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Preference of the recombination sites involved in the formation of extrachromosomal copies of the human alphoid *Sau*3A repeat family

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ABSTRACT

The human alphoid *Sau*3A repetitive family DNA is one of the DNA species that are actively amplified to form extrachromosomal circular DNA in several cell lines. The circularization takes place between two of the five ~170 bp subunits with an average of 73.1% homology as well as between identical subunits. To investigate the nature of the recombination reaction, we cloned and analyzed the subunits containing recombination junctions. Analysis of a total of 68 junctions revealed that recombination had occurred preferentially at four positions 10–25 (A), 40–50 (B), 85–90 (C) and 135–160 (D) in the 170 bp subunit structure. Two regions (B and C) were overlapped with the regions with higher homology between subunits, while other two regions (A and D) cannot be explained solely by the regional homology between the subunits. These regions were located at both junctions of the nucleosomal and the linker region, and overlapped with the binding motifs for α protein and CENP-B. Approximately 90% of the recombination occurred between the subunits located next but one (±2 shift), although the frequency of recombination between the adjoining subunits (±1 shift) was ~10%.

INTRODUCTION

Recombination of genomic DNA is of profound significance for the maintenance of genome diversity and molecular evolution of genes as well as somatic recombination employed by immunoglobulin genes (1–3). The nature of recombination events has been investigated by introduction of artificial substrates into cells, followed by recovery of the products by shuttle vectors, for example, for sequence analysis (4–7). This approach has advantages over selecting appropriate substrates for analysis and rapid recovery of the recombination products, while there is always dispute as to whether the observations actually reflect *in vivo* events. Another approach has been the analysis of naturally occurring recombination events. The recombination involved in rearrangements of chromosomes has been analyzed with respect to tumorigenesis, metastasis and specific genetic diseases such as Duchanne muscular dystrophy (8–10). More frequent recombination events have been shown to generate extrachromosomal (EC) DNA.

The presence of covalently closed circular DNA molecules has been reported in a wide variety of species from prokaryotes to higher eukaryotes and plants (11–20). These EC DNA species are generated either through self-reproduction of a plasmid or through excision of chromosomal DNA by homologous as well as nonhomologous recombination (21–24). From the evidence that the copy number of EC DNA increases by S-phase blocking reagents or at stationary phase, it has been postulated that multiple rounds of replication or replicon 'misfiring' generate additional copies of genomic DNA, forming an 'onionskin' structure (25). This structure is then circularized by recombination between homologous sequences to form EC DNA (see ref. 26 for a review). Therefore, the recombination for EC DNA formation is not reciprocal and the original copy is not lost through this process. This explains why active generation of extrachromosomal *Sau*3A family DNA would not cause the loss of the DNA in the genomic DNA.

We previously reported the nature of recombination involved in formation of extrachromosomal copies of a tandemly repeated sequence family, the alphoid *Sau*3A repeat (27). This family is one of the human alphoid repetitive family DNA and was originally cloned from the extrachromosomal (EC) DNA (28), and later, its location was mapped to the centromeric region of chromosome 11 (29). Similar sequences were found at the identical region of chromosomes 1, 17 and the X chromosome (29,30). This repeat has an 849 bp unit sequence which is divided into five homologous subunits of ~170 bp in length. This family DNA is actively amplified from chromosomal DNA to generate EC DNA in several human cell lines. In HeLa cells, ~1% of this family DNA is present extrachromosomally as circular DNA. The recombination for circularization occurs efficiently between any two subunits with homology of 68.4–77.2% as well as between identical subunits, creating a composite subunit. Sequence analysis of the recombination junctions suggested that a long stretch of homology is not required for these events. We present here a compilation of recombination junctions generated upon the formation of the extrachromosomal *Sau*3A family DNA to obtain insight into the mechanism of the recombination event.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides were synthesized using a Milligen Cyclone plus DNA synthesizer by the beta-cyanoethyl phosphoramidite

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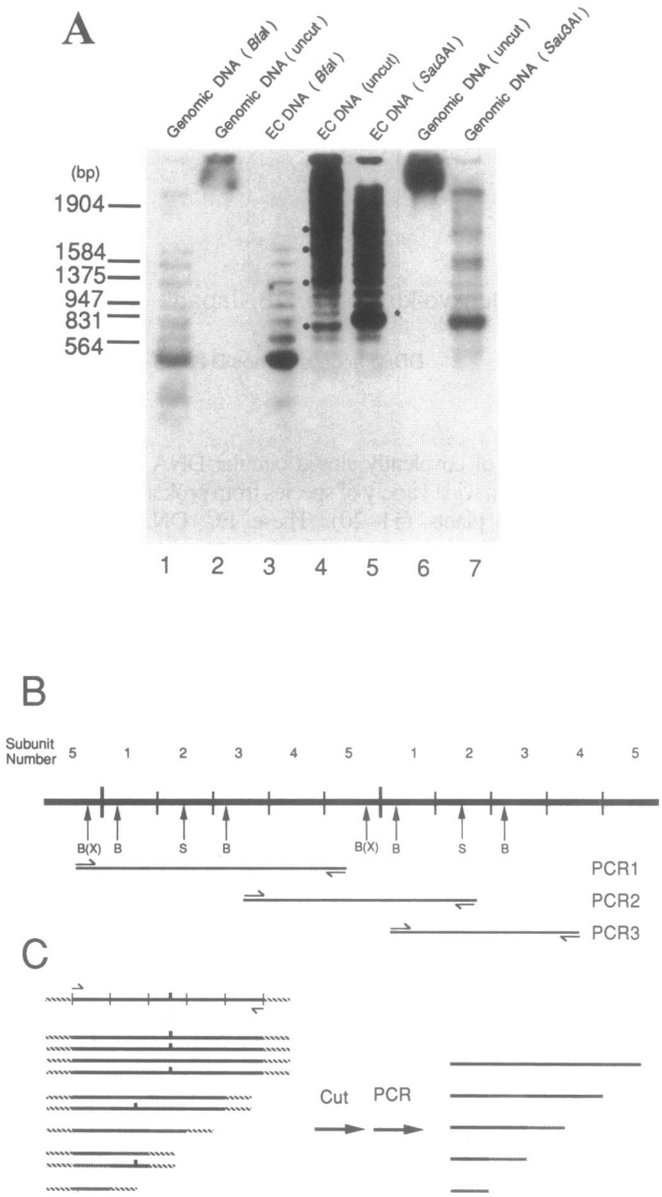


Figure 1. Cloning strategy of recombination junctions from the extrachromosomal *Sau*3A family DNA. (**A**) Southern blot patterns of the chromosomal and extrachromosomal *Sau*3A family DNA. The EC DNA purified from 3×10^8 cells (lanes 3–5) and 2 μg of genomic DNA (lanes 1, 2, 6 and 7), either digested with restriction enzymes (*Bfa*I for lanes 1 and 3 and *Sau*3AI for lanes 5 and 7) or uncut (lanes 2, 4 and 6), were electrophoresed in a 1.5% agarose gel and used for Southern blot analysis with a *Sau*3A family probe containing the whole unit. Monomers, and the multimers of the unit, exhibiting a ladder-like pattern in the uncut EC DNA (lane 4), were indicated by dots. (**B**) Map of the *Sau*3A family repetitive sequence. *Sau*3AI (S), *Bfa*I (B) and *Xba*I (X) sites are indicated by vertical arrows. PCR primers used to amplify the *Sau*3A family sequence are shown by horizontal arrows with the expected canonical PCR products. The positions of the primers were as follows: PCR1, 77–98 on subunit 5 and 99–120 on subunit 5; PCR2, 112–133 on subunit 2 and 95–117 on subunit 3; PCR3, 4–23 on subunit 1 and 104–123 on subunit 4. (**C**) Cloning strategy. Circular EC DNA was first treated with the restriction enzymes which cut the unit. The products were then subjected to PCR amplification with the primers (horizontal arrows) located in both sites of the restriction site. Therefore, only the fragments lacking the site by recombination or point mutation can be amplified. Composite subunits are shown by shadowed lines and restriction sites by filled boxes.

method, and purified with Milligen Oligo-Pak columns. The oligonucleotides used for amplification of chromosomal and EC DNA were: S-T, GATCCGCGGCCGCCCGAT: S-G, ATCGGGCGGCCGCG:B-C,GATCCGCGGCCGCCCGTC: B-G, GACGGGCGGCCGCG. *Sau*3AI adaptor, S-TG, was made by annealing S-T and S-G, and the *Bfa*I adaptor, B-GC, by annealing B-G and B-C. *Sau*3A family-specific primers were: TTCCTCTTGACAGAGCAGCTCT and AGTTCAATTCTTGAAGTGGAAC for PCR1, GAAAGTGGGTTTGGAAACTGCG and AACCTTGCTTTCATAGTTCAGC for PCR2, and GTGGTGGAAAAGGAAAATCC and TTCAAAACTGCTCCATCAGC for PCR3.

DNA isolation and purification

EC DNA was prepared from human (HeLa) cells by the method described by Hirt (31). Briefly, cells (6×10^9) were washed three times with phosphate-buffered saline and suspended in 120 ml of 10 mM Tris–HCl, 10 mM EDTA (pH 8.0). After the cells were lysed with 3.6 ml of 10% SDS and 32 ml of 5 M NaCl, the sample was incubated overnight at 4°C, and centrifuged at 30 000 r.p.m. for 2 h at 4°C. The supernatant was treated with proteinase K (final concentration 100 μg/ml) for 2 h at 37°C, followed by phenol extraction, chloroform/isoamylalcohol extraction and ethanol precipitation. The samples were further purified by three successive CsCl/ethidium bromide centrifugations at 55 000 r.p.m. for 19 h at 22°C, to completely remove chromosomal DNA from the covalently closed circular DNA fraction. Circular mitochondrial DNA was removed by linearization with *Pac*I, followed by treatment with *Micrococcus luteus* ATP-dependent deoxyribonuclease (0.0175 U/μl) for 1 h at 37°C (32). Chromosomal DNA was prepared from the pellets obtained by Hirt extraction by the conventional phenol extraction method.

PCR amplification of EC DNA

EC DNA was first digested with *Sau*3AI or *Bfa*I. Then, their 3′-ends were filled with dNTPs by the Klenow fragment, and *Sau*3AI adaptor S-TG for *Sau*3AI or *Bfa*I adaptor B-GC for *Bfa*I was ligated. The DNA fragments were amplified by PCR in 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 0.1% (w/v) Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.2 μM of the primer S-T (for *Sau*3AI) or B-C (for *Bfa*I) in a total volume of 100 μl with 0.05 U/μl of *Taq* DNA polymerase. The PCR program used was: 1 min at 92°C for denaturation, 2 min at 50°C for annealing and 3 min at 72°C for extension in each amplification cycle, with a final extension at 72°C for 10 min.

Cloning of recombination junctions from the extrachromosomal *Sau*3A family DNA

The *Sau*3A family DNA was amplified by PCR (PCR1, 2 and 3) with specific primers from PCR-amplified EC DNA or purified EC or chromosomal DNA digested with *Sau*3AI (PCR1 and 3), *Bfa*I (PCR2) or *Xba*I (PCR2 and 3) in advance (detailed in Fig. 4A). Therefore, only the fragments lacking the respective restriction sites can be amplified, removing most of the PCR products from complete units. The positions of the PCR primers and the canonical PCR products are illustrated in Figure 1B. The PCR conditions were the same as those described above. The fragments from EC DNA with sizes different from those of chromosomal DNA were recovered from a 1.5% agarose gel and cloned into the *Eco*RV site

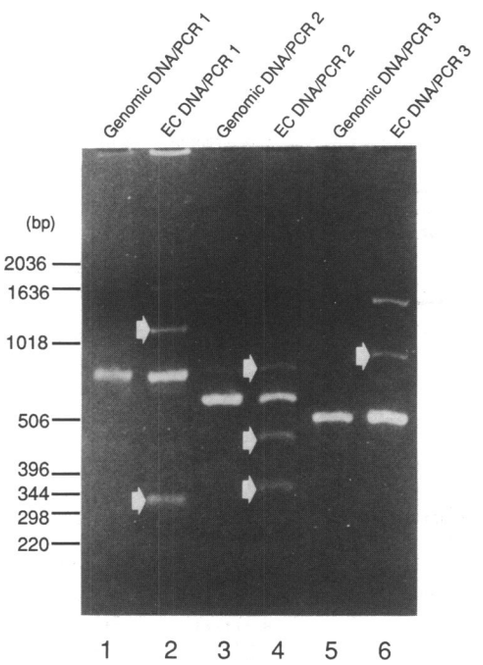


Figure 2. PCR amplification of the fragments containing recombination junctions. PCR-amplified EC DNA (lanes 2, 4 and 6) and chromosomal DNA (lanes 1, 3 and 5) were used for PCR amplification with specific primers shown in Figure 1B, and the PCR products were electrophoresed in a 1.5% agarose gel. PCR amplification was performed with *Sau*3AI fragments for PCR1 (lanes 1 and 2) and PCR3 (lanes 5 and 6), and *Bfa*I fragments for PCR2 (lanes 3 and 4) in a total volume of 100 µl containing 300 ng of DNA. Arrows indicate the PCR products with recombination junctions. The bands at 850 and 1700 bp (dimer) in PCR1, 680 bp in PCR2, 630 bp or 1480 bp (dimer) in PCR3 were derived from the canonical fragments.

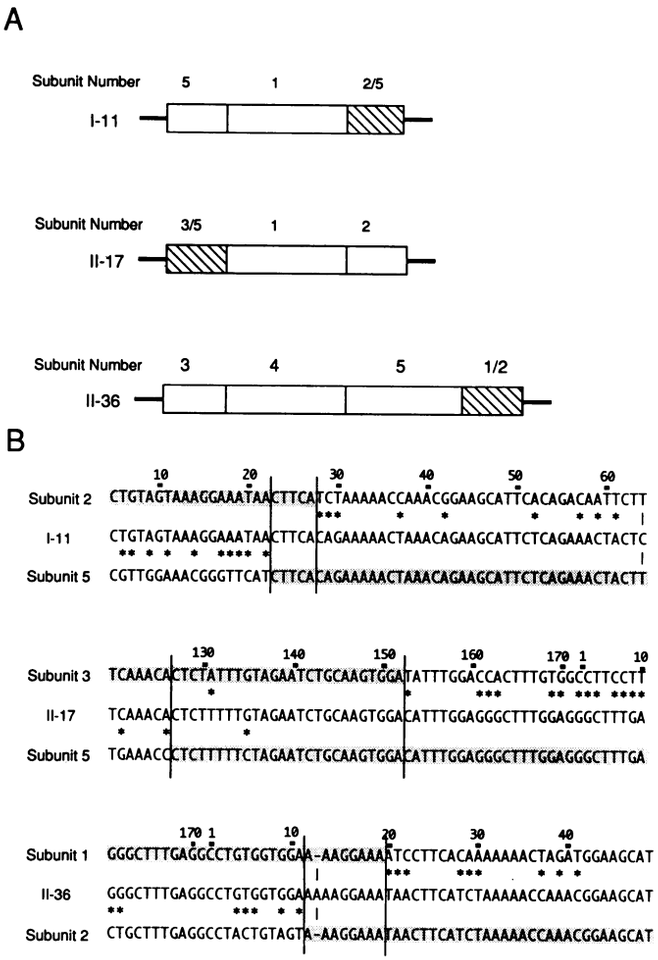


Figure 3. Clones containing a composite subunit (A), and the nucleotide sequences of their recombination junction (B). **(A)** Three clones, I-11, II-17 and II-36, contained composite subunits 2/5, 3/5 and 1/2 respectively. Composite subunits are indicated as striped boxes. **(B)** The sequences of the clones are aligned with those of the parental subunits and the regions from which the sequence was derived are shadowed. A vertical bar represents a mismatch to both of the parental subunits and an asterisk represents a mismatch to either of them. Accession numbers of the nucleotide sequences of these recombination junctions in DDBJ/GenBank/EMBL databases are: I-11, D49597; II-17, D49591; II-36, D49595.

of the pBluescript SK(–) vector (Stratagene, USA). The nucleotide sequences of the recombination junctions were determined by the dideoxynucleotide chain termination method (33) using 24mer reverse sequencing primer #1233 (New England Biolabs) or 17mer sequencing primer KS (Stratagene).

Southern blot analysis

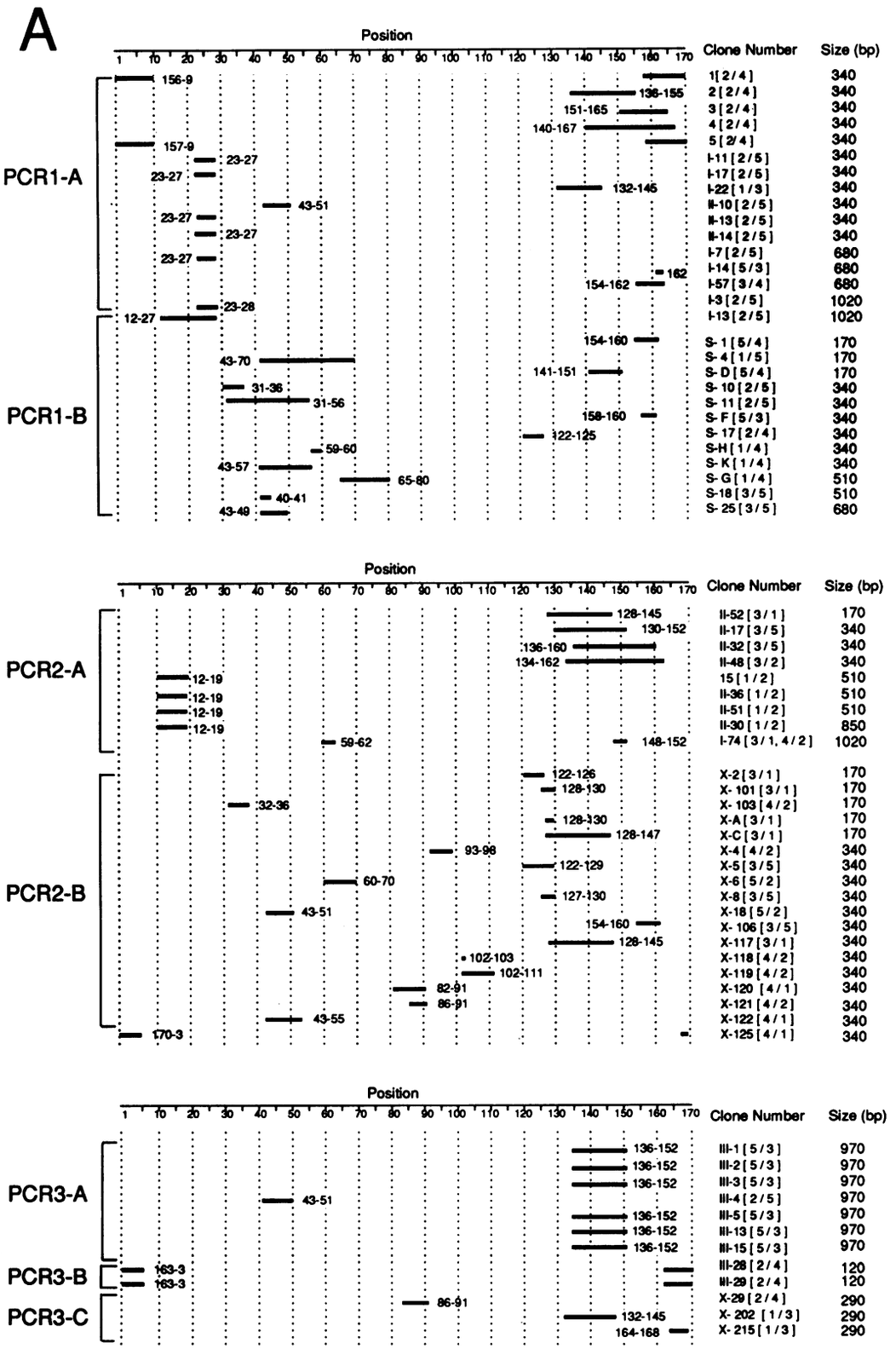
DNA samples were electrophoresed in a 1.5% agarose gel and transferred onto Hybond-N$^+$ nylon membranes (Amersham). Hybridization was performed overnight at 65°C with probes prepared with a random priming DNA labeling kit (Boehringer Mannheim) and, after hybridization, the membranes were washed three times in 2× SSC (SSC = 15 mM sodium citrate, 0.15 M NaCl), 0.1% SDS for 30 min at 65°C and twice in 0.2× SSC, 0.1% SDS for 20 min at 65°C, and autoradiographed.

RESULTS

Molecular cloning of the recombination junctions

Figure 1A shows the results of Southern blot analysis of the *Sau*3A family in the EC as well as chromosomal DNA. A typical ladder-like pattern was observed with purified uncut EC DNA (Fig. 1A, lane 4), indicating the presence of circular molecules with one, two or more unit(s) of the family sequence. As we reported previously (27,28), there existed four minor bands between the major bands (multimers of the 849 bp unit), suggesting the presence of a composite subunit. After digestion with *Bfa*I or *Sau*3AI, most of the bands converged to a 500 or 850 bp band (Fig. 1A, lane 3 or 5 respectively), whose patterns were identical to that of the chromosomal DNA digested with the respective enzymes (lane 1 or 7).

To clone the fragments containing the recombination junctions, we amplified the *Sau*3A family DNA with three different pairs of PCR primers (detailed in Fig. 1B) and obtained different size classes of fragments (PCR1–3). We first employed PCR amplification of EC DNA using adaptors (S-TG or B-GC, detailed in Materials and Methods) in order to supply enough materials for the analyses by Southern hybridization and others, and eventually for the cloning procedure. We then amplified the PCR products again, but this time, using *Sau*3A specific primers (PCR1–3). The PCR products in each scheme contained the fragments of the canonical length (shown in Fig. 1B) as well as fragments containing a composite subunit. As shown in Figure 1C, circular EC DNA was digested with the restriction enzyme (*Sau*3AI, *Bfa*I


A
Position
Clone Number
Size (bp)
PCR1-A
PCR1-B
PCR2-A
PCR2-B
PCR3-A
PCR3-B
PCR3-C

B

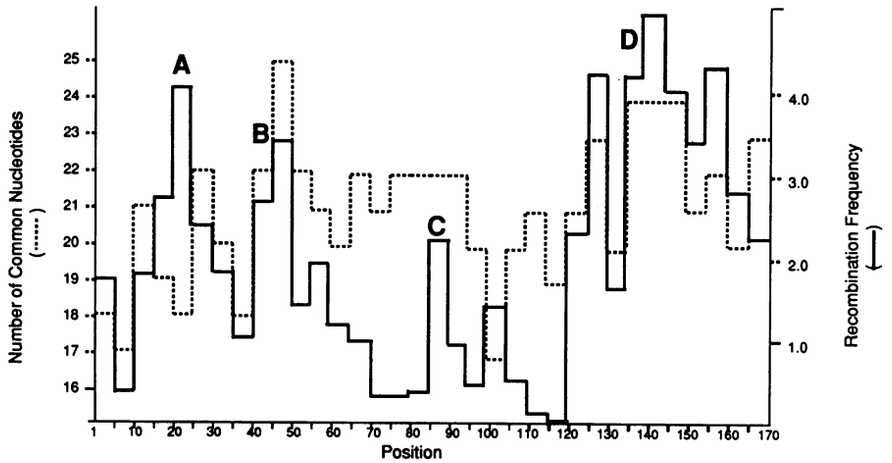


Figure 4. Distribution of the recombination junctions observed in PCR1–3 (A) and summary of recombination junctions arranged by the subunit structure (B). **(A)** The recombination junctions of the 67 clones (68 junctions) containing composite subunits are shown as horizontal bars. Clones in PCR1-A (16 clones), PCR2-A (nine clones), PCR3-A (seven clones) and PCR3-B (two clones) were obtained by two-step amplification using primers S-T and S-G (PCR1-A and PCR3-A) or B-C and B-G (PCR 2-A and PCR3-B) for the first step after *Sau*3AI or *Bfa*I digestion, respectively, and primers shown in Figure 1B for the second step. Clone I-74 contained two recombination junctions. Clones in PCR1-B (12 clones), PCR2-B (18 clones) and PCR3-C (three clones) were obtained by direct amplification with primers for PCR1 through PCR3 after digestion with *Sau*3AI (PCR1) or *Xba*I (PCR2-B and 3-C). The nucleotide sequences of the recombination junctions are available through DDBJ/GenBank/EMBL databases: accession numbers D49585–D49652. **(B)** Recombination frequencies were calculated by adding the probabilities of recombination for every 5 bp of the subunits for each recombination event. If the clone has the recombination junction between 21 and 27, its probabilities for the regions between 21 and 25, and 26 and 30, are 0.714 and 0.286. The frequencies of recombination within the subunits for PCR1 through PCR3 were combined and are shown by a solid line. Four recombination hotspots are indicated as A–D (A: 10–25, B: 40–50: C: 85–90 and D: 135–160). The numbers of common nucleotides (common for at least three subunits) among five subunits are shown as a dashed line.

or *Xba*I) before PCR amplification, which prevents amplification of most of the canonical fragments. Note that the canonical fragments without the restriction sites could be amplified, although they are minor species (see Fig. 1A).

Figure 2 shows that the products from EC DNA (Fig. 2, lanes 2, 4 and 6) contained additional fragments that were not observed in the products from chromosomal DNA (Fig. 2, lanes 1, 3 and 5). These additional fragments were cloned and their nucleotide sequences were determined. All of the 32 clones analyzed (16, 9 and 7 clones for PCR1-A, 2-A and 3-A respectively) contained at least one composite subunit. Figure 3A shows the subunit structure of three clones. Clone I-11 contained subunits 5 and 1, and a 2/5 composite. Clones II-17 and II-36 similarly contained the whole subunits and a composite, 3/5 or 1/2 respectively. The nucleotide sequences of their recombination junctions are shown in Figure 3B. Recombination seems to have occurred somewhere between positions 23 and 27 for I-11, 127 and 152 for II-17, and 12 and 19 for II-36.

Distribution of recombination junctions

We also employed direct amplification of recombination junctions from purified EC DNA digested with *Sau*3AI, *Bfa*I or *Xba*I (PCR1-B, 2-B, 3-B and 3-C) and added to the data shown above. The positions of recombination junctions observed in each PCR scheme (a total of 67 clones containing 68 junctions) are shown in Figure 4A. The results of three PCR schemes were combined and are summarized in Figure 4B. The distribution of the recombination junctions showed a marked centering at four regions, positions 10–25 (A), 40–50 (B), 85–90 (C) and 135–160 (D). When this was compared with the degree of sequence homology between subunits (shown as a dashed line in Fig. 4B), regions B and C overlapped the regions with a high sequence homology between the subunits, while regions A and D showed less apparent association between them, indicating that some factor other than sequence homology has a dominant effect on the events (see Discussion). We included region D in the latter case because, although the region is correlated with the homologous regions, the recombination frequency is apparently higher than expected when compared with regions B and C. Table 1 is the summary of the recombination events within the unit structure. There was a strong preference for ±2 subunit shift (87%), especially for 2/4 or 4/2 (22%), 3/5 or 5/3 (22%) or 2/5 or 5/2 (19%), while 13% of the cases occurred between the adjoining subunits (±1 shift).

DISCUSSION

We previously described the nature of the recombination involved in the rearrangement of a human repetitive sequence family, the alphoid *Sau*3A repeat family (27). From analysis of the composite subunits in these sequences in the chromosomal DNA, we and others proposed the presence of the recombination hotspots in the subunit structure (34,35). In the present study, hotspots were also observed for the formation of EC DNA having these sequences. To efficiently clone the fragments containing the recombination junctions, we used PCR amplification (Figs 1 and 2). The PCR products were then cloned and subjected to the sequence analysis. To avoid the amplification of the fragments with complete units, EC DNA was first digested with *Sau*3AI, *Bfa*I or *Xba*I which cut the family sequence, and the fragments

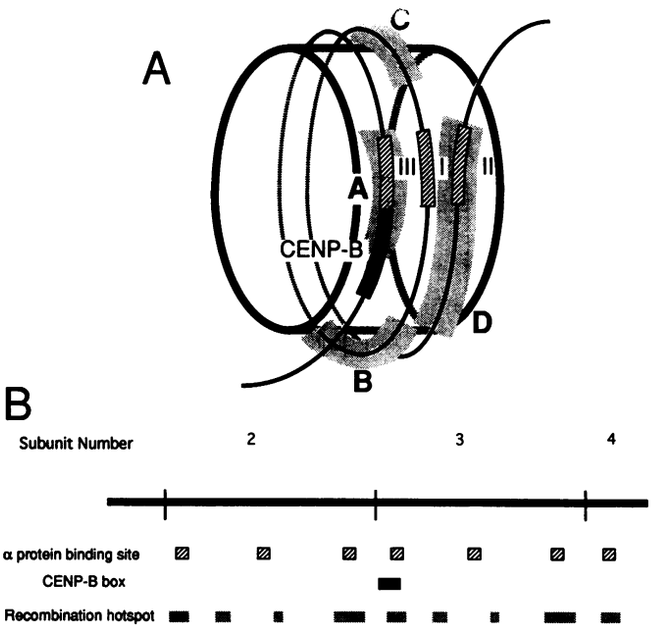


Figure 5. Schematic illustration of the recombination hotspots and the potential binding sites of α protein and CENP-B (A) and their map in the subunit structure (B). **(A)** Recombination hotspots (shadowed) are illustrated along with the α protein binding sites (striped) and the CENP-P box (solid) on the nucleosome structure. **(B)** Position of the α protein binding sites (74–80 for I, 151–157 for II and 17–23 for III), the CENP-B box (2–18) and the recombination hotspots are mapped on the *Sau*3A family subunits. The linker DNA of the nucleosome (positions 157–17) is located between the sites II and III.

Table 1. Subunits involved in recombination classified by the type of subunit shift

Type of recombination[a]	Type of subunit shift[b]	Number of junctions Each	(%)	Subtotal	(%)
1/2 or 2/1	±1	4	(6)		
2/3 or 3/2	±1	1	(1)		
3/4 or 4/3	±1	1	(1)	9	(13)
4/5 or 5/4	±1	2	(3)		
5/1 or 1/5	±1	1	(1)		
1/3 or 3/1	±2	10	(15)		
2/4 or 4/2	±2	15	(22)		
3/5 or 5/3	±2	15	(22)	59	(87)
1/4 or 4/1	±2	6	(9)		
2/5 or 5/2	±2	13	(19)		
Total		68	(100)	68	(100)

[a]Possible combination of subunits.
[b]Gain or loss of indicated numbers of subunit.

without these sites were amplified by PCR with the primers located on both sides of the restriction sites. Therefore, only the fragments which lacked the sites by rearrangement or point mutation could be amplified. As shown in Figure 2, all the cases of PCR amplification of EC DNA showed at least one additional band which was not present in the PCR products from chromosomal DNA. Since the amounts used for PCR were same between EC DNA and chromosomal DNA in Figure 2, the additional bands observed in the products from EC DNA were not the artifacts generated by PCR with specific primers. These bands were also observed in a separate experiment using the unamplified EC DNA as a source of PCR (Fig. 4A).

Since only the fragments generated by recombination that occurs in the region without the restriction site (*Sau*3AI, *Bfa*I or *Xba*I) or by the point mutation that removes the site was enriched, there might be a case of representing the recombination junctions from too small numbers of DNA fragments. To exclude this possibility, we used three restriction enzymes (*Sau*3AI, *Bfa*I or *Xba*I) which cut different sites, and three pairs of specific primers (PCR1–3). When the recombination junctions were analyzed, clones obtained by PCR1 contained many types of recombination junctions (Fig. 4A), indicating that they represented a wide variety of recombination junctions. On the other hand, clones from PCR2-A and especially PCR3 were likely to be derived from a few major species in the population, probably because both of the PCR schemes amplify a smaller region, and therefore, reduce the number of recombination events that could be detected.

Sequence analysis of the recombination junctions revealed that the subunit structure was retained through the homologous recombination and, based on comparison of scattered mismatches between the subunits, we mapped the recombination junctions (Figs 3 and 4). The distribution of the 68 junctions showed that most (55/68) of the sites were centered at positions 10–25 (A), 40–50 (B), 85–90 (C) and 135–160 (D). As expected, regions with high sequence homology between subunits exhibited higher recombination frequencies (regions B, C and D). Interestingly, region A did not correspond to those with high sequence homology between the subunits and region D showed higher recombination frequency compared with regions B and C, suggesting that another factor(s) affects the recombination frequency. The higher recombination frequency compared with the regional homology (shown in Fig. 4B) in both regions A and D is statistically significant as judged by $P < 0.05$. This tendency was absent in the regions B and C.

It has been shown that the alphoid (or α for primates) satellite DNA has several sequence features characteristic of protein binding motifs. Strauss and Varshavsky (36) reported that α protein binds preferentially to three regions (I–III), which are located close to each other spatially on the nucleosomes, and regions II and III are located next to the linker region. This protein is presumably essential for the function of positioning nucleosomes along the subunits. On the other hand, there are sequence motifs for CENP-B (37,38), a protein which is a part of kinetochore structure and binds specifically to alphoid satellite DNA, at positions 2–18 of subunits 3 and 5. This protein might play an important role in aligning the subunits. The preference of ±2 over ±1 subunit shift (Table 1) could be explained by this specificity. Meanwhile, the recombination hotspots described here were located next to the linker region and overlapped with these protein binding sites (Fig. 5). Furthermore, analysis of the recombination sites in the chromosomal DNA also indicated one of these regions (positions 135–160) to be recombinogenic (35). Therefore, these regions are not only susceptible to binding proteins such as α protein and CENP-B, but also to the proteins which mediate DNA rearrangements. Such preference of the recombination events at the linker region has been suggested from nonrandom size distribution of EC DNA in several cell types (26). In contrast, Kawasaki *et al.* (6) reported that no such

preference of the recombination sites was observed for artificially constructed tandem repeats of the primate α satellite DNA. This could be explained by the involvement of a protein specific to the human alphoid DNA which enhances the regional recombination frequency, or by differences between the artificial and the intact repeat constructs.

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