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Torsional Stress Stabilizes Extended Base Unpairing in Suppressor Sites Flanking Immunoglobulin Heavy Chain Enhancer†

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ABSTRACT: DNA sequences surrounding the immunoglobulin heavy chain (IgH) enhancer contain negative regulatory elements which are important for the tissue specificity of the enhancer. We have shown that sequences located both 5′ and 3′ of the enhancer, corresponding to the negative regulatory elements, become stably and uniformly unpaired over an extended length when subjected to torsional stress. These DNA sequences are also included within matrix association regions. The ability of the sequences to assume a stably unpaired conformation was shown by reactivity with chloroacetaldehyde which is specific for unpaired DNA bases, as well as two-dimensional gel electrophoresis of topoisomers. The sequences located 3′ of the enhancer induce base unpairing in the direction of the enhancer. This unpaired region progressively expands to include as much as 200 base pairs as the ionic concentration decreases or superhelical density increases. When an ATATAT motif within a negative regulatory element located 3′ of the enhancer was mutated, the extensive base-unpairing property was abolished. This base-unpairing property of DNA may be important for negative regulation of gene expression and attachement to the nuclear matrix.

The IgH enhancer sequence is located within the intron between the joining segments (J_H) and the constant regions ($C\mu$) of the murine $C\mu$ locus (Banerji et al., 1983; Gillies et al., 1983; Mercola et al., 1983; Neuberger, 1983). This enhancer is active in B cells but not in fibroblasts and was the first genetic element shown to confer cell-type specificity to a cellular gene (Banerji et al., 1983; Gillies et al., 1983). Evidence for negative control of this cell-type specificity was reported (Kadesch et al., 1986). Imler et al. (1987) have identified, within the surrounding the IgH enhancer, cis-acting negative regulatory elements which repress IgH enhancer activity in fibroblast but not in myeloma cells. Recently, a developmental-specific factor, NF-μNR, that binds to these elements has been reported (Scheuermann & Chen, 1989). Deletion of segments bound by NF-μNR from the enhancer results in an activation of the enhancer function in non-B cells.

Nuclear matrix association regions (MARs) or scaffold-attached regions (SARs) are often found in close vicinity to known enhancer sequences (Cockerill & Garrard, 1986; Gasser & Laemmli, 1986). These regions contain topoisomerase II cleavage consensus sequences and are thought to punctuate chromosomal DNA into functional units of topologically constrained loop domains. The J_H-$C\mu$ intron region has also been found to contain sites that are associated with the nuclear matrix. The MARs for this region were located around the IgH enhancer which contains these negative regulatory elements (Cockerill et al., 1987).

In this paper we report an unusual property of DNA sequences that correspond to the negative regulatory elements and the MAR sequences of the IgH enhancer. These sequences become entirely base-unpaired over an extended length even at room temperature when subjected to the torsional stress of negative supercoiling. This is not due to a high rate of DNA breathing, but instead they are stably base-unpaired. These sequences are A+T rich. However, in addition to being A+T

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rich, DNA sequence plays an important role to confer such base unpairing since only three point mutations within a NF-μNR binding site resulted in marked reduction of the unpairing property. Stable, unwound DNA structure induced by torsional stress has previously been reported for certain A+T-rich sequences (Kowalski et al., 1988). Recently, the DNA sequence with such a property has been correlated with DNA replication initiation (Umek & Kowalski, 1987, 1988; Kowalski & Eddy, 1989).

A possible role of DNA in regulation of gene expression utilizing its structural property is also suggested by the fact that multiple sites within transcriptional regulatory regions, when under torsional stress, readily adopt DNA structure deviated from the normal B-form DNA. These altered DNA structures have been probed by various methods, including the unpaired DNA specific chemical probes bromoacetaldehyde (BAA) and chloroacetaldehyde (CAA)[1] (Kohwi-Shigematsu et al., 1983, 1987; Kohwi-Shigematsu & Kohwi, 1985, 1989; Kohwi & Kohwi-Shigematsu, 1988; Hanvey et al., 1988; Furlong et al., 1989). Sites reactive with these chemicals in vivo have also been detected within transcriptional regulatory sequences of certain genes, including chicken adult β^A gene (Kohwi-Shigematsu et al., 1983), the human cytomegalovirus major immediately early gene (Kohwi-Shigematsu & Nelson, 1988), and human β interferon gene (Bode et al., 1986). These CAA- and BAA- reactive structures are presumably of biological significance since their occurrence correlates with transcriptional activity in vivo.

The in vivo CAA reactive sites are often reactive with CAA in supercoiled plasmid DNA as well, or if not, they occur in close vicinity to the in vitro sites, suggesting inherent potential of regulatory elements to adopt altered DNA structures. These results suggest an active role of DNA itself by utilizing its structural potential in biological functions, such as replication and transcription, rather than DNA being a passive participant by serving merely as the sequence information source. The extensive base-unpairing property of negative regulatory elements and matrix attachment regions in the IgH enhancer flanking sequences may also be of functional importance in vivo.

Experimental Procedures

Chemicals. Chloroacetaldehyde (CAA) was purchased from Fluka and doubly distilled (bp 78–80 °C) before use. Hydrazine, dimethyl sulfate, formic acid, and piperidine were purchased from Sigma.

Plasmid DNA. Supercoiled plasmid pSERCμ1kb (Gillies et al., 1983) was constructed by inserting the *Xba*I fragment (1–992) of the IgH enhancer region to the *Eco*R1 site of pSER vector plasmid with the use of the *Eco*R1 linkers. The pSER vector was constructed by removing the *Sph*1–*Pvu*II fragment containing the SV40 enhancer sequence from plasmid pSV2gpt (Gillies et al., 1983). The plasmid pSERCμ700bp contains the *Xba*I–*Eco*R1 (1–685) fragment of the IgH enhancer region inserted at the *Eco*R1 site of pSER vector plasmid with the use of *Eco*R1 linkers ligated to the *Xba*I site. Similarly, the plasmid pSERCμ300 bp contains the *Eco*R1–*Xba*I (685–992) fragment of the IgH enhancer region inserted into the *Eco*R1 site of the pSER vector. Supercoiled plasmids Cμ300TKCAT and Cμ1kbTKCAT contain either the *Eco*R1–*Xba*I (685–992) fragment or *Xba*I–*Xba*I (1–992), respectively, inserted at the *Sma*I site of pUC13 plasmid after the protruding ends were blunted by mung bean nuclease. The plasmids also contain the bacterial chloramphenicol acetyltransferase gene inserted at the *Hin*dIII site and the *Pvu*II (5′ end) to *Hin*cII (3′ end) of the herpes simplex virus promoter inserted at the *Pst*I site with the use of the *Pst*I linkers.

Chemical Modification and Mapping of CAA Modification Sites. Twenty-five micrograms of supercoiled plasmid DNA was incubated with either 2 μL (380 mM) or 0.1 μL (19 mM) of CAA per 100 μL of reaction volume in sodium acetate buffer at pH 5 for 1 h at 37 °C under varying salt concentrations as described in the figure legends. Four micrograms of CAA-modified DNA was digested with a restriction enzyme and then radiolabeled either at the 5′ end with T4 kinase (United States Biochemical) and [^{32}P]ATP (6000 Ci/mmol, New England Nuclear] or at the 3′ end with the Klenow fragment of DNA polymerase I (Boehringer Mannheim) or T4 polymerase (Pharmacia) and [α-^{32}P]dATP (ICN, 3000 Ci/mmol). The labeled DNA was digested at the second restriction site distal from the suspected CAA-modified sites. The DNA fragment containing CAA-modified sites was isolated from a 6% native polyacrylamide gel. The chemical cleavage of the DNA at the site of CAA modification is described in detail in our previous paper (Kohwi & Kohwi-Shigematsu, 1988). Briefly, the DNA was treated with either hydrazine or formic acid and reacted with piperidine as described by Maxam and Gilbert (1980).

For cleavage of the CAA-modified DNA with S1 nuclease, the CAA-modified DNA, after being radiolabeled, was digested with S1 nuclease (Pharmacia) at 0.5 unit/μg of DNA at 20 °C for 10 min. The DNA was then precipitated with ethanol and loaded onto a native acrylamide gel. The new bands generated by digestion with S1 nuclease were isolated from the gel and purified, heat-denatured, and loaded onto a urea-denaturing polyacrylamide gel for further analysis.

Two-Dimensional Electrophoresis. A mixture of topoisomers with different superhelical densities were prepared by relaxation of the DNA with calf thymus topoisomerase 1 (Bethesda Research Laboratories) in the presence of various amounts of ethidium bromide (Kochel & Sinden, 1988). The continuity of the distribution of topoisomers was examined by running the resulting topoisomers in agarose gels containing various amounts of chloroquine. Two micrograms of the topoisomer mixture was loaded onto a 1.2% agarose gel (20 × 20 cm) in 80 mM Tris–borate and 10 mM EDTA, pH 8.2, and the gel was electrophoresed at 80 V for 19 h at 22 °C. The gel was then soaked in the same buffer containing 50 μg/L chloroquine for 2 h. The gel was rotated 90° and reelectrophoresed in the circulating buffer containing the same amount of chloroquine for an additional 15 h. The gel was soaked in water for 6 h and stained in 1 μg/mL ethidium bromide solution.

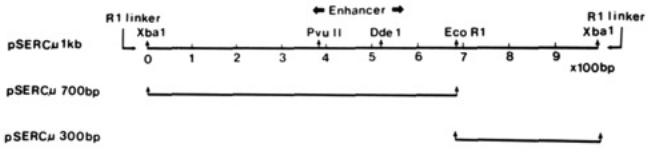
Site-Directed Mutagenesis. The Amersham system which is based on the method of Eckstein and his co-workers (Taylor et al., 1985) has been used to introduce base substitutions in the CAA-reactive region 3′ of the enhancer. This method involves a strand-specific selection step which eliminates the unwanted nonmutant sequence in vitro, generating a pure homoduplex mutant DNA sequence. Selective removal of the nonmutant strand is made possible by the incorporation of a thionucleotide into the mutant strand during in vitro synthesis, which makes the strand resistant to cleavages by certain restriction enzymes including *Nci*1 used in this experiment.

Results

DNA Sequences Surrounding the IgH Enhancer Are Reactive with Chloroacetaldehyde in an Ionic Condition Sensitive Manner. To determine whether the IgH enhancer region

[1] Abbreviation: CAA, chloroacetaldehyde.

Chart I



adopts an altered DNA conformation, we employed CAA which specifically reacts with unpaired adenine and cytosine and, to a limited extent, with guanine bases (Kochetkov et al., 1971; Barrio et al., 1972; Sattsangi et al., 1977). Chart I summarizes supercoiled plasmid DNA constructs used in this paper: pSERCμ1kb, pSERCμ300bp, and pSERCμ700bp (see details under Experimental Procedures). The plasmid pSERCμ1kb contains the *Xba*I restriction fragment (nucleotides 1–992) encompassing the IgH enhancer. The plasmid pSERCμ300bp is a deletion construct derived from the pSERCμ1kb which contains the 300bp *Eco*R1–*Xba*I(3′) fragment. The plasmid pSER700bp is another deletion construct containing the 700bp *Xba*I(5′)–*Eco*R1 fragment.

First, supercoiled plasmid DNA pSERCμ1kb, at the bacterial supercoiling density, was CAA modified to coarse map CAA-modified DNA regions within the entire plasmid DNA. For this purpose, the DNA was modified with CAA under varying salt concentrations at pH 5 since altered DNA structures are often sensitive to the changes of ionic conditions (Kohwi & Kohwi-Shigematsu, 1988; Sullivan & Lilley, 1987). The CAA-modified DNA was digested at the unique *Pvu*II site and end-labeled, and then the CAA-modified sites that remained as single-stranded regions in linear DNA were digested with S1 nuclease. The appearance of DNA fragments with one end labeled at the *Pvu*II site and the other end cleaved with CAA/S1 reflects the CAA-reactive regions were detected as shown by the appearance of bands not seen in the unmodified control in the native polyacrylamide gel (Figure 1, indicated by arrows). The band with the highest molecular weight appeared due to the CAA reaction with a DNA region in pBR322 containing inverted repeats and A+T-rich sequences located 1.5 kilobases (kb) counterclockwise from the *Pvu*II site. In addition to this band, two groups of bands with lower molecular weights were observed. These are indicated as I and II. Bands in region I were very intense, and their distribution of the molecular weight varied dramatically between 25 and 75 mM Na^+ (Figure 1, lanes 1–4). The bands in region II, on the other hand, were faint and only appeared at 50 and 75 mM Na^+ (Figure 1, lanes 3 and 4). Sequence analysis on isolated DNA fragments from region I and region II revealed that CAA-reactive elements are located immediately surrounding the enhancer sequence: bands in region I reflect CAA reactivity in the region 3′ of the enhancer and bands in region II in the region 5′. For convenience, we designate the former CAA-reactive region as "CAA region 3′" and the latter CAA-reactive region as "CAA region 5′". The results show that there exists a hierarchy between these two regions for adopting the CAA-reactive structure; the CAA region 3′ is much more reactive to CAA than the CAA region 5′ when both regions are present simultaneously in the same plasmid molecule. Once the CAA region 3′ is deleted as in the case of pSERCμ700bp plasmid, then the CAA region 5′ was as reactive to CAA as the CAA region 3′ in the plasmid pSERCμ1kb (data not shown; see also Figure 3C for more detail).

The fact that the width of the band in region I, corresponding to the CAA region 3′, broadens in the direction of the *Pvu*II site as the salt concentration decreased suggests that the DNA conformation alters progressively toward the *Pvu*II

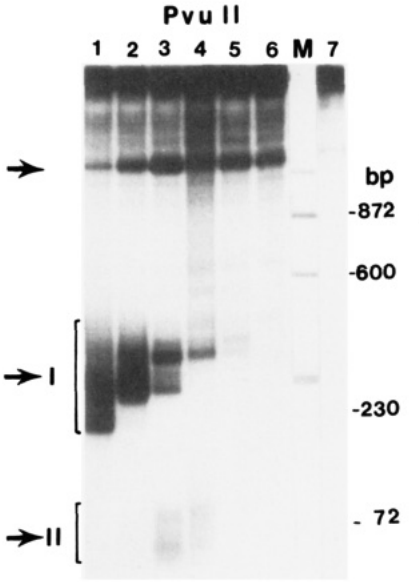


FIGURE 1: Ionic condition dependent CAA reactivity of pSERCμ1kb. The supercoiled pSERCμ1kb plasmid was reacted with 2 μL of CAA in 100-μL reaction volume in 25 mM sodium acetate buffer at pH 5 with varying amounts of sodium chloride. The total Na^+ concentrations are 25 mM Na^+ (lane 1), 37.5 mM Na^+ (lane 2), 50 mM Na^+ (lane 3), 75 mM Na^+ (lane 4), 25 mM Na^+ and 2 mM Mg^{2+} (lane 5), and 50 mM Na^+ and 2 mM Mg^{2+} (lane 6). The CAA-modified DNA was 3′-end-labeled with [α-^{32}P]dATP and T4 polymerase at the *Pvu*II site, cleaved with S1 nuclease, and separated on a 6% native acrylamide gel. Arrows indicate the bands which are present in the CAA-modified DNA but are absent in the control DNA without the CAA treatment (lane 7). Lane M shows labeled ϕX 174 DNA restricted with *Hae*III. The size of the restricted fragments is indicated. The appearance of the band at the highest molecular weight indicates the CAA reaction at the inverted repeat sequence of pBR322. Region I represents the CAA-reactive sequences 3′ of the IgH enhancer. Region II represents CAA reactive sequences 5′ of the enhancer.

site. The directionality of the base unpairing of the CAA region 3′ was more precisely studied by an alternative method described below.

IgH Flanking Sequences Are Uniformly Base-Unpaired in Supercoiled Plasmid DNA. Once CAA-reactive regions within the plasmid DNA were determined by the S1 nuclease technique, more precise mapping to identify bases that are CAA-modified in the CAA regions 5′ and 3′ was performed with a chemical cleavage method (Kohwi & Kohwi-Shigematsu, 1988). This method to map the CAA-modified bases relies on the difference in reactivity of CAA-modified bases vs unmodified bases to the first set of chemicals that are used for the Maxam–Gilbert sequencing reaction (Maxam & Gilbert, 1980), such as hydrazine and formic acid. For example, hydrazine which specifically reacts with cytosine bases under high salt also reacts with CAA-modified adenine residues. Formic acid which specifically protonates adenine and guanine bases reacts with CAA-modified cytosine bases as well. The CAA-modified bases can then be detected by the appearance of new bands which are not expected within a given chemical sequencing ladder (Kohwi & Kohwi-Shigematsu, 1988; Kohwi, 1989).

This method revealed a unique feature for the altered DNA structure adopted by the IgH enhancer flanking sequences in that every CAA-reactable base (adenine and cytosine residues) is CAA-modified. Within the population of plasmid DNA, each of these residues was cleaved by either hydrazine or formic acid when followed by the piperidine reaction to give

rise to new bands. The CAA reaction pattern of the region 3′ of the enhancer in the pSERCμ1kb plasmid was mapped from both directions, either from the *Pvu*II site (Figure 2A, left panel) or the *Xba*I site (Figure 2A, middle and right panels). A region between positions 795 and 655 was particularly reactive with CAA as judged by the appearance of very intense new bands on the sequencing gel (Figure 2A, arrows a–d).

The Base Unpairing in the Region 3′ of the Enhancer Extends Predominantly in One Direction as the Salt Concentration Decreases. The progressive expansion of the CAA-reactive region 3′ of the enhancer, as the Na^+ concentration decreases, was also observed by the chemical cleavage method. The CAA reactivity extended toward the 5′ direction up to 597 bp for the supercoiled plasmid modified with CAA in sodium acetate buffer at pH 5 containing 25 and 37.5 mM Na^+ (Figure 2A, left panel, lanes 2 and 3 shown by arrows). At 50 mM Na^+, the CAA reactivity was detected up to 655 bp (Figure 2A, left panel, lane 4 shown by arrow b). In contrast to the 5′ border of the CAA region 3′, its 3′ border varied at most by 24 bp between the plasmids treated with CAA at 25 and 50 mM Na^+ (Figure 2A, middle and right panels, shown by arrows c and d). This result indicates that, for CAA region 3′, the base unpairing extends unidirectionally toward the enhancer and, at low salt conditions of 25 and 50 mM Na^+, it can include as much as 200 bp in length.

Base Unpairing Still Remains at Discrete Sites under Conditions That Favor Double-Stranded Structure. The CAA reactivity of the CAA region 3′ was examined under relatively high salt conditions and in the presence of Mg^{2+} using the plasmid pSERCμ300bp previously described. The hydrazine/piperidine treatment of the CAA-modified DNA at 75 mM Na^+ revealed that the CAA reaction was restricted to the region 737–795 wherein every adenine base was CAA-reactive (Figure 2B, lane 3, indicated by arrow e). Also, every cytosine base in this region was CAA-reactive as revealed by the formic acid/piperidine treatment of the same plasmid DNA (data not shown). When 2 mM Mg^{2+} was added to the buffer containing either 25 mM (Figure 2B, lane 4) or 50 mM Na^+ at pH 5 (Figure 2B, lane 5), the CAA reactions were limited to only two bases: the two adenine bases at positions 747 and 749 of the top strand within the sequence ATATAT (745–750). For the bottom strand under the same conditions, the three adenine residues at positions 746, 748, and 750 were CAA-reactive (data not shown). The CAA reactivity of these adenine residues was greatly enhanced, as revealed by the higher intensity of new bands (see lanes 4 and 5 of Figure 2B), when the Na^+ concentration was increased from 25 to 50 mM in the presence of 2 mM Mg^{2+} (Figure 2B, lanes 4 and 5). These adenine residues within the ATATAT motif (in both strands) were CAA-reactive in a pH-independent manner while the rest of the sequences under different salt conditions were slightly more reactive at acidic pH than neutral pH, although CAA reactivity was clearly detected at the neutral pH as well (data not shown). When they were mapped from the 3′ *Xba*I site, there was no difference observed in their CAA-reactive patterns between the pSERCμ1kb plasmid and the pSERCμ300bp plasmids at 25–50 mM Na^+. At 75 mM Na^+, however, the CAA reactivity for the pSERCμ1kb plasmid was limited to adenine residues in the ATATAT motif (data not shown) instead of a defined region of 737–795, as observed for the pSERCμ300bp plasmid. In the presence of Mg^{2+}, both plasmids showed CAA reactivity confined to the adenine residue in the ATATAT motif.

The CAA-Reactive Region 5′ of the Enhancer Is Similar to but Different in Property from the CAA-Reactive Region 3′. The CAA region 5′ was analyzed by employing the pSERCμ700bp plasmid previously described (Figure 2C). The 5′ flanking sequence of the IgH enhancer also revealed that every adenine base in the 58 bp region between positions 272 and 329 (arrow g) was strongly CAA-reactive as reflected by intense new bands on the sequencing gel (Figure 2C, lanes 2–4 shown by arrow g; also see Figure 2D). In contrast to CAA region 3′, the typical CAA-reactive pattern in the CAA region 5′ did not change in a salt-dependent fashion (between 25 and 50 mM) but rather remained in a defined region. At 75 mM Na^+, the CAA reactivity was significantly reduced (Figure 2C, land 6). In the presence of 2 mM Mg^{2+} alone (Figure 2C, lane 7) or in the presence of both Mg^{2+} and 50 mM Na^+ (Figure 2C, lane 8), the signal nearly vanished with no specific bases remaining unpaired.

Propagation of Long-Range Conformational Effects Occurs in a Neighboring Sequence Dependent Manner. The region 30 bp from position 655 to the *Eco*R1 site (685) is strongly reactive with CAA in the pSERCμ1kb plasmid. When the same region was examined in the pSERCμ700bp plasmid construct by chemically cleaving the *Pvu*II–*Eco*R1 fragment prepared from the CAA-modified plasmid, no CAA reactivity was detected (Figure 2C, right panel). This indicates that this sequence (655–695) does not possess the inherent potential to adopt unusual DNA conformation. Thus, in the pSERCμ1kb plasmid, this sequence must have been induced to adopt the CAA-reactive structure by the sequence in the *Eco*R1–*Xba*I(3′) fragment. To examine if the base-unpairing effect that extends toward the enhancer is a neighboring sequence dependent or independent phenomenon, we examined the CAA reactivity of the 300 bp *Eco*R1–*Xba*I(3′) fragment placed adjacent to the herpes simplex virus thymidine kinase (TK) promoter. The data shown in Figure 3 (top panel) indicate that the CAA reaction is in fact extended beyond the *Eco*R1 site when mapped from the *Hin*dIII site located 3′ of the TK promoter (summarized in Figure 3, bottom panel). Therefore, the sequence in the *Eco*R1–*Xba*I(3′) is still capable of exerting base-unpairing effects on its 5′ sequence. Interestingly, however, the CAA reaction with DNA bases did not exceed the *Eco*R1 site by more than 20 bp (shown by a bracket in Figure 3) even at the lowest Na^+ concentration (25 mM) used, as opposed to more than 80 bp for the original construct when the native IgH sequence was adjacent to the *Eco*R1 site. Therefore, the induction of long-range base unpairing occurs in a sequence-dependent manner. This difference between the two different plasmid constructs is not due to a variation in supercoiled density of plasmids among different preparations, as several independent preparations gave rise to consistent results. Our unpublished observations using a deletion construct derived from the pSERCμ1kb plasmid show that as long as the sequence up to position 600 is intact, essentially the same extent of CAA reactivity is achieved as that of the pSERCμ1kb plasmid.

The Uniformly Base-Unpaired Structure Is Not Induced by Chemical Modification at Some DNA Sites. We asked if extensive base unpairing was induced as a consequence of the CAA reaction at any specific site. To address this question, we employed a low concentration of CAA (19 mM), by which a single nucleotide is estimated to be modified per plasmid molecule [Kohwi & Kohwi-Shigematsu (1988) and references therein]. If the structure originally contained unpaired bases over 150 bp in length, then even with the low CAA concentration, we should still detect a CAA reaction pattern identical

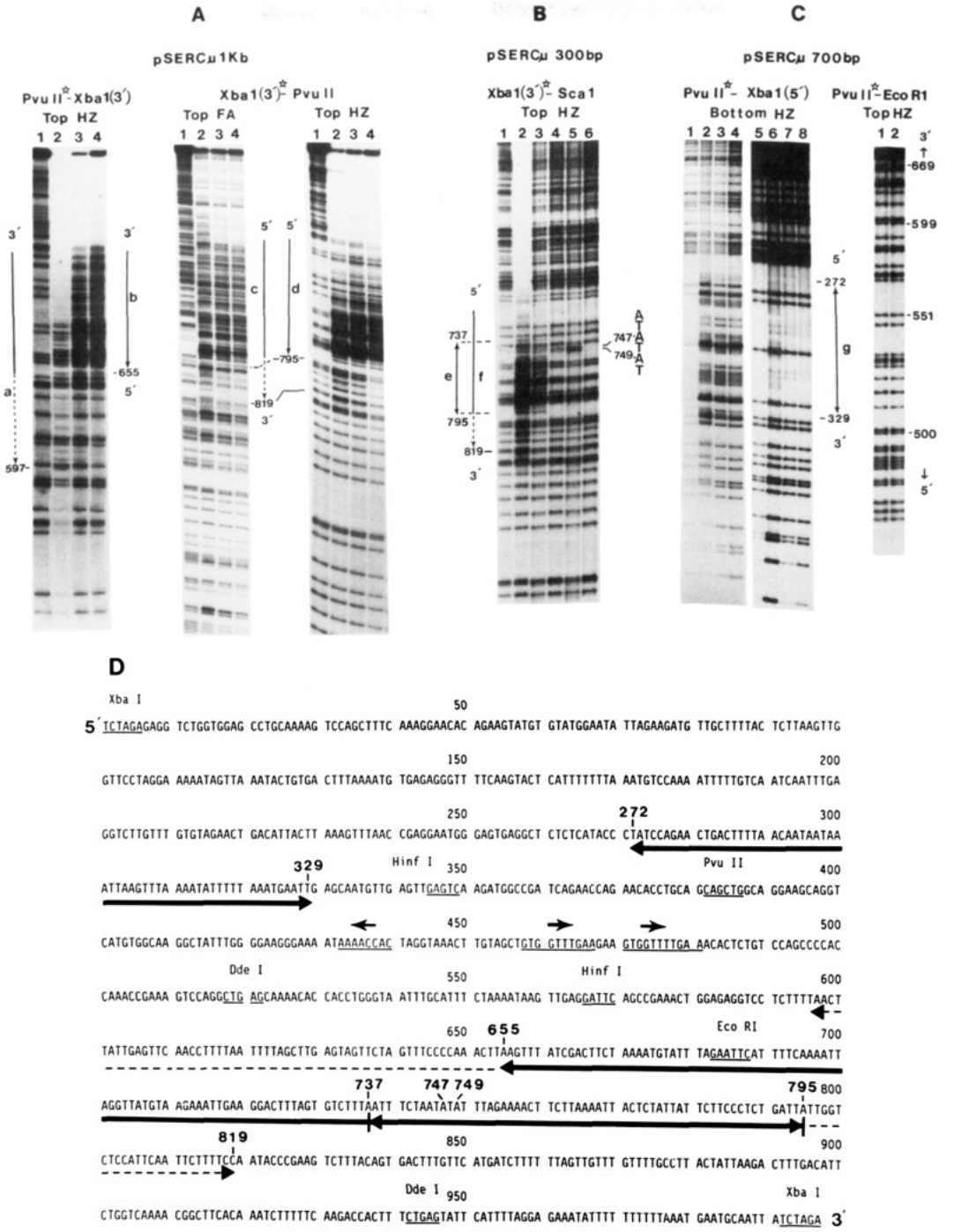


FIGURE 2: Fine mapping of the CAA-reactive sites surrounding the IgH enhancer. (Panel A) Supercoiled plasmid pSERCμ1kb was reacted with CAA in sodium acetate buffer at pH 5 containing 25 mM Na^+ (lane 2), 37.5 mM Na^+ (lane 3), and 50 mM Na^+ (lane 4). DNA in lane 1 was not CAA-modified. The CAA-modified and unmodified DNA were 5′-end-labeled at the *Pvu*II site and further digested with *Xba*I (left gel) or 3′-end-labeled at the *Xba*I site and digested with *Pvu*II (middle and right gels). The labeled DNA fragment containing the CAA-reactive sequence was isolated from a native acrylamide gel and subjected to chemical reaction, either with hydrazine (HZ) or with formic acid (FA). The DNA was then reacted with piperidine. The DNA sample was loaded on a 6% denaturing urea–polyacrylamide gel. Arrows a–d indicate regions of CAA reactivity. The region shown by broken lines (---) within the arrows indicates additional CAA reactivity resulting under low salt conditions. The DNA fragments are end-labeled at the restriction site indicated by a star. (Panel B) Supercoiled plasmid pSERCμ300bp containing the *Eco*R1–*Xba*I (3′) 300 bp fragment was reacted with CAA in sodium acetate buffer at pH 5 containing 25 mM Na^+ (lane 2), 75 mM Na^+ (lane 3), 25 mM Na^+ and 2 mM Mg^{2+} (lane 4), and 50 mM Na^+ and 2 mM Mg^{2+} (lane 5). Lanes 1 and 6 represent CAA-unmodified DNA. The DNA was 3′-end-labeled at the *Xba*I site and digested with *Sca*I (at position 157). The *Xba*I(3′)–*Sca*I fragment was isolated from a native acrylamide gel, subjected to hydrazine (HZ)/piperidine reaction, and loaded onto a 6% urea-denaturing polyacrylamide gel. The arrow f indicates the region of CAA reactivity under a low salt condition, and the double-headed arrow e indicates the region of CAA reactivity under a high salt condition. Positions 749 and 747 are indicated to show CAA reactivity at adenine residues at these positions in the presence of Mg^{2+} (see panel D for summary). (Panel C) Supercoiled plasmid pSERCμ700bp containing the *XBa*I(5′)–*Eco*R1 700 bp fragment was reacted with CAA in a solution acetate buffer at pH 5 containing 25 mM Na^+ (lane 2), 37.5 mM Na^+ (lane 3), 50 mM Na^+ (lane 4), 75 mM Na^+ (lane 6), 25 mM Na^+ and 2 mM Mg^{2+} (lane 7), and 50 mM Na^+ and 2 mM Mg^{2+} (lane 8). Lanes 1 and 5 represent CAA-unmodified DNA. The DNA was 5′-end-labeled at the *Pvu*II site and further digested with *Xba*I. The *Pvu*II–*Xba*I(5′) fragment (left and middle gels) and *Pvu*II–*Eco*R1 fragment (right gel) were isolated and subjected to hydrazine (HZ)/piperidine reaction. The double-headed arrow g indicates the region of CAA reactivity 5′ of the enhancer. (Panel D) Summary of chemical cleavage sites for CAA-modified IgH enhancer region. Solid double-headed arrows represent major CAA reactivity observed at either 50 mM Na^+ or higher. Broken lines represent extended CAA reactivity under 25 and 37.5 mM Na^+. Underlined sequences indicated by small arrows are those similar to the "core" elements common to most viral enhancers (Weiher et al., 1983).

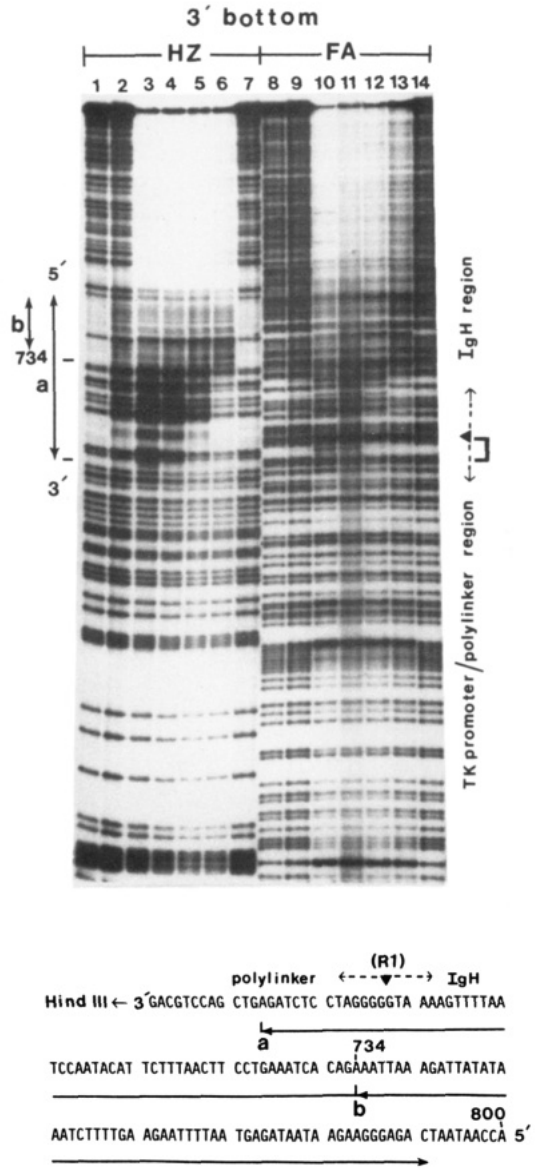


FIGURE 3: Conformational effect exerted by the CAA-reactive element of the IgH enhancer onto the vector sequence. (Upper panel) Supercoiled plasmid pTKCATCμ300bp containing the Cμ *Eco*R1–*Xba*I(3′) 300 bp fragment and the TK promoter was reacted with CAA in sodium acetate buffer containing 25 mM Na^+ (lanes 2, 3, 9, and 10), 37.5 mM Na^+ (lanes 4 and 11), 50 mM Na^+ (lanes 5 and 12), and 75 mM Na^+ (lanes 6 and 13). The DNA shown in lanes 2 and 9 was reacted with 0.1 μL of CAA (19 mM), and the DNA shown in lanes 3–6 and 10–13 was reacted with 2 μL (380 mM) of CAA in 100-μL reaction volume. The DNA shown in lanes 1, 7, 8, and 14 were not reacted with CAA. The DNA was 3′-end-labeled at the *Hin*dIII site and digested with *Bgl*I. The *Hin*dIII–*Bgl*I fragment was isolated and reacted with either hydrazine (HZ) or formic acid (FA). The DNA was then reacted with piperidine and loaded onto a 10% urea–denaturing polyacrylamide gel. Double-headed arrow a shows CAA-reactive region detected under 25–50 mM Na^+. Double-headed arrow b shows the CAA-reactive region under 75 mM Na^+. The solid triangle indicates the point of ligation of the *Eco*R1 of the IgH enhancer region and that of the pUC13 polylinker. The bracket indicates the CAA-reactive vector sequences. (Lower panel) The CAA-reactive sites indicated by double-headed arrows a and b are summarized.

with that for the plasmid modified at a higher CAA concentration (380 mM). Since the CAA reaction pattern obtained with CAA at 19 mM (Figure 3, lanes 2 and 9) was identical with that when CAA at 380 mM was employed (Figure 3, lanes 3–6 and 10–14) in the region that is CAA-reactive (shown by double-headed arrow a), we conclude that the structure with extensive unpairing existed prior to CAA reaction and was not induced by chemical reaction. When 19 mM CAA was used for reaction, a large percentage of the plasmid population remained unmodified. This is revealed by the presence of a ladder of bands distal to the CAA-reactive region. At a higher concentration of CAA, all plasmid molecules were modified with CAA and cleaved by the chemicals. This led to the absence of bands beyond the CAA-reactive region.

Effect of Superhelical Density of the Plasmid on the Extent of Base Unpairing in the pSERCμ1kb DNA. The CAA reactivity over an extensive stretch of DNA sequences could be attributed to an adopted stable non-B-form DNA structure in supercoiled plasmid DNA or possibly to a high rate of DNA "breathing" of DNA bases since the sequences are A+T-rich in nature. To distinguish between these alternatives we examined the mobilities of DNA topological isomers (topoisomers) with different linking numbers by two-dimensional agarose gel electrophoresis. This method allows detection of supercoiling-induced structural changes of the DNA double helix. For example, the B to Z transition and cruciform formation were previously analyzed by such a method (Wang et al., 1983). The negative supercoiling-induced DNA structural changes were revealed by a discontinuity in the curve that traces through the topoisomer spots. This discontinuance arises because the presence of an unwound structure within a given topoisomer reduces its negative superhelicity and thus reduces its mobility in the first dimension of electrophoresis. Once saturated with chloroquine phosphate, the topoisomer then becomes positively supercoiled and the negative supercoil-induced structure disappears. Therefore, the mobilities in the second dimension depend only on the linking number of the plasmid.

A complete mixture of topoisomers with varying linking numbers for a plasmid Cμ1kbTKCAT (4.7 kbp) containing the 992 bp *Xba*I fragment was electrophoresed in a 1.2% agarose gel in the Tris–borate buffer (80 mM Tris–borate and 10 mM EDTA, pH 8.2) in the absence of chloroquine phosphate at 22 °C. Electrophoresis in the second dimension was carried out after soaking the gel in the same buffer containing 50 μg/mL of chloroquine phosphate (Figure 4). This amount of chloroquine phosphate was employed to assure that all topoisomers prepared would revert to a positively supercoiled structure. The topoisomer mixture prepared for pBR322 (as a control without the *Xba*I fragment) revealed a continuous line that traces through the topoisomer spots under this electrophoresis condition (Figure 4, inset). This is consistent with observations made by others (Kowalski et al., 1988). However, in the case of the plasmid containing the 992 bp *Xba*I fragment, a sharp discontinuance in the topoisomer spots representing a structural transition was detected (Figure 4). This structural transition is due to the sequence 3′ of the enhancer changing its structure from the B-form DNA to the stably unpaired conformation; under conditions identical with those employed for the two-dimensional electrophoresis (80 mM Tris–borate and 10 mM EDTA, pH 8.2, 22 °C), CAA reactivity was detected only within the 3′ region of the enhancer and the CAA reaction pattern of this region on a sequencing gel showed continuous base unpairing (data not shown). The topoisomer number at the structural transition is approximately −23, corresponding to a superhelical density, $\rho = -0.05$, as determined by similar analysis using lower concentrations of chloroquine in order to resolve the topoisomers within that region (data not shown).

The 13 topoisomers shown by a bracket in Figure 4 contain stable unwound DNA structure as they all migrated slower

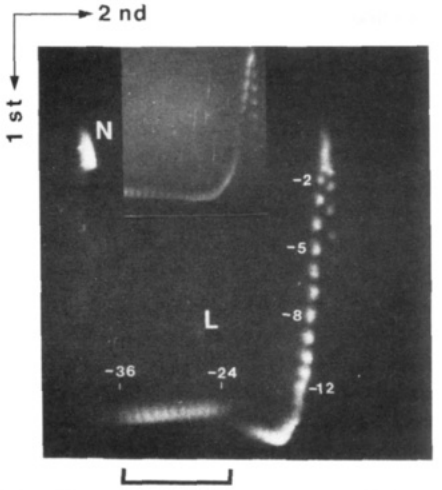


FIGURE 4: Two-dimensional gel electrophoresis of DNA containing the IgH *Xba*I 992 bp fragment. A mixture of topoisomers of different mean superhelix density for pTKCATCμ1kb and pBR322 (inset) was prepared using calf thymus topoisomerase I (Bethesda Research Laboratories) in the presence of various concentrations of ethidium bromide (Kochel & Sinden, 1988) and was electrophoresed from a single well in a 1.2% agarose gel in 80 mM Tris–borate and 10 mM M EDTA, pH 8.2 at 22 °C. The gel was then soaked in the same buffer containing 50 μg/mL chloroquine and rerun in this buffer in a direction 90° to the original dimension at 22 °C. The gel was stained in ethidium bromide and photographed under ultraviolet illumination. Number of topoisomers and the positions of nicked DNA (N) and linear DNA (L) are indicated. The bracket indicates topoisomers that contain stable unwound DNA structure.

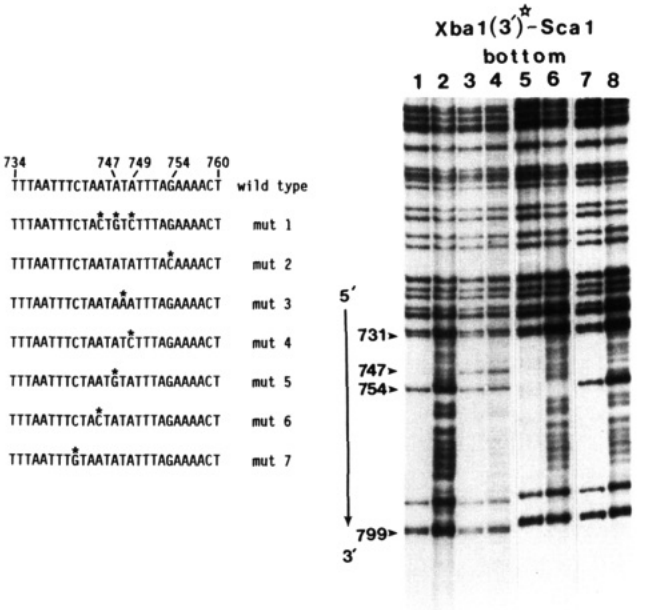


FIGURE 5: Effects of site-directed mutagenesis on the CAA reactivity in the IgH enhancer region. (Left panel) List of mutated DNAs employed in this experiment. These mutations were introduced to the pSERCμ1kb plasmid with the use of synthetic oligonucleotides shown here using the kit from Amersham. (*) indicates the nucleotide mutated. (Right panel) Supercoiled plasmid DNA, wild type (pSERCμ1kb) (lanes 1 and 2), mut 1 (lanes 3 and 4), mut 2 (lanes 5 and 6), and mut 4 (lanes 7 and 8) were either reacted (even-numbered lanes) or not reacted (odd-numbered lanes) with CAA in 25 mM sodium acetate buffer at pH 5. The DNA was then digested with *Xba*I and 5′-end-labeled with T4 kinase and [γ-^{32}P]ATP. The DNA was further digested with *Pvu*II and the *Xba*I(3′)–*Pvu*II fragment was isolated. The labeled DNA was then subjected to hydrazine (HZ)/piperidine reaction and electrophoresed on a 6% urea-denaturing polyacrylamide gel.

in the first dimension than the topoisomer at the structure transition. Mobility of these topoisomers was about the same in the first dimension with a very slight increase in mobility as the superhelical density increases. This indicates that beyond a superhelical density, $\rho = -0.05$, every additional increase in the absolute linking number difference results in approximately 10 more base pairs becoming unpaired, assuming the helical periodicity to be 10.4 base pairs per turn (Wang, 1979). Such progressive unpairing occurs at 22 °C and at a relatively high salt concentration. Even at 80 mM Tris–borate employed for the electrophoresis, a total of 200 bp was estimated to be unwound for the topoisomer with the highest absolute linking number (estimated to be −36) shown in the left end of the bracket (Figure 4). The topoisomer mobility in two-dimensional gels is very sensitive to the changes in salt concentrations employed for electrophoresis. With 50 mM Tris–borate buffer, for example, we observed a much larger degree of unwinding associated with each additional linking number difference after the structural transition (data not shown). The results shown here indicate that the CAA reactivity in the IgH enhancer surrounding sequences is due to a stable base-unpaired conformation adopted by the sequences rather than a high rate of DNA breathing of the region.

Site-Directed Mutagenesis of the Base-Unpairing Sequences. To examine the sequence responsible for the extensive unpairing, we focused on those sequences that persistently remained unpaired even under conditions that stabilize double-stranded DNA structure. Specifically, we considered that a potential core element for such an unwinding activity resides within the 58 base pairs (795–737) that were unpaired even under a high salt concentration. Within this region there exists two specific adenine residues, at 747 and 749 on the top strand, and three adenine residues, at 746, 748, and 750 on the bottom strand, that are the only bases within the entire 992 bp *Xba*I fragment that remain unpaired in the presence of Mg^{2+}. Thus we altered the sequence 5′-AATATATTT-3′ (744–752) that contains the CAA-reactive adenine residues by the method of site-directed mutagenesis. The mutated plasmid (mut 1), with a 5′-ACTGTCTTT-3′ (744–752) sequence, was constructed which has three point mutations (Figure 5, left panel).

The 3′ region of the enhancer for mut 1 was found virtually unreactive with CAA; chemical cleavage of the isolated *Xba*I(3′)–*Sca*I fragment from CAA-treated mut 1 revealed marked reduction of the intensity of new bands reflecting the CAA-reactive sites (see Figure 5, lane 4, for mut 1 DNA in comparison to lane 2 for the wild-type DNA). This data demonstrated that a 6 bp sequence 5′-ATATAT-3′ (745–750), when mutated, results in a near-complete abolishment of the extensive DNA-unpairing property, which includes more than 200 base pairs in the region 3′ at 25 mM Na^+ concentration.

We further examined whether a single-point mutation has any effect on the structure-forming potential of the 3′ flanking sequences. Various single point-mutations were constructed as shown in Figure 5, left panel, among which mut 4, mut 5, and mut 6 have one of the three-point mutations made for mut 1. When similar experiments on the CAA-treated mut 2–7 were performed, the CAA reaction patterns were essentially unchanged from those of the wild-type sequences. (The results for selected mutants are shown in Figure 5, right panel.) Therefore, none of these single-point mutations had an effect on the uniformly unpairing property of the DNA sequences in the 3′ flanking region.

In the presence of 2 mM Mg^{2+}, a cruciform structure may be formed which consists of a 7-base-pair stem with the loop region being CAA-reactive (5′-*TTTCTAA*TATAT*TTAGAAA*-3′). In this case, one would expect that the single-point

mutations within the 7-base-pair stem (mut 2 and 7) should inhibit the Mg^{2+} induced CAA reactivity while mutations within the loop region (mut 3–5) have no effect. Our unpublished results showed that all single-point mutations (mut 2–7) as well as the three-point mutation (mut 1) inhibited the formation of the Mg^{2+}-induced CAA-reactive structure. Although we cannot rule out that the wild-type sequence adopts a cruciform in the presence of Mg^{2+}, the existence of the 7-base-pair inverted repeat is clearly not sufficient to form the Mg^{2+}-induced CAA-reactive structure.

Discussion

We show here, with the use of an unpaired base-specific chemical probe and two-dimensional electrophoresis techniques, that the murine IgH enhancer region is surrounded by two distinct sets of DNA sequences that, when under torsional stress, become stably and uniformly unpaired. These CAA-reactive sequences occur within the matrix attachment regions and comap with the negative regulatory elements flanking the IgH enhancer.

Delimitation of Important Sequences. The sequences found either 5′ or 3′ of the enhancer are noticeably A+T-rich in nature. For example, the sequence 800–685 has 77% A+T content, and the sequence 272–329 has a 84% A+T content. It is important to emphasize, however, that sequences which are high in A+T content do not necessarily possess extensive CAA reactivity. A long stretch of DNA sequence with an 85% A+T content found within the spacer region of a *Xenopus* 5S RNA gene (Fedoroff & Brown, 1978) does not adopt a CAA-reactive structure (unpublished result). Therefore, the DNA sequence is crucial and a local, high A+T content is clearly not sufficient to cause supercoiled DNA to adopt a uniformly unpaired structure. For the purpose of delineating a possible core element for base unpairing, we have focused on the sequence ATATAT (746–750) in the CAA region 3′, which is most persistently CAA-reactive even under the conditions that disfavor melting of DNA. We found that mutation of the three adenine residues simultaneously in this motif to either cytosine or guanine bases, in fact, is sufficient to result in marked reduction in the CAA reactivity in the region 3′ of the enhancer. This dramatic change in unpairing property was observed with very little change in overall A+T content of the sequence 3′ of the enhancer. This indicates that this short sequence is important to confer base unpairing in this region. However, the mere presence of this sequence is not sufficient to cause this conformation. An identical A*ATA-TA*TTT motif, which occurs in the IgH enhancer region from position 744 to 752, is also found within the herpes simplex virus thymidine kinase (TK) promoter region. This motif, however, does not invoke base unpairing in the TK promoter region in supercoiled plasmid DNA (unpublished result). Thus, although an alternating dA·dT sequence motif is known to melt easily (Gotoh & Tagashira, 1981), this ATATAT sequence by itself cannot confer the extended base unpairing. Furthermore, single mutations made in the CAA region 3′ that destroyed the ATATAT motif had no effect on the long-range base unpairing in this region. Yet it is intriguing that three-point mutations within this sequence led to a near complete loss of base unpairing property of the region 3′ of the enhancer. The ATATAT motif in the CAA region 3′ may be "kinked" as the result of the neighboring-sequence cooperativity and may serve as the nucleation site for base unpairing. Multiple mutation in this motif may result in the loss of the possible "kinked" property.

Directional Preference of Base Unpairing and Effect of Neighboring DNA Sequence. The propensity of the sequence (*Eco*R1 685–795) inducing base unpairing toward its 5′ direction is conserved regardless of the nature of its neighboring sequence. The neighboring sequences, however, have an effect on the distance to which the unpairing effect is transmitted. The native J_H–Cμ intron flanking sequence becomes unpaired over a much more extended region upstream from the *Eco*R1 site (by approximately 100 bp under a low salt condition) than other vector sequences which became unpaired only by 20 bp immediately upstream of the *Eco*R1 site. The directional preference in the base unpairing is not readily explainable by the A+T content of the neighboring sequences. For example, the A+T contents of the 100 bp sequence 5′ of the *Eco*R1 site and that of the 3′ of the position 800 are both approximately 70%. Yet the DNA is preferred to be unpaired toward the 5′ direction than 3′. Specific arrangement of DNA nucleotide within the IgH enhancer surrounding region may be the determining factor for this directionality.

The CAA region 5′, which possesses a lower potential to be base-unpaired than the CAA region 3′, does not influence the structure of its neighboring regions. In contrast to the CAA region 3′, the CAA reactivity is all or none; the same defined region is base-unpaired only under relatively low ionic condition. Thus there are at least two different kinds of behavior in A+T-rich sequences. These two types of behavior may be due to the difference in base-unpairing potential of the sequences.

Other A+T-Rich Sequences That May Be Similar to the IgH Enhancer Flanking Sequences. The A+T-rich sequences in pBR322 DNA (Sheflin & Kowalski, 1985; Kowalski et al., 1988), yeast (Umek & Kowalski, 1987, 1988), and *Escherichia coli* replication origin sequences (Kowalski & Eddy, 1989) exhibit hypersensitivity to mung bean nuclease cleavages over a considerable length, and the two-dimensional gel electrophoresis study showed that they are stably unwound. Parallel CAA modifications of the yeast replicating sequence, near the copy 1 histone H4 gene (H4 ARS) (kindly provided from D. Kowalski) and the 3′ region of the IgH enhancer, showed that both sequences react with CAA in a similar manner. However, there was a marked difference in the CAA reactivity between the two DNA sequences: at 22 °C, only the sequence 3′ of IgH enhancer was CAA-reactive. At 37 °C, although the IgH sequence was very reactive with CAA, H4ARS sequence revealed very low CAA reactivity (unpublished data). Thus, the IgH enhancer surrounding sequence may be considerably more prone to unwind that yeast replication origin sequences. The ColE1 inverted repeat flanking sequences and alternating d(A-T) sequences seems to also possess some common property with the IgH enhancer surrounding sequences. These sequences extrude cruciform structure via a C-type pathway which is believed to proceed via a transition state represented by a large melted "bubble" in the DNA which may subsequently lead to a fully extruded cruciform in a single step (Sullivan & Lilley, 1986; McClellan & Lilley, 1987). These sequences that confer C-type cruciform extrustion are highly reactive toward chemical reagents that are normally reactive toward single-stranded DNA (Furlong et al., 1989).

Possible Biological Relevance of DNA with an Extensive Base-Unpairing Property. (a) Negative Regulatory Elements. The biological significance of the CAA-reactive sequences encompassing the IgH enhancer are suggested by the fact that the CAA-reactive domains coincide with the negative regulatory elements which represses IgH enhancer activity in fibroblast but not in B cells (Figure 6). There are four regions within the IgH enhancer surrounding sequence that are protected from DNase I digestion by NF-μNR binding

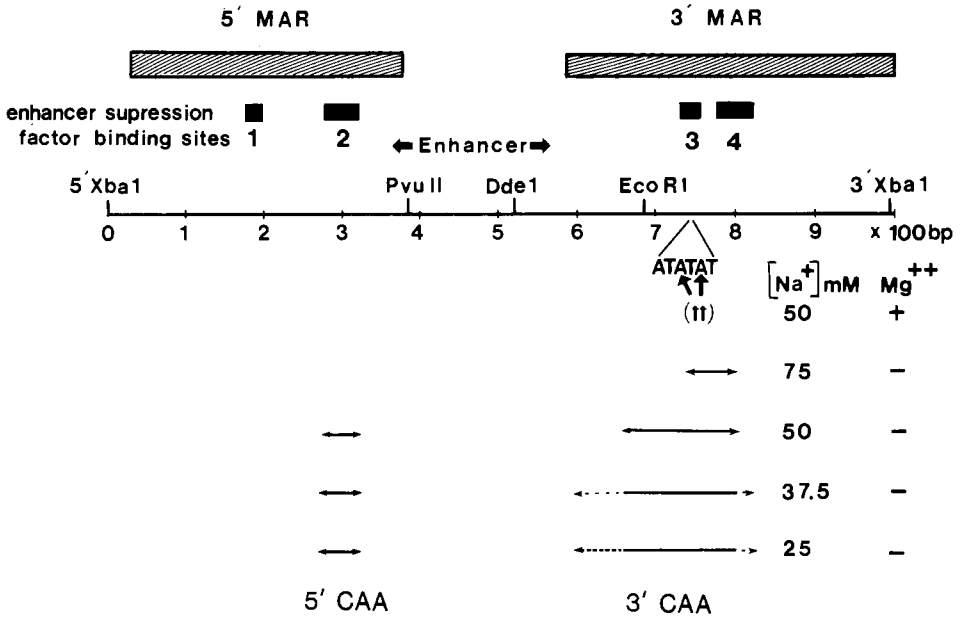


FIGURE 6: Relative location of CAA-reactive elements and sequences with known activities in the IgH enhancer region. The IgH enhancer region indicated by thick arrows represents the minimum sequence that confers the same enhancer activity as the 992 bp *Xba*I fragment (Wasylyk & Wasylyk, 1986; Gerster et al., 1987). The four regions that bind to the enhancer suppression factor NF-μNR are indicated by solid bars. Matrix association regions (MARs) that are located 5′ and 3′ of the enhancer are shown by hatched bars. The regions indicated by double-headed arrows represent CAA-reactive regions under the ionic conditions specified. The solid line represents the regions of strong CAA reactivity. The broken line represents relatively weak CAA reactivity which was observed under lower salt concentrations.

(Scheuermann & Chen, 1989), among which the locations of the three protection sites are in excellent agreement with that of the CAA-reactive elements. The CAA region 5′ (272–329) at 25–50 mM Na^+ corresponds to binding site 2 (285–333), and the CAA region 3′ (737–795) at 75 mM Na^+ corresponds with binding sites 3 (732–753) and 4 (787–826). The CAA region 3′ at lower salt concentrations include both binding sites 3 and 4. Among these two, binding site 3 (732–753) overlaps with the AATATATTT element (744–752). Deletion of either binding site 2 or 3 relieves the IgH enhancer suppression in a T-cell line (Scheuermann & Chen, 1989). Thus, the region determined to be CAA-reactive in the IgH enhancer surrounding sequences plays an important biological role in the regulation of the tissue-specific immunoglobulin heavy chain gene expression.

(*b*) *Nuclear Matrix Attachment.* Further evidence suggesting the biological relevance of the CAA-reactive sequences is the cohabitation of these sequences with the matrix attachment regions (Figure 6). The MARs located 5′ of the enhancer are mapped between the *Alu*I (34) and *Alu*I (383) region, while those located 3′ of the enhancer are the the *Ara*II (586)–*Xba*I (992) region, both of which contain negative regulatory elements and CAA-reactive regions.

There is a good correlation between sequences that adopt an extensive CAA-reactive structure under torsional stress and the MARs. Other sequences known to bind to the nuclear matrix, including the human β interferon gene flanking region (Bode & Maass, 1988), show continuous CAA reactivity as well (Kohwi-Shigematsu, Kohwi, and Bode, manuscript in preparation). Furthermore, when adenine residues of an AATATATTT motif which was also found within the MAR region of the human β interferon gene flanking region were mutated, the matrix attachment activity was decreased (Mielke et al., 1990) and the long-range CAA-reactive structure was abolished (unpublished result). It is highly possible that, in general, MARs exhibit this extensive base-unpairing property when studied in supercoiled plasmid DNA form.

Amati and Gasser (1988) reported that the yeast autonomous replication sequence, ARS, and a chromosomal centromere (CEN III) bind specifically with yeast nuclear scaffold. ARS consensus and sequences found in *Drosophila* SARs show significant sequence homology (Gasser & Laemmli, 1986). It is of interest to note that not only both ARS and SAR or MAR elements are A+T-rich, but also CEN elements are A+T-rich as well, containing multiple variations of the AATATATTT motif.

(*c*) *Autonomous Replication Origin.* The 3′ flanking sequence of the IgH enhancer and the ARSs are closely related in terms of their unwinding property. Such a structural property of particular A+T-rich sequences have been shown to be important for yeast and *E. coli* replication (Umek & Kowalski, 1988; Kowalski & Eddy, 1989). Another study suggested that ARS function depends on the presence of an exact match to the core ARS consensus and additional near matches in the 3′ flanking region (Palzkill & Newlon, 1988). The sequence TAATATATTTA, from position 743 to 753 of the IgH enhancer 3′ flanking region, has 9/11 matches to the ARS core consensus, 5′-(A/T)TTTAT(A/G)TTT(A/T)-3′. It is probable that a similar uniformly unpaired DNA structure exists in the ARS flanking sequence and that there is a directional preference for its base-unpairing property to effectively open up the ARS.

There appears to be a correlation between a particular type of A+T-rich sequences and certain biological functions. The extended base-unpairing property of these A+T-rich sequences may be important for their biological functions. For example, such a property may play a role in stabilizing binding of the sequences to the nuclear matrix as well as to suppressor factors, and the competition of binding might be one mechanism for negative regulation. It is also intriguing to speculate if such a structural property is effectively used in cells to modulate the topology of a chromatin loop domain. With the use of mutated DNA, which does not have the unpairing property, we will address the structure/function relationship for these A+T-rich sequences in the future.

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