliquots at −70°C.

Gel shift assays. Gel shift assays were performed essentially as described by Bramblett et al. (15). Reaction mixtures contained the amounts of nuclear extract indicated in Results, 0.1 M KCl, 20 mM HEPES (pH 7.9), 0.5 mM dithiothreitol, 4% glycerol, 0.5 mM PMSF, 0.2 μg of pepstatin A per ml, and 1 μg of poly(dI-dC) (Pharmacia) in a volume of 10 μl. The mixture was incubated at 4°C for 10 min prior to the addition of 10^4 cpm of an end-labeled probe (approximately 10^8 cpm/μg for the 120-bp fragment). Reaction mixtures were incubated for an additional 10 min prior to electrophoresis on 4% nondenaturing polyacrylamide gels containing 10% glycerol in a buffer containing 12.5 mM Tris-HCl (pH 8.05), 5 mM sodium acetate, and 0.5 mM EDTA. Electrophoresis was performed at 10 to 15 V/cm for 3.5 h at 4°C prior to autoradiography. Competition experiments with unlabeled DNA were performed as described previously (15). The DIST oligonucleotide (kindly provided by E. Neufeld) used for competition experiments is a 61-bp sequence containing a Cux/CDP-binding site from the promoter region of the phagocyte cytochrome oxidase gene (63). The MAR sequence used for gel shift assays was a fivefold repeat of a 25-bp oligonucleotide containing a SATB1-binding site from the 3′ MAR of the enhancer (62) (kindly provided by T. Kohwi-Shigematsu) located between the joining and constant regions of the immunoglobulin heavy chain (46). Distamycin A competition experiments were performed by using our standard gel shift conditions, except that various concentrations of distamycin were added simul-

taneously with the labeled probe. The NF-κB probe was prepared by end labeling the annealed oligonucleotides 5′ AATTCAGGGGAATTCCCCTAAGCTTGA GCT 3′ and 5′ CAAGCTTAGGGGAATTCCCCTG 3′ (55). For antibody ablation experiments, 2 μg of preimmune or immune serum was added to the reaction mixture, which was incubated for 20 min at 4°C prior to addition of the labeled probe.

Nuclear matrix preparation and matrix-binding assays. Nuclear matrix preparations were obtained from nuclei as described by Cockerill and Garrard (18). Nuclear matrix-binding assays also were performed as previously described (18).

In vitro transcription-translation. The complete murine SATB1 cDNA (kindly provided by T. Kohwi-Shigematsu) was linearized, and 1 μg was added to a coupled in vitro transcription-translation reaction (Promega, Madison, Wis.) in a total volume of 50 μl. A portion (8 μl) of the reaction mixture was used in a gel shift assay as described above.

Purification of NBP. Crude nuclear extracts were prepared from approximately 800 rat thymi (ca. 360 g [wet weight]). This extract was heated for 10 min at 47°C, and the insoluble material was removed by centrifugation at 10,000 rpm in an SS-34 rotor for 10 min. This step achieved approximately twofold purification of NBP, as judged by the amount of protein needed to achieve a 50% shift of an end-labeled 120-bp proximal NRE probe. The soluble material was adjusted to 30% (wt/vol) ammonium sulfate, and the precipitated protein was recovered by centrifugation at 14,500 rpm in an SS-34 rotor for 30 min. The ammonium sulfate precipitation also achieved approximately twofold purification of NBP. The pelleted material was dissolved in buffer D and then dialyzed for 4 to 5 h at 4°C in buffer D. The dialysate was applied to a DEAE-Sepharose (Sigma Chemical Co., St. Louis, Mo.) column equilibrated in buffer D, and then NBP was eluted with a 200-ml linear gradient of KCl (0.1 to 0.7 M) in buffer D. Fractions (1 ml) were collected, and adjacent fractions were pooled, dialyzed against buffer D, and assayed for NBP activity by gel shift assays. Fractions containing NBP were purified approximately 40-fold after this step.

Semipurified fractions from the DEAE-Sepharose column were pooled and subjected to magnetic bead affinity purification as described by Gabrielsen and Huet (36) and Gabrielsen et al. (35). The 22-bp proximal NRE oligonucleotides were modified by addition of GGG to the 5′ end of the positive strand and CCC to the 5′ end of the negative strand and then ligated with T4 DNA ligase (New England BioLabs). The concatemers were end labeled with biotinylated dCTP (GIBCO BRL) and cross-linked to streptavidin-coated magnetic beads (Promega). The complex formed was resistant to high concentrations of salt and urea (36). Semipurified NBP was bound to DNA affinity beads in the presence of buffer D and 30 μg of poly(dI-dC) per ml as a nonspecific competitor DNA, and the unbound material was removed with a magnetic separator (Stratagene, La Jolla, Calif.). The beads were washed with buffer D and eluted in buffer D containing 1.5 M KCl. The eluate was dialyzed against buffer D prior to gel shift assays, concentrated by Centricon filtration, precipitated with chloroform and methanol, and then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The final yield of NBP was approximately 15 pmol (2 μg).

High-performance liquid chromatography (HPLC) analysis and sequencing of NBP. After SDS-polyacrylamide gel electrophoresis, purified NBP was stained with Coomassie brilliant blue G (Sigma Chemical Co.). Under these conditions, only a single polypeptide of 115 kDa was apparent. This polypeptide was excised from the gel and digested in situ with *Achromobacter* protease I as described by Wang et al. (91), except that the Tween concentration was 0.05%. HPLC and sequencing analysis were performed as described previously (91).

Immunoprecipitations. Actively growing tissue culture cells (1×10^7 to 5×10^7) were incubated in methionine-free medium (GIBCO BRL) with 5% FCS for 1 h. Fresh, methionine-free medium containing 200 μCi of [^{35}S]methionine (New England Nuclear) was added subsequently, and the mixture was incubated for up to 5 h. Cells were washed once with medium without serum and lysed in radioimmunoprecipitation assay buffer (40) (25 mM Tris-HCl [pH 7.8], 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF, 0.32 μg of pepstatin A per ml, 10 μg of leupeptin per ml, 20 μg of aprotinin per ml). Particulate material was removed by centrifugation at $10,000 \times g$ for 10 min, and the supernatant was added to 150 μl of a 50% (vol/vol) suspension of formalin-fixed *Staphylococcus aureus* (Sigma Chemical Co.) and 10 μl of preimmune serum and incubated for a minimum of 1 h at 4°C. Beads were removed by centrifugation at $10,000 \times g$ for 10 min, and the supernatants were incubated with 5 μl of immune or preimmune serum and 150 μl of the protein A-Sepharose suspension on a rotating wheel for 1 h at 4°C. Immune complexes were collected by centrifugation and washed three times in radioimmunoprecipitation assay buffer. Samples were resuspended in SDS-gel electrophoresis buffer (31 mM Tris-HCl [pH 6.8], 5% thioglycerol, 1% SDS, 0.15 M β-mercaptoethanol, 0.0005% bromophenol blue), boiled, and centrifuged to remove the Sepharose. The supernatant was analyzed by electrophoresis on SDS–10% polyacrylamide gels followed by autoradiography.

In specific cases, SATB1 was immunoprecipitated from the nuclear extracts (500 μg) of unlabeled Jurkat T cells or rat thymus, subjected to electrophoresis on SDS-polyacrylamide gels, and stained with Coomassie brilliant blue, and the major 115-kDa band was excised from the gel and renatured by the method of Hager and Burgess (43). The renatured protein then was dialyzed against buffer D and used for gel shift assays.

Preparation of transgenic animals. DNA was injected as described previously (74). Swiss Webster mice (males and females) were purchased from the National Institutes of Health Frederick Cancer Research Facility (Frederick, Md.). Transgenic mice were identified by using tail DNA and either PCR or Southern blotting analysis (data not shown) as described previously (73). Chloramphenicol acetyltransferase (CAT) assays also have been described (75).

RESULTS

A 22-bp sequence is sufficient for NBP binding. We previously have shown that the promoter-proximal and -distal MMTV NREs contain binding sites for at least two nuclear protein complexes termed NBP and UBP (15). The locations of these binding sites corresponded well to those of regions implicated in negative regulation of MMTV transcription as detected by transfection assays and transgenic animal experiments (15, 75). The two NRE regions were mapped to 100-bp (promoter-proximal) and 180-bp (promoter-distal) fragments with an intervening 90 bp (15). UBP and NBP binding was demonstrated for the 100- and 180-bp fragments, but not by the 90 bp fragment, by direct binding and competition experiments (15). The promoter-proximal NBP-binding site was localized to imperfect inverted 7-bp repeats separated by 5 bp, and mutation of either half of the palindrome reduced or destabilized binding of NBP; mutation of both halves eliminated binding. All of these mutations in the context of the C3H MMTV LTR gave small, but highly reproducible, elevations of reporter gene activity in transient transfections of semipermissive mink lung cells (15). Moreover, NBP complexes were detected by using nuclear extracts from several cell lines (lung and T cells) that are semipermissive for MMTV transcription, but NBP was not detectable in mammary cells permissive for MMTV transcription (15).

A 22-bp oligonucleotide was synthesized that spanned the 7-bp imperfect inverted repeats and the intervening 5 bp (Fig. 1A). We observed previously that mutations in the proximal NRE (p909, p924, and p907/924) spanning this palindrome increased basal transcription (transcription in the absence of exogenous hormones) from the standard MMTV promoter in the U3 region (15). Initial gel retardation experiments indicated that the double-stranded 22-mer bound NBP, but not UBP, at high concentrations of nuclear extract from lung cell or T-cell extracts (Fig. 1B and data not shown). However, use of multimers of the 22-bp sequence dramatically increased NBP binding with these extracts (Fig. 1C).

Binding of UBP, but not NBP, to the 22-bp concatemer probe (pNRE4) was observed with mammary cell extracts (Fig. 1D). (Mammary cell extracts have been shown to lack NBP binding activity [15]). Protein binding to the 22-bp concatemer was specific, since it could be competed by an excess of the unlabeled homologous concatemer fragment or by the 120-bp fragment containing the single 22-bp proximal NRE-binding site (Fig. 1E), although the 120-bp fragment was a poor competitor for pNRE4 binding. Therefore, concatemers of the 22-bp proximal NRE contain specific binding sites for NBP and UBP.

A major component of the NBP complex is SATB1. Previous results indicated that the NBP complex was necessary for suppression of MMTV transcription from the standard U3 promoter since mutations that affected NBP, but not UBP, binding relieved this suppression (15). To understand the role of negative regulation in MMTV tissue-specific transcription, analysis of the NBP complex was critical. Therefore, starting with nuclear extracts from rat thymus, we purified the NBP complex by using a combination of conventional and affinity methods.

Nuclear extracts were prepared from a pool of rat thymi and heated at 47°C for 10 min. Previous data revealed that the DNA-binding activities of NBP and UBP were stable under

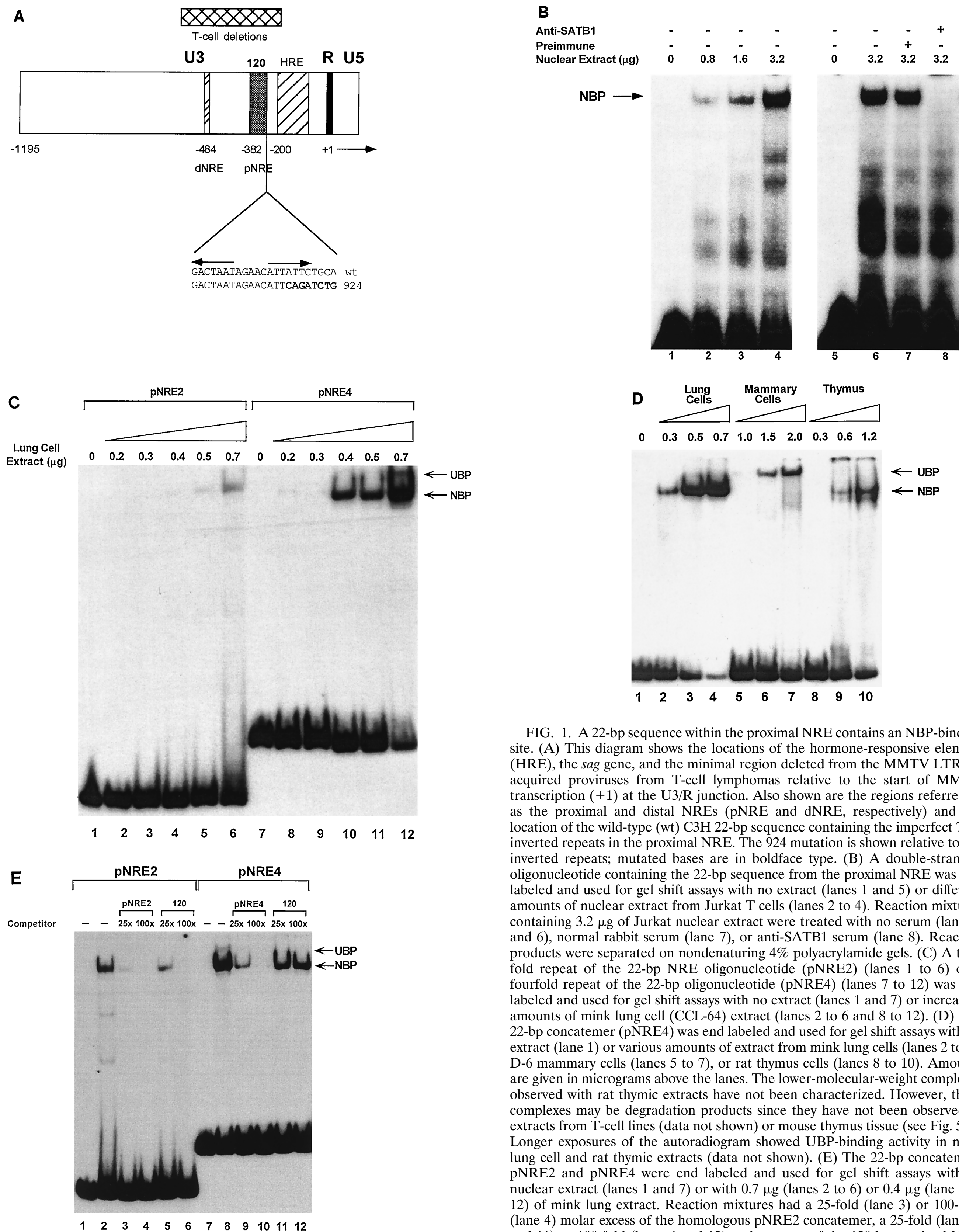

FIG. 1. A 22-bp sequence within the proximal NRE contains an NBP-binding site. (A) This diagram shows the locations of the hormone-responsive element (HRE), the *sag* gene, and the minimal region deleted from the MMTV LTRs in acquired proviruses from T-cell lymphomas relative to the start of MMTV transcription (+1) at the U3/R junction. Also shown are the regions referred to as the proximal and distal NREs (pNRE and dNRE, respectively) and the location of the wild-type (wt) C3H 22-bp sequence containing the imperfect 7-bp inverted repeats in the proximal NRE. The 924 mutation is shown relative to the inverted repeats; mutated bases are in boldface type. (B) A double-stranded oligonucleotide containing the 22-bp sequence from the proximal NRE was end labeled and used for gel shift assays with no extract (lanes 1 and 5) or different amounts of nuclear extract from Jurkat T cells (lanes 2 to 4). Reaction mixtures containing 3.2 μg of Jurkat nuclear extract were treated with no serum (lanes 4 and 6), normal rabbit serum (lane 7), or anti-SATB1 serum (lane 8). Reaction products were separated on nondenaturing 4% polyacrylamide gels. (C) A twofold repeat of the 22-bp NRE oligonucleotide (pNRE2) (lanes 1 to 6) or a fourfold repeat of the 22-bp oligonucleotide (pNRE4) (lanes 7 to 12) was end labeled and used for gel shift assays with no extract (lanes 1 and 7) or increasing amounts of mink lung cell (CCL-64) extract (lanes 2 to 6 and 8 to 12). (D) The 22-bp concatemer (pNRE4) was end labeled and used for gel shift assays with no extract (lane 1) or various amounts of extract from mink lung cells (lanes 2 to 4), D-6 mammary cells (lanes 5 to 7), or rat thymus cells (lanes 8 to 10). Amounts are given in micrograms above the lanes. The lower-molecular-weight complexes observed with rat thymic extracts have not been characterized. However, these complexes may be degradation products since they have not been observed in extracts from T-cell lines (data not shown) or mouse thymus tissue (see Fig. 5A). Longer exposures of the autoradiogram showed UBP-binding activity in mink lung cell and rat thymic extracts (data not shown). (E) The 22-bp concatemers pNRE2 and pNRE4 were end labeled and used for gel shift assays without nuclear extract (lanes 1 and 7) or with 0.7 μg (lanes 2 to 6) or 0.4 μg (lane 8 to 12) of mink lung extract. Reaction mixtures had a 25-fold (lane 3) or 100-fold (lane 4) molar excess of the homologous pNRE2 concatemer, a 25-fold (lanes 5 and 11) or 100-fold (lanes 6 and 12) molar excess of the 120-bp proximal NRE fragment, or a 25-fold (lane 9) or 100-fold (lane 10) molar excess of the homologous pNRE4 concatemer.

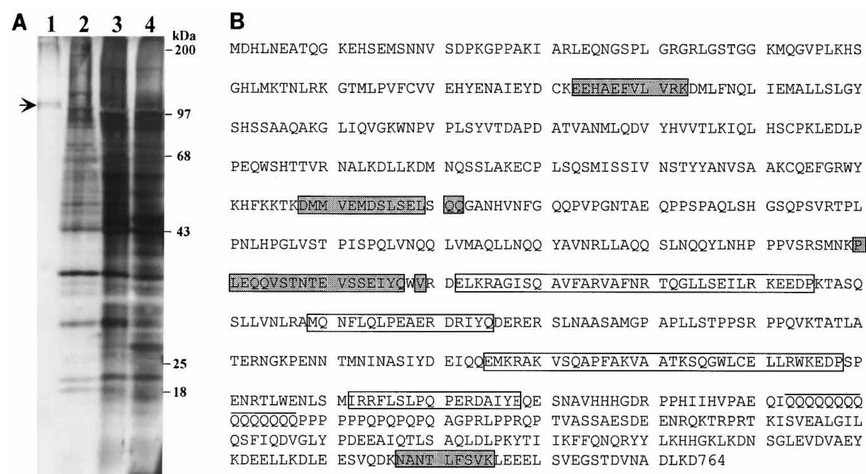

FIG. 2. Affinity-purified NBP contains SATB1. (A) Each lane shows the protein profile obtained at sequential stages of NBP purification after electrophoresis on an SDS–10% polyacrylamide gel and silver staining. The profile of soluble proteins in the original protein extract is shown in lane 4, precipitated proteins obtained after heating and 30% ammonium sulfate treatment are shown in lane 3, proteins obtained after DEAE-Sepharose chromatography are shown in lane 2, and proteins obtained after magnetic bead-DNA affinity purification are shown in lane 1. Equal amounts of protein (2 μg) were loaded in lanes 2 to 4, whereas ca. 50 to 100 ng was loaded in lane 1. The molecular masses of marker proteins are shown in kilodaltons on the right. The arrow indicates the position of purified NBP. (B) The entire sequence of murine SATB1 as determined by Nakagomi et al. (62) is shown. The dark boxes indicate the locations of four lysyl-containing peptides that were partially sequenced following elution from the HPLC column. The single-amino-acid gaps shown in two of the dark boxes indicate that the identities of the amino acids at those positions could not be determined unambiguously. The two longer open boxes indicate the positions of one set of homologous sequences, whereas the two shorter open boxes indicate the positions of a second set of homologous sequences (62). The overlined sequence shows the position of a 15-amino-acid continuous stretch of glutamines.

these conditions (data not shown). After removal of precipitated protein, the soluble fraction was precipitated by 30% ammonium sulfate and the precipitate containing UBP and NBP was redissolved and subjected to chromatography on DEAE-Sepharose. Both UBP and NBP eluted at approximately 1.0 M NaCl (data not shown). The eluted material then was used for magnetic bead affinity purification with the 22-bp concatemer from the proximal MMTV NRE. The material specifically bound to the magnetic beads contained a predominant band of approximately 115 kDa as visualized by SDS-polyacrylamide gel electrophoresis and silver staining (Fig. 2A).

Affinity-purified fractions were pooled, subjected to preparative SDS-polyacrylamide gel electrophoresis, and stained with Coomassie brilliant blue. The 115-kDa protein was removed from the gel and digested with *Achromobacter* protease I; the peptides then were separated by HPLC. The lysyl-containing peptides obtained from the 115-kDa band were consistent with those predicted from the amino acid sequence of the MAR-binding protein known as SATB1 (62) (Fig. 2B). Four of the HPLC-purified peptides were selected for amino acid sequencing. Sequence analysis revealed that all four of the peptides analyzed were identical to the published murine SATB1 sequence (62) (Fig. 2B). Therefore, the peptides obtained by HPLC and the sequencing data indicated that a major component of the purified NBP complex that binds to the MMTV NRE is SATB1.

Antibody to purified SATB1 abolishes the NBP complex. Antibody against SATB1 overexpressed and purified from *Escherichia coli* was used to determine whether the NBP or UBP complex contained SATB1. By using the 120-bp proximal NRE probe in gel shift assays, both the NBP and UBP complexes were formed with mink lung nuclear extracts (Fig. 3). However, the NBP complex was not present if several different rabbit anti-SATB1 sera were preincubated with nuclear extracts prior to addition of the probe (Fig. 3, lanes 4, 6, and 7). The ablation of the NBP complex by anti-SATB1 sera appeared to be specific, since addition of several different preimmune rabbit sera had no effect (lanes 3 and 5) compared to the complexes formed in the absence of serum (lane 2). Similar results were obtained by using the 22-bp double-stranded oligonucleotide containing the imperfect inverted repeats (Fig. 1B). No effect on either the UBP or NBP complex was observed by using antiglucocorticoid receptor sera (data not shown).

To confirm the identity of the NBP complex, we also per-

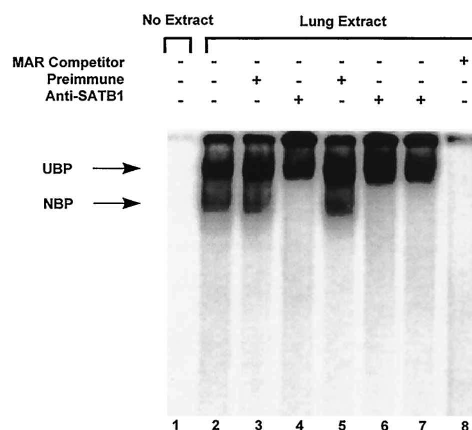

FIG. 3. Formation of the NBP complex is inhibited by antisera to SATB1 and by the MAR of the immunoglobulin heavy-chain intronic enhancer. The 120-bp proximal NRE probe was end labeled and incubated with no extract (lane 1) or 0.8 μg of mink cell nuclear extract (lanes 2 to 8) prior to electrophoresis on polyacrylamide gels. Preimmune rabbit sera (lanes 3 and 5) or different anti-SATB1 sera (lanes 4, 6, and 7) were added to some reaction mixtures prior to the labeled probe. In lane 8, a 100-fold molar excess of unlabeled DNA derived from a fivefold repeat of a 25-bp sequence representing a SATB1-binding site from the 3′ MAR of the immunoglobulin heavy-chain intronic enhancer (26) was added. To separate the high-molecular-weight complexes, the free probe was run off of the gel.

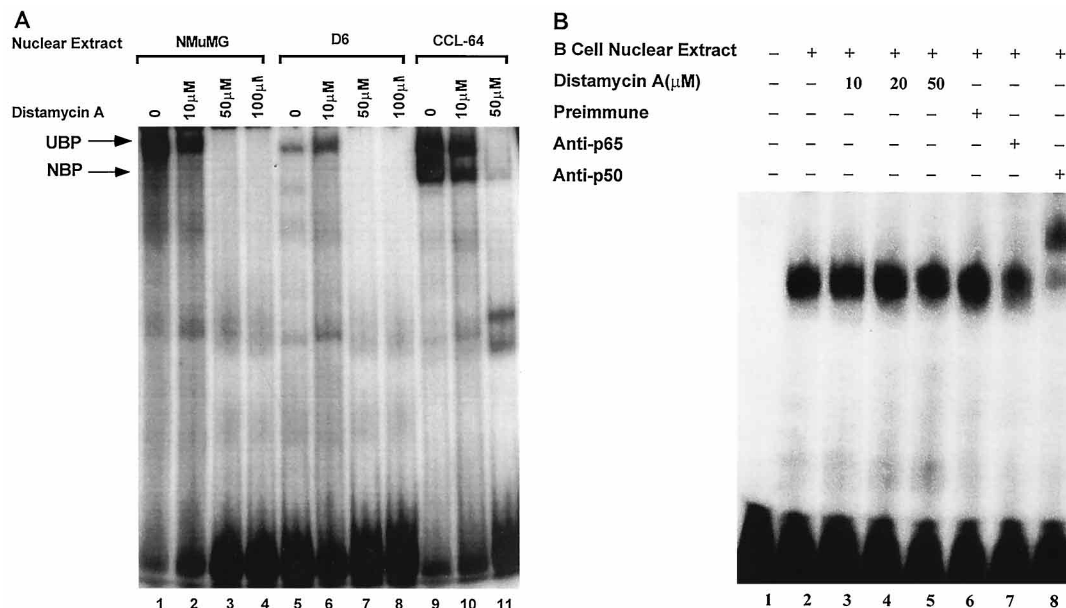

FIG. 4. Binding of NBP and UBP to the MMTV NRE is inhibited by distamycin A. (A) The 120-bp proximal NRE fragment was end labeled and incubated with nuclear extract from the mammary cell line NMuMG (lanes 1 to 4) or D6 (lanes 5 to 8) or the mink lung cell line CCL-64 (lanes 9 to 11) prior to electrophoresis. The amounts of nuclear extract used for the reactions were 1.5 μg for NMuMG and D6 and 1.0 μg for CCL-64. Some reaction mixtures contained distamycin A at 10 μM (lanes 2, 6, and 10), 50 μM (lanes 3, 7, and 11), or 100 μM (lanes 4 and 8). (B) An NF-κB probe was incubated with nuclear extract (8 μg) from the B-cell line LBB.A (lanes 2 to 8) in the presence of 10, 20, or 50 μM distamycin A (lanes 3 to 5) or in the presence of preimmune rabbit serum (lane 6), anti-p65 serum (lane 7), or anti-p50 serum (lane 8).

formed gel shift assays with the labeled 120-bp MMTV NRE probe in the presence of an excess of an unlabeled 25-bp concatemer containing a SATB1-binding site from the MAR of the intronic immunoglobulin heavy-chain enhancer (26). As expected, binding of the NBP complex was abolished in the presence of the competitor; however, UBP binding also was abolished (Fig. 3, lane 8). Together with the antibody ablation experiments, these results strongly suggested that SATB1 is a component of the NBP complex and that both NBP and UBP complexes contain MAR-binding proteins.

Binding of NBP and UBP to the MMTV NRE is inhibited by distamycin A. Although most transcription factors appear to make contact with the major groove of DNA, several of the characterized MAR-binding proteins bind to the minor groove (1, 46, 51, 62). Therefore, we used a minor-groove-binding drug, distamycin A (51), to compete for binding of UBP and NBP to the 22-bp concatemer of the MMTV NRE (Fig. 4A). By using nuclear extracts from mink lung cells or murine mammary cells in gel shift assays, various amounts of distamycin A were added to the DNA-binding reaction mixtures. We observed that the presence of 50 μM distamycin A gave nearly complete inhibition of UBP and NBP binding to the probe. These results are consistent with binding of both NBP and UBP complexes to the minor groove of DNA. As a control, we showed that the presence of 50 μM distamycin did not inhibit p50 binding to an NF-κB probe (Fig. 4B).

Tissue specificity of NBP and UBP complex formation. Previous results indicated that nuclear extracts from mink lung cells, T cells, and mammary cells contained UBP, whereas NBP was present in lung cells and T cells but was absent from mammary cells (15). To further define the tissue distribution of NBP, we prepared nuclear extracts from a variety of murine tissues, including lung, spleen, kidney, brain, thymus, heart, and lactating mammary gland (Fig. 5A and data not shown). These nuclear extracts were used in gel shift experiments with the 22-bp concatemer probe from the proximal MMTV NRE (pNRE4). The majority of tissue extracts contained NBP activity, as demonstrated by the ability of anti-SATB1 serum to abolish the complexes. The residual binding seen may represent UBP binding.

Brain, spleen, and thymic extracts reproducibly had the highest specific activity of NBP binding per microgram of protein, whereas lactating mammary gland extracts had little or no detectable NBP- or UBP-binding activity (Fig. 5A, lane 20). It is likely that tissue extracts were more degraded than those obtained from tissue culture cells, since the shifted bands obtained with tissues extracts were broader and small amounts of many complexes could be seen after gel electrophoresis. Nevertheless, extracts from multiple murine and human mammary gland cell lines (relatively undifferentiated) contained UBP-binding, but not NBP-binding, activity (15), and extracts from lactating mammary gland tissue (containing differentiated mammary cells) had no detectable binding activity but showed binding to Sp1 transcription factor probes (data not shown). The binding observed with different tissue extracts was specific, as judged by competition experiments (Fig. 5B). The presence of SATB1 in tissues other than thymus is consistent with results of others (22, 26).

To confirm the relative tissue distribution of SATB1, we performed immunoprecipitation experiments with [^{35}S]-methionine-labeled whole-cell extracts of several cell lines (Fig. 5C). SATB1 antibodies precipitated from extracts of mink lung cells and a human T-cell line (Jurkat) a major 115-kDa protein that was slightly smaller than a band observed in immunoprecipitates obtained with preimmune serum. We also detected several fainter bands in immunoprecipitates with SATB1 antisera that were not apparent with control sera. Strikingly, immunoprecipitates of a normal murine mammary line, NMuMG, with SATB1-specific antibody contained some of the fainter bands but not the 115-kDa protein (Fig. 5C, compare lanes 4 and 6).

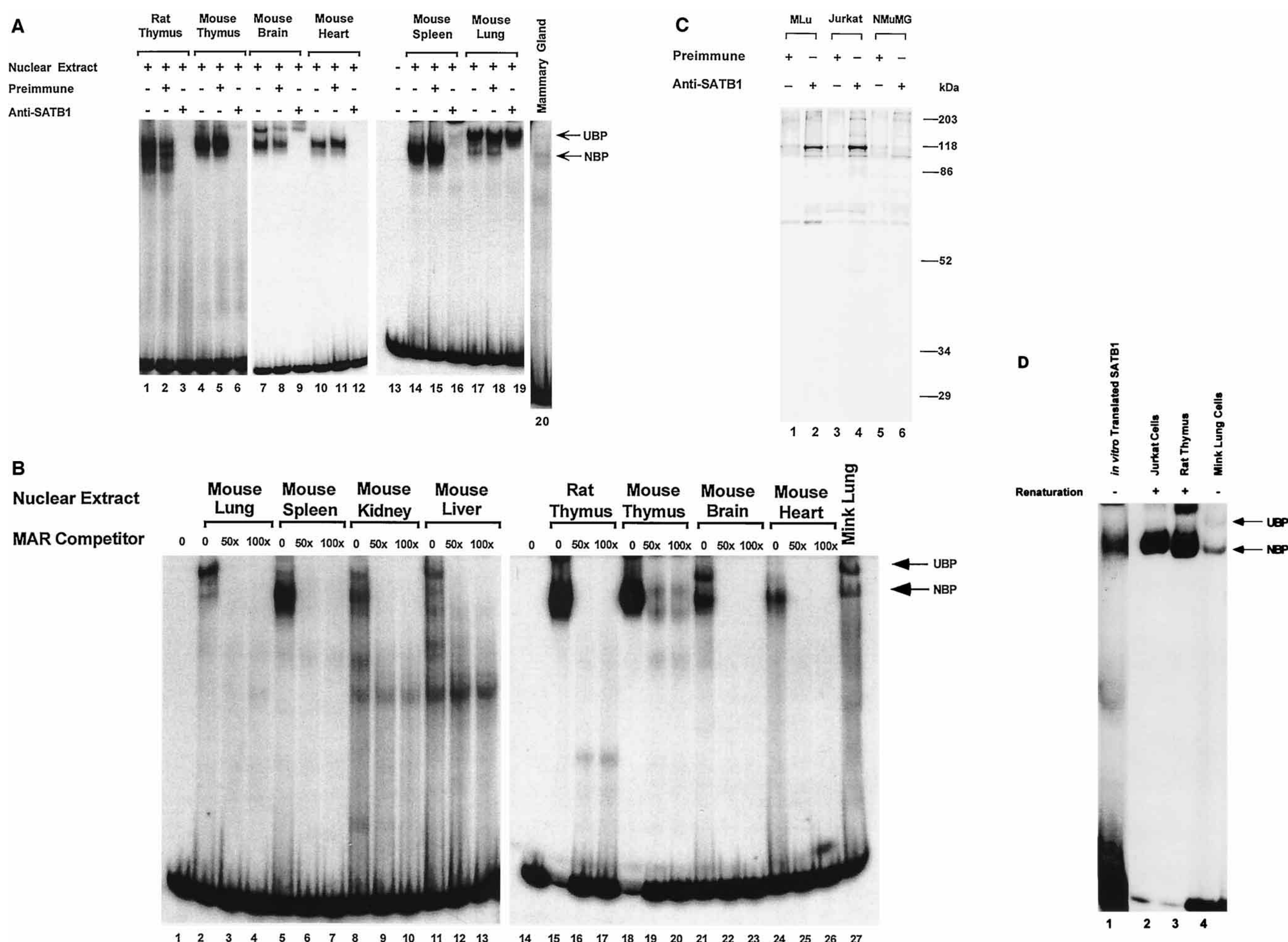

FIG. 5. Tissue specificity of NBP and UBP complex formation. (A) The pNRE4 probe was end labeled and incubated with no extract (lane 13), 0.2 μg of rat thymic extract (lanes 1 to 3), 0.3 μg of mouse thymic extract (lanes 4 to 6), 0.6 μg of mouse brain extract (lanes 7 to 9), 1.5 μg of mouse heart extract (lanes 10 to 12), 1.5 μg of mouse spleen extract (lanes 14 to 16), 1.5 μg of mouse lung extract (lanes 17 to 19), or 22 μg of lactating mammary gland extract (lane 20) prior to electrophoresis. Preimmune sera (lanes 2, 5, 8, 11, 15, and 18) or anti-SATB1 sera (lanes 3, 6, 9, 12, 16, and 19) were added to the indicated reaction mixtures prior to the labeled probe. The rat extract was slightly degraded due to the increased time used to process tissues from multiple animals. (B) The proximal NRE probe (pNRE4) was end labeled and incubated with no extract (lanes 1 and 14) or 1.5 μg of mouse lung extract (lanes 2 to 4), 1.5 μg of mouse spleen extract (lanes 5 to 7), 5.0 μg of mouse kidney extract (lanes 8 to 10), 14 μg of mouse liver extract (lanes 11 to 13), 0.4 μg of rat thymic extract (lanes 15 to 17), 0.6 μg of mouse thymic extract (lanes 18 to 20), 0.6 μg of mouse brain extract (lanes 21 to 23), 1.5 μg of mouse heart extract (lanes 24 to 26), or 1.0 μg of CCL-64 extract (lane 27). A 50-fold (lanes 3, 6, 9, 12, 16, 19, 22, and 25) or 100-fold (lanes 4, 7, 10, 13, 17, 20, 23, and 26) molar excess of the 25-bp concatemer of the MAR from the immunoglobulin heavy-chain intronic enhancer was added prior to the labeled probe. (C) Mink lung cells (CCL-64) (lanes 1 and 2), Jurkat T cells (lanes 3 and 4), or normal mouse mammary cells (NMuMG) (lanes 5 and 6) were labeled metabolically with [^{35}S]methionine. Whole-cell extracts were prepared and immunoprecipitated with normal rabbit serum (lanes 1, 3, and 5) or rabbit anti-SATB1 serum (lanes 2, 4, and 6). Immunoprecipitates were resolved on an SDS–10% polyacrylamide gel prior to autoradiography. The positions of molecular weight markers (Bio-Rad) are given at the right. (D) Murine SATB1 obtained by in vitro translation (lane 1) or SATB1 obtained by immunoprecipitation and renaturation from extracts of human Jurkat cells (lane 2) or rat thymus cells (lane 3) was incubated with the end-labeled pNRE4 probe prior to electrophoresis on a nondenaturing gel next to reaction mixtures obtained by incubation of the pNRE4 probe with crude nuclear extract from mink lung cells (lane 4). The reaction mixture in lane 1 was subjected to electrophoresis on the same gel as those in lanes 2 to 4. However, detection of the NBP complex in lane 1 required a longer period of autoradiography. An in vitro transcription-translation reaction also was performed in the absence of template DNA and used for gel shift assays (data not shown); a band with the same mobility as SATB1 was not observed.

These results are in close agreement with our earlier studies that indicated that the NBP complex is present in mink lung and T cells and absent from mammary cell lines (15).

To confirm the identity of the 115-kDa SATB1-related protein, we used a large amount of rat thymic or Jurkat T-cell nuclear extract for immunoprecipitation with SATB1 antibody. The immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, and the 115-kDa bands were removed from the gel and renatured. The renatured protein then was used in a gel shift assay with the 22-bp concatemer probe from the proximal MMTV NRE (Fig. 5D). This experiment showed that the 115-kDa protein produced a complex with a mobility comparable to that of NBP from crude nuclear extracts (compare lanes 2 and 3 with lane 4). Thus, it appears that the 115-kDa SATB1-related protein is sufficient to reconstitute a complex with electrophoretic properties similar to those of NBP. In other experiments, we used gel shift assays to show that in vitro-translated SATB1 also gave a complex with the same mobility as NBP from crude extracts (Fig. 5D, lane 1).

Transgenic mice carrying an MMTV LTR with a proximal NRE mutation have altered tissue-specific expression. Analysis of wild-type C3H MMTV reporter constructs revealed a tissue-specific distribution of expression that is highest in lac-

TABLE 1. CAT activities of tissue extracts from two mouse strains expressing the p924 CAT transgene

Tissue	CAT activity (cpm/μg/min)			
	Strain 924 CAT5		Strain 924 CAT7	
	Male	Female	Male	Female
Mammary gland	ND[a]	456	ND	1
Salivary gland	2,888	>16,675	183	3
Kidney	101	116	4	0
Lung	38	236	52	2
Spleen	5,312	5,097	1,070	338
Thymus	1,434	9,902	324	624
Lymph node	15,384[b]	7,970	28	136
Peyer's patch		>22,554	125	1,713
Seminal vesicle	0	ND	7	ND
Testis	0	ND	0	ND
Heart	0	24	0	0
Brain	0	7	0	0
Skeletal muscle	0	41	0	0
Liver	0	22	0	0

[a] ND, not done.
[b] This sample included both lymph node and Peyer's patch tissues.

tating mammary gland tissue, lower in lung, salivary gland, kidney, spleen, lymphoid, and reproductive tissues, and undetectable in the brain, heart, liver, and skeletal muscle (17, 75). Previous experiments have shown that deletions within the MMTV NRE increased basal MMTV expression in transient transfection assays and altered the tissue specificity of MMTV transcription (47, 75). Specifically, deletion of the proximal or distal NRE is sufficient to allow expression in tissues such as those of the brain, heart, liver, and skeletal muscles (75). However, these deletion mutations did not alter the relative distribution of MMTV-expressing tissues, since lactating mammary gland tissue showed the highest level of MMTV-based expression for both wild-type and deleted LTRs.

Substitution mutations in the proximal MMTV NRE that maintain the correct spacing between regulatory elements, e.g., p924, also have been shown to increase basal expression in transient transfection assays and to destabilize NBP binding (15) and therefore were tested for alterations in tissue-specific transcription. Two different strains of transgenic mice were constructed that contained the 924 mutation in the context of the C3H MMTV LTR upstream of the CAT reporter gene (Table 1). Intriguingly, both strains showed a dramatic and similar alteration in the pattern of CAT gene expression. The p924 mutant mice had very high CAT expression in all of the lymphoid tissues tested, including the spleen, thymus, lymph nodes, and Peyer's patches, as opposed to the deletion or full-length LTR constructs that showed intermediate levels of expression in these tissues. The lymphoid tissues of p924 mice had CAT levels that were 10 to 100 times greater than those in other tissues, such as lung and kidney tissues. Salivary gland expression also was very high in strain 924 CAT5 mice, particularly females. However, this may be an integration site effect since the salivary glands of strain 924 CAT7 mice had intermediate levels of CAT activity.

Although reporter gene expression from the wild-type C3H LTR has not been observed in the brain, heart, and liver, CAT expression in the tissues of strain CAT5 mice was comparable to that observed with NRE deletion constructs. Strain CAT7 mice did not have detectable CAT activity in these tissues, but the overall expression in this strain is much lower than that in strain CAT5, presumably due to integration site or copy number effects.

Surprisingly, both strains of p924 mice showed intermediate levels of CAT expression in mammary tissue, whereas all previous constructs, including NRE deletion mutants and wild-type constructs, had the highest level of reporter gene activity in mammary gland tissue relative to other tissues. Thus, compared to NRE deletion constructs, the 924 mutation that destabilizes SATB1 binding to the proximal NRE (15) causes a dramatic increase in LTR-driven transcription in tissues that normally suppress MMTV expression, particularly lymphoid tissues.

The UBP complex contains the homeodomain protein Cux/CDP. Our previous experiments indicated that the UBP complex binds to the 22-bp concatemer (pNRE4) (Fig. 1C and D) but not the monomer probe from the proximal MMTV NRE (Fig. 1B), that UBP is detectable as a high-molecular-weight complex in nuclear extracts from all of the cell lines that we have tested (15), that UBP binding to this probe occurs through the minor groove of DNA (Fig. 4), and that UBP binding can be competed with a MAR probe from the immunoglobulin heavy-chain intronic enhancer (Fig. 3). The homeodomain protein Cux (also known as Cut, Clox, or CDP), which is involved in the repression of end stage differentiation genes, has properties that are consistent with those observed for UBP (2, 8, 30, 57, 79). Therefore, we performed gel shift experiments with the 22-bp concatemer (pNRE4) with mink lung or mammary cell extracts (Fig. 6A) to determine whether the UBP complex contains the homeodomain protein Cux. Treatment of nuclear extracts with anti-CDP antibodies prior to addition of the labeled probe abolished the UBP complex. Gel shift assays with the 120-bp probe showed that anti-CDP, but not preimmune, sera abolished the formation of the UBP complex but not the NBP complex. Thus, these results suggest that the UBP complex contains the homeodomain protein Cux/CDP.

To further confirm the identity of UBP as Cux/CDP, we obtained an oligonucleotide from the phagocyte cytochrome oxidase (PHOX) gene promoter containing a Cux/CDP-binding, but not a CP-1-binding, site (63). As expected, the PHOX promoter oligonucleotide (DIST) competed for UBP binding to the proximal MMTV NRE (Fig. 6B).

The MMTV proximal NRE region binds to the nuclear matrix. Because SATB1 and Cux/CDP have been implicated in binding to MARs from the immunoglobulin heavy-chain intronic enhancer (46, 62, 91a), a 120-bp fragment spanning the proximal MMTV NRE was used to determine whether this region could bind to the nuclear matrix by standard methods (18). Incubation of the labeled 120-bp fragment with nuclear matrix obtained from Jurkat T cells showed specific binding (Fig. 7, lane 2), and this binding could be competed by excess unlabeled homologous DNA (lanes 3 and 4). Binding could not be competed by excess unlabeled nonhomologous competitor DNA (lane 5), and in agreement with data reported by others (93), binding to the nuclear matrix was not tissue specific (lanes 6 to 9). Together, these results indicated that the region including the SATB1- and Cux-binding sites in the proximal NRE of the MMTV LTR had the characteristics of a MAR element.

DISCUSSION

The NBP complex that binds to the proximal MMTV NRE contains SATB1. MMTV variants that cause T-cell lymphomas have large deletions in their LTRs that remove two or more negative regulatory elements, allowing high-level transcription in T cells (47, 75). These NREs normally suppress MMTV transcription from the full-length LTR, and they bind at least

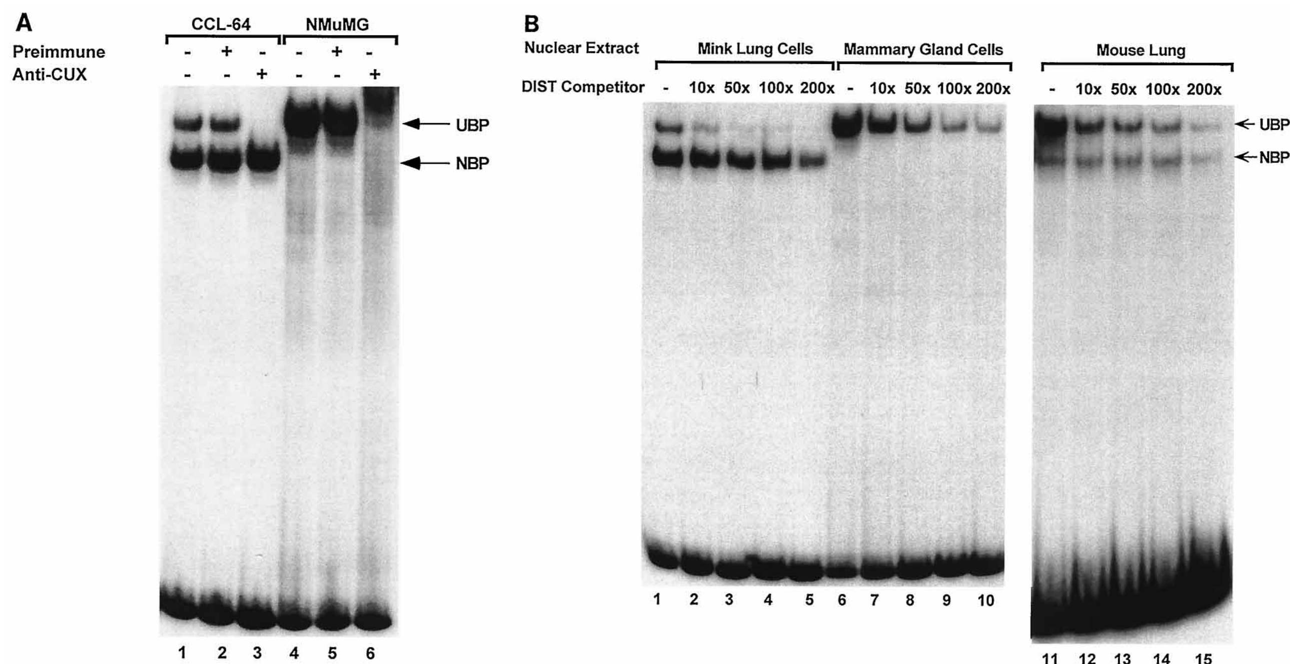

FIG. 6. The UBP complex contains the homeodomain protein Cux/CDP. (A) Nuclear extract from mink lung cells (CCL-64) (lanes 1 to 3) or normal mammary NMuMG cells (lanes 4 to 6) was incubated with no serum (lanes 1 and 4), preimmune rabbit serum (lanes 2 and 5), or anti-CDP serum (lanes 3 and 6) prior to addition of the end-labeled pNRE4 fragment. Reactions were analyzed on 4% nondenaturing polyacrylamide gels. (B) Nuclear extracts from mink lung cells (CCL-64) (lanes 1 to 5), normal mammary cells (NMuMG) (lanes 6 to 10), or mouse lung tissue (lanes 11 to 15) were incubated with no unlabeled DNA (lanes 1, 6, and 11), a 10-fold molar excess of the unlabeled DIST competitor (lanes 2, 7, and 12), a 50-fold molar excess of the unlabeled DIST competitor (lanes 3, 8, and 13), a 100-fold molar excess of the unlabeled DIST competitor (lanes 4, 9, and 14), or a 200-fold molar excess of the unlabeled DIST competitor (lanes 5, 10, and 15) for 10 min prior to addition of the end-labeled pNRE4 probe.

two nuclear protein complexes known as UBP and NBP (15). At least one substitution mutation (924) within the inverted repeat of the proximal MMTV NRE results in the elevation of basal transcription from the MMTV promoter in transient transfections of mink lung cells, and this same mutation increases the rate at which NBP can be displaced from a protein-DNA complex by an unlabeled NRE probe (15). Intriguingly, the NBP-binding site is located between nucleosomes B and C within the MMTV LTR (72). In this paper, we have shown that the 924 mutation in the context of the full-length LTR transgene results in a dramatic elevation of MMTV-initiated transcription in all of the lymphoid tissues tested (Table 1). Reporter gene expression initiated from the MMTV promoter also was increased in the nonpermissive tissues (brain, heart, and liver) of strain 924 CAT5 mice (Table 1), since wild-type LTR constructs never show expression in these tissues (75). These data suggest that NBP is responsible, in part, for suppression of MMTV expression in nonmammary tissues.

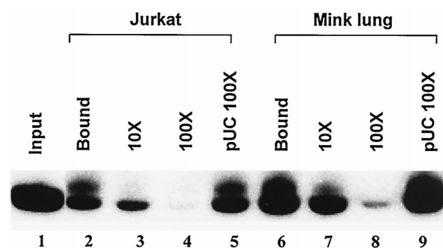

FIG. 7. Binding of the proximal NRE region in the MMTV LTR to the nuclear matrix. Nuclear matrix extracted from Jurkat T cells (lanes 2 to 5) or mink lung cells (CCL-64) (lanes 6 to 9) was incubated with a ^{32}P-labeled 120-bp fragment from the MMTV proximal NRE in the absence of a competitor (lanes 2 and 6) or in the presence of a 10-fold molar excess of homologous DNA (lanes 3 and 7), a 100-fold molar excess of homologous DNA (lanes 4 and 8), or a 100-fold molar excess of heterologous pUC9 (lanes 5 and 9) unlabeled DNA prior to digestion with proteinase K, phenol extraction, ethanol precipitation, and analysis on a 4% polyacrylamide gel. The labeled DNA (10% of input radioactivity) is shown in lane 1. The slower-migrating band seen after incubation of labeled DNA with nuclear matrix appears to result from binding of proteins resistant to proteinase K digestion and phenol extraction. Proteins resistant to these extraction conditions have been observed by others (66).

NBP from rat thymus was subjected to affinity purification with a concatemerized 22-bp oligonucleotide derived from the proximal MMTV NRE (Fig. 2). NBP was shown to be identical to MAR-binding protein SATB1 (26, 62) based on a variety of criteria. (i) Purified NBP has the lysyl peptides predicted for murine SATB1 (62), and partial amino acid sequencing of four of these peptides indicated that the sequence of rat NBP was the same as that of murine SATB1. (ii) NBP and SATB1 both are minor-groove-binding proteins, as demonstrated by distamycin A inhibition of NRE binding. (iii) NBP and SATB1 have similar tissue distributions, since the highest levels of both occur in thymus tissue but they are absent in mammary gland tissue. (iv) SATB1-specific antibodies abolish the NBP, but not the UBP, complex, as detected by gel shift assays with NRE-specific probes and various nuclear protein extracts. Preimmune and anti-GR sera have no effect on NBP binding to the MMTV NRE. (v) NBP and SATB1 binding to the MMTV NRE can be competed with a probe derived from the MAR of the intronic enhancer of the immunoglobulin heavy-chain locus. (vi) The NBP complex with NRE probes has a mobility on polyacrylamide gels similar to that of the protein-DNA complex generated with in vitro-translated SATB1. (vii) The major 115-kDa protein immunoprecipitated with anti-SATB1 sera was renatured to form a complex with the same mobility on

gels as the NBP-DNA complex. Thus, by these criteria, the NBP complex contains SATB1.

Evidence that the UBP complex contains Cux/CDP. The MMTV NRE contains binding sites for at least two protein complexes called NBP and UBP (15). Our experiments to characterize the NBP complex suggested that the UBP complex contains the homeodomain protein Cux/CDP. First, Cux (also known as CDP, Clox, and Cut) is a protein of approximately 180 to 200 kDa (2, 8, 63, 87) and the UBP complex has slower mobility than the NBP complex (15) that contains the SATB1 protein (estimated as 115 kDa on SDS-polyacrylamide gels). Second, use of CDP-specific antisera from guinea pigs (Fig. 6) or from rabbits (data not shown) abolished the UBP complex but not the NBP complex and normal guinea pig or rabbit sera had no effect on UBP or NBP binding. Third, an oligonucleotide containing the Cux/CDP-binding site from the PHOX promoter (79) competed for UBP, but not NBP, binding to a proximal MMTV NRE probe. Fourth, like Cux/CDP, the components of the UBP complex are expressed in a large number of tissues and cell lines (2, 15; this paper).

Other experiments also are consistent with the conclusion that the UBP complex contains Cux. For example, gel shift experiments indicated that a 25-bp oligonucleotide from the MAR of the intronic immunoglobulin heavy-chain enhancer (26, 62) could compete for the binding of both UBP and NBP to the proximal NRE (Fig. 3) and both UBP and NBP complexes are disrupted by an excess of the minor-groove-binding drug distamycin (Fig. 4). Although this is, to our knowledge, the first description of the minor-groove-binding properties of Cux, this property is consistent with the DNA-binding characteristics of other MAR-binding proteins, such as SATB1 and Bright (26, 46).

Interestingly, Cux did not bind to the monomeric or dimeric form of the 22-bp sequence containing the inverted repeat in the proximal NRE (Fig. 1A, B, and C) yet Cux clearly bound to tetrameric forms of this sequence (Fig. 1C, D, and E). These results suggested that the 22-bp sequence contains a weak binding site for Cux and that multimerization of this sequence may facilitate Cux binding in a cooperative manner. Nevertheless, Cux bound with relatively high affinity to proximal NRE sequences upstream of the 22-bp inverted repeat (15), as well as to the distal NRE (data not shown). Therefore, because of the redundancy of the LTR, there appeared to be at least four high-affinity and two low-affinity binding sites for Cux within the MMTV provirus.

Role for MAR-binding proteins in the MMTV life cycle. Milk-borne MMTV enters the guts of newborn mice via the milk of infected mothers, and the ingested virus is believed to infect B cells and, subsequently, T cells by using the virally encoded superantigen (45, 50). Both lymphoid cell types are required for transmission of MMTV to the mammary gland (13, 41, 44). Interestingly, we have evidence that B cells (data not shown) and mammary epithelial cells (Fig. 5C) lack SATB1 detectable by immunoprecipitation and gel shift experiments whereas thymus or T-cell lines have the highest levels of SATB1 (Fig. 5C). Since the NBP complex contains SATB1, these and other results suggest that SATB1 is partially responsible for suppression of MMTV expression from the full-length LTR and that deletion or mutation of these sequences allows higher levels of MMTV transcription in tissues that express SATB1. Therefore, it appears that MMTV transcription is repressed specifically during viral replication in T cells whereas transcription of MMTV proviruses is not suppressed by SATB1 in B cells or mammary cells. This is supported by our data obtained with the 924 LTR-CAT transgene, indicating that reporter gene expression is elevated, relative to that in mammary gland tissue, in all lymphoid tissues that contain T cells (Table 1).

In the T-cell lines that we have examined that have both Cux and SATB1, the endogenous MMTVs (all of which have full-length LTRs) are transcriptionally silent yet these same proviruses are transcribed in most B-cell lines (21, 78, 97). MMTV transcription in B-cell lines may be related to the similar organization of the transcriptional elements in the MMTV U3 region and those in the immunoglobulin heavy-chain enhancer between the J and C regions (46). For example, the intronic heavy-chain enhancer is flanked by two MAR elements that bind both SATB1 (46) and a complex called NF-μNR that contains Cux/CDP (91a). NF-μNR activity is detectable in undifferentiated, but not in differentiated, B-cell lines and is inversely related to immunoglobulin heavy-chain transcription (77). Like NF-μNR, SATB1 also appears to be absent from differentiated B cells, as demonstrated by immunoprecipitation experiments (unpublished data). Moreover, SATB1 and Cux are undetectable in lactating mammary gland tissue (Fig. 5A), a tissue that shows the highest levels of MMTV transcription. Therefore, tissues that lack expression of these MAR-binding proteins correlate well with tissues that display high levels of MMTV transcription and replication.

MAR-binding proteins as suppressors of MMTV transcription. The MMTV proximal NRE contains a region that allows nuclear matrix association (MAR) (Fig. 7), and this region also has binding sites for SATB1 and Cux/CDP (Fig. 3 and 6). Binding of both proteins to the proximal NRE probe is inhibited by a MAR-binding site from the immunoglobulin heavy-chain intronic enhancer (Fig. 3). SATB1 is expressed in a tissue-specific manner (26, 62), whereas Cux/CDP is expressed in a wide variety of tissues (2, 14). Interestingly, in T cells that express both SATB1 and Cux, only SATB1 binding is detectable with the 22-bp sequence (Fig. 1B). This strengthens our conclusion that the 924 mutation in the proximal NRE affects primarily SATB1 binding.

It has been suggested that binding to the nuclear matrix is a prerequisite for gene transcription and that MARs function to anchor genes to the nuclear matrix, a structure rich in transcription factors (39, 82). In one possible model for MMTV transcription, MARs in both LTRs bind to the nuclear matrix and, as in models proposed for other transcriptional units (39, 82), loop out the intervening transcribed region. Cux/CDP and SATB1 bind to the MAR-associated NREs in the MMTV LTR. Cux/CDP has been documented as a repressor of transcription (30, 79, 87). However, previous experiments have produced little evidence to support a biological role for SATB1 (62). Our data strongly suggest that SATB1 represses transcription by binding to the proximal NRE, since mutations that alter the SATB1-binding site also elevate reporter gene expression from the MMTV promoter in transient assays (15). At least one of these mutations (924) also gives a dramatic elevation of CAT gene transcription in all of the lymphoid tissues tested (Table 1).

We propose that SATB1 is the dominant suppressor of MMTV transcription in T cells, since mutation of the 924 binding site has a striking effect on transcription in lymphoid tissues. However, it is possible that Cux participates in negative regulation of MMTV, as it does for other genes (30, 79, 87), particularly since SATB1 and Cux have related homeodomains (25). In any event, we propose that activation and/or differentiation of T cells leads to the displacement of SATB1 by positive-acting transcription factors that have not been identified. Failure of such positive factors to bind to the 924 mutant sequence in the proximal NRE would explain the low-to-intermediate levels of MMTV expression in mammary glands of

924-CAT transgenic mice (Table 1). The highest level of expression from the wild-type MMTV LTR is observed in lactating mammary gland tissue (75), a tissue that apparently lacks both Cux- and SATB1-binding activities (Fig. 5A). Thus, loss of Cux and SATB1 activities in the differentiated mammary gland may relieve the transcriptional repression that exists in undeveloped mammary tissue. Various aspects of this model are being tested.

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