A Nuclear Matrix Attachment Region Upstream of the T Cell Receptor β Gene Enhancer Binds Cux/CDP and SATB1 and Modulates Enhancer-dependent Reporter Gene Expression but Not Endogenous Gene Expression*

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The rearrangement and expression of the T cell receptor β (TCRβ) gene is essential to early T lymphocyte development (1). Prior to TCRβ gene rearrangement, germline transcripts are initiated upstream of the Dβ1 gene segment in CD4–CD8– (double negative, DN) thymocytes (2, 3). Recombination of TCRβ gene occurs exclusively during the DN stage of thymocyte development by an ordered process wherein Dβ-Jβ rearrangement occurs prior to joining of a Vβ gene segment. Allelic exclusion operating at the level of Vβ to Dβ-Jβ rearrangement ensures that only one of the two possible TCRβ alleles are expressed by an individual T cell (4, 5). After VβDβ-Jβ rearrangement, a mature transcript is initiated from the Vβ promoter in a T cell-specific manner (6). To achieve the lineage-, stage-, and allele-specific TCRβ gene rearrangement and transcription, many cis-acting elements and their associated transacting factors are likely to be involved (4, 5).

To date, the TCRβ gene enhancer (Eβ) is the only cis-regulatory element demonstrated to be required for both the lineage- and stage-specific transcription and rearrangement of the TCRβ gene (7–12). Although the Vβ promoter is required for lineage-specific TCRβ transcription, its role in regulating Vβ gene rearrangement remains unclear (13–17). In addition to Vβ promoters and Eβ, there are likely other cis-regulatory elements involved in the control of various aspects of TCRβ gene rearrangement and transcription. In particular, nuclear matrix attachment regions (MAR) are a class of cis-regulatory elements found in many genetic loci that are distinct from transcriptional promoters and enhancers, and yet are often closely associated with these regulatory elements (18–20). MARs are typically AT-rich DNA sequences that bind to the nuclear matrix, often contain topoisomerase II cleavage sites, and exhibit a propensity for base unpairing when subjected to superhelical strain (21, 22). They have been proposed to be involved in transcription, DNA recombination, replication, and repair (23). In the immunoglobulin heavy chain (IgH) locus, MARs flank the intronic enhancer Eα and are in close proximity to VH promoters (22–26). Reporter gene assays in cell lines and transgenic mice have suggested that these MARs exert both positive and negative effects on IgH gene transcription and promote long range chromatin accessibility (27–31). A highly conserved MAR is also found 200 base pairs (bp) upstream of the intronic immunoglobulin κ (Igκ) enhancer in mouse, human, and rabbit (22, 32). Together, the Igκ MAR and enhancer promote demethylation, transcription, recombination, and somatic hypermutation of the locus although no specific function has been attributed to the MAR alone (33–36). The presence of MARs at other antigen receptor loci has not been reported.

To characterize novel cis-regulatory elements involved in controlling TCRβ gene rearrangement and/or transcription, we previously screened a 100-kb region of the TCRβ locus and identified along with Eβ 10 additional DNase I-hypersensitive sites (HS) (37). HS1 was previously shown to be just 400 bp upstream of Eβ and to be strongly induced during DN to DP thymocyte differentiation. In this report, we localize nuclear factor binding sites within HS1, characterize two nuclear factors that bind to HS1, demonstrate that HS1 is a nuclear matrix attachment region, and reveal the potential pitfalls of functional analyses of MARs outside of their natural chromosomal context.

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The abbreviations used are: TCR, T cell receptor; Eβ, Vβ-TCR gene enhancer; IgH, immunoglobulin heavy chain; Igκ, immunoglobulin κ light chain; MAR, nuclear matrix attachment region; HS, DNase I-hypersensitive sites; EMSA, electrophoretic mobility shift assay; DN, CD4–CD8– double negative; DP, CD4+CD8+ double positive; bp, base pairs; kb, kilobase pairs; DTT, dithiothreitol.
Experimental Procedures—A 780-bp BstXI-BgII DNA fragment containing HS1 was blunt-ended by Klenow fragment of DNA polymerase I, gel-purified, and cloned into the Smal site of Bluescript SK(−) (p780; Stratagene, La Jolla, CA). Probe II (300-bp AccI-BgII fragment), probe II* (300-bp AccI-BgII fragment with a deletion of 30 bp from the BgII end), probe III (128-bp AccI-BgII fragment), and probe IV (170-bp BgII fragment) were isolated from p780 plasmid and subcloned into the Smal site of pSP72 (Fig. 1A; Promega, Madison, WI). DNA fragments containing these probes (II, II*, III, and IV) were isolated from the resulting plasmids by Xhol-EcoRI digestion. Probes V and VIII were isolated from plasmid containing probe II* by BsgI-EcoRI or MboII-EcoRI digestion, respectively. Probes VI, VII, and IX were obtained from plasmid containing probe IV by digestion with MboII-BglII, BglII, or XhoI-BamHI, respectively.

Luciferase reporter constructs were prepared using the pGL2 promoter vector (Promega, Madison, WI). The existing SV40 promoter in pGL2 was deleted by BglII-HindIII digestion, and a 424-bp EcoRI-NcoI fragment containing the V6 intron was inserted to generate construct 1 (Fig. 8A). Construct 1 was then used to generate constructs 2 and 3. An 630-bp BglII-NcoI fragment containing Eκ was cloned into the BamHI site located downstream of the poly(A) site of the luciferase gene, generating construct 2. A 1000-bp BsgI-NcoI fragment containing HS1 and Eκ in their natural configuration was cloned into the same position of construct 1 to generate construct 3. Therefore, construct 3 differed from construct 2 only by having the 170-bp HS1 (Fig. 8A).

Nuclear Extracts—Nuclear extracts were prepared from DN and DP thymocytes of RAG-deficient mice and RAG-deficient mice complemented with lch, respectively (37). Nuclear extracts from DN thymocytes were prepared following the method of Forrester et al. (38). Nuclei were resuspended in 0.2 ml of buffer A containing 10 mM Tris–Cl, pH 7.5, 7.5 mM HEPES, 10 mM MgCl2, 1 mM EDTA, 50 mM NaCl, and 20% glycerol. An equal volume of buffer B supplemented with 420 mM NaCl was then added slowly to the nuclei suspension and mixed immediately. The resulting mixture was incubated on ice for 10 min and then centrifuged at 14,000 g for 5 min at 4 °C. Supernatants at 4 °C were dialyzed against a low salt buffer containing 20 mM Tris–Cl, pH 7.5, 10 mM MgCl2, 1 mM EDTA, and 10% glycerol, and then dispersed into small aliquots and stored at −80 °C. Protein concentrations of nuclear extracts were determined using a protein assay kit from Bio-Rad.

Electrophoretic Mobility Shift Assays (EMSA)—All DNA probes for EMSA were end-labeled with 32P-dCTP using Klenow fragment of DNA polymerase I. Reaction mixtures were passed through a Nuc-trap column (Stratagene, La Jolla, CA) and precipitated. The labeled DNA fragments were further purified on a 5% polyacrylamide gel. For EMSA, 0.2–20 μg of nuclear extract or 1.25–5 ng of purified Cux/CUDP protein were incubated with 2 ng of labeled DNA probe (∼20,000 cpm) in 24 μl of reaction buffer containing 10 mM HEPES–KOH, 10 mM Tris–HCl, pH 7.4, 50 mM NaCl, 2 mM EDTA, and 0.25 μg/ml bovine serum albumin. The mixture was centrifuged for 5 min and the pellet was resuspended in 1 ml washing buffer containing 10 mM Tris–Cl, pH 7.5, 50 mM NaCl, 2 mM EDTA, and 0.25 μg/ml bovine serum albumin. The nuclear complex was treated with proteinase K in the presence of 10 mM Tris–Cl, pH 7.4, 2 mM EDTA, and 5 μg/ml salmon sperm DNA at 37 °C overnight. The mixture was extracted with phenol–chloroform, and DNA was recovered by alcohol precipitation. Bound DNA was resolved on a 4.5% acrylamide gel and visualized by autoradiography.

Southern Blotting Assay—DNA from DN thymocytes was digested with HindIII, and Southern blots were prepared as described by Zong and Scheuermann (39) with minor changes. Briefly, 10 μg of nuclear matrix (corresponding to 1 × 108 nuclei) was incubated with 2 ng of each labeled probe, and 20 or 40 μg of sonicated E. coli DNA at the room temperature for 1 h in a total volume of 100 μl containing 10 mM Tris–Cl, pH 7.4, 50 mM NaCl, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 0.25 mg/ml bovine serum albumin. The mixture was centrifuged for 5 min and the pellet was resuspended in 1 ml washing buffer containing 10 mM Tris–Cl, pH 7.4, 50 mM NaCl, 2 mM EDTA, and 0.25 mg/ml bovine serum albumin. The nuclease complex was treated with proteinase K in the presence of 10 mM Tris–Cl, pH 7.4, 2 mM EDTA, and 5 μg/ml salmon sperm DNA at 37 °C overnight. The mixture was extracted with phenol–chloroform, and DNA was recovered by alcohol precipitation. Bound DNA was resolved on a 4.5% acrylamide gel and visualized by autoradiography.

Northern Blotting Assay—Total RNA was isolated from thymocytes of wild type and homozygous mutant mice that were deleted of HS1 from the TCRβ locus (36). Total RNA was fractionated on a formaldehyde gel and transferred onto Zeta-probe filters. Filters were hybridized individually with a C2 probe, a V6 probe, or a D1-J1 intronic probe and exposed to x-ray film. The probes used were as follows: C2 probe, a 430-bp cDNA fragment; V6 probe, a 190-bp EcoRI-StuI fragment containing the V6 gene segment; and D1-J1 intronic probe, a 663-bp PCR product.

Results—Localization of Two Nuclear Factor Binding Sites Within HS1—HS1 was previously mapped to a 780-bp BstXI-BgII fragment located 400 bp upstream of Eκ (Fig. 1A) (37, 42). To identify protein factors that bind to HS1, we performed EMSAs with nuclear extracts from DP thymocytes because HS1 was most prominent in these cells (37). Two subfragments of HS1 were used as probes in EMSAs, a 480-bp BstXI-AciI fragment (probe I) and a 300-bp BglII fragment (probe II) (Fig. 1A). While probe I failed to yield any complex, probe II hybridized to two shifted complexes in EMSA (Fig. 1B, lanes 1–4). The same complexes were also observed when the entire 780-bp BstXI-BgII fragment was used as a probe, although at a substantially reduced level probably due to the large size of the probe (data not shown).

We next sought to narrow down the location of the factor...
binding sites within probe II. Previously, sequence analyses showed the presence of consensus motifs of AP-1, Oct-1, and c-Myb binding sites in the 5′ region of the 300-bp probe II in both mouse and human (37, 42). Therefore, two subfragments of probe II, a 128-bp AccI-BglI fragment (probe III) and a 170-bp BsgI-BglII fragment (probe IV), were used in EMSA to test if factor binding occurred at any of these consensus sites. Despite the presence of the consensus nuclear factor binding sites within probe III, it did not generate any complex in EMSA (Fig. 1B, lanes 5 and 6). Likewise, a slightly larger AccI-BsmAI fragment did not produce any complex (Fig. 1A and data not shown). In contrast, probe IV detected the two shifted complexes as seen when probe II was used (Fig. 1B, lanes 7 and 8). Thus, although the 128-bp fragment (probe III) contained the various sequence motifs (42), the complexes detected under our reaction conditions resulted from nuclear factor binding to cis elements in the 170-bp BsgI-BglII region of HS1 (probe IV). The observed nuclear protein-DNA interactions were specific. First, the two complexes were stable in the presence of an excess amount of nonspecific competitor poly(dI-dC) (100 μg/ml). Second, a greater amount of complexes was formed with increasing amounts of DP thymocyte nuclear extract (Fig. 1B). Finally, the formation of both complexes was specifically abolished by non-radiolabeled probe IV but not probe III in a competition experiment (Fig. 2).

To further delineate the location of the nuclear factor binding sites within probe IV, smaller probes were generated by deleting sequences from the 5′ and 3′ flanks of probe II and used in EMSA (Fig. 1). Probe V, which was 30 bp shorter than probe IV at the 3′ end formed the lower complex at a level comparable to that of probe IV, but did not efficiently form the upper complex, as only a miniscule amount was detected (Fig. 1B, lanes 9 and 10). This observation suggested that the upper and lower complexes were formed by factor binding to different regions of probe IV. Factor binding at the far 3′ region of the probe produces the upper complex and factor binding somewhere else in the probe produces the lower complex. Supporting this notion, probe VI, which has 21 bp removed from the 5′ end of probe IV, formed only approximately a third as much of the lower complex as probe IV (Fig. 1B, lanes 11 and 12). An additional 3-fold reduction in the formation of the lower complex resulted with probe VII, which had additional 33 bp removed from the 5′ end of probe VI (Fig. 1A and B, lanes 13 and 14). In contrast, in both of these cases, the upper complex was not significantly reduced, confirming its probable binding to the far 3′ region of probe IV. The binding site for the formation of the lower complex was probably broad because deletion from 5′ end of probe IV substantially reduced the amount of the complex and probe IX containing only the 5′ third of probe IV did not form a complex (Fig. 1, A and B, lanes 17 and 18). The 3′ region of probe IV appears to stabilize the lower complex.
Because no complexes were formed using probe VIII that had 30 bp deleted from the 3' end of probe VI (Fig. 1B, lanes 15 and 16). In summary, these data suggest that there are two major factor binding sites in the 170-bp BsgI-BglII fragment (probe IV) of HS1.

**Correlation of Factor Binding with the Induction of HS1 during DN to DP Thymocyte Differentiation**—We have previously shown that HS1 is induced during DN to DP thymocyte differentiation. In DN thymocytes of RAG2-deficient mice, HS1 is barely detectable, whereas HS1 is a major DNase I-hyper-sensitive site in DP thymocytes of RAG2-deficient mice that have been complemented with a functional TCRβ or activated lck transgene, or have been treated with anti-CD3ε antibody (37). We reasoned that if the formation of the complexes in EMSA correlated with HS1 formation, we should detect less complex formation when nuclear extracts from DN thymocytes are used in EMSA. To test this, we performed EMSA using probe IV and nuclear extracts prepared from DN thymocytes of RAG2-deficient mice or activated lck transgene; whether it binds to HS1 specifically is currently under investigation.

**Identification of Proteins That Bind to Cis Elements in HS1**—To determine the identity of protein factors in the upper and lower complexes, we screened an expression cDNA library constructed in λ phage using mRNA from DP thymocytes with the 170-bp probe IV. Five positive plaques were obtained from screening $6.5 \times 10^5$ phages. DNA was prepared from all five phage clones and analyzed by restriction enzymes. Of the five clones, two contained cDNA inserts that gave distinct restriction patterns (data not shown), indicating that they were unique. The other three clones contained related cDNA inserts, as indicated by the same sizes and restriction patterns (data not shown). Sequence analysis revealed that one of the unique cDNA inserts encoded the deoxycytidine kinase. Considering the function of the deoxycytidine kinase, its binding to probe IV was probably nonspecific. The second unique phage clone contained a 4-kb cDNA insert identical to the 3' region of the mouse Cux gene (CDP in human) (43–46). The other three phage clones contained a cDNA whose sequences did not match any known genes, although identical sequence fragments were found in EST data base. Thus, this cDNA encodes a novel protein; whether it binds to HS1 specifically is currently under investigation.

**Specific Binding of Cux/CDP to HS1**—Cux/CDP is a homeodomain protein originally identified in Drosophila (called Cut) and later in mouse and human (43–46). In addition to its homeodomain, Cux/CDP also contains three cut repeats that can independently bind DNA of relatively degenerate consensuses sequences (47–49). To determine whether Cux/CDP binds to HS1 specifically, we first performed a competition assay using DIST oligonucleotide derived from the promoter of cytochrome b heavy chain gene (gg91phox), which was previously characterized to efficiently and specifically bind Cux/CDP in EMSA (50). As shown in Fig. 4A (lanes 1–6), increasing amounts of the unlabeled DIST oligonucleotide progressively abolished the formation of both the upper and lower complexes detected by probe IV. Under the same conditions, an oligonucleotide derived from the γ-globin promoter that was previously shown to only weakly bind Cux/CDP (51) had minimal effect on the formation of the complexes (Fig. 4A, lanes 7–10). These data indicate that the 170-bp HS1 contains binding sites for the same protein complexes as gg91phox fragment and the binding affinity is relatively high.

To directly determine if both complexes detected by probe IV contained Cux/CDP, we examined if these complexes were immunoreactive with anti-Cux/CDP antiserum (46). As shown in Fig. 4A (lanes 12–14), the upper complex was first supershifted and then abolished completely in the presence of increasing amounts of specific antiserum. In contrast, the formation of the lower complex was enhanced initially with increasing amounts of antiserum, and only at the highest antiserum concentration was this complex supershifted. The observed immunoreactivity was specific since the same amounts of control preimmune serum did not supershift or abolish the complexes (Fig. 4A, lanes 15–17). These data were most consistent with the interpretation that the upper complex contains Cux/CDP and a different but probably related protein contributes to the formation of the lower complex. Supporting this conclusion, when probe IV was incubated with purified Cux/CDP protein, only the upper complex was generated (Fig. 4B, lanes 1–6). In contrast, no complex was detected when the purified Cux/CDP was incubated with probe II*, which has deleted 30 bp from the 3' end of probe II and preferentially forms the lower complex when incubated with DP thymocyte nuclear extract (Fig. 4B, lanes 7–9, and data not shown). Finally, thymocyte nuclear extract was prepared from a mutant mouse that expresses a truncated Cux lacking the strongest cut binding repeat. When used in EMSA with probe IV, the mobility of the upper complex was altered and the amount of the complex was significantly reduced, whereas the formation of the lower complex was enhanced (Fig. 4B, lanes 10–12). Together, these data suggest that Cux/CDP specifically binds to HS1 to from the upper complex.

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2 Tufarelli, C., Fujiwara, Y., Zappulla, D. C., and Neufeld, E. J. (1998) *Dev. Biol.* 200, 69–81.
Previous studies have shown that Cux/CDP binds to elements located within MARs. For example, Cux/CDP binds to a MAR in the CD8α promoter, in the long terminal repeat of mouse mammary tumor virus, and the MARs flanking the enhancer Eₘ where SATB1 binds to nearby sites (39, 53–55). SATB1 is a major MAR-binding protein and is most abundantly expressed in thymocytes (53).

Although within HS1 there were no long stretches of AT-rich sequences as found in many MARs, a stretch of 150-bp sequence was present with high ATC on one strand that may permit SATB1 binding (Fig. 10) (57). Moreover, the region resided entirely within the 170-bp probe IV, and the putative core element was contained within the 115-bp probe VII, the smallest probe shown to form the lower complex (Fig. 1).

To determine whether SATB1 binds to HS1, EMSA was carried out with probe IV and DP thymocyte nuclear extracts in the presence of anti-SATB1 antiserum. With increasing amounts of anti-SATB1 antiserum, the formation of the lower complex was progressively abolished, while the formation of the upper complex was increased initially and reduced only when a large amount of antiserum was used (Fig. 5, lanes 3–8).

In contrast, a comparable amount of preimmune serum did not abolish the lower complex formation, indicating that the result was due to specific anti-SATB1 immunoreactivity. We also conducted competition EMSA with unlabeled DNA fragments containing MAR from the CD8α promoter region, which has been previously shown to bind SATB1 as well as Cux/CDP (55).

With the increasing amounts of unlabeled CD8α MAR, both the upper and the lower complexes were competed away while the lower complex formation was not significantly reduced (Fig. 5, lanes 10–12).

Cux/CDP protein; lanes 10–12, probe IV plus 5, 10, or 20 μg of nuclear extract from mutant thymocytes.

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Footnote 2: Wang, Z., Goldstein, A., Neufeld, E. J., Scheuermann, R. H., and Tucker, P. W. (1998) Mol. Cell. Biol., in press.
As a positive control, a 200-bp MAR binding assay with radiolabeled probe IV of HS1 (39, 55). MARs (20, 22). Nuclear matrices were prepared and used in a specific binding to nuclear matrix, a characteristic inherent to conducted to determine whether the 170-bp probe IV exhibits lower (and SATB1 and specifically competed away both the upper (m) or 0.4 lanes 2 with the nuclear matrix (Fig. 6), indicating that in the absence of E. coli/CDP is known as a transcriptional repressor. Many studies have shown that the binding of Cux/CDP and/or SATB1 to a MAR can alter the transcriptional activity of a nearby transcriptional regulatory element (50, 54, 55, 58–62).3 SATB1 to a MAR can alter the transcriptional activity of a MAR. Together with the finding that CD8α MAR competed away the complex formation a MAR from the CD8 gene. Two ng of probe IV or CD8α MAR and 20 ng of pUC18 were used in a 100-μl reaction. Twenty or 40 μg of unlabeled E. coli DNA was added per reaction as nonspecific competitor. DNA bound to nuclear matrix was extracted with phenol-chloroform, precipitated, and run on a 4.5% polyacrylamide gel. Lanes 1–4, control binding assays with CD8α MAR as specific probe and pUC18 as nonspecific probe; lanes 5–8, MAR binding assays with probe IV as specific probe and pUC18 as nonspecific probe.

**FIG. 6.** Complexes formed with HS1 are specifically competed away by a MAR from the CD8α gene. Two ng of 32P-labeled probe IV were incubated with 20 μg of nuclear extract from DP thymocytes in the presence of unlabeled pUC18 DNA or CD8α MAR. Lane 1, probe alone; lanes 2 and 9, probe plus extract; lanes 3–8, probe plus extract in the presence of 3.12-, 6.25-, 12.5-, 25-, or 50-fold excess of nonspecific pUC18 DNA; lanes 10–13, probe plus extract in the presence of 3.12-, 6.25-, 12.5-, 25-, or 50-fold excess of a 200-bp PstI-EcoRI fragment containing CD8α MAR (55). CD8α MAR is known to bind both Cux/CDP and SATB1 and specifically competed away both the upper (up) and the lower (low) complexes.

nonspecific pUC18 DNA did not have any effect (Fig. 6). Together, these data indicate that SATB1 binds specifically to HS1 and contributes to the formation of the lower complex.

**HS1 Is a MAR**—Given the above findings, experiments were conducted to determine whether the 170-bp probe IV exhibits specific binding to nuclear matrix, a characteristic inherent to MARs (20, 22). Nuclear matrices were prepared and used in a MAR binding assay with radiolabeled probe IV of HS1 (39, 55). As a positive control, a 200-bp PstI-EcoRI fragment containing the CD8α MAR was used and the linearized pUC18 DNA was included in the assays to measure nonspecific binding. Fig. 7 shows that in the absence of E. coli competitor DNA, significant levels of pUC18, probe IV, and the CD8α MAR were associated with the nuclear matrix (lanes 2 and 6). In the presence of 0.2 or 0.4 μg/ml E. coli competitor DNA, pUC18 binding was abolished, consistent with its nonspecific binding to nuclear matrix. Under the same condition, the binding of probe IV and the CD8α MAR persisted (Fig. 7, lanes 3, 4, 7, and 8), indicating their specific binding to the nuclear matrix. Together with the finding that CD8α MAR competed away the complex formation by probe IV and nuclear extract (Fig. 6), these data show that the 170-bp HS1 is a MAR.

**HS1 Represses Eβ-dependent Transcription in Reporter Gene Assays**—Cux/CDP is known as a transcriptional repressor. Many studies have shown that the binding of Cux/CDP and/or SATB1 to a MAR can alter the transcriptional activity of a nearby transcriptional regulatory element (50, 54, 55, 58–62).3 Considering that HS1 is just 400 bp upstream of the Eβ enhancer, we tested whether HS1 modulates transcriptional activity of the Eβ by transient transfection assays using reporter gene constructs. Construct 1 was a basal vector in which the luciferase gene was placed behind a 424-bp promoter derived from the Vβ13 gene segment. Construct 2 had an additional 830-bp BglII-NcoI fragment containing the Eβ inserted downstream of the luciferase gene. Construct 3 had a 1.0-kb BglII-NcoI fragment containing HS1 and Eβ in their natural configuration inserted downstream of the luciferase gene. Transient transfection assays of construct 1 gave only low levels of luciferase activity, indicating a strict requirement for Eβ in transcriptional activation. Construct 2 consistently resulted in much higher levels of luciferase activity, ranging from 80- to 160-fold increases over the construct 1 in four thymoma lines tested (Fig. 7B). In contrast, construct 3 yielded luciferase activities that were approximately 2.5-fold lower than those of construct 2 in all four thymoma lines (Fig. 7B). Thus, in this assay system, HS1 and the associated trans factors appear to be able to interact with Eβ enhancer and repress its transcriptional activity.

**Mutational Analysis of HS1 in TCRβ Transcription at the Endogenous Locus**—Simultaneous to our present studies, we have generated mutant mice in which the 780-bp BsrXI-BglII fragment containing HS1 was deleted from the TCRβ locus (37). To examine the effect of the deletion on TCRβ gene transcription, we isolated RNA from thymocytes of wild type and homozygous mutant mice and assayed for the levels of transcripts initiated from different regions of the TCRβ locus by Northern blotting. As shown in Fig. 9, no apparent difference was detected in the level of mature TCRβ transcripts by a Cγ2 probe, a Vβ8 probe, or a Vβ14 probe (data not shown). Similarly, no difference was detected in the level of germline transcripts by a Dγ1-Jγ1 intron probe. Analyses of methylation status in the promoter region of the Vβ14 gene segment revealed similar levels of demethylation between wild type and mutant mice (data not shown), correlating to the similar levels of Vβ14 transcription. These data suggest that HS1 is not required for transcription and demethylation at the endogenous TCRβ locus under physiological conditions.
binding sites, one for Cux/CDP and the other for SATB1, to within a 170-bp region of HS1. Of the two nucleoprotein complexes detected, the upper complex is most likely due to Cux/CDP binding to the 3' end of the 170-bp HS1. First, deletion of 30 bp from the 3' end of the probe IV almost completely abolished the formation of the upper complex without significantly reducing the lower complex formation (Fig. 1). Second, the formation of the upper complex was preferentially abolished by the presence of anti-Cux/CDP antiserum in EMSA (Fig. 4).

Finally, only the upper complex was detected in EMSA using the purified Cux/CDP protein and probe IV (Fig. 4). Cux/CDP is a homeodomain protein containing a homeodomain and three cut repeats, all of which can independently bind DNA (43–46). Based on binding of individual cut repeat polypeptides to oligonucleotides in vitro, cut repeats 2 and 3 share similar consensus binding sequences while cut repeat 1 displays a more restricted pattern of DNA sequence recognition (Fig. 10) (47, 48). Sequence comparison revealed a stretch of sequence at the 3' end of the 170-bp HS1 that shares a high homology (83%) to the cut repeats 2 and 3 consensus sequence (Fig. 10B). In addition, an overlapping stretch of sequence shares a modest homology (50%) to cut repeat 1 consensus binding site. Thus, Cux/CDP may interact with cis DNA elements in HS1 through its multiple DNA-binding domains. Supporting this notion, an altered upper complex was observed when probe IV was incubated with thymocyte nuclear extract from mutant mice that express a truncated Cux lacking cut repeat 1 (Fig. 4B).

SATB1 contributes to the formation of the lower complex, as revealed by its preferential abolishment by anti-SATB1 antiserum. The core SATB1 binding site apparently does not overlap substantially with the Cux/CDP binding site at the 3' end of the 170-bp probe IV because deletion of 30 bp from the 3' end abolished the upper complex but not the lower complex (Fig. 1B). SATB1 binding in HS1 is probably broad because deletion of 54 bp from the 5' end did not abolish but substantially reduced the formation of the lower complex (Fig. 1B). SATB1 is known to lack strict sequence specificity and prefers a stretch of ATC-rich sequence confined to one strand of DNA (21, 57, 63). Sequence analysis revealed that the leading strand (5' to 3') of probe IV is highly ATC-rich (156 of 174 nucleotides) (Fig. 10). In particular, there is a stretch of 37 ATC nucleotides that probably forms the core unwinding element for SATB1 binding. The characteristic feature of ATC-rich on one strand in this region is highly conserved between mouse and human. In the rest of the sequence, mouse and human share 89% identity.

As discussed above, binding of HS1 by Cux/CDP and SATB1 appears to be relatively independent; however, their binding sites may overlap. Supporting this notion, inhibition of SATB1 binding to HS1 by anti-SATB1 antiserum actually caused an increase in the amount of the Cux/CDP-containing upper complex in EMSA (Fig. 5). Conversely, inhibition of Cux/CDP binding to HS1 by anti-Cux/CDP antiserum promoted the formation of the SATB1-containing lower complex (Fig. 4A). Moreover, when truncated Cux/CDP that lacks the cut repeat 1 was used in EMSA the formation of Cux/CDP-containing upper complex was reduced, whereas the formation of the SATB1-containing lower complex was markedly enhanced (Fig. 4B). Thus, Cux/CDP and SATB1 appear to interfere with each other in binding to HS1. In addition to its originally defined MAR-binding domain, SATB1 was recently found to contain a separate homeodomain and two cut-like repeats that share homology to those in Cux/CDP (57, 63). These structural homologies between the
two proteins may provide an explanation for competitive binding of overlapping sites in HS1 and the minor cross-reactivities of antisera.

HS1 Is a MAR—Cux/CDP and SATB1 binding sites. A, the 170-bp DNA region in HS1 (probe IV) from mouse and human are aligned. Both mouse and human sequences are ATC-rich on the top (leading) strand. The stretch of 37 ATC (overlined) could potentially serve as the core unpairing region for SATB1 binding. Cux/CDP binding sites may reside in the second overlined region (dotted line). B, putative Cux/CDP binding sequences from MARs are compared with cut repeat 1 (CR1) and cut repeat 2/cut repeat 3 (CR2/CR3) consensus binding sequences.

|  | T | C | G | G | T | T | C | C | C | G | A | T | T | T | G | A | T | G |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mouse | T | C | G | G | T | T | C | C | C | G | A | T | T | T | G | A | T | G |
| human | C | T | T | C | C | C | C | C | A | A | A | C | T | A | A | C | A | A |

|  | T | G | T | T | A | T | C | A | C | C | C | G | T | C | C | C | G | T | A | G | G |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mouse | T | G | T | T | A | T | C | A | C | C | C | G | T | C | C | C | G | T | A | G | G |
| human | C | T | T | C | C | C | C | C | A | A | A | C | T | A | A | C | A | A |

|  | G | A | T | T | C | A | T | G | C | C | G | C | T | T | T | T | T | T | T | T | T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mouse | G | A | T | T | C | A | T | G | C | C | G | C | T | T | T | T | T | T | T | T | T |
| human | C | T | T | C | C | C | C | C | A | A | A | C | T | A | A | C | A | A |

|  | G | A | T | C | A | T | G | C | C | G | C | T | T | T | T | T | T | T | T | T | T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| mouse | G | A | T | C | A | T | G | C | C | G | C | T | T | T | T | T | T | T | T | T | T |
| human | C | T | T | C | C | C | C | C | A | A | A | C | T | A | A | C | A | A |

|  |  | C | C | A | A | T | A | A | T | C | G | A | T | C | A | C | C | A | A | T | C |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MARp homology: | C | C | A | A | T | A | A | T | C | G | A | T | C | A | C | C | A | A | T | C |
| MARp homology: | C | C | A | A | A | A | T | C | A | C | C | A | A | T | C |

MARp did not affect TCRβ transcription at the endogenous locus in developing T cells. Moreover, in the absence of MARp, demethylation in the promoter region of the Vb14 gene segment and recombination of the TCRβ gene also occurred to the same extent as on the normal allele (Ref. 37 and data not shown). Thus, our findings demonstrate that MARp is not absolutely required for these processes at the TCRβ locus under physiological conditions.

The contrasting results obtained from transcriptional analyses of MARp in reporter gene assays and at the endogenous locus could have different underlying mechanisms. One possibility is that MARp does modulate the contribution of Eκ to transcriptional activity in the endogenous locus; however, its function is compensated for by additional MARs within the locus in the mutant mice. It is also possible that the 780-bp region deleted in the mutant mice may contain an as yet unidentified cis-regulatory element that has an opposite function as MARp. Deletion of both simultaneously might result in functional neutralization and no apparent phenotype. Alternatively, MARp may have no role in the regulation of TCRβ gene transcription. Its effect on Eκ-dependent transcription in transient reporter gene assays may be an artifact resulting from taking MARp out of its natural chromosomal context. In this regard, it is worthwhile to consider the putative functions of MARs at the IgH and Igκ loci. By reporter gene assays in cell lines or transgenic mice, MARs flanking the enhancer Eκ were shown to modulate IgH gene chromatin accessibility (31) and transcription in both positive and negative manners (27–30). Similarly, MAR associated with the Igκ intronic enhancer is thought to be involved in Igκ gene transcription, demethylation, recombination, and somatic hypermutation (18, 33–36). However, similar to targeted deletion of MARp, targeted deletion of MAR from Igκ locus did not have any obvious effect on Igκ gene recombination and transcription.4

Although MARs are widely found in diverse eukaryotic organisms and have been implicated in the regulation of transcription, DNA recombination, replication, and repair, their true function in most of these processes is still unclear (56). The uncertainty arises because putative functions of MARs are usually studied using reporter gene assays. Targeted mutagenesis of MARp represents a first attempt to elucidate a MAR's function in its endogenous locus. The different results we have obtained from assaying MARp in reporter gene assays and at

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4 Y. Xu, personal communication.
the endogenous locus clearly point out the potential pitfalls of analyzing the function of MARs outside of their natural chromosomal context.

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