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Sequence analyses of extrachromosomal *Sau*3A and related family DNA: analysis of recombination in the excision event

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SUMMARY

Previously, we reported a recombination-prone human alphoid-like repetitive DNA (Sau3A family) which is characterized by abundance in the extrachromosomal fraction and restriction fragment length polymorphism (1, 2). We suggested a specific homologous recombination to be responsible for the DNA excision from the chromosomes and also the sequence rearrangement in the chromosomes (3). In order to investigate the nature of the recombination further, 8 different clones were obtained which hybridized with Sau3A probe among over 1,500 extrachromosomal DNA clones. Restriction mapping and nucleotide sequence analyses showed two to be Sau3A monomers and dimers, four Sau3A recombinants, as observed previously, one a recombinant of the Sau3A-related sequence on chromosome 17, and one a new Sau3A-related sequence. Sequence analyses of the recombination junctions in the recombinant clones indicated a specific homologous recombination also to be responsible for all but one clone. The molecular mechanism and biological significance associated with the recombination are discussed.

INTRODUCTION

Recent studies have revealed the structures of eukaryotic chromosomes to be more dynamically organized than originally supposed. The transposition of movable genetic elements and the integration of retroviruses create DNA rearrangements in a wide range of chromosomal locations (4-6). Besides these, several repetitive sequences such as Alu and L1 families, summarily called retroposons, are likely to be transposed via RNA intermediates (for review, see ref. 7). These movable elements may have contributed to the species specific chromosome complexity and to the ubiquitous existence of several repetitive sequences.

The Sau3A family is a human alphoid-like tandem-repetitive sequence with a basic 850 bp unit which further comprises five approximately 170 bp subunits (1). We reported the sequence to be unstable in the chromosomes, causing a considerably high degree of restriction-fragment length polymorphism (RFLP) among individuals, and generating extrachromosomal covalently closed circular (ccc) basic unit oligomers (1, 2). Further analysis of the excision event

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suggested the recombination, which is totally reciprocal in the subunit structure, to have occurred even between subunits with only 70-80% homology. We also obtained evidence of the same type of recombination being responsible for the rearrangement of the Sau3A-related sequences in the chromosomes (3).

In order to obtain more information on the nature of the recombination, we screened extrachromosomal DNA clones and analyzed the structure of several recombinant clones. In the present paper, we describe the results of the analyses which suggest a unique type of recombination to be associated with the repetitive DNA sequence.

MATERIALS AND METHODS

Cell Culture

HeLa cells (S3) were cultured in MEM (calcium free, Nissui) supplemented with 10% (v/v) newborn calf serum (Irvine Scientific) with constant stirring at 37°C.

Purification of Extrachromosomal ccc DNA for Cloning

Seven liters of HeLa cells in the exponentially growing stage (~5 x 10^5 cells/ml) were harvested and washed three times with phosphate-buffered saline (PBS), and the extrachromosomal fraction containing low molecular weight DNA was obtained according to Hirt's procedure (8). The fraction was first treated with 100 μg/ml of proteinase K (Sigma) at 37°C for 2 hr and extracted with phenol/chloroform (1:1, v/v); this was followed by ethanol precipitation. The precipitate was dissolved in CsCl solution (1.57 g/cm^3) containing 600 μg/ml ethidium bromide, and subjected to centrifugation (55,000 rpm, at 22°C, for 20 hr) in a TFT65.13 rotor (Kontron). ccc DNA fractions were collected from the bottom of the tube. A total of three successive centrifugations were preformed to remove linear DNA completely; the absence of linear DNA was confirmed by electron microscopy. To remove mitochondrial DNA, the extrachromosomal ccc DNA was further centrifuged by sucrose density gradient (10-40% sucrose in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl) centrifugation (30,000 rpm, 20 hr, 4°C) in a TST41.14 rotor (Kontron), and smaller molecular weight DNA fractions (purified extrachromosomal ccc DNA) were collected.

Cloning of Sau3A Family Derived Extrachromosomal DNA

The purified extrachromosomal ccc DNA was partially digested with the restriction enzyme XbaI (Takara Shuzo Co), and ligated to an XbaI-digested pUC19 vector. Upon transformation, with Escherichia coli HB101 as recipient, a total of 1,613 ampicillin-resistant transformants was obtained. In an

independent experiment using E. coli JM109, 3% of the transformants (4 out of 130 colonies) contained an insertion sequence in pUC19. For screening DNA clones with Sau3A family-derived extrachromosomal DNA, a subclone (pXR1) of the previously isolated Sau3A family DNA clone (pUCXba2) was used as a probe. The pXR1 contained an XbaI fragment of a complete Sau3A family DNA basic unit.

Restriction Mapping and Nucleotide Sequencing

From the 1,613 colonies analyzed by colony hybridization using pXR1 as a probe, 18 positive clones were obtained. After analysing their structures with several restriction enzymes (XbaI, StuI, EcoRI, HindIII, SphI, PstI, AluI, Sau3AI), the 18 clones were classified into 8 different types (summarized in Table 1). Duplication of more than one identical clone probably arose during the transformation process. Portions or whole DNA sequences of these clones were determined by the dideoxynucleotide-chain termination method (9). When necessary, the clones were subcloned into pUC19 (10). The sequencing strategy for each clone is indicated in the respective map (Fig. 1, 3A, 4A and 5A).

Southern Hybridization

Hybridization was performed according to Southern (11). In essence, DNA samples were subjected to electrophoresis in 1.0% agarose gel and transferred to a nylon membrane (Biodyne, Pall). After having been dried at 80°C in a vacuum oven and prehybridized (prehybridization solution: 5 x SSC, 4 x Denhardt's and 50 µg/ml heat-denatured salmon sperm DNA) overnight at 65°C, the membranes were subjected to hybridization (hybridization solution: prehybridization solution, with 1/5 volume 50% (w/v) dextran sulfate and heat-denatured probe DNA added). After hybridization, the membranes were washed in 2 x SSC, 0.1% SDS (3 times, 30 min), 0.5 x SSC, 0.1% SDS (once, 1 hr) and 0.3 x SSC, 0.1% SDS (once, 3 hr), all at 65°C. The ^{32}P-labeled DNA probe was prepared by nick-translation with α-^{32}P-dCTP (3,000 Ci/mmol, ICN) with a specific activity of ~ 3 x 10^8 cts/min per µg DNA (12). The autoradiography was carried out using Kodak X-Omat RP films with an intensifying screen for 3 days at -80°C.

RESULTS

Extrachromosomal ccc DNA Hybridizing with Sau3A Family DNA

We cloned extrachromosomal DNA in HeLa cells after its partial digestion with XbaI. Since Sau3A family DNA has one XbaI site in its basic unit, the majority of the extrachromosomal Sau3A family DNA was presumably linearized by this treatment. Among the total 1,613 colonies tested, 50 colonies had an

Table 1. Summary of Clones (pUCXba Series) from Extrachromosomal DNA

Clone no.[a]	Length of insertion (kb)	Classification	Map appeared in:
1	2.4	Recombinant[b]	Fig. 1, ref. 3
2 (10, 13)	2.2	Recombinant	Fig. 1, ref. 3
25 (26, 28, 33)	1.8	Related[c]	Fig. 4
30	1.3	Recombinant	Fig. 3
31 (32, 41[d])	0.85	Monomer	Fig. 1
34 (35)	1.2	Related	Fig. 5
36 (37)	1.2	Recombinant	Fig. 1
38 (42)	1.7	Dimer	Fig. 1

[a]Clones with identical restriction maps are in parentheses (see text).

[b]"Recombinant" is used for clones in which a fusion of two subunits of the Sau3A family was observed.

[c]"Related" is used for clones which had a considerably high (80~90%) but distinguishable sequence homology to the Sau3A family sequence and a subunit order similar to that of the Sau3A family.

[d]Clones nos. 31 and 32, with a HindIII site, are distinguishable from clone no. 41, with no HindIII site.

insertion at the cloning site of pUC19, and 18 of them hybridized with the Sau3A family DNA probe (pXR1). Restriction mapping analysis indicated the clones to be classifable into eight groups. Some appeared to be products duplicated during the cloning process, having identical restriction maps (for example, clone nos. 2, 10 and 13, see Table 1). Six clones (nos. 1, 2, 30, 31, 36 and 38) exhibited relatively strong hybridization patterns with the Sau3A probe, while two (nos. 25 and 34) hybridized weakly despite long insertion sequences. The eight clones were subjected to further characterization by the restriction mapping and/or nucleotide sequencing summarized in Table 1. It appeared that the former group (six clones) had a direct correlation with the Sau3A family in the nucleotide sequence, whereas the latter group (two clones) was distinct from the Sau3A family, although their sequences and subunit orders were similar to those of the Sau3A family.

Analyses of Clones with Sau3A Family Sequences

Figure 1 shows the restriction maps of pUCXba1, pUCXba2, pUCXba36, pUCXba31 and pUCXba38, all classified under the Sau3A family. These clones have at least one complete Sau3A family basic unit (849 bp) with five subunits (sub1 to sub5). Clones pUCXba31 and pUCXba38 comprise one (monomer) and two (dimer) basic units, respectively. On the other hand, pUCXba1, pUCXba2 and

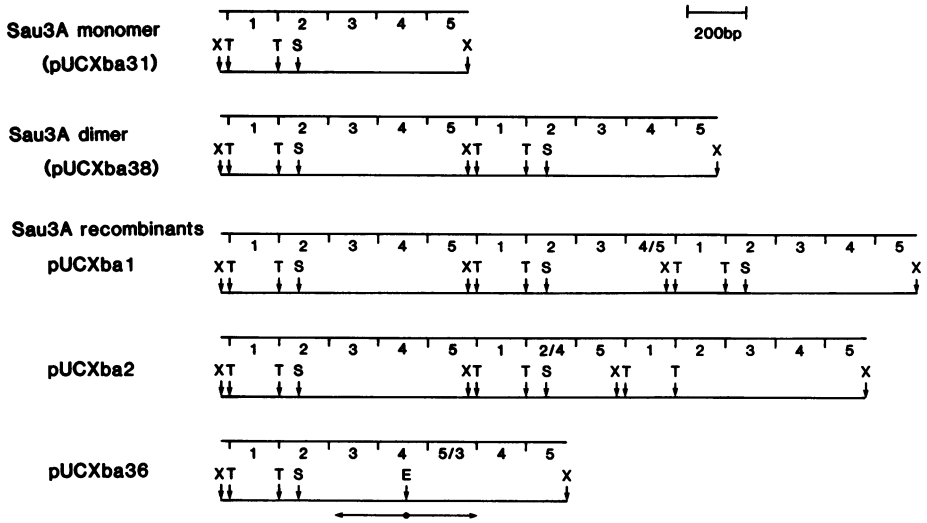

Figure 1. Restriction maps of clones of Sau3A monomer and dimer, and three Sau3A recombinants. For details, see text and Table 1. Subunit numbers of each clone were determined by restriction mapping and/or nucleotide sequencing. Among the restriction sites examined, XbaI (X), StuI (T), Sau3AI (S), and EcoRI (E) are depicted. The sequencing strategy for the recombination region in pUCXba36 is shown by horizontal arrows.

pUCXba36 are not complete oligomers but have an extra incomplete basic unit. The clones to be excised from the chromosomes by a recombination between two heterologous subunits in the tandemly repeated basic sequence have been suggested previously (1). Sequence analysis indicated the recombination to have occurred between sub4 and sub5 at nucleotides 40 to 57 of the subunit in pUCXba1, and between sub2 and sub4 at nucleotides 107 to 111 in pUCXba2.

The newly obtained clone, pUCXba36, has seven subunits in the order: 1, 2, 3, 4, 5/3, 4, 5, with one composite subunit (5/3) among them. Sequence analysis indicated the subunits other than the 5/3 composite to have approximately 97% homologies to the pUCXba1 and pUCXba2 subunit sequences.

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                30        40        50        60        70        80        90       100       110
sub5   ATCTTCACAGAAAAACTAAACAGAAGCATTCTCAGAAACTACTTTGTGATGTTTGTGTTCCACTTCAAGAAATGAACTTTCCTCTTGACA
pUCXba36 ATCTTCACAGAAAAACTAAACAGGAGCATTCTCAGAAACTGCTTTGTGATGTTTGCATTCAACTCACAGAGTTGAACCTTGCTTT-CATA
sub3   AACTTCCC---AGAACTACACGGAAGCATGCTGAGAAACTTCTTTGTGATGTTTCCATTCAACTCACAGAGTTGAACCTTGCTTT-CATA
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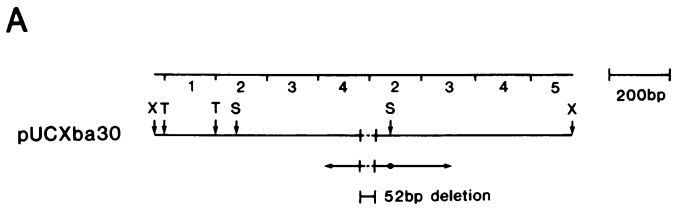
Figure 2. Nucleotide sequence of the recombination junction (5/3 recombination) compared with Sau3A family subunits, sub5 and sub3. Mismatched bases are shown by asterisks but mismatches to both parental subunits are shown by dots. The estimated recombination junction is indicated by a triangle.



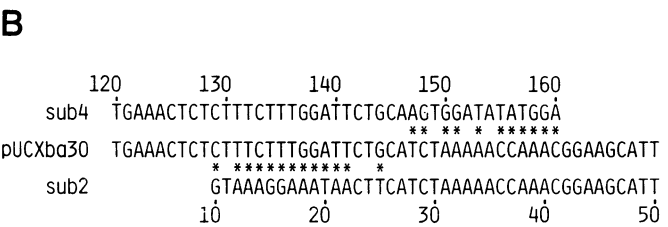


Figure 3. Restriction map (A) and nucleotide sequence of the recombination junction (B) of pUCXba30. For details, see text. Restriction sites: X, XbaI; T, StuI; S, Sau3AI. For nucleotide sequences, see legend to Figure 1.

Figure 2 shows the nucleotide sequence of the possible recombination junction in the 5/3 composite. The recombination seems to have occurred at nucleotide no. 76. It should be noted that no homology is present in the nucleotide sequences adjacent to the recombination site. The recombination event seems to have been totally reciprocal since no deletion or insertion was observed at the junction. On the other hand, we have found a long homologous region (nucleotide nos. 54 to 74 except for a mismatch at no. 61) between sub5 and sub3. This region may have been the initial recognition site for a putative recombinase in homologous recombination and, after the strands were displaced within this homologous region, a recombination-intermediate structure, such as a Holliday structure, may have moved to the position of nucleotide no. 76 before the heteroduplex was resolved.

We characterized another Sau3A family clone (pUCXba30) with a possible recombination site. Restriction mapping and subsequent nucleotide sequencing, however, indicated the mode of recombination to be different from the one observed in the three clones: pUCXba1, pUCXba2 and pUCXba36. As shown in Figure 3A, this clone did not retain the 170 bp subunit structure. It appears from sequence analysis, that a recombination event occurred between nucleotide nos. 145 or 146 of sub4 and 26 or 27 of sub2 (Fig. 3B). We have found a short homologous sequence (CTNCA, underlined in Fig. 3B) at both possible recombination sites.

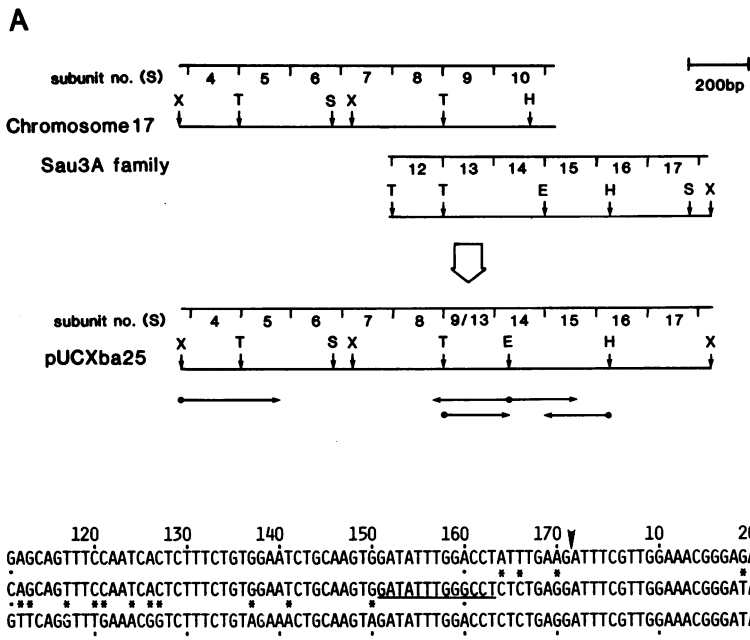


Figure 4. Restriction maps of a clone (pUCXba25) from a Sau3A-related sequence and its chromosomal sequence (A), and nucleotide sequence of the recombination junction (B). For details, see text and legend to Figure 1. Abbreviations for restriction sites are as follows: X, XbaI; T, StuI; S, Sau3AI; E, EcoRI; H, HindIII. The sequencing strategy is shown by horizontal arrows. A possible recombination junction is underlined in (B). A vertical arrow indicates the subunit junction.

Analysis of Clone pUCXba25 Derived from a Sau3A-Related Sequence

Figure 4 shows the structure of clone pUCXba25. This clone has a relatively long insertion (1.8 kb) with a structure apparently different from that of the Sau3A family. Restriction mapping and nucleotide sequencing of a part of the sequence (also shown in Fig. 4A) indicated this clone to have been derived from the Sau3A-related family on chromosome 17 (3). When we compared the structure of pUCXba25 with that of the family on chromosome 17, four subunits were found to have been apparently deleted in pUCXba25. As shown in Figure 4B, sequence analysis of the recombination junction strongly suggests that a recombination had occurred somewhere between nucleotides 151 to 163 between subunits S9 and S13 of the chromosome 17 sequence, deleting a portion of S9 and S13 and whole units of S10, S11 and S12 in chromosome 17. The recombination is totally reciprocal in subunits S9 and S13.

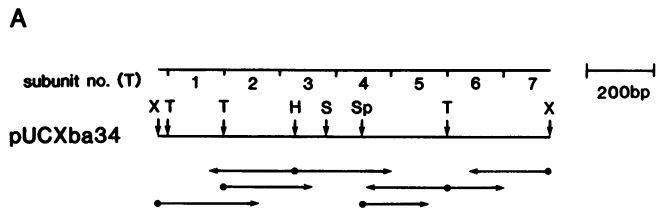


Figure 5. Restriction map (A) and nucleotide sequence (B) of a clone (pUCXba34) from a new Sau3A-related sequence. For details, see text and legend to Figure 1. Abbreviations for restriction sites are as follows: X, XbaI; T, StuI; H, HindIII; S, Sau3AI; Sp, SphI. The total nucleotide sequence (1,194 bp) of pUCXba34 is shown in a 171 bp-periodic table in (B). The nucleotide numbers of seven subunits (T1-T7) are indicated at the right of the figure.

Analysis of Clone pUCXba34 Derived from a New Sau3A-Related Sequence

The clone, pUCXba34, has an approximately 1.2 kb insertion corresponding to seven subunits (Fig. 5A). Using both restriction mapping and nucleotide sequencing, we failed to find any relation of the clone to the previously reported Sau3A and Sau3A-related sequences. Figure 5B shows the total 1,194 nucleotides of the pUCXba34 insertion in 170 bp periodic order. We designated the seven subunits, T1-T7. The 37 nucleotides at the extreme left and the 135 nucleotides at the extreme right combine into subunit T7 since these two regions are likely to be linked directly in the chromosomes (see below). The sequences of the seven subunits were compared with those of the five Sau3A family subunits (sub1 to sub5) (Fig. 6A). The Sau3A family subunit order is retained in the clone's sequence. The most homologous subunit numbers are 1, 2, 3, 4, 5, 1/4, and 5, from T1 to T7, there being one composite subunit (1/4 of T6) among them. Figure 6B shows the nucleotide sequence of the possible recombination junction in T6. When the T6 sequence was compared with those of both sub1 and sub4, it seemes that a recombination may have occurred somewhere between nucleotides 106 and 126. In this case, we could detect neither a base insertion nor deletion at the recombination junction. The overall homology of

A

Sequence Homology (%)

	T1	T2	T3	T4	T5	T6	T7
sub 1	80.1	75.4	70.8	74.9	71.3	76.0	75.4
sub 2	73.1	78.9	69.0	74.3	69.0	73.7	71.9
sub 3	70.2	68.4	82.5	72.5	75.4	70.8	79.5
sub 4	70.2	71.3	68.4	82.5	70.8	75.4	70.8
sub 5	71.9	69.0	77.8	74.3	78.9	70.8	88.9
homologous sub no.	1	2	3	4	5	1/4	5
possible recombination site						▲	

B

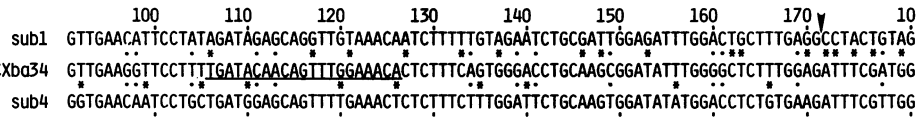


Figure 6. Sequence homology of subunits, T1-T7, of pUCXba34 to the Sau3A family subunits, sub1 - sub5 (A), and nucleotide sequences for the recombination junctions (B). The sequence homologies of both subunits in pUCXba34 (T1-T7) and in the Sau3A family (sub1 - sub5) are indicated in (A). The subunit numbers most homologous for each of subunits T1-T7 (percentages underlined) are indicated below the comparison table. A composite subunit (T6) is shown by a vertical arrow. The nucleotide sequence of the recombination junction in T6 is compared with sub1 and sub4 in (B). Details are indicated in the legends to Figures 1 and 4.

the sequence to that of the Sau3A family is 81.1%, suggesting this clone to have been derived from a new Sau3A-related sequence in the chromosomes.

Figure 7A shows the Southern blot analysis of the chromosomal DNA using pUCXba34 as a probe. Contrary to expectation, we were not able to detect any hybridized band at the 1.2 kb position after the XbaI digestion of the chromosomal DNA, whereas several discrete bands appeared at the upper region of the gel, suggesting a deletion of the subunits to have occurred during the excision event. The XbaI/HindIII digested chromosomal DNA gave the same 770 bp band as the clone DNA while the 430 bp band which emerged in the clone DNA did not appear. A subunit deletion may, therefore, have occurred somewhere between the XbaI site (in T7) and the HindIII site (in T3) in pUCXba34 (the

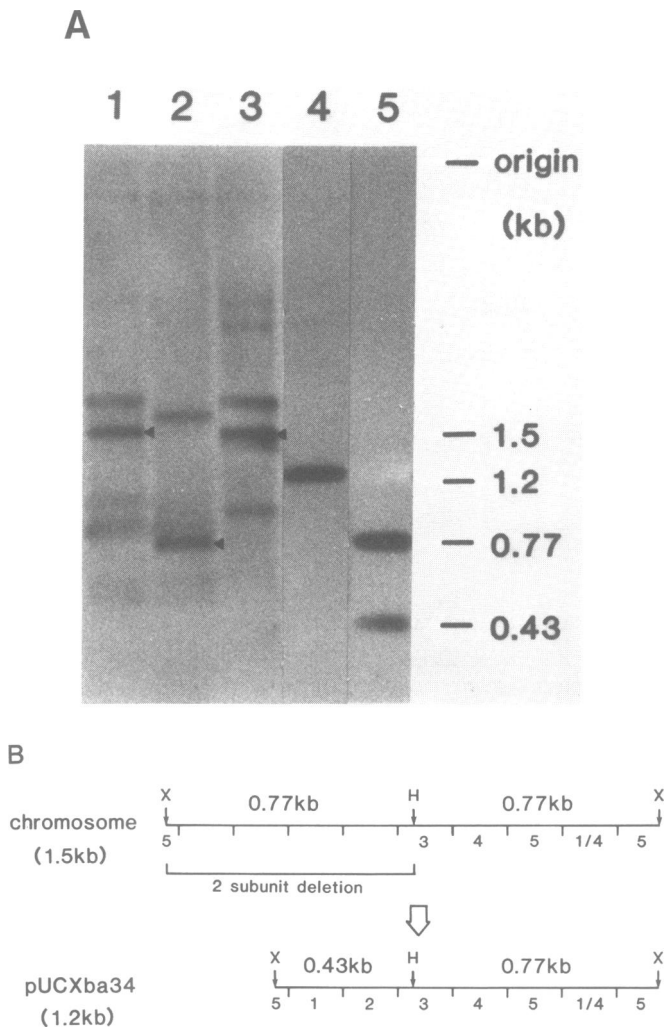


Figure 7. Southern blot analysis of chromosomal DNA with the clone, pUCXba34 (A), and the structures of pUCXba34 and its chromosomal counterpart (B). (A) Ten μg of HeLa chromosomal DNA was digested with XbaI (lane 1), XbaI and HindIII (lane 2), or HindIII (lane 3) before electrophoresis. The clone DNA (0.71 ng each lane), corresponding to 100 copies/cell, was digested with XbaI (lane 4), or XbaI and HindIII (lane 5). All DNAs were subjected to electrophoresis in 1.0% agarose gel and transferred to a nylon membrane. Southern hybridization was carried out with a nick-translated pUCXba34 insertion (1.2 kb). The experimental procedure is detailed in Materials and Methods. The chromosomal sequences from which clone pUCXba34 was derived are indicated by triangles. (B) For details, see text.

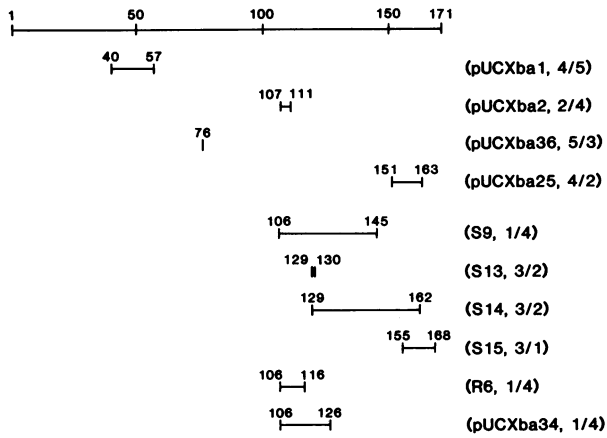


Figure 8. Summarized recombination junctions appeared in the Sau3A and Sau3A-related family sequence. For details, see text. Recombination junction data are described in ref. 3 (for pUCXba1, pUCXba2, S9, S13, S14, S15 and R6) and the present report (for pUCXba36, pUCXba25, and pUCXba34).

map appears in Fig. 5A). As shown in Figure 7A, we were able to detect a 1.5 kb band in both XbaI and HindIII digestions, and the intensity of several other bands decreased under more stringent washing conditions. These suggest the pUCXba34 chromosomal structure to have one XbaI and one HindIII site at an approximate distance apart of 770 bp in a 1.5 kb unit. Based upon these results, we constructed a map of the chromosomal counterpart of pUCXba34 (Fig. 7B). A two subunit deletion may have occurred somewhere between the XbaI site (in T7) and the next HindIII site (in T3). The recombination must have occurred between two derivatives from the same sub number, for example, between two sub2-derivatives, to show no trace of the recombination junction in the sequence. One could speculate on the existence of at least one more recombination junction in the chromosomal sequence apart from the 1/4 composite in T6.

DISCUSSION

Sau3A and Sau3A-related families can be classified as one sub-group of human alphoid satellite DNA. They have two unique characteristics, however: RFLP and frequent excisions from chromosomes (1). In the previous report, we discussed the issue of the same type of recombination being associated with both the rearrangement and excision of the family sequence (3). The recombi-

nation results in a total reciprocal exchange of homologous subunits. It occurs, however, not only between the same subunits but also between the heterologous subunits with only 70~ 80% sequence homology. The results presented here also confirmed the finding that complete homology between the two parental DNA molecules is not required for recombination, even although the process is totally reciprocal. The most significant example of the unique recombination mechanism is found in the case of pUCXba36, where homology is detected at neither recombination site (nucleotide no. 76, Fig. 2). As reported previously (Kiyama et al., unpublished results), although we could exclude the possibility of the DNA sequences in the clones being artefacts rearranged during the process of cloning rather than recombinants produced by excision, the possibility still exists of the recombinants being produced at the extrachromosomal DNA level after excision.

We previously reported seven composite subunits: 4/5 and 2/4 (from extrachromosomal DNA, ref. 3), 1/4, two 3/2s and 3/1 (from the chromosome 17 family, ref. 3) and 1/4 (from the X chromosome family, ref. 3). Of the 8 types of clones examined in this report, a total of three junctions have been newly found: 5/3 in pUCXba36, S9/S13 (equivalent to 4/2) in pUCXba25 and 1/4 in pUCXba34. Possible recombination sites determined to date are summarized in Figure 8. Subunits subl, sub2 and sub3 were used four times, sub4 six times and sub5 twice, suggesting all the subunits to be more or less equal. On the other hand, the recombination sites were apparently concentrated in the distal part of the subunits. At present, we do not know why the recombination site distribution is skewed toward that location. Recombination signals, such as the chi sequence (for review, see ref. 13) in bacteria, or specific secondary (hairpin) structures prone to recombination, may be present in that region and responsible for the degree of skew.

We have described one case (pUCXba30) in which the recombination apparently resulted in a non-reciprocal subunit exchange. This type of recombinant may represent another type of recombination involved in the excision of DNA from the chromosome although its frequency seems to be relatively low. Jones and Potter (14) showed a circular extrachromosomal DNA to be formed by homologous recombination in 9 bp repeat sequences separated from each other by stretches of 3.7 kb. The four base homology is present at the pUCXba30 recombination region (see Results).

There are several possibilities to consider regarding the mechanism by which the Sau3A family becomes prone to excision. These include (i) the tandem repetitiveness of the family sequence, (ii) the presence of specific

recombination signals, or (iii) the specific chromosomal location susceptible to excision. If (i) is correct, the frequency of recombination should show an increase when the homologous sequences are generally found located within short distances in the chromosomes. Riabowol et al. (15), however, showed the alphoid EcoRI family to be stable and to represent only a small portion (less than 1%) of the extrachromosomal DNA, even although it occupied 2~3% of the human chromosomal DNA. The fact that the recombination junctions are not randomly distributed in the subunits may suggest the second possibility to be correct. At present, little information is available for evaluation the third possibility of the regional instability of the chromosome, such as it possessing fragile sites, being responsible for the excision of the Sau3A family DNA.

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