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Identification of a stimulus-dependent DNase I hypersensitive site between the Iα and Cα exons during immunoglobulin heavy chain class switch recombination

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Abstract The complete humoral response to foreign antigen depends upon two distinct recombination events within the heavy chain locus of immunoglobulin. The first recombination event takes place in what will become the antigen combining site of the antibody molecule, encoded by V, D and J segments. The second recombination event involves the looping-out of large spans of DNA which separate the various clusters of heavy chain exons which define the different immunoglobulin isotypes, or classes. While a great deal has been learned about the nature of the VDJ recombinase, very little is known about the nature of the class-switch recombinase. Using a cell system where class-switch recombination occurs primarily to the IgA locus, we have looked for stimulus-dependent changes in the chromatin structure of the IgA locus which might result from interactions between components of the recombinase and *cis*-elements within the region. We present evidence that strongly suggests that the class-switch recombinase interacts between the Iα and Cα exons of IgA, just upstream of the highly reiterated DR1 and DR2 elements. However, although multiple potential SMAD-4 sites are located precisely within the DNase I hypersensitive site and 160 bp upstream of that site, we failed to detect any evidence of DNA/protein interactions near the hypersensitive site. Moreover, recombinant SMAD-3/4 proteins fail to interact with these sites with appreciable affinity in vitro. These data suggest that some other structural alteration at this site (e.g. RNA/DNA hybrid) may mediate the nuclease sensitivity.

Key words: Immunoglobulin gene; Class switching; Hypersensitive site; IgA

1. Introduction

The humoral response to soluble antigen initiates when surface IgM-positive B lymphocyte clones bearing an antigen combining site complementary to a conformational determinant on the antigen receive signals via the B lymphocyte receptor for antigen in conjunction with signals from a T helper lymphocyte. The signals from the T helper lymphocyte stimulate the growth and subsequent differentiation of antigen-reactive clones to generate plasma cells which secrete antibody molecules with the same specificity (although of higher affinity), but which have distinct effector functions as determined by the nature of the Fc portion of the immunoglobulin produced. The phenomenon of isotype class-switching from IgM to the other antibody classes is referred to as immunoglobulin heavy chain class switch recombination [1–4].

Despite the general similarities in recombination events which engineer the Fab portion of the antibody molecule (VDJ recombination) and isotype class-switching, it is now clear that the molecular mechanisms mediating these recombination events are distinct. First, the recombination events occur at very different stages of B cell differentiation. Second, distinct extracellular signals are required for VDJ recombination and isotype class-switching.

Far more is known about the molecular basis of VDJ recombination than isotype class-switching. For example, it is now known that the RAG-1 and 2 proteins as well as the Ku70 and Ku80 proteins act in concert to recombine VDJ segments 'marked' by 12/23 DNA elements [5–7]. Definitive demonstration that particular proteins are components of the antibody class-switch recombinase lag far behind what is known for VDJ recombination. In addition, the *cis*-elements mediating class-switch recombination do not involve the 12/23 rule, but rather rely upon clusters of direct repeats located upstream of the first heavy chain exon for each isotype [8,9]. Transcription from an upstream I region exon appears to be very important for class-switching, probably by altering the chromatin structure of 'downstream' class-switch recombinase targets [10–12]. Ultimately, class-switch recombination requires the bridging of much of the DNA between the VDJ 'cassette' and the 'targeted' heavy chain exon. The intervening loop of DNA is excised and the VDJ and selected heavy chain exons are brought into proximity to each other [13,14].

In this report, we have scanned the region immediately upstream of the IgA region exons for stimulus-dependent DNase I hypersensitivity sites which are likely to coincide with recombinase recognition elements. We have also compared two isogenic cell lines CH12 F3-2 and CH12 D7, which are competent or mutant for class-switch recombination to IgA, respectively. The data suggest that a region immediately upstream of the DR1/DR2 stretches found between the Iα and Cα exons is likely to be an important recognition site for the IgA recombinase.

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2. Materials and methods

2.1. Cells and tissue culture

CH12 D7 and CH12 F3-2 cells were generated by limited dilution and assessment of capacity to class-switch to IgA [15,18]. These cells were derived from the CH12 LX cell line of Warren Strober (NIH, Bethesda, MD, USA) [16]. The cells are cultured in RPMI 1640 supplemented with inactivated 10% fetal calf serum, 50 μM mercaptoethanol, 2 mM L-glutamine, 5% NCTC 109 and 20 mM HEPES (pH 7.4). The -D7 and F3-2 sublines of CH12 were generated by limiting dilution.

2.2. In vitro isotype class-switching

Both the CH12 D7 and CH12 F3-2 cells were induced to class-switch using the following stimuli: soluble CD40 ligand (a fusion protein of CD40 ligand and CD8α produced from the J558L cell line), mouse recombinant IL-4 and recombinant TGF-β 1.

2.3. Flow cytometric analysis

At various stages after treatment with cytokines, the CH12 cells were stained with PE-α-mouse IgM polyclonal antibody and/or FITC-α-mouse IgA goat polyclonal antibody. After appropriate washes with 1×PBS, the cells were analyzed on flow cytometers equipped with multiple laser detectors.

2.4. Isolation of nuclei and Southern blot hybridization analysis

1×10^8 cells were centrifuged at 1000 rpm for 10 min to pellet CH12 lymphocytes. These were then resuspended in 50 ml ice-cold 1×PBS and centrifuged at 1000 rpm for 10 min. The pellet was then resuspended in 25 ml ice-cold nuclear isolation buffer (60 m KCl, 15 mM NaCl, 5 mM MgCl 2, 0.1 mM EGTA, 15 mM Tris–HCl, pH 7.4, 0.5 mM DTT, 0.1 mM PMSF, 0.3 M sucrose). After centrifugation for 10 min, the pellet was then resuspended in 1 ml NIB containing 0.5% NP40. The cells were then allowed to swell in this buffer for 6 min. The nuclei were then layered over 500 μl NIB containing 1.7 M sucrose and 5% glycerol. This was then centrifuged at 15 000 rpm for 15 min. The nuclear pellet was resuspended in 1 ml NIB containing 5% glycerol. This was then split into four microcentrifuge tubes into which no or increasing concentrations of DNase I were delivered in 25 μl 5 mM $CaCl_2$, 1 mM $MgCl_2$. The following range of units of DNase I were delivered: 0, 2.75, 5.5 and 11.0. The tubes were mixed immediately after addition of DNase I by inversion and incubated at room temperature for 6 min. The enzymatic reactions were terminated by addition of 275 μl 20 mM EDTA, 1% SDS. An additional 2.7 μl proteinase K (10 mg/ml), was added to each tube and incubated overnight at 37°C. Genomic DNA was then isolated by multiple chloroform/isoamyl-alcohol extractions followed by phenol/chloroform extractions. Approximately 5.25 μl of aqueous phase were recovered from each microcentrifuge tube and 2.5 μl RNase A (10 mg/ml) were added and incubated at 37°C for 2 h. The DNA was then precipitated by addition of 3 M sodium acetate, pH 5.2 and ethanol. After resuspension of genomic DNA, the DNA was digested to completion with the restriction enzyme *Eco*RI prior to agarose gel electrophoresis and Southern blot hybridization using standard techniques.

2.5. Hybridization probes and washing conditions

The Cα probe was a 782 bp *Pst*I fragment encompassing the entire Cα first exon and a small region of the Cα second exon. The Iα hybridization probe contained that entire exon amplified from genomic DNA using oligonucleotide primers. After hybridization at high stringency, Southern blots were washed twice in 2×SSC, 0.1% SDS at room temperature, 2× in 1×SSC, 0.1% SDS at 65°C, and twice in 0.1×SSC, 0.1% SDS at 65°C.

3. Results and discussion

With the aim of identifying regions within the IgA heavy chain locus which might interact with components of the isotype class-switch recombinase, we searched for DNase I hypersensitive sites induced in this region in response to extracellular signals which drive class-switching from IgM to IgA. Our system of choice was the CH12 system, where wild type

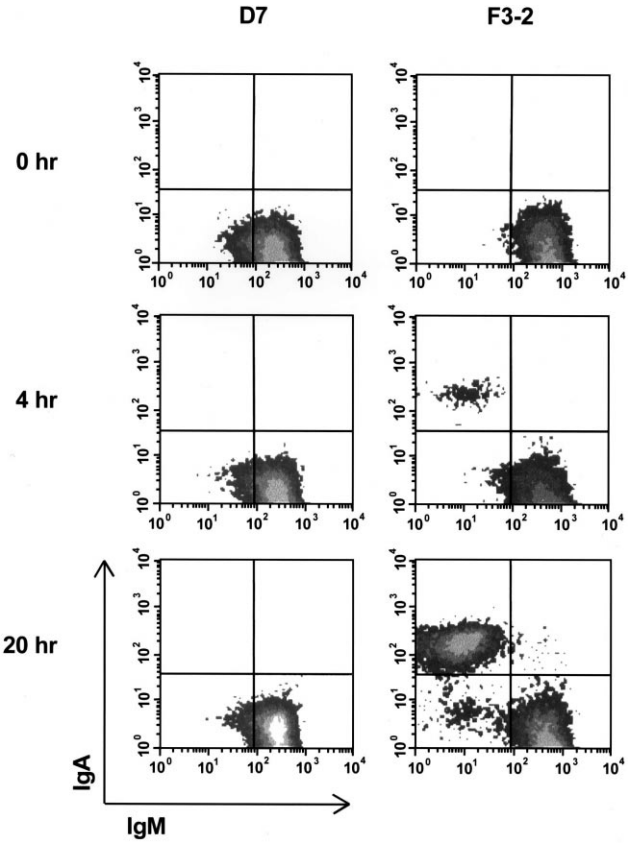

Fig. 1. Two color flow cytometric analysis of CH12 D7 and CH12 F3-2 surface immunoglobulin expression during in vitro isotype class-switching. Both cell lines were treated with interleukin-4, TGFβ1 and CD40 ligand and the proportion of cells expressing surface IgM or IgA was evaluated using antibodies specific for IgM or IgA. The *x*-axes indicate fluorescence intensity as a measure of surface IgM antibody molecules during in vitro differentiation. The *y*-axes indicate the proportion of cells and the fluorescence intensity of B cells expressing cell surface IgA. The limits of auto-fluorescence detected on unstained cells are shown via the bold lines which demarcate the plot. Fluorescence intensity is shown as a logarithmic scale.

cells switch from surface IgM positive to IgA positive in response to treatment with interleukin-4, CD40 ligand and transforming growth factor β1 (TGFβ1) [15]. This system was chosen since the cells selectively class-switch to IgA, minimizing the complexity of the experimental situation. Two sublines of CH12 were generated by limiting dilution and analyzed, F3-2: which is class-switch competent, and D7: which fails to class-switch to IgA (despite inducible Iα region transcription). Fig. 1 illustrates the distinct phenotypes of the two CH12 sublines as assessed by flow cytometry.

To scan the IgA locus for changes in chromatin structure which might correlate with class-switching, we treated both the D7 and F3-2 cell lines with interleukin-4, CD40 ligand and TGF-β1 for varying lengths of time prior to isolation of intact nuclei. These nuclei were then incubated in the presence of different concentrations of DNase I, prior to isolation of high molecular weight genomic DNA. The DNA samples were then digested to completion with the restriction enzyme, *Eco*RI. A 9.1 kb *Eco*RI fragment contains the Iα and Cα exons as well as a highly repetitive intervening sequence. To detect stimulus-dependent DNase I hypersensitive sites, we first used a 782 bp *Pst*I fragment spanning Cα first exon as

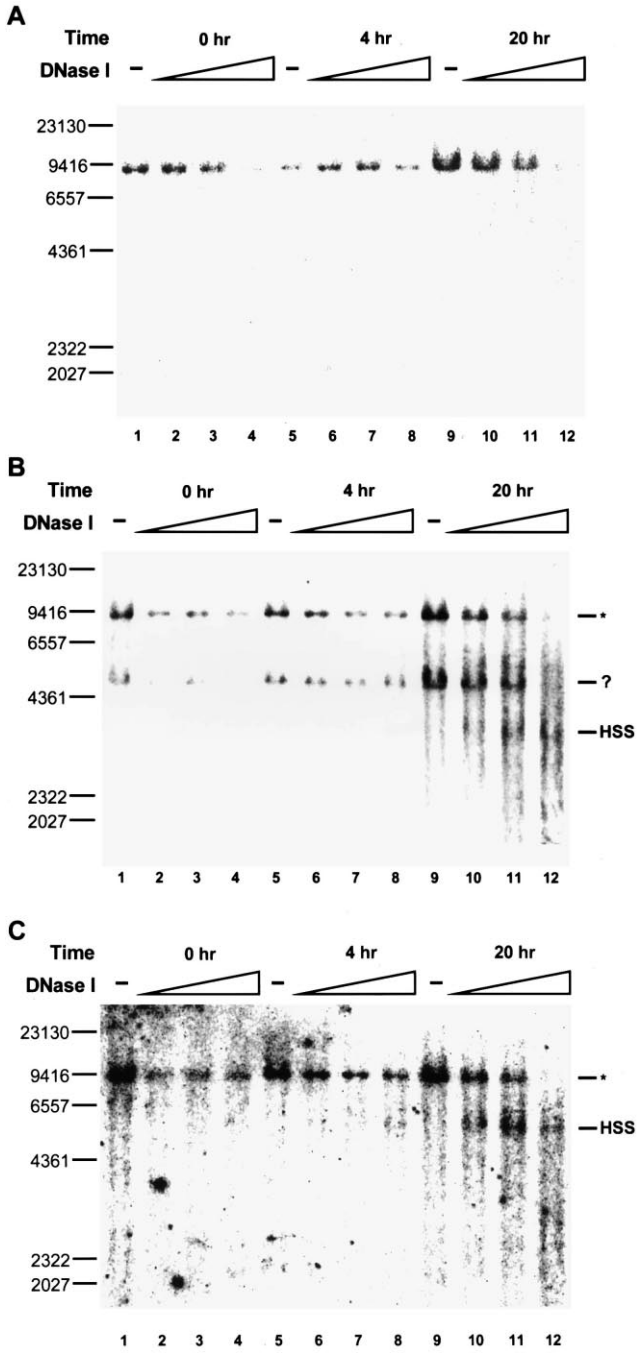

Fig. 2. Southern blot analyses of genomic DNA isolated from CH12 D7 and CH12 F3-2 cells during in vitro class-switch recombination. Nuclei from untreated and cytokine-treated cells were isolated at the indicated time points and were then incubated in the absence of/or in the presence of increasing concentrations of DNase I. Genomic DNA was then isolated from the nuclei and cleaved to completion with the restriction endonuclease *Eco*RI. The molecular size markers shown on the left hand border of each blot refer to λ *Hin*dIII, which was run on the agarose gel. A: Southern blot hybridization of CH12 D7 derived genomic DNAs. The Cα hybridization probe was used. A single 9.1 kb hybridizing fragment is detected in both untreated and cytokine-treated cells. B: Southern blot hybridization of CH12 F3-2 derived genomic DNAs using the same Cα probe used in (A). In addition to the 9.1 kb fragment, a smaller fragment of approximately 4.5 kb in length is also detected using probe. A stimulus-dependent DNase I hypersensitive fragment is detected in this class-switch competent cell line. C: Southern blot hybridization of genomic DNase extracted from CH12 F3-2 cells using the Iα hybridization probe. In addition to the 9.1 kb fragment, a stimulus-dependent DNase I hypersensitive site is observed using this probe. No hypersensitive sites were detected in the CH12 D7 mutant cell line.
←

a hybridization probe of Southern blots of the DNase I/*Eco*RI digested DNA samples.

Fig. 2A illustrates the results of the Southern blot hybridization using the Cα probe on DNA samples isolated from the CH12 D7 subline. A single hybridizing band of approximately 9.1 kb in length is detected in all lanes, regardless of degree of digestion with DNase I, and regardless of time of treatment with cytokines. Although the intensity of the hybridizing band decreases with the concentration of DNase I used (as would be expected, since DNase I would cleave the hybridizing fragment non-specifically), no specific hypersensitive site is detected. This is despite the fact that Iα region transcription is induced by cytokine treatment in this cell line (Nakamura and Honjo, data not shown).

A very different result is obtained when genomic DNA isolated from the CH12 F3-2 subline is analyzed. Once again, a 9.1 kb fragment is observed in all samples (see Fig. 2B). The intensity of this band decreases with increasing concentrations of DNase I in both non-stimulated and stimulated cells. We also detect a smaller hybridizing band in all samples of approximately 4.8 kb in length. This stimulus-independent fragment likely results from a deletion upstream of Cα in one allele of the IgA locus in this particular subline of CH12. This genetic alteration may explain why the CH12 F3-2 cell line class-switches exclusively to IgA. Most interestingly, a 3.57 kb DNase I hypersensitive site is observed in cytokine-treated F3-2 cells at 4 and 20 h. The appearance of this fragment is entirely dependent upon DNase I digestion, indicating that the fragment is generated by DNase I hypersensitivity and not recombination at the locus.

To more finely map the DNase I hypersensitive site, and to get an idea of the size of the hypersensitive region, we also performed Southern blot hybridizations using an Iα exon probe generated by polymerase chain reaction. Once again, a single DNase I hypersensitive site was observed at 4 and 20 h post-cytokine treatment (see Fig. 2C). The size of the DNase I hypersensitive fragment generated was 5.56 kb in length. Since the sum of the Cα and Iα hypersensitive fragments equals the entire length of the 9.1 kb *Eco*RI fragment

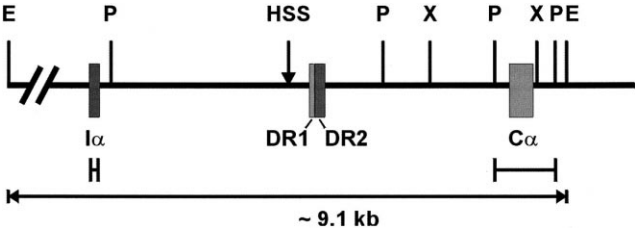

Fig. 3. Genomic organization of the Iα/Cα locus. The location of the 9.1 kb *Eco*RI fragment is indicated as are the positions of the Iα and Cα hybridization probes. The general locations of the Iα and Cα are shown as are the positions of the highly reiterated DR1 and DR2 sequences found intermediate between these two exons. The position of the stimulus-dependent hypersensitive site is indicated by an arrow, as are the positions of the restriction endonuclease sites for E: *Eco*RI, P: *Pst*I, and X: *Xba*I.

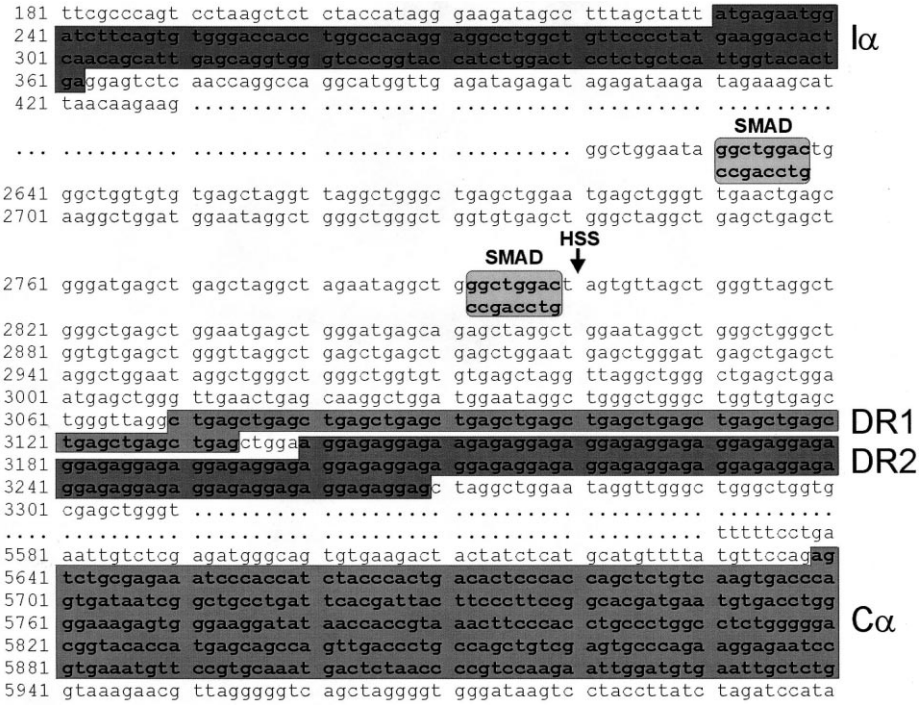

Fig. 4. Nucleotide sequence of the region surrounding the DNase I hypersensitive site and the Iα and Cα exons. Nucleotide sequence is omitted for large gaps between Iα and the hypersensitive site and between the hypersensitive site and Cα. The location of the hypersensitive site and of two perfect SMAD-4 recognition elements are indicated.

containing both the Iα and Cα exons, these data strongly suggest that a single protein or (more likely) set of proteins interacts with a discrete region within the locus. Fig. 3 illustrates our interpretation of the DNase I hypersensitivity experiments. The stimulus-dependent hypersensitive site which occurs exclusively in the class-switch competent F3-2 subline maps upstream of the DR1/DR2 repetitive regions located in-between Iα and Cα [17]. We calculate that a region of 10–100 bp in the vicinity of this hypersensitive site interacts with a single or a set of proteins which are likely to be component(s) of the IgA recombinase.

Since we know that interleukin-4, CD40 ligand and TGF-β1 are required for class-switching to IgA [19–21], we immediately inspected the nucleotide sequence in the vicinity of the DNase I hypersensitive site for binding sites for transcription factors mobilized by these extracellular signals. While we were unable to detect binding sites for IL-4 mobilized STAT-6 proteins, we were able to identify two potential (imperfect) SMAD-4 binding sites in this region [22,23] (Fig. 4). Additional sequences with significant similarity to SMAD binding sites are also reiterated multiple times in this region (although they frequently occur on the wrong strand of DNA). However, despite exhaustive electrophoretic mobility shift experiments, we were unable to detect any stimulus-dependent DNA/protein complexes forming on these sites (data not shown). Moreover, recombinant SMAD-3/4 proteins were unable to complex with these sites in vitro (while they bound strongly to bona fide SMAD sites) (data not shown). Therefore, we feel it is unlikely that the SMAD-4 protein (or another member of this family) interacts near the DNase I hypersensitive site. Other structural alterations (e.g. DNA/RNA hybrid formation) need to be investigated to explain the basis of the nuclease sensitivity [24].

The data obtained in this study are consistent with our recently published transfection studies in the CH12 F3-2 cell line which also indicate that the precise sequence of this region is not critical for class-switch recombination [25].

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