Identification of tissue specific nuclear proteins: DNA sequence and protein binding regions in the T cell receptor beta J-C intron

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ABSTRACT

We have determined the DNA sequences in the J2-C2 intron of the T cell receptor (TCR) beta gene and analyzed nuclear proteins binding to this region. Previously, we identified two tissue-specific DNase I hypersensitive regions, potential regulatory regions, in the J-C intron. The DNA sequence of the J2-C2 intron revealed that both DNase I hypersensitive regions have similar DNA sequences, suggesting that these regions are evolutionarily conserved. We have also identified tissue-specific nuclear-protein binding regions downstream of the DNase hypersensitive regions. Although transcriptional enhancer activity was not observed in the hypersensitive regions or the adjacent protein binding regions in the J-C intron, our findings suggest that the TCR-beta J-C intron may contain some other type of regulatory element.

INTRODUCTION

B and T cells participate in the generation of humoral and cellular immune responses, respectively. B cell surface immunoglobulins function as foreign antigen-receptors, while a comparable structure on T cells recognizes foreign antigen combined with a histocompatibility complex derived protein (1, 2). Both the structure and regulation of immunoglobulin (Ig) genes have been clearly defined. Studies of genes encoding the alpha, beta, gamma and delta chains of T cell receptors (TCR) have revealed a high level of structural similarities to the Ig genes. Like Ig genes, TCR genes consist of variable (V), joining (J), and constant (C) regions (3–11). These segments are separated in the germline DNA, but are combined during differentiation to generate functionally diverse receptor genes which encode the heterodimeric T cell surface receptor proteins. The functional similarities between Ig and TCR gene may extend to comparable regulatory systems needed for gene activation.

The Ig genes have unique regulatory regions, specifically, enhancer elements, within the J-C intron (12–15). Recently, the second enhancer element of the Ig k gene was identified downstream of the Ck region (16). Enhancer elements of the TCR-alpha and -beta were also identified downstream of the C regions (17–19). We have recently shown that the TCR-beta enhancer region displays nuclease hypersensitivity in a tissue-specific manner (20). The Ig enhancer regions were also shown to be nuclease hypersensitive. Such nuclease hypersensitive sites occur in most eukaryotic genes which are actively transcribed in cells. These regions coincide with protein binding sites, and in a few cases, disappear concomitantly with transcription as a result of site directed point mutations (21–30). We previously identified DNase I hypersensitive sites in the TCR-beta-2 J-C intron which were found in T cells but not B cells or fibroblasts (L cell) and are thus putatively tissue specific (31). Although transcriptional enhancer activity of the TCR-beta gene was identified downstream of the C region but not in the J-C intron (18,19), the tissue-specificity of DNase hypersensitivity in the J-C intron suggests that this region may have other regulatory functions.

To determine whether the J-C intron contains regulatory elements, we have determined the DNA sequence proximal to the DNase I hypersensitive sites in the TCR-beta-2 J-C intron, and analyzed nuclear proteins binding to this region. The nucleotide sequence of the TCR-beta-2 J-C intron shows significant similarity to human TCR beta 5'D-beta and 3'C-beta regions. A few sequences characteristic of regulatory elements were found in the J-C intron, but not as many as those found in the enhancer region. Interestingly, we have found that nuclear proteins obtained from T cell lines bind to regions downstream of the nuclease hypersensitive regions, whereas nuclear extracts obtained from the B cell line do not. Therefore our data suggest that the J-C intron may play a role in tissue-specific gene activation, distinct from that of conventional transcriptional enhancer activity.

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METHODS

Cloning

The TCR-beta gene was cloned by construction of a liver genomic DNA library (32) in a phage vector, and screening with a C-beta-2 cDNA clone (31) as described (33). The probe used for screening the genomic library was an isolated C-beta-2 insert from pUC25 (31), labeled with ^{32}P by nick-translation (34) to a specific activity of 2×10^8 cpm per μg . Plaques hybridizing with this probe were screened twice more at low plaque density and verified by Southern analysis using the same C-beta-2 probe. Two restriction fragments were separated on agarose gel, electroeluted, and subcloned. Subclone pC1-2 contains a 4.5 kb HindIII fragment in pUC9. Subclone pGXS contains a 2.5 kb XbaI-SacI fragment in the pGEM2 vector (Promega Biotec).

Mapping

Restriction sites in the T-beta-2 J-C intron were mapped by partially digesting end-labeled DNA and separating the nested set of fragments by gel electrophoresis (35). The subclone pGXS (32) was digested with XbaI, labeled at the 5' ends with gamma [^{32}P]-ATP and redigested with Pst I, results in two single-end-labeled restriction fragments: a small 16 bp polylinker, and the cloned insert, which was used without isolation for partial restriction cleavage. Portions of the fragment and unlabelled carrier DNA (total 1 μg) were partially digested for 10, 15, 20 and 40 minutes with 0.5 units of the restriction enzymes HindIII, AvaI, BamHI and EcoRI (New England Biolabs), and run on acrylamide gels.

Sequencing

All fragments were labeled at their 5' ends with gamma [^{32}P]-ATP and polynucleotide kinase (36), except the fragments made with XmnI, which were labeled at their 3' ends with alpha [^{32}P]-ddATP and terminal transferase (Amersham). End-labeled fragments were sequenced by the chemical method (36). Each region was sequenced more than four times and all sequences were read by more than two individuals. In addition, the EcoRII site [CC(A/T)GG] was confirmed by restriction enzymes. This was necessary because in the chemical method, 5-methyl cytosine is not cleared at that position and can be missed. Similarity with known sequences was determined by comparing the J-C intron DNA sequence with GenBank (February, 1990) sequences using the 'fasta' program.

Electrophoretic mobility shift assay

A DNA fragment [Hind III-Hpa II restriction fragment, # 1058 - # 1448 (the position numbers are taken from Figure 1)] was end-labelled with alpha [^{32}P]-dCTP and the large fragment of *E. coli* DNA polymerase I. Nuclear protein extracts were prepared according to Dignam et al. (37). Approximately one ng (10,000 cpm) of DNA fragment was incubated with 10 μg of nuclear protein extract and 1.2 or 2.4 μg poly(dI-dC)-poly(dI-dC) in 12% glycerol, 12 mM HEPES-NaOH (pH 7.9), 4mM Tris-Cl (pH 7.9), 60 mM KCl, 1 mM EDTA, and 1mM dithiothreitol for 30 min. at room temperature. Protein binding complexes were analyzed in low ionic strength 4% polyacrylamide gels (acrylamide: bisacrylamide weight ratio of 80:1) containing 6.7 mM Tris-Cl (pH 7.9), 3.3 mM Na-acetate, 1 mM Na-EDTA, and 2.5% glycerol. The gel was pre-electrophoresed for 30 min at 11 V/cm. Electrophoresis was carried out at the same voltage gradient for 2 hr, with buffer

recirculation. The gel was then dried and autoradiographed at -70°C with an intensifying screen.

DNase I footprinting assay

Nuclear extracts were fractionated on DNA cellulose columns according to the method of Emerson et al. (38). Seven to fifteen ng of end-labelled DNA was incubated with 2 to 10 μg of a 0.25M $(\text{NH}_4)_2\text{SO}_4$ DNA cellulose fraction in the presence of 2.4 μg of poly(dI-dC)-poly(dI-dC). Final buffer concentrations were 25mM Hepes (pH 7.9), 80mM NaCl, 5mM MgCl_2 , 1mM dithiothreitol, 10% glycerol, and 100 $\mu\text{g}/\text{ml}$ BSA in a reaction volume of 25 μl . Binding proceeded for 60 min at 0°C . After binding, complexes were cleaved by DNase I (0.1 $\mu\text{g}/\text{ml}$) for 1 min at 25°C . Reactions were terminated by the addition of 15mM EDTA (pH 7.5), 0.2% SDS, and 40 $\mu\text{g}/\text{ml}$ *E. coli* DNA. DNA fragments were then purified by extraction with phenol/ CHCl_3 and precipitation with ethanol. After resuspension in 90% formamide and heating at 95°C for 5 min, the samples were electrophoresed through 6% polyacrylamide, 8M urea gradient sequencing gels in $1 \times$ TBE buffer. The gel was exposed to Kodak DAR-5 film with intensifying screens at -70°C .

RESULTS AND DISCUSSION

DNA cloning and sequence in the J-C intron

A BALB/c liver DNA phage library was prepared and screened with a C-beta-2 cDNA clone. Three TCR-beta genomic clones were isolated from approximately 1×10^6 independent recombinants and two subclones were obtained (details described in Materials and Methods). Using one of the subcloned DNA fragment we next analyzed restriction sites in the J-C intron. Our restriction analysis revealed two BamHI sites instead of the single site previously described (39). This difference may be due to the mouse strain from which the genomic clones were derived, since these authors obtained genomic DNA clones from B10 mice, while our map was generated from genomic clones obtained from Balb/c mice.

Partial DNA sequences of the TCR-beta-2 J-C intron have been published (39-41). To study the DNA sequences surrounding the DNase I hypersensitive sites, and to compare the DNA sequences of the J-C region to other defined sequences, and to other regulatory regions, we determined the DNA sequences of the entire TCR-beta-2 J-C region (Fig. 1a).

The TCR-beta-2 J-C intron extends 2872 base pairs from the last J segment (J-beta-2-6) to the first C region exon (C-beta-2 exon 1) (Fig. 1a). The J-C intron contains DNA sequences with significant similarity to sequences in the human TCR 5' D-beta-1 and 3' C-beta-2 regions. One sequence (# 840-1090 in Fig. 1) bears 65% similarity with sequences in the human 3' TCR C-beta-exon 4 region while another sequence (# 2160-2360 in Fig. 1) shares 68% similarity with the human 3' TCR D-beta-2 region. The function of these regions in the murine or human TCR loci remains to be determined.

At approximately 150 bases upstream of the minor DNase I hypersensitive site (referred to as DHI-2), strongly symmetrical sequences (CTGGGAAATCTAGAGTTCCAG) are observed. These sequences have not been described previously as regulatory sites or protein binding sites. However their unusual symmetry suggests that this region might represent a locus for regulatory protein interactions. The proximity of this symmetrical stretch

Fig. 1. Sequence and features of the beta-2 TCR J-C intron. (a) DNA sequence of the J-C intron from the last J-beta-2 segment (J-beta-2-6) to the beginning of C-beta-2 (exon 1). Sequences in the upper (1-769) and lower (1939-2872) segments have been reported by others as indicated on the figure; sequences in the middle portion (742-1987) were determined in this investigation, and overlaps with previously identified sequences are indicated. Like most introns, this one begins with GT and ends with AG (underlined). (b) Feature map of the beta-2 J-C intron, showing the positions of DNase I hypersensitive sites and potentially significant regulatory elements.

to the DHI-2 site suggests that its physiological function may be related to the DHI-2 site. Other potentially significant elements found in the J-C intron included a span of 15 cytosine-guanidine

base pairs [poly(dC)-(dG)] and several Z-DNA like nucleotide sequences (Fig.1b). We mapped the CpG location because CpG clusters are thought to serve as binding regions for ubiquitous

Fig. 2. Sequences proximal to nuclease hypersensitive sites in the TCR-beta J-C intron. Nucleotide alignment between homologous regions in the DHI-1 and DHI-2 regions are indicated by hatched vertical lines.

nuclear factors (42) (Fig. 1b). A unique distribution of the CpG nucleotides was not found in the J-C intron.

Additional Exons in the J-C2 intron

Previous studies revealed that 5% of thymocytes contain TCR-beta messenger RNA that encodes an extra 24 amino acids between J-beta-1 and C-beta-1. This additional exon is called 'C-beta-0' (43). It was of interest to study whether or not a region equivalent to C-beta-0 exists in the TCR-beta-2 J-C region. DNA sequences with approximately 70% similarity to the C-beta-0 region were found in the bottom strand of the TCR-beta-2 J-C region, although an open reading frame with proper splicing signals was not found in this region. Furthermore since no open reading frames with proper splicing signals were identified in the entire J-C2 region. Therefore, it is unlikely that the TCR-beta-2 J-C region contains an authentic C-beta-0 exon.

Homology with Ig J-C Introns

Since the organization and sequences of TCR and Ig coding segments are homologous, their introns and flanking regions might be expected to be related by ancestry, if not by function. We compared the TCR and Ig J-C introns for sequence homology. Unexpectedly, the J-C intron DNA sequences of Ig genes, although structurally related to the TCR genes, did not show significant homology to that of the TCR-beta-2 J-C intron. This result suggests that the TCR-beta gene intron region may have evolved without interacting with other genes for long periods of evolutionary time. In contrast, comparison of DNA sequences around the DNase I hypersensitive sites in the TCR genes revealed that the sequences of DNase I hypersensitive site I (DHI-1) and site II (DHI-2) are strongly homologous to one another (Fig. 2).

Identification of nuclear protein binding in the J-C intron

The discovery of tissue-specific nuclease hypersensitive regions with conserved DNA sequences led us to search for the presence of functional elements adjacent to these regions. We initially analyzed DNA fragments from the J-C intron for transcriptional activity in CAT assays but were unable to detect any effects of these sequences on CAT transcriptional activity (not shown). The CAT assay has traditionally been used to detect promoter or enhancer function; thus this assay may not be sufficient to reveal other regulatory elements. Since regulatory elements often require interaction with trans-acting factors in order to function, another way to pinpoint sequences with potential regulatory activity is to identify DNA regions that specifically bind to nuclear proteins, using the electrophoretic mobility shift assay. This technique was developed to analyze nuclear protein-DNA interactions (44–46). We used this approach to analyze nuclear protein binding to the hypersensitive sites and their flanking regions. Our initial

Fig. 3. Electrophoretic mobility shift assay. (a) DNA fragment used for this assay. The end labelled site is indicated by the asterisk mark. (b) Crude nuclear extracts were obtained from B cell (A20, lanes 1,2), fibroblast cell (NIH 3T3, lanes 3,4) and T cell (AKRB, lanes 5,6) lines. Two concentrations of poly (dI-dC) were used as nonspecific competitor DNA with each nuclear extract (2.4μg for lanes 1,3,5 and 3.6μg for lanes 2,4,6). Detailed procedure are described in Materials and Methods.

experiments focused on the DHI-1 region: we found that nuclear proteins do appear to bind to this region. However, neither the methylation interference assay nor the DNase I footprinting confirmed the presence of specific nuclear-protein binding regions, suggesting that nuclear proteins may bind to this region with low affinity (not shown). Next we examined the flanking region of the DHI-1 site (Fig. 3a). The electrophoretic mobility shift assay of the DNA fragment downstream of the DHI-1 region revealed two distinct shifted bands. These bands were observed using nuclear protein extracts obtained from a T cell line but not from B cell or fibroblast cell lines (although very faint bands were visible at a different position when B cell or fibroblast extracts were used) (Fig. 3b). Similar results were obtained with nuclear extracts prepared from other T cell (KgV and KKB) and fibroblast cell (L cell) lines (not shown). Note that the KgV and KKB cell lines express the TCR-beta gene (detailed description in ref. 47). These results suggest that tissue-specific nuclear proteins bind to the region downstream of DHI-1.

To define the DNA sequence to which these nuclear proteins bind, we partially purified the nuclear proteins using DNA-cellulose columns and determined the protein binding sites using

Fig. 4. DNase I footprinting assay. (top) DNA-cellulose column fractions of the T-cell (AKRB) and B-cell (A20) nuclear extracts were analyzed for binding the DNA fragment described in Figure 3a by the DNase I footprinting assay (detailed procedure is described in Materials and Methods). The amount of nuclear extract used in each reaction is indicated above each lane. Binding regions are indicated by brackets A-D, and enhanced bands are shown by arrows. (bottom) DNA sequence of nuclear protein binding regions. Binding regions are indicated by brackets as in the autoradiograph (top). Palindromic sequence is indicated by the arrow.

the DNase I footprinting assays (Fig.4). T-cell nuclear extracts protected several regions from DNase I digestion; these regions were clearly identified with the addition of increasing amounts of nuclear protein. In contrast, purified nuclear proteins from B-cells did not protect any regions, thereby confirming the data obtained by the electrophoretic mobility shift assay shown in figure 3b. T cell derived nuclear proteins appear to bind several regions within the DNA fragment located downstream of the DHI-1 region in a tissue-specific manner. First, the poly (dC)-(dG) and its flanking regions were protected by T cell-derived nuclear proteins (Fig.4). Second, a long stretch of palindromic DNA sequences, downstream of the poly (dC)-(dG) region, was also found to be protected (Fig.4). Such sequences are common among nuclear protein binding regions and are believed to facilitate the interaction of nuclear protein dimers with DNA.

Poly-C and Potential Z DNA Regions

Poly (dC)-(dG) regions from several genes have been identified as regulatory or nuclear protein binding regions (summarized in Fig.5a). The 5' poly (dC)-(dG) region of the c-myc gene, for example, contains both DNase I hypersensitive and protein binding sites (48). In the promoter region of the chicken beta-globin gene, nuclear protein(s) binding to poly (dC)-(dG) sequences have also been demonstrated (38). These data strongly suggest that poly (dC)-(dG) regions in the TCR J-C intron may also have a regulatory function. The conservation of this region during evolution suggests that it may have been protected from base changes because of its specificity for regulatory DNA binding proteins.

The physiological function of the poly (dC)-(dG) region is not known. One possible function for this region is in changing DNA conformation. DNA modification experiments using bromoacetaldehyde (BAA) have demonstrated that poly (dC)-(dG) sequences induce an altered DNA conformation in neighboring DNA sequences (49). It is possible that such altered DNA conformations may be involved in the generation of DNase I hypersensitive sites. Our data indicate that nuclear proteins also bind to the Z-DNA like sequences (CACACACATGGATAT) located next to poly (dC)-(dG) stretches (Fig.4). Such DNA sequences are known to cause conformational changes in neighboring stretches of DNA. Note that the observed polarity between poly (dC)-(dC) and potential regulatory sites is always in the same direction (Fig.5b).

Furthermore, DNA modification experiments show the same polarity is required for conformational changes. Although the actual role of poly (dC)-(dG) sequences is not clear, these observations suggest that poly (dC)-(dG) stretch may cause distortion of DNA structure which in turn may make regulatory sites accessible.

Roles of the J-C intron for TCR-beta gene regulation

Our data demonstrate that the J-C intron displays nuclease hypersensitivity in a tissue-specific manner and that the sequences of the two DNase hypersensitive sites are homologous. Furthermore, tissue-specific nuclear proteins bind near the hypersensitive regions, and the nature of the protein binding sites suggests that these regions may be involved in conformational changes of DNA. These findings suggest that the TCR-beta J-C

Fig. 5. Correspondence of chromatin and base sequence features in several genes may reflect regulatory elements. (a) DNA sequences neighboring poly (dC)-(dG) sequences. Protein binding, DNase I, II and S₁ sensitive regions are indicated by bars above or below DNA sequences. (b) Location of DNase I hypersensitive regions and poly (dC)-(dG) sequences in the TCR beta J-C intron and the 3' regulatory regions in the c-myc and beta-globin genes. The polarity of poly (dC)-(dG) sequences are always toward the transcriptional start sites (indicated as RNA →).

intron contains significant regulatory elements, distinct from classic enhancer elements. Further analysis should provide direct evidence of the functional capacity of this region.

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