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Both CTCF-dependent and -independent Insulators Are Found between the Mouse T Cell Receptor α and *Dad1* Genes*

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The T cell rearrangement of the T cell receptor (TCR) genes *TCR α* and δ is specifically regulated by a complex interplay between enhancer elements and chromatin structure. The α enhancer is active in T cells and drives *TCR α* recombination in collaboration with a locus control region-like element located downstream of the *C α* gene on mouse chromosome 14. Twelve kb further downstream lies another gene, *Dad1*, with a program of expression different from that of *TCR α* . The ~6-kb locus control region element lying between them contains multiple regulatory sites with a variety of roles in regulating the two genes. Previous evidence has indicated that among these there are widely distributed regions with enhancer blocking (insulating) activity. We have shown in this report that one of these sites, not previously examined, strongly binds the insulator protein CTCF-binding factor (CTCF) *in vitro* and *in vivo* and can function in an enhancer blocking assay. However, other regions within the 6-kb element that also can block enhancers clearly do not harbor CTCF sites and thus must reflect the presence of a previously undetected and distinct vertebrate insulator activity.

The T cell receptor genes (TCR)¹ play a central role in the development of T lymphocytes. Rearrangement within the TCR α , β , γ , and δ gene segments lead to specific recognition and immunological response to foreign antigens. The heterodimeric $\alpha\beta$ TCR is expressed in the major subset of circulating peripheral blood T cells, whereas the $\gamma\delta$ TCR is expressed in a minor population of T cells. The α and δ genes are located on the same locus in human, mouse, and chicken genomes, and a separate enhancer has been identified for each of the TCR genes (Fig. 1A; reviewed in Ref. 1). During mouse embryonic development, the δ genes are rearranged at 14 days of gestation, whereas the rearrangement of the α genes starts at day 16. Consequently, TCR ontogeny is tightly regulated by a complex array of *cis*-acting elements and targeted changes in chromatin structure exerting either positive or negative regulatory effects on V(D)J recombination (reviewed in Ref. 2).

In T cell lines, nine hypersensitive sites (HS) have been identified in the 3' region of the *TCR α* locus (Fig. 1C). HS1 maps to the *TCR α* enhancer (3), HS7 and 8 are located within

the *C α* gene (4) and HS1' (5) and HS2–6 (4) lie downstream of *E α* . Constructs containing HS2–6 are required for cell-specific, copy number-dependent *TCR α* expression in transgenic mice, independent of the integration site of the transgene (4). Thus, it was suggested that this region may be the locus control region (LCR) of the *TCR α* locus, consisting of a set of important elements that control the locus accessibility to regulatory factors by opening the chromatin structure as described for other LCRs (6–8).

Using a gene targeting approach to study the role of the *TCR α* LCR *in vivo*, Hong *et al.* (9) have identified a new gene located 12 kb downstream of the α constant region and the region containing the hypersensitive sites (Fig. 1, A and C). This gene, *Dad1*, is expressed ubiquitously (9), and the coding sequences of *Dad1* are highly conserved between species even in organisms that do not have TCR genes, such as yeast (10) or *Caenorhabditis elegans* (11). The *Dad1* protein has been shown to play a role in preventing apoptosis in certain cell types (10, 12–13) and is also a subunit of the oligosaccharyltransferase enzyme complex that initiates N-linked glycosylation (10, 14).

The nine DNase I hypersensitive elements within the 12-kb region separating the *TCR α* locus and *Dad1* form distinct patterns in lymphoid tissues where both *TCR α* and *Dad1* are expressed and in non-lymphoid tissues where only *Dad1* is expressed. The replacement of HS2–6 in its natural context impairs *Dad1* expression, resulting in early lethality of mice (9). Indeed, mice carrying this deletion die at 7 days post coitum, before *TCR α* activation, suggesting that this region is important for both *TCR α* and *Dad1* expression. Moreover, a deletion of *E α* that leaves HS2–6 intact abolishes V(D)J recombination and transcription of the *TCR α* gene, indicating that HS2–6 cannot control *TCR α* expression in the absence of the enhancer (15). Nonetheless, a deletion of HS2–6 that leaves *E α* intact affects *TCR α* expression (9). Therefore, Zhong and Kranzel (16) suggested that the HS2–6 is a boundary element that has an LCR-like activity, able to confer copy number-dependent and integration site-independent expression on an *E α* -containing transgene (4–5) but also able to protect both against ectopic activation of the *TCR α* by *Dad1* regulatory elements and, reciprocally, against *E α* activation of *Dad1* expression in T cells. They also showed that HS2–6 blocks an enhancer from activating a promoter when located between the two (16). However, in addition to enhancer-blocking activity, HS2–6 also shows a synergistic activation property when located upstream of the enhancer, suggesting that this region is a mosaic of regulatory elements involved in a complex regulation system for both *TCR α* and *Dad1* genes.

Known insulator elements vary greatly in their DNA sequences and the specificity of proteins that bind to them. However, they share at least one of the two following properties: (i) they have the ability to act as an enhancer blocker when placed between an enhancer and the promoter, and (ii) they have the

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¹ The abbreviations used are: TCR, T cell receptor; HS, hypersensitive site; TAD1, TCR α -*Dad1*; MAR, matrix attachment region.

ability to protect against position effects due to the chromosomal environment (17–19). The first vertebrate insulator to be described is located near the 5' end of the chicken β -globin locus (20). A number of insulators have now been identified in other vertebrates, located between genes with independent patterns of expression and consistent with a role in preventing inappropriate interaction between the regulatory elements of the neighboring loci. The insulators at the ribosomal RNA genes of *Xenopus* (21), the *Bead-1* element at human *TCR α / δ* locus (22), the chicken β -globin gene 5'HS4 element (22), and the imprinted *Igf2/H19* locus (23–24) are bound by the CTCF protein. Moreover, CTCF sites have been found recently at the *Tsix* locus, suggesting also a role for CTCF in X inactivation (25). The CTCF protein is only present in higher eukaryotes, and its sequence is highly conserved among species. CTCF is a multivalent protein that binds to different targets through the combinatorial use of its 11 zinc fingers and can be involved in gene activation or repression and chromatin insulation (26).

In this report, we describe a search for potential binding sites for CTCF within the region between the *TCR α* locus and the *Dad1* gene. We began by comparing this DNA sequence to all the known CTCF sites. Despite the presence of several close homologies, *in vitro* binding studies followed by *in vivo* chromatin immunoprecipitation analysis revealed only a single CTCF binding site, located downstream of the enhancer of the *TCR α* locus and possessing a strong enhancer blocking activity in our assay. We also undertook a quantitative study of the pattern of histone acetylation across the region that revealed that this enhancer blocking site may be associated with a more complex array of regulatory elements. Surprisingly, although we confirmed the enhancer blocking activity of the region containing sites HS2–6 as previously described (16), we found no evidence of additional CTCF sites in this region. This indicates the presence of novel enhancer blocking insulator elements that remain to be characterized. Our results suggest that the CTCF protein participates in the insulator activity of this region, allowing proper expression of *TCR α* and *Dad1* genes, but is working in concert with previously unrecognized mechanisms of insulation.

EXPERIMENTAL PROCEDURES

Cell Culture—The human erythroleukemia cell line K562 was maintained in improved minimal essential medium. The mouse fibroblast cell line NIH3T3 was grown in Dulbecco's minimal essential medium. Media were supplemented with 10% fetal calf serum. AKR1.G.1.Ovar.1.26 T lymphocytes were obtained from ATCC and grown in Dulbecco's minimal essential medium supplemented with 4 mM L-glutamine and 10% horse serum. All cell lines were grown at 37 °C in a humidified atmosphere of 5% CO₂.

Preparation of Nuclear Extracts—Cells (5×10^9) were harvested, rinsed in 1× phosphate-buffered saline, resuspended in 0.67 × phosphate-buffered saline, and incubated on ice for 10 min. After centrifugation, the pellet was resuspended in ice-cold 10 mM Hepes (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol in the presence of proteases inhibitors, and nuclei were released by Dounce homogenization. After centrifugation, the pellet was resuspended in 20 mM Hepes (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM EDTA, 1 mM dithiothreitol. Samples were Dounce-homogenized, stirred on ice for 30 min, and centrifuged at 25,000 × *g* for 30 min. The supernatant was frozen in a dry ice/ethanol bath and stored at –80 °C.

Oligos and Gel Mobility Shift Assay—In a first attempt to search for potential CTCF sites, the *TCR α -Dad1* sequence was compared with the 14-bp consensus binding site for CTCF insulator activity (22–23). The following sequences were tested for CTCF binding by gel mobility shift assay: 730–743, GTGTCTTAAGGTAGCCACACGGGGGCGAGTACTCCACC; 2296–2309, GCACCGT-TTAATCCAGTACTTGGAGGCAGAGGCAGTCTGA; 2850–2863, AATCCACCTGCCTCTGCCTCCCAAGT-GCTGGGATTAAGC; 2878–2891, GCTGGGATTAAGCACTGCCACATGCTGGCGCAAGACA; 3753–3766, GGGTTTTACTCTCTGAGCCACCTTGCAGCCCCGTGGTC; 5533–5572, CCCATGTAGGTGGT-GCCTCCCTGAGCAAGCTGGCCAGCT (Fig. 1B). Putative CTCF

sequences were compared with the chicken 5'HS4FII sequence or chicken 5'HS4FII mutants described in Ref. 22.

Because CTCF can bind different DNA sequences, other potential CTCF sites within the *TCR α -Dad1* intergenic region were searched and tested for binding activity: 147–195, ACTCGTCACGGCTGTGACATGGGCAAACAGGTCCCCCTTTGAAGCTCTCCCGCAGAAGCCACATCCTCTGGAAAGAGGAGT; 2780–2810, CTCTGTGTAGCCCTGGCGCTGCTGTCTGGAACCTACTCTGTAGACCAGAC; 1860–1850, ACGTCGGATGCACCCATGCTGCGATAAAACGAGCCTGTGCGTGAGGATGCCGGCGCTCACATA; 3607–3655, ACTGACTATTGAAGTTCTCTGACCGTACAGGCACGCGCCACACCATCCCGCCATGAGACTGAGCCTACAGTTTAT; 3647–3724, CGCGTGCCTGTGACGGTCAGAGAGCTTAATAGTCAAGTTCTACTCTGCGTGGAGCTGGGGGGGGGGGTCATCTCAGATCACCGGGGCTGCTCAGA. The corresponding homologies are indicated in Fig. 1C.

Gel mobility shift assays were carried out in a binding buffer composed of 20 mM HEPES (pH 7.9), 150 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 5% glycerol, 0.5% Triton X-100. DNA binding was carried out at room temperature for 30 min in the presence of 1–2 μ l of nuclear extract, 20–40 fmol of end-labeled probe, and 100 μ g/ml poly(dI/dC). Cold competitors were added simultaneously with the labeled probes at 50–100-fold molar excess. For supershift experiments, nuclear extracts were preincubated in binding buffer at 4 °C for 2 h with anti-CTCF antibody (Upstate Biotechnology) followed by a 30-min incubation at room temperature with the DNA as described above.

Southwestern Experiments—The Southwestern procedure is based on a protocol previously described (27). Nuclear extracts (25 μ g) and recombinant CTCF proteins (0.5 μ g) were resolved by 4–20% SDS-PAGE electrophoresis and transferred at 4 °C for 5 h at 350 mAmps to polyvinylidene difluoride membranes. After transfer, immobilized proteins were denatured for 5 min in binding buffer (20 mM Hepes (pH 7.5), 3 mM MgCl₂, 40 mM KCl, and 10 mM 2-mercaptoethanol) containing 6 M guanidine hydrochloride, followed by four successive 2-fold dilutions with binding buffer only and two additional washes with binding buffer alone. The filters were then blocked for 10 min with 2% nonfat dried milk in binding buffer and washed with binding buffer alone. The filters were incubated for 1 h at room temperature in binding buffer with 0.1% Triton X-100 in the presence of labeled probe (2×10^6 cpm/ml) and nonspecific competitor DNA (20 μ g/ml native *Escherichia coli* DNA, 2 μ g/ml denatured *E. coli* DNA). Finally, the filters were washed four times in binding buffer supplemented with 0.01% Triton X-100. After air drying for 5 min, the filters were exposed to film.

Formaldehyde Cross-linking and Chromatin Immunoprecipitation—*In vivo* protein-DNA cross-linking was carried out as described (28) with some modifications (29). Generally, $1.5\text{--}2 \times 10^8$ cells were harvested and rinsed one time in phosphate-buffered saline, and nuclei were prepared in ice-cold RSB buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 5 mM MgCl₂) in the presence of a mixture of protease inhibitors. After centrifugation, nuclei were resuspended in RSB buffer containing 0.1% Nonidet P-40. Proteins were then cross-linked to DNA for 10 min at room temperature, followed by 40 min at 4 °C with a final concentration of 1% in 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Tris (pH 8). After lysis in the presence of SDS, nucleoprotein complexes were sonicated to reduce DNA fragments to 400–600 bp. To reduce nonspecific background, the chromatin solution was precleared with salmon sperm DNA/protein A-agarose beads for 1 h at 4 °C. At this point DNA was prepared from a sample of protein A-purified chromatin and used as the input sample. Antibodies specific for acetylated histones H3 or CTCF (Upstate Biotechnology) were incubated with protein A-clarified chromatin overnight at 4 °C with gentle rocking. After immunoprecipitation, immune complexes were collected by adding 60 μ l of salmon sperm DNA/protein A-agarose beads for 1 h at 4 °C. The protein A-agarose and unbound chromatin were separated, and the protein A-agarose was washed. Complexes were then eluted in 1% SDS, 0.1 M NaHCO₃, and cross-links were reversed by heating. DNA was recovered by proteinase K digestion, phenol extraction, and ethanol precipitation. DNA samples were quantified using picogreen fluorescence (Molecular Probes, Eugene, OR) and spectrophotometry.

Primers and TaqMan Probes—Primers and TaqMan probes were selected from the region between the mouse *TCR α* and the *Dad1* gene (GenBank™ accession numbers AF000941 and X14895) using the PE Applied Biosystems Primer Express software. Primers and TaqMan probes were obtained from Invitrogen and PE Applied Biosystems, respectively. The list is given in Table I.

Real-time PCR and Data Analysis—DNA from input and antibody-bound chromatin were analyzed by real-time PCR using the TaqMan universal PCR master mix protocol (PE Applied Biosystems) and an ABI prism sequence detector as described previously (30–32). Each

TABLE I
Primers and TaqMan probes used

Position	Primers and probes
21–200	Primers: 5'–GCCAGAAGTAGAACAGGAAATGGA–3' 5'–GGGACCTGTTTGCCCATGT–3'
671–830	TaqMan probe: 6FAM–CCACTTCCCTCCAGGTGTTTGGGTC–TAMRA Primers: 5'–GGGCAGCAGTACTCCACCAA–3' 5'–CGTAGGATGCAGGGATTTTCTTTA–3'
1321–1500	TaqMan probe: 6FAM–CTCCCTCGGCGTGTATTATTCGGG–TAMRA Primers: 5'–GGTAGATGCCTGTCAAAATGCA–3' 5'–TGACTTTAGGCAATCTTGAGTTTAATTTA–3'
2001–2180	TaqMan probe: 6FAM–CCAGCTGGAAGCCTGGGTTTGC–TAMRA Primers: 5'–CCCCTGATGCTTCTCACTGTATC–3' 5'–CTACTGGCACAAGGAATCCTACAA–3'
2491–2670	TaqMan probe: 6FAM–TTTCCCTCCTCTCTGGCACCCCA–TAMRA Primers: 5'–ATGGAAGAAGCACAGACAGACGTA–3' 5'–CTCCACAACCTAAACCATGTTATT–3'
3101–3280	TaqMan probe: 6FAM–ATGGGACTGGCAGACTGAGAGTGAAGTGG–TAMRA Primers: 5'–AGTGGTGGCGCATCGTTTA–3' 5'GCTGGCCTCGAAGTCAGAAA 3'
3821–4000	TaqMan probe: 6FAM–CCCAGCACTTAGGAGACAGAGCCAGGT–TAMRA Primers: 5'–AGAACCGACTCCTGCCAAGAT–3' 5'CCTCTGACAGGGTGTGGTTTTC 3'
4501–4680	TaqMan probe: 6FAM–ACAAACGCGCACGCACGCATAC–TAMRA Primers: 5'–CACGCCCGCAATATTTAAT–3' 5'–CTCACTAGGGACCCATTCTTGCT–3'
5871–6050	TaqMan probe: 6FAM–TTTTAAAGCAAAGTGGTCCATCCCTGTGAG–TAMRA Primers: 5'–TGTCACCTCCCAAGTATGTTCCA–3' 5'–CCTGCACCTCTGCTCTTGATC–3'
6119–6298	TaqMan probe: 6FAM–AACAAGACCGCTTTGTGGCCAAGAT–TAMRA Primers: 5'–CCCACAGATTGAACACAGGAAAT–3' 5'–GAGGGATGGATGCTGCTGTTT–3'
	TaqMan probe: 6FAM–CACACACAGAGGAGGTGTGAGCTGAAGC–TAMRA

amplification was carried out in triplicate to control for PCR variation on 2 ng of DNA at 50 °C for 2 min and 95 °C for 15 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The *Ct* values were collected at 60 °C. The *Ct* is the number of PCR cycles necessary to reach a predetermined fluorescence intensity and is a function of the amount of target DNA in the samples analyzed. Quantification was determined by applying the comparative *Ct* method as described previously (31). The concentration of primers and TaqMan probes used was determined by following the optimization procedure described in PE Applied Biosystem protocol.

Constructs and Enhancer Blocking Assay—Different fragments within the *TCRα-Dad1* region were generated by PCR on genomic DNA and cloned into the pNI vector at the *Ascl* site using the following primers: F311–330, 5'–AGGCGCGCTACGGAGAGCACATTGGGTG–G–3'; R1287–1307, 5'–AGGCGCGCCTCTCCTTTCCCATCAGTCCCTT–3'; R1614–1633, 5'–AGGCGCGCTAAAATGGAGA GAGACAGGGG–3'; F1450–1469, 5'–AGGCGCGCCTGTCAAGGCACAGACAGTCCG–3'; R4011–4030, 5'–AGGCGCGCCTCTGGGTTGTGCGATAGATCCG–3'; F3846–3866, 5'–AGGCGCGCCTTGGTGACCCA AGCTTGAACCT–3'; R6266–6285, 5'–AGGCGCGCCTGGTTTTGTGCTGACTCAGGTAC–3'. We deleted the CTCF binding site using the following additional primers: F756–775(AflII), 5'–ACCCTTAAGAAAAGGCTTTCTCCCTCGGC–3'; R754–775(AflII), 5'–CCCTTAAGGCCGAGGGAGAAAGCCTTTTGG–3'. Enhancer blocking assays were performed as previously described (20, 33).

In Vivo Matrix Assay—Low ionic strength matrices from AKR1 cells were prepared as described (34). Cells were washed in phosphate-buffered saline, incubated for 10 min on ice in isolation buffer (3.75 mM Tris (pH 7.4), 20 mM KCl, 0.5 mM EDTA/KOH, 0.05 mM spermine, 0.125 mM spermidine, 0.1% digitonin, phenylmethylsulfonyl fluoride), and homogenized with a Dounce homogenizer. Nuclei were pelleted by centrifugation and washed three times with isolation buffer. Nuclei were resuspended in isolation buffer without EDTA and incubated in a 37 °C water bath for 20 min. Extraction buffer (5 mM HEPES (pH 7.4), 2 mM KCl, 2 mM EDTA, 0.25 mM spermidine, 0.1% digitonin, 25 mM 3,5-diiodosalicylic acid, lithium salt) was slowly added to a final volume of 7 ml; extracted nuclei were incubated for 5 min at room temperature. Following protein extraction, nuclei were digested with 100 µg/ml RNase-free DNase I (Roche Applied Science) for three hours at room temperature. Matrices were centrifuged for 5 min at 4000 rpm, washed twice with digestion buffer, and resuspended in 10 mM Tris, 1 mM EDTA, pH 8, 0.1% SDS, 1 mg/ml proteinase K. Matrices were digested at 50 °C O/N, phenol:chloroform extracted, and EtOH precipitated. Matrix DNA was analyzed by quantitative PCR.

RESULTS

Search for CTCF Binding Sites within the *TCRα-Dad1* Region—Because the DNA region between the *TCRα* locus, which is only expressed in T cells, and the ubiquitous *Dad1* gene (Fig. 1A) has been shown to possess an insulator activity (16), we searched for CTCF binding sites across the region flanked by the enhancer of the *TCRα* (*Eα*) and exon 3 of *Dad1*. We searched a 6298-bp sequence derived from the mouse T cell receptor α locus enhancer element (GenBankTM accession number X14895) and the mouse DNase I hypersensitive sites 2–6 of the LCR for the T cell receptor α chain gene (GenBankTM accession number AF000941) containing HS1, HS1', and HS2–6. In a first attempt, the consensus binding site for insulator activity at the chicken β -globin and the *Igf2/H19* loci (22–23) was used for this search (Fig. 1B). The best match to the consensus sequence was found at position 730–743. Over this region, 13 of the 14 bases are identical to the canonical site. At position 2878–2891, 12 of the 14 bases are identical to the consensus, whereas at positions 2296–2309, 5545–5558 and 2850–2863, and 3753–3766 the homologies are either 11/14 or 10/14.

CTCF is a versatile protein that can bind to different DNA targets depending on the combinatorial use of the 11 zinc fingers. Therefore, we compared the *TCRα-Dad1* sequence to the other known CTCF binding sites and found five additional potential sites (Fig. 1C). At position 147–195, a match of 25 of 47 bases to the chicken lysozyme gene silencer (35) was found. Another potential site was found at position 1806–1850, displaying a match of 26 of 46 bases to the CTCF repressor site located downstream of the human c-Myc P2 promoter (36). At position 2780–2810, the match of 19 bases/32 corresponds to the promoter of the amyloid β -protein precursor where CTCF activates transcription (37). Two other candidate sites were also found: a match at position 3607–3655 homologous to the chicken c-myc 5'-flanking sequence (34/46) (38) and at position 3647–3724 homologous to the CTCF methylation-sensitive insulator site at the human myotonic dystrophy locus (39).

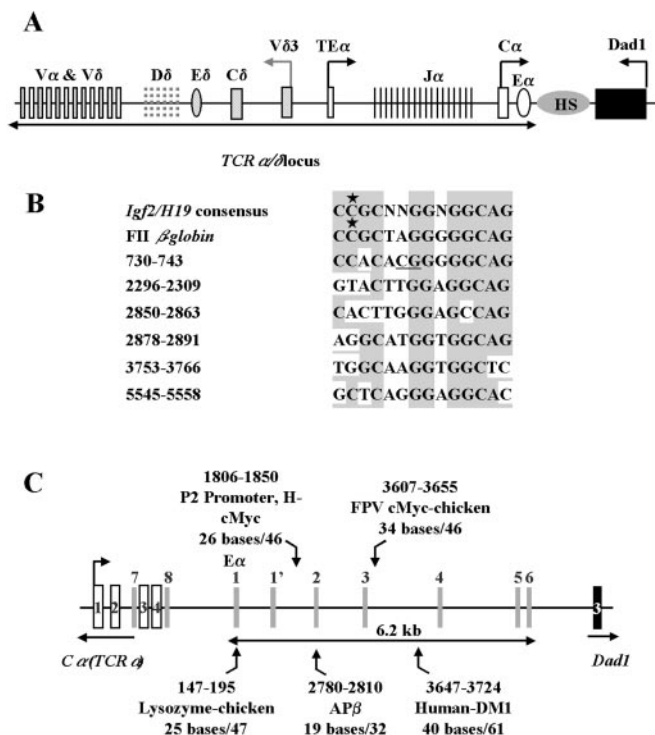


FIG. 1. Sequences of the putative CTCF sites in the intergenic region between the *TCRα/δ* locus and *Dad1*. A, schematic diagram of the *TCRα/δ* locus on mouse chromosome 14. The variable (V), joining (J), diversity (D), constant (C) regions and enhancers (E) are shown. The *Dad1* gene is shown by a black box. The region containing hypersensitive sites HS1', HS2-HS6 is depicted by a gray oval. Transcriptional orientations of the genes are shown by arrows. B, alignment of the region located between *Cα* and *Dad1* with the *Igf2/H19* and chicken β-globin *FII* consensus sites. Shading indicates conservation with the consensus sites. Stars indicate the position of the CpG sites sensitive to DNA methylation. The second CpG site, present in the CTCF site at position 730-743, is underlined. The position of the different sites is based on GenBank™ sequence numbers X14895 and AF 000941 and are indicated by arrows. C, alignment of the *Cα-Dad1* region with other known CTCF sites. The position of the sites and the degree of homology are indicated. The previously characterized sites for CTCF are the chicken lysozyme promoter, the human c-Myc P2 promoter, the human amyloid β protein promoter (*APβ*), the chicken c-myc promoter, the human muscular dystrophy locus (*DM1*). The region between the *Cα* and the *Dad1* genes is drawn to scale. The nine hypersensitive sites are represented in gray. The four exons of the *Cα* gene are indicated by white boxes. Exon 3 of *Dad1* is indicated by a black box.

In Vitro Binding of the Various Sequences to CTCF—To determine whether these candidate sequences are actually capable of binding CTCF, gel retardation assays were carried out. A set of oligonucleotides containing the potential CTCF site at position 15-28 within the 40-base pair oligonucleotides (see "Experimental Procedures") were end-labeled and tested for the ability to bind CTCF in nuclear extracts. For each DNA fragment, the mobility was compared with the mobility of the CTCF-chicken 5'S4FII complex (FII) (22). Using increasing amounts of AKR1 nuclear extract, direct binding was only observed for the site at position 730-743 (Fig. 2A, lanes 1-6), which shows a similar mobility to the FII site (Fig. 2A, lanes 7-12). These CTCF-DNA complexes could be supershifted by incubation with a CTCF antibody (Fig. 2B), suggesting that the site at position 730-743 is a *bona fide* site for CTCF. Because this site is located downstream of the transcriptional enhancer of the *TCRα* and upstream of the ubiquitously expressed *Dad1* gene, we asked whether the binding of CTCF was cell-specific by comparing T cells (AKR1) and fibroblasts (NIH 3T3). We observed similar binding of CTCF to the 730-743 site in AKR1 extracts (Fig. 2C, lanes 1-4) and

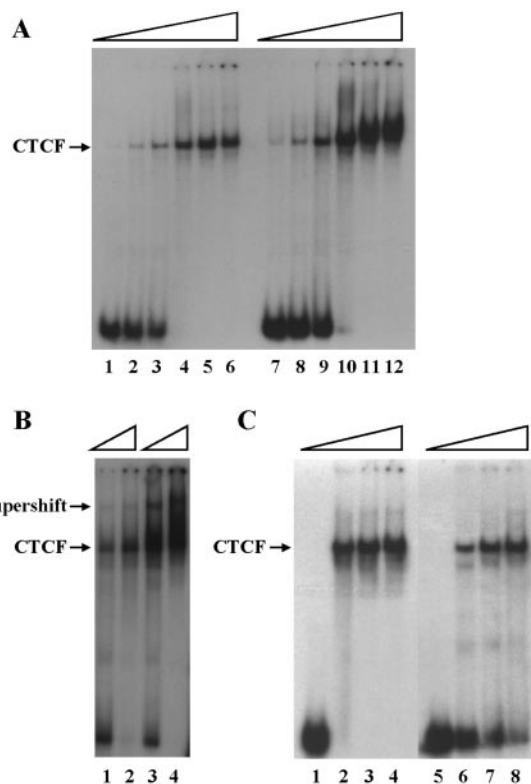


FIG. 2. Identification of a putative binding site for CTCF within the *TCRα-Dad1* region. A, the oligonucleotide at position 730-743 was tested for gel mobility with increasing amounts of AKR1 nuclear extract (lanes 1-6) and compared with the mobility of the chicken 5'S4FII site for CTCF (lanes 7-12). B, supershift experiments using an antibody directed against CTCF on increasing amounts of AKR1 extract with the 730-743 probe (lanes 1-2) and the FII probe (lanes 3-4). C, the binding of CTCF to the 730-743 probe was compared on increasing amounts of AKR1 extracts (lanes 1-4) with increasing amounts of NIH 3T3 nuclear extracts (lanes 5-8).

NIH3T3 extracts (lanes 5-8). We named this new CTCF binding site TAD1, for TCR Alpha-Dad1. No significant mobility shift was observed for the other potential CTCF sites either in direct binding assays or competition experiments (data not shown).

Given the large variation of sequences among the known CTCF binding sites, the new site was further characterized by competition experiments (Fig. 3). Fixed amounts of AKR1 extracts were incubated in the presence of labeled TAD1 (Fig. 3A, lane 1) or labeled FII (Fig. 3B, lane 1) alone or in the presence of 100-fold molar excess unlabeled double strand FII (Fig. 3, A and B, lanes 2) or TAD1 competitor (Fig. 3, A and B, lanes 5). The addition of this excess of unlabeled DNA (FII or TAD1) is sufficient to compete the binding of CTCF to the FII- or TAD1-labeled probe with approximately the same efficiency, suggesting a strong affinity of CTCF for the TAD1 site. However, when mutant versions of the chicken FII were used (22), no competition was observed (TAD1: Fig. 3A, lanes 3-4; FII: Fig. 3B, lanes 3-4). We also tested the binding of CTCF to some mutants of TAD1 (Fig. 3). Increasing amounts of AKR1 nuclear extracts were incubated with TAD1 (Fig. 3C, lanes 1-3) or TAD1 oligonucleotides where the CTCF site was deleted (Fig. 3C, lanes 4-6). In the absence of the CTCF site, no or very little DNA-protein complex can be seen on the gel, suggesting that the sequence is specific for CTCF. At some loci (notably the *Igf2/H19* locus), the binding of CTCF to its target is sensitive to the methylation at CpG sites. The TAD1 sequence contains one CpG site (though not at the same place as in *Igf2/H19*). We asked whether its methylation could abolish CTCF binding. We

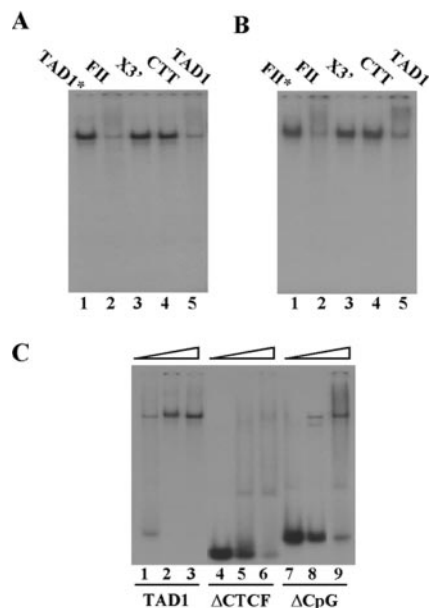


FIG. 3. Competition of binding of CTCF to the TAD1 sequence. A, gel retardation analysis of complexes between labeled TAD1 (40 fmol) and AKR1 nuclear extracts (lane 1) following competition with a 100-fold excess of unlabeled competitor duplexes. Lane 2, chicken 5'HS4FII (FII); lane 3, X3' chicken 5'HS4FII mutant (X3); lane 4, chicken 5'HS4FII CTT deletion mutant (CTT); lane 5, unlabeled TAD1. Asterisk denotes labeled probe. B, gel retardation analysis of complexes between labeled chicken 5'HS4FII (40 fmol) and AKR1 nuclear extracts (lane 1) following competition with a 100-fold excess of unlabeled competitor duplexes. Lane 2, FII; lane 3, X3; lane 4, CTT; lane 5, unlabeled TAD1. Asterisk denotes labeled probe. C, gel mobility shift assay of TAD1 or TAD1 mutants and AKR1 nuclear extracts. Triangles above gel indicate increasing amounts of AKR1 nuclear extracts. Lanes 1–3, TAD1; lanes 4–6, TAD1 sequence with a deletion of the CTCF site (Δ CTCF); lanes 7–9, TAD1 sequence with a deletion of the CpG site within the CTCF binding site (Δ CpG).

did not observe a decrease in the affinity of CTCF binding when this site was methylated on both strands (data not shown), although deletion of the CpG site diminished the binding (Fig. 3C, lanes 7–9), suggesting that these nucleotides are nonetheless important for CTCF targeting.

We also used the TAD1 mutant oligonucleotides for competition studies. As expected from previous experiments, the molar excess of unlabeled wild type TAD1 DNA can displace, at least partially, the binding of CTCF to the labeled wild type probe, whereas the mutant versions of TAD1 cannot (data not shown).

The binding of CTCF to the chicken 5'HS4FII (Fig. 4, lanes 1–3), TAD1 (lanes 4–6), and TAD1- Δ CTCF sequences (lanes 7–9) was also investigated by Southwestern analysis of AKR1 and K562 nuclear extracts (the cells in which the enhancer blocking assays are performed) as well as purified recombinant CTCF from baculovirus extracts. Migration patterns were identical for the recombinant CTCF and the endogenous protein from human or mouse extracts. However, no binding was observed for the TAD1- Δ CTCF probe, as expected. These results suggest the presence of a single, high affinity binding site for CTCF in a region corresponding roughly to the previously identified HS1' binding site.

Enhancer Blocking Activity of the TAD1 CTCF Site—Earlier work by Zhong & Krangel (16) described the enhancer blocking activity of the HS2–6 sequence. However, at that time the HS1' site had not been identified and was not included in the study. The results described above show that there is potentially a strong and previously undetected CTCF binding site downstream of the E α , close to and perhaps coincident with the HS1' site. CTCF is responsible for enhancer blocking activity at the

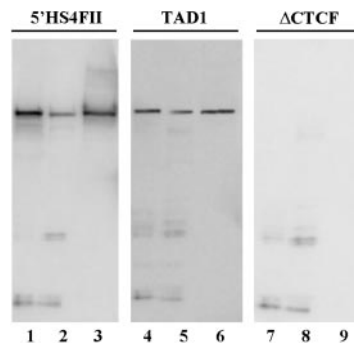


FIG. 4. Southwestern experiments to measure binding site interactions with CTCF. Nuclear extracts (AKR1, lanes 1, 4, 7; K562, lanes 2, 5, 8) or recombinant CTCF (lanes 3, 6, 9) were analyzed. Filters were probed with chicken 5'HS4FII (lanes 1–3), TAD1 (lanes 4–6), or TAD1- Δ CTCF (lanes 7–9).

chicken β -globin locus and other vertebrate insulators (22, 26, 40). Using a colony assay, we therefore tested the ability of the TAD1 site to block the activation of a promoter by an enhancer (20). At the same time, we re-examined the remainder of the region upstream of *Dad1* for similar properties. In each colony assay, a construct containing a fragment of λ DNA integrated between the reporter gene (γ -Neo) and the mouse 5' HS2 enhancer (pJC-3.4) was used as a reference to determine the relative colony number. The pJC5–4 construct, which contains one copy of the 1.2-kb 5'HS4 chicken insulator sequence on each side of the reporter gene, was used as a control for enhancer blocking activity. Three overlapping genomic fragments (Fig. 5A, HS1'–2, HS2–4, and HS4–6) within the *TCR α -Dad1* region and the HS1' site alone (HS1') were subcloned into the testing vector in both orientations (Fig. 5B). As a control, the pNI empty vector was also included in our study. As described earlier (16) in a different experimental system (Jurkat T cells), both HS2–4 and HS4–6 strongly reduced the colony number in an orientation-independent way, suggesting that these enhancer blocking activities are not T cell-specific. When the HS1'–2 fragment was used, the number of Neo-resistant clones was significantly reduced to a level similar to that for the chicken β -globin insulator element (4.7-fold). Similar results were obtained when a shorter fragment containing only the HS1' site was used (5-fold insulation). These results suggest that the TAD1 site for CTCF participates in the enhancer blocking activity of the *TCR α -Dad1* DNA region.

To confirm this observation, we tested for insulating activity a construct in which 14 bp of the CTCF site were deleted (pNI Δ CTCF). When the mutant version was used, the enhancer blocking activity was lost (1.1-fold insulation), indicating that the new CTCF binding site located downstream of the T cell receptor α enhancer acts as a positional enhancer blocking element when placed between an enhancer and a promoter.

In Vivo Distribution of Histone H3 Acetylation and CTCF Binding within the TCR α -Dad1 Region—To determine the histone acetylation status of the *TCR α -Dad1* locus *in vivo*, high resolution chromatin immunoprecipitation assays were performed on mouse T cells (AKR1) and fibroblasts (NIH 3T3). Samples were treated with formaldehyde to cross-link proteins to DNA, sonicated for chromatin fractionation, and immunoprecipitated with antibodies to acetylated histone H3 tails. For each cell line, the input (before immunoprecipitation) and bound fractions of the no-antibody and immunoprecipitated samples were analyzed using TaqMan real-time PCR. For this purpose, we designed 10 probes targeted evenly across the domain (Fig. 6). In AKR1 lymphoid cells, histone tails were

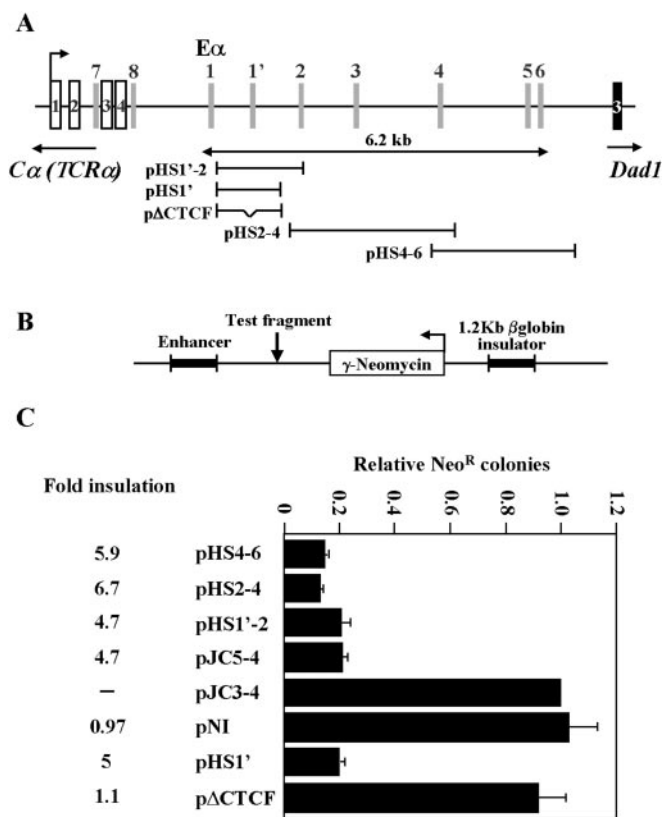


FIG. 5. Enhancer blocking activities of the region located between the *TCRα* locus and *Dad1*. *A*, map of the region between the *Cα* gene and *Dad1*. The position of the test fragments used for enhancer blocking assays is indicated. *HS1'*, position 311–1307. *pΔCTCF*, *HS1'* fragment with a deletion of the 14 bp corresponding to the CTCF site. *HS1'-2*, position 311–1614. *HS2-HS4*, position 1450–3866. *HS4-HS6*, position 4011–6285. *B*, constructs contain a neomycin reporter gene driven by the human *Aγ-globin* promoter (*γ-neomycin*) flanked with the mouse 5' *HS2* enhancer and the chicken *β-globin* 1.2-kb insulator. The arrow indicates the position of the test fragments. *C*, for each construct the relative numbers of colonies obtained from four independent assays relative to the *pJC3-4* construct are plotted. The mean values with S.E. are indicated. The following constructs were used as controls: *pNI*, no insert; *pJC3-4*, λ DNA; *pJC5-4*, chicken *β-globin* 1.2-kb insulator.

acetylated (3.7–6.25-fold enrichment of the immunoprecipitated fraction over the input) across the entire locus with a peak (17.6-fold) at the *HS1'* site. In non-lymphoid cells (NIH 3T3), the overall level of H3 acetylation was lower than in the AKR1 site with a peak only at the *HS1'* site (8.1-fold enrichment). Of particular note, high levels of acetylation were also observed around the *HS4* site in AKR1 cells. DNA at this site is unmethylated in T cells but methylated in non-lymphoid cells (41). Correspondingly, the level of acetylation is low at *HS4* in non-lymphoid cells.

Using antibody to CTCF, we carried out similar chromatin immunoprecipitation assays across the 6.2-kb region between the *TCRα* and *Dad1* genes. Although this region displays strong and rather broadly distributed enhancer blocking activities, the experiments described above revealed only the single CTCF binding site in the vicinity of *HS1'*. Because CTCF is the only known vertebrate insulator, we searched by chromatin immunoprecipitation to determine whether we might have overlooked additional sites of CTCF binding across the region in AKR1 and NIH 3T3 cells (Fig. 6). In confirmation of the *in vitro* binding results, CTCF is found *in vivo* only at the *HS1'* hypersensitive site in both lymphoid (AKR1) and non-lymphoid (NIH 3T3) cells. Moreover, the high acetylation levels described

above are found mainly at that site.

The CTCF Site at HS1' Is Associated with a Nuclear Matrix Attachment Region—Nuclear matrix attachment regions (MARs) have been associated variously with regions of altered chromatin conformation and histone modification and with the ability to influence transcriptional activity of nearby genes. Previous reports have also suggested that some MARs are associated with enhancer blocking elements (42–43). In addition, MARs can function as boundary elements to alleviate position effect in transgenic animals (44–46). Thus, there appear to be different classes of MARs with different sequence specificities, functions, and mechanisms of action. This variability in reported properties may reflect the operational definition of a MAR, which involves its ability to co-purify with an insoluble, DNaseI-resistant and salt- or detergent-extracted nuclear fraction.

Recent results from our laboratory (47) have shown that CTCF forms a complex with the nucleolar and nuclear matrix-binding protein nucleophosmin. Furthermore, the CTCF insulator site at the 5' end of the chicken *β-globin* locus co-purifies with the nuclear matrix fraction in a CTCF-dependent manner.² It has also been reported that CTCF behaves as a matrix protein (49). Given these results, the suggested association of MAR activity with insulators and the presence of non-CTCF enhancer blocking activity over extended portions of the 6.2-kb region, we surveyed this region for associations with the nuclear matrix. In this experiment, we measured the amount of endogenous DNA remaining in the nuclear matrix fraction of AKR1 cells prepared with detergent in a low ionic buffer (lithium salt method) (34) after digestion with DNase I, using the TaqMan probes across the *HS1-HS6* region. We found that following DNase I extraction, the CTCF site at the *HS1'* hypersensitive site is highly enriched (22-fold) in the nuclear matrix fraction compared with bulk DNA. A slight enrichment of 4.6-fold can also be seen between the *HS3* and the *HS4* sites, suggesting that this region is also associated with the nuclear matrix, whereas other regions are accessible to enzymatic digestion with DNase I and cannot be amplified (Fig. 7). Similar results were observed when *in vitro* nuclear matrix assays were performed or when the nuclear matrix was extracted with a high salt procedure (data not shown).

DISCUSSION

The *TCR δ*, *α*, and *Dad1* genes share a complex genomic locus. However, the three genes have distinct temporal and spatial expression patterns, and transcriptional regulation by *cis*-acting elements is tightly constrained. An LCR active at the double positive stage in T cells enhances *TCRα* recombination and drives the *αβ* lineage recombination. As discussed in the Introduction, the LCR at the *TCRα-Dad1* locus is a bifunctional element regulating the tissue specificity of the *TCRα* rearrangement but also expression of the ubiquitous *Dad1* gene (4, 9). Within this LCR, *HS1* is specific for T cells. Downstream of this element, six additional sites, *HS1'* and *HS2-6*, have been identified within a 6.2-kb region (5, 9, 50); these sites alone cannot provide chromatin accessibility for V(D)J recombination and transcription of the *TCRα* genes (15) but are involved in *Dad1* regulation (9). Thus, *HS2-6* differs from classical LCRs because it does not provide absolute copy number dependence and does not function in single copy, indicating that although this region may have partial LCR function, it may have other roles as well. Indeed, Zhong & Krangel (16) showed that the *HS2-6* region displayed an enhancer blocking activity suggesting that this region acts as a boundary element allowing the

² T. M. Yusufzai and G. Felsenfeld, unpublished data.

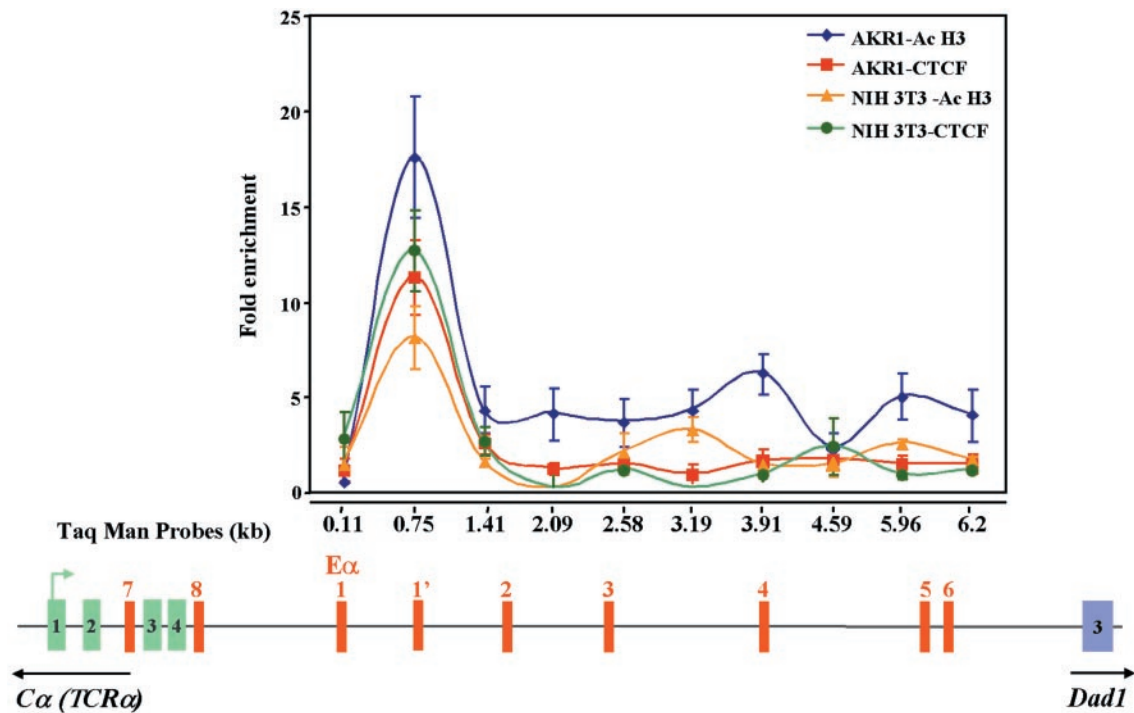
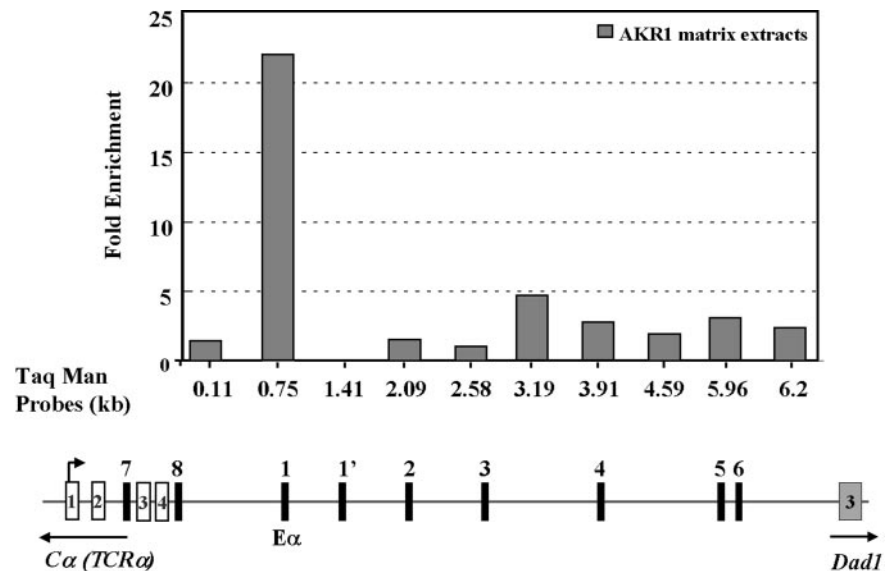


FIG. 6. **Histone H3 acetylation and CTCF binding across the *TCRα-Dad1* region.** Chromatin immunoprecipitation samples with antibodies to acetylated histone H3 or CTCF were analyzed by real-time PCR methods. Primer sets with TaqMan probes spanning every 500 bp of the region were used to amplify the bound and input DNA. The TaqMan probe numbers correspond to the map position. The y-axis shows the fold enrichment of analyzed proteins in the bound fraction versus input chromatin. Each data point indicates the average of at least three independent PCR analyses of immunoprecipitation with the S.D. shown by error bars.

FIG. 7. **Attachment to the nuclear matrix across the *TCRα-Dad1* region.** Genomic DNA that remained associated with lithium salt-extracted nuclear matrix samples digested with DNase I were analyzed by real-time PCR methods using primer sets with TaqMan probes spanning every 500 bp of the *TCRα-Dad1* region. The y-axis shows the relative enrichment of matrix-associated DNA.



expression of *Dad1* in non-lymphoid cells and protecting against inappropriate activation of *Dad1* by *Eα*.

CTCF is a highly conserved, ubiquitously expressed protein that has been found to bind to all identified vertebrate insulator sequences (Introduction). Given the strong insulation activity of the HS2–6 region, we decided to search for the presence of putative CTCF binding sites, first by comparing the region between HS1 and HS6 at the *TCRα-Dad1* locus to all the known binding sites for CTCF. Several candidate sites were identified, but direct measurement of binding *in vitro* revealed that only one of these corresponded to a high affinity CTCF binding site, located at the HS1' hypersensitive site. Chromatin immuno-

precipitation assays confirmed that CTCF was bound to this site *in vivo* and that this was the only such site occupied by CTCF in the entire HS1–6 region.

Using our enhancer blocking assay in erythroleukemia cell lines (K562), we were able to confirm, as shown previously in Jurkat T cells (16), that multiple fragments tested within the HS2–6 region confer enhancer blocking activity. This indicates that proteins or mechanisms mediating this activity are not T cell-specific and is consistent with a role for HS2–6 in both *TCRα* and *Dad1* control. We also report here the presence of a new site harboring enhancer blocking activity, located at the previously identified HS1' site (5) and corresponding to a bind-

ing site for CTCF. Taken together, these results suggest that the CTCF protein participates in the enhancer blocking activity within the DNase I hypersensitive region between the mouse *TCR α* and *Dad1* genes. However, additional enhancer blocking in the HS2–6 region appears to arise from the presence of multiple enhancer blocking elements dispersed among the different HS sites. The data shown in Fig. 5, and especially the detailed earlier analysis by Zhong and Krangel (16) of the distribution of enhancer blocking activity, make it clear that this activity must be dispersed at sites throughout the region between HS2 and HS6. Our data clearly rule out a role for CTCF in this insulator activity.

Although a variety of enhancer blocking elements have been reported in *Drosophila*, CTCF is the only example in vertebrates where an identified protein and binding site have unambiguously been implicated in this activity. MARs have been found in some cases to interfere with enhancer-promoter interactions when placed between these elements (42); they can also function as boundary elements to alleviate position effects in transgenic animals (44, 46, 51). Similar, though as yet poorly defined, mechanisms could explain some of the enhancer blocking activity between the *TCR α* enhancer and the *Dad1* gene. We have recently observed that CTCF mediates nuclear matrix attachment of the 5'HS4 insulator at the chicken β -globin locus and interacts with proteins known to be associated with the nuclear matrix, notably the nucleolar protein nucleophosmin (47).² Nucleophosmin co-localizes with CTCF at the nucleolar surface, suggesting that it can tether such sites to make a loop domain structure that prevents enhancer-promoter interaction (47). Quite similar models (in this case involving interaction with the nuclear envelope) have been proposed for the action of Suppressor of Hairy wing protein at gypsy insulator sites in *Drosophila* (48). Thus, enhancer blocking activity may be connected directly or indirectly with the ability of DNA sequences to be sequestered at sites on the nuclear matrix. However it should be noted (Fig. 7) that not all of the regions within HS2–6 associated with enhancer blocking activity are enriched in MAR-bound sequences. Furthermore, loop domain models consistent with insulating activity do not require that binding sites be tethered to fixed points within the nucleus but only that these sites interact with each other.

The pattern of histone acetylation at HS4 (Fig. 5) is consistent with the previously reported pattern of DNA methylation over this site: HS4 is not methylated in lymphoid tissues, where it presumably is involved in T cell differentiation, but it is methylated in non-lymphoid tissues, where it probably is inactive. Correspondingly, we find hyperacetylated histones, a mark of activity, only in AKR1 cells and not in fibroblasts. We note that deletion of HS1' leads to the hypermethylation of HS4 in lymphoid cells (41), which suggests that there is some interaction between these two sites.

A particularly interesting feature of the histone acetylation pattern is the peak of acetylation over HS1', centered on the CTCF binding region and present in both the lymphoid and fibroblast cell lines. We have reported elsewhere that peaks of acetylation are not necessarily associated with CTCF binding: the CTCF enhancer blocking site at the 3' end of the chicken β -globin locus is not a peak of acetylation. However, the 5'HS4 element at the β -globin locus does coincide with a peak of acetylation. We have attributed this (31) to the presence of binding sites for other regulatory factors also found at 5'HS4 that recruit acetylases and are associated with barrier activity. We have searched for similar binding motifs in the neighborhood of the TAD1/HS1' site but have so far been unsuccessful in identifying any. There is at least good reason to suspect that the TAD1 site harbors additional regulatory elements that may

either contribute to barrier insulator activity or have some other regulatory function for this extremely complicated gene system.

We have also not yet been able to identify the critical sequences within the HS2–6 region that contain no CTCF sites and are nonetheless responsible for strong enhancer blocking activity. By analogy with previously described sites such as those for CTCF or Suppressor of Hairy wing, we suspect that the sites themselves will be relatively small and bind specific proteins that in turn can interact with some element of the nuclear architecture to create a loop domain structure. The identification of such sites within this region will be important both for understanding insulator function and the regulation of the *TCR α /Dad1* locus.

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Both CTCF-dependent and -independent Insulators Are Found between the Mouse T Cell Receptor α and *Dad1* Genes

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