Hum Genet (1994) 93:383–388

human genetics

Chromosomal localization of human satellites 2 and 3 by a FISH method using oligonucleotides as probes

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Received: 30 July 1993

Abstract. Classical satellites I, II and III are composed of a mixture of repeated sequences. However, each of them contains a simple family of repeated sequences as a major component. Satellites 2 and 3 are simple families of repeated sequences that form the bulk of human classical satellites II and III, respectively, and are composed of closely related sequences based on tandem repeats of the pentamer ATTCC. For this reason, extensive cross-hybridizations are probably responsible for the similar in situ hybridization patterns obtained for satellites II and III. We have used a fluorescent in situ hybridization method with highly specific oligonucleotides for satellites 2 and 3, respectively, as probes. Our results show that satellite 2 is mainly located on chromosomes 1, 2, 10 and 16, whereas the major domain of satellite 3 is on chromosome 9. Furthermore, minor sites of satellites 2 and 3 are shown. Two-colour in situ hybridizations have enabled us to define the spatial relationships existing between the major domains of both satellites and centromeric alpha satellite sequences. These experiments indicate that the heterochromatin regions of chromosomes 1, 9 and 16 have different molecular organizations.

Introduction

Human DNA can be fractionated by ion-$CsSO_4$ gradients to reveal three major satellites, designated I, II and III. These satellites are known as the classical satellites (for a review, see Jones 1973).

It has been demonstrated by a variety of criteria that satellites II and III are closely related. Melting profiles of both satellites are essentially indistinguishable and they show identical $C_0t_{1/2}$ values (Mitchell et al. 1979). Furthermore, they exhibit similar restriction patterns when they are digested with certain restriction enzymes (Mitchell et al. 1979; Frommer et al. 1982; Prosser et al. 1986). From the point of view of sequences, a significant extent of homology exists between satellites II and III, since both of them are based on the simple repeat ATTCC (Mitchell et al. 1979; Frommer et al. 1982; Prosser et al. 1986; Hollis and Hindley 1988). Consequently, approximately 40% of the molecules present in satellite III cross-react with about 10% of the molecules of satellite II (Mitchell et al. 1979).

With a few exceptions, ^{3}H-RNAs synthesized on templates of satellites II and III hybridize in situ to the same chromosome regions, particularly to the heterochromatic blocks of chromosomes 1, 9, 16, acrocentrics and Y (Jones and Corneo 1971; Jones et al. 1973, 1974; Evans et al. 1974; Gosden et al. 1975). The similarity between satellites II and III could explain their overlapping in situ hybridization patterns, i.e. these patterns are the result of cross-hybridization reactions. However, it is also possible that different satellites occur within the same chromosomes (as different blocks or as interspersed arrays).

Each of classical satellite DNAs (I, II and III) is composed of a mixture of different repeated sequences. However, all of them contain a single family of simple repeated sequences as a major component. The three simple sequence families are called satellites 1, 2 and 3 to indicate the enrichment of each, in each of the classical satellites I, II and III (Prosser et al. 1986).

Here, we have used a fluorescent in situ hybridization (FISH) procedure with oligonucleotides as probes to determine the distribution of satellites 2 and 3 on human chromosomes. Furthermore, by means of two-colour FISH, we have studied the spatial relationship existing between alpha satellite sequences and the major domains of satellites 2 and 3.

Materials and methods

Chromosome preparations obtained from cultured lymphocytes of four unrelated individuals were treated with RNase A (100 µg/ml) in 2 × SSC (1 × SSC = 150 m*M* NaCl/15 m*M* sodium citrate, pH 7.0) at 37°C for 60 min. The slides were then incubated with 0.005% pepsin in 10 m*M* HCl for 10 min at 37°C.

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The probes used in the present study, designated S.2, S.3 and A.0, were single-stranded oligonucleotides obtained by automated synthesis. S.2 and S.3 comprised highly specific regions of the consensus sequences of satellites 2 and 3 respectively (Table 1). A.0 contained a highly conserved region found within the 171-bp alphoid monomer (base positions 23–51 in the consensus sequence derived by Choo et al. 1991). Their sequences are: S.2: 5′-TCG AGT CCA TTC GAT GAT-3′ (18-mer); S.3: 5′-TCC ACT CGG GTT GAT T-3′ (16-mer); A.0: 5′-ATG TGT GCA TTC AAC TCA CAG AGT TGA AC-3′ (29-mer).

Oligonucleotides were labelled at the 3′ end with biotin-16-dUTP (S.2 and S.3) or digoxigenin-11-dUTP (A.0), by means of terminal deoxynucleotidyl transferase (TdT), as described elsewhere (Tagarro et al. 1994). Chromosomes were denatured for 2 min in 70% formamide, 2 × SSC, at 80°C. After being dehydrated in an ethanol series, hybridization was performed for 18 h at 35°C (S.2) or 33°C (S.3). The hybridization solution contained 2 ng/μl probe, salmon sperm DNA (50 μg/μl), yeast RNA (50 μg/μl), 25% formamide, 4 × SSC, 50 m*M* NaH_2PO_4/Na_2HPO_4 pH 7.0, 1m*M* EDTA. After hybridization, the slides were washed three times for 5 min in 2 × SSC at room temperature.

For detection, preparations were incubated with fluorescein-isothiocyanate (FITC)-avidin conjugate (Vector Laboratories) (5 μg/ml in 4 × SSC plus 5% non-fat dry milk) for 30 min at room temperature. They were then washed three times for 5 min with 4 × SSC plus 0.05% Tween-20. Signal amplification was performed by treating the slides with a biotinylated goat anti-avidin (Vector Laboratories) (5 μg/ml in 4 × SSC plus 5% non-fat dry milk) for 30 min at room temperature. Preparations were washed again three times for 5 min with 4 × SSC plus 0.05% Tween-20, and a new incubation with FITC-avidin conjugate was carried out for 30 min at room temperature. Counterstaining was performed with propidium iodide (5 μg/ml in PBS) or DAPI (4,6 diamidino-2-phenyl-indole) (1 μg/ml). Chromosomes were identified by DAPI/AMD (actinomycin D) staining (Schweizer 1976).

For two-colour FISH, the hybridization solution contained two different probes (either S.2 and A.0, or S.3 and A.0) at the same concentration (2 ng/μl). Detection was performed as described above but, in the last incubation with FITC-avidin conjugate, tetramethylrhodamine-isothiocyanate (TRITC)-sheep anti-digoxigenin antibody (Boehringer-Mannheim, Germany) (4 μg/μl) was added. Microphotographs were taken on Kodak Ektachrome 160 film (tungsten illumination), by performing multiple exposures with adequate filter combinations on a Zeiss Standard microscope equipped for epi-illumination. Since there was a danger of image shifts between the different exposures, the results described here for each chromosome were checked in both homologues in more than 100 metaphases. Futhermore, two-colour hybridizations on chromosomes with 5-azacytidine-undercondensed heterochromatin were performed. Undercondensation of the major heterocromatin regions was performed according to Schmid et al. (1983).

Results and discussion

The simple repeated components 2 and 3 are clearly related in sequence, since both appear to have originated from 5-bp sequence ATTCC. Thus, to prevent cross-hybridizations, the oligonucleotides that we have chosen to detect satellites 2 and 3 comprise highly specific regions of the consensus sequences of satellites 2 and 3, respectively. In other words, our oligonucleotides do not contain tandem repeats of the sequence ATTCC, common to both satellites 2 and 3 (Table 1). We can assume that S.2 and S.3 are representative of the whole satellites 2 and 3, respectively, since differences between subfamilies of specific satellites are known to be based on higher order repeat units and not on the basic repeat (Beauchamp et al. 1979; Willard 1991; Jackson et al. 1992). Even so, the stringency conditions that we have used allow about 2–3 mismatches (Sambrook et al. 1989). Thus, for example, when A.0 is used as a probe, labelling occurs on all the centromeres and not merely on a certain subset (Tagarro et al. 1994).

Table 1. Consensus sequences of satellites 2 and 3 (Prosser et al. 1986). The underlined segments of the consensus sequences correspond to the sequences of oligoprobes S.2 and S.3, respectively

Satellite 2	ATTCCATTCG$\underset{G}{\overset{A}{}}G\underset{T}{\overset{A}{}}$CCATTCGATGAT
Satellite 3	$(ATTCC)_n$ATTCCA$\underset{C}{\overset{T}{}}$TCGGGTTGATTCC$(ATTCC)_n$

FISH with the probe S.2 revealed that satellite 2 was localized on the large heterochromatin regions of chromosomes 1 and 16, and on the pericentromeric regions of chromosomes 2 and 10 (Fig. 1a–c). This pattern was consistently observed in 96% of metaphases (122 metaphases scored). On interphase nuclei, the four large signals corresponding to chromosomes 1 and 16 could be consistently seen, whereas the minor signals frequently appeared masked by the counterstaining (Fig. 1e). Furthermore, when a higher probe concentration (10 ng/μl) and a dull counterstaining were used (DAPI observed with the filter for FITC), additional minor sites could be observed (chromosomes 7, 15, 17 and 22) (Fig. 2a).

On the other hand, FISH with probe S.3 revealed that satellite 3 was localized on the large heterochromatin block of chromosome 9 (Fig. 1d). Both chromosomes 9 were labelled in 100% of metaphases (112 metaphases scored). Interphase nuclei also showed two clear hybridization signals (Fig. 1f). Moreover, in this case, the use of higher probe concentrations and DAPI counterstaining allowed the detection of additional minor sites (chromosomes 1, 5, 10, 17, 20 and acrocentrics) (Fig. 2b). The coincidence of the localization of satellite 3 and the specific chromosome staining with Giemsa at pH 11 (Giemsa-11) (Bobrow et al. 1972) is of particular interest; perhaps alkaline Giemsa stains a fraction of proteins that specifically binds to satellite 3 DNA.

Our results demonstrate that both satellites 2 and 3 are present in the heterochromatin block of chromosome 1. However, whereas satellite 2 constitutes a prominent block (Fig. 1a, c), satellite 3 occupies a minor domain (Fig. 2b). Satellites 2 and 3 do not co-exist within the large heterochromatin regions of chromosomes 9 and 16. Previously reported hybridizations of both satellites II and III to such chromosome regions can be ascribed to cross-hybridization reactions occurring between ATTCC-based sequences. Alternatively (or additionally), certain subsets of alpha satellite sequences that form part of satellites II and III (Mitchell et al. 1979; Prosser et al. 1986) could account for the cross-hybridizations.

Classical satellites II and III (2% and 1.5% of the genome, respectively) are much more abundant than satellite I (0.5% of the genome) (Jones et al. 1974; Gosden et al. 1975; Mitchell et al. 1979). This is also shown by our results since, whereas satellites 2 and 3 constitute the most conspicuous autosomal heterochromatin blocks,

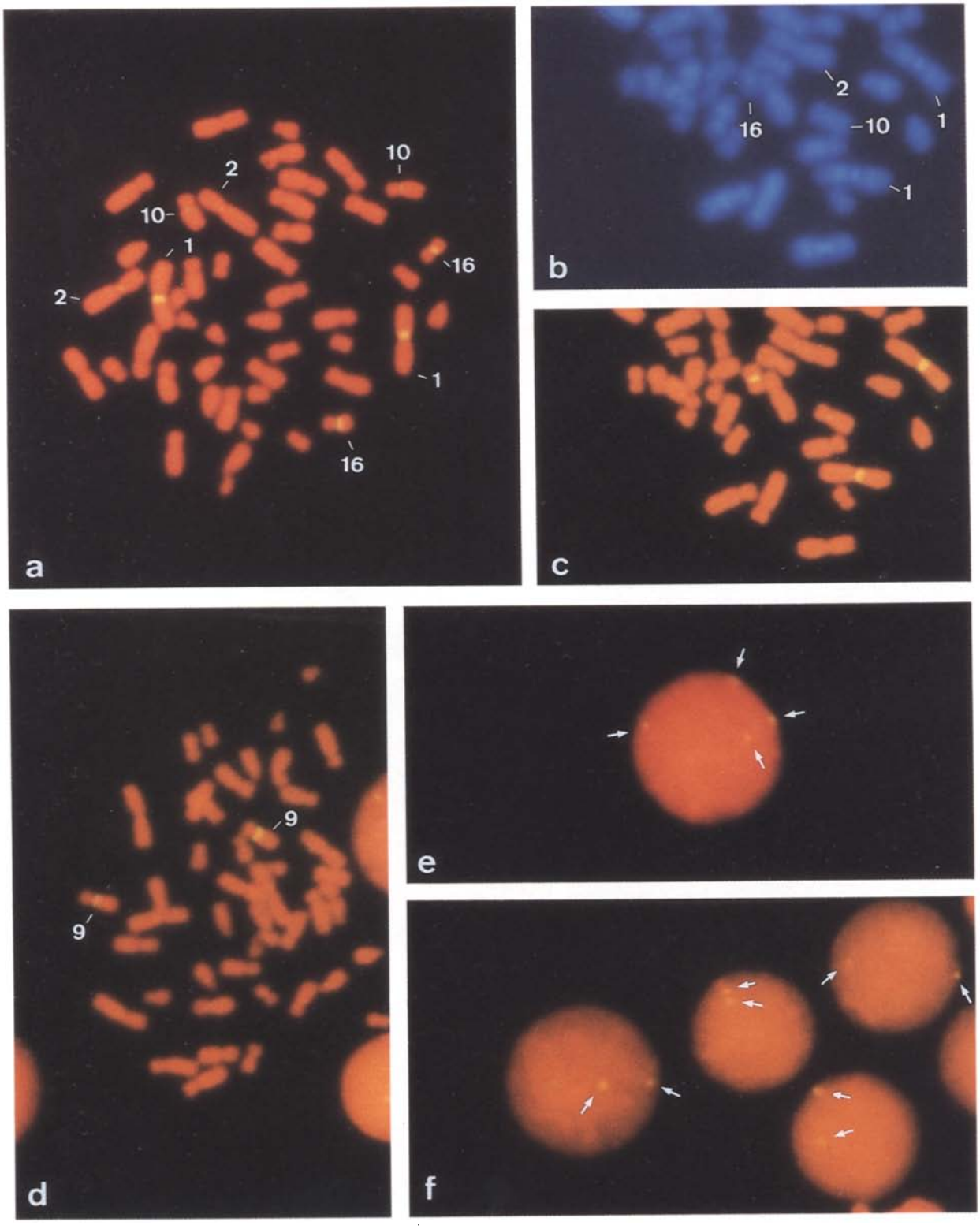

Fig. 1. **a** Fluorescent in situ hybriditation (FISH) of the probe S.2 on human chromosomes. **b** DAPI/AMD banding performed for chromosome identification. **c** FISH of S.2 on the same metaphase. **d** FISH of probe S.3 to a metaphase. **e** Hybridization of the oligonucleotide S.2 on an interphase nucleus. Signals corresponding to chromosomes 1 and 16 are indicated (*arrows*). **f** Hybridization of the oligonucleotide S.3 on interphase cells. Signals corresponding to both chromosomes of pair 9 are indicated (*arrows*)

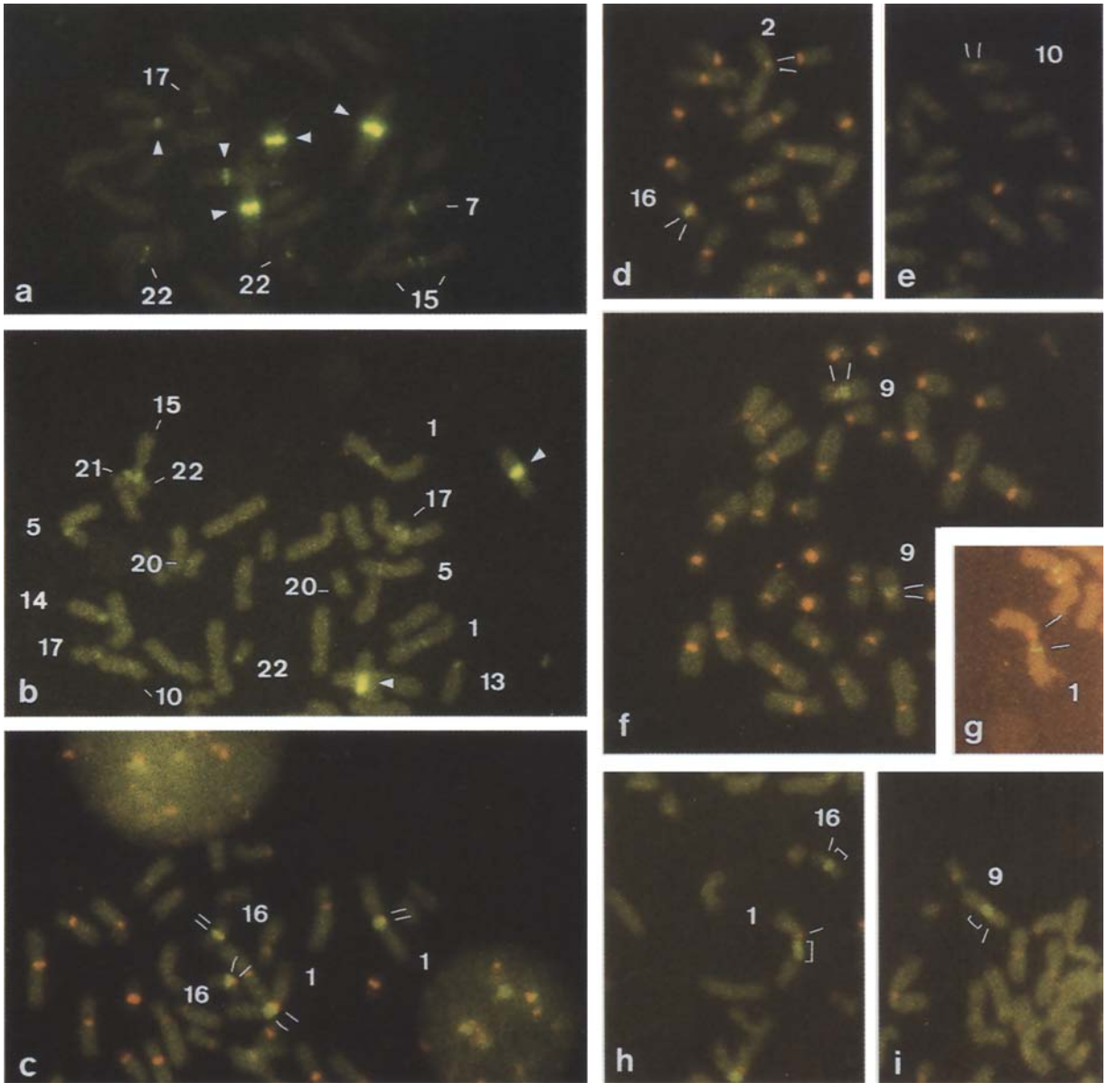

Fig. 2. **a** Minor hybridization sites of S.2 on chromosomes 7, 15, 17 and 22. Major sites (chromosomes 1, 2, 10 and 16) are indicated (*arrowheads*). **b** Minor hybridization sites of S.3 on chromosomes 1, 5, 10, 17, 20 and acrocentrics. Major domains (chromosome 9 homologues) are indicated (*arrowheads*). **c–e** Two-colour FISH performed with S.2 and A.0. *Green* signals (FITC) correspond to S.2, and *orange* signals (TRITC) to A.0 (alpha satellite). *Bars* indicate the different hybridization domains. **f–g** Two-colour FISH performed with S.3 and A.0. *Green* signals (FITC) correspond to S.3, and *orange* signals (TRITC) to A.0 (alpha satellite). Both signals on chromosomes 9 **f** and 1 **g** are indicated (*bars*). **h** Two-colour FISH with S.2 and A.0 on chromosomes with 5-azacytidine-undercondensed heterochromatin. **i** Two-colour FISH with S.3 and A.0 on chromosomes with 5-azacytidine-undercondensed heterochromatin

satellite 1 is restricted to small pericentromeric domains (Tagarro et al. 1994).

The long arm of chromosome Y appears strongly labelled when in situ hybridizations with ^{3}H-cRNAs obtained from satellites II or III are performed (Evans et al. 1974; Gosden et al. 1975). The absence of labelling when S.2 or S.3 are used as hybridization probes implies that, although Yq12 is composed of sequences contained in classical satellites II and III, they are distinct from satellites 2 or 3. Thus, if DYZ1 sequences are not part of satellites 2 and 3, an alternate classification should be considered.

Moyzis et al. (1987) isolated two repetitive DNA clones, pHuR 98 and pHuR 195, specific for the heterochromatin of chromosomes 9 and 16, respectively. On the basis of sequence studies, they concluded that clone pHuR 98 was a variant satellite 3 sequence, and pHuR 195 a variant satellite 2 sequence. Our data on the locations of satellites 2 and 3 reinforce such assumptions. On the other hand, Cooke and Hindley (1979) cloned a 1.77-kb *Eco*RI fragment (pUC1.77), specific for the heterochromatin of chromosome 1 (Gosden et al. 1981). pUC1.77 was initially considered as a satellite III sequence. However, this assignment is controversial; where-

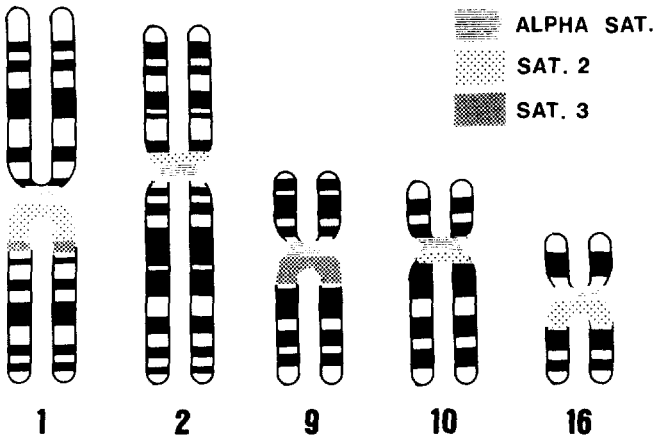

Fig. 3. Schematic representation of the distribution of the major domains of satellites 2 (*SAT. 2*) and 3 (*SAT. 3*) in relation to alpha satellite sequences (*ALPHA SAT.*)

as Mitchell et al. (1979) have reported that 1.77-kb *Eco*RI fragments are present in both satellites II and III, Frommer et al. (1982) have shown that they are present exclusively in satellite II. Thus, satellites isolated by different authors, although being essentially the same, may not contain identical sequences (Singer 1982). Since non-isotopic in situ hybridization with pUC1.77 shows specific binding to the large heterochromatin block of chromosome 1 (Guttenbach and Schmid 1991), our data suggest that it must be a fragment of satellite 2 DNA. On the other hand, the obtainment of satellite III fragments from chromosomes 10, 13, 14, 15, 21 and 22 from rodent-human hybrid cells (Higgins et al. 1985; Choo et al. 1990, 1992; Jackson et al. 1992; Vissel et al. 1992) agrees with the pattern of hybridization signals on these chromosomes when S.3 is used as a probe (Fig. 2b).

Two-colour FISH experiments with simultaneously either A.0 and S.2 or A.0 and S.3 have allowed us spatially to resolve the molecular composition of the largest autosomal heterochromatin blocks. On chromosome 1, satellite 2 constitutes a prominent block adjacent to alpha satellite sequences (Fig. 2c), whereas satellite 3 is confined to a distal narrow region (Fig. 2g). On chromosome 16, satellite 2 is located at the secondary constriction (16q11.2) adjacent to the strictly centromeric alpha satellite sequences (Fig. 2c, d). On chromosome 2, satellite 2 is localized on the short arm, adjacent to alpha satellite blocks (Fig. 2d), whereas it is localized on the long arm, adjacent to the alpha satellite block on chromosome 10 (Fig. 2e).

However, satellite 3 is localized on the secondary constriction of chromosome 9 (9q12), close to alpha satellite sequences (Fig. 2f). Previous hybridizations with cloned sequences have also suggested this type of organization (Mitchell et al. 1986; Rocchi et al. 1991).

The distribution of satellites 2 and 3 in relation to the alpha satellite was confirmed by performing two-colour FISH experiments on chromosomes with 5-azacytidine-undercondensed heterochromatin (Fig. 2h, i). Results obtained by two-colour FISH are summarized in Fig. 3.

It is noteworthy that, whereas satellites 2 and 3 are susceptible to undercondensation by 5-azacytidine, the centromeric alpha satellite does not seem to be affected by this nucleoside analogue (Fig. 2h, i). In a similar way, the chromosome domains that we have described as containing satellite 1 (Tagarro et al. 1994) are not uncoiled after treatments with 5-azacytidine. The autosomal regions described as containing the bulk of satellites 2 and 3 are precisely those that are more intensely methylated (Miller et al. 1974). Thus, the idea that the uncoiling of heterochromatin induced by 5-azacytidine is a consequence of the demethylation of intensely methylated regions is reinforced. Furthermore, the different methylation levels of the distinct satellites are the consequence of their base compositions. We should note that satellites 2 and 3, being based on the pentamer ATTCC, are sources of CpG sites caused by the accumulation of random point mutations C→G (Fowler et al. 1988). However, satellite 1, being the most A + T rich fraction in the human genome (72.4%), must be less susceptible to the generation of CpG sites. Automated synthesized probes, such as S.2, S.3 and A.0, should be useful in studies of chromosome rearrangements, heteromorphisms affecting the large autosomal heterochromatin blocks and aneuploidies of chromosomes 1, 9 or 16 in interphase nuclei, as the researcher would not be limited by the availability of adequate cloned sequences. Furthermore, the high sensitivity of this method is demonstrated by the fact that the minor hybridization sites of satellite III that have been demonstrated by cloned fragments (Frommer et al. 1988; Fowler et al. 1989) are shown when using S.3.

Acknowledgements. This work was supported by the Comunidad Autónoma de Madrid (C255/91) and the FISss (93/0179). Ignacio Tagarro was supported by a fellowship from the Comunidad Autónoma de Madrid.

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