The Genomic Sequences Bound to Special AT-rich Sequence-binding Protein 1 (SATB1) In Vivo in Jurkat T Cells Are Tightly Associated with the Nuclear Matrix at the Bases of the Chromatin Loops

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Abstract. Special AT-rich sequence-binding protein 1 (SATB1), a DNA-binding protein expressed predominantly in thymocytes, recognizes an ATC sequence context that consists of a cluster of sequence stretches with well-mixed A's, T's, and C's without G's on one strand. Such regions confer a high propensity for stable base unpairing. Using an in vivo cross-linking strategy, specialized genomic sequences (0.1–1.1 kbp) that bind to SATB1 in human lymphoblastic cell line Jurkat cells were individually isolated and characterized. All in vivo SATB1-binding sequences examined contained typical ATC sequence contexts, with some exhibiting homology to autonomously replicating sequences from the yeast *Saccharomyces cerevisiae* that function as replication origins in yeast cells. In addition, LINE 1 elements, satellite 2 sequences, and CpG island–containing DNA were identified. To examine the higher-order packaging of these in vivo SATB1-binding sequences, high-resolution in situ fluorescence hybridization was performed with both nuclear "halos" with distended loops and the nuclear matrix after the majority of DNA had been removed by nuclease digestion. In vivo SATB1-binding sequences hybridized to genomic DNA as single spots within the residual nucleus circumscribed by the halo of DNA and remained as single spots in the nuclear matrix, indicating that these sequences are localized at the base of chromatin loops. In human breast cancer SK-BR-3 cells that do not express SATB1, at least one such sequence was found not anchored onto the nuclear matrix. These findings provide the first evidence that a cell type–specific factor such as SATB1 binds to the base of chromatin loops in vivo and suggests that a specific chromatin loop domain structure is involved in T cell–specific gene regulation.

For large eukaryotic chromosomes to be compacted into a small volume inside the nucleus and yet remain fully functional, chromosomal DNA must be effectively packaged by a series of higher-order structures. Much of the available evidence strongly supports the model that the 30-nm chromatin fiber is segregated into a series of discrete and topologically independent loop domains of ~50–100 kb in size, restrained at their bases by interaction with the nuclear scaffold or matrix (Berezney and Coffey, 1974; Benyajati and Worcel, 1976; Cook and Brazell, 1976; Paulson and Laemmli, 1977; Igo-Kemenes and Zachau, 1978; Vogelstein et al., 1980; Lebkowski and Laemmli, 1982*a*,*b*). Histone-depleted metaphase chromosomes revealed a halo of DNA loops converging on a central skeletal structure, referred to as the scaffold (Paulson and Laemmli, 1977). A similar loop domain organization of chromosomes is thought to exist in interphase nuclei by periodic attachment of genomic DNA onto the nuclear matrix (Mirkovitch et al., 1984; Cockerill and Garrard, 1986). The nuclear matrix has been operationally defined as the insoluble structure left in the nucleus after a series of biochemical extraction steps (for review see Nelson et al., 1986), and such structures can be visualized by high-resolution electron microscopy (Penman, 1995; Nickerson et al., 1997).

Specific genomic DNA segments that exhibit high affinity to the nuclear matrix in vitro have been identified from various species and are called scaffold or matrix attach-

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ment regions (SARs or MARs).[1] Such DNA regions have been postulated to form the base of chromosomal loops, thereby organizing the chromatin loop structure in both interphase nuclei and metaphase chromosomes (for review see Gasser and Laemmli, 1987). The loop domain structure of chromatin would not only help to compact genomic DNA, but it may also have a significant biological function. Each loop domain constrained by the matrix attachment sites may define a unit of genetic function, and the sites of attachment may have a role in regulating DNA transcription and replication. DNA segments containing matrix attachment sites (MARs/SARs) are often found at the boundaries of transcription units and near enhancer-like regulatory sequences (Mirkovitch et al., 1984; Cockerill and Garrard, 1986; Gasser and Laemmli, 1986*a,b*; Cockerill et al., 1987; Bode and Maass, 1988; Dijkwel and Hamlin, 1988; Jarman and Higgs, 1988; Phi-Van and Strätling, 1988; Levy-Wilson and Fortier, 1990). For MARs surrounding the immunoglobulin μ heavy chain IgH enhancer, it has been shown that these elements are essential for the transcription of a rearranged μ gene in transgenic B lymphocytes (Forrester et al., 1994), and these MARs have also been reported to be responsible for chromatin accessibility (Jenuwein et al., 1997). Also, both flanking MARs have been shown to be required for B cell–specific demethylation of the gene (Kirillov et al., 1996). Evidence that MARs are biologically significant is now accumulating.

While no primary consensus sequence has been found for MARs except that they are generally A + T rich, MARs typically contain a small region of not more than 150–200 bp that is highly potentiated for base unpairing when examined under negative superhelical strain (Kohwi-Shigematsu and Kohwi, 1990; Bode et al., 1992). The base-unpairing region (BUR) can be readily detected with the use of a chemical probe that specifically reacts with unpaired DNA bases (for review see Kohwi-Shigematsu and Kohwi, 1992). Within a BUR, a core unwinding element can often be identified, and upon mutation of this element, the base-unpairing propensity of the BUR region within a MAR is abolished (Kohwi-Shigematsu and Kohwi, 1990). The high unwinding capability of MARs is important for conferring high-affinity binding to the nuclear matrix in vitro and for augmenting the activity of a reporter gene in a stably transformed cell line, while the mutated versions that have lost the unwinding capability lack these activities (Bode et al., 1992).

With the use of a specific sequence containing the core unwinding element derived from the IgH enhancer MAR, we previously cloned a gene encoding special AT-rich sequence-binding protein 1 (SATB1), which is predominantly expressed in thymocytes (Dickinson et al., 1992). SATB1 can thus clearly distinguish AT-rich sequences that are highly potentiated to unwind from those that lack this property. We have shown that SATB1 binds to the BURs of MARs with high affinity and specificity, exhibiting dissociation constants in the range of 10^{-9} M, which is comparable to many sequence-specific transcription factors. In fact, SATB1 is a homeodomain protein (Dickinson et al., 1997), and evidence for SATB1 being a transcriptional suppressor has been reported (Kohwi-Shigematsu et al., 1997*a*; Liu et al., 1997). Unlike many DNA-binding proteins that recognize specific primary DNA sequences, SATB1 recognizes and binds in a specific AT-rich sequence context where one strand consists of mixed A's, T's, and C's excluding G's (ATC sequences). In general, clustered ATC sequences have a strong tendency to unwind by extensive base-unpairing when subjected to negative superhelical strain (Kohwi-Shigematsu and Kohwi, 1990; Bode et al., 1992). Although the number is still small, there seems to be a class of ATC sequence context-binding proteins (Dickinson and Kohwi-Shigematsu, 1995; Herrscher et al., 1995; Yanagisawa et al., 1996).

As a first step to understanding cell type–specific gene regulation at the level of higher-order chromatin structure, we asked whether the genomic DNA sequences that bind to SATB1 in vivo are tightly attached to the nuclear matrix and thus serve as the base of chromatin loops in vivo. If this is the case, it can be concluded that SATB1 binds to the base of chromatin loops in vivo. We isolated individual in vivo SATB1-binding sequences from human lymphoblastoid Jurkat cells based on a new strategy. In situ hybridization of Jurkat nuclear matrices and nuclear "halos" with representative in vivo SATB1-binding sequences revealed that these sequences remained tightly bound to the nuclear matrix even after loop domains were digested and removed, indicating that they were hybridizing to DNA at the base of the chromatin loops in Jurkat cells. Attachment of chromatin to the nuclear matrix at one of these sites was found to be cell type dependent.

Materials and Methods

Cells and Cell Culture

Jurkat cells were grown in RPMI medium containing 10% FBS in a humidified atmosphere of 5% CO_2. For cross-linking, the cells were grown to a density of 1–2 × 10^6 cells/ml. Thymocytes were obtained from 4–5-wk-old C57/B mice. The thymus was removed and rinsed in ice-cold PBS buffer, minced with fine scissors, and filtered through two layers of gauze. The cells were collected by centrifugation and washed twice with ice-cold PBS.

To label Jurkat cell DNA and protein, cells were incubated with 10 μCi/ml of [^{3}H]thymidine (20 Ci/mmol; New England Nuclear, Boston, MA) and 10 μCi/ml of [^{14}C]leucine (320 mCi/mmol; ICN Biomedicals, Costa Mesa, CA) for a period of 24 h before in vivo cross-linking. To label RNA, 10 μCi/ml of [^{3}H]uridine (15 Ci/mmol; ICN Biomedicals) was added to the cultures under similar conditions.

In Vivo Cross-Linking

The method used for cross-linking has been previously described by Orlando and Paro (1993). We made the following modifications: 1 × 10^8 cells were incubated in medium containing 1% buffered formaldehyde at 37°C for 10 min and then placed at 4°C for 2 h. The length of exposure to cross-linker was optimized in in vitro experiments using bacterially produced SATB1 and a labeled synthetic IgH enhancer 3′ MAR. After cross-linking, the cells were washed twice with ice-cold PBS. The cells were then lysed in a solution of 4% SDS in 10 mM Tris, pH 8.0, 1 mM EDTA. Cross-linked DNA and proteins were then purified from free uncross-linked proteins by centrifugation through a gradient of 5–8 M urea prepared in 10 mM Tris, pH 8.0, 1 mM EDTA. 1–2 × 10^7 cells were loaded on the top of a 12-ml gradient and centrifuged in a rotor (model SW 41; Beckman Instruments, Fullerton, CA) at 30,000 rpm for 16 h. The gradients were then fractionated by removing fractions from the top of the gradient. These samples of the fractions were assayed for [^{3}H]thymidine and [^{14}C]leucine.

1. *Abbreviations used in this paper*: BUR, base-unpairing region; GST, glutathione S-transferase; MAR, matrix attachment region; ORF, open reading frame; SATB1, special AT-rich sequence-binding protein 1; SAR, scaffold attachment region; SBS, SATB1-binding sites.

Cross-linked chromatin, which sedimented at the bottom of the gradient, was collected, dialyzed overnight, and stored in aliquots at −80°C as described by Orlando and Paro (1993).

Immunoprecipitation

Samples of 30–50 μg of cross-linked chromatin were digested with 12 U of Sau3AI (New England Biolabs, Beverly, MA) in 500 μl of 1× reaction buffer recommended by the manufacturer, at 37°C overnight. This digestion resulted in the fragmentation of genomic DNA to an average size of ~600 bp (in the range of 300–1,000 bp) and created the appropriate DNA ends for subsequent linker ligation. The cross-linked and digested chromatin was adjusted to 1% NP-40 and precleared by incubation with 50 μl of a 50% suspension of protein A–Sepharose beads (Sigma Chemical Co., St. Louis, MO) for 1 h at 4°C with mixing. The beads were removed by centrifugation, and supernatants were transferred to a new microfuge tube. Samples in 500 μl of the buffer described above were immunoprecipitated by the addition of 10 μl of preimmune serum and were incubated at 4°C for 1 h with mixing, followed by additional incubation with 40 μl of protein A–Sepharose beads. After centrifugation, the supernatant was divided into two equal aliquots. Samples were immunoprecipitated by the addition of 5 μl of either preimmune serum or polyclonal anti-SATB1 antibody together with 50 μl of protein A–Sepharose beads and incubated at 4°C overnight with gentle mixing.

Immunoprecipitates were collected by centrifugation and washed as described in Orlando and Paro (1993). Samples of the immunoprecipitates were removed for Western blot analysis, and the remainder was treated for the reversal of cross-links by proteinase K digestion and heat treatment (Orlando and Paro, 1993).

PCR Amplification of Immunoprecipitated DNA

After the reversal of formaldehyde cross-links and purification, we routinely obtained ~1–5 ng of DNA. For cloning of these sequences, ligation-mediated PCR was performed. The following oligonucleotides were annealed and used as linkers after 5′ phosphorylation of the 24-mer: 5′ GATCAGAAGCTTGAATTCGAGCAG 3′, 5′ CTGCTCGAATTC-AAGCTTCT 3′.

After linker ligation, the 20-mer oligonucleotide was used as a primer in a PCR reaction with Pfu polymerase (Stratagene, La Jolla, CA) using the following parameters: 95°C/45 s, 55°C/1 min, and 72°C/1.5 min for the indicated number of cycles followed by a final incubation at 72°C for 5 min. The number of amplification cycles was determined by examining the PCR products produced by both preimmune and SATB1 immunoprecipitates after increasing number of cycles. The number of amplification cycles was chosen such that the anti-SATB1–immunoprecipitated DNA yielded PCR-amplified DNA, whereas the preimmune sample contained little or no amplification product.

Cloning of PCR Products

Oligonucleotide linkers were removed from the PCR-amplified DNA by digestion with 8 U of Sau3AI at 37°C for 2 h. PCR products were then purified on an agarose gel followed by electroelution and ethanol precipitation. Purified DNA was then ligated into pBluescript vector (Stratagene), which had been digested with BamHI. Cloned DNA was then transformed into competent XL1Blue cells (Stratagene). Transformants were grown in liquid cultures of Luria-Bertani Medium (LB broth) containing 50 μg/ml ampicillin.

Purification of SATB1-binding Sites from Cloned DNA

Plasmid DNA was isolated from liquid cultures by centrifugation through CsCl gradients. We obtained the best results using CsCl purification rather than purifying plasmid DNA by minipreparation. Large-scale plasmid preparation also provided sufficient DNA to allow many screenings for SATB1-binding sites from a single transformation. Insert DNA was harvested by digesting plasmid DNA with XhoI and NotI. These enzymes were chosen to preserve the KS and SK vector primer sites within pBluescript, which were used in the subsequent PCR amplification. Insert DNA was purified from the vector by agarose gel electrophoresis followed by electroelution and ethanol precipitation. Insert DNA was visualized as a smear of DNA sizes ranging from ~200 bp to 2 kbp.

Insert DNA was labeled using Klenow polymerase and [α-^{32}P]dATP (3,000 Ci/mmol; New England Nuclear). SATB1-binding sites were isolated from the mixture of labeled DNA by the gel shift assay using purified, recombinant SATB1. Up to 10 lanes of the gel shift assay were loaded on a gel, and after electrophoresis, the shifted DNA was visualized by exposing the wet gels to a film at room temperature. Shifted protein/DNA was isolated by excising the shifted band from the wet gels and eluted from the gel slices in a minimal volume of 10 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl at 37°C for 2 h. The eluted DNA was purified by phenol/chloroform extraction and ethanol precipitation. The shifted DNA was then further purified by passage through an Elutip D minicolumn (Schleicher & Schuell, Keene, NH).

Amplification and Recloning of SATB1-binding Sites

To enable recloning of the SATB1-binding sites, PCR amplification of the shifted DNA was necessary. Amplification was performed using both KS and SK primers from pBluescript and Pfu polymerase (Stratagene). 20 cycles of amplification were performed using the following parameters: 95°C/45 s, 50°C/30 s, and 72°C/1 min. PCR products were purified by phenol/chloroform extraction and ethanol precipitation. PCR-amplified DNA was then digested using the appropriate restriction enzymes for recloning into pBluescript. The enzymes selected for recloning were determined by performing preliminary experiments to determine whether internal restriction sites for the enzymes were present. If the amplified DNA contained an internal site(s), then alternate restriction enzymes were used. The digested DNA was purified by agarose gel electrophoresis followed by electroelution and ethanol precipitation. DNA was then cloned into pBluescript, transformed into XL1Blue-competent cells (Stratagene) and plated onto LB agar plates containing 50 μg/ml of ampicillin. Colonies were picked and grown in LB broth containing ampicillin. Plasmid DNA was prepared from individual colonies, and inserted DNA was isolated. The insert DNA was tested for SATB1-binding activity and specificity by gel shift assays.

Analysis of SATB1-binding Sites

Clones that were positive for specific SATB1-binding activity were retained for sequence analysis. Sequencing of SATB1-binding sites was performed by the Sanger method using Sequenase (United States Biochemical Corp., Cleveland, OH) and the T3 and T7 primers from pBluescript. Homology searches were performed against nucleotide databases using the BLAST program (Altschul et al., 1990).

Gel Shift Assay

The gel shift assay was performed as described (Dickinson et al., 1992), using recombinant SATB1. The recombinant SATB1 was a glutathione S-transferase (GST) fusion protein consisting of amino acids 346–763 of human SATB1. (We refer to this construct as GST–SATB1 in this study.) This protein contains both the MAR-binding domain and homeodomain and was referred to as GST (MD + HD) in Dickinson et al. (1997). To estimate the K_d values for SATB1-binding sites, gel shift assays were run with increasing concentrations of SATB1 under conditions of saturating DNA concentration. The dried gels were exposed to a PhosphorImager screen, and the amounts of remaining free probe in each lane were quantitated. K_d values were determined by calculating the concentration of SATB1 required to shift 50% of the probe DNA. For the determination of SATB1-binding specificity, gel shift assays were performed in the presence of a 50-fold molar excess of unlabeled wild-type concatemer of the IgH enhancer 3′ MAR or the mutated version of this sequence, as previously reported (Dickinson et al., 1992).

Western Blotting

Western blotting was performed as previously described (Dickinson and Kohwi-Shigematsu, 1995), except that a 5,000-fold dilution of polyclonal anti-SATB1 antibody was used.

Nuclear Halo Preparation

The previously described procedure (Gerdes et al., 1994) was followed with minor modifications. Briefly, Jurkat cells or SK-BR-3 cells were pelleted, washed two times in PBS, and resuspended in PBS at 1×10^6 cells/ml. Cells were incubated in 0.5% Triton X-100 in isotonic cytoskeleton CSK buffer (100 mM KCl, 300 mM sucrose, 10 mM Pipes, pH 6.8, 3 mM $MgCl_2$, 1 mM EGTA, 1.2 mM PMSF) (Fey et al., 1984) at 4°C for 5 min.

Then 2×10^5 nuclei were cytospun onto slides for 5 min at 500 *g*. The slides were extracted with 2 M NaCl buffer (2 M NaCl, 10 mM Pipes, pH 6.8, 10 mM EDTA, 0.1% digitonin, 0.05 mM spermine, 0.125 mM spermidine) for 4 min. After extraction of histones, nuclear halos were prepared by sequential 1-min rinses in 10, 5, and 2× PBS and a 2-min rinse in 1× PBS followed by sequential 1-min rinses with 10, 30, 50, 70, and 95% ethanol. Slides were air dried, and nuclear halos were fixed by baking at 70°C for 2 h.

Nuclear Matrix Preparation

Nuclear matrices were prepared essentially according to the method of Fey et al. (1984). Jurkat cells or SK-BR-3 cells either on slides or in Eppendorf tubes (Madison, WI) were treated with 0.5% Triton X-100 in CSK buffer at 4°C for 5 min, and the supernatant (either directly from the monolayer or after a 5-min centrifugation at 600 *g*) was removed as the "soluble" fraction. The Triton X-100–insoluble structures were extracted with an extraction buffer (42.5 mM Tris-HCl, pH 8.3, 8.5 mM NaCl, 2.6 mM $MgCl_2$, 1.2 mM PMSF, 1% Tween 40, and 0.5% deoxycholic acid) for 5 min at 4°C. Extracted proteins in the "cytoskeleton" fraction were removed in supernatant. The "chromatin" fraction (DNA and histones) was released from nuclei by digestion with 100 μg/ml bovine pancreatic DNase 1 (Boehringer Mannheim Corp., Indianapolis, IN) in a buffer identical to the CSK buffer except that 50 mM NaCl was present instead of 100 mM KCl, and digestion proceeded for 20 min at 25°C. Ammonium sulfate was added to a final concentration of 0.25 M for 5 min at 4°C. The chromatin fraction was removed as a supernatant, leaving the nuclear matrix on the slides. The slides were fixed in cold methanol and acetone for 3 min each. Before hybridization, nuclear matrix preparations were baked at 70°C for 2 h.

In Situ Hybridization and DNA Probes

For DNA probes, the U2 snRNA gene cluster (pTP18) and the 5S RNA gene cluster (pH5SB) sequences were kindly provided from Alan M. Weiner (Yale University, New Haven, CT) and Randall D. Little (Genome Therapeutics, Waltham, MA), respectively. SBS-2, SBS-3, and SBS-11 are in vivo SATB1-binding DNA sequences that were cloned by us (this study).

Hybridization was performed according to Lawrence et al. (1988). For fluorescence in situ DNA hybridization (FISH), the DNA probes were labeled with biotin-14-dATP (GIBCO BRL) using a BioPrime DNA labeling system (Life Technologies, Gaithersburg, MD). The samples were incubated for 10 min in 0.1 M triethanolamine and 0.25% acetic anhydride at room temperature, denatured by incubation at 70°C for 2 min in 70% formamide, 2× SSC for 2 min, dehydrated in cold 70 and 100% ethanol for 3 min each, and allowed to air dry. For each sample, 10–50 ng of probe, mixed with competitor nucleic acids (10 μg sonicated salmon sperm DNA, 10 μg *Escherichia coli* tRNA) was resuspended in 10 μl deionized formamide and denatured at 70°C for 5 min. Hybridization was allowed to proceed overnight in a buffer of 50% formamide, 20% dextran sulfate, 1% BSA, and 2× SSC (1× SSC is 150 mM NaCl, 30 mM sodium citrate) at 37°C. Samples were rinsed for 10 min each in 50% formamide/2× SSC, 2× SSC, and 1× SSC. To eliminate the possibility of contamination with cytoplasmic and nuclear RNA, samples were treated with 100 μg/ml RNase A (Sigma Chemical Co.) for 1 h at 37°C before hybridization.

Samples were incubated in 1:500 diluted FITC-conjugated ExtrAvidin (2.7 mg/ml) or rhodamine-conjugated ExtrAvidin (3.5 mg/ml) (Sigma Chemical Co.) in 4× SSC, 1% BSA for 30 min at room temperature. Samples were then rinsed for 10 min each in 4× SSC, 4× SSC/0.1% Triton X-100, then 4× SSC. Slides were stained with propidium iodide (30 μg/ml) (Sigma Chemical Co.) or 4,6-diamidino-2-phenylindole (DAPI; 1 μg/ml) (Sigma Chemical Co.) to visualize DNA in halos or nuclei and mounted in antibleach mounting medium (Vector Laboratories, Burlingame, CA). Samples were examined at 1,000 magnification (model BX60 microscope; Olympus America, Inc., Melville, NY). Color photographs were taken using PMC ASA 400 film (Eastman Kodak Corp., Rochester, NY).

Subcellular Localization of SATB1

As described above in detail in the nuclear matrix preparation section, the soluble fraction, the cytoskeleton fraction, and the chromatin fraction prepared from Jurkat cells or SK-BR-3 cells were extracted in 1 ml each of the corresponding buffer. The nuclear matrix fraction was directly suspended in 1 ml of 1× SDS sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 10 mM DTT, 0.001% bromophenol blue). 20 μl was removed from each sample, and 4 μl of 6× SDS sample buffer was added to all fractions except the nuclear matrix fraction, boiled, and analyzed by SDS-PAGE.

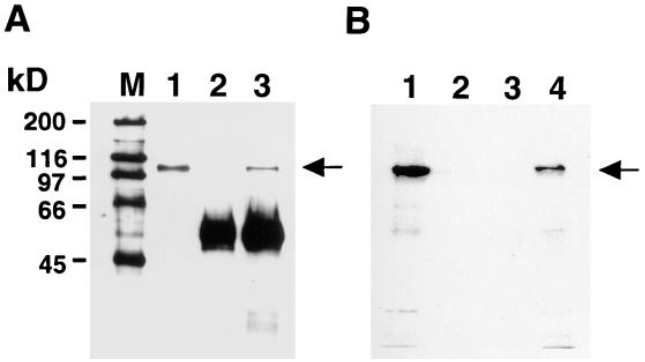


Figure 1. SATB1 is expressed in Jurkat cells and is a component of the nuclear matrix. (*A*) Western blot with anti-SATB1 antibody from Jurkat cell extracts. Cell extracts from Jurkat cells were either untreated (lane *1*) or immunoprecipitated with preimmune (lane *2*) or anti-SATB1 antibody (lane *3*). The protein extracts and immunoprecipitates were then resolved by 10% SDS-PAGE and electrotransferred to an immobilon-P membrane. The membrane was then probed using anti-SATB1 antibody and was visualized using an HRP-conjugated secondary antibody followed by ECL detection. Bio-Rad biotinylated molecular mass markers are indicated on the left, and the position of SATB1 migration is shown by an arrow on the right. (*B*) Subcellular localization of SATB1. Mouse thymocytes (5×10^6) were fractionated as described in Materials and Methods: Lane *1*, supernatant of 0.5% Triton X-100–CSK extraction (soluble fraction); lane *2*, the "cytoskeleton" fraction; lane *3*, the "chromatin" fraction; lane *4*, the nuclear matrix fraction as described in Materials and Methods.

Results

Isolation of Genomic DNA Bound to SATB1 In Vivo

SATB1 is a T cell factor that is predominantly expressed in thymocytes and found at a high level in certain T cell lines (Dickinson et al., 1992; Cunningham et al., 1994). The human and mouse SATB1 proteins are highly conserved with 98% homology at the amino acid sequence level (Nakagomi et al., 1994). We used, for the present study, the human T cell lymphoblastic Jurkat cell line in which we found SATB1 expressed (Fig. 1 *A*). SATB1 can be immunoprecipitated by rabbit polyclonal anti–human SATB1 antibody from cell extracts of Jurkat cells, while a matching preimmune serum did not immunoprecipitate SATB1. SATB1 is a component of the nuclear matrix in both Jurkat nuclei and thymocyte nuclei. Sequential extraction of mouse thymocyte nuclei (Fig. 1 *B*) and Jurkat cells (data not shown) according to the method by Fey et al. (1984) revealed a significant amount of SATB1 (~30%) remaining in the nuclear matrix fractions (Fig. 1 *B*, lane *4*).

Based on the strategy for identifying in vivo SATB1-binding genomic sequences as outlined in Fig. 2, we isolated genomic sequences bound to SATB1 in Jurkat cells (see Kohwi-Shigematsu et al., 1997*b* for a detailed protocol). Previous in vivo protein/DNA binding studies were designed to detect DNA target sequences within a pool of

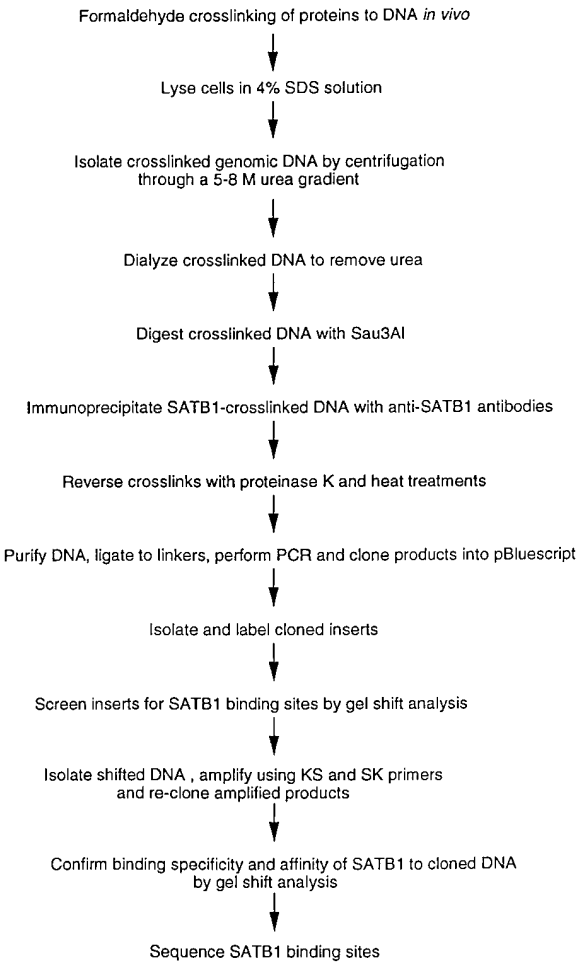
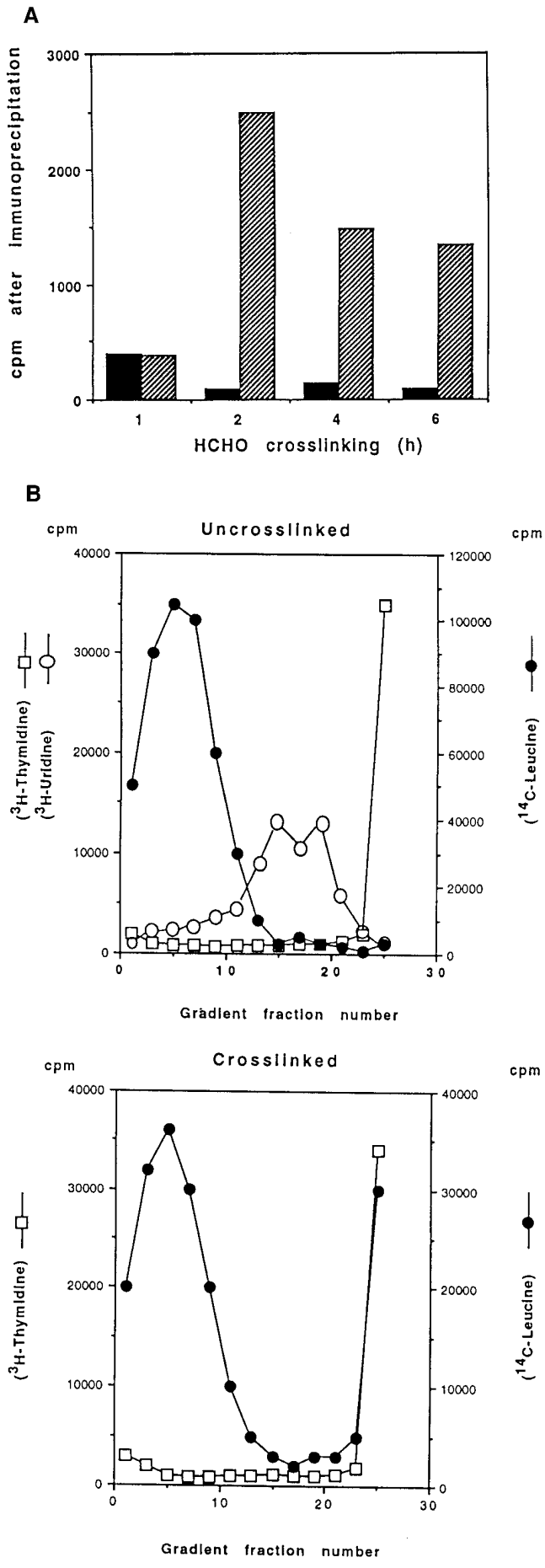


Figure 2. Outline of in vivo cross-linking and binding site cloning protocol. The general scheme for the cross-linking of DNA and proteins and subsequent isolation and cloning of SATB1-binding sites is shown. For a detailed description of the protocol see the Materials and Methods section.

in vivo cross-linked genomic DNA by hybridization analysis. A similar method using a specific DNA probe for hybridization cannot be used in the case of in vivo SATB1-binding sequences because SATB1 recognizes a specific DNA sequence context rather than a primary sequence consensus based on in vitro studies (Dickinson et al., 1992). To identify SATB1-binding sites, it was necessary to isolate and analyze individual sequences that were cross-linked to SATB1 in vivo. For this reason, a new strategy was devised, except that formaldehyde was used as the cross-linking reagent as previously published (Solomon and Varshavsky, 1985; Orlando and Paro, 1993). In our study, one important inclusion was to use preimmune serum as a control. First, optimal conditions for formaldehyde (HCHO) cross-linking of SATB1 to a binding target sequence were determined in vitro with the criteria that an anti-SATB1 antibody, but not preimmune serum from the same rabbit, coprecipitated the highest amount of DNA after cross-linking with formaldehyde. Whether a radiolabeled 3′ MAR of the IgH enhancer or labeled genomic DNA from Jurkat cells was used, the optimal conditions for cross-linking to SATB1 was found to be a 2-h incubation at 4°C in the presence of 1% formaldehyde (Fig. 3 *A*). The optimal cross-linking conditions determined by the in vitro study were applied to Jurkat cells prelabeled with [^{3}H]thymidine and [^{14}C]leucine. After cross-linking, cells were lysed in buffer containing SDS and layered on top of a gradient of 5–8 M urea. Upon centrifugation, under conditions described in Materials and Methods, genomic DNA was pelleted at the bottom of the gradient and was completely separated from free proteins. It was also free from RNA, which sedimented between free protein and pelleted DNA under this centrifugation condition (Fig. 3 *B*, *top*). Only in the formaldehyde cross-linked sample did ^{14}C counts cosediment with ^{3}H counts, indicating the presence of DNA/protein cross-links (Fig. 3 *B*, *bottom*). Under these conditions, all nuclear matrix–associated proteins, including SATB1, were apparently removed from genomic DNA unless they were cross-linked. The cross-linked chromatin was collected, dialyzed to remove urea, and digested with Sau3AI at 37°C overnight. The resulting DNA fragments, with an average size of 300–1,000 bp, were then immunoprecipitated using either preimmune or anti-SATB1 antibody. In the case of the in vitro study, immunoprecipitation with anti-SATB1 antibody was done without preclearing with preimmune serum, which contributed to a background of ~4%, under optimal conditions (Fig. 3 *A*, lane *2*). Therefore, in the subsequent in vivo studies, preclearing steps were used. Before immunoprecipitation, samples were precleared with both preimmune serum and protein A–Sepharose (for a more precise protocol, see Kohwi-Shigematsu et al., 1997*b*). Histones are known to cross-link with genomic DNA under similar conditions (Fragoso and Hager, 1997), but apparently Sau3A1 restriction digestion was not severely affected.

A single immunoprecipitation quantitatively depleted the SATB1/DNA complexes from DNA fragments cross-linked to proteins (data not shown). Typically, between 1–5 ng of DNA was present in the SATB1 cross-linked and immunoprecipitated sample. Western blot analysis of anti-SATB1 antibody immunoprecipitates revealed multiple fuzzy bands instead of a single 103-kD SATB1 band, indicating the presence of SATB1 cross-linked to genomic DNA sequences (data not shown).

To isolate the DNA cross-linked to SATB1, proteins cross-linked to DNA were removed by protease digestion and further digested with Sau3AI to ensure complete digestion. Purified DNA was subject to various cycles of ligation-mediated PCR to determine the optimal conditions (typically 17–18 cycles), allowing detection of DNA signals from anti-SATB1, but not preimmune immunoprecipitates. Representative data is shown in Fig. 4 *A*. These PCR products, which are shown by brackets, were cloned into the Bluescript cloning vector. A pool of cloned inserts was prepared from bacterial transformants, and the inserts were separated on an agarose gel. Inserts of different size ranges were isolated and examined for their SATB1-binding activity by gel shift assays using bacterially produced GST–SATB1. A clear SATB1-binding activity was observed for inserts of various size ranges. Typical gel shift results are shown in Fig. 4 *B*. It should be noted that with increasing PCR cycles, nonspecific DNA segments will also be amplified, and they represent Bluescript inserts lacking SATB1-binding activity (data not shown). There-



fore, it is advisable to perform this gel shift analysis to isolate the shifted DNA fragments for subsequent subcloning. However, if the number of PCR cycles is well controlled as shown in Fig. 4 *A*, where essentially all anti-SATB1 antibody immunoprecipitated and amplified-DNA fragments derived from the immunoprecipitated DNA confer SATB1 binding as shown in Fig. 4 *B*, the extra step of recloning from shifted bands may be omitted.

Various control experiments were performed to ascertain that the above DNA sequences that bound SATB1 by gel shift assay indeed represented sequences bound to SATB1 in vivo. First, we tested whether preimmune precipitates contained any SATB1-binding sequences. Upon PCR amplification to 35 cycles to reach saturation, the preimmune samples also generated PCR products (Fig. 4 *C*) because of a trace amount of DNA nonspecifically coprecipitated. These 35-cycle preimmune precipitates were cloned into pBluescript cloning vector. No SATB1-binding signal was detected for the pool of DNA inserts derived from preimmune serum precipitates (Fig. 4 *D*). Furthermore, we also used anti-SATB1 antiserum precleared with exogeneously added SATB1 followed by immunoprecipitation. Such precleared serum gave identical results as preimmune serum and did not give rise to DNA inserts that have SATB1-binding activity upon immunoprecipitation (Fig. 4 *E*). As yet another control, protein cross-linked DNA fractions, after a prior single immunoprecipitation with anti-SATB1 antiserum to deplete SATB1–cross-linked DNA, also failed to give rise to any SATB1-binding sequences upon 35-cycle PCR amplification (data not shown). These results demonstrate that genomic DNA bound to SATB1 in vivo can be specifically immunoprecipitated from a pool of cross-linked DNA with anti-SATB1 antiserum. These data were completely reproducible by using either crude anti-SATB1 serum with a 160,000 titer by ELISA assay or with affinity-purified SATB1 antibody (data not shown).

Identification of SATB1-bound Sequences In Vivo

To identify individual SATB1-binding sequences, the shifted DNA was isolated from acrylamide gels, PCR am-

Figure 3. Immunoprecipitation of SATB1 and urea gradient purification of genomic DNA. (*A*) In vitro cross-linking of GST–SATB1 to a radiolabeled synthetic MAR. The GST–SATB1 protein was mixed with a radiolabeled MAR probe representing a concatemerized IgH enhancer MAR. After exposure to 1% formaldehyde for the indicated period of time, samples were immunoprecipitated with either preimmune (*solid histograms*) or anti-SATB1 antibody (*hatched histograms*). The cpm associated with the immunoprecipitates was determined by liquid scintillation counting. (*B*) Urea gradient purification of genomic DNA. Jurkat cells were grown in the presence of [^{3}H]thymidine and [^{14}C]leucine for a period of 24 h. Cells were then either untreated (*top*) or subjected to in vivo formaldehyde cross-linking for a period of 2 h (*bottom*). Cells were lysed and loaded onto 5–8 M urea gradients and centrifuged as described in the Methods section. After centrifugation, the gradients were fractionated and ^{3}H and ^{14}C counts were determined by liquid scintillation counting. As a separate experiment, similar urea gradient centrifugation was performed with Jurkat cells grown in the presence of [^{3}H]uridine to monitor RNA distribution in the gradient (*top*).

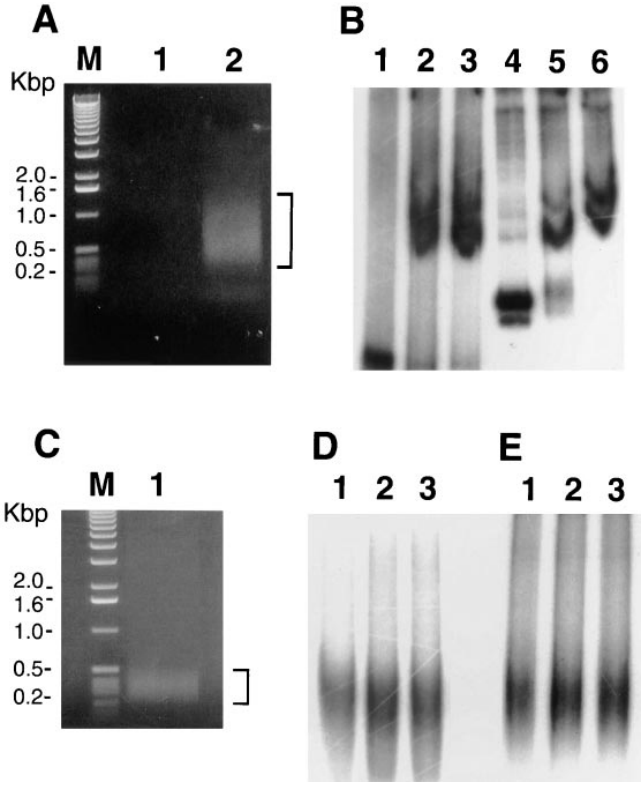


Figure 4. SATB1 binds to genomic DNA in vivo. (*A*) PCR amplification of immunoprecipitated DNA. After purification of immunoprecipitated DNA and linker ligation, PCR amplification was performed as described in the Materials and Methods section using 17 cycles. PCR products from preimmune (lane *1*) and anti-SATB1 immunoprecipitated DNA (lane *2*) were examined by 1% agarose gel electrophoresis followed by staining with ethidium bromide. Lane *M*, 1-kb molecular size markers. (*B*) Gel mobility shift with anti-SATB1 immunoprecipitated DNA. PCR products shown by a bracket in *A* were isolated from the gel and cloned into pBluescript. Insert DNA in different size ranges was tested for SATB1-binding activity. DNA in the range of 0.6 kb (lanes *1–3*) and 1-kb ranges (lanes *4–6*) are shown. Lanes *1* and *4* contain 0 nM; lanes *2* and *5*, 8 nM; lanes *3* and *6*, 16 nM GST–SATB1 protein. (*C*) PCR amplification of preimmune immunoprecipitated DNA. PCR products were generated using 35 cycles of amplification, and the resulting DNA was visualized in 1% agarose gel stained with ethidium bromide (lane *1*). The region shown by a bracket was cloned into pBluescript. Lane *M*, 1-kb molecular size markers. (*D*) Gel mobility shift assay with preimmune immunoprecipitated DNA. Inserts derived from DNA shown by the bracket from *C* were isolated and used in a gel mobility shift assay with GST–SATB1 protein. Lanes *1–3* contain 0, 8, and 16 nM GST–SATB1 protein, respectively. (*E*) Gel mobility shift assay with precleared anti-SATB1 antibody. A similar experiment as described for *C* and *D* was performed with precleared anti-SATB1 antiserum, which was prepared by adding SATB1 to the serum, followed by immunoprecipitation. After a 35-cycle amplification, the cloned DNA failed to demonstrate a gel shift with GST–SATB1. Lanes *1–3* contain 0, 8, and 16 nM GST–SATB1, respectively.

plified, and recloned into Bluescript. A total of 16 such cloned DNA were sequenced. In the present study, we focused on relatively short DNA sequences, mostly under 500 bp, except for one which was 1.1 kb. These sequences had a common characteristic of 60–70% in A + T content and clustering of ATC sequence stretches. The typical ATC sequence context found for the in vivo SATB1-binding sites is shown in Fig. 5. Most of the cloned SATB1-binding sites (11 out of 16 clones) displayed no extensive homology to any known sequence found by the BLAST search tool (Altschul et al., 1990). However, it is important to note that some specific regions within these sequences, containing long stretches of ATC sequences, typically showed significant homology with autonomously replicating sequences (ARSs) from the yeast *Saccharomyces cerevisiae* and mitochondrial replication origins. ARSs confer on plasmids the ability to be maintained autonomously in yeast cells as minichromosomes and have been shown, through a combination of genetic and physical mapping techniques, to function as in vivo origins of replication (for review see Benbow et al., 1992). This sequence characteristic with an ATC sequence cluster is most typical of SATB1-binding sequences found in various MARs previously determined in vitro. In one clone (SBS-13), a stretch of a d(CA) repeat was found within an ATC sequence cluster. Repeating elements such as this, as well as other DNA sequences that readily form non-B DNA structure, including homopurine-homopyrimidine stretches (Kohwi and Kohwi-Shigematsu, 1988, 1993), are frequently found within MAR segments (Stockhaus et al., 1987; Jarman and Higgs, 1988; Spitzner Muller, 1989).

Amongst the remaining five clones, one clone (SBS-11) was identified as satellite 2, a collection of simple sequences that constitute a large part of classical satellite II, which is one of the three classical satellites fractionated by ion-$CsSO_4$ gradients (for review see Prosser et al., 1986). The SBS-11 sequence contains the simple repeat 5′-ATTCC-3′, but the repeat is poorly conserved, and Taq I and Hinf I sites are both formed from apparently random base changes in the 5-bp repeat. This is typical for satellite 2, which is distinct from satellite 3, the simple sequence component of classical satellite III. The simple repeat 5′-ATTCC-3′ in satellite 3 is well conserved, and satellite 3 is digested by Hinf I to generate a ladder based on the repeated elements 5′-ATTCC-3′, but lack Taq I sites. SBS-11 exhibits 95% homology with a human satellite 2 sequence located in chromosome 1 (HSSAT2) and another satellite 2 sequence identified as CpG DNA (Cross et al., 1994). In fact, by in situ hybridization, the SBS-11 homologous sequence was localized to the pericentrometric region of chromosome 1 as a prominent site (data not shown). Another short clone (SBS-16) contained ATC sequences and also showed high homology to sequences identified as CpG island containing DNA (for review see Cross and Bird, 1995). While CpG islands are frequently found at the 5′ ends of genes, no gene linked with this particular CpG island has yet been identified. Satellite sequences are composed of a collection of variants of the sequence ATTCC, which can potentially formulate a typical ATC sequence if positioned properly. In the case of SBS-11, multiple ATC sequence stretches of 22–24 bp were identified. The remaining three clones (SBS-2, SBS-4, and SBS-14) exhibited homology between 77–85% to a full-length LINE-1 (L1) sequence (Skowronski et al., 1988) over the entire length of each clone. One clone (SBS-2) exhibited a 98% homology over 300 bp in length with the particular L1 sequence found in the Huntington Disease gene region

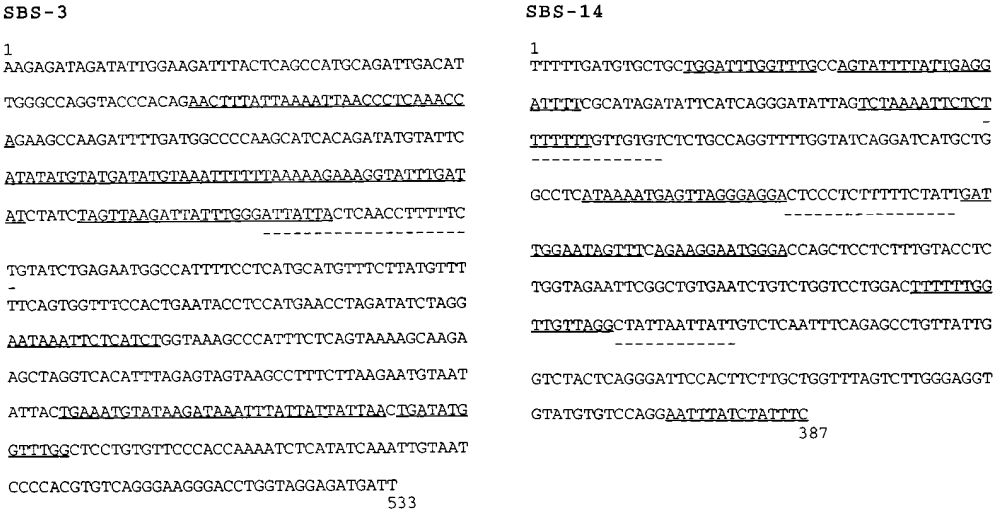


Figure 5. In vivo SATB1-binding sites contain ATC sequences. The nucleotide sequence of two of the cloned SATB1-binding sites, SBS-3 and SBS-14, is shown. The presence of ATC/ATG sequences are indicated by underlining or a dotted line. These clones are representative, and all sequences contained similar ATC sequences. In SBS-14, the central region with an ATC sequence stretch juxtaposed with surrounding ATC sequence stretches is likely to be a preferential site for SATB1 binding.

(Baxendale et al., 1993). L1 elements are an extremely abundant class of long, highly repetitive DNA elements of mammalian retrotransposons that lack long terminal repeats (for review see Boeke et al., 1997). Although most L1 elements are truncated and internally rearranged, a full-length L1 contains two open reading frames (ORF), the second of which encodes a reverse transcriptase (Mathias et al., 1991). Homology comparison of the cloned L1 sequences to the full-length L1 placed all of these SATB1-binding sites within the second ORF. Two regions within the second ORF of the full-length L1 (Skowronski et al., 1988), nucleotides 2427–2887 and 3238–3626, were identified as the homologous regions to the in vivo binding sequences for SATB1. Significantly, these two regions contain ATC sequence stretches (Fig. 5, *right*).

SATB1-binding Specificity to Cloned SATB1–cross-linked DNA

For each of 16 isolated cloned sequences, binding specificity to bacterially produced GST–SATB1 using specific and nonspecific competitor DNA was examined. As a specific competitor, a synthetic MAR (wild-type $[25]_5$) derived from 25 bp containing the core unwinding element of 3′ MAR of the IgH enhancer (Kohwi-Shigematsu and Kohwi, 1990; Bode et al., 1992) was used. As a nonspecific competitor, a mutated version of this sequence (mutated $[24]_8$) was used, which is still A + T–rich but no longer binds SATB1 and lacks affinity to the nuclear matrix in vitro (Dickinson et al., 1992; Bode et al., 1992). In all cases, competition with a 50-fold molar excess of unlabeled wild-type $(25)_5$ was able to compete out the shift, while the same amount of the mutated MAR was completely ineffective as competitor. Also, the naturally occurring IgH enhancer MAR was equally effective in competing for SATB1 binding (data not shown). These results indicate that despite the fact that the primary sequences are different, there exist specific DNA contexts that SATB1 recognizes in cross-linked genomic DNA common to synthetic and naturally occurring MARs. A representative gel shift assay using two different cloned SATB1-binding sites (SBS-3 and SBS-14) with a positive control of wild-type $(25)_5$ is shown in Fig. 6. Although most of the sequences have affinities comparable to those measured for the synthetic wild-type $(25)_5$ concatemer, some sequences show lower affinity to SATB1 (Table I). This is probably due to the consequence of deleting important flanking regions by Sau3AI restriction digestion. SATB1 often requires the concomitant presence of neighboring ATC sequences for strong binding (our unpublished results).

In Vivo SATB1-binding Sequences Are Tightly Bound to the Base of Chromatin Loops

We first confirmed that in vivo SATB1-binding sequences have a strong affinity in vitro to isolated nuclear matrix preparations (data not shown). Therefore, by the operational definition, these SATB1-binding sequences are classified as MARs. The most critical question is whether SATB1-binding sites are at the base of chromatin loop domains, at which position they are tightly attached to the nuclear matrix in interphase nuclei. To examine this point, we used high-resolution in situ hybridization as described by Gerdes et al. (1994). We used in vivo SATB1 sequences as probes for in situ hybridization and examined their signals with intact nuclei, nuclear halos, and the nuclear matrix from Jurkat cells. Nuclear halos were prepared by extracting nuclei in situ with 2 M NaCl to remove histones and most of the nuclear proteins from nuclei to distend the DNA loops, and the nuclear matrices were prepared according to the method described in Fey et al. (1984) that contains an enzymatic digestion step to remove distended chromatin loop regions. The 2 M NaCl extraction procedure was used for the halo preparations because this method has been successfully used for the similar in situ hybridization experiments and is well characterized. This method gives reproducible structures in which a uniform halo of DNA circumscribes the residual nucleus, and the resulting structures retain several aspects of native nuclear morphology (Gerdes et al., 1994).

Three in vivo SATB1-binding sequences, SBS-2 (ATC sequence cluster), SBS-3 (L1), and SBS-11 (satellite 2), were chosen as DNA probes for in situ hybridization studies. With these DNA probes, in situ hybridization revealed single condensed spots of fluorescent signals in the nuclei and these spots remained tightly condensed in the residual

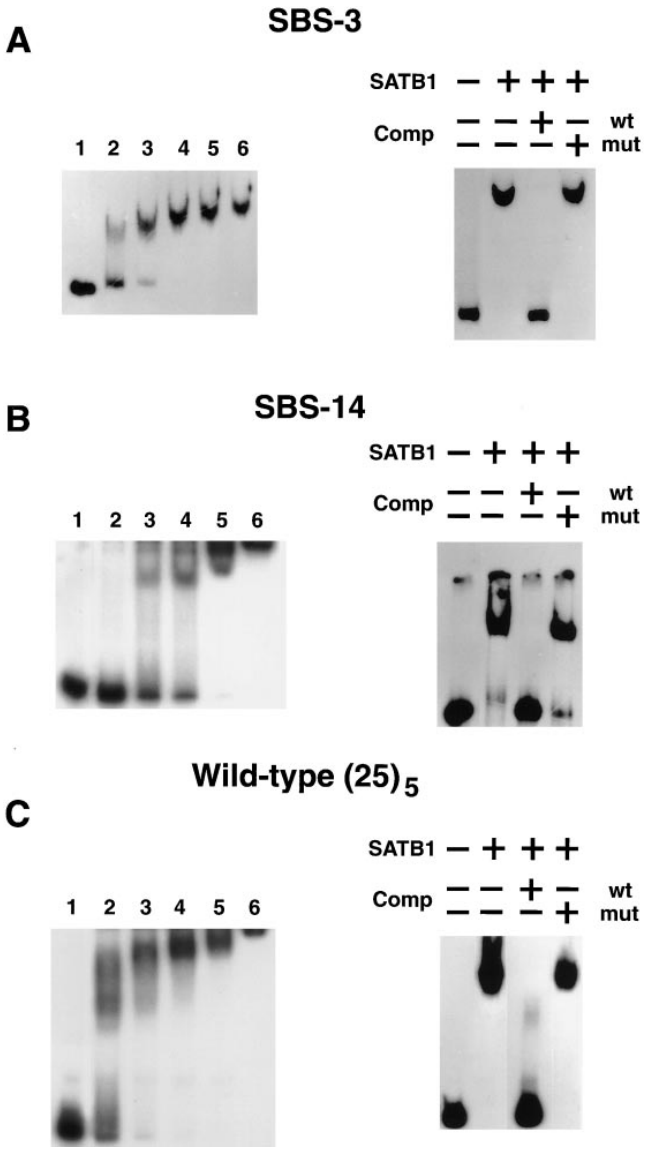


Figure 6. SATB1 binds specifically and with high affinity to cloned binding sites. Gel mobility assay was performed with cloned in vivo SATB1-binding sequences SBS-3 (*A*) and SBS-14 (*B*) as well as synthetic MAR wild-type $(25)_5$ (*C*) and GST–SATB1 protein. DNA binding activity was visualized after electrophoresis through a 6% acrylamide gel followed by autoradiography. In the left panels, lanes *1–6* contain 0, 0.5, 1, 2, 4, and 8 nM GST–SATB1 protein. The right panel shows a gel mobility shift competition using a 50-fold molar excess of unlabeled wild-type $(25)_5$ (*wt*) or a nonbinding mutated version mutated $(24)_8$ (*mut*). The concentration of SATB1 used was 4 nM.

nucleus of the halo preparations (Fig. 7). These results are consistent with the notion that SATB1-binding sites might be located at the base of chromatin loops. To examine this possibility further, in situ hybridization was performed for each SATB1-binding site with the nuclear matrix prepared from nuclei cytospun onto slides by the method, described in Materials and Methods, that included an extensive digestion with DNase 1. This treatment removed most of the genomic DNA from the resulting nuclear matrix, as indicated by the absence of staining with propidium iodide. In

Table I. Table of the Affinities and Sequence Features for Cloned SBS from Jurkat Cells

SATB1-binding site	K_d (nM) (clone size in bp)	Sequence features
wild-type $(25)_5$	1.0 (125)	ATC
SBS-1	29.2 (273)	ATC
SBS-2	6.4 (514)	ATC/L1
SBS-3	1.1 (533)	ATC
SBS-4	13.2 (465)	ATC/L1
SBS-5	12.4 (179)	ATC
SBS-6	6.7 (290)	ATC
SBS-7	4.0 (249)	ATC
SBS-8	8.4 (446)	ATC
SBS-9	13.5 (176)	ATC
SBS-10	5.9 (214)	ATC
SBS-11	3.8 (1,105)	ATC/Satellite/CpG
SBS-12	2.8 (449)	ATC
SBS-13	11.6 (497)	ATC/Microsatellite
SBS-14	2.0 (387)	ATC/L1
SBS-15	10.0 (308)	ATC
SBS-16	12.1 (110)	ATC/CpG

situ hybridization with the resulting nuclear matrix still exhibited single spot signals in all three cases of SATB1-binding sites (Fig. 7). This result indicates that these sequences, which bind to SATB1 in vivo, remained tightly associated with the nuclear matrix even after distended chromatin was removed. These in vivo SATB1-binding sequences, therefore, constitute the base of chromatin loops. We examined at least 300 nuclei, nuclear matrices, and halos for each in situ hybridization. Although typical data are shown by a single example, similar in situ hybridization signals were observed for the majority of the population (>90%) examined when the single spots were carefully focused under a microscope. In most cases, a similar number of fluorescent signals, ranging from two to four per nucleus, was obtained.

As controls, we used the 5S ribosomal gene and the U2 snRNA gene cluster as hybridization probes. These human genes are actively transcribed (Reddy et al., 1981). However, it was previously found that the organization of these gene clusters relative to DNA halos is markedly different (Gerdes et al., 1994). In humans, most 5S RNA genes are localized in a cluster of ~100 tandem copies of 2.3-kb sequence (Little and Braaten, 1989; Sorensen and Frederiksen, 1991) and are transcribed by RNA polymerase III (for review see Geiduscheck and Kassavetis, 1992). The U2 gene cluster contains ~20 copies of 6.2-kb repeat (Van Arsdell and Weiner, 1984; Westin et al., 1984) and is transcribed by RNA polymerase II (Lobo et al., 1990). Using halos from human fibroblasts, it was previously shown that the 5S RNA gene cluster is consistently released on a single distended DNA loop, whereas the U2 snRNA gene cluster remains as a condensed spot within the residual nucleus (Gerdes et al., 1994). These results were reproduced for these two probes with halos prepared from human Jurkat cells (Fig. 7). We have further examined the in situ hybridization signals after the removal of the genomic DNA. The resulting in situ prepared nuclear matrix still showed single spots for the U2 snRNA gene cluster, indicating that this sequence is, at least in part , situated at the base of the chromatin loops. On the other hand, there were no single spots detected for the 5S RNA

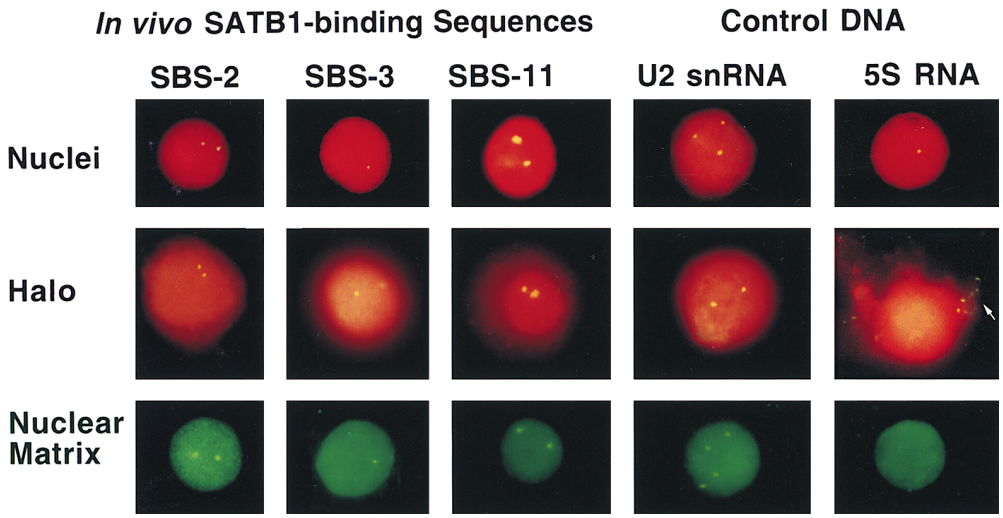


Figure 7. SATB1-binding sites are localized to the base of chromatin loops. In situ hybridization with Jurkat nuclei (*top*), nuclear halo (*middle*), and nuclear matrix (*bottom*) was performed using in vivo SATB1-binding sequences, SBS-2, SBS-3, and SBS-11, and control DNA, pTP18 (for U2 snRNA) and pH5SB (for the 5S rRNA gene), as DNA probes. These probes were labeled with biotin-11-dATP. Total DNA was stained with propidium iodide (*red*), and specific sequence hybridization was detected with FITC-conjugated extravidin (*yellow-green*). Since most of the genomic DNA has been removed from the nuclear matrix preparations, no propidium iodide staining was detectable in the nuclear matrix. In situ hybridization signals to the 5S rRNA within the distended chromatin halo are indicated by a white arrow.

gene cluster probe, indicating that this gene cluster was located in the extended chromatin loop region.

Anchoring of In Vivo SATB1-binding Site to the Nuclear Matrix Is Cell Type Dependent

We chose a cell line in which SATB1 is absent and tested whether the localization of in situ hybridization signals is altered in comparison to that detected in Jurkat cells. In situ hybridization with SBS-11 in the human breast cancer cell line, SK-BR-3 cells, was performed. Although bright intense signals were detected for in situ hybridization with SK-BR-3 nuclei, the signals were totally undetectable for the nuclear matrix preparations after chromatin was released into the distended loop region, the SBS-11 sequence apparently became more accessible to hybridization with the DNA probe. With a 30-s exposure, signals in the Jurkat halo preparations became more intense but remained condensed in the residual nucleus (data not shown). SK-BR-3 cells are aneuploid and giving more hybridization spots on nuclei.

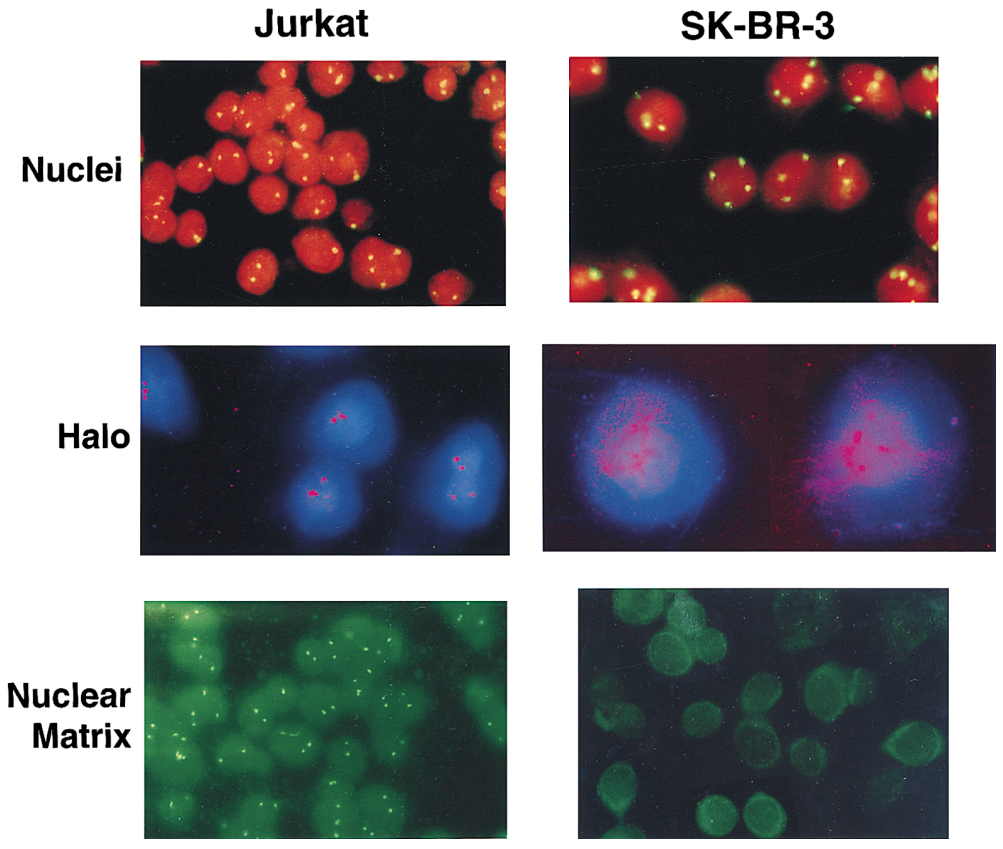


Figure 8. Anchoring of a SATB1-binding genomic sequence to the nuclear matrix is cell type dependent. In situ hybridization with SBS-11 was performed on nuclei, halos, and nuclear matrices prepared from Jurkat cells and breast cancer SK-BR-3 cells. The detection method was identical to that described in the legend to Fig. 7 for nuclei and the nuclear matrix. To enhance visualization of hybridization signals within the nuclear halos, the hybridization signal was detected with rhodamine-conjugated extravidin (*red*) over total DNA staining with DAPI (*blue*). The sizes of nuclei, halos, and nuclear matrix from SK-BR-3 are in general larger than those of Jurkat cells as shown. The exposure time was extended for the SK-BR-3 halos to 30 s (15 s for the Jurkat halos) to reproduce all rhodamine signal detected within the halo region in the final photograph. Because of the release of the sequences

moved by nuclease digestion (Fig. 8). This observation is in direct contrast with data obtained from the Jurkat nuclear matrix, in which hybridization signals were always detected. Consistent with the results from nuclear matrices, the nuclear halo preparations also showed a large difference in the distribution of in situ hybridization signals between Jurkat and SK-BR-3 cells. Although the signals were condensed as single spots that remained in the residual nucleus for Jurkat cells, the signals in the SK-BR-3 halo preparations were more diffuse and spread toward the DNA halos, providing more area for in situ hybridization. When in situ hybridization was performed with the U2 snRNA gene cluster on the halo preparation from SK-BR-3 cells, the signals remained condensed as single spots in the residual nucleus, as observed in the Jurkat halo preparation (Fig. 7 and data not shown). Apparently, SBS-11 is not tightly fixed onto the SK-BR-3 nuclear matrix and is therefore easily removed upon nuclease digestion. These results show that the chromatin loop organization, which is dictated by the loop attachment site, is different depending on cell type. It is of interest to note that satellite 2 sequences appear to serve as loop attachment DNA in a cell type–dependent manner.

The experiments described here were conducted as an important control to eliminate the possibility that SBS-11 hybridization to the Jurkat nuclear matrix was due to incompletely digested chromatin, rather than to its tight association with the nuclear matrix. This control experiment was important particularly for a sequence like satellite DNA, which represents a highly repetitive element. The Southern blot hybridization with the SBS-11 probe after pulsed field electrophoresis analysis of restriction enzyme (SmaI)-digested genomic DNA revealed an ~5.5-Mbp band containing ~3,600 copies per haploid genome (data not shown). This high copy number explains the bright and intense signals for in situ hybridization with SBS-11. The sequence organization of SBS-11 hybridized genomic DNA appears highly complex judging from the results of the pulsed field electrophoresis experiments optimized to examine different size ranges of restriction enzyme–digested genomic DNA fragments. So far, no clear repeating units have been detected, except for the simple repeat 5′ATTCC-3′ in a highly diverged form.

The in situ hybridization technique used here does not allow the detection of a single copy gene using DNA probes in the range of 500 bp. Therefore, we expect that the target sites we detected are tandemly repeated sequences, or at least clustered within a small locus. Because highly repeated L1 elements are interspersed, the SBS-2 probe cannot detect each individual L1 element by in situ hybridization. The SBS-2 in situ hybridization signals most likely detect this particular L1 sequence occurring more than once within a short distance from one another. Unfortunately, the absolute copy number of this particular L1 element can only be determined if its unique 5′ and 3′ genomic sequences become available. For SBS-3, the copy number was determined to be ~10 copies per diploid nucleus by Southern blot analysis (data not shown).

Discussion

We have demonstrated that SATB1, predominantly expressed in thymocytes and the T cell lymphoblastic Jurkat cell line, was bound in vivo to genomic sequences located at the base of the chromatin loops that are tightly attached to the nuclear matrix. This was shown by first isolating genomic sequences that are bound to SATB1 in human Jurkat cells by a combination of in vivo cross-linking, immunoprecipitation, and PCR amplification methods. All of the isolated in vivo SATB1-binding sites displayed high affinity and specificity to SATB1 in vitro and contained an ATC sequence context. These sequences were then used as probes for high resolution in situ fluorescence hybridization of nuclear halos, and in situ prepared nuclear matrices after the chromatin loop regions had been digested and removed. Genomic DNA that was tightly anchored onto the matrix, corresponding to the base of the chromatin loops, hybridized with the in vivo SATB1-binding sequences in Jurkat cells. This hybridization at the base of chromatin loops with a SATB1-binding sequence was totally undetectable in breast cancer cells in which SATB1 is absent, indicating that chromatin loop organization depends on cell type.

Determination of SATB1-binding Sequences In Vivo

The cross-linking approach to confirm target binding sites in vivo for a number of DNA-binding proteins with primary sequence specificity has been described (Orlando and Paro, 1993; Zhao et al., 1995). Because SATB1 recognizes a specific nucleotide sequence context instead of a primary sequence, each SATB1-binding sequence is different and no two are identical. This mandated that our experiments extend beyond the step of Southern blot hybridization of pooled immunoprecipitated cross-linked DNA to individually isolate SATB1–cross-linked genomic sequences. To ensure that each isolated sequence indeed represents the SATB1-binding sequence in vivo, multiple control experiments with preimmune antisera and extra gel shift steps were performed. Among 16 sequences characterized by this strategy, it is of interest that sequences with homology to L1 elements and CpG islands were found in addition to expected ATC sequence clusters that resemble ARS and mitochondrial replication origins with similar sequence context (Table I). One clone, SBS-11, was identified as satellite 2. L1 sequences appear to be a significant target for SATB1 binding because 3 out of 16 characterized in vivo SATB1-binding sequences contain significant homology to L1 elements. Jackson et al. (1996) reported a method for cloning genomic DNA bound to the nuclear matrix prepared in agarose-embedded cells and found one short cloned sequence homologous to an L1 element. L1 insertions have been identified in several genes, resulting in their disruption and contributing to various diseases including muscular dystrophy and cancer (Kazazian et al., 1988; Morse et al., 1988; Miki et al., 1992; Narita et al., 1993). Our results show that L1 elements, especially within the second ORF, are direct binding targets of SATB1 and are tightly bound to the nuclear matrix in Jurkat cells. It has been estimated that there are 30–60 transpositionally active L1 copies per diploid human genome (Sassaman et al., 1997). Whether SATB1 only binds to the second ORF of these active L1 or to any full-length or truncated L1 in a Jurkat cell is an important question that

remains to be answered. L1 elements provide the first example of SATB1 binding directly to a coding sequence. The second ORF itself contains ATC sequence stretches and exhibits strong and specific binding to SATB1 in vitro and in vivo. It is noteworthy that the second ORF of an L1 element and satellite DNA have been previously identified to be integration sites in the human genome for the human immunodeficiency virus (HIV) (Vincent et al., 1990). HIV may be preferentially integrating into those L1 and satellite DNA sites that correspond to MARs in vivo in T cells.

In Situ Fluorescence Hybridization: In Vivo SATB1-binding Sites Are at the Base of Chromatin Loops

The most significant characterization of isolated SATB1-binding sequences was the direct visualization of the sequences within the in situ prepared "halo" of histone-depleted nuclei and in nuclear matrices after extensive digestion with nuclease to remove the loop region of chromatin. Our results, showing that hybridization signals remained as condensed single spots in the residual nucleus rather than in the distended chromatin loop regions (DNA halos) and the fact that these spots remained in the nuclear matrix, provide strong evidence that these SATB1-binding sequences are localized at the base of chromatin loops. Because these are the sites to which SATB1 was cross-linked in Jurkat cells, we conclude that SATB1 binds in vivo to the genomic sequences tightly attached to the nuclear matrix or MARs. Similar in situ hybridization data have been obtained for mouse thymocytes using the mouse SATB1-binding sequences as probes (data not shown).

One of the clones, SBS-11, which is highly homologous to the satellite 2 sequences, displayed exceptionally strong in situ hybridization signals because based on the copy number estimation, the SBS-11 homologous sequence is expected to span ~4 Mbp of the genomic region. Such a large region appears to remain condensed as single spots in the residual nucleus of the Jurkat halo preparation, and the single spots remained on the nuclear matrix. In contrast, using SBS-11 as a probe, we found that there was no detectable hybridization signal for the nuclear matrix prepared from the human breast cancer cells, SK-BR-3, a cell line that does not express SATB1. This observation has significance for the following three reasons: First, original in situ hybridization signals for the Jurkat nuclear matrix are not due to the high copy number of this sequence, which could not be completely digested during the nuclear matrix preparations, and were thereby nonspecifically retained. Second, these results indicate that anchoring of specific sequences onto the nuclear matrix is cell type dependent. Identification of satellite 2 sequences as specific targets of SATB1 in Jurkat cells, and their cell type–dependent anchoring onto the nuclear matrix, suggests a new biological significance for this repetitive element. Third, the in situ hybridization of the nuclear matrices from Jurkat cells and SK-BR-3 cells strongly suggests that the matrix anchorage sites of chromosomes (base of chromatin loops) can be very large and not only limited to several hundred base pairs typically identified as MARs in vitro after genomic DNA digestion. It remains to be examined whether SATB1 binds to all MARs or only to a selected subset of MARs in T cells, leaving other MARs to be bound by other MAR-binding proteins. Recently, a MAR-binding protein SAF-A (hnRNP U), which is ubiquitously expressed, was found to directly bind to genomic DNA in vivo (Göhring and Fackelmayer, 1997). Individual SAF-A–bound DNAs must be characterized to address the above question.

Biological Significance of SATB1 Binding to the Matrix Attachment Sites of Chromatin In Vivo

SATB1 contains a divergent homeodomain, and the homeodomain together with the previously characterized MAR-binding domain (Nakagomi et al., 1995; Wang et al., 1995) confers specificity to the core unwinding element of a MAR (Dickinson et al., 1997). Homeodomain proteins are sequence-specific transcription factors and play important regulatory roles in development (for reviews see Gehring, 1987; Treisman et al., 1992). SATB1 strongly suppresses transcription of a stable transfected reporter gene flanked by MARs (Kohwi-Shigematsu et al., 1997*a*), which, in the absence of SATB1, exhibits high transcriptional activity (Klehr et al., 1991; Bode et al., 1992). SATB1 has also been shown to bind to the mouse mammary tumor virus (MMTV) long terminal repeat and negatively regulate MMTV transcription in lymphoid tissues (Liu et al., 1997). On the other hand, Banan et al. (1997) suggested that SATB1 binding to a regulatory sequence of the mouse CD8a gene promotes its transcription by replacing a known suppressor CDP/Cux. The nuclear matrix has been shown to provide the sites for active transcription (for review see Stein et al., 1996). In the case of SATB1, it may be important for negative and potentially positive regulation of certain genes in T cells by binding to the nuclear matrix at the base of chromatin loops.

Our observation that SATB1 binds in vivo to the genomic DNA segments at the base of chromatin loops suggests that transcriptional regulation takes place at these sites on nearby genes. Also, the data that attachment of an in vivo SATB1-binding sequence onto the nuclear matrix is a cell type–dependent phenomenon supports the idea that higher-order chromatin organization itself is an important component of cell type–specific regulation. SATB1 and genomic sequences that bind to SATB1 inside cells will provide unique tools to investigate an as yet unexplored area of cell type–specific gene regulation at the level of higher-order chromatin structure.

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Note Added in Proof. Sequence data for SBS are available from GenBank/EMBL/DDBJ under accession numbers: SBS-1 (AF051674), SBS-2 (AF051675), SBS-3 (AF051676), SBS-4 (AF051677), SBS-5 (AF051678), SBS-6 (AF051679), SBS-7 (AF051680), SBS-8 (AF051681), SBS-9 (AF051682), SBS-10 (AF051683), SBS-11 (AF051684), SBS-12 (AF051685), SBS-13 (AF051686), SBS-14 (AF051687), SBS-15 (AF051688), and SBS-16 (AF051689).

References

Altschul, S.F., W. Gish, W. Miller, E.W. Meyers, and D.J. Lipman. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403–410.

Banan, M., I.C. Rojas, W.-H. Lee, H.L. King, J.V. Harriss, R. Kobayashi, C.F. Webb, and P.D. Gottlieb. 1997. Interaction of the nuclear matrix-associated region (MAR)-binding proteins, SATB1 and CDP/Cux, with a MAR element (IL2α) in an upstream regulatory region of the mouse CD8a gene. *J. Biol. Chem.* 272:18440–18452.

Baxendale, S., M.E. MacDonald, R. Mott, F. Francis, C. Lin, S.F. Kirby, M. James, G. Zehetner, H. Hummerich, J. Valdes, et al. 1993. A cosmid contig and high resolution restriction map of the 2 megabase region containing the Huntington's disease gene. *Nat. Genet.* 4:181–186.

Benbow, R.M., J. Zhao, and D.D. Larson. 1992. On the nature of origins of DNA replication in eukaryotes. *BioEssays.* 14:661–670.

Benyajati, C., and A. Worcel. 1976. Isolation, characterization, and structure of the folded interphase genome of *Drosophila melanogaster*. *Cell.* 9:393–407.

Berezney, R., and D. Coffey. 1974. Identification of a nuclear protein matrix. *Biochem. Biophys. Res. Commun.* 60:1410–1419.

Bode, J., and K. Maass. 1988. Chromatin domain surrounding the human interferon-β gene as defined by scaffold-attached regions. *Biochemistry.* 27:4706–4711.

Bode, J., Y. Kohwi, L. Dickinson, T. Joh, D. Klehr, C. Mielke, and T. Kohwi-Shigematsu. 1992. Biological significance of unwinding capability of nuclear matrix-associating DNAs. *Science.* 255:195–197.

Boeke, J.D. 1997. LINEs and *Alus*-the poly A connection. *Nat. Genet.* 16:6–7.

Cockerill, P.N., and W.T. Garrard. 1986. Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. *Cell.* 44:273–282.

Cockerill, P.N., M.-H.Yuen, and W.T. Garrard. 1987. The enhancer of the immunoglobulin heavy chain locus is flanked by presumptive chromosomal loop anchorage elements. *J. Biol. Chem*. 262:5394–5397.

Cook, P.R., and I.A. Brazell. 1976. Conformational constraints in nuclear DNA. *J. Cell Sci.* 22:287–302.

Cross, S.H., and A.P. Bird. 1995. CpG islands and genes. *Curr. Opin. Genet. Dev*. 5:309–314.

Cross, S.H., J.A. Charlton, X. Nan, and A.P. Bird. 1994. Purification of CpG islands using a methylated DNA binding column. *Nat. Genet.* 6:236–244.

Cunningham, J.M., M.E. Purucker, S.M. Jane, B. Safer, E.F. Vanin, P.A. Ney, C.H. Lowrey, and A.W. Nienhuis. 1994. The regulatory element 3′ to the A gamma-globin gene binds to the nuclear matrix and interacts with special A-T-rich binding protein 1 (SATB1), an SAR/MAR-associating region DNA binding protein. *Blood.* 84:1298–1308.

Dickinson, L.A., and T. Kohwi-Shigematsu. 1995. Nucleolin is a matrix attachment region DNA-binding protein that specifically recognizes a region with high base-unpairing potential. *Mol. Cell. Biol*. 15:456–465.

Dickinson, L.A., T. Joh, Y. Kohwi, and T. Kohwi-Shigematsu. 1992. A tissue-specific MAR/SAR DNA-binding protein with unusual binding site recognition. *Cell*. 70:631–645.

Dickinson, L.A., C.D. Dickinson, and T. Kohwi-Shigematsu. 1997. The nuclear matrix attachment region (MAR)-binding protein SATB1 contains a homeodomain that promotes specific recognition of the core unwinding element of a MAR. *J. Biol. Chem.* 272:11463–11470.

Dijkwel, P.A., and J.C. Hamlin. 1988. Matrix attachment regions are positioned near replication initiation sites, genes, and an interamplicon junction in the amplified dihydrofolate reductase domain of Chinese hamster ovary cells. *Mol. Cell. Biol.* 8:5398–5409.

Dombrowski, B.A., A.F. Scott, and H.H. Kazazian, Jr. 1993. Two additional potential retrotransposons isolated from a human L1 subfamily that contains an active retrotransposable element. *Proc. Natl. Acad. Sci. USA*. 90:6513–6517.

Fey, E.G., K.W. Wan, and S. Penman. 1984. Epithelial cytoskeletal framework and nuclear matrix-intermediate filament scaffold: three-dimensional organization and protein composition. *J. Cell Biol.* 98:1973–1984.

Forrester, W.C., C. van Genderen, T. Jenuwein, and R.Grosschedl. 1994. Dependence of enhancer-mediated transcription of the immunoglobulin m gene on nuclear matrix attachment regions. *Science*. 265:1221–1225.

Fragoso, G., and G.L. Hager. 1997. Analysis of in vivo nucleosome positions by determination of nucleosome-linker boundaries in crosslinked chromatin. *METHODS*: *A Companion Methods Enzymol.* 11:246–252.

Gasser, S.M., and U.K. Laemmli. 1986*a*. Cohabitation of scaffold binding regions with upstream/enhancer elements of three developmentally regulated genes of *D. melanogaster*. *Cell*. 46:521–530.

Gasser, S.M., and U.K. Laemmli. 1986*b*. The organization of chromatin loops: characterization of a scaffold. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:511–518.

Gasser, S.M., and U.K. Laemmli. 1987. A glimpse at chromosomal order. *Trends Genet.* 3:16–22.

Gehring, W.J. 1987. Homeo boxes in the study of development. *Science*. 236: 1245–1252.

Geiduschek, E.P., and G.A. Kassavetis. 1992. RNA polymerase III transcription complexes. *In* Transcriptional Regulation. S.L. McKnight and K.R. Yamamoto, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 247–280.

Gerdes, M.G., K.C. Carter, P.T. Moen, Jr., and J.B. Lawrence. 1994. Dynamic changes in the higher-level chromatin organization of specific sequences revealed by in situ hybridization to nuclear halos. *J. Cell Biol.* 126:289–304.

Göhring, R., and F.O. Fackelmayer. 1997. The scaffold/matrix attachment region binding protein HnRNP-U (SAF-A) is directly bound to chromosomal DNA in vivo: a chemical cross-linking study. *Biochemistry*. 36:8276–8283.

Herrscher, R.F., M.H. Kaplan, D.L. Lelsz, C. Das, R. Scheurmann, and P.W. Tucker. 1995. The immunoglobulin heavy-chain matrix-associating regions are bound by Bright: a B cell-specific *trans*-activator that describes a new DNA-binding protein family. *Genes Dev*. 9:3067–3082.

Igo-Kemenes, T., and H.G. Zachau. 1978. Domains in chromatin structure. *Cold Spring Harbor Symp. Quant. Biol.* 42:109–118.

Jackson, D.A., J. Bartlett, and P.R. Cook. 1996. Sequences attaching loops of nuclear and mitochondrial DNA to underlying structures in human cells: the role of transcription units. *Nucleic Acids Res*. 24:1212–1219.

Jarman, A.P., and D.R. Higgs. 1988. Nuclear scaffold attachment sites in the human globin gene complexes. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:3337–3344.

Jenuwein, T., W.C. Forrester, L.A. Fernandez-Herrero, G. Laible, M. Dull, and R. Grosschedl. 1997. Extension of chromatin accessibility by nuclear matrix attachment regions. *Nature*. 385:269–272.

Kazazian, H.H., Jr., C. Wong, H. Youssoufian, A.F. Scott, D.G. Phillips, and S.E. Antonarakis. 1988. Haemophilia A resulting from de novo insertion of L1 sequences represents a novel mechanism for mutation in man. *Nature*. 332:164–166.

Kirillov, A., B. Kistler, R. Mostoslasky, H. Cedar, T. Wirth, and Y. Bergman. 1996. A role for nuclear NF-κB-cell-specific demethylation of the *Igκ* locus. *Nat. Genet*. 13:435–441.

Kohwi, Y., and T. Kohwi-Shigematsu. 1988. Magnesium ion-dependent, triple-helix structure formed by homopurine-homopyrimidine sequences in supercoiled plasmid DNA. *Proc. Natl. Acad. Sci. USA*. 85:3781–3785.

Kohwi, Y., and T. Kohwi-Shigematsu. 1993. Structural polymorphism of homopurine-homopyrimidine sequences at neutral pH. *J. Mol. Biol.* 231:1090–1101.

Kohwi-Shigematsu, T., and Y. Kohwi. 1990. Torsional stress stabilizes extended base unpairing in suppresser sites flanking immunoglobulin heavy chain enhancer. *Biochemistry*. 29:9551–9560.

Kohwi-Shigematsu,T., and Y. Kohwi. 1992. Detection of non-B DNA structures at specific sites in supercoiled plasmid DNA and chromatin with haloacetaldehyde and diethylpyrocarbonate. *Methods Enzymol.* 212:155–180.

Kohwi-Shigematsu, T., K. Maass, and J. Bode. 1997*a*. A thymocyte factor SATB1 suppresses transcription of stably integrated MAR-linked reporter genes. *Biochemistry*. 36:12005–12010.

Kohwi-Shigematsu, T., I. de Belle, L.A. Dickinson, S. Galande, and Y. Kohwi. 1997*b*. Identification of base-unpairing region (BUR)-binding proteins and characterization of their in vivo binding sequences. *Methods Cell Biol.* 53: 323–354.

Lawrence, J.B., C.A. Villnave, and R.H. Singer. 1988. Sensitive, high-resolution chromatin and chromosome mapping in situ: presence and orientation of two closely integrated copies of EBV in a lymphoma line. *Cell*. 52:51–61.

Lebkowski, J.S., and U.K. Laemmli. 1982*a*. Evidence for two levels of DNA folding in histone-depleted HeLa interphase nuclei. *J. Mol. Biol.* 156:309–324.

Lebkowski, J.S., and U.K. Laemmli. 1982*b*. Nonhistone proteins and long range organization of HeLa interphase DNA. *J. Mol. Biol.* 156:325–344.

Levy-Wilson, B., and C. Fortier. 1990. The limits of the DNase I-sensitive domain of the human apolipoprotein β gene coincide with the locations of the chromosomal anchorage loops and define the 5′ and 3′ boundaries of the gene. *J. Biol. Chem.* 264:21196–21204.

Little, R.D., and D.C. Braaten. 1989. Genomic organization of human 5S rDNA and sequence of one tandem repeat. *Genomics*. 4:376–383.

Liu, J., D. Bramblett, Q. Zhu, M. Lozano, R. Kobayashi, S.R. Ross, and J.P. Dudley. 1997. The matrix attachment region-binding protein SATB1 participates in negative regulation of tissue-specific gene expression. *Mol. Cell. Biol.* 17:5275–5287.

Lobo, S.M., S. Ifill, and N. Hernandez. 1990. cis-acting elements required for RNA polymerase II and III transcription in the human U2 and U6 snRNA promoters. *Nucleic Acids Res.* 18:2891–2899.

Mathias, S.L., A.F. Scott, H.H. Kazazian, Jr., D. Boeke, and A. Gabriel. 1991. Reverse transcriptase encoded by a human transposable element. *Science*. 254:2891–2899.

Miki, Y., I. Nishisho, A. Horii, Y. Miyoshi, J. Utsunomiya, K.W. Kinzler, B. Vogelstein, and Y. Nakamura. 1992. Disruption of the *APC* gene by a retrotransposial insertion of L1 sequence in a colon cancer. *Cancer Res.* 52:643–645.

Mirkovitch, J., M.-E. Mirault, and U.K. Laemmli. 1984. Organization of the higher-order chromatin loop: specific DNA attachment sites on nuclear scaffold. *Cell*. 9:223–232.

Morse, B., P.G. Rothberg, V.J. South, J.M. Spandorfer, and S.M. Astrin. 1988.

Insertional mutagenesis of the *myc* locus by a LINE-1 sequence in a human breast carcinoma. *Nature*. 333:87–90.
Nakagomi, K., Y. Kohwi, L.A. Dickinson, and T. Kohwi-Shigematsu. 1994. A novel DNA-binding motif in the nuclear matrix attachment DNA-binding protein SATB1. *Mol. Cell. Biol.* 14:1852–1860.
Narita, N., H. Nishio, Y. Kitoh, Y. Ishikawa, R. Minami, H. Nakamura, and M. Matsuo. 1993. Insertion of a 5′ truncated L1 element into the 3′ end of exon 44 of the dystrophin gene resulted in skipping of the exon during splicing in a case of Duchenne muscular dystrophy. *J. Clin. Invest.* 91:1862–1867.
Nelson, W.G., K.J. Pienta, E.R. Barrack, and D.S. Coffey. 1986. The role of the nuclear matrix in the organization and function of DNA. *Annu. Rev. Biophys. Chem.* 15:457–475.
Nickerson, J.A., G. Krockmalnic, K.W. Wan, and S. Penman. 1997. The nuclear matrix revealed by eluting chromatin from a cross-linked nucleus. *Proc. Natl. Acad. Sci. USA*. 94:4446–4450.
Orlando,V., and R. Paro. 1993. Mapping polycomb-repressed domains in the bithorax complex using in vivo formaldehyde cross-linked chromatin. *Cell*. 75:1187–1198.
Paulson, J.R., and U.K. Laemmli. 1977. The structure of histone-depleted metaphase chromosomes. *Cell*. 12:817–828.
Penman, S. 1995. Rethinking cell structure. *Proc. Natl. Acad. Sci. USA*. 92: 5251–5257.
Prosser, J., M. Frommer, C. Paul, and P.C. Vincent. 1986. Sequence relationships of three human satellite DNAs. *J. Mol. Biol.* 187:145–155.
Phi-Van, L., and W.H. Strätling. 1988. The matrix attachment regions of the chicken lysozyme gene co-map with the boundaries of the chromatin domain. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:655–664.
Reddy, R., D. Henning, P. Epstein, and H. Busch. 1981. Primary and secondary structure of U2 snRNA *Nucleic Acids Res.* 9:5645–5658.
Sassaman, D.M., B.A. Dombroski, J.V. Moran, M.L. Kimberland, T.P. Naas, R.J. DeBerardinis, A. Gabriel, G.D. Swergold, and H.H. Kazazian, Jr. 1997. Many human L1 elements are capable of retrotransposition. *Nat. Genet.* 6:37–43.
Skowronski, J., T.G. Fanning, and M.F. Singer. 1988. Unit-length Line-1 transcripts in human teratocarcinoma cells. *Mol. Cell. Biol.* 8:1385–1397.
Solomon, M.J., and A. Varshavsky. 1985. Formaldehyde-mediated DNA-protein crosslinking: a probe for in vivo chromatin structures. *Proc. Natl. Acad. Sci. USA*. 82:6470–6474.
Sorensen, P.D., and S. Frederiksen. 1991. Characterization of human 5S rRNA genes. *Nucleic Acids Res.* 19:4147–4151.
Spitzner, J.R., and M.T. Muller. 1989. Application of a degenerate consensus sequence to quantify recognition sites by vertebrate DNA topoisomerase II. *J. Mol. Recognit.* 2:63–74.
Stein, G.S., J.L. Stein, J.B. Lian, A.J. van Wijnen, and M. Montecino. 1996. Functional interrelationships between nuclear structure and transcriptional control: contributions to regulation of cell cycle- and tissue-specific gene expression. *J. Cell Biochem*. 62:198–209.
Stockhaus, J., P. Eckes, A. Blau, J. Schell, and L. Willmitzer. 1987. Organ-specific and dosage-dependent expression of a leaf/stem specific gene from potato after tagging and transfer into potato and tobacco plants. *Nucleic Acids Res.* 15:3479–3491.
Treisman, J., E. Harris, D. Wilson, and C. Desplan. 1992. The homeodomain: a new face for the helix-turn-helix? *BioEssays*. 14:145–150.
Van Arsdell, S.W., and A.M. Weiner. 1984. Human genes for U2 small nuclear RNA are tandemly repeated. *Mol. Cell. Biol.* 4:492–499.
Vincent, K.A., D. Yoork-Higgins, M. Quiroga, and P.O. Brown. 1990. Host sequences flanking the HIV provirus. *Nucleic Acids Res*. 18:6045–6047.
Vogelstein, B., D.M. Pardoll, and D.S. Coffey. 1980. Supercoiled loops and eucaryotic DNA replication. *Cell*. 22:79–85.
Wang, B., L.A. Dickinson, E. Koivunen, E. Ruoslahti, and T. Kohwi-Shigematsu. 1995. A novel MAR binding motif identified using a random phage peptide library. *J. Biol. Chem.* 270:23239–23242.
Westin, G., J. Zabielski, K. Hammarstrom, H.-J. Monstein, C. Bark, and U. Pettersson. 1984. Clustered genes for human U2 RNA. *Proc. Natl. Acad. Sci. USA*. 81:3811–3815.
Yanagisawa, J., J. Ando, J. Nakayama, Y. Kohwi, and T. Kohwi-Shigematsu. 1996. A matrix attachment region (MAR)-binding activity due to a p114 kilodalton protein is found only in human breast carcinomas and not in normal and benign breast disease tissues. *Cancer Res.* 56:457–462.
Zhao, K., C.M. Hart, and U.K. Laemmli. 1995. Visualization of chromosomal domains with boundary element-associated factor BEAF-32. *Cell*. 81:879–889.