Interaction of the Nuclear Matrix-associated Region (MAR)-Binding Proteins, SATB1 and CDP/Cux, with a MAR Element (L2a) in an Upstream Regulatory Region of the Mouse CD8a Gene*

(Received for publication, April 11, 1997, and in revised form, May 20, 1997)

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Matrix-associated regions (MARs), AT-rich DNA segments that have an affinity for the nuclear matrix, have been shown to play a role in transcriptional regulation of eukaryotic genes. The present study demonstrates that a DNA element, called L2a, which has been implicated in the transcriptional regulation of the mouse CD8a gene encoding an important T cell coreceptor, is a MAR. Moreover, the identities of two nuclear proteins, L2a-P1 and L2a-P2, previously shown to bind to the L2a element, have been determined. The L2a-P1 protein found to be present in all CD8-positive T cell lines tested is SATB1, a known MAR-binding protein. The widely expressed L2a-P2 protein is CDP/Cux, a MAR-binding protein that has been associated with repression of gene transcription. Interaction of both proteins with the L2a element was studied using the missing nucleoside approach, DNase I footprinting, and electrophoretic mobility shift assays with wild type and mutant L2a elements. The data suggest that CDP/Cux bound to the L2a element is displaced by binding of SATB1 and the accompanying conformational change in the DNA lying between the primary binding sites of SATB1 and CDP/Cux. We suggest that displacement of CDP/Cux by SATB1 favors transcription of the CD8a gene, possibly by enhancing or altering its association with the nuclear matrix.

Among the DNA elements implicated in the regulation of gene transcription are matrix-associated regions (MARs), which are defined as AT-rich DNA sequences that are preferentially retained by the nuclear matrix (1, 2). The nuclear matrix is a proteinaceous subfraction that remains after extraction of nuclei with high salt concentrations, and it is thought to form a scaffold for chromosome attachment (3, 4). MARs are typically 200–300 bases in length, contain topoisomerase II cleavage sites, and occur on the average of one for every 30 kb of eukaryotic DNA (3, 5–8). MARs have been increasingly observed near enhancer and promoter regions of genes (2, 8–10), and the participation of MARs in regulating transcription of the immunoglobulin κ light chain (1) and μ heavy chain (11–13) genes has been described. Because MARs can often be shown to bind to nuclear matrices of a wide variety of cells, their ability to regulate transcription of specific genes might be questioned. However, cell type-specific MAR-binding proteins (12, 14–18) could modulate the association of specific MAR sequences and their adjacent genes with the nuclear matrix and thereby repress or activate those genes.

The present study demonstrates that a DNA element (L2a; Ref. 19) implicated in the regulation of transcription of the mouse CD8a gene is a MAR. The CD8 molecule on the surface of T lymphocytes functions as an important coreceptor for class I major histocompatibility/peptide complexes on stimulator and target cells (20–22). In the mouse, CD8 is expressed on class I major histocompatibility complex-specific T cells as a heterodimer of CD8α and CD8β chains that are encoded by the CD8α (or Lyt-2) and CD8β (or Lyt-3) genes, respectively (23, 24), but as CD8α homo dimers on some intraepithelial T lymphocytes (25).Little is known concerning the regulation of the mouse CD8α and CD8β genes, but some insights have come from studies of T-T cell hybrids produced by fusing cytotoxic T lymphocytes with the BW5147 thymic lymphoma. Such hybrids are invariably CD8-negative (26) due to shut-off of CD8α gene transcription (27), but interestingly the CD8β gene, which is located 36 kb upstream of the CD8α gene (28) (see Fig. 1), continues to be transcribed (27, 29). Working on the hypothesis that the shut-off of CD8α gene transcription in T-T cell hybrids was due to cis-acting DNA elements upstream of the CD8α gene, stable clonal transfec tants of BW5147 were produced using constructs of the CD8α gene containing varying amounts of 5′-flanking DNA and surface expression was monitored by flow microfluorometry. Initial results suggested that DNA sequences located between nucleotides −1,400 and −4,700 (the transcription start site is nucleotide +1) (see Fig. 1) were sufficient to provide the negative effect (19). This same region is part of a DNase I-hypersensitive site specific to CD8α positive T cells and contains two binding sites for the GATA-3 transcription factor (30).

Attention focused on a Acc/1HpaI subfragment of this region (nucleotides −3800 to −4700) that lay upstream of the GATA-3 binding sites and that negatively affected CD8α gene expression (Fig. 1). A 270-nucleotide Acc/1HpaI fragment contained an element, designated L2a, which was protected in two places (called L and S) from DNase I digestion in vitro by nuclear extract of the CD8+ T cell tumor line, VL3. A fragment (called 200(L+S)) containing both pro-
SATB1 and CDP/Cux Bind to the L2a MAR Upstream of Mouse CD8a

EXPERIMENTAL PROCEDURES

Cell Lines—The VL-3.B4 (VL-3) (the kind gift of Dr. Irving Weissman, Stanford University School of Medicine, Stanford, CA) and BW5147 (34) T cell lines were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% bovine calf serum and 5 x 10^{-5} M 2-mercaptoethanol. The hypoxanthine/aminopterin/thymidine medium-sensitive μ-BW5147 cell line (35) was maintained in the same medium, and the SATB1-positive transfectant, A11 (see below), was maintained in the same medium containing G418 (250 μg/ml of active drug).

Probes and DNA Constructs—The 0.9-kb AccI/HpaI fragment located 3.8–4.7 kb upstream of the mouse CD8a gene was treated with Klenow fragment and cloned into the Smal site of pUC18 (p2211-1). The same fragment containing a deletion of 50 base pairs at its 5′ end generated by BglII digestion is present in plasmid p2211-1.5′-43. The various fragments and subfragments used in EMSAs were generated by digestion of these two plasmids with enzymes that cut within the polylinker site of the plasmid and/or within the fragment.

The 200(L+S) probe used in gel retardation assays consists of a 200-nucleotide genomic AccI/BstXI fragment that contains the regulatory element described by Lee and co-workers (19). This fragment was subcloned into pUC18 and was excised using restriction sites in the polylinker. The 158-base pair fragment used for the DNase I footprinting of the bottom strand is included in the 200(L+S) probe and was generated by digesting p2211-1.5′-43 with BamHI and BstXI followed by labeling with [α-32P]dNTPs at the BamHI end using Sequenase (Amersham Corp.). Similarly, the 150(L+S) fragment used for the missing nucleoside and DNase I footprinting experiments was obtained by digesting p2211-1.5′-43 and BstXI followed by labeling at the HindIII end with Sequenase. The 100(L+S) fragment was generated by subcloning a HindIII/Refsite fragment from p2211-1.5′-43 into the Smal site of pUC18, digesting the resulting plasmid with EcoRI and PstI, and end labeling at the EcoRI end with Sequenase.

Preparation of Nuclear Extracts—Nuclear extracts of cell lines were prepared essentially as described by Dignam et al. (38). All steps were performed at 4°C or on ice. Cells (2–5 x 10^6) were washed with ice-cold phosphate-buffered saline and resuspended in 5 ml of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF)). DTT and PMSF were added to all buffers immediately before use. After incubation for 10 min, the cells were pelleted (1000 g for 10 min) and resuspended in 2 ml of buffer A. The cells were then lysed using 10 strokes in a homogenizer (Kontes homogenizer with a B pestle; Kontes, Vineland, NJ), and nuclei were pelleted (1000 x g for 10 min), washed with 6 ml of buffer A, and pelleted again (30,000 x g for 20 min). The nuclei were resuspended in 1–2 ml of buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.4 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 2.5 μg/ml pepstatin A, 5 μg/ml leupeptin, 12 μg/ml aprotinin, and 12 μg/ml soybean trypsin inhibitor), homogenized (30 strokes), and stirred on a magnetic stirrer for 30 min. The nuclei were pelleted (30,000 x g for 20 min), and the supernatant was dialyzed against 75 volumes of buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 mM KCl, 0.2 mM EDTA, 1 mM DTT, and 0.7 mM PMSF) for 3 h. After dialysis, extracts were centrifuged (30,000 x g, 20 min), and the supernatant was stored as aliquots in liquid nitrogen. The protein concentration in each extract was measured using the Bradford assay (37).

Antisera—Rabbit polyclonal anti-SATB1 antisem was kindly provided by Dr. T. Kohwi-Shigematsu (Burnham Institute, University of California, Berkeley, CA) and also produced in our laboratory as described (15). Rabbit polyclonal anti-CDP/Cux was the kind gift of Dr. Ellis Neufeld (Harvard Medical School, Boston, MA).

EMSA—EMSA assays were performed essentially as described previously (38, 39). Nuclear extracts (1–10 μg) were mixed with poly(dI-dC)poly(dI-dC) (1–2 μg) in 10–30 μl of binding buffer (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 10 mM DTT, and 30 mM PMSF). End-labeled probe (0.002–0.008 pmol/5000 cpm) was added 5 min later, and after incubation for 20 min at room temperature, 1 μl of dye was added to each tube. The mixtures were applied to a 4% polyacrylamide gel (20:1) in 1 x TEB buffer (0.09 M Tris, 0.09 M boric acid, 0.002 M EDTA) and subjected to electrophoresis for an appropriate period at 10 V/cm. The gel was dried and autoradiographed overnight at ~70°C using an intensifying screen.

For competition experiments, specific and nonspecific competitor DNAs (0.002–0.8 pmol) were added to the nuclear extracts before addition of radiolabeled probe. For distamycin A studies, distamycin A (Sigma) was added to nuclear extracts to the desired concentration before the addition of radiolabeled probe. For antibody inhibition ex-
acrylamide gel. 

**Purification of an L2a-P1-like Protein from Calf Thymus**—Calf thymus nuclear extract (600 mg) was prepared from fresh calf thymus using a modification of the procedure of Nathan (37) and Secondale (38). 

Using 4% 2-mercaptoethanol inhibitors orthovanadate (0.1 mM) and β-glycerophosphate (50 mM) and immunoprecipitated with preimmune or anti-SATB1 antisem were added to the 

nuclear extracts before the addition of the radiolabeled probe.

**Electrophoretic Mobility Shift Assay (EMSA)**— 

**DNA Binding Assay**— 

** DNasel Footprinting**— 

**Site-specific Mutagenesis**—Site-directed mutagenesis was performed using the pALTER system (Promega, Madison, WI) essentially as described by the manufacturer. The SfiI site in the polycloning site of pALTER-1 was changed to HpaI by site-directed mutagenesis. A 1.2 kb XbaI/HpaI fragment containing the L2a element was cloned into the XbaI and HpaI sites of pALTER-1. Site-directed mutagenesis was then performed, and AccI/BstXI fragments representing mutant 200(L+S) regions were subcloned into pUC18 and subjected to 

**RNA Isolation and Northern Hybridization**—RNA isolation using guanidine isothiocyanate and Northern hybridizations were performed essentially as described previously (18,43).

**Production of a SATB1-positive Transfectant of BWS1547—An EcoRV/XbaI fragment containing the full-length mouse SATB1 cDNA was excised from the pmAT plasmid (47), made blunt ended using Klenov fragment, and cloned into the SmaI site of the pECH expression vector to produce the pECH-SATB1 construct. Both the pmAT and pECH vectors were the kind gifts of Dr. T. Kohwi-Shigematsu. The pECH vector contains the major PvuII fragment from pBluescript and the SV40 sequences and multicloning site from the pECE plasmid (48).

Electroporation was performed essentially as described previously (49). The hypoxanthine/aminopterin/thymidine medium-sensitive a/β BWS147 cell line (35) (1 × 10^6 cells/ml) was plated in DMEM containing 10% FCS and 0.1 mg/ml colcemid 12–16 h before electroporation. Cells were then pelleted and resuspended (5 × 10^6 cells/ml) in 12 × 75 mm polyurethane tubes. To each tube was then added linearized pECH-mSATB1 (40 μg) and linearized pSV2neo (1 μg; Ref. 50). After incubation for 10 min on ice, each mixture was transferred to a cuvette (Evergreen Scientific, Los Angeles, CA) containing electrodes 1 cm apart, and 1200 V were applied using a Zapper electroporation unit (University of Wisconsin Medical Electronic Lab, Madison, WI) with capacitors charging at the high voltage for 2.5 ms. After incubation on ice for 10 min, cells were transferred to a 100 × 20 mm tissue culture dish containing 10 ml of DMEM/10% FCS and incubated for 48 h (37 °C, 5% CO₂ in air). Cells were then plated in 24-well plates (2.5 × 10^6 cells/well) in 1 ml of DMEM/10% FCS containing G418 (600 μg/ml of active drug). Every 3–4 days an additional 0.25 ml of G418 containing medium was added. Clones expressing SATB1 were 

peptidase, 2 μl of various dilutions of preimmune rabbit serum, anti-

SATB1 antiserum or anti-CDP/Cux antiserum were added to the nu-

clear extracts before the addition of the radiolabeled probe.

**Radiolabeling and Immunoprecipitation**—For radiolabeling with 

**DNasel Footprinting**—DNasel I footprinting of the isolated bands was performed essentially as described by Landolphi et al. (46). Ten EMSA reactions were performed as described above using approxi-

mately 100 kDa upon SDS-PAGE and silver staining. The peak 

fraction (fraction 54) was used for EMSA analysis and Western blotting (see "Results"), and fraction 54 was subjected to microsequence analysis as described below.

**Microsequence Analysis of Fraction 54**—Following SDS-PAGE and detection of the stained bands with Coomassie Blue, fraction 54 was subjected to in-gel digestion, and peptides were prepared for HPLC separation essentially as described previously (43). The peptides were separated on a reverse phase HPLC column (see "Results") and identified using on-line HPLC analysis of phenylthiohydantoin derivatives.

**Radiolabeling and Immunoprecipitation**—For radiolabeling with 

**Nucleosome Interference Assay—** The nucleosome experiment was performed essentially as described by Hayes and Tullius (44). Each DNA fragment was radiolabeled at one end of one strand only as indicated in the figure legends and subjected to hydroxyradical treatment by adding 10 μl of 0.2 mM (NH₄)₂FeSO₄/0.4 mM EDTA/3% H₂O₂/10 mM 1-ascorbate, to 70 μl (300 ng) of labeled fragment. The reaction mixture was incubated at 37 °C for 30 min. The reaction was quenched with 15 μl of 0.1% thiosurea/92 μl of 0.2 mM EDTA. The reaction mixtures were then digested by proteinases K, S1 nuclease, and RNase A, and the retarded bands and free probe were excised from nine lanes essentially as described previously (45). One lane was kept as reference. The probes were eluted from the gel by the crush and soak method using 0.2 M NaCl-TE as described by Dickinson et al. (15). The samples were elec-

phosphatase (Sigma, St. Louis, MO) prior to electrophoresis on an 8% sequencing gel together with probes subjected to hydroxyradical treatment (control lane) or Maxam-Gilbert sequencing reactions (45) at 55 W for the appropriate amount of time. The gel was fixed in 10% methanol/acetic acid, dried, and exposed for 1–2 days to x-ray film at −70 °C.

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mRNA were identified by Northern hybridization using a SATB1-specific probe.

**Nuclear Matrix Isolation and MAR Binding Assay**—Nuclei of the EL-4 T cell line were obtained by hypotonic lysis and purified by centrifugation through a cushion of 2 M sucrose, and nuclear matrices were isolated as described previously (1, 8). Nuclear matrix binding was determined in an *in vitro* DNA-binding assay (8). Briefly, plasmids containing the wild type or mutant L2a elements were linearized with *Nde*I and end-labeled with [γ-32P]ATP. 20–50 ng of each plasmid were incubated with nuclear matrices (2 h, room temperature) in the presence of 37–40 μg of unlabeled *E. coli* carrier DNA. Insoluble matrix proteins were pelleted and washed extensively to remove unbound DNA, treated with proteinase K, and extracted with phenol-chloroform, and the bound DNA was precipitated and electrophoresed on 0.7% agarose gels. Gels were dried on nylon membranes for autoradiography and the bound DNA was precipitated and electrophoresed on 0.7% DNA, treated with proteinase K, and extracted with phenol-chloroform, proteins were pelleted and washed extensively to remove unbound DNA.

The ratio of binding of wild type L2a element to BrMAR was taken as 1.0, and the relative binding of mutant L2a elements was calculated on this basis.

**RESULTS**

**An L2a-binding Protein Isolated from Calf Thymus Shows Homology to SATB1**—EMSA analysis performed with radiolabeled L2a probe and nuclear extracts from mouse (19) or calf thymus (see below) yields a retarded band with the same mobility as band 1 shown by Lee et al. (19) to reflect binding of protein L2a-P1. A protein with properties similar to L2a-P1 was purified from calf thymus nuclear extract as described under “Experimental Procedures.” The final purification step involved affinity purification on a portion of the 200(L+S) fragment (called 100(L)), which binds L2a-P1 and gives rise to retarded band 1 in EMSA assays with VL3 nuclear extract (19). Three partially resolved bands of similar mobility (100–110 kDa) were observed upon Coomassie Blue staining of the affinity-purified fractions, consistent with the estimated molecular mass of mouse L2a-P1 (19). EMSA analysis using the 100(L) probe and purified protein that had been subjected to SDS-PAGE and renatured from guanidine HCl gave rise to a specific retarded complex (Fig. 2A). These results suggested that the isolated protein could be the calf homologue of L2a-P1.

Though only a very small amount of purified protein was obtained, the partial amino acid sequence of a peptide obtained from two incompletely resolved bands of fraction 54 showed significant homology in a data base search to SATB1, a protein whose cDNA was originally cloned from a testis cDNA library but that is reported to be expressed only in thymus (15, 47) (Fig. 2B). Although the SATB1 cDNA encodes a protein of molecular mass of 87 kDa, it is reported to migrate on SDS-PAGE as several closely spaced components of molecular mass of approximately 103 kDa, possibly due to phosphorylation or other post-translational modifications (47). This is comparable with the M₀ observed for the L2a-P1 protein (19). To test whether the isolated calf thymus protein was SATB1, Western blot analysis was performed using a polyclonal anti-human SATB1 antiserum. As shown in Fig. 2C, all three partially resolved components were detected by the anti-SATB1 antiserum but not by normal rabbit serum. Finally, to determine whether the retarded bands of mobility comparable with band 1 were due to calf SATB1, an EMSA assay was performed in the presence of polyclonal anti-SATB1 antiserum. As shown in Fig. 2D, anti-SATB1 antiserum abolished the appearance of band 1 and gave rise to a supershifted band. Taken together, these results demonstrate that calf SATB1 gives rise to ret-
2. These results suggest that mouse L2a-P1 is antigenically related to SATB1 and that the L2a-P2 protein that gives rise to band 2 is not (19).

Because the above experiment shows only that L2a-P1 is antigenically related to SATB1, it was necessary to test whether authentic mSATB1 gives rise to band 1 in an EMSA. The mouse BW5147 thymic lymphoma cell line, which gives no band 1 in EMSA assays (19) and does not produce mSATB1 mRNA (Fig. 3B), was therefore stably transfected with pECHmSATB1, an expression construct encoding mSATB1. Subclone A11 and a number of additional subclones expressed an appreciable amount of mSATB1 mRNA (though less than VL3) (Fig. 3B), and A11 nuclear extract gave rise to a complex of similar mobility to band 1 in an EMSA assay performed with the 200(L+S) probe (Fig. 3A). Furthermore, anti-SATB1 antiserum but not normal rabbit serum abolished and supershifted the band 1 complex obtained with both A11 and VL3 nuclear extracts (Fig. 3A). These results strongly suggest that L2a-P1 is mSATB1. Importantly, band 2 was not supershifted by anti-SATB1 antiserum.

**Binding of L2a-P1 to MAR Probes**—The binding specificity of the L2a-P1 protein in VL3 nuclear extract was compared with that reported for human SATB1 (hSATB1) (15) in direct binding and competition EMSA assays. The hSATB1 was shown to bind a pentamer of the wild type binding site ((25)5), referred to as WT, derived from the 3′ MAR of the immunoglobulin heavy chain enhancer, but not to a mutated version ((24)4) (referred to as MUT) of the WT probe (15). In EMSA assays using VL3 nuclear extract and radiolabeled WT probe, all bands were efficiently competed by a 50-fold excess of unlabeled WT fragment (Fig. 4A) and incompletely competed by as much as a 500-fold excess of the 200(L+S) fragment. The less efficient competition by the 200(L+S) fragment may reflect the fact that it is a monomer, whereas the WT probe is a pentamer, but it is also possible that the affinity of L2a-P1 for the WT probe binding site is greater than that for its binding site in the 200(L+S) fragment. No competition was obtained with the MUT fragment. In EMSA assays using radiolabeled 200(L+S) fragment as probe (Fig. 4B), unlabeled 200(L+S) and WT fragments but not MUT fragment competed for both bands with WT competing somewhat more efficiently than 200(L+S) fragment.

The ability of L2a-P1 to bind to the WT probe and the ability of the WT but not the MUT probe to compete for L2a-P1 binding to WT and 200(L+S) probes suggest that on the basis of

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**FIG. 3.** Panel A, effect of preimmune rabbit serum or anti-SATB1 antiserum (1:20 dilution) on EMSA assays performed with the 200(L+S) probe and nuclear extracts from VL3 (2 μg protein/lane) and A11 (6 μg protein/lane) cells. Panel B, Northern hybridization of total RNA (20 µg/well) from various cell lines with a ScaI/XmnI SATB1 cDNA probe (approximately 700 nucleotides in length) derived from the pmAT construct (see “Experimental Procedures”).

**FIG. 4.** Panel A, competition EMSA performed with VL3 nuclear extract (2 μg protein/lane), radiolabeled wild type MAR probe and various unlabeled competitor DNAs. Band 3 is likely to represent binding of more than one protein to the concatenated probe that is a pentamer of the binding site. Panel B, competition EMSA performed with VL3 nuclear extract (2 μg protein/lane), radiolabeled 200(L+S) probe, and various unlabeled competitor DNAs. Panel C, EMSA assays performed with VL3 nuclear extract (1.5 μg protein/lane) and radiolabeled 200(L+S) or wild type MAR probe as indicated beneath the figure. Anti-SATB1 antiserum or preimmune serum (1:20 dilution) was added as indicated above the figure.
of these measures of binding specificity, L2a-P1 behaves like SATB1. This conclusion is strengthened by the finding that anti-SATB1 antiserum supershifts the band 1 complexes formed by VL3 nuclear extract and the WT MAR probe (Fig. 4C). Importantly, band 2 formed with either the 200(L+S) or the WT MAR probe was not supershifted by anti-SATB1 antiserum (Figs. 3A and 4C).

Anti-SATB1 Antiserum Precipitates a Protein with a Molecular Mass of 110 kDa from VL-3 Nuclear Extract—Proteins present in VL-3 and A11 nuclear extract were labeled with either [35S]methionine/[35S]cysteine or [32P]orthophosphate and precipitated with the anti-SATB1 or preimmune rabbit serum (see “Experimental Procedures”). Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions followed by autoradiography. Each lane contains the immunoprecipitate from approximately 5 × 10⁶ cells.

L2a-P1 and SATB1 Have Similar DNA Sequence Requirements for Interaction with the WT Probe—Dickinson et al. (15) used the missing nucleotide assay to determine the nucleotides required for interaction of bacterially produced SATB1 with a dimer of the WT MAR sequence. We have used both the missing nucleoside approach and DNase I protection assays to study the interaction of L2a-P1 and L2a-P2 with the L2a element.

In the missing nucleoside approach (44), double-stranded probe molecules are radiolabeled at one end of one strand only, and an average of one single-stranded nucleoside gap is introduced into each probe molecule. An EMSA is then performed with limiting amounts of probe, retarded bands are recovered, and the distribution of probe molecules in each band is displayed on a DNA sequencing gel. Failure of a probe of a certain length to appear in a retarded band suggests that a gap at that region interfered with binding of the protein that gives rise to that band. Generally, probes that fail to bind protein are over-represented in the remaining unbound probe, which is also run on the sequencing gel (referred to as “free probe”).

To compare the binding of L2a-P1 with that previously observed for SATB1, we performed the missing nucleoside assay using VL3 nuclear extract and the (25)₂ WT probe, which is a dimer of the SATB1 binding site (47). The under-representation of certain bands in the retarded band and the over-representation of the same bands in the free probe lane indicate that loss of a nucleoside at those positions interferes with protein binding.
obtain a footprint with bacterially produced mSATB1 in our laboratory for direct comparison, and the A11 cell line (see above) produced too little mSATB1 protein for use in the missing nucleoside experiment.

Use of the Missing Nucleotide Experiment to Study the Binding of L2a-P1 (mSATB1) to the L2a Element—Previously, Lee and co-workers (19) had performed DNase I footprinting of the L2a element by mixing VL3 nuclear extract with the L2a probe and performing EMSA analysis without isolating the individual retarded bands 1 and 2. The two protected regions (L and S) and the intervening DNase I-hypersensitive region (referred to here as the INTER-LS region) identified in that study are shown in Fig. 1B. Also highlighted is a palindromic 12-mer present in the INTER-LS region.

In the present study using the missing nucleoside approach, the complex formed by L2a-P1 (i.e., band 1) was isolated before electrophoresis on the sequencing gel. As shown in Fig. 7A and summarized in Fig. 7E, nucleosides in the lower strand of the previously described L site (bracket I) were found to be important for L2a-P1 binding as seen by the absence of probe molecules in this region of the band 1 lane and enhancement of the corresponding fragments in the free probe lane. Loss of nucleosides in the L region of the upper strand (bracket VI in Fig. 7, B and E) also appeared to reduce binding. Interestingly, gapping at nucleosides in the middle of the previously described S site in the bottom strand also interfered with binding of L2a-P1 (bracket III in Fig. 7, A and E).

The bands corresponding to gapping at nucleosides in the INTER-LS region of both strands were enhanced in the band 1 lane as compared with the free probe and control lanes (bracket II of Fig. 7, A and E, bracket VII of Fig. 7, B and E). We interpret this to mean that gapping of probes in the INTER-LS region favors L2a-P1 binding, possibly by facilitating a conformational change in the DNA adjacent to the L2a-P1 interaction sites.

Use of DNase I Footprinting to Determine the Interaction of L2a-P1 (mSATB1) with the L2a Element—VL3 nuclear extract was used to footprint the L2a element, and in contrast to the previous study (19), the individual retarded bands were isolated before electrophoresis on the sequencing gel. The complex formed by L2a-P1 (retarded band 1) yielded a strong footprint in the L site of the bottom strand and a weak footprint in the top strand (brackets I and VI of Fig. 8, A and B, respectively; summarized in Fig. 8E), demonstrating that bound L2a-P1 contacted nucleotides in this region. Consistent with the results of the missing nucleoside experiment showing some binding in the S region, a short DNase I-protected region was apparent in the S region of the bottom strand (bracket III, Fig. 8, A and E).

The footprint of the complex formed by L2a-P1 demonstrated DNase I hypersensitivity in the INTER-LS region, which ex-
tended several nucleotides into the S site (brackets II and VII of Fig. 8, A and B, respectively; summarized in Fig. 8E). This DNase I-hypersensitive region overlapped with the enhanced region seen in the missing nucleoside assay (brackets II and VI of Fig. 7E), and its presence is consistent with a local alteration of DNA conformation that is induced by L2a-P1 binding. In summary, the results of the DNase I footprinting studies of the complex of L2a-P1 with the L2a element demonstrate that most of the nucleosides required for L2a-P1 binding to the L site, as implicated by the missing nucleoside assay, are protected by L2a-P1 binding.

The Requirements for Binding of L2a-P2 to the L2a Element Are Distinct from Those of L2a-P1—Previous studies (19) suggested that a protein(s) of 200 kDa or greater gave rise to retarded band 2 in EMSA assays performed with VL3 nuclear extract and an L2a probe. Simultaneous with the studies of the band 1 complex described above, the interactions with the L2a element of protein(s) in retarded band 2 were studied using the missing nucleoside experiment and DNase I footprinting.

In the missing nucleoside experiment performed with VL3 nuclear extract, the requirement for binding of L2a-P2 appear different from L2a-P1. Nucleoside gapping in the lower strand of the S site significantly interferes with band 2 formation (brackets IV and V, Fig. 7A), whereas gapping in the L site, which interfered with L2a-P1 binding (bracket I, Fig. 7A), appears to have no effect. Also, unlike the case with band 1, nucleoside gapping in the INTER-LS region does not appear to favor formation of band 2. As summarized in Fig. 7E, these results suggest that the S site of the L2a element is the primary interaction site of protein L2a-P2.

However, previous studies had suggested that L2a-P1 binds more strongly to the L2a probe than L2a-P2 (19). Under the conditions of limiting amounts of probe employed for the missing nucleoside experiment, the L2a-P1 and L2a-P2 proteins are competing for probe molecules, and in this situation an incomplete assessment of the binding specificity of the L2a-P2 protein may be obtained. We therefore performed the missing nucleoside experiment using nuclear extract derived from the BW5147 cell line, which does not express L2a-P1 and thus gives rise to only band 2 in the EMSA assay. As shown in Fig. 7C for the lower strand and summarized in Fig. 7E (brackets VIII and X), in the absence of L2a-P1 and band 1 formation, gapping in both the L and S regions interferes somewhat with the formation of band 2 complexes. Gapping in the L and S sites of the upper strand has little or no obvious effect on band 2 formation (Fig. 7D). However, gapping in either strand in the INTER-LS region appears to favor formation of band 2 complexes (bracket IX, Fig. 7C and bracket XI, Fig. 7D, summarized in Fig. 7E). Thus in the absence of competing L2a-P1, the L2a-P2 protein appears to show interaction with the L site as

Fig. 8. Panels A–D. DNase I footprinting was performed on retarded bands formed by VL3 or BW5147 nuclear extracts on probes containing the L2a element. Regions delineated by brackets to the right of the figures correspond to regions implicated in protein binding are are summarized in Panel E. In Panels A and B where both bands 1 and 2 formed by VL3 nuclear extract are analyzed, the brackets closest to the autoradiogram refer to band 1 and the right-most brackets (labeled in italics) refer to band 2. Nuclear extract, probe and radiolabeled strand for each panel are as follows: A, VL3, 150(L+S), top strand; B, VL3, 100(L+S), bottom strand; C, BW5147, 150(L+S), bottom strand; D, BW5147, 100(L+S), top strand. Panel E, summary of results of DNase I footprinting experiments. Nuclear extract and band analyzed are shown to the left of the L2a sequence in each line. Regions of DNase I protection (solid lines) or hypersensitivity (dotted lines) are labeled with the same Roman numerals that appear in panels A–D. The L, S, and DNase I-hypersensitive region (labeled INTER-LS) described previously (19) are shown above the figure for reference. The 12-nucleotide palindrome lying in the latter region is underlined.
shown in Fig. 10, anti-CDP antiserum but not normal rabbit serum abolished formation of retarded band 2 in EMSA assays performed with VL3, BW5147, and A11 nuclear extracts but had no effect on band 1, suggesting that the L2a-P2 protein may be CDP/Cux. Taken together with the finding that formation of band 2 was inhibited by distamycin A (Fig. 9), these results suggest that binding to the narrow groove contributes significantly to interaction of CDP/Cux with the 200(L+S) probe.

**EMSA Performed with Mutant L2a Elements Further Suggest That L2aP1 and L2a-P2 Bind Preferentially to the L Site and S Site, Respectively.—**To further analyze the sites in the L2a element required for formation of bands 1 and 2, mutations or deletions were introduced in regions implicated in L2a-P1 or L2a-P2 binding (Fig. 11A). In an EMSA performed using VL3 nuclear extract and wild type or mutant L2a elements as probes, mutations in the L site and in the middle of the S site had little effect on formation of band 2 but effectively abolished band 1 (Fig. 11B, lane M1). In results not shown, mutations in the L site alone nearly abolished band 1, although not as completely as when mutations in S were present as well. This suggests that L2a-P1 binds preferentially to the L site, whereas L2a-P2 binds to regions in the S site unaffected by the mutations. Also, as shown in Fig. 11B (lane M4), leaving the L site intact and deleting the S site and most of the INTER-LS region virtually abolished band 2 formation and appeared to enhance band 1 formation. These results support the above conclusion that L2a-P1 and L2a-P2 preferentially bind to the L and S sites, respectively.

**The L2a Element Exhibits Properties of a MAR by Binding to the Nuclear Matrix.—**A direct nuclear matrix-binding assay was performed to test whether the L2a element behaves as a MAR (Fig. 12). A linearized pUC18 construct containing the wild type L2a element was radiolabeled, and its binding to nuclear matrices prepared from the mouse T cell line, EL4, was compared with that of a known MAR (8) and with vector alone as internal positive and negative controls, respectively (see “Experimental Procedures” and the legend to Fig. 12). The construct containing the wild type L2a element bound as strongly as the BrMAR positive control to the nuclear matrix, whereas the vector control showed no binding (Fig. 12A, lane B under the WT bracket). This was a highly reproducible observation and strongly suggests that the wild type L2a element contains a MAR.

To determine whether the MAR involved sequences within the L2a element implicated in the above studies to interact
with the L2a-P1 and L2a-P2 proteins, nuclear matrix binding of L2a elements containing mutations or deletions that affected binding of L2a-P1 and/or L2a-P2 (Fig. 11, A and B) was compared with binding of the wild type L2a element. As summarized in Fig. 12B, alterations in sites affecting either L2a-P1 or L2a-P2 binding reduced nuclear matrix binding to 15–28% of that observed for the wild type L2a element. Thus sequences required for binding of both L2a-P1 and L2a-P2 contribute to the nuclear matrix-binding properties of the L2a element.

**DISCUSSION**

Previous studies in which **CD8a** gene constructs containing varying amounts of 5′-flanking regions were stably transfected into BW5147 cells suggested the presence of an element between −3,800 and −4,700 nucleotides upstream of the gene, which was a target of negative regulation in that cell line (19). Negative regulatory activity appeared to reside in a 270-nucleotide subfragment of this region (nucleotides −4,430 to −4,700), which contained an element (L2a) that interacted with nuclear proteins. The L2a element lies within a DNase I-hypersensitive site specific to CD8-positive cells (30). Lee and co-workers (19) observed two retarded complexes (bands 1 and 2) in EMSA studies with the L2a element and nuclear extracts from all CD8-positive cell lines tested, mouse thymus, and two of four double-negative T cell lines. In contrast, only the larger complex (band 2) was observed in EMSA assays using nuclear extract from a variety of CD8-negative cell lines. Evidence was presented that the L2a-P1 protein (molecular mass, approximately 100 kDa), which gives rise to retarded band 1, competes more strongly for binding to the L2a element than L2a-P2 (molecular mass, >200 kDa), which gives rise to band 2. A model was proposed in which interaction of the ubiquitously expressed L2a-P2 protein with L2a negatively affects **CD8a** gene expression, and its displacement by L2a-P1 has a positive effect on **CD8a** gene expression.

The present studies strongly suggest that the L2a element is a MAR and that the L2a-P1 and L2a-P2 proteins described by Lee et al. (19) are the MAR-binding proteins SATB1 and CDP/Cux, respectively. That the L2a element is a MAR is suggested by its binding to T cell nuclear matrix preparations (Fig. 12A), and the observation that matrix binding is greatly reduced by mutants and deletions throughout the L2a element (Fig. 12, A and B) suggest that the MAR properties reside in several regions of the L2a element. That L2a-P1 is SATB1 was demonstrated by results of EMSA supershift and immunoprecipitation studies using anti-SATB1 antiserum (Figs. 3A and 5, A and B), direct binding and competition EMSA studies using wild type and mutant MAR probes (Fig. 4, A and B), and

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**FIG. 11.** Panel A, nucleotide sequences of wild type (WT) and mutant (M1–M4) L2a elements used for EMSA and nuclear matrix-binding assays. The L, S, and INTER-LS regions are as shown in Figs. 1B, 7E, and 8E. The palindromic 12-mer in the INTER-LS region is underlined. All nucleotide sequences were verified by nucleotide sequence analysis of both DNA strands. The restriction sites indicated at the bottom of the figure are present in the following mutants: BglII, M1, M2, and M3; ClaI, M1; BamHI, M1; StyI, WT and M1. Panel B, EMSA performed with VL3 nuclear extract and radiolabeled wild type or mutant L2a elements. Lanes containing WT (200/L+S) or mutant probes are labeled at the top of the figure.

**FIG. 12.** Panel A, nuclear matrix binding assay demonstrating that the L2a element is a MAR. Linearized, radiolabeled plasmids (L2a-Vec) containing the wild type (WT) or one of four mutant L2a elements (M1–M4, see Fig. 10A for their nucleotide sequences) were incubated with nuclear matrix prepared from the EL-4 T cell line, a known MAR (BrMAR, positive control; Ref. 8) and vector (negative control) as described under *Experimental Procedures.* Lanes labeled L contain 25% of the radioactivity input into each MAR assay, and the adjacent lanes labeled B contain the bound DNA recovered from the corresponding assay. The BrMAR probe has been shown to undergo nicking over time, causing it to migrate with an aberrant mobility (shown by an asterisk). Panel B, relative binding of wild type and mutant L2a elements to EL-4 nuclear matrices. Radioactivity of the test sample (L2a-Vec) is normalized in each lane to the binding of the positive MAR control, BrMAR, and the value obtained with the wild type L2a element is taken as 1.0. Values shown for M1–M4 represent percentage of wild type binding, and each represents the average of 5–10 determinations. Nuclear matrix binding of the wild type L2a element was comparable with that of the BrMAR positive control.
inhibition of binding by distamycin A (Fig. 9). That L2a-P2 is CDP/Cux is suggested by EMSA supershift experiments using anti-CDP antiserum (Fig. 10), the general size of the protein that gives rise to the band 2 complex, and the similarity of the L2a-P2-binding sequences in the L2a element with the binding specificity reported for DNA-binding cut domains of the CDP/Cux protein (51, 52). The distamycin A sensitivity reported here for band 2 (Fig. 9) suggests that like SATB1, the CDP/Cux protein interacts significantly with the minor groove of DNA probes. Based on results of the present studies, the names SATB1 and CDP/Cux will be used below in place of L2a-P1 and L2a-P2, respectively.

The SATB1 protein was described as a special AT-rich DNA-binding protein present in high concentration in thymus (15, 47). SATB1 was shown to bind in the shallow groove of DNA sequences in which one of the strands is ATC-rich. The site to which SATB1 binds in the 3' MAR of the IgH enhancer shows a propensity to become stably base-unpaired under conditions of torsional stress (53). Besides containing a proposed unique DNA-binding motif (47), SATB1 also contains a homeodomain that may also participate in its interaction with DNA. Although no function for SATB1 has yet to be definitively shown, the studies of Lee and co-workers (19) together with the present results suggest that SATB1 may play a positive role leading to transcription of the mouse CD8a gene.

The CDP/Cux protein is named for the human (CDP; Ref. 54) and mouse (Cux; Ref. 55) homologues of the Drosophila homeodomain protein, Cut, which determines cell fate of several tissues of different embryonic origins (56, 57). The CDP/Cux proteins contain a single homeodomain and three conserved DNA-binding repeats (called cut repeats) approximately 70 amino acids in length that exhibit subtle differences in DNA binding specificity (51, 52, 54). Although cut repeats 2 and 3 generally bind AT-rich sequences, they discriminate among similar nucleotide sequences. The CDP/Cux protein was suggested to negatively regulate the sea urchin histone H2B gene by displacing or competing for binding of a positively acting CCAAT box-binding factor (58). Negative regulation by CDP was demonstrated for the human cytochrome gene, gp91-phox (31), and CDP binding sites are present in the genes encoding human γ-globin (59, 60) and rat neural cell adhesion molecule (55). Because not all CDP binding sites contain CCAAT boxes (31), the mechanism of repression by CDP/Cux is likely to have a more general basis.

We suggest that the L2a element, located in a region shown to be DNase I-hypersensitive and thus in an active and presumably exposed state in CD8-positive T lymphocytes (30), performs its function in regulating transcription of the mouse CD8a gene by behaving as a MAR. Based on studies of the interactions of SATB1 and CDP/Cux with the L2a element using the missing nucleoside experiment, DNase I footprinting, and EMSA studies using mutant L2a elements, we suggest that CDP/Cux represses CD8a gene transcription by binding to the L2a element and that its displacement by SATB1 favors transcription of the CD8a gene.

The L site defined previously by DNase I footprinting (19) appears to be the preferred binding site for SATB1 on the L2a element. This is suggested by missing nucleoside and DNase I protection experiments (summarized in Figs. 7E and 8E, respectively) and by EMSA assays using mutant L2a elements (Fig. 11B). Although SATB1 shows some evidence of interaction with the S site in the missing nucleoside experiment (region III in Fig. 7E) and in an EMSA assay performed with an L2a mutant probe with a wild type S region and altered L region (data not shown), the L site is its primary site of interaction with the L2a element. The CDP/Cux protein binds primarily to the S site as shown by the missing nucleoside (summarized in Fig. 7E) and DNase I protection experiments (summarized in Fig. 8E), but it can interact with the L site as shown in missing nucleotide experiments with BW5147 nuclear extract (Fig. 7E) and in DNase I protection experiments with BW5147 and VL3 nuclear extracts (Fig. 8E). When SATB1 is present, however (i.e., in VL3 nuclear extract), missing nucleoside experiments demonstrate that no binding of Cux/CDP to the L site is observed (Fig. 7E). Importantly, EMSA assays suggest that simultaneous binding of SATB1 and CDP/Cux does not occur because only two retarded bands are observed, one of which is shifted by anti-SATB1 antiserum and the other by anti-CDP/Cux antiserum. It cannot be ruled out, however, that both proteins may bind simultaneously to the L2a element under other conditions (e.g., in an intact chromosome).

The conformation of the region between the L and S sites appears to be affected by binding to the L site. The results of missing nucleoside experiments performed with VL3 nuclear extract suggest that gaps through the entire length of this region but especially in the palindromic 12-mer at the end of the INTER-LS region favor binding of SATB1 to the L site (summarized in Fig. 7E). In this experiment, such gaps appear to have little or no effect on Cux/CDP binding to the S site, but this could be misleading because under conditions of limiting probe, SATB1 may have bound to all such gapped molecules. Of interest in this regard is the finding that in studies with BW5147 nuclear extract that lacks SATB1, gaps in the intermediate region closest to the S site do appear to favor CDP/Cux binding (summarized in Fig. 7E). Nucleoside gaps in the IN-

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3 T. Kohwi-Shigematsu, personal communication.
TER-LS region may favor binding to the L or S site by facilitating a conformational change in the DNA that occurs upon SATB1 binding. That a major conformational change occurs upon SATB1 binding to the L site is indicated by the DNase I hypersensitivity of the entire INTER-LS region but especially the region closest to the S site in footprints of SATB1 (summarized in Fig. 8E). Similarly, DNase I hypersensitivity studies suggest that CDP/Cux binding can result in a significant but less dramatic conformational change in the INTER-LS region adjacent to the S site (summarized in Fig. 8E).

A model to explain the interaction of the SATB1 and CDP/Cux proteins with the L2a element is shown in Fig. 13. In the absence of SATB1, we believe CDP/Cux binds primarily to the S site, but that it may also interact with the L site with one or more of its multiple cut domains (51, 52). That CDP/Cux does not use the L site as its primary binding site is suggested by the significant reduction in band 2 in the EMSA performed with the M4 mutant L2a element (Fig. 11B). Some distortion of the INTER-LS region closest to the S site may result from binding of CDP/Cux. The interaction of CDP/Cux with the L site appears to be favored by gaps in the INTER-LS region as seen in the missing nucleoside experiment with BW5147 nuclear extract (Fig. 7D) and may lead to the distortion and resulting DNase I hypersensitivity observed with BW5147 nuclear extracts (Fig. 8, C and D). That such distortion is not a major factor in CDP/Cux association with its primary binding site is suggested by the missing nucleoside experiment with VL3 nuclear extract where CDP/Cux binds well to the S site of probe molecules that are not gapped in the INTER-LS region (Fig. 7A).

In the presence of SATB1, any CDP/Cux bound to or interacting with the L site is displaced by binding of SATB1. The present results suggest that the displacement of CDP/Cux is not due to simple competition for a common binding site, but results from the appreciable structural distortion induced by SATB1 throughout the INTER-LS region, particularly in the palindromic 12-mer adjacent to the S site (Fig. 8, A and B). Thus binding of SATB1 to the L site creates a structural distortion in the INTER-LS region that displaces CDP/Cux from the S site. We have called this mechanism a “displacement switch,” and it may represent a general mechanism for switching some MAR elements from a repressed to an active state (Fig. 13). In this instance, the presence of SATB1 on the L2a element and the structural distortion resulting from SATB1 binding may increase the element’s affinity for the nuclear matrix, thereby bringing the adjacent CD8α gene into an environment favorable to its transcription.

It is interesting that SATB1 binding is especially favored by palindromic gaps in the palindromic 12-mer (Fig. 7, A, B, and E) and that DNase I hypersensitivity induced by either SATB1 or CDP/Cux binding is greatest in this region as well (Fig. 8). Because palindromes are frequently sites for protein interaction, it is possible that an as yet unidentified protein interacts with this site and may function in concert with SATB1 and/or CDP/Cux to modulate CD8α gene expression. For example, such a protein may bind to the L2a element, either alone or together with CDP/Cux, and be displaced by binding of SATB1 and the conformational change it induces in the palindromic binding site. Such a protein could function to further modulate association of the L2a element with the nuclear matrix, or it could affect CD8α gene transcription in some other way. Recently, EMSA analyses using a probe containing the palindromic 12-mer but lacking L and S sites suggest the presence in BW5147 nuclear extracts of a protein that specifically binds to this region. The identity and binding properties of this protein are under investigation.

The model predicts that alterations in the L2a element that affect binding of SATB1 and/or CDP/Cux should have effects on transcription of the CD8α gene. We have recently shown that surface CD8α molecules are expressed by BW5147 cells stably transfected with a CD8α gene construct containing up to nucleotide −6,900 of the 5′-flanking region but lacking a 1.2-kb fragment containing the L2a element. Thus the transfected CD8α gene is not subject to the negative regulation seen when this region is present (19). Interestingly, results of studies on mice in which the SATB1 gene has been “knocked out” by homologous recombination have shown that within the abnormal and greatly reduced population of T cells present in lymph nodes of SATB1−/− mice, significant numbers of CD4-positive T cells are present but CD8-positive T cells are virtually absent. These results suggest that SATB1 is required for production of mature CD8-positive T lymphocytes and are consistent with our suggestion that binding of SATB1 to the L2a element plays a role in regulating expression of the CD8α gene.

Further information on the role of the L2a element and its associated proteins in CD8α expression should be forthcoming from stable transfection studies with constructs in which the wild type L2a MAR element has been replaced by mutant elements impaired in their interactions with SATB1 and/or CDP/Cux and from mouse knockout studies in which the L2a element has been deleted or modified. The model proposed for the replacement of CD8α from the L2a element by SATB1 can be further tested by in vitro studies with additional mutant L2a probes and with modified SATB1 and CDP/Cux proteins and/or domains.

Acknowledgments—We thank Terumi Kohwi-Shigematsu for providing SATB1-related materials and for allowing us to cite unpublished studies. We thank Jaquelin Dudley and Elisa Neufeld for anti-CD8 antibodies and helpful discussions, Phil Tucker and Henry Bose for helpful advice and comments during the course of this work, and Randy Goldblum for helpful suggestions and critical reading of the manuscript.

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