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High Frequency of Matrix Attachment Regions and Cut-Like Protein x/CCAAT-Displacement Protein and B Cell Regulator of IgH Transcription Binding Sites Flanking Ig V Region Genes

Peter Goebel,* Alina Montalbano,* Neil Ayers, † Elizabeth Kompfner,* Liliane Dickinson,* Carol F. Webb, † and Ann J. Feeney 2 *

A major component in controlling V(D)J recombination is differential accessibility through localized changes in chromatin structure. Attachment of DNA to the nuclear matrix via matrix attachment region (MAR) sequences, and interaction with MAR-binding proteins have been shown to alter chromatin conformation, promote histone acetylation, and influence gene transcription. In this study, the flanking regions of several human and mouse Ig V genes were analyzed extensively for the presence of MARs by in vitro matrix-binding assay, and for interaction with the MAR-binding proteins cut-like protein x/CCAAT-displacement protein (Cux/CDP), B cell regulator of IgH transcription (Bright), and special AT-rich sequence-binding protein (SATB1) by EMSA. Cux/CDP and SATB1 are associated with repression, while Bright is an activator of Ig transcription. Binding sites were identified in the vicinity of all analyzed Ig V genes, and were also found flanking TCR Vβ genes. We also show that the binding sites of the different factors do not always occur at MAR sequences. MAR sequences were also found within the Ig V loci at a much higher frequency than throughout the rest of the genome. Overall, the frequency and location of binding sites relative to the coding regions, and the strength of DNA-protein interaction showed much heterogeneity. Thus, variations in factor binding and MAR activity could potentially influence the extent of localized accessibility to V(D)J recombination and thus could play a role in unequal rearrangement of individual V genes. These sites could also contribute to effective transcription of Ig genes in mature and/or activated B cells, bringing both the promoter as well as the enhancer regions into close proximity at the nuclear matrix. The Journal of Immunology, 2002, 169: 2477–2487.

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3 Abbreviations used in this paper: RSS, recombination signal sequence; CDP, CCAAT-displacement protein; Cux, cut-like protein x; Bright, B cell regulator of IgH transcription; Eμ, Ig H chain intronic enhancer; iEμ, Ig e-chain intronic enhancer; MAR, matrix attachment region; NF-μNR, NF-μ negative regulator; SATB1, special AT-rich sequence-binding protein; TE, tris-EDTA.

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MARs, Cux/CDP, AND BRIGHT BINDING SITES FLANK Ig V GENES

and distribution have been much less well characterized. For example, the V \(_{\gamma}S107/V1\) gene was shown to contain a matrix attachment region (MAR) within 500 bp 5' of the transcription start site, and two MAR-binding proteins, NF-\(\mu\) negative regulator (NF-\(\mu\)NR) and B cell regulator of IgH transcription (Bright), also interact with this region (24, 25). MARs are AT-rich regions located throughout the genome and have been shown to be involved in organizing chromatin into topological loops by anchoring DNA to the nonhistone proteins of the nuclear matrix (26, 27). Additional studies of MARs have shown that they are also associated with more complex functions (reviewed in Refs. 28 and 29). For example, actively transcribed genes have been shown to be associated with the nuclear matrix, and MAR sequences have recognition sites for topoisomerase II (27), which could result in the introduction of torsional stress making nearby genes more accessible. The two MARs flanking the H chain intronic enhancer \(E_{\mu}\) have been shown to extend the local accessibility induced by the core enhancer alone over a larger region (30, 31), while deletion of the Ig \(\kappa\)-chain intronic enhancer iEx-associated MAR resulted in hyperrecombination of the \(J_k\) genes closest to the deleted MAR (32). Furthermore, mice lacking the iEx-MAR showed premature onset of V\(\kappa\)-J\(\kappa\) recombination and a decreased level of somatic hypermutation (33). These observations suggest that MAR sequences may contribute to changes in chromatin structure and accessibility and therefore may be relevant for studies of control of V(D)J recombination and Ig gene transcription.

Our preliminary observations that other MARs may be present in the Ig \(V_H\) locus (34) and our sequencing of the upstream promoter region of another \(V_H\) gene revealing a previously unknown AT-rich stretch led us to investigate whether extended areas surrounding Ig V gene coding regions would contain MAR sequences as a general feature, because this could certainly influence Ig functions. We also wanted to determine whether these flanking regions could be recognized by some of the MAR-binding proteins such as cut-like protein x (Cux)/CCAAT-displacement protein (CDP), Bright, and special AT-rich sequence-binding protein (SATB1), which differ in their expression pattern. Most of our investigation focused on the ubiquitously expressed Cux/CDP. The CCAAT-displacement protein Cux/CDP has been shown to be a component of NF-\(\mu\)NR (35), one of the proteins binding to the MAR in the V1 promoter and the \(E_{\mu}\) enhancer region. Cux/CDP is a homeodomain protein that recognizes MARs and also regulates genes at many different stages of development and cell differentiation (36–39). Cux/CDP can cause gene repression by competing with transcriptional activators for binding sites (40, 41), or through its recruitment of the histone deacetylase 1 (42). Furthermore, Cux/CDP binds to MAR DNA, but not to the nuclear matrix, thus preventing matrix association of the MAR DNA (37, 38). Because individual IgV genes contribute unequally to the Ab repertoire, we wanted to determine whether the occurrence and distribution of Cux/CDP binding sites and/or MARs in the vicinity of individual V genes would be related to the frequency with which a particular gene undergoes V(D)J recombination. It is possible that repression by Cux/CDP, possibly by histone deacetylase recruitment or prevention of matrix binding, could be one mechanism to restrict V gene accessibility.

We have extensively analyzed several kilobases of 5' and 3' flanking region from 13 mouse and human IgH and Igk V region genes. Of these genes, 70% showed Cux/CDP binding sites within 1 kb of the coding region on either the 5' or the 3' side, while 50% had Cux/CDP recognition sites on both sides. We have also analyzed flanking sequences from three frequently recombining TCR V\(\beta\) genes and here too we identified Cux/CDP binding regions. Several fragments were also tested for Bright binding and matrix attachment by in vitro MAR assay. Previous studies had shown that the binding sites for SATB1, Cux/CDP (NF-\(\mu\)NR), and/or Bright partially overlap within the MARs flanking \(E_{\mu}\), CD8, and TCR\(\beta\) intronic enhancer E\(\beta\) (35, 37, 38). However, the results from our analysis of Ig V gene flanking regions presented in this study differ from these previous observations. Specifically, our binding studies revealed that on several occasions the protein recognition sites and MAR regions did not colocalize, but rather were identified in different fragments.

Overall, we detected much heterogeneity in the frequency and location of binding sites and the capability to bind to the nuclear matrix within the flanking sequences of these genes, suggesting that variations in Cux/CDP and Bright binding and MAR activity could possibly contribute to localized control of accessibility and therefore nonrandom gene use during V(D)J recombination. In addition, the presence of MARs and Bright binding sites in the promoter-associated regions of most Ig V genes is also likely to affect transcription of Ig genes in mature B cells.

**Materials and Methods**

**Preparation of nuclear extracts and nuclear matrix**

EL4 cells were grown in RPMI medium supplemented with 10% FCS, 200 \(\mu\)M glutamine, 2-ME, and gentamicin sulfate. The B cell line, BCg3R-1d, was grown as previously described (25). Preparations of nuclear extracts were performed as previously described. For larger numbers of cells, the protocol by Dignam et al. (43) was used, while for smaller cell numbers nuclear extracts were prepared following Schreiber et al. (44). EL4 extracts were used for all EMSA experiments, because they are a rich source of Cux/CDP. Nuclear matrix preparations from EL4 and BCg3R-1d cells were isolated following the procedure by Cockerill and Garrard (27). Matrix preparations from EL4 and BCg3R-1d cells were used interchangeably throughout the in vitro matrix-binding assay with similar results. The final nuclear matrix preparations were resuspended in buffer at a concentration of 2 \(\times\) 10\(^{12}\) cell equivalents/\(\mu\)l.

**Probes**

All DNA fragments for which the genomic DNA sequence was available were isolated by PCR using primers designed based on the published sequence. The sizes of all PCR fragments were in the range of 300–600 bp. The \(V_{\gamma}S107/V1\) upstream fragments were isolated by restriction digestion through subcloning of genomic fragments from a bacterial artificial chromosome (BAC) clone spanning the \(V1/V3\) region (generously provided by R. Riblet, Torrey Pines Institute for Molecular Studies, La Jolla, CA). All PCR fragments were end labeled using \(\gamma\)-\(\alpha\)-\(P\)ATP and polynucleotide kinase (New England Biolabs, Beverly, MA), according to the manufacturer’s instructions. Restriction fragments were end labeled by either using polynucleotide kinase in a nucleotide-exchange buffer (Life Technologies, Rockville, MD) or by fill-in reaction of the overhanging ends using \(\gamma\)-\(\alpha\)-\(P\)ATP and Klenow (New England Biolabs). As the negative control in the matrix-binding assay, pBluescript was linearized by digesting with an appropriate volume of water. Probes were used at 5,000 cpm/\(\mu\)l for EMSA experiments and at 10,000 cpm/\(\mu\)l for matrix-binding assays.

**EMSA**

A total of 3 \(\mu\)l nuclear extract was preincubated with 1–2 \(\mu\)g poly(dIdC) in buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT) for 5 min at 37°C. For supershift experiments, 1 \(\mu\)l 1/50 diluted anti-CDP guinea pig serum (Life Technologies, Rockville, MD) or, as negative control, 1 \(\mu\)l 1/10 diluted preimmune guinea pig serum was added to the preincubation. A total of 5000 cpm probe was then added to each reaction tube and
the incubation continued for 10 min at 37°C, followed by 15 min at room temperature. Samples were loaded onto 4% polyacrylamide gels, containing 5% glycerol in 1× Tris-acetate-EDTA, and were run at ~120 V at 4°C. Gels were dried and exposed to film overnight at ~70°C. In vitro translated Bright was prepared using a full-length Bright cDNA clone and TNT rabbit reticulocyte lysates (Promega, Madison, WI), and 1 μl reaction mixture was used for binding.

SATB1 binding was assessed by incubating a constant amount of labeled probe with increasing amounts of rSATB1 protein. Briefly, rSATB1 in the range from 0.15 to 20 nM final concentration was preincubated with 1 μg poly(dIC/dC) in buffer C (10 mM HEPES, pH 7.9, 50 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, 10% glycerol) supplemented with 2.5 mg/ml BSA in 20 μl reaction volume. After 5 min of preincubation at room temperature, 5,000–10,000 cpm probe was added and incubated for an additional 15 min at room temperature. Samples were loaded on 4 or 6% polyacrylamide gels with 5% glycerol in 1× TBE (90 mM Tris-borate, 1 mM EDTA) and run at ~120 V at 4°C. Gels were dried and either exposed to film overnight at ~70°C or exposed to a phosphor imager overnight at room temperature.

Matrix-binding assay

For each binding reaction, nuclear matrix equivalent to 2 × 10⁶ cells equaling 10 μl nuclear matrix preparation was used. Matrix preparations from EL4 and BCG3R-1d cells were used interchangeably throughout the in vitro matrix-binding assay with similar results. The nuclear matrix was washed three times in matrix wash buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM MgCl₂, 0.25 M sucrose, 0.25 mg/ml BSA) with spinning between washes at 4000 rpm for 4 min at 4°C. The matrix was resuspended in 25 μl assay buffer (10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.25 M sucrose, 0.25 mg/ml BSA), and sonicated Escherichia coli DNA was added to a final concentration of 100 or 200 μg/ml reaction volume. Matrix was preincubated with the E. coli DNA for 30 min at room temperature on an orbital shaker before adding 20,000 cpm labeled probe and 20,000 cpm labeled pBluescript, followed by incubation 90 min at room temperature with shaking. Thereafter, 500 μl assay buffer was added to the reaction and mixed by inverting. The matrix was pelleted by spinning at 12,000 rpm for 1 min at 4°C, and the supernatant was saved for determination of the unbound fraction. The matrix was washed once more, the supernatant was discarded, and the matrix pellet was resuspended in 25 μl TE (10 mM Tris, 1 mM EDTA, pH 8.0), 0.5% SDS, and 400 μg/ml proteinase K, and incubated at 37°C overnight. After adding loading dye, the matrix-bound DNA was separated on agarose gels. The unbound DNA from the first wash was precipitated overnight and resuspended in 100 μl TE, 0.5% SDS, of which 25 μl equivalent to one-fourth of the total amount, was loaded. To compare bound and unbound fractions with the amount of input DNA, 20,000 cpm of the same probe and 20,000 cpm pBluescript were diluted in 100 μl TE, 0.5% SDS, of which one-fourth was loaded. Samples were separated by electrophoresis on 0.8% vertical agarose gels, and gels were dried and exposed to film overnight at ~70°C. The strong MAR 5′ of the V1 gene was used as a positive control.

Results

CDP binding sites surround the V₁S107/V₁ gene

A region from ~574 to ~125 bp upstream of the V₁S107/V₁ transcription start site had been shown to harbor one nuclear MAR and to contain two binding sites of the MAR-binding proteins Bright and Cux/CDP/NF-κB (24, 25). However, it had not been determined how far 5′ the MAR extended, or whether the repressor protein Cux/CDP could bind to this extended promoter flanking region. To obtain additional sequence information, a genomic clone was isolated from a λ-phage library, and the flanking region was sequenced up to the EcoRI site ~4.4 kb upstream of the transcription start site. The sequence information was used to design PCR primers, and the amplified PCR products were analyzed for Cux/CDP binding by EMSA. Fig. 1 shows a representative Cux/CDP-EMSA experiment for some of these fragments. Cux/CDP-binding sites were found in intervals of several kilobases upstream of the transcription start site. The sequence information was used to design Cux/CDP-binding fragments as shown on the map, indicating that the V1 coding region is flanked by Cux/CDP binding sites on both sides over an extended region.

CDP binding is not limited to MARs

Cux/CDP binds to many MARs (35, 37, 38). To determine whether all Cux/CDP binding sites found in the V1 flanking sequences were also located in MARs, we assayed these same regions for MAR activity using an in vitro MAR assay. A representative experiment is shown in Fig. 3. The location of MAR-positive fragments in the V₁S107/V₁ gene is illustrated in Fig. 2. In the region from the promoter up to the EcoRI site, we found a good correlation between Cux/CDP binding and matrix attachment; however, in the area from the EcoRI to the 5′ BamHI site, Cux/CDP binding and matrix attachment did not correlate. Specifically, two Cux/CDP-binding fragments were negative in the MAR assay, while...
two other matrix-binding fragments did not bind Cux/CDP, indicating that matrix attachment and Cux/CDP binding do not always occur within the same region.

Several Bright binding sites are located surrounding V_{H}S107/V1

Because Cux/CDP and Bright binding sites overlap significantly in the intronic enhancer and promoter-proximal V1 MARs (24, 25, 35), and because we identified multiple Cux/CDP binding sites over a larger region surrounding the V_{H}S107/V1 gene, we asked whether additional Bright binding sites might also exist distant from the core promoter. Thus, several of the fragments identified as MARs were tested for Bright binding. In addition to the previously described promoter-proximal Bright binding sites, we identified one Bright binding site 2.5 kb upstream of the transcription start site and another one 300 bp downstream of the RSS. We also found a Bright binding site 5.8 kb upstream of the V1 coding region that did not bind Cux/CDP or have MAR activity. Furthermore, the 600-bp fragment immediately 5’ to the EcoRI site did not bind Bright, although it is a MAR and bound Cux/CDP by EMSA. A few additional fragments that were tested for Bright binding during our investigation based on their Cux/CDP reactivity were also found to be negative and are shown in Fig. 2. Thus, Bright binding sites were observed both 5’ and 3’ of V1, but Bright-binding activity was not always correlated with Cux/CDP binding or with MAR activity.
The A/T profile alone does not allow prediction of binding sites

One of the prominent characteristics of MARs is the increased occurrence of (A + T) nucleotides clustered in longer AT-rich stretches. DNA binding by Cux/CDP occurs through three cut repeats, CR1, CR2, and CR3. CR1 binds to DNA with a consensus core sequence of AATAAT, while CR2- and CR3-binding motifs contain a shorter ATA core flanked by one or two additional A/T nucleotides (45). To determine whether there might be a correlation between the occurrence of Cux/CDP binding sites, matrix attachment, and the percentage of A/T nucleotides, we analyzed our entire V1 sequence for its A/T profile. We arbitrarily chose to test for the presence of five consecutive A/T nucleotides in the sequence by using the “window” program from the GCG package (Wisconsin Package Version 10.2; Genetics Computer Group, Madison, WI), using the search parameter “WWWWW,” with W representing either an A or a T nucleotide. Although a high W5 profile does not allow accurate prediction of Cux/CDP or Bright binding or matrix attachment, long stretches with a low W5 profile correlated with fragments that were completely negative for MARs and MAR-binding proteins (Fig. 2 and data not shown).

One of the challenges, however, of predicting MAR sequences or recognition sequences for proteins such as Cux/CDP or SATB1 is that there are no well-defined recognition sites, contrary to what is often found for enzymes or transcription factors. There are a limited number of computer programs that claim to predict potential MAR sequences. To investigate whether these programs and their approach to identify MARs would be more successful than our approach with the W5 profile, we analyzed our 5-kb V1 sequence using three of the MAR prediction computer programs: MARFinder (http://www.futuresoarf.org/MAR-Wiz), SMARTTest (http://genomatix.gsf.de/cgi-bin/smartest_pd/smartest.pl), and ChrClass (ftp://ftp.bionet.nsc.ru/pub/biology/chrclass) (47). However, none of the three programs was capable of predicting our experimentally identified MARs by their algorithms.

LINE elements do not correlate with protein binding or matrix attachment

Previous studies of the mouse Ig H locus had revealed that the C region carries many copies of repetitive sequences and that these may represent LINE elements (48). An extensive analysis of the entire murine Ig C region identified the presence of three distinct MAR clusters (49). The same study also showed, based on hybridization patterns, a very strong correlation between the presence of MAR sequences and the occurrence of repetitive elements, most likely LINE elements, within the same genomic fragments. Based on these findings, we analyzed our sequence of V\textsubscript{H} S107/V1 for the presence of repetitive sequences using the computer program RepeatMasker (http://repeatmasker.genome.washington.edu/) (50). The positions of identified repetitive elements within the entire sequence are indicated by black lines directly underneath the W\textsubscript{5} profile (Fig. 2). The position of a “simple repeat” is further marked with an asterisk below the line. In contrast to the findings for repetitive elements in the Ig C locus, the identified LINE elements and simple repeats in the V\textsubscript{H} S107/V1 flanking region did not correlate with MAR sequences or Cux/CDP, Bright, and SATB1 binding sites.

Murine V\textsubscript{H} genes show differences in their Cux/CDP binding

The murine V\textsubscript{H} gene V\textsubscript{H} 81x is unique in being the most 3′ functional mouse V\textsubscript{H} gene, and it is the most frequently rearranged gene (51, 52). We sequenced the flanking regions of V\textsubscript{H} S107x to obtain more sequence information than was currently available. The entire region, ~4 kb in total, was first analyzed by calculating the W\textsubscript{5} profile (Fig. 4). The A/T-rich region correlating with the spike in the W\textsubscript{5} profile within the intron between the 81x leader sequence and the coding region was already known from the published sequence, and this sequence contains a stretch of ~40 bp that is mostly composed of T nucleotides. Such homopolymeric stretches are very uncommon in the regions we analyzed. However, we found that the 5′ flanking region beyond the published sequence also showed an increased A/T content. In addition, we identified another A/T-rich segment starting ~500 bp 3′ of the RSS with even greater concentration of A/T nucleotides. The V\textsubscript{H} 81x flanking regions were therefore analyzed for Cux/CDP binding by EMSA. Surprisingly, only one segment located >1 kb upstream of the 81x coding region was identified as being Cux/CDP positive. Because 81x rearranges with such high frequency, this result was in accordance with our hypothesis that frequently rearranging genes might have a paucity of flanking Cux/CDP sites.

We also tested a member of the J558 family for promoter-associated Cux/CDP recognition sites. J558 represents the largest of all murine V\textsubscript{H} families and is located at the 5′ end of the locus, while 7183 represents the most 3′ located V\textsubscript{H} family. However, the studies on the J558 genes were limited by the small amount of flanking sequence information available. The promoter-associated regions of the gene tested, 186.2, had one very strong peak in its W\textsubscript{5} profile that correlated exactly with Cux/CDP binding (Fig. 4). These limited results indicate that the Cux/CDP binding sites were not restricted to flanking regions in the V\textsubscript{H} S107/V1 gene, but can be found in the flanking regions of other mouse V\textsubscript{H} genes too. The location and distance relative to the individual coding region nonetheless varied among genes.

Many human Ig V\textsubscript{H} genes are flanked by Cux/CDP binding sites

Our study of murine V\textsubscript{H} genes for binding sites of Cux/CDP and Bright was limited by the sequence information available for the mouse Ig loci. However, the human Ig H and L chain loci have recently been sequenced (53–55). This sequence information provided us with the opportunity to study more extensive flanking regions for the presence of repressor and/or activator binding sites, and the potential capability of these flanking regions to interact with the nuclear matrix.

V genes contribute to the Ab repertoire in a nonrandom fashion in both mice and humans (1–5). We therefore wished to investigate whether the presence, number, and relative location of Cux/CDP binding sites would correlate with the recombination frequency of
individual genes to extend our observation with 81x. In addition, testing additional V gene flanking sequences for Cux/CDP binding would also address the question of whether clustering of repressor/MAR binding sites and their proximity to the promoter region are common among human V\(_H\) genes.

The human V\(_H\) locus contains 123 genes distributed over ~950 kb that belong to one of seven families (53). We first chose to analyze three genes from the V\(_H\)3 family that rearrange at different levels: V\(_H\)3-23, which has been reported to be the single most frequently rearranging V\(_H\)3 gene; V\(_H\)3-33, which recombines at a moderate frequency; and V\(_H\)3-13, which contributes at a very low level to the V\(_H\)3 repertoire (1). The most frequently rearranging V\(_H\)3-23 gene was found to be flanked by Cux/CDP binding sites both close to the promoter and immediately 3’ of the RSS (Fig. 5A). V\(_H\)3-33 showed Cux/CDP-reactive fragments 5’ and 3’ of the coding region, but at some distance from the regulatory elements (Fig. 5A). However, we were unable to amplify one AT-rich region within the 5’ flank that might carry additional binding sites. Finally, V\(_H\)3-13, the gene recombining the least frequently showed strong Cux/CDP binding close to and upstream of the promoter, while the downstream Cux/CDP-reactive fragment was located at a distance of >1 kb from the RSS (Fig. 5A). Two fragments isolated from the V\(_H\)3-23 flanking region that were Cux/CDP positive also showed binding to the nuclear matrix by in vitro MAR assay and were positive for binding of Bright. Interestingly, the fragment directly 3’ of the V\(_H\)3-23 RSS was positive in all three assays, although it did not have a particularly high A/T content.

We also analyzed two genes from the V\(_H\)4 family that differed drastically in their recombination frequencies, namely V\(_H\)4-34, which is the most frequent rearranging V\(_H\)4 gene, and V\(_H\)4-28, a gene that encodes an apparently functional gene, but for reasons not yet known, does not appear to recombine (1). The W\(_5\) profile was determined and PCR fragments were analyzed by Cux/CDP-EMSA. V\(_H\)4-34, like V\(_H\)3-23, was flanked by Cux/CDP-positive regions very close to the V\(_H\) coding region (Fig. 5B). Two more Cux/CDP-binding fragments were identified ~2 kb upstream and 3.3 kb downstream, respectively, relative to the V\(_H\)4-34 coding region. However, V\(_H\)4-34 is surrounded by two other V\(_H\) genes at relatively close distances. V\(_H\)7-34.1 is located only 3.2 kb 5’ of V\(_H\)4-34, while V\(_H\)3-33.2 is situated only 4 kb 3’ of the V\(_H\)4-34 RSS. Therefore, the two distant Cux/CDP-positive fragments may be functionally more closely associated with the neighboring genes than with V\(_H\)4-34. This is especially pronounced for the most 3’ Cux/CDP-binding fragment, which would have to be characterized.

**FIGURE 5.** MAR and MAR-binding protein data for human V\(_H\) genes. The representation of the results obtained from mobility shift assays for Cux/CDP and Bright binding and from the in vitro matrix-binding assay is similar to Fig. 2. The V\(_H\) genes shown in A belong to the human V\(_H\)3 family and recombine with different frequencies: V\(_H\)3-23 is the most frequently recombining V\(_H\)3 gene, V\(_H\)3-33 recombines moderately frequently, while V\(_H\)3-13 only rearranges at a low frequency. The two genes depicted in B belong to the V\(_H\)4 family: V\(_H\)4-34 rearranges with high frequency, while V\(_H\)4-28 has not been observed rearranged in the peripheral repertoire. The two genes represented in C represent two genes located on opposite ends of the human V\(_H\) locus: V\(_H\)6-1 is the most 5’ gene; V\(_H\)1-69 is among the most 5’-situated human V\(_H\) genes. Numbers above the W\(_5\) profiles indicate the distance in kilobases to the nearest neighbor V gene.
as promoter associated with regard to the next gene, V\textsubscript{H}3-33.2. Three promoter-associated fragments of V\textsubscript{H}4-28, a gene that does not rearrange, were also tested for Cux/CDP binding. Two of the three fragments were positive in Cux/CDP-EMSA experiments; however, the first 200 bp closest to the V\textsubscript{H}4-28 coding region did not carry Cux/CDP recognition sites.

We also asked whether Cux/CDP binding sites might be more prevalent within certain regions of the human V\textsubscript{H} locus. We therefore chose to analyze two V\textsubscript{H} genes situated at opposite ends of the V\textsubscript{H} locus, namely V\textsubscript{H}1-69, which is located almost 800 kb upstream of the D\textsubscript{H} locus, and V\textsubscript{H}6-1, the most 3' V\textsubscript{H} gene. Fig. 5C shows our results obtained from Cux/CDP-EMSA experiments for these two genes in correlation with their W\textsubscript{S} profile. Several fragments located upstream of the V\textsubscript{H}1-69 coding region were positive for Cux/CDP binding, and their location correlated with spikes or plateaus in the W\textsubscript{S} profile (Fig. 5C). One fragment directly downstream of the RSS and another fragment \~4 kb 3' of the RSS also showed Cux/CDP binding, separated by \~3 kb that did not show any Cux/CDP reactivity. Surprisingly, several of the fragments that did not bind Cux/CDP colocalized with relatively high spikes in the W\textsubscript{S} profile. We therefore chose four fragments to assess their capability to bind to the nuclear matrix and to bind the transcriptional activator Bright. As shown in Fig. 5A, three of the four fragments carried MARs and one fragment also bound Bright. The fragment that was MAR positive and Bright positive also showed the highest A/T level in the W\textsubscript{S} profile. In contrast to the other human V\textsubscript{H} genes, V\textsubscript{H}6-1, the gene located most 3' in the locus, had the lowest overall W\textsubscript{S} profile of all genes analyzed (Fig. 5C), showing only two distinct regions of increased A/T content. The only detected Cux/CDP-binding fragment in the 5' flanking sequence was located 2 kb from the coding region, while the two fragments immediately adjacent to the RSS also bound Cux/CDP. Because V\textsubscript{H}6-1 showed a fairly good correlation between the W\textsubscript{S} profile and Cux/CDP binding, and because the RSS-associated fragment bound strongly, we chose this particular fragment for further examination. However, it did not bind the nuclear matrix or Bright.

Thus, all human V\textsubscript{H} genes that we analyzed in this study showed Cux/CDP binding sites in their flanking regions. Furthermore, as seen in the murine V\textsubscript{H}S107/V1 gene, fragments flanking the human V\textsubscript{H} genes also showed extensive variability in the different assays, in that some fragments were positive in all three binding assays, while others were only positive for one or two and in different combinations. However, the flanking regions showed much heterogeneity, and no easily recognizable differences were detected between human V\textsubscript{H} genes that either recombine with very different frequencies or which are located far apart within the locus. Interestingly, the two V\textsubscript{H} genes with the fewest adjacent MARs and Cux/CDP binding sites are the most 3' V\textsubscript{H} genes in mice (81x) and humans (V\textsubscript{H}6-1).

**CDP binding also occurs in Ig Vk genes**

We also investigated whether a similar occurrence of Cux/CDP binding sites would be found in the human Vk locus. We chose three genes at different locations within the proximal arm of the locus and from different families for analysis: V\textsubscript{k}2-54 (A17), the most Jk-proximal gene; V\textsubscript{k}1-19 (L8), \~150 kb upstream of the Jk cluster; and V\textsubscript{k}2-54 (A17), \~400 kb from the Jk genes and close to the 5' end of the proximal arm. All three genes rearrange fairly frequently (3, 4). Fig. 6 shows the W\textsubscript{S} profiles for each of these genes together with the results obtained from the Cux/CDP-EMSA experiments. All three V\textsubscript{k} genes had segments that bound Cux/CDP on either side of the coding region. The Cux/CDP-binding fragments in the V\textsubscript{k}II gene A17 were at some distance from the coding region, while the VkI and the VkIV gene showed Cux/CDP binding somewhat closer to the coding region.

A sequence analysis of the A/T content performed on several other VkI genes showed a common increase in the W\textsubscript{S} profile \~2 kb downstream of the RSS that was observed for V\textsubscript{k}2-54. Similarly, several genes of the VkI family exhibited a peak in the W\textsubscript{S} profile \~2.3 kb 3' of the RSS seen in V\textsubscript{k}1-19 (data not shown). Interestingly, however, while this A/T-rich fragment associated with the VkI (L8) gene bound Cux/CDP strongly, the similar fragment 3' of the VkII (A17) gene was only weakly positive.

The intron between the leader and the coding region of all VkII genes is \~400 bp in length; the intron of the single VkIV gene is \~200 bp. Both introns are much larger than the short \~50 bp intron observed in VkI genes. The W\textsubscript{S} profile of the VkII (A17) and VkIV (B3) gene showed a spike in the intronic sequence. Surprisingly, when tested for Cux/CDP binding, only the VkIV coding region, while the VkI and the VkIV gene showed Cux/CDP binding somewhat closer to the coding region.

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intron was weakly positive, while the A17 intron fragment did not bind Cux/CDP at all (Fig. 6).

TCR Vβ genes also have Cux/CDP-binding fragments in close proximity

The TCR and Ig loci share the same overall gene organization and use the same enzymatic mechanisms to undergo V(D)J recombination, and both exhibit allelic exclusion. We therefore asked whether TCR V region genes would also contain Cux/CDP sites. We chose to analyze the regions surrounding two commonly rearranged TCR Vβ genes, namely Vβ5.1 and Vβ8.2, that are in close proximity to each other and to another gene, Vβ8.3. These three genes are located within a genomic region of only 6 kb (Fig. 7), and only 5.6 kb upstream of the pseudo gene Vβ5.3. The Ws profile showed a higher A/T content in sequences between Vβ8.3 and Vβ8.1 than between Vβ5.1 and Vβ8.2. All of the fragments analyzed bound Cux/CDP, except for the fragments located directly 5′ of both the Vβ5.1 and Vβ8.2 coding region. Furthermore, fragments directly 3′ of the RSS of all three closely located genes bound Cux/CDP, as was observed for many Ig V genes (Fig. 7).

SATB1 binds to Ig VH and Vk gene flanking regions

SATB1 is another MAR-binding protein whose expression, in contrast to Cux/CDP and Bright, is limited to thymocytes (56). It has been shown to bind to flanking regions of the intronic enhancers of the Ig H-chain, Eμ, as well as the enhancer of the TCRβ locus, Eβ (38). Furthermore, SATB1 has been demonstrated to be involved in the repression of many T cell-specific genes, in that SATB1-null mice exhibit inappropriate ectopic up-regulation of several genes (57). Because the MARs flanking the Ig and TCR enhancers bound SATB1, we wanted to investigate whether the same correlation would hold up for the promoter-associated MARs identified in Ig V genes. We therefore screened the promoter region sequences of several VH and VK genes specifically for the presence of ATC motifs, the preferred binding sites for SATB1, in addition to our Ws analysis. Regions that were identified as potential ATC sequences were then analyzed for SATB1 binding.

SATB1-EMSA experiments were performed in a semiquantitative way, in that a constant amount of 32P-labeled DNA fragment was incubated with increasing quantities of rSATB1 and the relative affinity was estimated based on DNA-SATB1 complex formation. Of the eight ATC fragments tested, only one was SATB1 negative, while the others showed binding to varying degrees. Interestingly, when the same or overlapping fragments had been tested for Cux/CDP binding, only three bound Cux/CDP, while four SATB1-positive fragments did not bind Cux/CDP. This indicates that competition between the two proteins is not the only mechanism of down-modulating Cux/CDP binding, and therefore proposes an even more complex regulation of Cux/CDP binding and associated V gene repression.

Low Ws profiles suggest absence of protein binding sites from the vicinity of mouse Cμ region exons

An earlier study by Cockerill (49), extensively analyzing the murine Ig C region for the presence of MARs, revealed three distinct MAR clusters located in the intergenic regions between Ig Cμ region exons. However, all fragments that facilitated binding to the nuclear matrix in the in vitro assay were found at distances of at least several kilobases away from the nearest exon. Our previous results had shown that in several instances, the location of repressor/activator protein binding sites did not colocalize within the same fragment that also bound to the nuclear matrix. We therefore wanted to investigate whether potential protein recognition sites could be found in closer proximity to the Cμ region exons. Because our Ws profile showed reasonable correlation in many assayed fragments, specifically in that the absence of any noticeable peaks in the Ws profile correlated with the absence of binding sites, the profiles for several Cμ region exons and their flanking sequence were calculated. To further confirm this approach, the sequence surrounding the Eμ enhancer was also evaluated (data not shown). Two peaks present in the Ws profile and situated on either side of the Eμ core enhancer sequence correlated with the well-known MARs flanking the enhancer region. Unfortunately, the majority of the mouse Ig Cμ region sequence, including the reported MAR-containing regions in the Cμ locus, is not yet publicly available, thus limiting our analysis to the few publicly available genomic sequences. Our analysis showed an overall very low Ws profile in the surrounding sequences of Ig μ/δg δ, Ig γ3, and Ig α. Only one relatively high peak was found in the intron between the first and second Ig e exon. However, this sequence contains long stretches of poly(A) homopolymers. Because a similar homopolymeric region had been found in the intron of the 81x coding region, which did not bind Cux/CDP or SATB1, it was also assumed that this homopolymer sequence would not bind to any repressor and/or activator protein. Based on the absence of any Ws predictable binding sites, we assumed that no Cux/CDP, Bright, and SATB1 binding sites would be present in the immediate vicinity of these Cμ region exons; therefore, no fragment from the Ig Cμ locus was analyzed experimentally.

In addition to the Ws profile, all five sequences were also analyzed for the presence of repetitive elements using RepeatMasker. Of all the identified repetitive elements, only two short stretches in the Ig μ(m) region were actual LINE elements, while all other segments were simple di-, tri-, or tetranucleotide repeats. These theoretical findings correlate well with the absence of MAR activity in the immediate flanking sequences of the Ig C region exons reported earlier (49).

Discussion

It is estimated that attachment of chromosomal DNA to the non-histone proteins of the nuclear matrix through MARs occurs every 30–90 kb, thereby organizing DNA into well-defined domains in the form of chromosomal loops (26, 58). These A/T-rich sequences therefore occur fairly infrequently, are widely dispersed throughout the genome, and vary considerably in their distance relative to each other (58). A study of ~200 kb of the IgH C region locus, reaching from the last Dμ gene to ~20 kb past the Cε region, had identified three clusters of MARs in addition to the ones flanking the Eμ, but even here the distances between them varied from 20 to over 70 kb (49), and therefore do not occur at any higher frequency than throughout the rest of the genome. Recently, a 100-kb region encompassing the CD8 gene was analyzed for the presence of MARs, and four strong MARs, along with some weaker ones, were observed. Three of the strong MARs were clustered around two DNase-hypersensitive sites within a 9-kb region; two of them also contained SATB1 binding sites. The fourth strong MAR was located ~60 kb 5′ of this region (59). In contrast to these findings, we identified MARs and/or binding sites for MAR-binding proteins near all Ig V genes analyzed. Moreover, the analyzed V genes were not limited to a specific region within the loci and consequently cannot represent a localized MAR cluster. The average distance between neighboring VH genes in the human locus is less than 8 kb (53), while the average distance between neighboring VK genes is even shorter, less than 4 kb (55). Thus, based on our findings, the average distance between MARs in the human Ig H and Ig κ V gene locus also can be assumed to be less than 8 or even 4 kb, respectively, which is much shorter than the previously reported minimum distance between adjacent MARs of
20–30 kb (49, 58). Therefore, MARs in the Ig V loci occur at a frequency much higher than throughout the rest of the genome. Ig V genes have been shown to contribute in a nonrandom frequency to the Ab repertoire. Although there are naturally occurring variations among the different RSSs, which can affect the frequency of rearrangement of individual genes, there are clearly other factors in addition to the RSS potency influencing V(D)J recombination and therefore the contribution of the various genes to the peripheral repertoire (5, 7–9). One other mechanism by which nonrandom V(D)J recombination could occur is through differential and localized accessibility of individual genes to the recombinase enzymes. MAR sequences themselves may directly affect V gene accessibility. For example, it has been shown that deletion of the iEκ MAR results in premature Vκ-Jκ recombination (33). Local changes in chromatin could be imposed through histone acetylation and DNA demethylation, both of which have been associated with MARs (60, 61). For example, it has been shown that demethylation of the Igκ locus resulted in an increase in gene rearrangement, and this demethylation has been linked to the iEκ-associated MAR sequences, as well as the core enhancer. A recent study by Fernandez et al. (62) also showed that histone acetylation of nucleosomes distant from the Eμ was augmented by the Eμ-associated MAR sequence, and histone acetylation has been tightly linked to accessibility for V(D)J rearrangement (62). Matrix attachment is also associated with transcription, and germ-line transcription is strongly correlated with the onset of recombination (51).

One of the MAR-binding proteins, Cux/CDP, had been reported to be frequently involved in the transcriptional repression of genes that are developmentally regulated (39). Cux/CDP is evolutionarily conserved, and it is expressed in a wide variety of tissues (39). In most reported cases, only single Cux/CDP binding sites were described in promoters. In this study, we demonstrate that Cux/CDP binding sites are present in fairly high number and located throughout the intergenic regions between Vfi coding sequences, presenting the possibility that Cux/CDP binding may be involved in keeping the chromatin and thereby the different V genes within the loci in an inaccessible state. We show that Cux/CDP binding does not always colocalize with sequences that can facilitate binding to the nuclear matrix. Cux/CDP-mediated repression could therefore be imposed by discrete mechanisms: 1) in cases in which Cux/CDP binding does colocalize with MARs, Cux/CDP binding could prevent these MAR regions from interacting with the nuclear matrix and/or matrix-associated proteins, such as Bright or topoisomerase II; 2) under the circumstance that Cux/CDP binding occurs at a region with no MAR activity or in which the nearest MAR is located at some distance, recruitment of histone deacetylase complexes by Cux/CDP might be responsible for keeping DNA in a closed inaccessible conformation.

Cux/CDP has been shown to be a component of NF-μNR (35), a protein first isolated because of its capability to bind to the Eμ-flanking MARs (63). This study also showed that NF-μNR activity as detected by EMSA experiments was present in many murine non-B lineage cell lines and in cell lines representing the pro-B stage. However, NF-μNR activity was not detectable in any μ-expressing pre-B or B cell lines. It was therefore proposed that NF-μNR, and thus Cux/CDP, may function as a lineage-specific repressor of the Eμ enhancer activity in non-B cells by binding to the Eμ-flanking regions, consequently repressing Ig V(D)J recombination in T cells (63). The high number of Cux/CDP binding sites that we have identified in this study near Ig V genes, and their location not only associated with the promoter regions, but also 3′ of the analyzed genes, support the hypothesis that Cux/CDP may be involved in accessibility control of recombining loci. However, if Cux/CDP binding would be involved in specifically preventing accessibility of Ig genes, but not TCR genes in T cells, one might predict that we would find Cux/CDP binding sites associated only with the Ig gene loci and not the TCR loci. However, we identified Cux/CDP binding sites in the flanking sequence of all three TCR Vβ genes examined. This suggests that Cux/CDP-mediated repression may contribute to both TCR and Ig loci inaccessibility. Recently, Cux/CDP-deficient mice were generated. Although, as expected, in the absence of the repressor Cux/CDP, Ig and TCR rearrangement occurs, the mice were lymphopenic presumably due to misregulation of other genes normally controlled by Cux/CDP (64).

Finding Cux/CDP binding sites surrounding genes of the TCR locus, however, does not affect its potential involvement in control of accessibility at the Ig locus because repression of Ig gene rearrangement only persists until the appropriate time point in B cell development. Although EMSAs on normal bone marrow B cell progenitors have not been done, analysis of murine pre-B cell lines and normal mouse splenic B cells would suggest that Cux/CDP-binding activity appears to be lost as lymphocytes pass through the stages of V(D)J rearrangement (Refs. 63 and 65 and unpublished data). This might occur either by decreasing expression levels of Cux/CDP or by inactivating its DNA-binding capability as the cells differentiate. Several posttranslational modifications of Cux/CDP have been shown to influence its capability to bind to DNA, such as serine phosphorylation and/or acetylation of the cut homolog domain (66, 67). If Cux/CDP-binding activity does diminish as VH gene rearrangement progresses, it may contribute to the nonrandom gene accessibility throughout the IgH locus. In one potential model, a decrease in Cux/CDP concentration or its lowered affinity for binding sites would then remove Cux/CDP, and therefore Cux/CDP-mediated repression from V genes that are flanked by low affinity binding sites, while high affinity Cux/CDP recognition sequences would remain occupied. As a result, some V genes would become locally derepressed, while other genes even within the same locus could remain inaccessible.

In another model, repression could be modulated through the competition of Cux/CDP with other proteins for DNA binding. It has been demonstrated that several other MAR-binding proteins can specifically compete or interfere with Cux/CDP for MAR binding (35, 37, 38, 68). Interestingly, one of these proteins, Bright, functions as a transcriptional trans-activator (35), while another protein that can compete with Cux/CDP, SATB1, exhibits predominantly repressive properties (69, 70), although SATB1 displacement of Cux/CDP has also been associated with activation (37). Importantly, while Cux/CDP is expressed in many cell types, Bright expression is limited to two specific stages during B cell development (65) and SATB1 is expressed predominantly in thymocytes (56). Bright shares homology with mating type switching (SWI), a component of the sucrose nonfermenting/mating type switching complex involved in chromatin remodeling (71). Thus, because Bright outcompetes Cux/CDP for binding to the MAR sequences (35), expression of Bright in bone marrow progenitors and binding to its recognition sites could result in Bright-mediated chromatin remodeling. SATB1 has also been reported to interact directly with Cux/CDP in a DNA-independent fashion (70), resulting in inactivation of DNA binding in both proteins. It was therefore hypothesized that the ratio of the two proteins could contribute to modulation of their net function.

If competition between Cux/CDP and other proteins is an important factor in modulating Cux/CDP binding, one should frequently observe binding sites for Cux/CDP and the competing proteins within the same flanking fragment. To date, only a few MARs have been analyzed extensively (28, 37, 59, 68). In all these
MARs, Cux/CDP, AND BRIGHT BINDING SITES FLANK Ig V GENES

previous studies, it was found that the binding sites of MAR-binding proteins such as Cux/CDP, Bright, SATB1, and MAR-BP1 occurred within the same sequence that also facilitated direct binding of DNA to the nuclear matrix (35, 37, 38). It was therefore assumed that most of these proteins can be characterized as MAR binding. In this study, we analyzed a selected number of fragments for matrix attachment, Cux/CDP, Bright, and SATB1 binding. Surprisingly, and in contrast to expected results, we found several fragments that showed dissociation of binding any of these proteins and binding to nuclear matrix preparations. This indicated that the binding sites of the different repressors/activators not always overlap and/or colocalize. Moreover, this also ascribes additional properties to these proteins separate from their MAR-binding activity. Thus, individual V genes may be more or less likely to have Cux/CDP binding successfully competed out by binding of other MAR-binding proteins, depending upon whether their flanking DNA has sites for those proteins. It is also very likely that other MAR-binding proteins that were not addressed in this study may also interact with or displace Cux/CDP, or that they may directly affect matrix binding, which could influence Ig V gene accessibility and/or Ig transcription.

It is possible that the frequent occurrence of MARs and/or Cux/CDP binding sites flanking recombining genes could potentially be involved in preferentially targeting recombination to some genes and not others. In the case of Cux/CDP, a simple hypothesis would be that genes that exhibit a lower recombination frequency might be more repressed by having more Cux/CDP sites in their vicinity, while more frequently rearranging genes would have few binding sites. Although this seemed to be true for 81x, which rearranges at very high frequency and was almost deficient in Cux/CDP binding sites on either side, this was not the general case. One therefore could hypothesize that a differential impact of Cux/CDP on RAG accessibility would be most profound if the proteins would be bound in close range to either the RSS, and directly exclude RAG binding, or within the promoter regions. The latter site may be important in influencing germline transcription or transcription factor binding whose role in V(D)J recombination has not yet been fully established (17, 51). We therefore analyzed the presence of Cux/CDP-binding fragments 5′ and 3′ of the Ig V gene coding regions. However, there was not a simple correlation between clustering and/or relative location of Cux/CDP binding sites and the frequency with which the corresponding V gene recombinates, as one would expect if only Cux/CDP occupancy influenced local accessibility. Instead we found much heterogeneity in the number and location of the individual flanking Cux/CDP binding sites. We also detected heterogeneity in the location of recognition sites for other MAR-binding proteins and for matrix binding as well as divergence in the colocalization of these binding sequences. Thus, the affinity and frequency with which Cux/CDP binds in the vicinity of individual V genes, and its interaction with MARs and MAR-binding proteins could result in a net chromatin conformation that has the potential for influencing V(D)J recombination by altering localized accessibility to the recombinase enzymes. The importance of these recognition sites for the individual V gene segments is further strengthened by the predicted absence of MAR-binding protein recognition sites from the murine Ig C region locus. The number of C regions is much smaller than the number of V region genes, and importantly, isotype switch recombination requires completely different mechanisms of control than V(D)J recombination. In addition, it has been hypothesized that an important function of MARs in the Ig C region could be to separate the highly recombining V, D, and J genes from the C regions, and to segregate the early Ig µ- and δ-chains from the other isotype C regions (49).

Because it is becoming progressively clearer that chromatin remodeling and histone acetylation are associated with the increase in accessibility to V(D)J recombination (10–15), and because the Eµ MARs have been shown to promote histone acetylation over extended distances (62), the sites that we describe in this work may also play a role in chromatin changes augmenting V(D)J recombination in the V region loci. It has also recently been shown that the Ig locus is redirected during B cell development from the nuclear periphery to the center of the nucleus (72). The Vγ region in particular shows more nuclear peripheralization than the C region portion of the locus, leading to the suggestion that the more distal portion of the V gene locus, but not the C gene locus, may preferentially facilitate this subnuclear compartmentalization. It is thus also possible that these V gene-associated MARs could play a role in the subnuclear localization of the Ig loci during B cell development. Finally, the frequent occurrence of MARs, and binding sites for transcriptional activators such as Bright, could also affect Ig gene transcription at later stages of B cell development. Bright has been shown to activate Ig transcription in activated cells (65), so the presence of Bright sites in the 5′ flank of many V genes could influence levels of Ig transcription. Mature B cells may use promoter-flanking and enhancer-flanking MARs to colocalize these regions of rearranged Ig H and L chain genes at the nuclear matrix, thus promoting Ig transcription. Thus, the occurrence of MARs, and binding sites for MAR-binding transcriptional activator and repressor proteins may be relevant for multiple aspects of B cell function.

References


