Hum Genet (1993) 91:489–495

human genetics

Analysis of human extrachromosomal DNA elements originating from different β-satellite subfamilies

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Received: 4 August 1992 / Revised: 30 October 1992

Abstract. By screening total human DNA with probes derived from the small polydisperse circular (spc) DNA fraction of cultured human cells, we identified three clones that carry long stretches of β-satellite DNA. Further experiments have shown that the three sequences belong to at least two different β-satellite subfamilies, which are characterized by different higher order subunits. Members of one of these subfamilies are located in the cytological satellites of all acrocentric chromosomes, whereas members of another are located on the short arms of the acrocentrics on both sides of the stalk regions and also in the centromeric regions of chromosomes 1 and 9. This is the first time that β-satellite sequences obtained from the spcDNA of human cells have been assigned to β-satellite subfamilies that are organized as long arrays of tandemly arranged higher order monomers. This indicates that β-satellite sequences can be excised from their chromosomal loci via intrastrand-recombination processes.

Introduction

Numerous families of tandemly repeated DNA sequences are located within the human genome. Examples are the classical satellite DNA (Prosser et al. 1986), the alphoid sequence family (for a review, see Willard and Waye 1987) and the β-satellite sequences, also called *Sau*3A sequences (Meneveri et al. 1985; Waye and Willard 1989). The alphoid sequence family is by far the best characterized and can therefore, with certain reservations, serve as a model for other families of short clustered repetitive elements. This family (unit length 171 bp) can be divided into numerous subfamilies characterized by higher order monomers that are clustered to build up long sequence arrays within the centromeric regions of human chromosomes. Alphoid sequences have also been found within the small polydisperse circular DNA fraction (spcDNA fraction) of human cells (Jones and Potter 1985; Kunisada and Yamagishi 1987; Kiyama et al. 1987), where they seem to be enriched relative to their chromosomal copy numbers.

Comparatively less well-characterized is the family of β-satellite DNA. The basic unit is 68 bp long and is defined by the presence of one *Sau*3A restriction site within nearly every monomer. Clusters of β-satellite sequences have been shown to be located in heterochromatic regions of different human chromosomes, especially on the acrocentric chromosomes and on chromosomes 1 and 9 (Agresti et al. 1987; Waye and Willard 1989). Different subclasses of β-satellite sequences have been found that are structured like the alphoid subfamilies and that also build up long clusters. In addition to these long clusters, short stretches of β-satellite sequences are located within other DNA sequences (Hollis and Hindley 1986; Agresti et al. 1989). β-Satellite sequences are enriched within the spcDNA fraction of human lymphocytes (Hollis and Hindley 1986). Up to now, it has not been clear whether these extrachromosomal β-satellite sequences are derived from long clusters or from short dispersed family members. Now we have isolated three clones carrying up to 2.2-kb-long fragments of β-satellite sequences out of an spcDNA library derived from human cells. These three sequences belong to at least two obviously clustered β-satellite subfamilies. Together with the findings of alphoid sequences, this shows that the arrangement in long clusters might be a prerequisite for the preferred circularization of members of these sequence families.

Materials and methods

DNA techniques

The isolation and cloning of spcDNA from cultured cells was carried out as described elsewhere (Assum et al. 1989). Total human

This paper is dedicated to Prof. Dr. Ulrich Wolf on his 60th birthday, January 1993

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DNA was isolated from blood cells according to the method described by Miller et al. (1988). Southern blot hybridization and the radioactive labeling of DNA probes were performed according to standard procedures (Southern 1975; Feinberg and Vogelstein 1983). Prehybridization and hybridization solutions contained 5 × SSC, 5 × Denhardt's solution, 10% (w/v) dextran sulfate, and 10 µg/ml sonicated and heat-denatured human genomic DNA isolated from blood. After hybridization overnight at 65°C, membranes were washed in 1 × SSC. 0.1% (w/v) SDS for 30 min, 0.5 × SSC, 0.1% (w/v) SDS for 30 min, and 0.1 × SSC, 0.1% (w/v) SDS for 1 h at 65°C.

Probe preparation and in situ hybridization

Total DNA from clones JoG6, JoN10 and JoD8 was labeled by nick translation using biotin-11-dUTP (Brigati et al. 1983; Langer et al. 1981) or digoxigenin-11-dUTP. These probes were used for chromosomal in situ suppression (CISS) hybridization to elongated metaphase chromosomes as described (Lichter et al. 1990).

Aliquots of 200 ng biotinylated and digoxigenin labeled DNA were combined in cohybridization experiments with 10 µg total human Cot1 DNA (BRL), ethanol-precipitated and resuspended in 10 µl hybridization cocktail containing 50% formamide, 10% dextran sulfate and 2 × SSC. Following heat denaturation at 75°C for 5 min, the preannealed mixture was applied to prewarmed slides. Following hybridization at 37°C overnight and post-hybridization washes to a stringency of 0.1 × SSC at 60°C, the biotinylated probe was detected by incubation with avidin conjugated to fluorescein isothiocyanate (FITC) (Vector Laboratories) and the digoxigenin labeled probe via rhodamine-conjugated anti-digoxigenin antibodies (Boehringer, Mannheim). The location was also established by chromosome banding with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) and digital images were generated by a cooled charge-coupled device (CCD) camera (Photometrics, Tucson, Ariz., USA). The digitized images were processed and carefully aligned using Apple hardware and the software package NIH Image 1.41.

Results

In order to obtain information about the sequence composition of the spcDNA fraction of human cells, we isolated extrachromosomal DNA elements from primary cell cultures derived from a neurofibrosarcoma. Total spcDNA was digested with *Bam*HI and *Bgl*II, and ligated into the *Bam*HI site of pGEM3Z. Out of the approximately 200 spcDNA clones obtained, 20 were randomly selected and hybridized to total human DNA digested with *Bam*HI or *Bgl*II. The resulting banding patterns allow a preliminary characterization of copy numbers and structures of their chromosomal counterparts. Using one of these clones, JoG6, with a DNA insert of 2.2 kb, we obtained complex banding patterns (Fig. 1a). Using JoG6 as a probe for colony filter hybridization of the spcDNA library, we identified two further clones with inserts crosshybridizing to JoG6. These two clones, JoN10 (with a DNA insert of 1 kb) and JoD8 (with a DNA insert of 0.9 kb) also showed complex banding patterns when hybridized to digested human DNA (Fig. 1b, c). Therefore, the three clones should contain copies of the same repetitive DNA family.

To analyze further to which family of repetitive DNA the inserts belonged, we established partial DNA sequences from both ends of the three DNA fragments. In addition, we cut the JoG6 insert at single *Kpn*I and *Pst*I restriction sites and subcloned the resulting fragments to obtain DNA sequence information from internal parts of JoG6. Figure 2 shows the DNA sequences from the three clones. The DNA sequences revealed a 68-bp periodicity with a *Sau*3A restriction site present within nearly every monomer, the monomers being arranged regularly in a head-to-tail fashion. This is the typical structure of the human β-satellite family. The consensus sequences derived from the 68-bp monomers of the three clones are identical to each other and are also identical to the consensus sequence of the β-satellite family (Fig. 2).

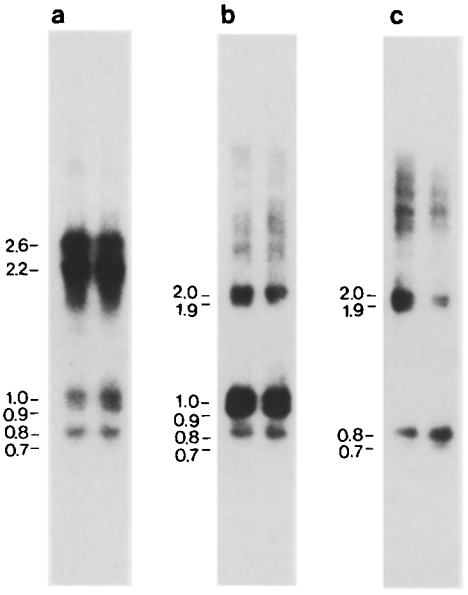
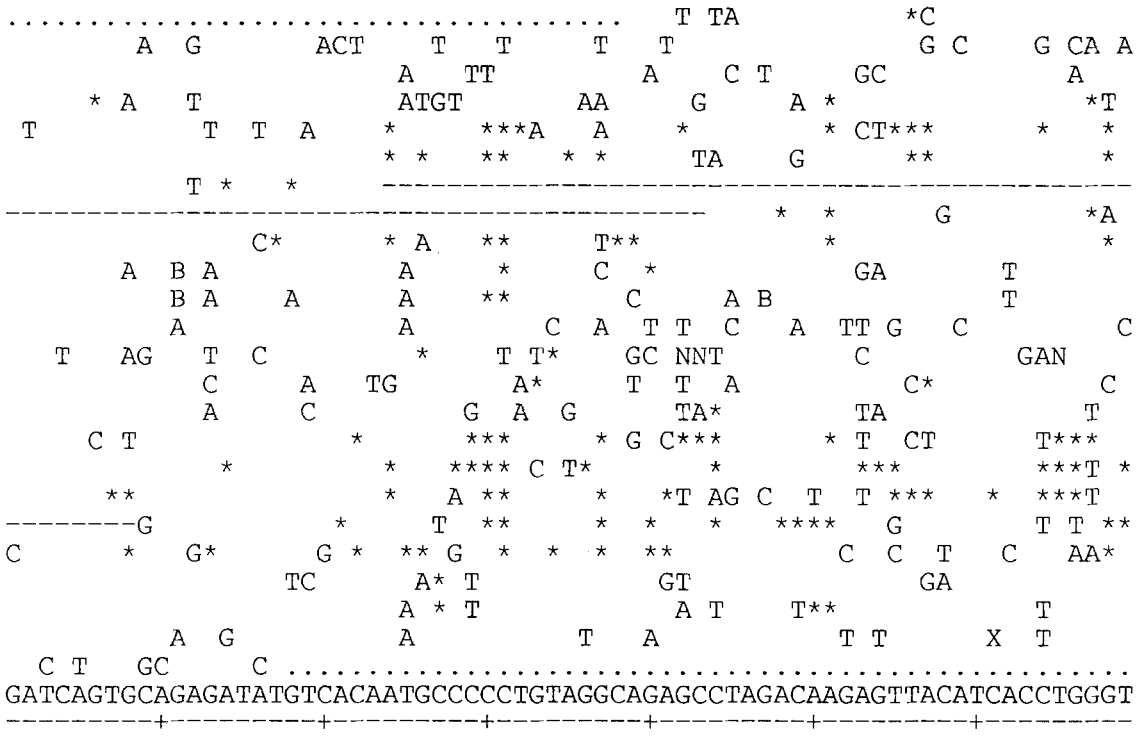


Fig. 1a–c. Hybridization of **a** JoG6, **b** JoN10 and **c** JoD8 to Southern blots of human genomic DNA (female and male, *left* and *right lane*, respectively) cut with *Bgl*II. *Numbers* indicate fragment sizes in kilobases

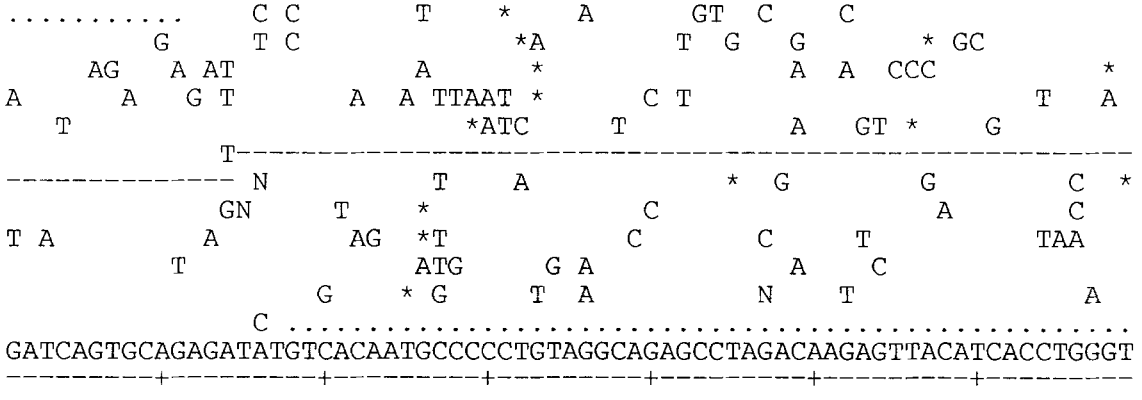
In order to analyze whether the DNA inserts of JoG6, JoN10 and JoD8 were composed only of members of the *Sau*3A family, or whether they contained sequences not related to this family, we digested the isolated inserts of the three clones with *Sau*3A and separated the resulting DNA fragments on an agarose gel (Fig. 3). In the case of JoG6 and JoD8, we only obtained the monomer 68-bp unit and oligomers thereof. Within the pattern of JoN10, an additional band appeared that corresponded to an approximately 100-bp-long DNA fragment. This fragment possibly also contained *Sau*3A-specific DNA sequences originating from a dimer of the 68-bp unit that contained one mutated in-frame *Sau*3A restriction site and an additional *Sau*3A site not fitting into the 68-bp frame. There-

Fig. 2. Sequence data of clones JoG6, JoN10 and JoD8, aligned according to β-satellite monomer structure. The three consensus sequences are identical to each other and differ from the consensus sequences of Hollis and Hindley (1986) and Agresti et al. (1989) only by an additional C at position 31. *Asterisks,* deletions introduced to maximize the homology; *points,* boundaries of the clones; *dashes,* parts inside the clones for which sequence data are not available. The *X* JoG6 corresponds to CAACCT, i.e. a duplication of 4 bp

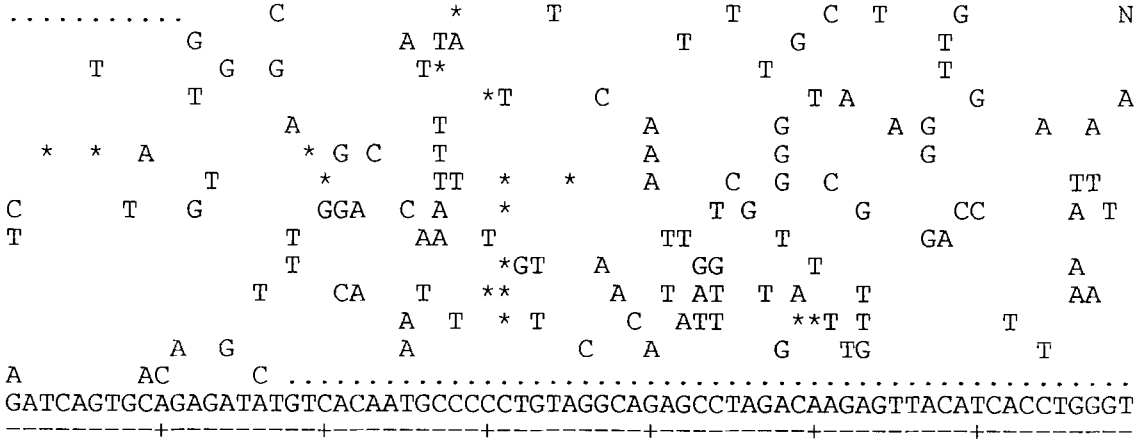
JoG6; partial sequence



JoN10; partial sequence



JoD8; complete sequence



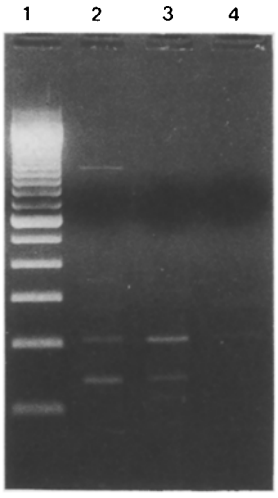


Fig. 3. *Sau*3A-digested inserts of clones JoG6 *(lane 2)*, JoN10 *(lane 3)* and JoD8 *(lane 4)*, separated on an agarose gel (2% w/v), and stained with ethidium bromide. *Lane 1*, 100-bp ladder

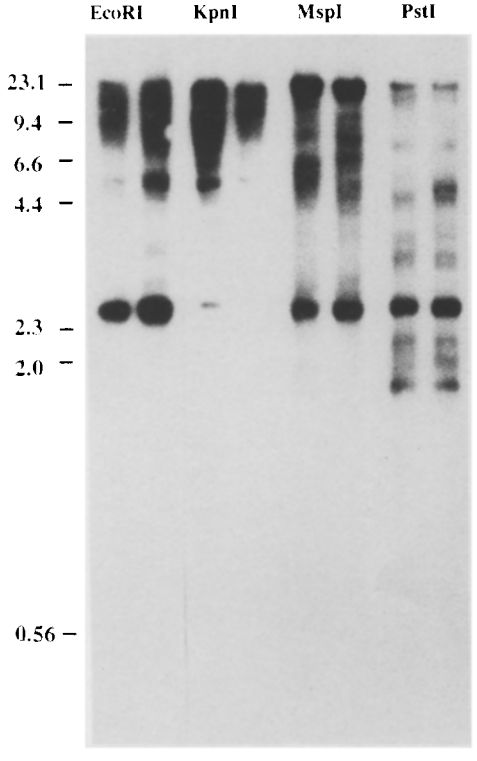


Fig. 4. Hybridization of JoG6 to Southern blots of human genomic DNA (female and male, *left* and *right lane*, respectively) cut with the restriction endonucleases indicated above. *Numbers* indicate fragment sizes in kilobases

fore, the human DNA sequences present in JoG6 and JoD8, and probably also in JoN10, seem to be composed entirely of β-satellite family related sequences. The fact that each of the three clones shows a different banding pattern when hybridized to *Bgl*II-digested human DNA, and the fact that the inserts of at least two clones contain only sequences related to the β-satellite family indicate that JoG6, JoN10 and JoD8 may define distinct subfamilies of the β-satellite family. These subfamilies are called the G6, N10 and D8 subfamily below.

The β-satellite family and other tandemly arranged repetitive DNA families, such as the alphoid DNA sequences, are defined by different higher order periodicities. To analyze whether this is also true for our β-satellite subfamilies, we hybridized the inserts of JoG6, JoN10 and JoD8 to total human DNA digested with different restriction enzymes. The results are shown in Fig. 4. The banding patterns of JoG6, obtained after the hybridization of DNA digested with *Eco*RI, *Msp*I, *Kpn*I, and *Pst*I are dominated by a JoG6-specific band corresponding to an approximately 2.8 kb DNA fragment. Moreover, strong JoG6-specific fragments with a length of approximately 5.6 kb also appeared after digestion of human DNA with *Kpn*I and *Eco*RI. This clearly indicates that the G6 subfamily indeed has a higher order periodicity with a basic unit of 2.8 kb with one restriction site for the enzymes *Eco*RI, *Msp*I, *Kpn*I, and *Pst*I present in nearly every unit and the 2.8 kb units tandemly arranged in a head-to-tail fashion. A similarly structured β-satellite subfamily has been described as the pB3 subfamily by Waye and Willard (1989). A 2.8-kb G6-specific band also appeared after *Bgl*II digestion of human DNA (Fig. 1) but, in this case, the most prominent G6-specific band corresponded to a 2.2-kb fragment. Therefore, most of the basic units of the G6 subfamily seem to contain two *Bgl*II restriction sites. The length of this 2.2-kb *Bgl*II fragment corresponds well to the length of the DNA fragment cloned in JoG6, indicating that this clone contains only a part of the G6 repetition unit. Two of the four restriction sites (*Kpn*I and *Pst*I) that define the higher order structure of G6 can be found in the cloned part of G6, whereas the other two (*Eco*RI and *Msp*I) must be located in the remaining 600-bp fragment.

Both JoN10 and JoD8 hybridize almost exclusively to large DNA fragments when each of the four enzymes is used to cut human DNA (data not shown). Therefore total human DNA was digested with another set of enzymes (*Alu*I, *Hae*III, *Hin*fI, *Rsa*I) and hybridized with both clones (Fig. 5). The restriction patterns obtained with JoD8 and JoN10 are composed of clone-specific bands and of bands hybridizing with both probes. This can be explained either by the assumption that JoD8 and JoN10 define different but closely β-satellite subfamilies, or that JoD8 and JoN10 represent two different *Bgl*II restriction fragments derived from the same subfamily. Unfortunately, the restriction patterns do not provide any information about the length of their repetitive units. As the alternative explanation of assuming a large repetitive unit for D8 and N10 seems to be unlikely, we favor the hypothesis that N10 and D8 are arranged as clusters within the genome, and that all of the enzymes used either do not cut (as in the case of *Eco*RI) or have more than one restriction site (as in the case of *Alu*I, *Hae*III, *Hin*fI, *Rsa*I) within the higher order units.

In this study, a more detailed chromosomal localization of the three members of the β-satellite family was carried out using fluorescent in situ hybridization. The signals of JoN10 could only be detected in the terminal regions of the short arms of the acrocentric chromosomes (Fig. 6a;

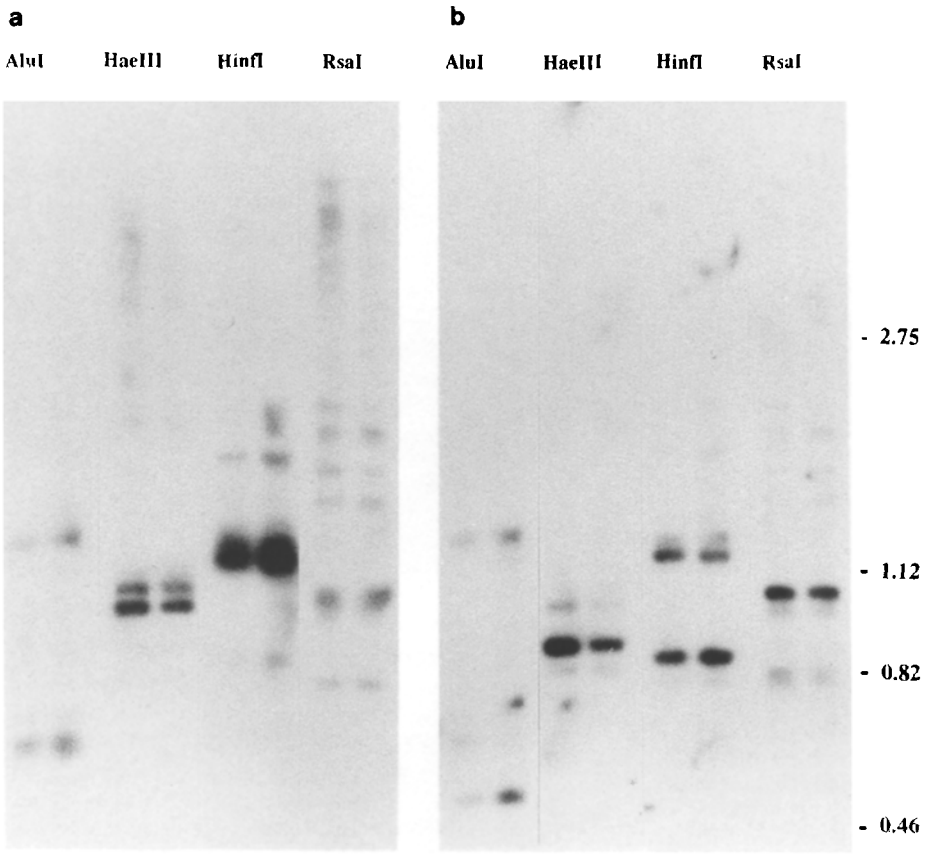


Fig. 5a, b. Hybridization of **a** JoN10 and **b** JoD8 to Southern blots of human genomic DNA (female and male, *left* and *right lane*, respectively) cut with the restriction endonucleases indicated above. *Numbers* indicate fragment size in kilobases

arrows), whereas JoG6 in addition hybridized to the pericentric region of all acrocentric chromosomes and to the centromeric regions of chromosomes 1 and 9 (Fig. 6b; arrowheads). Thus, the signals on the centromeric regions appear on both sides of the stalk regions. In another experiment, cohybridization of differentially labeled JoD8 with JoN10 revealed no differences in the hybridization pattern of these clones (data not shown).

In contrast to our results obtained for the G6 subfamily, Waye and Willard (1989) found their pB3 subfamily (which is probably identical to the G6 subfamily) to be located only in the heterochromatic region of chromosome 9. Our in situ hybridization experiments showed that G6 is located on the seven pairs of chromosomes listed above, where we obtained clear signals under conditions of high stringency in seven independent hybridization experiments. We have, at present, no explanation for this discrepancy, but probably JoG6 and pB3 define different β-satellite subfamilies, despite the similaries obtained in Southern blot hybridization.

Discussion

In this paper, we describe the isolation and characterization of three clones carrying β-satellite-related DNA sequences out of the spcDNA fraction of human cell cultures. Within the human genome, β-satellite sequences are located in the heterochromatin of chromosomes 1 and 9, and on the short arms of the acrocentrics, on both sides of the rDNA region. In addition to the G6 subfamily and other subfamilies described by Greig and Willard (1992), a subfamily or subfamilies defined by D8 and N10 have been localized in the telomeric regions of the short arms of all acrocentric chromosomes. Therefore, various β-satellite subfamilies, like other families of repetitive DNA elements (Assum et al. 1991; Kurnit et al. 1984; Worton et al. 1988) can be found at the same loci on all acrocentrics; this provides additional support for the high degree of sequence homology found within these chromosomal regions. In addition to the β-satellite sequence, another family of repetitive DNA elements, the chAB4 family (Assum et al. 1991), is located within the heterochromatic blocks of the acrocentrics and on chromosomes 1 and 9. Therefore, the heterochromatic sequences of these seven chromosomes might be closely related; this can be explained by a frequent exchange of sequences between these heterochromatic regions. These sequence exchanges supposedly take place during the aggregation of the heterochromatin of different chromosomes in interphase (Haaf et al. 1986). Alternatively, one could speculate that extrachromosomal DNA elements, like those that we have isolated, are able to mediate the interchromosomal exchange of DNA sequences.

This is not the first report concerning the isolation of β-satellite sequences from the spcDNA fraction of human cells (Hollis and Hindley 1986). The three clones that we have isolated are the first to carry longer β-satellite-specific inserts, and are related to at least two different subfamilies of clustered elements. This is important, since β-satellite sequences are not only found in long arrays; short

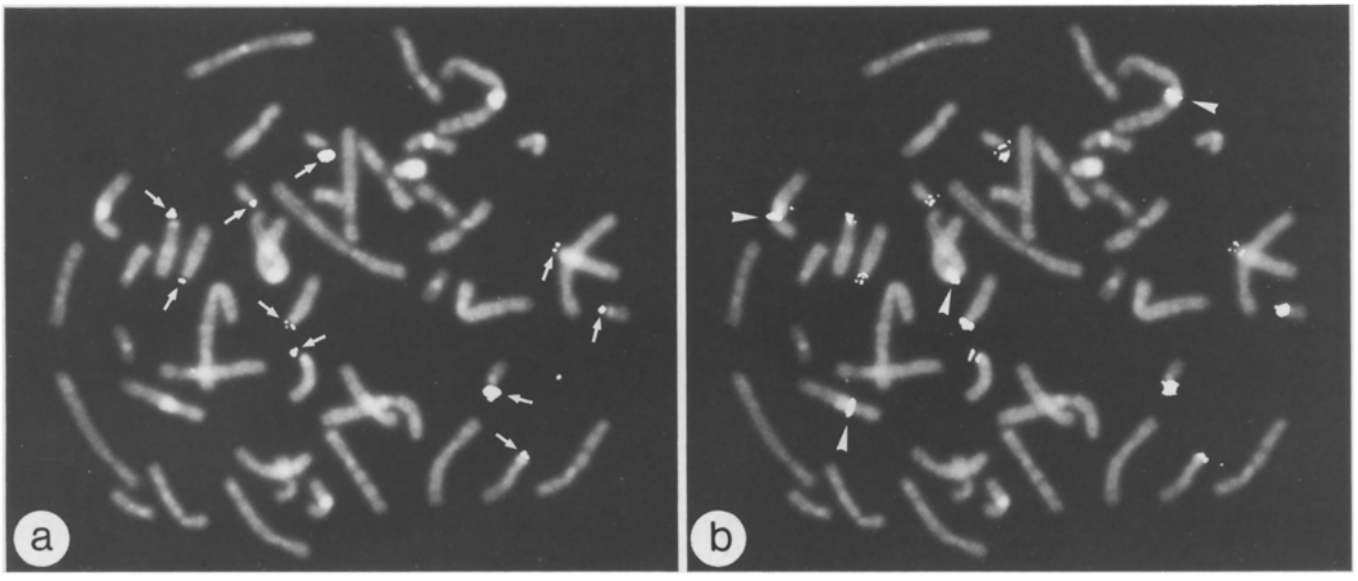


Fig. 6a, b. Mapping of differentially labeled β-satellite probes JoG6 and JoN10 cohybridized to a human metaphase spread (46,XY). **a** The digoxigenin labeled probe JoN10 was visualized via rhodamine fluorescence and is located in the terminal regions of the short arms of the acrocentric chromosomes *(arrows)*. **b** The same metaphase as in **a** hybridized with the biotinylated probe JoG6 and detected via fluorescein (FITC) fluorescence. JoG6 is not only located in the terminal regions of the short arms and in the pericentric regions of the acrocentric chromosomes but also in the centromeric regions of chromosomes 1 and 9 *(arrowheads)*. Separately recorded digitized images of FITC, rhodamine and 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) fluorescence were generated with the use of a CCD camera and processed and overlaid electronically. Pictures were taken directly from the video screen

stretches comprising only a few β-satellite monomers have also been found dispersed among non-satellite DNA (Hollis and Hindley 1986) or accompanied by other repetitive DNA elements (Agresti et al. 1989). The fact that all of our three clones, isolated from spcDNA, are derived from the first class of β-satellite DNA suggests that the arrangement in long arrays with higher order periodicities might be a prerequisite for the excision and circularization of β-satellite sequences. The structure of clustered elements with higher order periodicities and the small divergence rates between the higher order monomers perhaps enables the circularization of these sequences via intrastrand recombination. SpcDNA molecules carrying one or a number of complete higher order monomers could result from this process. β-Satellite sequences represent at least 1%–2% of the sequences of our spcDNA library, and therefore seem to be overrepresented relative to their contribution to human genomic sequences (0.1%). This is in good agreement with the finding of Hollis and Hindley (1986) for human lymphocytes. Kiyama et al. (1987) have found that a substantial part (1%) of alphoid sequences, which seem to belong to various subfamilies, are located extrachromosomally in HeLa cells. Thus, clustered repetitive sequences seem indeed to be prone to becoming excised from their chromosomal loci, a fact that in relation to their ability to frequent unequal crossovers, can explain the rapid evolutionary changes found within these sequence classes. Further analysis of spcDNA sequences will establish whether this holds true for families of clustered repetitive elements that may not as yet be known.

Acknowledgements. We gratefully acknowledge Professor Dr. Winfrid Krone for a critical review of the manuscript and many helpful suggestions, and Dr. Peter Lichter for support in digital imaging microscopy. Hannelore Halm and Brigitte Assum have contributed to this work by preparing the manuscript. This project was supported by the Deutsche Forschungsgemeinschaft.

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