Nuclear matrix attachment occurs in several regions of the IgH locus

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ABSTRACT

The genome is thought to be divided into domains by DNA elements which mediate anchorage of chromosomal DNA to the nuclear matrix or chromosome scaffold. The positions of nuclear matrix anchorage regions (MARs) have been mapped within the 200 kb mouse immunoglobulin heavy chain constant region locus, thereby allowing an estimate of the size of DNA domains within a segment of the genome. MARs were identified in four regions, which appear to divide the locus into looped DNA domains of 30, 20, 30 and greater than 70 kb in length. These DNA domain sizes fall within the range of DNA loop sizes observed in histone-extracted nuclei and chromosomes. In two regions, large clusters of MARs were identified, and many of these MARs lie on DNA fragments that include repetitive DNA elements, perhaps indicating that repetitive DNA integrates into the genome close to MARs, or that some classes of repeats could themselves act as MARs.

INTRODUCTION

Chromosomes manifest a hierarchy of structural organization (1), the nature of which remains controversial at almost every level of resolution. Chromosomal DNA is coiled around histone octamers, forming nucleosomes, which assemble as larger and larger diameter chromatin fibres (2). Within the nucleus there exists a skeletal element termed the nuclear matrix (3, 4), which resembles the cytoskeleton (5, 6), and is operationally defined as the structure that remains after DNase I digestion and high salt extraction of nuclei. One approach taken to unravel the higher order structure of chromatin has been to dissociate the histones, allowing the DNA to partially unfold. Such studies suggest that the genome is divided into a series of looped DNA domains (7–11) anchored to the interphase nuclear matrix (3, 4) or metaphase chromosome scaffold (9, 12, 13). In a study of histone-depleted nuclei (8), the average DNA domain size was estimated to be 85 kilobases (kb), as determined by the rate of DNase I-mediated relaxation of discrete supercoiled DNA loops, while

electron microscopy of histone-extracted metaphase chromosomes determined a mean DNA loop length of 42 kb (9).

Recent studies have focused attention on the nature of the DNA sequences that mediate attachment of DNA looped domains to the nuclear matrix or chromosome scaffold (14, 15). These elements are termed MARs (matrix association regions), or SARs (scaffold attachment regions), and generally consist of several hundred base pairs of (A+T)-rich DNA. Significantly, MARs are often found close to regulatory elements (15–19) and, in at least two instances, MARs demarcate active chromatin domains (20, 21). Little data are available regarding the spacing between MARs at specific loci. The limited mapping that is available (14, 18–23) indicates that they can occur in the genome at intervals of as little as 5 kb, as in the *Drosophila* histone gene cluster (14), or at intervals greater than 140 kb (18).

The aim of this study is to use a DNA-binding assay which predicts the endogenous sites of nuclear matrix anchorage (15) to map MARs across a large locus for which all of the DNA has been cloned. The mouse heavy chain immunoglobulin (IgH) gene locus has been chosen for this purpose. During the development of the B cell, the IgH locus undergoes recombination whereby one of multiple upstream variable (V_H) and diversity (D) regions are recombined with one of the four joining (J_H) regions to form a complete (VDJ) gene (24). When IgH class-switching occurs, further rearrangement places alternate downstream C regions next to the J_H region locus. The 200 kb portion of the IgH locus studied here is in the unrearranged form, and includes one D region, the J_H region locus, an enhancer in the $J_H/C\mu$ intron and all eight C regions (μ to α) (Fig. 2A, 25). The present study identifies several MARs in the IgH C region locus, many of which co-reside with repetitive DNA elements.

METHODS

Nuclear matrix preparation

Histone and DNA-depleted nuclear matrices were prepared from MPC11 mouse plasmacytoma and mouse liver nuclei, as previously described (15), except that 10 μ g/ml aprotinin, 5 μ g/ml leupeptin and 0.5mM EGTA (ethylene glycol-bis-tetracetic acid)

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were included in all buffers to prevent proteolysis (reagents supplied by Sigma). Briefly, nuclei sedimented through 2M sucrose, were digested with DNase I (Cooper Biomedical), and extracted with 2M NaCl.

Nuclear matrix binding assays

Nuclear matrix attachment sites were identified using an *in vitro* DNA-binding assay, in which ^{32}P -labelled fragments of cloned DNA are incubated with preparations of nuclear matrix in the presence of unlabelled *E. coli* competitor DNA. Binding was assayed as described elsewhere (15), except that 0.25M sucrose was omitted from the incubation mixture, and 0.5mM phenyl methyl sulphonyl fluoride (PMSF), 10 $\mu\text{g}/\text{ml}$ aprotinin and 5 $\mu\text{g}/\text{ml}$ leupeptin were included in the pre-wash and incubation solutions (but not the final washing solution). Following binding, and removal of non-bound DNA, the pellets of matrix (with bound DNA) were solubilised in 30 μl of electrophoresis buffer containing 0.5 $\mu\text{g}/\text{ml}$ proteinase K (Boehringer Mannheim), 0.5% SDS and 5 μg of sonicated carrier DNA. After overnight incubation in a 37°C incubator, half of each sample was electrophoresed alongside a sample of probe DNA (in 15 μl solubilisation buffer) representing 25% of that used in the initial reaction. Electrophoresis was performed for 16 hr at 30V on 20 cm long 0.8% agarose gels in 0.1% SDS, 30mM sodium phosphate, 2mM EDTA, pH 7.8, with recirculation of buffer. Following electrophoresis, gels were fixed for 1 hr in 1% hexadecyltrimethylammonium bromide (CTAB), 30mM sodium acetate, pH 5.5, and dried for autoradiography.

DNA probes

Plasmid pR19.2 was the gift of Emil Kakkis and Kathryn Calame (26), CH4-142.7 was the gift of Phil Tucker (27) and the other phage clones were the gifts of Tasuku Honjo (25) and the Japanese Cancer Research Resources Bank. Plasmid pG19/45 contains the 2.85 kb HindIII/BamHI fragment of the Ig kappa gene, which includes the J_{κ} - C_{κ} intron MAR, cloned into pBR322 (15). Plasmid pEH contains 2.7 kb of DNA, spanning the IgH enhancer and MARs (Box 1, Fig. 2B), cloned as a BamHI/HindIII segment in pBR322 (16). DNA probes used in binding assays were prepared by ^{32}P -end-labelling the restriction enzyme digests, using either T4-polynucleotide kinase, or the Klenow fragment of *E. coli* DNA polymerase I. Following labelling, probes were purified by phenol extraction, and passage over a Sephadex G-50 column.

Several of the larger MARs identified in this study were subcloned into the relevant sites of pUC19 (Fig. 3). pB7.0 = MAR #3, 7.0 kb BamHI; pR6.0 = MAR #6, 6.0 kb EcoRI; pSK3.2 = MAR #10, 3.2 kb SacI/KpnI. pH5.2 contains a 5.2 kb HindIII fragment which overlaps MAR #4, and pX3.65 contains a 3.65 XbaI fragment which spans MAR #11.

Analysis of repetitive DNA

Approximately 0.5 μg of each phage DNA was restriction enzyme digested, and electrophoresed on a 0.8% agarose gel. The DNA was transferred to a 'Zeta-Probe' nylon membrane (Bio-Rad), and hybridized to ^{32}P -labelled mouse cell DNA, in the presence of 0.75M NaCl (5 $\times$ SSC) at 60°C. Following hybridization, the filter was washed in the presence of 0.15M NaCl (1 $\times$ SSC) at 60°C for 1 hr.

RESULTS

An *in vitro* DNA binding assay has proved to be a very efficient means of identifying MARs within large numbers of cloned DNA

Figure 1. Matrix binding assay of pooled kappa and EH MARs. Approximately equal amounts of ^{32}P -labelled XbaI pEH (1.0 kb EH + pBR), HindIII pEH (2.3 kb EH + pBR), BamHI/HindIII pG19/45 (2.8 kb κ + pBR) and HindIII pG19/45 (6.8 kb κ = whole linear plasmid) were pooled, and assayed for nuclear matrix binding in the presence of 100 and 200 $\mu\text{g}/\text{ml}$ *E. coli* competitor DNA, with MPC11 nuclear matrices.

segments (15). This assay is also the only means to readily study MARs in segments of the genome such as the IgH locus (25), which are known to harbour repetitive DNA elements. There are, however, few guidelines that allow estimation of the relative binding affinities for MARs within mixtures of DNA fragments. As a preliminary aid to the long range mapping study, the effect of DNA fragment size in the assay was examined, by assaying well-characterised MARs in mixtures of different sized DNA fragments. MARs are located near the Ig kappa and IgH enhancers (15, 16), and these were chosen for this purpose, as they possess different binding affinities (16). They were combined as 1.0 and 2.3 kb fragments spanning the IgH enhancer MAR (EH), and 2.8 and 6.8 kb DNA fragments containing the Ig kappa MAR (κ and $\kappa\kappa$) (Fig. 1). When this mixture of fragments, together with their respective vector fragments (pBR), were assayed in the presence of 100 $\mu\text{g}/\text{ml}$ *E. coli* competitor DNA, all four MAR fragments exhibited strong binding relative to the vector fragments (Fig. 1). Under the more stringent conditions of 200 $\mu\text{g}/\text{ml}$ competitor DNA, the 2.8 kb kappa MAR bound to a greater degree than the similarly sized 2.3 kb EH MAR, confirming earlier observations (16). This difference became distorted, however, when the 1.0 kb EH fragment was compared to the 6.8 kappa DNA fragment. Although the kappa MAR has a greater affinity, in this instance it exhibited less binding than the smaller EH fragment. This indicates that fragment size does indeed influence binding, and close inspection of Fig. 1 suggests that binding efficiency decreases steadily as fragment length

Figure 2. Mapping MARs in the IgH locus. **A.** Map of the IgH constant region locus, showing the D_{Q52} diversity region (D), the joining region locus (J), and the enhancer (E), and the eight constant region genes (μ , δ , γ_3 , γ_1 , γ_{2b} , γ_{2a} , ϵ and α) aligned along the 200 kb axis defined by Shimizu *et al.* (25). **B.** Alignment of each assayed DNA clone with the IgH map (25). Boxes indicate the DNA restriction enzyme fragments which exhibited specific binding in the nuclear matrix assays in Fig. 2D. Digest fragments containing portions of lambda DNA are not included. **C.** Graphical representation of the bound fragments depicted in Fig. 2B. Fragments are aligned along the same axis, and column heights indicate the relative strengths of binding. + equals binding equivalent to the EH MAR (#1 in Fig. 2B), ++ is equivalent to the Ig kappa MAR, and +++ represents matrix binding significantly greater than the kappa MAR. **D.** Matrix binding assays. Each set of DNA fragments has been combined with either the kappa MAR (K, pK), the IgH EH MAR (EH, pEH), or both, and assayed for nuclear matrix binding. The MAR and vector fragments used as the internal standards are HindIII pG19/45 (pK), BamHI/HindIII pG19/45 (K + p), XbaI pEH (EH + p) and BamHI pEH (pEH). Matrix-bound DNA is shown on the right, while a sample of the input probe is on the left of each pair of lanes. Dots to the left of each panel show the positions of the HINDIII- λ markers, with sizes indicated on the far left. The bound fragment indicated by an asterisk includes portions of both MAR #6 and the vector.

increases above about 1 kb. For fragments below 1 kb, however, binding may decrease with size (15). In subsequent assays in this study the influence of size has been taken into account when making visual estimates of relative binding affinities.

For long range mapping of MARs within the C region locus, seventeen overlapping DNA clones were assayed with MPC11 nuclear matrices (Fig. 2B). In each case, at least two different sets of restriction enzyme fragments were used, to ensure that no MARs were overlooked as a result of enzyme cleavage within MARs. Either the kappa MAR or the EH MAR, or both, were included in each assay as internal references. To further aid in determining the relative strengths of binding, assays were performed at a range of *E. coli* DNA competitor concentrations. Fig. 2D shows just one point of one assay of each clone that contains a MAR. The data from such assays have been summarised in Fig. 2B, where boxed segments indicate MARs within the individual DNA clones. The relative strength of attachment for each MAR was visually estimated from each series of assays, and shown in graphical form in Fig. 2C. On the scale used here, '+' indicates binding equivalent to the EH MAR, '++' is equivalent to the kappa MAR, and '+++' represents binding significantly greater than the kappa MAR. MARs are operationally defined here as the restriction enzyme-cleaved fragments that exhibit binding equal to or greater than the EH MAR. In this study, binding of the lambda vector was often noted (Fig. 2D), and bound DNA fragments containing portions of lambda DNA were not included in Fig. 2B. The right arm of the lambda genome has several stretches of (A+T)-rich DNA which contribute to matrix binding.

In the first panel of Fig. 2D, the 1 kb XbaI EH fragment is seen to be the only MAR within the first 20 kb of the locus (#1, Figs. 2B and D), confirming earlier observations (16). This fragment was previously subdivided into two smaller MARs which exactly flank the enhancer (16). The next MAR identified (#2, δ -16) lies about 30 kb downstream of EH, 3' to the μ and δ constant regions. The δ -16 MAR, however, is only the first of a large group of MARs, which occupy a 30 kb stretch of DNA (DNA fragments 2 to 8). Several of these MARs are of high affinity. In γ_3 -32, for example, there are four segments which bind the matrix (#4 to 7), by comparison with pBR322 DNA (p) which does not (Fig. 2D). Three of these fragments (#4, 6, 7) exhibited binding equal to or greater than the kappa MAR (κ). This cluster of MARs lie within the δ/γ_3 intergenic region, and represents the largest concentration of MARs identified to date. Another concentration of 3 MARs, spanning about 10 kb, lies in the γ_3/γ_1 intergenic region (γ_1 -71, #9 to 11), about 20 kb 3' to the δ/γ_3 region MARs. The remaining MAR identified in this study was in the γ_1/γ_{2b} intergenic region (#12), about 30 kb 3' to the γ_1 -71 MARs. The γ_1/γ_{2b} MAR (#12) appeared to have different affinities in the two assays shown here (XbaI- γ_{2b} -69 and HindIII/XbaI- γ_{2b} -2), and so has been shown as having an intermediate +++ binding affinity. For all the MARs identified here, identical matrix binding data were obtained with liver nuclear matrices, suggesting that these MARs act as constitutive anchorage elements (data not shown).

The evidence presented in Fig. 2 suggested that the two large clusters of MARs might in fact be two very large MARs that have been fragmented by the restriction enzymes used in this study. To examine this question, and also to determine whether the larger MAR fragments could be further subdivided, a more detailed analysis was made in these two regions. To this end, five of the large high affinity MARs were subcloned into pUC19

Figure 3. Mapping of MARs within subcloned DNA. The top portions of each panel show phage clones with MARs, as in Fig. 2B. The bottom portions of each panel indicate subcloned segments of DNA, with MARs shown as boxes, with binding affinities above (+, ++ or +++). Restriction enzyme sites are shown as vertical lines: B, BamHI; H, HindIII; K, KpnI; N, NdeI; R, EcoRI; S, SacI; X, XbaI. Axes are as in Fig. 2. The HindIII site at 58.2, the KpnI site at 91.3 and the XbaI site at 91.5 are sites not identified in a previous study (25).

(MARs #3, 4, 6, 10 and 11) and subdivided further by restriction enzymes, as outlined in Fig. 3. The phage clones and MAR fragments used for subcloning are shown in the top half of each panel (A and B). The subclones are shown below, together with the enzyme sites (vertical lines) used to prepare binding assay probes. For reference purposes, kappa MAR DNA was included in each matrix assay of these subclones (data not shown), and the positions of MARs are indicated on the subclone maps by boxes, together with estimates of binding affinities (+, ++ or +++). From this summary of the binding assay data (Fig. 3) we note that most of these MARs can be further subdivided into 2 or more MARs, and that there are in fact several regions of DNA within each of the two clusters of MARs that do not bind nuclear matrices. Nevertheless, these intervening segments are relatively small, and so each of the two large clusters of MARs (Fig. 2) may be best considered as a single functional unit. Note that for γ_1 -71, several assays were required to obtain the data summarized in Figs. 2 and 3. EcoRI and XbaI digests of γ_1 -71 not only confirmed the existence of 3 MARs (#9, 10 and 11), but demonstrated that all of the MAR activity found in fragment #9 is to the right of the EcoRI site (Fig. 3B, data not shown). Note also that the 0.7 kb KpnI fragment of γ_1 -71, which separates MARs #10 and 11, exhibits some matrix-binding activity (Figs. 2D and 3B).

Testing the endogenous activity of MARs requires DNA hybridization analysis, which does not permit analysis of any single locus that harbours repetitive DNA elements. As the IgH locus contains many copies of repetitive DNA elements (25), the endogenous activities of the IgH locus MARs were not tested in this study. To test whether any of these MARs actually co-reside with repetitive DNA elements, the same set of DNA fragments that were used for matrix assays, were also probed for the presence of repeat sequences. In Fig. 4, a sample of each restriction digest (A) has been analysed by Southern blot analysis (B), using 32 P-labelled total mouse DNA as a hybridization probe, which only permits detection of highly repeated DNA

Figure 4. Analysis of repetitive elements in the IgH locus. **A.** Approximately 0.5 μ g of each phage DNA was digested, electrophoresed on an agarose gel, and stained with ethidium bromide. The lanes correspond to: λ , HindIII λ ; 1, XbaI 142.7; 2, EcoRI δ -16; 3, BamHI γ_3 -201; 4, EcoRI γ_3 -32; 5, HindIII γ_3 -32; 6, EcoRI γ_3 -25; 7, BamHI/EcoRI γ_3 -30; 8, KpnI/SacI γ_1 -71; 9, EcoRI γ_1 -71; 10, KpnI γ_1 -13; 11, KpnI γ_1 -6; 12, EcoRI γ_1 -3; 13, XbaI γ_{2b} -69; 14, HindIII/XbaI γ_{2b} -2; 15, BamHI γ_{2a} -9; 16, BamHI ϵ -12; 17, HindIII ϵ -6; 18, HindIII α -27. Lane 18 was underloaded. **B.** Hybridization pattern of the DNA, after being transferred to a membrane, and probed with 32 P-labelled mouse cell DNA. Weakly hybridizing bands require longer exposures to see clearly (3.9 kb band, lane 2; 1.8 kb band, lane 4). **C.** Alignments of repetitive elements and MARs with the IgH axis. For the repeats, filled boxes represent strongly hybridizing fragments, while open boxes indicate weakly hybridizing fragments.

elements. Those fragments that contain repetitive DNA (Fig. 4B) have been shown in Fig. 4C as either strongly (filled boxed), or moderately hybridizing (open boxes), and are aligned with the MARs identified in Fig. 2. It is immediately apparent that there is almost a direct correspondence between those fragments that contain MARs and ones that bear repeat elements. Note however that the EH (#1) and γ_1/γ_{2b} (#12) MARs do not contain repeats, and that the α -gene 3' repeat element does not bind the matrix. The α -27 digest was greatly underloaded (Fig. 4, lane 18), but other analyses of both α -27 and pR1R19.2 confirms the presence of a repeat sequence downstream of the α -gene (data not shown, 26).

DISCUSSION

This study identified MARs that divided the 200 kb IgH C region locus into four domains. One 30 kb domain spans the μ and δ C regions, which are the earliest to be expressed during B cell development. These C regions are segregated by MARs from the upstream V_H , D and J_H regions, which undergo extensive recombination, and from the downstream C regions which are

activated at later stages of B cell development. These downstream C regions are divided into three domains of 20 kb (γ_3), 30 kb (γ_1) and greater than 70 kb (γ_{2b} , γ_{2a} , ϵ and α). The suggestion here that MARs can in some instances act to divide the genome into functional units is supported by other studies which have found MARs at the boundaries of active chromatin domains (20, 21). The DNA domain sizes determined in this study, and in parallel studies in *Drosophila* (22) and hamster cells (23), are similar to the DNA loop sizes observed in histone-extracted nuclei and chromosomes (7–11), suggesting that MARs do indeed define these DNA loops. Supporting this view is the observation that the genome is divided into domains of 10–40 kb by (A+T)-rich elements (28). It was, however, somewhat unexpected to find such a large cluster of MARs in the δ/γ_3 intergenic region. Such a high density of MARs has not been previously recorded. This group of at least 11 MARs spanning 30 kb may be functionally equivalent to one single strong MAR in the genome. It is likely that the MARs identified here also act as endogenous MARs, as previously studies have found MARs to act in a constitutive fashion and found them to bind to common binding sites (15, 16, 20). There have, however, been some instances

where alternative MAR assay procedures have appeared to identify different subsets of DNA elements (23).

In this study there was a striking correlation between DNA fragments bearing MARs and those that bear repetitive elements. Preliminary studies suggest that there are several copies of the L1-Md long interspersed repetitive element (LINE) (29) in the vicinity of these IgH MARs (data not shown). It has previously been noted that some classes of repetitive elements are enriched in the nuclear matrix (30, 31), and that some of these elements may function as MARs. Studies are in progress to determine whether LINES can mediate nuclear matrix attachment. If such mobile repetitive elements do contain MARs, then they have the potential to influence genome evolution. An alternative possibility is that MARs act as hot spots for insertion of repetitive DNA elements roaming the genome. A recent study supporting this hypothesis found several copies of Alu-like repetitive elements interspersed with long lengths of (A+T)-rich DNA, in a MAR-like recombinogenic region near the adenylate deaminase gene (32). Variable numbers of repetitive elements have also been found near similar (A+T)-rich elements in avian globin loci (33). As it has been shown that topoisomerase II preferentially interacts with MARs, this enzyme could be acting in association with MARs to mediate some recombination and insertion events within the genome (34).

There are several other functions which MARs could fulfill within the genome. MARs are likely to be involved in the replication and assembly of chromosomes. Topoisomerase II is a major chromosome scaffold protein (35), and the preferential binding of MARs to this protein suggests that MARs do anchor DNA within metaphase chromosomes. Also, the requirement for topoisomerase II during mitosis suggests that it co-operates with MARs in the DNA decatenation and chromatin assembly that occurs following DNA replication (36, 37). It has also been noted that origins of DNA replication are associated with the nuclear matrix (38). The IgH MARs described here, however, do not appear to correspond to origins of replication, as the whole constant region locus has been shown to lie within a single replicon (39).

In immunoglobulin loci, and several other loci, MARs either reside close to, or overlap, regulatory elements (15–19), suggesting some role, direct or indirect, in the transcription process. For example, some MARs might act via a looping mechanism to bring different regulatory elements closer together. In the mouse Ig kappa gene, the MAR adjacent to the enhancer is required for maximal expression (40, 41) while in the chicken lysozyme gene, flanking MARs are required for efficient position-independent expression (42). It is, however, unlikely that MARs themselves act as regulatory elements, since anchorage appears to occur regardless of gene activity. Nevertheless, a particular domain-organization for regulatory regions of genes may in some cases be necessary for correct activation and control.

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