An Atypical Homeodomain in SATB1 Promotes Specific Recognition of the Key Structural Element in a Matrix Attachment Region*

(Received for publication, October 11, 1996, and in revised form, January 29, 1997)

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SATB1 is a cell type-specific nuclear matrix attachment region (MAR) DNA-binding protein, predominantly expressed in thymocytes. We identified an atypical homeodomain and two CACGTCG repeats in SATB1, in addition to the known MAR-binding domain. The isolated MAR-binding domain recognizes a certain DNA sequence context within MARs that is highly potentiated for base unpairing. Unlike the MAR-binding domain, the homeodomain when isolated binds poorly and with low specificity to DNA. However, the combined action of the MAR-binding domain and the homeodomain allows SATB1 to specifically recognize the core unwinding element within the base-unpairing region. The core unwinding element is critical for MAR structure, since point mutations within this core abolish the unwinding propensity of the MAR. The contribution of the homeodomain is abolished by alanine substitutions of arginine 3 and arginine 5 in the N-terminal arm of the homeodomain. Site-directed mutagenesis of the core unwinding element in the 3’ MAR of the immunoglobulin heavy chain gene enhancer revealed the sequence 5’-GCATATAT-3’ to be essential for the increase in affinity mediated by the homeodomain. SATB1 may regulate T-cell development and function at the level of higher order chromatin structure through the critical DNA structural elements within MARs.

Eukaryotic chromosomes are thought to be separated into topologically independent loop domains by periodic attachment onto an intranuclear frame known as the nuclear matrix or skeleton, defined as the insoluble material left in the nucleus after a series of biochemical extraction steps (1). Specific DNA sequences that bind to the nuclear matrix in vitro are called matrix attachment regions (MARs), and these sequences have been postulated to form the base of chromosomal loops (reviewed in Refs. 2 and 3). MARs may be important to organize chromosomes and regulate DNA transcription and replication within the nucleus. In support of this notion, MARs often colocalize or are located in close proximity to regulatory sequences including enhancers (4–9), and some MARs can augment transcription from heterologous promoters in stable transformants (5–7, 10, 11). Recent evidence shows that MARs play a role in tissue-specific gene expression. The MARs associated with the immunoglobulin \( \mu \) heavy chain locus are essential for transcription of a rearranged \( \mu \) gene in transgenic B lymphocytes (12). Identification of the cell type-specific MAR-binding protein SATB1, which is predominantly expressed in thymocytes, shows that MARs can be specific targets for a cell type-specific factor (13).

SATB1 defines a novel class of DNA-binding proteins that recognize a specific sequence context that exhibits a high base unpairing or unwinding propensity. MARs are generally AT-rich and typically contain a subregion(s) that exhibits a strong potential to base-unpair under negative superhelical strain (10, 14). A high AT content, however, is not sufficient to confer high affinity binding to SATB1; specific mutations within MARs, which maintain the AT-richness but eradicate the unwinding capability, substantially reduce or abolish SATB1 binding (15). Analysis of SATB1 binding sites in MARs revealed that binding is restricted to the subregion of MARs that has a high unwinding propensity. This base-unpairing region consists of a cluster of sequence stretches with a special AT-rich DNA sequence context, in which Cs are sequenced exclusively on one strand and Gs on the other (ATC sequences) (13). A short core unwinding element can be present within one of these ATC sequences, which can be detected by virtue of its most persistent base unpairing even under conditions that favor the double-stranded DNA configuration; mutation of this element abolishes the base-unpairing propensity of MARs (14). The unwinding potential was demonstrated to be essential for MAR function; a concatemer, wild-type (25)\(_{\alpha}\), of the core unwinding element of the 3’ MAR of the immunoglobulin heavy chain (IgH) enhancer displays high binding affinity to the nuclear matrix, unwinds under superhelical strain, and enhances transcription from a linked reporter gene. A corresponding mutated version, mutated (24)\(_{\alpha}\), has lost all of these properties (10).

To date, three proteins with similar binding specificity have been identified in addition to SATB1: nucleolin, a major nucleolar protein with multiple functions (15), p114, isolated from breast carcinoma (16), and Bright, a protein that is predominantly expressed in B-cells (17). These proteines bind with high affinity to MARs, and we showed that nucleolin and p114 can distinguish wild-type (25)\(_{\alpha}\) from mutated (24)\(_{\alpha}\). Unlike other proteins known to bind MARs such as lamin B1 (18) and topoisomerase II (19), SATB1 binds MARs with very high affinity, exhibiting dissociation constants (\( K_d \)) in the range of 10\(^{-9}\) to 10\(^{-10} \) M, comparable to many sequence-specific transcription factors.

To understand the biological role of SATB1, it is important to delineate the functional domains in this protein. A minimum 150-amino acid MAR-binding domain that contains novel DNA-
binding motifs was previously identified (20). We report here that SATB1 contains an additional domain that shares homology with known homeodomains. Homeodomains are 60-amino acid DNA-binding domains, and their amino acid sequence is highly conserved, as well as their three-dimensional structure. Homeodomain proteins function in vitro and in vivo as sequence-specific transcription factors, and they are important developmental regulators that determine position or cell-type specification (reviewed in Refs. 21 and 22). Unlike known homeodomains that directly and independently bind DNA, the homeodomain in SATB1 does not bind to the MAR probes analyzed here nor does it bind to a dimerized sequence (RPJ) that resembles the homeodomain consensus sequence (23). When associated with the MAR-binding domain, however, the SATB1 homeodomain enhances binding specificity toward the core unwindig element of a MAR.

**EXPERIMENTAL PROCEDURES**

**Protein Domain Analysis**—We performed searches of the SWISS-PROT data base (release 26.0, August, 1993) using the program Blast (24) and Blitz (25). Computations were performed using the Blast server on the Blitz server at EMBL. Best results were cloned in frame in the BamHI or BamHI/EcoRI site of the vector pGEX2T (Pharmacia Biotech Inc.). Deletion of the homeodomain was achieved by first synthesizing a fragment ranging from the N-terminal residue of the MAR domain (position 346) to the N-terminal residue of the homeodomain (position 641) and cloning it into BamHI/EcoRI-digested pGEX2T. In a second step, a fragment ranging from the C-terminal residue of the homeodomain (position 702) to the end of the cDNA was amplified and ligated in frame in the EcoRI site downstream of the insert made in the first step. Glutathione S-transferase (GST)-fusion proteins were overexpressed in Escherichia coli (XL1 Blue) and purified on glutathione-Sepharose according to standard procedures (26). Protein concentrations were determined using a Bradford protein assay (Bio-Rad), which was followed by quantitation of the fusion proteins on glutathione-Sepharose according to standard procedures (26). Protein concentrations were determined using the Bradford protein assay (Bio-Rad), which was followed by quantitation of the fusion proteins on glutathione-Sepharose according to standard procedures (26).

**DNA-binding Assays**—Gel mobility shift assays were carried out as described (13), with no poly(dI-dC)poly(dI-dC) added or with 0.5 μg/20 μl. The 3′ MAR is identical to the IgH 3′-end fragment described previously (13). The wild-type 3′ MAR and the mutated fragments were subcloned in the EcoRI site of Bluescript (Stratagene), and the fragments were isolated by EcoRI restriction enzyme digestion and purification from an agarose gel. Pentamer repeats of binding sites V and VI were made exactly as described for wt(V)5, (13), using the following oligonucleotides: 5′-CTTAAATTCTTATTATTCTGAAATc-3′ with its complementary strand 5′-TTCGAAATATAGATTAATTTAGGaa-3′ for wt(V)5, and 5′-TTCCTCTCTGATTATTGGTCTCCATGAAttc-3′ with 5′-TTCATGGGAGACCAATAATCATGAGGGAGGaag-3′ for wt(V)6. The lowercase letters indicate single-stranded overhangs used for end to end ligation of the double-stranded oligonucleotides.

Probes for gel mobility shift analysis were prepared by labeling isolated oligonucleotide fragments at both ends using Klenow polymerase and [32P]dATP. Under conditions of protein excess, the concentration required for half-maximal binding may be considered an estimate of the equilibrium binding coefficient (27). Autoradiographs of the gel mobility shift experiments were scanned by laser densitometry, and the percentage of free probe remaining was plotted against the protein concentration in nM.

DNA titration experiments were performed as described (28) with some modifications. The concentration of the DNA fragment to be labeled was determined using a TRO 100 minifluorometer (Hoefer Scientific Instruments), followed by agarose gel electrophoresis and ethidium bromide staining using a plasmid of known concentration as a standard. The concentration of protein that gave rise to a 40–70% shift at the lowest DNA concentration was determined empirically. All the DNA titrations were done in the presence of 0.5 μg/20 μl of poly(dI-dC).

The binding reaction was incubated for 30 min at room temperature to ensure that equilibrium was reached. After electrophoresis the gels were dried and analyzed by a PhosphorImager (Bio-Rad).

**RESULTS**

**SATB1 Contains a Homeodomain and Cut-like Repeats**—In addition to the MAR-binding domain (residues 346–495) previously reported (20), computer-aided homology searches of the Swiss-Prot data base (30) identified a homeodomain homology with the C terminus of SATB1 (residues 641–702) (Fig. 1A). Many of the residues that are most conserved among homeodomains are also found in the SATB1 homeodomain, which shares 33% identity with the engravled class of homeodomains (reviewed in Ref. 31, Fig. 1B). Identities are found with residues that in the x-ray structure of other homeodomains contribute to the hydrophobic core and residues that directly interact with DNA (32). This putative homeodomain is, however, divergent. Major differences include a single amino acid insertion at the end of the first helix and a substitution of the highly conserved WFQ motif in the third helix of known homeodomains by FFQ in both human and mouse SATB1.

In addition to the homeodomain homology, a set of two repeats was found near the center of SATB1 (residues 370–445 and 493–568), similar to the Cut repeats of the Cut- and Clox homeo-proteins of Drosophila and mammals, respectively. Cut proteins contain a homeodomain and three additional DNA-binding domains of 73 amino acids, called Cut repeats (33–36). The two Cut-like repeats in SATB1 (named here A and B) contain the previously documented repeats box I (residues 382–415 and 505–538) and box II (429–445 and 552–568), respectively (20) (Fig. 1, A and C). Repeat A occurs at the center of the MAR-binding domain of SATB1, but it does not include the N- and C-terminal amino acids that are mandatory for MAR binding (20). Two copies of SATB1 were identified in 75% of repeat in box II, each with at least 27–35% identity with the Cut repeat.

**The Homeodomain Increases Binding Affinity of SATB1 to a MAR**—Most homeodomain proteins contain a homeodomain as the sole DNA-binding domain. A group of homeodomain proteins have additional domains that assist the homeodomain in DNA binding specificity (reviewed in Refs. 37 and 22). In the
case of SATB1, the MAR-binding domain by itself is sufficient to recognize and bind a specific region (base-unpairing region) within MARs that has a high propensity for base unpairing, and the homeodomain may have a new role in DNA recognition. To explore this possibility, glutathione S-transferase (GST)-SATB1 fusion proteins were constructed; one protein contained the MAR domain and homeodomain linked together in their natural protein context (GST(MD1HD)); one protein had the 60-amino acid homeodomain specifically deleted (GST(MDHD)), and one fusion protein contained the homeodomain separately (GST(HD)) (Fig. 2A). These purified fusion proteins were used in quantitative gel mobility shift experiments with a fixed concentration of a synthetic MAR probe, wild-type (25)5, and increasing protein concentrations. This probe was derived from the core unwinding element of the MAR located 3' of the IgH enhancer, and it has the same properties as a natural MAR (10). Fig. 2B shows the gel mobility shift experiments and the binding curves that were derived from these autoradiographs. Each of these and the following gel shift experiments were repeated at least three times giving similar results. The isolated HD showed virtually no binding activity for the wt(25)5 probe; however, when HD was associated with MD (MD1HD), the binding affinity was approximately 10 times higher ($K_d \approx 0.1 \text{nM}$) than for MDHD ($K_d \approx 1.0 \text{nM}$). The affinity of MDHD toward wt(25)5 was virtually identical to that of the isolated MD alone (GST(MD)), indicating that the C-terminal amino acids from 496 to 763 besides HD have no additional contribution toward binding to wt(25)5 (data not shown). HD weakly bound to longer MAR fragments, but this activity was mainly nonspecific, since it could be competed by nonspecific competitors (data not shown). This effect of the homeodomain on binding affinity was confirmed by additional DNA titration experiments, in which the dissociation constants were determined using a fixed protein concentration and increasing concentrations of the DNA probe (Fig. 2C). Dissociation constants determined in this manner are independent of minor variations in the protein concentration determination or the amount of active protein in the protein preparation. The results obtained from gel mobility shift experiments were quantitated using a PhosphorImager, and
The Kd values were calculated from the least squares fit of a Scatchard plot of bound/free DNA as a function of bound DNA. The approximate Kd values were estimated from the negative reciprocal of the slope, and the Kd for MD + HD (0.06 nM) was approximately 7 times lower than for MDΔHD (0.4 nM) (Fig. 2C). The dissociation constants determined by protein titration or DNA titration were similar, indicating that nearly all the protein in the protein sample was in an active form.

The SATB1 Homeodomain Promotes Binding of the MAR Domain to the Core Unwinding Element of the IgH 3' MAR. A, schematic representation of the IgH enhancer flanked by two MARs. The SATB1 binding sites are indicated by black bars and roman numerals. The ATC sequence cluster in the IgH 3' MAR is shown. Each ATC sequence is indicated by a bracket, and the SATB1 direct contact sites are shown by double-headed arrows and roman numerals. Residues that constitute the core unwinding element within site IV are indicated by filled dots. The sequences of the mutated binding sites are shown below, with an asterisk to mark the mutated residues. B, gel mobility shift experiments and binding curves comparing the affinities of (MD + HD) and (MDΔHD) to wild-type 5' MAR, wild-type 3' MAR, and mut IV.

FIG. 2. The homeodomain increases binding affinity of SATB1 to the wild-type (25) 5' MAR, but by itself it binds poorly to this sequence. A, GST-fusion protein constructs used in gel mobility shift assays. GST(MD + HD) contains both MAR domain and homeodomain in their natural protein context; GST(MDΔHD) is identical to the above except for specific deletion of the 60-residue homeodomain. GST(HD) contains the homeodomain. The wavy line indicates the GST moiety of the fusion proteins. Q indicates the polyglutamine stretch found at this position. B, protein titration experiments. Gel mobility shift experiments and binding curves comparing the binding activities of GST-fusion proteins (shown in A) to the wt(25) 5' MAR. Protein concentrations (in nM) are indicated at the top of the lanes. (Note that the concentration range differs with each construct.) The results were quantitated by laser densitometer scanning, and the amount of free DNA was plotted against the protein concentration. The Kd value was estimated as the concentration of protein that results in a 50% shift. The experiments were repeated at least three times and gave similar results.

FIG. 3. The SATB1 homeodomain specifically increases binding affinity to the core unwinding element among three SATB1 direct contact sites in the 3' MAR. A, schematic representation of the IgH enhancer flanked by two MARs. The SATB1 binding sites are indicated by black bars and roman numerals. The ATC sequence cluster in the IgH 3' MAR is shown. Each ATC sequence is indicated by a bracket, and the SATB1 direct contact sites are shown by double-headed arrows and roman numerals. Residues that constitute the core unwinding element within site IV are indicated by filled dots. The sequences of the mutated binding sites are shown below, with an asterisk to mark the mutated residues. B, gel mobility shift experiments and binding curves comparing the affinities of (MD + HD) and (MDΔHD) to wild-type 5' MAR, wild-type 3' MAR, and mut IV.
The concatemer wt(IV)5 is identical to the previously described the core unwinding element was further confirmed by employing (MD
Hill coefficient of 2.2 (data not shown). On the contrary, the weaker bind-

Both MARs contain a base-unpairing region, but only the IgH core unwinding element, it should specifically increase affinity to wt(IV)5 but not to wt(V)5 or wt(VI)5. Indeed, the increase in binding affinity to the 39-

The role of SATB1 homeodomain in MAR recognition arose; to determine whether the homeodomain in SATB1 contrib-
utes to this preferential recognition of site IV, we used mutated MAR fragments as probes in gel mobility shift experiments with GST(MD + HD) and GST(MDΔHD). Each of these mutated MARs had one of the three sites destroyed by mutation and two sites intact (Fig. 3A). The affinity of the MAR-binding domain alone (MDΔHD) to each of the three mutated fragments was nearly the same as to wild-type 39-

The homeodomain recognizes a short (C/A)TAATA motif that colocalizes with the core unwinding element—To examine if specific residues in binding site IV are necessary for homeodomain recognition, we analyzed a series of single point mutations as shown in Fig. 4B, left panel. Among these, mut 4, mut 5, and mut 6 each had one of the three base substitutions made in mut IV. These single point mutations did not alter the high unpairing propensity of DNA sequences in the 39-

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The isolated SATB1 homeodomain exhibits only very weak nonspecific binding activity to base-unpairing sequences. The MAR domain, on the other hand, can bind independently with high affinity and specificity; it distinguishes MARs that can unwind from mutated MARs that have lost this capability. Thus, the homeodomain initially appeared to be nonsignificant in DNA binding.

**HOMEOomain Contribution to MAR BINDING**—The isolated SATB1 homeodomain exhibits only very weak nonspecific binding activity to base-unpairing sequences. The MAR domain, on the other hand, can bind independently with high affinity and specificity; it distinguishes MARs that can unwind from mutated MARs that have lost this capability. Thus, the homeodomain initially appeared to be nonsignificant in DNA binding. However, a unique function is now attributed to this homeodomain. When associated with the MAR domain in the natural protein context, the SATB1 homeodomain directs the MAR domain to the core unwinding element of a MAR. This distinguishes SATB1 from the way by which paired protein, the Drosophila Cut, and the mammalian Cut-like proteins recognize their target DNA. In these proteins, the homeodomains bind DNA independently, and the associated domains contribute to binding specificity by making additional DNA contacts (33, 35, 44, 45). The SATB1 homeodomain is similar to the homeodomains in the POU transcription factors, which cannot bind independently or bind with low affinity and relaxed specificity (reviewed in Ref. 46). In the case of the POU transcription factors, both the POU domains and the homeodomains are equally required for high affinity binding, and together they form a bipartite binding domain (38). For SATB1, on the other hand, the MAR domain alone displays fully functional MAR-binding activity, and the contribution of the homeodomain results in further selection of specific elements embedded within a MAR sequence context. The contribution of the homeodomain is small, however, and was previously missed when the minimum domain that confers MAR binding was delineated (20). This in part could be due to the active protein component in the full-sized bacterially produced SATB1 not being accurately determined.

The dissection of SATB1 protein in individual components has brought to light how these multiple levels of recognition are ultimately put together to achieve a high degree of binding site specificity that is unprecedented among MAR-binding proteins. This is illustrated in Fig. 5. We had previously shown that SATB1 does not bind MARs merely on the basis of their high AT content but that it specifically recognizes AT-rich regions in MARs that have a high propensity for base unpairing, and within these base-unpairing regions it exhibits a preference for binding to the core unwinding element (13). First, we showed in a separate study using a phage display library of random peptides that a short peptide homologous to the N-terminal arm of the MAR-binding domain can effectively recognize AT-rich DNA (47). This suggests that the short homologous N- and C-terminal amino acid stretches of the MAR-binding domain are individually sufficient for recognizing AT-rich DNA, but to
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The isolated MAR-binding domain is shown to bind to the base-unpairing region of the IgH 3′ MAR, contacting each of the three binding sites with comparable affinities. Site IV contains the core unwinding element (indicated by an open rectangle). The MAR-binding domain together with the homeodomain specifically bind to the core unwinding element within the base-unpairing region of the MAR fragment.

distinguish between AT-rich DNA with high unwinding propensity and DNA that lacks this property, the entire 150-amino acid MAR-binding domain is required. Within an AT-rich DNA sequence with high unwinding propensity, the specific recognition of the core unwinding element that is critical in affecting overall DNA structure of the MAR (14) is achieved by the combined action of a unique homeodomain and a MAR-binding domain. Core unwinding elements have been identified in several other MARs, such as in the MAR at the 5′ boundary of the human β-globin locus control region (48). These elements are remarkably similar to the SATB1 homedomain recognition site of the IgH 3′ MAR, which suggests that SATB1 may exhibit preference for core unwinding elements in general.

Unusual Mode of Binding of the MAR Domain and Homeodomain in SATB1—The MAR-binding domain in SATB1 binds DNA in the minor groove, making little contact with DNA bases. SATB1 presumably recognizes DNA sequences indirectly by binding to the altered sugar phosphate backbone structure dictated by a specific DNA sequence context (13). Although the homeodomain in SATB1 does not bind DNA independently, mutagenesis of the target DNA revealed that a specific sequence 5′-TAATATA-3′, in the SATB1 binding site IV, is necessary for the increase in affinity mediated by the homeodomain. Furthermore, the increase in affinity was almost completely abolished by alanine substitutions of arginine residues in the N-terminal arm of the SATB1 homeodomain, which is known in other homeodomains to bind the minor groove. The corresponding region for other homeodomains was found to be flexible and lack any secondary structure as shown by NMR and x-ray crystallography (reviewed in Ref. 49). Therefore, the effect resulting from alanine substitutions of the two arginine residues is unlikely to be a consequence of the subsequent change in the overall protein folding. These results taken together suggest, but do not prove, that the homeodomain, in the context of the SATB1 protein, may directly contact the target DNA site in the minor groove. Unlike other homeodomains, mutagenesis of residues in the third helix, which is known to interact with the major groove, has only a minor effect on SATB1 binding. This finding is consistent with previous results showing that SATB1 is a minor groove binding protein.

The SATB1 homeodomain recognition sequence found in site IV is similar to the homeodomain binding site consensus, TAAT core (22, 50), and it overlaps with the direct SATB1 contact site IV. Missing nucleoside experiments revealed no additional contacts with (MD + HD) compared with (MDΔHD) (data not shown). This result, taken together with the fact that the sequence 5′-TAATATA-3′ in site IV is responsible for the positive effect of the homeodomain in SATB1 binding, may suggest that upon binding to a MAR, the SATB1 homeodomain and the MAR domain contact the same site simultaneously, possibly from opposite sides of the DNA helix. Crystal structural analysis must be done to determine whether the SATB1 homeodomain in its natural protein context directly makes contact with DNA. It is of interest that the crystal structure of the even-skipped homeodomain showed that two homeodomains are bound by one 10-bp consensus sequence on both faces of the DNA, without any steric hindrance (51). This simultaneous occupation of one site from both sides of the DNA helix could provide significant stability to the protein-DNA complex. This protein-DNA interaction is unusual, however. The multiple DNA-binding domains found in the POU, Cut, and the Paired proteins bind to sites that are juxtaposed. Similarly, in the transcription factor oct-1, the POU-specific domain and the homeodomain were suggested to occupy adjacent positions in the major groove (52).

Biological Significance of SATB1 Recognition of MARs—Homeodomains represent the hallmark of developmental regulatory proteins (reviewed in Ref. 21), and the presence of this domain in a MAR-binding protein is unprecedented. In this regard, SATB1 is unique among several other proteins that preferentially bind MARs in vitro including nucleolin (15), topoisomerase II (19), histone H1 (53), the high mobility group proteins HMG I(Y) (54), lamin B1 (18), ARBP (55), and hnRNPu (56–58). In fact, a recent study of SATB1 knockout mice showed that SATB1 ablation results in a major defect in T-cell development and alterations in expression of multiple genes. Genomic DNA sequences that are bound to SATB1 in vivo have recently been characterized based on cross-linking techniques. This study revealed that in the nucleus SATB1 actually binds DNA sequences containing ATC sequence clusters and that these sequences are tightly bound to the nuclear matrix, representing MARs. This result, together with the results from the SATB1 knockout experiments, suggests that higher order chromatin structure may be involved in T-cell-specific gene regulation. Such regulation could be directed toward MARs at the base of chromatin loops, in particular toward the core unwinding elements, as specified by the combined action of the MAR-binding domain and the homeodomain of SATB1.

Acknowledgments—We thank Dr. Yoshinori Kohwi for valuable discussions, Dr. Craig Hauser for helpful comments and critical reading of

3 J. D. Alvarez, H. Niida, T. Kohwi-Shigematsu, and D. Y. Loh, unpublished results.
4 I. de Belle, S. Cai, and T. Kohwi-Shigematsu, unpublished results.
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