FINAL REPORT: INDIVIDUALISATION OF HUMAN BODY FLUID STAINS RELATED TO THE INVESTIGATION OF SEXUAL AND OTHER ASSAULTS.

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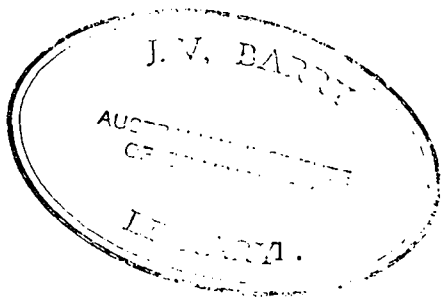

The principal original findings are as follows:

1.1 There is a complex TaqI-specific restriction fragment length polymorphism associated with Satellite III DNA sequences in the human genome. The primary probe sequence that was developed to demonstrate this polymorphism is coded 228S.

1.2 This polymorphism has its greatest variability amongst the TaqI restriction fragments in the size range about 25 to 4kb. Such fragments are herein called the TaqI-deficient-polymorphic-sequences (TDPS). Fragments smaller than this show much less inter-individual variation.

1.3 The TDPS are highly discriminating of individual genomes and family studies show them to be inherited in a Mendelian manner.

1.4 The primary cause of the Satellite III polymorphism appears to be a specific C to G point mutation in the pentameric repeat unit, 5' TTCCA 3', typical of Satellite III. However less frequent mutational events such as non-reciprocal recombination cannot be excluded, although this is unlikely to be responsible for the bulk of the polymorphism.

1.5 At limiting probe concentration and extreme stringency, the primary probe 228S specifically locates chromosomal region 9qh. Despite this, the genomic location of the TDPS, based upon the examination of a limited range of somatic cell hybrids, appears to be predominantly the centromeric heterochromatin of chromosomes 20, 21 and 22. These regions may therefore represent the largest blocks of homogeneous Satellite III in the genome and are thus likely to be the locations for more recent Satellite III amplification events. Chromosomal region 9qh is deduced to be predominantly composed of small TaqI fragments, ie. the non TDPS or TaqI-rich regions.

The TaqI-deficient polymorphic regions differ from previously described Satellite-III-enriched 'domains' in the genome where the TaqI site frequency is far greater, namely the long arm of the Y chromosome and so-called 'K-domain' regions located on the short arm of chromosome 15.

A minor contribution to the TDPS may arise from chromosomes 9 (9qh), 15 and possibly chromosomes 13, 14, 5 and 1.

1.6 The blocks of Satellite III sequence from which the TDPS are derived are highly homogeneous in sequence being completely or largely resistant to digestion with other common restriction endonucleases. The TDPS are thus apparently only rarely and intermittently interrupted by either non-Satellite III sequences or regions of mutationally degraded Satellite III. This suggests the TDPS are evolutionary recent amplimers, having incurred only limited levels of transposon invasion and having accumulated, by random drift, little degeneracy in their sequence. These conclusions do not necessarily and,

indeed, probably do not apply to the TaqI-rich regions of Satellite III.

1.7 Digestion of human DNA with HaeIII and separation of the products by field inversion electrophoresis shows the blocks of homogeneous Satellite III DNA to range in length between 0.2 Mb and up to at least 2.0 Mb. This contrasts with the high HaeIII susceptibility of normal genomic DNA and re-confirmed the unusual sequence homogeneity of such sequences. The results also revealed megabase-scale inter-individual length variations, as did digestion with a number of other restriction enzymes such as BamHI.

1.8 Within human cells, the TDPS show a marked stability in mitotic division. Comparison of the DNA from a number of clonal lines, each derived from a single human lymphocyte from the same individual, showed no detectable alteration in the polymorphic patterns associated with each individual. Further, comparison of the DNA from primary sites of malignant tissue and the DNA from normal tissue from single individuals likewise showed no detectable alteration in the polymorphic fragments. There was however suggestive evidence indicating that Satellite III is not so stable in mouse/human hybrids.

2. This work confirms the observations of other groups on the following points:

2.1 The Satellite III-like family of sequences is widespread in the human genome. The heterochromatin of chromosome 1 and 16 is, for example, a major source of Satellite II DNA but contains little Satellite III DNA in the size range covered by the TDPS.

2.2 It was confirmed that the so-called 'K-domain' Satellite III - typified by 3.6 and 1.8 kb KpnI Satellite III tandem repeating fragments - segregate from chromosome 15. The same fragments were also reported to segregate from chromosome region 9qh, but this could not be confirmed. Evidence was obtained which suggested these 'K-domain' fragments may not however be confined to chromosome 15, one example arising from another genomic region (either chromosome 20, 21 or 14). This tended to support previous reports of chromosomal instability of 'K-domain' Satellite III DNA.

The KpnI derived 'K-domain' fragments are of recent evolutionary origin, being specific to humans and absent in non-human higher primates. (Note: this particular finding does not appear to have been reported by other groups). Another Satellite III sequence related repeat - the 3.5 kb HaeIII fragments segregating from the long arm of the Y chromosome - was confirmed to be also of recent evolutionary origin being specific to human males only.

2.3 Extensive cloning and sequencing of Satellite III and other tandemly repeated sequences confirmed the generality of

previously published consensus sequences of Satellite III, Satellite II and satellite alphoid. A slight modification of the previously reported consensus Satellite III sequence is presented. Examples of these sequences were isolated by creating an endonuclease-resistant repetitive sequence library. This was created by digesting DNA with selected endonucleases, the larger restriction fragments - about 5kb or more - then being isolated and cloned. The enzyme-resistant DNA fraction was found to contain an enriched proportion of the major tandem repeats. Some clones were found to exhibit chromosome-specific in situ-hybridization properties eg. alphoid (chromosomes 7, 9 and 18) and Satellite II (chromosome 1).

2.4 Satellite III sequences were found to be hypomethylated in germline tissue in comparison to somatic tissue. This result is consistent with the methylation status of other major satellite DNA sequences. This relative de-methylation of Satellite III sequences in germline tissue includes the TDPS.

3. This work also clarifies previously conflicting reports regarding the phylogeny of major satellite sequences:

3.1 It confirms the existence of the Satellite III-like family of sequences and alphoid DNA sequences in the higher primates. In particular, it clarifies that large amounts of Satellite III DNA and satellite alphoid are relatively characteristic of higher primates and also clarifies Satellite II DNA to be human specific amongst the primates examined. This, as well as sequence information, implies Satellite II probably evolved very recently and likely after the divergence of humans from the higher primates.

3.2 The results from examination of Satellite III sequences in the higher primates are consistent with the view that the molecular evolution of Satellite III has proceeded far faster than primate speciation has and, secondly, that the radiation of the human race has occurred in orders of magnitude less time than primate divergence.

4. A number of inconclusive results arise from this work. These are as follows:

4.1 Attempts were made to locate those sequences which form junction points between Satellite III and other DNA sequences in the genome, either being the boundary or edge sequences to Satellite III, or as a consequence of sequence invasion by transposons. No unequivocal evidence or examples of such sequences were found. A few potential junction sequences were isolated, notably a series of AT rich sequences. However, their association with Satellite III in vivo is unconfirmed.

4.2 Investigations were made as to the existence of closed circular extrachromosomal (CCC) Satellite III DNA in the genome but this could not be confirmed. This may be either

because its extrachromosomal existence is short-lived or it is in quantities too small to be found by the isolation and detection method employed. However, the extrachromosomal existence of such species might be inferred from the finding by in situ methods of small marker chromosomes. The chromosomal origin of two such marker chromosomes has been located using the chromosome-specific in situ hybridization properties of probes such as; 228S (Satellite III) and RR17 (alphoid) - both to chromosome 9 - and RR6 (Satellite II) to chromosome 1.

4.3 Sequence comparisons made between isolated Satellite II and satellite alphoid containing clones, showed the longest commonality to be only nine base pairs in length: 5' TGCATTCAA 3'. (Note: this finding also does not appear to have been reported by other groups). This sequence identity is however potentially important, as this exact sequence has previously been suggested, but never confirmed, as a possible centromere-specific protein binding site in alphoid DNA. Its co-presence in Satellite II, otherwise quite different in sequence to satellite alphoid, may be significant.

5. The following conclusions with practical applications can be drawn from this work.

5.1 The Satellite III "macrosatellite" polymorphism represents an alternative method to those based upon "minisatellite" hypervariation for either the discrimination of individual genomes in criminal forensic studies or in establishing parentage. Conservatively estimated, the average chance of two individuals having the same DNA fingerprint with probe 228S in 1 in 100,000 people.

The probe 228S (patented and marketed by Bresatec Limited as Polysat 3™) has its principal practical advantage in the use of the same probe for either sexing human genomes (HaeIII digests) or for discriminating between individuals (TaqI digests). The probe is only known to hybridise to higher primate DNA and thus may be used for quantifying human DNA in mixtures of human and non-human e.g. microbial DNA. Results may be achieved using non-isotopic probe methods, using photobiotinylated probes.

The principal practical disadvantages are the complexity of the polymorphic pattern, its interpretation and comparison. The size range of the SatelliteIII/TaqI fragments require the source DNA to have a high initial integrity, whereas both the quantity and quality of DNA recoverable from typical forensic specimens may often be limiting. There is also no potential for in-vitro amplification of the satellite III polymorphism as is now possible with some single locus 'micro' satellite sequences.

In addition, sequence 216S also isolated and used in this study, is marketed by Bresatec Ltd. as an invariate control probe suitable for examination of band-shifting in the

Satellite III fingerprint patterns, or in other DNA typing systems. Probe 216S is also a commercial product, PolyCon™.

ACKNOWLEDGEMENTS.

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PUBLISHED WORK.

1. Fowler JCS, Drinkwater RD, Burgoyne LA, Skinner JD (1987) Hypervariable lengths of human DNA associated with a human Satellite III sequence found in the 3.4-kb Y-specific fragment. Nucleic Acids Res. 15:3929.

2. Fowler JCS, Drinkwater RD, Skinner JD, Burgoyne LA (1988a) Human Satellite-III DNA: an example of a "macrosatellite" polymorphism. Hum. Genet. 79:265-272.

3. Fowler JCS, Skinner JD, Burgoyne LA, McInnes JL (1988b) Improved separation of multi-locus hypervariable DNA restriction fragments by field inversion gel electrophoresis and fragment detection using a biotinylated probe. Appl. and Theoret. Electrophoresis 1:23-28.

4. Fowler JCS, Burgoyne LA, Baker EG, Ringenbergs ML, Callen DF (1989a) Human Satellite III DNA: Genomic location and sequence homogeneity of the TaqI-deficient polymorphic sequences. Chromosoma 98:266-272.

5. Fowler JCS, Skinner JD, Burgoyne LA, Drinkwater RD (1989b) Satellite DNA and higher primate phylogeny. Mol. Biol. Evol. 6:553-557.

The following refereed review has also been published:

1. Fowler JCS, Burgoyne LA, Scott AC, Harding HWJ. (1988c) Repetitive deoxyribonucleic acid (DNA) and human genome variation - A concise review relevant to forensic biology. J For. Sci. 33:1111-1126. Reply to letter. J For. Sci. (1989c) 34:1299-1300.

The following unrefereed information has been published.

1. Fowler JCS, Harding HWJ, Burgoyne L (1988d) A protocol using an alkali blotting procedure for the analysis of restriction length fragments of human DNA. Advances in Forensic Haemogenetics 2. Ed. WR Mayr, Springer Verlag, Berlin, Heidelberg 339-346.

2. Fowler JCS. (1989d) Polymorphism in the major human repetitive DNA sequences: Theory, practice and comparison with other methods of measuring human genome variation. (to be published in "DNA in Forensic Science: Theory, Techniques and Applications" Ellis Horwood Series Limited, London UK.)

The following conference presentations have been made:

1. Fowler JCS. (1987) Investigation of the human genome for polymorphisms associated with repetitive sequence DNA. 9th Annual Genome Conference, Lorne, Victoria, Australia (Poster).

2. Fowler JCS, Harding HWJ, Burgoyne LA (1987) Discrimination of individuals using hypervariable lengths of human DNA associated with a human Satellite III sequence. 11th Meeting International Association of Forensic Sciences, Vancouver, Canada. (delivered by HWJ Harding).

3. Fowler JCS, Harding HWJ, Burgoyne LA (1988) 228S, A Satellite III related DNA probe for the discrimination of individuals. 10th Australian International Symposium on the Forensic Sciences, Brisbane, Australia. (delivered by HWJ Harding).

4. Fowler JCS, Williams K, Dunaiski V (1990) Human genome variation in satellite sequences: PCR amplification of VNTR "micro-satellite" DNA for forensic purposes. 12th Annual Genome Conference, Lorne, Victoria, Australia (Poster).

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Hypervariable lengths of human DNA associated with a human satellite III sequence found in the 3.4kb Y-specific fragment

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Human Satellite III DNA contains a degenerate, pentameric tandem repeat sequence TTCCA (1). A probe (Seq.228S below) isolated from a human sperm library enriched in repeat sequences, has disclosed that such sequences are associated with hypervariable lengths of DNA upon Taq 1 digestion of human genomes. The resulting restriction fragments are highly discriminating of individuals, (Fig. 1) a major cause apparently being random C-G point mutations in the pentamer (TTCCA) thus creating Taq 1 sites (TCGA), though further mutations of the resultant (CpG) dinucleotide may be contributory. Equality of Taq 1 restrictions was checked by reprobing with centromeric tandem repeat sequences where invariate patterns were obtained. Probe 228S also identifies the 3.4 kb Y-specific fragment (2) and other unspecified autosomal fragments upon EcoRI digestion of human genomes. Comparison of Taq 1/228S patterns with mini-satellite probe patterns (3) produced different but similarly complex restriction patterns for individual genomes. Mini-satellite hypervariation is revealed by enzymes restricting in regions flanking the short tandem repeats. By contrast, this hypervariation is revealed only by Taq 1 apparently restricting within the pentameric repeat.

References: (1) Prosser,J.,et.al., (1986) J.Mol.Biol. 187, 145-155.
(2) Nakahori,Y.,et.al., (1986) Nuc.Acids Res. 14, 7569-7580.
(3) Jeffreys,AJ.,et.al., (1985) Nature. 314, 67-73.

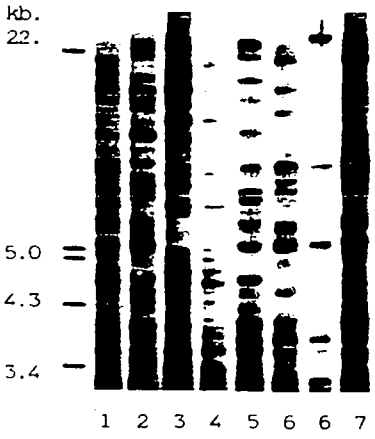

Fig 1. Taq 1/228S analysis of individuals 1-7: persons 1-4 are related, 5-7 are unrelated and duplicate 6 is germline/ somatic tissue compared.

SEQ-228S: Satellite III sequence related DNA.

G,G,A,A,T,G,G,A,A,T,G,G,A,A,T,G,G,A,A,T,G,G,A,A,T, (30)
G,G,A,A,T,G,G,A,T,T,C,A,A,C,C,C,G,A,A,T,G,G,A,A,T,G,G,A,A,A, (60)
G,G,A,A,T,G,G,A,A,T,A,A,A,C,C,G,A,G,T,G,G,A,A,T,G,G,A,A,T, (90)
G,G,A,A,T,G,G,A,G,T,G,G,A,A,T,G,G,A,A,T,G,T,A,A,T,G,G,A,A,G,(120)
G,G,A,A,T,G,G,T,A,C,G,G,A,A,T,G,G,A,A,T,G,G,A,A,T,G,G,A,A,C,(150)
T,A,A,A,T,G,A,A,A,T,G,G,A,A,T,G,G,A,A,T,G,G,A,A,T,G,G,A,A,T,(180)
G,G,A,A,T,G,G,A,A,T,G,G,A,A,T,G,G,A,A,T,G,A,A,C,C,C,G,T,G,T,(210)
G,C,A,A,T,T,G,A,A,T,G,G,A,A,T,C,G,A,A,T,G,G,A,A,T,G,G,A,A,T,(240)
G,C,C,A,A,T,G,G,A,A,T,G,G,A,T,T,C,A,C,T,A,G,A,T,G,G,A,A,T,G,(270)
G,A,A,G,G,A,A,T,G,G,A,A,T,C,A,A,C,C,A,G,T,G,G,A,A,T,G,G,C,A,(300)
A,T,G,G,C,A,T,G,G,A,A,T,G,G,A,A,T,C,G,A,A,T,G,G,A,A,T,G,C,A,(330)
G,T.

Hum Genet (1988) 79:265–272

Human Genetics

Human satellite-III DNA: an example of a "macrosatellite" polymorphism

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Summary. Human satellite III DNA contains a complex polymorphism, which appears to be *Taq*I-specific. Its likely cause is a two-step point mutation in the pentameric repeat TTCCA, typical of satellite III. Hybridization of the satellite-III sequence-related probe that demonstrates this polymorphism is directly attributable to clusters of "pure" pentameric TTCCA repeats in the genome. The sites of such repeats include the 3.4-kb fragment specific to the Y chromosome and a limited number of autosomes. The polymorphism arises from the latter and is likely to include chromosomes containing so-called K domain satellite III sequences found, for example, in chromosomes 9 and 15. Segregation of the polymorphic fragments appears to follow orthodox genetics.

Introduction

Eukaryotic DNA contains many types of repeated sequences (for a review see Hardman 1986). These consist of either individual repeat elements interspersed throughout the genome (Singer and Skowronski 1985), or as tandem arrays of repeat sequences such as the major human satellites I, II, III and alphoid DNA (Singer 1982). Tandem sequences may demonstrate long-repeat-length structures often within which occur shorter tandem repeats of simple but divergent base sequences (Prosser et al. 1986). This divergence means each major satellite generally consists of a family of like sequences, the members of which, as in the case of the alphoid family, are associated with specific chromosomes (Devilee 1986; Lund Jorgensen et al. 1987; Way and Willard 1986). Human repetitive sequences are a source of exceptional genomic variation, many such examples arising from a variation in the number of short tandem repeats (variable number tandem repeats, VNTR) (Nakamura et al. 1987a), also called "minisatellites" (Jeffreys et al. 1985a). These may either be site-specific in the genome, (see examples listed in Nakamura et al. 1987a; Simmler et al. 1987) or dispersed at multiple sites in the genome (Jeffreys et al. 1985a; Jarman et al. 1986; Vassart et al. 1987) or site-specific but within a larger (i.e. greater than 250 kb) "midisatellite" (Nakamura et al. 1987b; Das et al. 1987).

Other tandemly repeating DNA sequences have also been shown to be polymorphic in human genomes, e.g. centromeric alphoid tandem repeats (Willard et al. 1986; Jabs et al. 1986), the alphoid related *Sau*3A repeat sequences (Kiyama et al. 1987), Bkm simple quadruplicate repeat sequences (Ali et al. 1986; Singh and Jones 1986) and satellite III (Fowler et al. 1987).

The family of sequences classified as satellite-III-like are characterized by the presence of a tandem pentameric repeat sequence TTCCA (Deininger et al. 1981; Frommer et al. 1982; Prosser et al. 1986; Nakahori et al. 1986). Classic satellite III sequences are interspersed with regular *Hin*fI sites, which produce a laddered array of bands 25–250 bp in size in 5-bp increments (Frommer et al. 1982). Satellite II sequences are strongly related to satellite III in that they also contain the tandem pentameric repeat TTCCA. They are more degenerate, containing more and much less regular *Hin*fI sites and less orderly tandem repeating (Frommer et al. 1982; Prosser et al. 1986).

In many instances of polymorphism in human repetitive sequences it is still unclear how much of the variability is due to simple accumulation of point mutations with reciprocal recombination, and how much is due to non-reciprocal events. The complex satellite-III polymorphism (Fowler et al. 1987) is suited to investigating this question. This work also investigates the genomic location of this polymorphism and examines its segregation.

Materials and methods

Preparation of DNA

Isolation of placental DNA was carried out by a published method (Wolf et al. 1980). Sperm cells were washed in aqueous buffer (10 m*M* Tris, 10 m*M* EDTA, 100 m*M* NaCl, pH 8.0) and demembranized in the same buffer containing 2% SDS. The sperm head preparation was digested at 37°C for 14 h in the above buffer to which was added 1% SDS, proteinase K (50 mg/ml) and 2-mercaptoethanol (0.04 *M*). The digest was extracted with phenol and chloroform, and the DNA precipitated in ethanol. DNA from standard Utah families (extended family pedigrees maintained as cultures by the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, N.J) was obtained from the Department of Histopathology, Adelaide Children's Hospital, Adelaide. DNA isolated from a mouse/human hybrid cell panel (Callen 1986) was a gift from Dr. D. Callen, Cytogenetics Unit, Adelaide Children's Hospital, Adelaide.

Digestion of DNA

The DNA was digested with restriction enzymes *Eco*RI, *Pst*I, *Xba*I, *Bam*HI, *Hae*III, *Kpn*I, *Alu*I, *Rsa*I, *Hpa*II, *Hin*fI,

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*Msp*I, *Taq*I and *Cla*I (Boehringer Mannheim) and *Nsp*I (Amersham). The digests were precipitated with ethanol and then washed (with 80% ethanol, dried and redissolved in suitable volumes (generally 8 µl) of an aqueous buffer (10 m*M* Tris, 1 m*M* EDTA, pH 8.0), and loaded into each gel slot.

Electrophoresis

Electrophoresis was in agarose gels ranging in concentration from 0.7% to 1.0% using 1 × TAE buffer (10 m*M* Tris, 5 m*M* sodium acetate, 0.5 m*M* EDTA, pH 7.8) at 5 V/cm for 2–5 h, or in 4 × TAE buffer at 2 V/cm for 21–24 h at ambient temperature. DNA molecular size markers were nick-translated with [^{32}P]dATP and included in each gel.

Southern blotting

Gels were blotted by Southern transfer to nylon membranes (Bio-Rad Zeta Probe) using the method of Reed and Mann (1985) modified as follows. After depurination in acid (0.25 *M* HCl) gels were soaked in NaOH (0.4 *M*) for about 10 min and then blotted by Southern transfer in NaOH (0.02 *M*) for about 14 h. Gels of 0.8% concentration or less were additionally soaked in 40 m*M* NaCl for 15 min prior to acid depurination.

Probe preparation

The sequences 228S, 233S, 262F, 227S and 216S were obtained from human DNA libraries (sperm S, fibroblast F) enriched in repetitive sequences. They were cloned into M13, sequenced and classified by comparison to sequence data banks (Fig. 1). The minisatellite probe 33.15 (Jeffreys et al. 1985a), also cloned into M13, was a gift from Dr. A. J. Jeffreys.

Radioactive probes were prepared by primed synthesis of the complementary strand of M13 using [^{32}P]dATP (1,800 Ci/mmole, Bresa S.A.) or using [^{35}S]dATP (1,000 Ci/mmole, Amersham), and dCTP, dGTP, dTTP (0.5 m*M* each) and the Klenow fragment of DNA Polymerase I. This synthesis leaves the insert sequences single-stranded and free for hybridization. The whole construct was precipitated with ethanol, dried and resuspended in approximately 3 ml of hybridization buffer (0.7 *M* NaCl, 50 m*M* NaH_2PO_4, 4 m*M* EDTA, 1% SDS, 0.5% non-fat powdered milk, pH 7.7).

The synthetic oligonucleotide satellite sequence was obtained by synthesis of two complementary single strands each of 25 bp (Applied Biosystems Model 381A), one being 5′ [(TGGAA)$_5$]3′ and the other 5′CCA,[(TTCCA)$_4$)], TT3′. A probe was prepared by the method of Hellman and Petterson (1987), mixing the TGGAA sequence (5 ng) with the TTCCA sequence (12 ng) and immediately end-filling the hybridizing strands using [^{35}S]dATP, and dCTP, dGTP and dTTP (0.5 m*M* each) and the Klenow enzyme. The probe (predominant size approximately 25 bp with minor species up to 40 bp) was suspended in the same volume of hybridization buffer as above, but denatured before use by boiling at 100°C for 4 min.

Hybridization, washing and exposure

Membranes were prehybridized for 3 h at 68°C in hybridization buffer and hybridized for 16 h. Hybridization and washing was conducted in perspex cassettes (chamber of 0.8 mm width) rotating at 15° to the vertical. The membranes were washed in four solutions of increasing stringency: wash A, 2 × SSC (0.3 *M* NaCl, 0.03 *M* trisodium citrate), 0.1% SDS, 15 min; wash B, 0.5 × SSC, 0.1% SDS, 90 min; wash C, 0.1 × SSC, 0.1% SDS, 15 min; wash D, 0.1 × SSC, 1.0% SDS 15 min, with addition of proteinase K (250 µg/ml) to wash B. Washes A, B, C were at 37°C and wash D at 55°C. Hybridization of the oligonucleotide probe was conducted at 60°C. Unreported experiments using this probe on a serial dilution of whole human DNA, and washing as above, produced a linear response to DNA concentration, the limit of detection being about 100 pg using [^{35}S]dATP.

Membranes were dried and exposed at ambient temperature to X-ray film (Kodak XOMat RP Film). Probes were stripped from membranes essentially as described by Reed and Mann (1985).

SEQ-216S

G,T,T,T,C,A,T,A,G,C,T,T,C,T,A,T,C,T,C,C,A,A,A,C,G,A,A,A,G,T, (30)

T,C,A,A,C,T,C,T,G,T,G,A,G,T,T,G,A,A,T,G,C,A,A,G,C,A,T,C,A,C, (60)

A,A,A,G,A,A,G,T,T,T,C,T,G,A,G,A,A,T,G,C,T,T,C,T,G,T,T,T,A,G, (90)

C,T,T,T,C,C,T,G,T,G,A,A,G,A,T,T,C,T,C,C,C,G,T,T,T,C,C,A,A,C, (120)

G,A,A,A,T,C,T,T,C,A,A,A,A,T,A,G,G,T,C,C,A,A,A,T,A,T,C,C,A,C, (150)

T,T,G,C,A,G,A,T,T,C,C,A,C,A,C,A,A,G,A,G,T,G,A,T,T,G,G,A,A,A, (180)

C,T,G,C,T,C,T,T,G,A,A,A,G,G,A,A,C,C,T,C,A,C,T,C,T,G,T,G,A,G, (210)

SEQ-233S

C,T,A,A,G,G,G,T,A,A,T,G,C,A,G,T,C,A,G,C,G,A,A,T,G,G,A,A,T,T, (30)

G,A,A,T,G,G,A,A,T,G,G,A,A,T,G,G,A,A,T,T,A,A,C,C,C,G,A,A,T,A, (60)

C,A,A,T,G,G,A,A,T,G,G,A,A,T,G,G,A,G,T,G,G,A,A,C,G,G,A,A,T,G, (90)

G,A,G,T,G,G,

SEQ-262F

T,T,C,A,T,T,C,C,A,T,T,C,C,A,G,A,C,C,A,T,T,C,C,A,T,T,T,X,A,C, (30)

T,C,C,A,T,T,C,C,T,T,T,C,G,A,G,C,C,C,T,T,T,C,A,A,T,T,T,X,A,G, (60)

T,C,C,A,T,T,C,C,T,T,T,C,C,A,G,T,C,C,A,T,T,T,C,A,C,T,C,C,A,G, (90)

T,C,C,A,T,T,

SEQ-228S (see Fowler et al. 1987)

SEQ-227S (see Drinkwater et al. 1986)

Fig. 1. Nucleotide base sequences

Results

Restriction patterns using the satellite III probe (228S)

The probe 228S (Fowler et al. 1987) is sequence-related to human satellite-III DNA (Deininger et al. 1981). DNA from male tissue digested with either *Hae*III or *Eco*R1 enzymes and probed with 228S showed a restriction fragment of 3.4 kb. This was absent in female tissue. Both sources of DNA showed unresolved high molecular weight satellite-III sequence-related material resistant to *Hae*III digestion and fragments at 1.3, 1.8, 2.0 kb common to both after *Eco*R1 digestion. The 228S sequence is not, therefore, sequence-specific to the 3.4-kb Y-chromosome repeat previously described (Cooke et al. 1983) but is also present on autosomes.

The *Kpn*I/228S restriction pattern showed two major bands at 3.6 and 1.8 kb, consistent with the results of Burk et al. (1985) and Jeanpierre et al. (1985).

The *Taq*I/228S restriction patterns showed complex banding. Most fragments were less than about 2 kb in size. The 3.4 kb male-specific fragment, evident after *Hae*III digestion, was removed by *Hae*III *Taq*I double digestion. This confirmed the existence of multiple *Taq*I sites within it (Nakahori et al. 1986). However many fragments in the 2–20 kb size range were low in *Taq*I sites and these provided complex patterns, which appeared to be highly discriminating of individuals (Fowler et al. 1987). The equality of *Taq*I restriction digests was checked by stripping membranes of 228S and reprobing them with sequence 216S (Fig. 1), which is related in sequence to centromeric repetitive DNA (Wu et al. 1980). As long as digestion was complete this probe gave an "invariate" banding patterns for each genome (demonstrated in the family studies below).

The *Hin*fI/228S restriction pattern showed a laddered array of fragments ranging in size from 5 kb to less than 1 kb, but with no discernible variation between individuals. When the minisatellite probe 33.15 (Jeffreys et al. 1985a) was used to probe the same membrane, polymorphic patterns were revealed. Comparison of the banding patterns achieved by *Taq*I/228S with those obtained using *Hin*fI/33.15 showed them to be different when testing the same individual. An analysis of 15 individuals using the *Taq*I/228S method showed each to have between 12 and 20 fragments in the 4–20 kb region, with a distribution and complexity of banding similar to that of one minisatellite probe (Jeffreys et al. 1985b). However the patterns appeared to differ in that the *Taq*I/228S method displays some fragments common to most individuals and others that are highly discriminating.

Digestions using *Cla*I (ATCGAT) and *Nsp*I (TTCGAA), both of which have an internal *Taq*I (TCGA) sequence, did not show polymorphic patterns with probe 228S. The bulk of these digests remained as unresolved (>20 kb) fragments in 0.7% agarose gels. Attempts to resolve the *Cla*I-digested DNA by using field inversion gel electrophoresis gave smears in the 15–30 kb region with no clear banding pattern.

Digestions using the enzymes *Pst*I, *Xba*I, *Bam*H1, *Cfo*I, *Alu*I, *Hpa*II gave unresolved smears, while other enzymes, e.g. *Msp*I, *Rsa*I gave smears with some discrete banding which appeared to be constant between different individuals.

Comparison of other "satellite-III-like" sequences as probes for the TaqI polymorphism

Blots of *Taq*I-digested human DNA samples were probed with other satellite-III-like sequences (Fig. 1). The sequences 233F and 262F both revealed a polymorphic pattern similar to that of 228S. However neither was as effective as 228S, with the extent of labelling and the fine resolution of bands being inferior. Sequence 262F also gave different relative band intensities from the sequence 228S. Sequence 227S did not reveal the polymorphism. The sequences 228S, 262S, 233F and 227F were compared with sequences pPD9 and pPD17 (Deininger et al. 1981) and their relative homologies plotted (Fig. 2). pPD 9 is a satellite III sequence, 65% pure in the pentamer TTCCA and pPD17 is described by Prosser et al. (1986) as typical of their "consensus" satellite II sequence. The sequence 228S shows strongest homology to satellite III (pPD9) and lesser homology to satellite II (pPD17). Sequences 233S, 262S, and 227S show an approximately linear decline in their

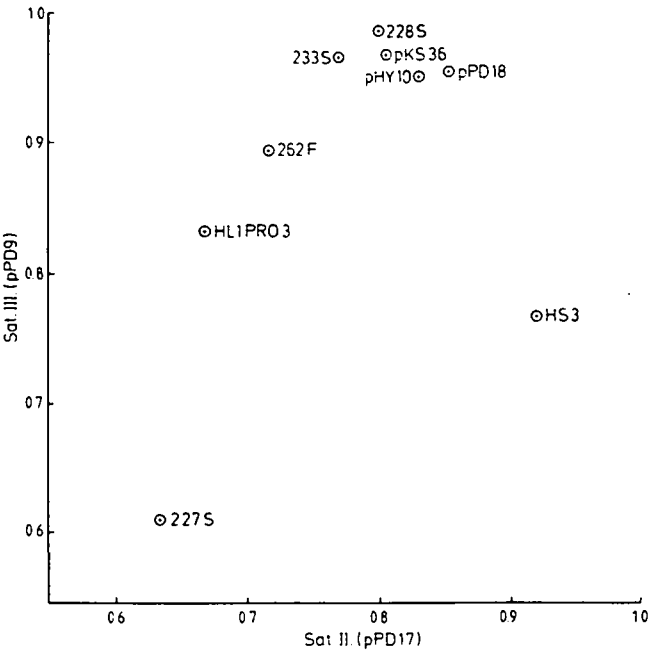

Fig. 2. Comparison of a number of satellite-III-like DNA sequences. Sequence homologies were calculated by the method of Bucholtz and Reisner (1986) giving homology coefficients with respect to two extreme sequence types (see text)

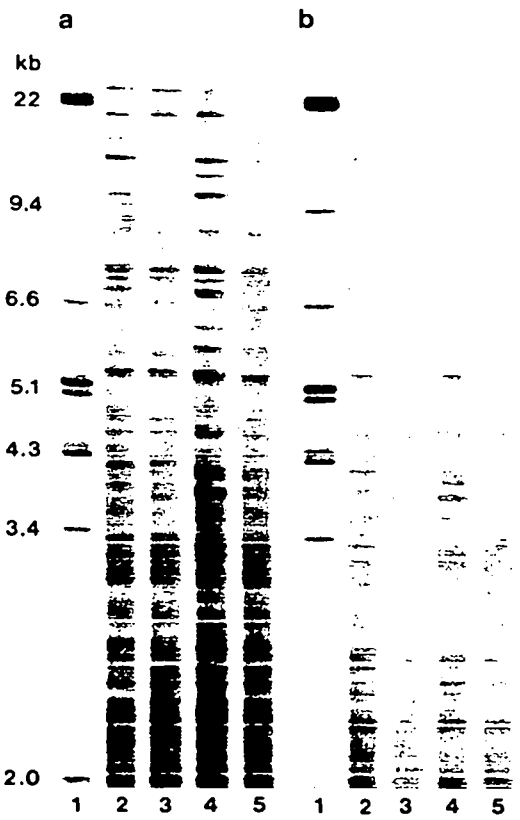

Fig. 3a, b. Comparison of different probes for the satellite III polymorphism. DNA was digested with *Taq*I and probed with either ^{35}S-labelled 228S (**a**), or probed with a ^{35}S-labelled "pure" satellite III oligonucleotide probe (**b**). *Lane 1* DNA size standard, *lanes 2, 3, 4* individual lymphoblastoid DNA samples (3 µg), *lane 5* mixed human sperm DNA (3 µg). Film exposure 30 h

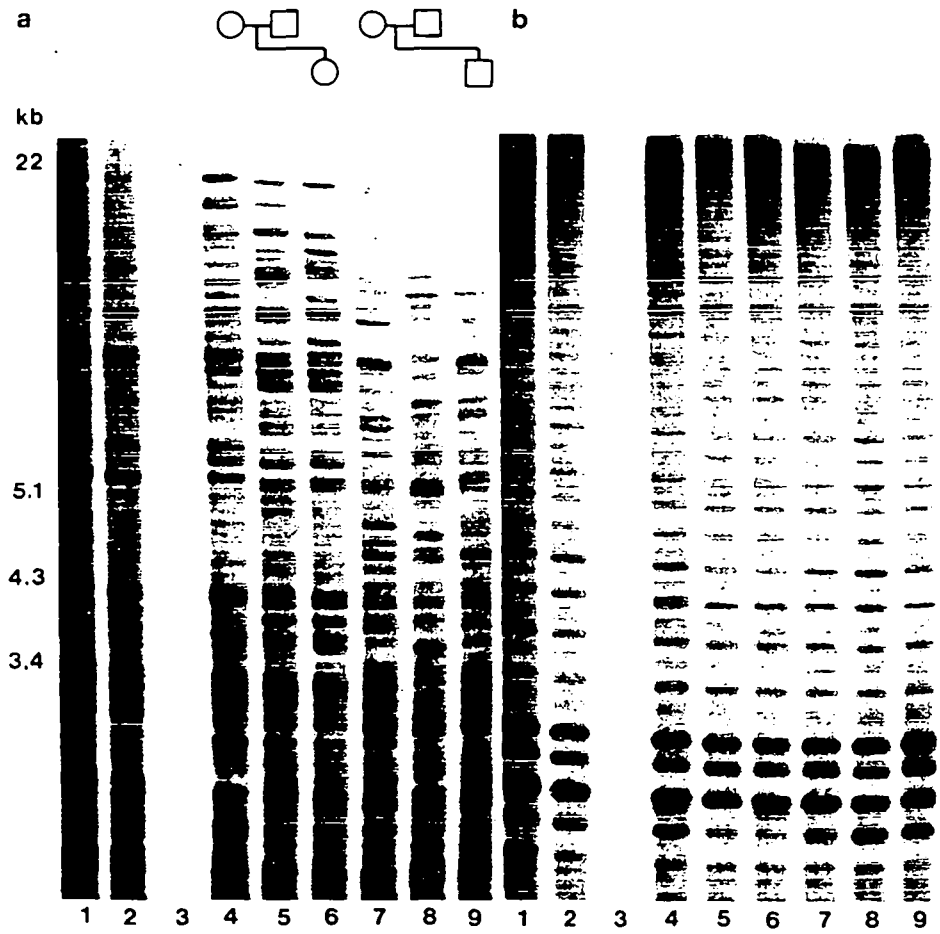

Fig. 4a, b. Examination of related individuals for the satellite III polymorphism. **a** DNA was digested with *Taq*I and probed with ^{35}S-labelled 28S. **b** The membrane was then stripped and probed with ^{35}S-labelled 216S. *Lanes 1, 2* mixed human sperm DNA (5 and 3 µg). *lane 3* DNA size standard. *lanes 4–9* lymphoblastoid DNA (3 µg). Film exposure 30 h

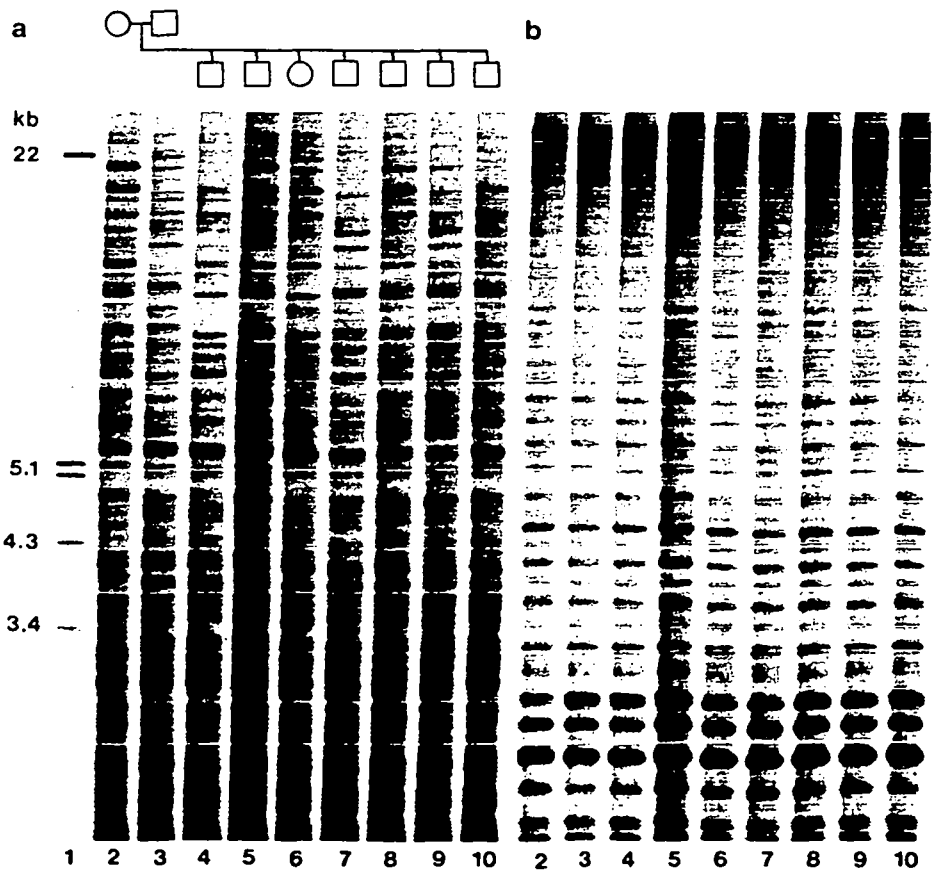

Fig. 5a, b. Examination of related individuals for the satellite III polymorphism. **a** DNA was digested with *Taq*I and probed with ^{35}S-labelled 28S. **b** The membrane was then stripped and probed with ^{35}S-labelled 216S. *Lane 1* DNA size standard, *lanes 2–10* lymphoblastoid DNA (3 µg). Film exposure 30 h

homology to either pPD9 or pPD17 consistent with a decline in their effectiveness as probes for the *Taq*I polymorphism. This suggested that the number and distribution of the pentamer 5′TTCCA3′ (or its complement) in each sequence was significant in revealing the polymorphism. Thus the synthetic oligonucleotide sequence of pure pentamers was hybridized to fresh membranes of *Taq*I digests and compared directly to 28S hybridization patterns. They gave identical results up to the 20-kb region of fragment size (Fig. 3a, b). Note that the fragments greater than 20 kb seen in Fig. 3a but weak to absent in Fig. 3b were no stronger when the membrane in Fig. 3b was stripped and reprobed with 28S, indicating that handling and/or blotting is setting the upper size-limits to the *Taq*I fragments. Both 28S and the oligomeric probe highlighted the same set of predominant restriction fragments (within a smeared hybridization signal) in a DNA sample isolated from a mixture of human sperm (> 100 donors) (Fig. 3a, b).

Family studies

DNA from related individuals was digested with *Taq*I and the resultant blots hybridized with the principal satellite-III probe (28S). The polymorphic restriction patterns and their pedigrees are shown (Figs. 4a, 5a). The segregation of restriction fragments in these pedigrees indicated that, within the present limits of band resolution achieved across the most variable region (5–20 kb), there were no fragments in the progeny that were not present in either or both of the parents, but the intensity variations at the lower molecular weights indicated that these bands were the results of contributions from multiple loci.

As expected, mixed sperm DNA (> 100 donors) showed a smeared pattern, within which were a number of predominant consensus restriction fragments (Fig. 4a).

Membranes were then stripped of probe 28S and reprobed with sequence 16S, to establish that the digestions were complete and that the amounts of DNA in each track were approximately equal (Figs. 4b, 5b). The patterns from the mixed DNA samples were then unsmeared and indistinguishable from individual DNA samples (Fig. 4b).

Analysis of a mouse/human hybrid cell panel

Autosomal sequences homologous to the human Y-chromosome 3.4-kb repeat have been described as existing in three separate "domains", one of these being centered on chromosome 16, the D "domain" (Burk et al. 1985; Jeanpierre et al. 1985 – see summary of their results in Table 1). Examination was therefore made of a mouse/human cell panel useful in mapping chromosome 16 (Callen 1986).

DNA from each cell line (Table 2) was digested with *Eco*RI and the Southern blots probed with 28S. DNA from

Table 1. Autosomal location of the Y 3.4-kb repeat homologues

Probe source	Hybridization to autosomes	Restriction fragments	Reference
Fragment (3.4 kb) from hybrid containing Y chromosome only	Major: 1, 9, 13, 14, 16, 21, 22 Minor: 11, 12, 17 20	*Kpn*-I: 3.6, 1.8 (chromosome 15) *Eco*RV: 6.2 chromosome 16), 3.6, 1.8 *Eco*RI: 3.4 (Y chromosome), 1.8 and others	Burk et al. (1985)
Fragment from chromosome 22 library hybridizing with Y-chromosomal 3.4-kb fragment	Major: 9, 13, 14, 15, 17, 20, 21, 22 Minor: 1, 5, 10, 16	*Kpn*I: 3.6, 1.8 (chromosome 9 or 15) *Eco*RV: 6.1 (chromosome 16), 3.6, 1.8	Jeanpiere et al. (1985)

Table 2. Mouse/human hybrid cell panel[a]

Clone	Selected chromosomes present	Other human chromosomes
CY2	der(X)t(X;16)(q26;q24)	Nil
CY3	der(16)t(x;16)(q26;q24)	Nil
CY5	der(10)t(10;16)(q26;q22)	Nil
CY7	der(3)t(3;16)(q13.2;q13)	10, 12
CY9	del(16q)	4, 13, 21
CY11	der(16)t(11;16)(p11;q13)	Nil
CY13	der(16)t(1;16)(q44;p13.11)	3, 11, 14, 17, 20, 21, 22
CY15	del(16p)	14, 17, 21
CY18	16	Nil
A9	Mouse parent line	

[a] The hybrid cell panel was that described by Callen (1986)

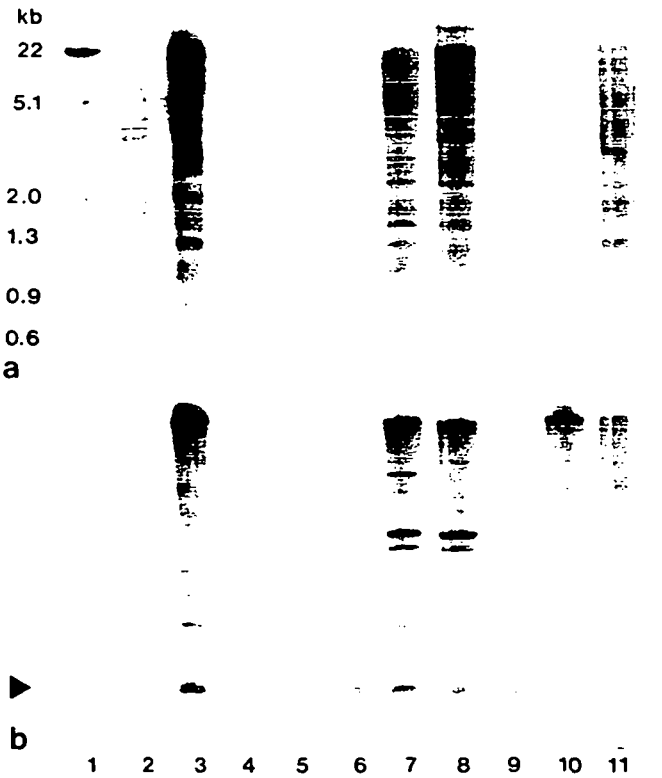

Fig. 6a, b. Examination of a mouse/human cell panel. **a** DNA was digested with *Eco*RI and probed with ^{35}S-labelled 28S. **b** The membrane was then stripped and probed with ^{35}S-labelled 16S. *Lane 1* DNA size standard. *lane 2* CY7. *lane 3* CY9. *lane 4* CY11. *lane 5* A9, *lane 6* CY18, *lane 7* CY15, *lane 8* CY13, *lane 9* CY3, *lane 10* CY2, *lane 11* CY5 (5 µg each). A 700-bp fragment correlating with the presence of extensive regions of chromosome 16 is marked by an *arrowhead*. Film exposure 24 h

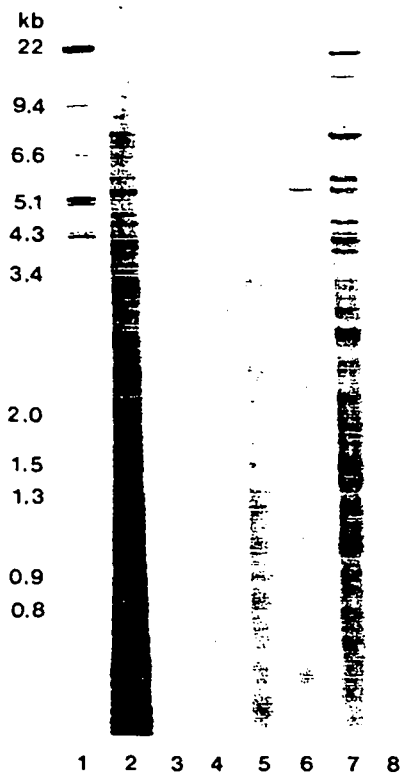

Fig. 7. Examination of mouse/human cell lines containing DNA positive for hybridization to 228S. DNA was digested with *Taq*I and probed with ^{35}S-labelled 228S. *Lane 1* DNA size standard. *lane 2* mixed human sperm DNA (3 μg), *lane 3* A9, *lane 4* CY5, *lane 5* CY13, *lane 6* CY15, *lane 7* CY9, *lane 8* CY7 (5 μg each). Film exposure 30 h

the cell lines CY7, CY9, CY15, CY13 and CY5 showed multiple restriction fragments but the DNA from the lines CY11, CY18, CY3 and CY2 showed very little hybridization with 228S (Fig. 6a). The presence of extensive regions of chromosome 16 was found to correlate with a fragment of approximately 700 bp (arrowed in Fig. 6b) when the same membrane was reprobed with sequence 216. The results show that 228S does not hybridize with chromosomes 16, X or part of chromosome 11.

DNA from the cell lines positive with 228S were then digested with *Taq*I and the blot probed with 228S. Many fragments in the size range 2–20 kb were found in all these lines except CY7 (Fig. 7). Some fragments were of similar size to the predominant *Taq*I/228S fragments in the mixed human sperm DNA sample.

Discussion

The satellite III DNA family is a graded series of sequences. These range from "pure" satellite III containing no *Taq*I sites, to examples with a high proportion of pure pentamer and some *Taq*I sites, through to Satellite II, which contains low proportions of the TTCCA pentamer and many *Taq*I sites. Examples of such sequences are compared in Fig. 2. This shows that the sequences 228S, pPD18 (Deininger et al. 1981), pHY10 (Nakahori et al. 1986), and pKS36 (Sol et al. 1986), are all closely related. The sequence HS3 (Cooke and Hindley 1979) is poorly related to these examples, showing strongest homology to pPD17 and failing to hybridize with another clone HS5 derived from the Y chromosome (Cooke and Hindley 1979).

Hybridization of the probe 228S

The *Taq*I patterns displayed by both 228S and the oligomeric probe indicate that the hybridization of 228S to genomic DNA is caused predominantly by the "clusters" of its pure pentameric repeats. Thus the polymorphic fragments themselves are either pure satellite III sequences, or clusters of such sequences within more degenerate forms of satellite III or other unrelated DNA sequences. It is predicted that sequences closely related to 228S (related by the method of Fig. 2) will be useful as probes for this *Taq*I polymorphism.

Genomic location of the TaqI/228 S polymorphism

Location of the polymorphism is made unusually difficult by the fact that satellite III is common, diverse and much of it is not polymorphic. However, the three domains identified as containing autosomal homologues to the Y chromosome 3.4-kb repeat are: domain K (*Kpn*I 3.6-, 1.8-kb fragment pattern, e.g. chromosomes 15 and 9), domain D (*Eco*RV 6.2-kb fragment from chromosome 16) and domain R (Burk et al. 1985). The latter domain was considered by Burk et al. (1985) to reside on chromosome 1, this being the site of a previously mapped satellite-III-like sequence HS3 (Gosden et al. 1981).

The finding that 228S gives a *Kpn*I restriction pattern characteristic of domain K-type sequences and that chromosome 16 is negative for 228S (Fig. 6a), indicates that domain K (e.g. chromosomes 9 and 15) is the primary genomic site of hybridization for sequence 228S. The poor homology between sequence 228S and the sequence HS3 (Fig. 2) from chromosome 1, suggests also that 228S may not hybridize strongly with R-domain chromosomes.

The presumptive source of the *Taq*I/228S polymorphism is therefore autosomes containing K domains, e.g. chromosomes 9 and 15. However autosomes other than these may also be contributory. This is because large (>3 kb) *Taq*I fragments were observed (Fig. 7) in the DNA from some of the cell lines in the chromosome 16 panel examined. These lines included chromosomes 13, 14, 17, 20, 21 and 22, all of which are listed (see Table 1) as containing autosomal homologues to the 3.4-kb Y-specific repeat. Thus results therefore implicate chromosomes 9, 15, 13, 14, 17, 20, 21 and 22 as the likely complement of autosomes that make up the complex *Taq*I/228S polymorphic patterns. Their relative contribution to these patterns is as yet unclear.

Aetiology of the TaqI/satellite III polymorphism

This polymorphism appears to be *Taq*I-specific, suggestive of some point mutational mechanism peculiar to *Taq*I. A possible cause is the accumulation of random point mutations (C→G) in the sequence TTCCA, thus creating *Taq*I sites, *TTCGA*. However the CpG dinucleotide created by such a mutation is itself considered to be highly mutable (C→T) (Cooper et al. 1985). This would convert the sequence to TTTGA, deleting *Taq*I sites with a consequent re-escalation in the size of restriction fragments. Examination of published satellite-III sequences shows that an increase in the frequency of the pentamer TTCGA in a sequence is generally accompanied by a proportionate increase in the pentamer TTTGA in the same

Table 3. Number of pentamers TTCCA, TTCGA and TTTGA (average per kb) of satellite-III-like sequences

Sequence	TTCCA	TTCGA	TTTGA	Reference
pPD9	131	–	–	Deininger et al. (1981)
228S	108	6	–	Fowler et al. (1987)
pPD18	84	18	6	Deininger et al. (1981)
233S	73	–	–	
262F	42	10	–	
pHY10	64	16	6	Nakahori et al. (1986)
pKS36	72	10	5	Sol et al. (1986)
HS3	40	16	14	Cooke and Hindley (1979)
pPD17	53	36	13	Deininger et al. (1981)

sequence (Table 3). This is in accord with a point mutational two-stage "decay" mechanism.

If the *Taq*I polymorphism is not generated by point mutational events, then it must arise from some alteration in DNA lengths between the *Taq*I sites. Recombination by integration of foreign DNA into satellite III sequences is known, e.g. integration of the HL1 virus (Seiki et al. 1982). The 33-bp sequence (HL1PRO3) forming the junction between the 3'long terminal repeat of this virus and the host satellite III shows strong homology to other satellite III sequences (Fig. 2). Hepatitis B virus has also been reported to integrate into satellite III (Shaul et al. 1986), though the same virus integrates as readily with GC-rich host sequences (Zerial et al. 1987).

Some polymorphisms in human repetitive DNA are attributed in part to the mobility of such sequences as extrachromosomal DNA, e.g. *Sau*3A polymorphism (Kiyama et al. 1987), and possibly the Bkm polymorphism (Traut 1987). One Satellite III sequence (clone pKS36) was isolated by virtue of its cross hybridization with clones of the *Drosophila* P element (Sol et al. 1986). This hybridization, though conducted at relatively low stringency, was not simply a result of AT composition as a viral sequence of similar AT proportion to the P element failed to hybridize to the same 1.8-kb *Eco*R1 human fragment. The sequences 228S and pKS36 are very closely related (Fig. 2) and both show *Kpn*I restriction fragment patterns of 3.6 and 1.8 kb, typical of K-domain autosomal satellite-III sequences (Burk et al. 1985). The three longest regions of homology between clone pKS36 and the P element were within open reading frames 1 and 2 of the P-element-encoded transposase (Sol et al. 1986). P elements are mobile genetic elements causing hybrid dysgenesis and chromosomal instability in selected *Drosophila* strains (Engels 1983). However the direct evidence is that satellite III sequences are not extremely mobile, as no detectable hybridization of sequence pPD18 (closely related to 228S, Fig. 2) was found in closed circular extrachromosomal DNA (Riabowol et al. 1985).

If any of these non-reciprocal recombination events are predominantly responsible for the *Taq*I polymorphism, then the spacing of restriction sites, other than *Taq*I, in satellite III should also alter. If such alternative sites were of similar frequency to *Taq*I then a different presentation of the same polymorphism should occur. This has not been found. Thus although non-reciprocal recombination events cannot be entirely excluded, they are unlikely to be responsible for the bulk of the polymorphism. Thus the satellite III polymorphism appears to be quite distinct in its aetiology from that of the (GC-rich) minisatellites (Jeffreys et al. 1985a).

In summary the *Taq*I/satellite III polymorphism is carried by approximately a third of the chromosomal complement, it is probably caused by point mutational events, it is highly discriminating of individual genomes, and the restriction fragments segregate in an orthodox manner with no evidence thus far for production of new bands by recombination.

Examination of larger populations is required to establish the frequency of the major restriction fragments. The ease of its detection from small quantities of DNA (3 µg) using a [^{35}S] labelled probe makes it potentially useful for forensic analyses.

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References

Ali S, Müller CR, Epplen JT (1986) DNA finger printing by oligonucleotide probes specific for simple repeats. Hum Genet 74: 239–243

Bucholtz CA, Reisner AH (1986) MBIS-an integrated system for the retrieval and analysis of sequence data from nucleic acids and proteins. Nucleic Acids Res 14:238–265

Burk RD, Szabo P, O'Brien S, Nash WG, Yu L, Smith KD (1985) Organization and chromosomal specificity of autosomal homologs of human Y chromosome repeated DNA. Chromosoma 92:225–233

Callen DF (1986) A mouse-human hybrid cell panel for mapping human chromosome 16. Ann Génét (Paris) 29:235–239

Cooke HJ, Hindley J (1979) Cloning of human satellite III DNA: different components are on different chromosomes. Nucleic Acids Res 6:3177–3197

Cooke HJ, Fantes J, Green D (1983) Structure and evolution of human Y chromosome DNA. Differentiation [Suppl] 23:S48–S55

Cooper DN, Smith BA, Cooke HJ, Niemann S, Schmidtke J (1985) An estimate of unique DNA sequence heterozygosity in the human genome. Hum Genet 69:201–205

Das HK, Jackson CL, Miller DA, Leff T, Breslow JL (1987) The human apolipoprotein C-II gene sequence contains a novel chromosome 19-specific minisatellite in its third intron. J Biol Chem 262:4787–4793

Deininger PL, Jolly DJ, Rubin CM, Friedmann T, Schmid CW (1981) Base sequence studies of 300 nucleotide renatured repeated human DNA clones. J Mol Biol 151:17–33

Devilee P, Cremer T, Slagboom P, Bakker E, Scholl HP, Hager HD, Stevenson AFG, Cornelisse CJ, Pearson PL (1986) Two subsets of human alphoid repetitive DNA show distinct preferential localization in the pericentric regions of chromosomes 13, 18, and 21. Cytogenet Cell Genet 41:193–201

Drinkwater RD, Burgoyne LA, Skinner JD (1986) Two human repetitive elements: a new interspersed repeat found in the factor IX gene, and a satellite II tandem repeat sequence. Nucleic Acids Res 14:9541

Engels WR (1983) The P family of transposable elements in *Drosophila*. Annu Rev Genet 17:315–344

Fowler JCS, Drinkwater RD, Burgoyne LA, Skinner JD (1987) Hypervariable lengths of human DNA associated with a human satellite III sequence found in the 3.4-kb Y-specific fragment. Nucleic Acids Res 15:3929

Frommer M, Prosser J, Tkachuk D, Reisner AH, Vincent PC (1982) Simple repeated sequences in human satellite DNA. Nucleic Acids Res 10:547–563

Gosden JR, Lawrie SS, Cooke HJ (1981) A cloned repeated DNA sequence in human chromosome heteromorphisms. Cytogenet Cell Genet 29:32–39

Hardman N (1986) Structure and function of repetitive DNA in eukaryotes. Biochem J 234:1–11

Hellman L, Pettersson U (1987) Analysis of closely related genes by use of synthetic oligonucleotide probes labelled to a high specific activity. Gene Anal Tech 4:9–13

Jabs EW, Meyer DA, Bias WB (1986) Linkage studies of polymorphic, repeated DNA sequences in centromeric regions of human chromosomes. Am J Hum Genet 38:297–308

Jarman AP, Nicholas RD, Weatherall DJ, Clegg JB, Higgs DR (1986) Molecular characterization of a hypervariable region downstream of the human alpha-globin gene cluster. EMBO J 5: 1857–1863

Jeanpierre M, Weil D, Gallano P, Creau-Goldberg N, Junien C (1985) The organization of two related subfamilies of human tandemly repeated DNA is chromosome specific. Hum Genet 70: 302–310

Jeffreys AJ, Wilson V, Thein SL (1985a) Hypervariable "minisatellite" regions in human DNA. Nature 314:67–73

Jeffreys AJ, Wilson V, Thein SL (1985b) Individual-specific fingerprints of human DNA. Nature 316:76–79

Kiyama R, Matsui H, Okumura K, Oishi M (1987) A group of human repetitive DNA families that is characterized by extrachromosomal oligomers and restriction-fragment length polymorphism. J Mol Biol 193:591–597

Lund Jorgensen A, Bostock CJ, Leth Bak A (1987) Homologous subfamilies of human alphoid repetitive DNA on different nucleolus organizing chromosomes. Proc Natl Acad Sci USA 84:1075–1079

Nakahori Y, Mitani K, Yamada M, Nakagome Y (1986) A human Y-chromosome specific repeated DNA family (DYZ1) consists of a tandem array of pentanucleotides. Nucleic Acids Res 14:7569–7580

Nakamura Y, Leppert M, O'Connell P, Wolff R, Holm T, Culver M, Martin C, Fujimoto E, Hoff M, Kumlin E, White R (1987a) Variable number of tandem repeat (VNTR) markers for human gene mapping. Science 235:1616–1622

Nakamura Y, Julier C, Wolff R, Holm T, O'Connell P, Leppert M, White R (1987b) Characterization of a human "minisatellite" sequence. Nucleic Acids Res 15:2537–2548

Prosser J, Frommer M, Paul C, Vincent PC (1986) Sequence relationships of three human satellite DNAs. J Mol Biol 187:145–155

Reed KL, Mann DA (1985) Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res 13:7207–7221

Riabowol K, Shmookler Reis RJ, Goldstein S (1985) Interspersed repetitive sequences are differentially represented in extrachromosomal covalently closed circular DNA of human diploid fibroblasts. Nucleic Acids Res 13:5563–5584

Seiki M, Hattori S, Yoshida M (1982) Human adult T-cell leukemia virus: molecular cloning of the provirus DNA and the unique terminal structure. Proc Natl Acad USA 79:6899–6902

Shaul Y, Garcia PD, Schonberg S, Rutter WJ (1986) Integration of hepatitis B virus DNA in chromosome-specific satellite sequences. J Virol 59:731–734

Simmler MC, Johnsson C, Petit C, Rouyer F, Vergnaud G, Weissenbach J (1987) Two highly polymorphic minisatellites from the pseudoautosomal region of the human sex chromosomes. EMBO J 6:963–969

Singer MF (1982) Highly repeated sequences in mammalian genomes. Int Rev Cytol 76:67–112

Singer MF, Skowronski J (1985) Making sense out of LINES: long interspersed repeat sequences in mammalian genomes. Trends Biochem Sci 10:119–122

Singh L, Jones KW (1986) Bkm sequences are polymorphic in humans and are clustered in pericentric regions of various acrocentric chromosomes including the Y. Hum Genet 73:304–308

Sol K, Lapointe M, MacLeod M, Nadeau C, DuBow MS (1986) A cloned fragment of HeLa DNA containing consensus sequences of satellite II and satellite III DNA hybridizes with the *Drosophila* P-element and with the 1.8 kb family of *Kpn*I fragments. Biochim Biophys Acta 868:128–135

Traut W (1987) Hypervariable Bkm DNA loci in a moth, *Ephestia kuehniella:* does transposition cause restriction fragment length polymorphism? Genetics 115:493–498

Vassart G, Georges M, Monsieur R, Brocas H, Lequarre AS, Christophie D (1987) A sequence in M13 Phage detects hypervariable minisatellites in human and animal DNA. Science 235:683–684

Way JS, Willard HF (1986) Structure, organization, and sequence of alpha satellite DNA from human chromosome 17: evidence for evolution by unequal crossing over and an ancestral pentamer repeat shared with the human X chromosome. Mol Cell Biol 6: 3156–3165

Willard HF, Waye JS, Skolnick MH, Schwartz CE, Powers VE, England SB (1986) Detection of restriction fragment length polymorphisms at the centromeres of human chromosomes by using chromosome-specific alpha satellite DNA probes: implications for development of centromere-based genetic linkage maps. Proc Natl Acad Sci USA 83:5611–5615

Wolfs SF, Mareni CE, Migeon BR (1980) Isolation and characterization of cloned DNA sequences that hybridize to the human X chromosome. Cell 21:95–102

Wu JC, Manuelidis L (1980) Sequence definition and organization of a human repeated DNA. J Mol Biol 142:363–386

Zerial M, Salinas J, Filipski J, Bernardi G (1987) Genomic localization of hepatitis B virus in a human hepatoma cell line. Nucleic Acids Res 14:8373–8386

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Improved separation of multi-locus hypervariable DNA restriction fragments by field inversion gel electrophoresis and fragment detection using a biotinylated probe

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DNA probes to multiple hypervariable human genomic loci are potentially highly informative as regards individual genome identification and parentage studies. However the resultant Southern blot patterns are generally highly complex and their interpretation difficult, with one important limitation being the extent of fragment separation and resolution. The separation of large DNA restriction fragments in agarose gels may be improved, in comparison to separations obtained by conventional electrophoresis, by the use of field inversion techniques. This is demonstrated by the analysis of the complex human Satellite III related DNA polymorphism. Detection of the Satellite III related restriction fragments is achieved either by using a [^{35}S]-labelled probe (228S) or by using the same probe in a convenient non-isotopic form constructed by the photobiotin process. In addition, the probe 228S is useful for sexing the human genome, by the identification of a Y-specific restriction fragment.

Introduction

Pulsed field gel electrophoresis (PFGE) and field inversion gel electrophoresis (FIGE) have made possible the separation of extremely large DNA fragments e.g., PFGE up to 10 Mb (Anand 1986) and FIGE up to 1 Mb (Carle *et al.*, 1986). These techniques will greatly benefit the study of complex genomes (Barlow & Lehrach 1987; McPeek *et al.*, 1986, Southern *et al.*, 1987; Gemmill *et al.*, 1987; Holzwarth *et al.*, 1987; Lalande *et al.*, 1987). The field inversion method is relatively simple since the electrode geometry is the same as that for conventional electrophoresis but improvement in DNA separation is achieved simply by switching the field polarity and by controlling the rate of this inversion (Carle *et al.*, 1986). Distinct 'windows' of enhanced fragment separation are obtained by altering the so-called forward and reverse pulse times. Generally, the faster the switching rate, the further the 'window' of enhanced separation moves to smaller size fragments (Carle *et al.*, 1986). Although PFGE and FIGE will have their most important application in the analysis of extremely large DNA fragments, the field inversion method also has significant influence upon the separation of DNA fragments less than 30 kb in size (Carle *et al.*, 1986).

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Restriction fragment length polymorphisms which arise from multiple loci in the human genome generally present complex banding patterns over a broad size range e.g., (Jeffreys *et al.*, 1986; Fowler *et al.*, 1987) and are associated with tandemly repeated DNA. Length variations are revealed by either the restriction of DNA *external* to the block of variable number tandem repeats – 'minisatellites' – (Jeffreys *et al.*, 1986), or by restriction *within* a long tandemly repeating sequence at point mutable sites which are distributed unevenly along its length. The TaqI specific human Satellite III related DNA polymorphism is believed to be an example of the latter type (Fowler *et al.*, 1987; Fowler *et al.*, 1988a, 1988b). The most informative region of this genomic variation occurs in the range of about 30 to 5 kb, with the restriction fragments apparently following orthodox segregation based on family studies (Fowler *et al.*, 1988a). The field inversion technique is adapted here to improve the separation of the most informative subset of fragments in the Satellite III polymorphism.

Since Satellite III related sequences are abundant in the human genome and the polymorphic fragments are in multiple copies (Fowler *et al.*, 1988a), their detection using a non-isotopic method is an attractive and safe alternative to the use of isotopically labelled probes. Thus the Satellite III sequence related probe used previously (Fowler *et al.*, 1987, 1988a) has been recloned into a PUC vector, photo-chemically labelled with biotin (Forster *et al.*, 1985; McInnes *et al.*, 1987) and used as a probe for the TaqI/Satellite III polymorphism.

Materials and methods

DNA samples

Frozen blood samples which had previously been drawn and stored in tubes containing a heparin anticoagulant were thawed in 2 volumes of 1 × SSC (150 mM NaCl, 15 mM trisodium citrate). Nucleated cells were recovered by centrifugation at about 8000*g* for 20 min. The pellet was digested at 37°C for 14 h in aqueous buffer (10 mM Tris, 10 mM disodium EDTA, 100 mM NaCl, pH 8.0) to which was added 1% SDS and proteinase K (50 μg ml^{-1}). The digests were extracted with phenol and chloroform and the DNA precipitated with ethanol. The DNA was redisolved in TE buffer (10 mM Tris, 1 mM EDTA pH 8.0) and quantitated by spectrofluorimetry.

DNA was digested with TaqI or HaeIII (Boehringer Mannheim) using the buffer and conditions specified by the manufacturer and at a five fold excess calculated to

give complete digestion. The DNA was precipitated and redissolved in TE buffer (generally 8 µl) prior to loading into the gel as described previously (Fowler *et al.*, 1988a).

Electrophoresis

Conventional electrophoresis was performed at conditions and in buffers described previously (Fowler *et al.*, 1988a).

Electrophoresis using the field inversion technique was generally in 1.0% agarose gels (Sigma grade 1a) using TAE buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.5) at approximately 6 V/cm for defined periods (see below). Electrophoresis was conducted at ambient temperature (24°C) with rapid recycling of the buffer in the direction of anode to cathode (total electrophoresis tank volume was 1.9 l, pumping rate was 200 ml min^{-1}, temperature differential outlet minus inlet was less than 2°C). The cast gel was approximately 4.5 mm thick and 21 cm long. A pre-cast strip (5 mm wide and 4.5 mm thick) of agarose (1.0% in the same buffer) containing 0.5% clean DEAE sephadex beads was positioned perpendicular to the path of the DNA across the extreme anode end of the gel. This trapped small DNA fragments (about 2.0 kb and less) which would otherwise have migrated out of the gel and been re-cycled by the buffer flow. The purpose of this was to minimize background contamination of the Southern blots.

Field inversion was attained by interfacing between the power pack and the tank electrodes, a transistorised switch controlled by a Commodore PC Model CBM 4016 N. The rate of switching was controlled by one of either two programmes: either general ramp or mini-ramp. General ramp increased both the forward and reverse pulse rates from a selected start to a selected end time, ramping evenly over the full anticipated duration of electrophoresis. Mini-ramps completed the same ramp process but in a series of continuous cycles of 15 min duration. Electrophoresis is then continued to reach a defined number of mini-ramps.

Molecular size markers (intact lambda 48.5 kb) and fragments from combined HindIII and HindIII/EcoRI digests (23.1, 21.2, 9.4, 6.5, 5.1, 4.9, 4.4, 4.3, 3.5, 2.3 kb and smaller fragments) were included in each gel, either being stained with ethidium bromide and photographed, or being nick-translated with 35S dATP and appearing on the auto-radiographs themselves.

Gels were brought to a stable and uniform temperature by electrophoresis at operating voltages for 30 min prior to loading the DNA samples.

Sourthern blotting

Samples intended for subsequent analysis by isotopically labelled probes were blotted to nylon membranes (Bio-Rad Zeta Probe) as described previously (Fowler *et al.*, 1988a). Samples intended for analysis by the biotin labelled probes were blotted to nitro-cellulose membranes (Schleicher & Schuell BA 85) as described by McInnes *et al.*, 1987.

Probe preparation

Probes of the Satellite III related sequence 228S (Fowler *et al.*, 1987) were prepared, using 35S dATP, and used as described previously (Fowler *et al.*, 1988a). Biotinylated probes of the sequence 228S (Fowler *et al.*, 1987) and the alphoid related sequence 226S (Fowler *et al.*, 1988a) were prepared as follows.

The sequences 228S and 216S previously cloned into the single- stranded phage M 13 vector were both recloned into the SmaI site of vector PUC 19. The purified plasmid was then biotinylated by the photobiotin method (Forster *et al.*, 1985, McInnes *et al.*, 1987) as detailed in the BRESATEC protocol. The purpose of placing the inserts into PUC 19 was that the biotinylation of double stranded plasmids gives about 5 × greater sensitivity of sequence detection than that of the biotinylated single stranded M 13 phage when used as probes (C. Fowler, unpublished observations). The PUC biotinylated probes, containing either the insert 228S or 216S, were denaturated by heating at 95°C for 4 min, snap cooled and added to establish a final probe concentration of 50 ng ml^{-1} of hybridization solution.

Hybridization, washing and development

Both nylon and nitro-cellulose membranes were pre-hybridized for 2 h at 68°C in the same hybridization buffer (0.7 M NaCl, 50 mM NaH_2PO_4, 4 mM disodium EDTA, 1% SDS and 0.5% non-fat powdered milk; pH 7.7). The membranes were then hybridized in the hybridization buffer above containing the appropriate probe: nylon membranes with 35S dATP 228S as described previously (Fowler *et al.*, 1988a), and nitrocellulose membranes with biotin labelled probes. Both were hybridized at 68°C for 16 h.

The membranes were then washed in four solutions of increasing stringency (wash A 2 × SSC/0.1% SDS for 15 min, wash B 0.5 × SSC/0.1% SDS for 90 min, wash C 0.1 × SSC/0.1% SDS for 15 min, wash D 0.1 × SSC/1% SDS for 15 min. Wash A, B and C were at 37°C and wash D was at 55°C.

Membranes probed with 35S dATP 228S were dried and exposed at ambient temperature to X-Ray film (Kodak XOMat RP film).

Fragments complementary in sequence to the biotinylated probes were detected by an avidin-alkaline phosphatase conjugate following the method of Forster *et al.*, 1985 and the protocol of the probe manufacturer (BRESATEC photobiotin-labelling and detection kit). Membranes were incubated in the substrate reaction for up to 6 h in the dark, before being stored in the stopping buffer.

Results and discussion

Establishing a field inversion protocol

Switching rates: Published results indicated switching rates of 0.5 sec forward and 0.25 sec backwards to be appropriate for the separation of fragments in the size range 30 to 15 kb (Carle *et al.*, 1986). Based on the stability and accuracy of the switch constructed, our switching was generally ramped between 0.3 sec forward, 0.15 sec reverse at the start of ramping, and 0.7 sec forward to 0.35 sec reverse at its end. Other settings were also tested (see below). Ramping of the forward and reverse pulse times is necessary so as to optimize the field inversion process over the size range of fragments which

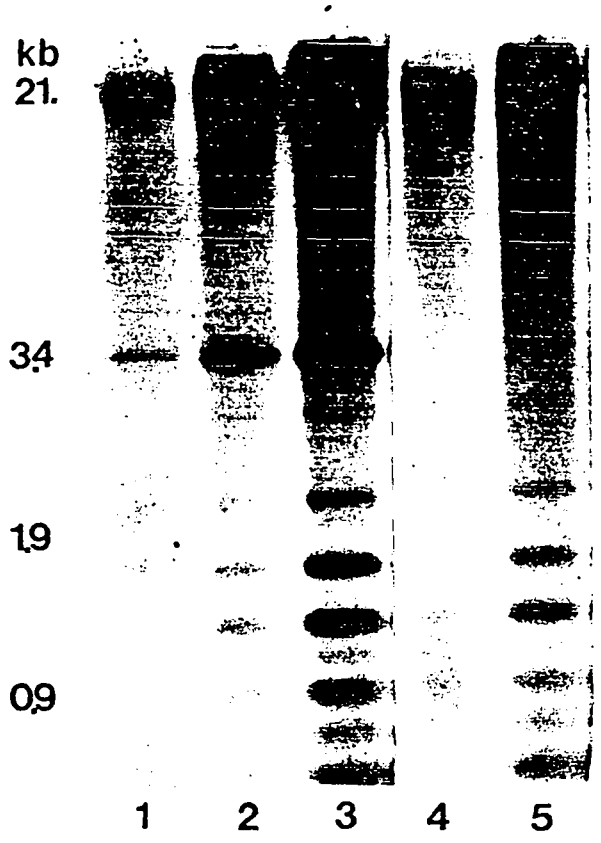

Figure 2 Digestion of male and female DNA with HaeIII. Southern blotting to nitro-cellulose and probing with the biotinylated probes 228S and 216S simultaneously. Male DNA: tracks 1, 2 and 3 (5, 20, 100 ng respectively) Female DNA: tracks 4 and 5 (5, 20 ng respectively)

Demonstration of the combined use of field inversion electrophoresis and the biotinylated probe 228S to detect the TaqI/Satellite III polymorphism

This is demonstrated by the Southern analysis of 5 unrelated individuals for the TaqI/Satellite III polymorphism (Fowler *et al.*, 1987, 1988a) (Figures 3 and 4). The analysis was made firstly using a blot to a nylon membrane (Zeta-probe) and probing with 35S dATP/228S (Figure 3). Electrophoresis time was 22.5 h or 88 miniramps. Separation of the same lambda standard markers by conventional electrophoresis is shown for comparison (Figure 3, track 1). Electrophoresis here was in 0.8% agarose for 27 h. This result was so chosen as to show coincidence in the 23.1 kb and 5.1 kb bands, but the clear differences in fragment separation both within this range and fragments smaller than this (Figure 3, compare tracks 1 and 2).

The analysis was repeated using the same five individuals, but this time blotting to a nitro-cellulose membrane and probing with the biotinylated 228S probe (Figure 4). The results were identical to those obtained earlier (Figure 3). Electrophoresis time for the biotin result was 24 h (96 miniramps).

Comparison of these two methods showed that band resolution was marginally superior in the 35S probed membrane when compared to that of the biotin result. This may be due in part to the greater speed and simplicity of the alkali blotting protocol (Fowler *et al.*, 1988a), compared to that of the high salt method (McInnes *et al.*, 1987), thereby reducing band diffusion. Either method requires DNA samples of between 2 to 4 μg lots, although the isotopic method was slightly more sensitive. However, the biotinylated probe 228S was used without sonication. If this is done, it is known to improve the sensitivity of the biotin/alkaline phosphatase detection at least five fold (Habili *et al.*, 1987). Any difference in sensitivity between these methods may not indeed be directly related to their detection efficiencies, but may rather be a difference in the quantity of the target DNA transferred by the different blot protocols and/or the nature of DNA binding to the different membranes. Although it is possible to use nylon membranes such as Zeta Probe with biotinylated probes, nitro-cellulose membranes generally do not show as much background colour development. The colour development of the banding patterns on nitro-cellulose mem-

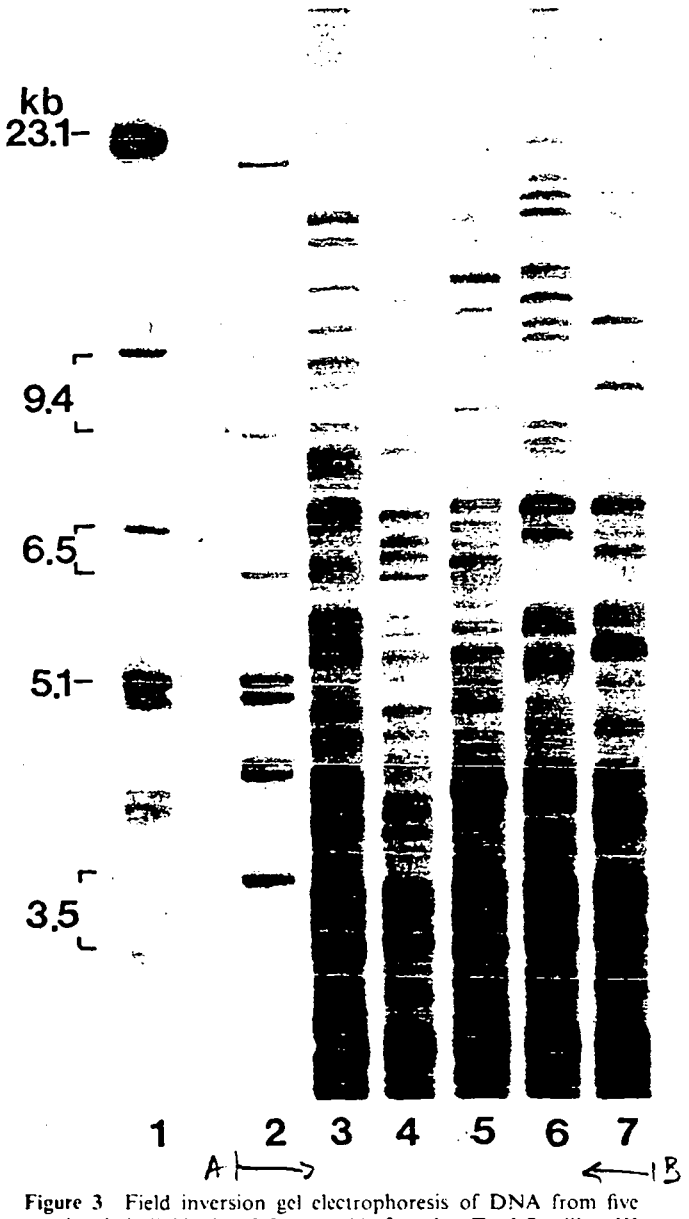

Figure 3 Field inversion gel electrophoresis of DNA from five unrelated individuals (2.5 μg each) for the TaqI/Satellite III polymorphism (tracks 3 to 7). The separation of lambda standard kb fragments (tracks 1 and 2) by conventional electrophoresis (track 1, 0.8% agarose, 2 V/cm, 27 h) and field inversion (track 2, 1% agarose, 5.2 V/cm, 22.5 h or 88 mini-ramps, 0.3 to 0.7 sec forward, 0.15 to 0.35 sec reverse) may be compared. Blot was made to a nylon membrane and probed with the radio-isotopically labelled probe 228S. 60 h film exposure

are of interest (Carle *et al.*, 1986). By using mini-ramps, all selected conditions are met every 15 min, and thus fragment separation is representative of all selected conditions should mid-run power failure occur.

Buffer choice and buffer concentration: So as to obtain sufficient total migration of DNA, the voltage gradients in FIGE need to be greater than those in conventional electrophoresis if comparable separations are to be obtained in similar times. Such gradients generate higher currents, and thus better temperature controls are needed. The complete absence of sodium ions from the buffer used (see Anand 1986 and Materials and methods) greatly reduced the current from when sodium ions were present, thus minimising gel heating.

Lowering the Tris ion concentration from 40 mM to 20 mM also lowered currents and reduced heating, but this generally worsened band shape and resolution.

Agarose concentration: Reported agarose concentrations used were between 1.0 and 1.5% (Anand 1986; Carle *et al.*, 1986). This is advantageous, providing greater mechanical stability for both gel handling and in the blotting process when compared to the lower concentration gels (0.8% and less) normally used in the separation of DNA in approximately the size range 30 to 5 kb (Jeffreys *et al.*, 1986; Baird *et al.*, 1986).

Figure 1 (Graph) shows the results from the trials made. The plots are of log kb DNA size against the square root of the band fragment mobility, for intact lambda and combined fragments from lambda/HindIII EcoRI and lambda/HindIII digestions.

Conventional electrophoresis (1% agarose) shows the plot to be linear about as far as 10 kb with DNA larger than this being poorly separated, eventually becoming unresolved as a 'reptating' (Carle *et al.*, 1986) band. Field inversion at the switching and ramp conditions specified greatly eliminates the 'reptating' behaviour of DNA fragments greater than about 15 kb, producing a linear plot beyond 23 kb (Figure 1). Small DNA fragments, about 5 kb and less, are less separated than in conventional electrophoresis, becoming by comparison bunched, and thus the gradient of the plot is steeper (Figure 1). An increase of agarose concentration from 1.0 to 1.5% lessened the overall migration of DNA fragments, for similar voltage gradients and run times (Figure 1). Despite this higher concentration, separation of large DNA fragments, e.g., 48.5 to 15 kb, was still possible (Figure 1). Halving the reverse voltage compared to the forward voltage, but maintaining the same pulse and ramp conditions gave poorer separation of the large fragments and a greater tendency to 'reptation' behaviour, more similar to that of conventional separations (Figure 1). Faster switching, e.g., 0.3 sec to 0.5 sec forward and 0.15 sec to 0.25 sec reverse was found to reduce the separation in the 48.5 to 23 kb region.

As regards temperature control, most attention was paid to minimizing any temperature gradients through the 4.5 mm thick gel. This was best achieved by rapid recycling of the buffer, and by casting and supporting the gel level, and on a thin (3 mm) glass plate which was raised (3 mm) in the electrophoresis tank so as to allow the buffer to circulate rapidly both above and below the gel. Although some trials were made controlling buffer temperatures to between 10 and 14°C, lower temperatures slows DNA mobility (West 1987), thus lengthening analysis time. Electrophoresis at ambient temperature (24°C) gave good results provided that the precautions noted above were made and that voltage gradients did not exceed about 7.5 V/cm.

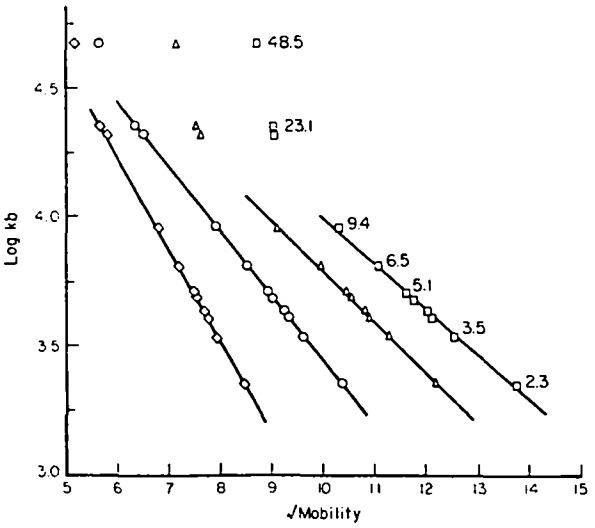

Figure 1 Plot of log kb of the DNA band/fragment against the square root of its mobility (mm). □ Conventional electrophoresis at 5 V/cm, 16 h, 1% agarose. ○ F.I.G.E. 7.5 V/cm, 16 h (64 mini-ramps), 1% agarose, 0.3 sec forward and 0.15 sec reverse at start of the ramp and 0.7 sec forward and 0.35 sec reverse at the end of the ramp. ◇ F.I.G.E., as ○, but using 1.5% agarose. △ F.I.G.E., as ○, but reverse voltage reduced to 3.75 V/cm, half of the forward voltage

Properties of the probe 228S

The probe 228S is a Satellite III sequence related fragment containing a high proportion of the repeated pentameric sequence 5'TGGAA3' typical of Satellite III (Fowler *et al.*, 1987). It has two useful properties. Firstly, it detects a 3.4 kb Y-specific human specific fragment (Cooke *et al.*, 1983), and therefore may be used to sex human genomes. This is demonstrated by the conventional electrophoresis and Southern analysis of HaeIII digested human male and female DNA, where the probe 228S detects the 3.4 kb Y-specific fragment in males and an unresolved band, greater than 20 kb, in both male and female DNA (Figure 2). Simultaneous use of the biotinylated, alphoid probe 216S is advised as an internal control upon the adequacy of HaeIII digestion and to provide an estimate of the quantity of DNA present. The probe 216S detects a 'ladder' of autosomal fragments of an alphoid related sequence (Figure 2).

The second and more important property of the Satellite III probe 228S is that it detects, upon TaqI digestion of human genomes, a series of hypervariable fragments (Fowler *et al.*, 1987, 1988a). The region of greatest inter-individual variation is a subset of bands in the size range approximately 25 to 5 kb. The fragments in this subset are of autosomal origin only (Fowler *et al.*, 1988a), forming the unresolved DNA seen as the intense band, greater than about 20 kb, in the HaeIII digests used for sexing (Figure 2).

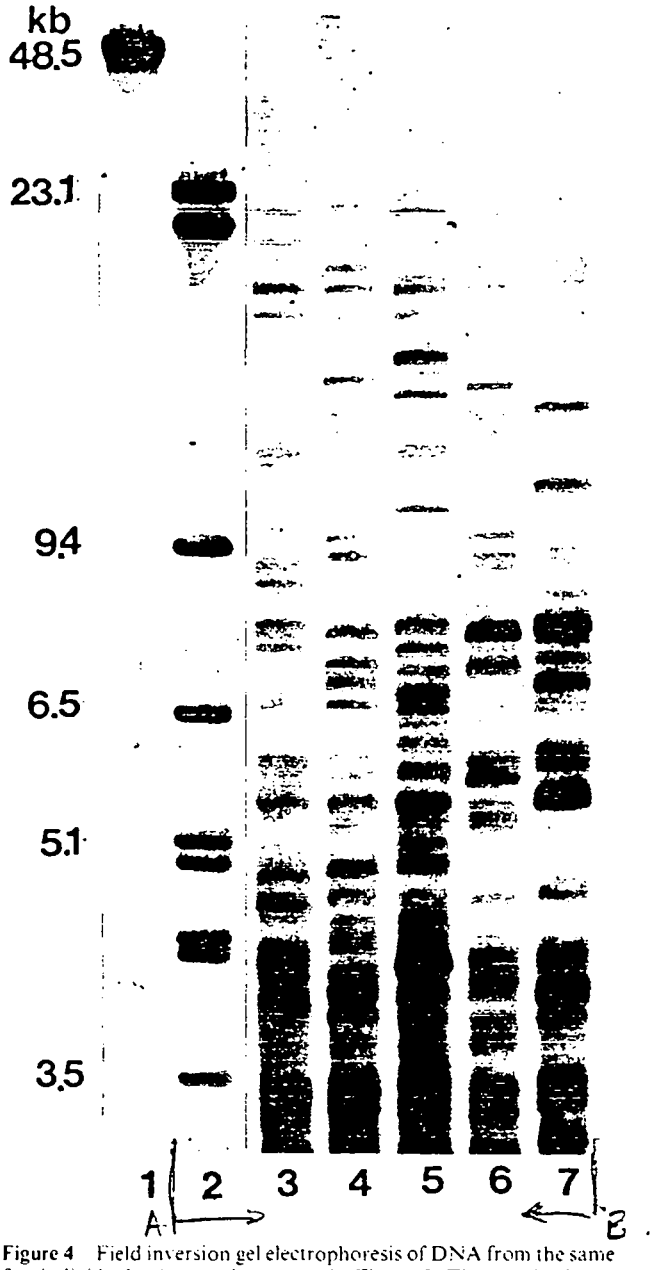

Figure 4 Field inversion gel electrophoresis of DNA from the same five individuals (4 μg each) as seen in Figure 3. The standards are intact lambda (track 1) and lambda standard kb fragments (track 2). Conditions as in Figure 3 (except electrophoresis at 6 V cm for 24 h or 96 mini-ramps). Blot was made to a nitro-cellulose membrane and probed with the biotinylated probe 228S. Detection time was 5 h

branes is normally evident after 30 min, fully developed after about 5 h but should not be prolonged beyond about 8 h when background colour becomes unacceptable.

Concluding remarks

Multi-locus hypervariable probes are potentially highly informative genetic markers for parentage studies and discrimination between individuals (Jeffreys *et al.*, 1986; Gill *et al.*, 1985; Gill *et al.*, 1987). However, the complex fragment patterns developed are relatively difficult to interpret, and this is partly because conventional electrophoresis separates DNA less effectively the larger the fragments, and particularly if fragments are greater than about 10 kb. These sizes of fragments may be highly informative. Field inversion electrophoresis may be used to separate DNA fragments in a wide size range, especially very large DNA which is 'reptating' as an unresolved band as seen in conventional electrophoresis. The technique is also adaptable to improving the separation of DNA fragments in the size range about 30 to 5 kb. This is achieved in 1% (or greater) agarose gels which are of greater mechanical strength in handling and blotting procedures than the lower concentration gels (0.8% and less) normally used to separate large DNA fragments. The field inversion method presented would also provide better separation of hypervariable single locus sequences whose fragment distribution falls into the 30 to 15 kb size range (Baird *et al.*, 1986). Such sequences have been used, for example, in the analysis of forensic samples (Kanter *et al.*, 1986). The improved separations using field inversion techniques, rather than conventional electrophoresis, are of benefit in establishing the automated image capture, computer analysis and comparison of the fragment patterns. These methods are currently being evaluated.

Non-isotopic probe methods have the advantage of being convenient and safe, as well as being more rapid than isotopic methods. No unduely long film exposure times are required. However a disadvantage is that the nitro-cellulose membranes cannot be stripped of one probe and reprobed with another, but this may be possible with nylon membranes (Gebeyehu *et al.*, 1987). Biotinylated probes have been used in the detection of single copy restriction fragment length polymorphisms (Dykes *et al.*, 1986). However, the resulting information is limited. The advantage of human Satellite III is its abundance in the genome and the complexity of its polymorphism. Its detection by a biotin labelled probe would serve as an advantage in its clinical or forensic application when compared to other methods (Baird *et al.*, 1986, Gill *et al.*, 1985, Gill *et al.*, 1987), although the quantity and quality of DNA recoverable from samples of forensic size and significance may limit the use of present non-isotopic probe methods.

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References

Anand, R. (1986). *Trends in Genet.*, **2**, 278–283.

Baird, M., Balazs, I., Giusti, A., Miyazaki, L., Nicholas, L., Wexler, K., Kanter, E., Glassberg, J., Allen, F., Rubinstein, P. & Sussman, L. (1986). *Am. J. Hum. Genet.*, **39**, 489–501.

Barlow, D.P. & Lehrach, H. (1987). *Trends in Genet.*, **3**, 167–171.

Carle, G.F., Frank, M. & Olson, V. (1986). *Science*, **232**, 65–68

Cooke, H.J., Fantes, J. & Green, D. (1983). *Differentiation*, **23** *(Supp.)*, S48–S55.

Dykes, D., Fondell, J., Watkins, P. & Polesky, H. (1986). *Electrophoresis*, **7**, 278–282.

Forster, A.C., McInnes, J.L., Skingle, D.C. & Symons, R.H. (1985). *Nucleic Acids Res.*, **13**, 745–761.

Fowler, J.C.S., Drinkwater, R.D., Burgoyne, L.A. & Skinner, J. (1987). *Nucleic Acids Res.*, **15**, 3929.
Fowler, J.C.S., Drinkwater, R.D., Skinner, J. & Burgoyne, L.A. (1988a). *Hum. Genet.*, (in press).
Fowler, J.C.S., Burgoyne, L.A., Harding, H.W.J. & Scott, A.C. (1988b). *J. For. Sci.*, (in press).
Gebeyehu, G., Rao, P.Y., SooChan, P., Simms, D.A. & Klevan, L. (1987). *Nucleic Acids Res.*, **15**, 4513–4534.
Gemmill, R.M., Coyle-Morris, J.F., McPeek, F.D., Ware-Uribe, L.F. & Hecht, F. (1987). *Gene Anal. Tech.*, **4**, 119–131.
Gill, P., Jeffreys, A.J. & Werrett, D.J. (1985). *Nature*, **318**, 577–579.
Gill, P., Lygo, J.E., Fowler, S.J. & Werrett, D.J. (1987). *Electrophoresis*, **8**, 38–44.
Habili, N., McInnes, J.L. & Symons, R.H. (1987). *J. Virol Meth.*, **16**, 225–237.
Holzwarth, G., McKee, C.B. Steiger, S. & Crater, G. (1987). *Nucleic Acids Res.*, **15**, 10031–10044.
Jeffreys, A.J., Wilson, V., Thein, S.L., Weatherall, D.J. & Ponder, B.A.J. (1986). *Am. J. Hum. Genet.*, **39**, 11–24.
Kanter, E., Baird, M., Shaler, R. & Balazs, I. (1986). *J. For. Sci.*, **31**, 403–408. 1986.
Lalande, M., Noolandi, J., Turmel, C., Rousseau, J. & Slater, G.W. (1987). *Proc. Nat. Acad. Sci. USA*, **84**, 8011–8015.
McInnes, J.L., Dalton, S., Vize, P.D. & Robins, A.J. (1987). *Biotechnology*, **5**, 269–272.
McPeek, F.D., Coyle-Morris, J.F. & Gemmill, R.M. (1986). *Anal. Biochem.*, **156**, 274–285.
Southern, E.M., Anand, R., Brown, W.R.A. & Fletcher, D.S. (1987). *Nucleic Acids Res.*, **15**, 5925–5944.
West, R. (1987). *Biopolymers*, **26**, 607–608.

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CHROMOSOMA

Human Satellite III DNA: Genomic location and sequence homogeneity of the TaqI-deficient polymorphic sequences

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Abstract. Human Satellite III DNA is a major tandem repeat in the human genome and presents a TaqI-specific hypervariable restriction fragment length polymorphism when a Satellite III related sequence (228S) is used as a probe. In situ examination shows this sequence to be near specific for the region 9qh on chromosome 9 when it is used at low probe concentrations. However the region 9qh does not appear to be the only or even the primary source of the TaqI-deficient polymorphic sequences (TDPS). Rather, such sequences appear to be mostly present in chromosomes 20, 21, and 22, and these represent the largest regions of homogeneous Satellite III in the genome; they are also resistant to digestion with a range of other restriction endonucleases. The TDPS do not arise from either of the two currently recognized Satellite III-enriched genomic regions, namely autosomal 'K-domains', which form part of 15p in chromosome 15 or the heterochromatin of chromosome Y.

Introduction

The human Satellite III like family of sequences comprises major tandem repeats located typically in the pericentric and centric heterochromatin of about one-third of the chromosome complement (Burk et al. 1985). They are diverged in sequence, the major recognised division being between Satellite III and Satellite II (Prosser et al. 1986). The former has a high proportion of the 'core' pentameric tandem repeat sequence 5'TTCCA 3', whilst the latter is believed to be a degenerate and more recently diverged form of this (Hollis and Hindley 1988). The proportion of these two related sequences within individual chromosomes differs, and thus chromosome-specific 'domains' enriched in one or the other sequence type exist (Burk et al. 1985; Cooke and Hindley 1979; Higgins et al. 1985; Jeanpierre et al. 1985; Moyzis et al. 1987).

Satellite III enriched domains are a source of extreme interindividual variation (Fowler et al. 1987, 1988a). The principal cause of this appears to be a C-G point mutation in the pentameric repeat 5'TTCCA 3', thereby creating a TaqI site (TCGA). These TaqI restriction sites are randomly located in the tandemly repeating blocks of Satellite III and thus provide the observed polymorphism (Fowler et al. 1988a).

This TaqI-specific Satellite III polymorphism is best observed in fragments of size range about 4 to 25 kb. About 10 to 20 such fragments exist in any one individual and these show Mendelian inheritance (Fowler et al. 1988a). There are in addition abundant numbers of Satellite III related fragments smaller than this in size, but these become increasingly difficult to resolve electrophoretically. Therefore the fragments greater than about 4 kb in size are the most informative. These sequences are only a minor portion of total Satellite III DNA and are here called the TaqI-deficient polymorphic sequences (TDPS).

It has been suggested (Fowler et al. 1988a) that the TDPS arose from autosomes, and probably those containing so-called 'K-domain' (Burk et al. 1985) Satellite III regions. These are typified by 3.6 and 1.8 kb KpnI tandem repeat fragments which have been mapped to chromosome 15 (Burk et al. 1985; Higgins et al. 1985) and chromosome 9 (Jeanpierre et al. 1985). This study further resolves the genomic location and properties of the TDPS.

Materials and methods

Tritium-labelled probes. A probe was prepared from the Satellite III related sequence 228S (Fowler et al. 1987) by primed synthesis of the complementary strand of the M13 template as previously described (Fowler et al. 1988a), but for in situ techniques [^{3}H]dATP (65 Ci/mmol) and [^{3}H]dCTP (50 Ci/mmol) were incorporated rather than [^{35}S]dATP.

Biotin-labelled probe. The single-stranded M13 vector containing the sequence 228S (Fowler et al. 1987) was biotinylated by the photobiotin method (McInnes et al. 1987) following the Bresatec protocol (Bresatec, South Australia).

Preparation of metaphase chromosomes. Phytohaemagglutinin-stimulated lymphocyte cultures were prepared from individuals of normal karyotype. The lymphocyte cultures were treated with 200 µg/ml bromodeoxyuridine for 16 h, then the cells were washed and incubated in fresh medium containing 2.4 µg/ml thymidine for 6 h. Colchicine (5 ng/ml) was added 20 min before harvest. Cells to be probed with the biotin-labelled probe were not pre-treated with

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Table 1. Human rodent hybrid cell panel[a]

Cell line	Human chromosomes present
64063[b]	9q
HORL 1[b]	15
CY14[c]	16(p13.3→qter), 4(q31→4qter), 1, 4, 12, 14, 20, 21
PK14[c]	46, XY,-16, +der(16)t(4;16)(q31;p13.3)
CY9[d]	del(16q), 4, 13, 21
CY13[d]	16(p13.11→qter), 1(q44→1qter), 3, 11, 14, 17, 20, 21, 22
CY15[d]	16(p12 or p13→qter), 11, 14, 17, 21
T85-43[c]	46,XX,del(16)(p12 or p13)
CY4[c]	der(13)t(13;16)(q34;q22.1)

[a] All hybrids are mouse/human except 64063 which is a hamster/human hybrid
[b] Obtained from Dr. P. Goodfellow, Imperial Cancer Research Fund, London, UK
[c] D.F. Callen (unpublished)
[d] Callen (1986)

bromodeoxyuridine and thymidine, but rather incubated with colchicine only, added 60 min prior to harvest. The cells were fixed and spread on slides, treated with RNAse and the chromosomes denatured and dehydrated (Simmers et al. 1986).

In situ hybridization using tritium-labelled probes. The probe (0.01 ng/μl) was hybridized to metaphase spreads, the slides then being dipped in Kodak NTB-2 emulsion and developed after 1 to 4 days (Simmers et al. 1986). Chromosomes were then banded as previously described (Callen et al. 1987).

In situ hybridization using biotinylated probe. Hybridization was for 17 h at 37° C in a humid chamber. Probe concentration was between 0.01 and 0.02 ng/μl dissolved in 25 μl hybridization buffer, pH 7.0 [2 × SSC (300 mM NaCl, 30 mM sodium citrate), 50% deionized formamide, 5% dextran sulphate, 0.1 mM disodium EDTA, 0.05 mM Tris-HCl, pH 7.3, 50 μg/ml sheared salmon sperm DNA]. Washing of the slides, and detection and amplification of the biotin were done essentially by the method of Burns et al. (1985).

Source and isolation of genomic DNA. DNA was isolated from nucleated cells in human blood and human sperm cells by standard methods described previously (Fowler et al. 1988a, b) as was the DNA from rodent/human hybrids. DNA from two rodent/human hybrids (64063 and HORL 1) was a gift from Dr. P. Goodfellow, The Imperial Cancer Research Fund, Lincoln's Inn Fields, London, UK. Hybrids examined are detailed in Table 1.

Digestion of DNA. The DNA was digested with restriction enzymes TaqI, HaeIII, KpnI, MspI, HpaII, AvaI, PstI, MboI, AluI, RsaI, or EcoRI (Boehringer Mannheim), precipitated and prepared for electrophoresis as described previously (Fowler et al. 1988a). DNA was recovered from preparative agarose gels using Gene-Clean (Bio-101).

Electrophoresis and Southern blotting. The DNA was separated by either conventional electrophoresis (Fowler et al. 1988a), or by field inversion gel electrophoresis (FIGE). Polarity switching, at constant voltage, was ramped in time intervals from 0.3 s forwards, 0.15 s reverse at the start, to 0.7 s forwards, 0.35 s in reverse at the finish, in continuous 15 min cycles so as to reach a defined number of 'miniramps' as described in detail elsewhere (Fowler et al. 1988b). Gels were blotted to nylon membranes as described previously (Fowler et al. 1988a). DNA molecular size standards were labelled with [^{35}S]-dATP and included in each gel or measured after staining the gel with ethidium bromide.

Probe preparation, hybridization, washing and exposure. The Satellite III sequence related probe 228S and the satellite

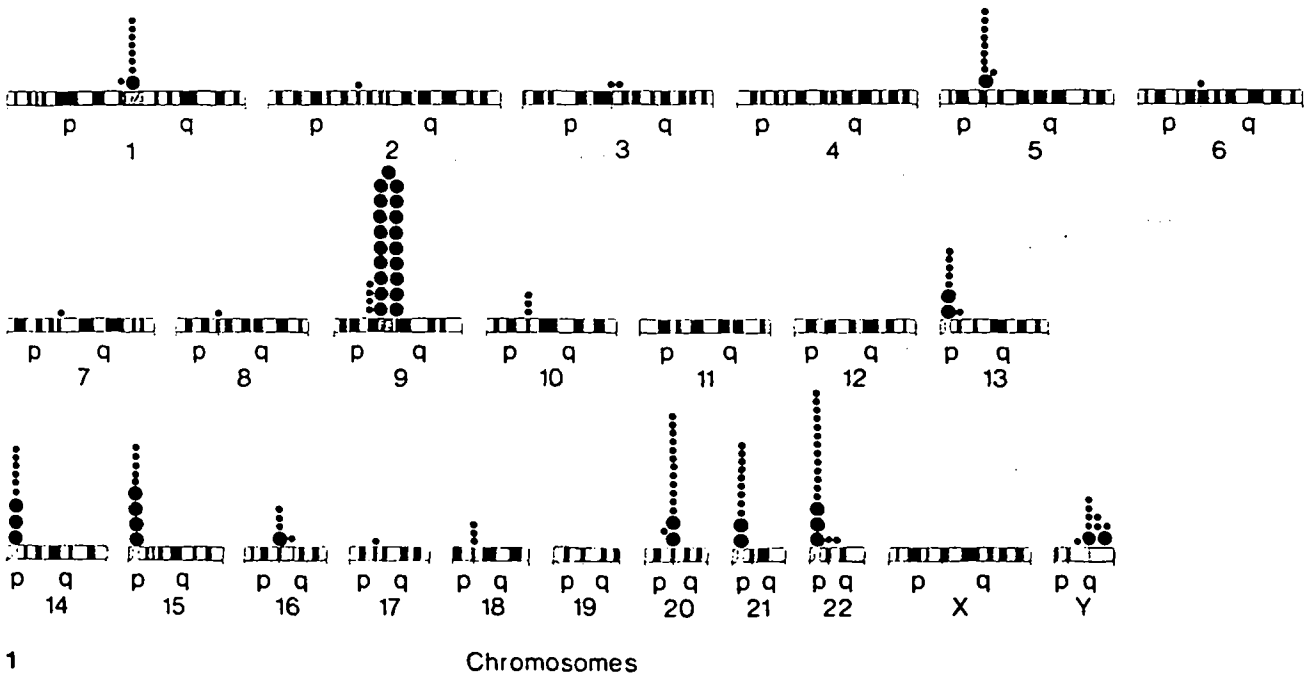


Fig. 1. Chromosomal location and distribution of sequences homologous to (^{3}H-labelled) clone 228S by in situ hybridization. Final wash stringency 2 × SSC, 50% formamide, 39° C. • single grain; ● multiple grain clusters

alphoid sequence related probe 216S were labelled with [^{35}S]dATP and used as previously described (Fowler et al. 1988a).

Results

In situ hybridization using probe 228S

The distribution of grain counts on 15 metaphases is shown for probe 228S (final wash stringency 2 × SSC, 50% formamide at 39° C; Fig. 1). The major region of in situ hybridization is region 9qh. Other genomic regions also contribute. These are: the short arms of chromosomes 13, 14, 15, 21 and 22; the centromeric heterochromatin of chromosomes 1 and 20; and region Yq (Fig. 1). The biotinylated probe 228S is near specific for region 9qh (Fig. 2) when used at probe concentrations less than about 0.01 ng/μl and high stringency (0.1 × SSC at 65° C). This suggests that the TaqI/Satellite III polymorphism revealed by probe 228S in Southern analyses (final wash stringency 0.1 × SSC at 55° C) may predominantly arise from region 9qh. Alternatively, this 9qh-specific result may be due to the reduced sensitivity of the biotin 228S probe compared with the same sequence labelled with tritium and the relatively large quantity of Satellite III in region 9qh.

The in situ hybridization results indicate only the genomic distribution of 228S homologous sequences. They do not necessarily correlate with the genomic location of the TDPS. Previous evidence has indicated that the Satellite III polymorphism is of autosomal origin, excluding Yq, and also excluding chromosome 16 and probably chromosome 1 (Fowler et al. 1988a). Therefore attempts were made better to define the location of the TDPS.

Genomic location of the Satellite III polymorphism

Examination of K-domains. Human DNA was digested with KpnI. The DNA from the 3.6 and 1.8 kb regions was recovered from a preparative agarose gel. Part of the recovered DNA was digested further with TaqI. The resultant fragments were examined by Southern analysis using 228S as a probe (Fig. 3). The initial KpnI digest showed 3.6 and 1.8 kb K-domain Satellite III fragments. The same fragments were however extensively digested with TaqI, and therefore neither can be included in the TDPS, since these are represented by sequences whose TaqI spacing is greater than about 4 kb. Furthermore, if such KpnI tandem repeats were to represent all the Satellite III component of chromosome 15 (Burk et al. 1985) and chromosome 9 (Jeanpierre et al. 1985), this would tend to exclude these chromosomes from contributing to the Satellite III polymorphism.

Examination of rodent/human somatic cell hybrids. The DNAs from seven different somatic cell hybrids (Table 1) were digested with TaqI and the fragments separated by FIGE and then blotted and probed with 228S. Background mouse and hamster genomic DNA do not hybridize with the satellite III related probe 228S (Fowler et al. 1988a; see also Discussion).

Hybrid 64063 (Fig. 4, lane 8) showed about six distinct TaqI fragments greater than about 4 kb (within a moderately intense background smear) but by comparison an increasing number of fragments smaller than this size, particularly below about 2 kb. Hybrid 64063 contains 9q as the

Fig. 2. In situ hybridization of biotinylated probe 228S to unbanded metaphase chromosomes. Hybridization (*arrowed*) is near specific for region 9qh (established in Fig. 1). Final wash stringency 0.1 × SSC, 65°. Bar represents 10 μm

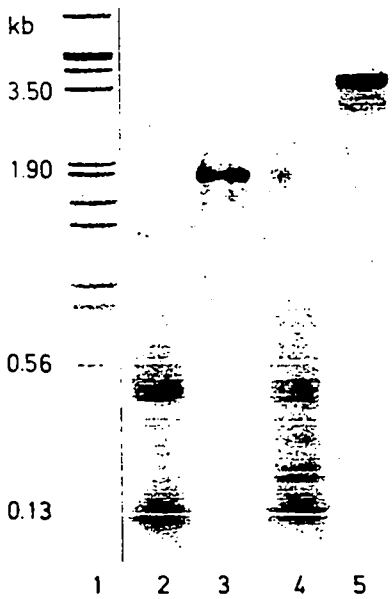


Fig. 3. TaqI digestion of isolated 1.8 and 3.6 kb KpnI 'K-domain' Satellite III fragments. Electrophoresis (1.8% agarose) and Southern analysis using ^{35}S-labelled 228S. *Lane 1* lambda molecular size standard; *lane 2* KpnI + TaqI double digestion of KpnI 1.8 kb isolate; *lane 3* original KpnI 1.8 kb isolate; *lane 4* KpnI + TaqI double digestion of KpnI 3.6 kb isolate; *lane 5* original KpnI 3.6 kb isolate. Film exposure 48 h

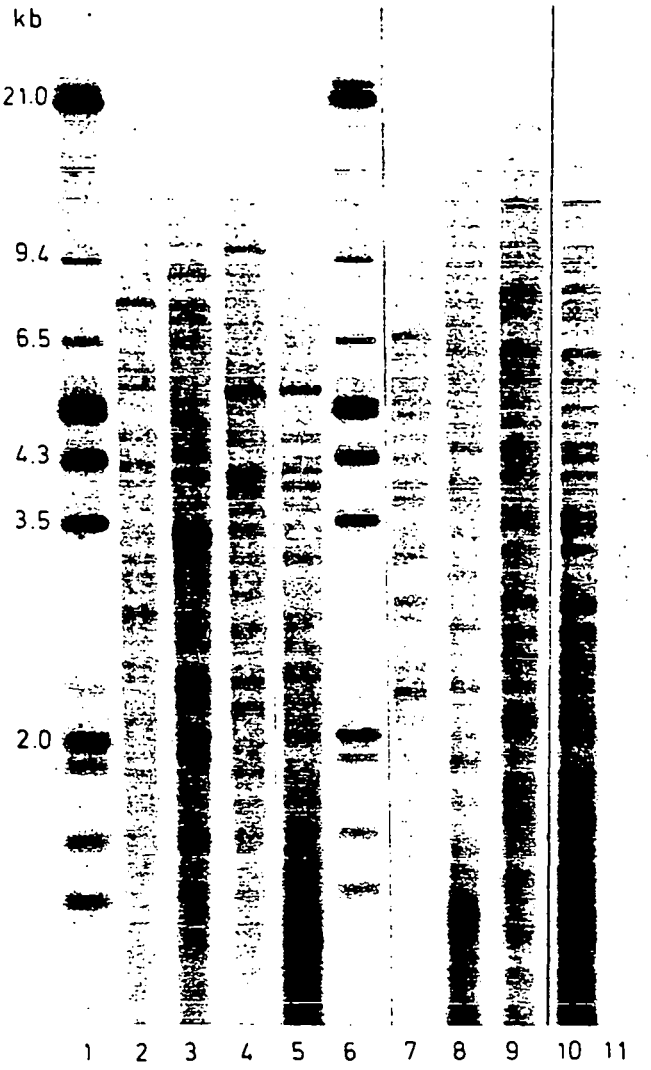


Fig. 4. TaqI digestion, field inversion gel electrophoresis (FIGE, 54 miniramps), and Southern analysis of somatic cell hybrids (Table 1) using ^{35}S-labelled 228S. *Lane 1* lambda molecular size standard; *lane 2* CY9; *lane 3* CY13; *lane 4* CY15; *lane 5* T85-43; *lane 6* as 1; *lane 7* HORL I; *lane 8* 64063; *lane 9* CY14; *lane 10* PK14; *lane 11* CY4. Each lane, 5 μg except T85-43 and PK14, 2 μg each. Film exposure 60 h

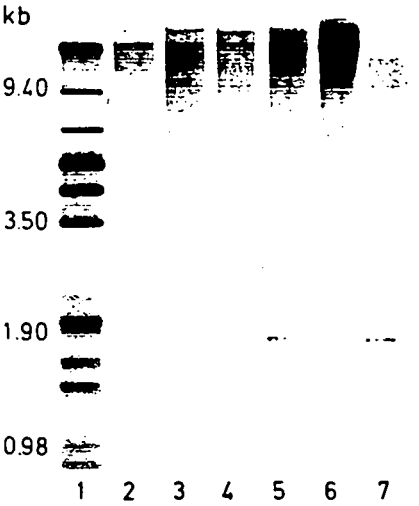


Fig. 5. KpnI digestion and Southern analysis of selected somatic cell hybrids using ^{35}S-labelled 228S. *Lane 1* lambda molecular size standard; *lane 2* CY9; *lane 3* CY13; *lane 4* CY15; *lane 5* CY14; *lane 6* 64063; *lane 7* HORL I. Each lane, 4 μg; film exposure 20 h

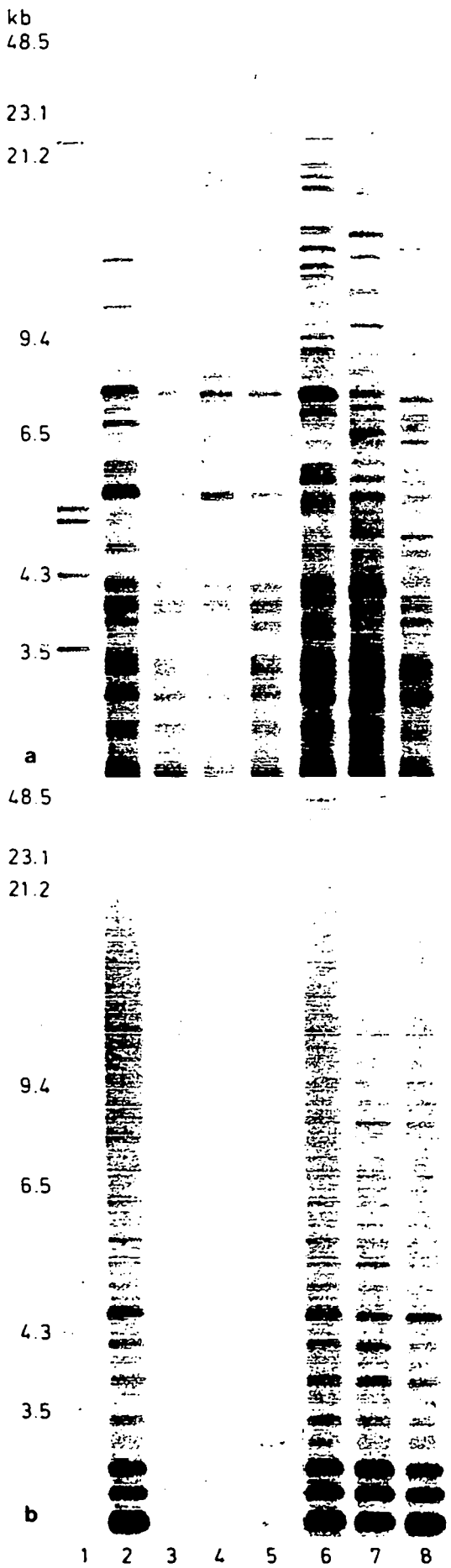
only human chromosome. About ten fragments greater than about 4 kb (with seven being smaller than 6 kb) were present in hybrid HORL I (lane 7). This hybrid contains chromosome 15 as its only human chromosome. There were also far fewer fragments smaller than 4 kb in hybrid HORL I than were seen in hybrid 64063, particularly below 2 kb (Fig. 4).

Hybrids CY9, CY13, CY14 and CY15 (Table 1) showed a larger proportion of their TaqI fragments to be greater than 4 kb in size compared with either hybrid 64063 or hybrid HORL I (Fig. 4). For example, 17 fragments were seen in hybrid CY14, 14 in hybrid CY9, 10 in hybrid CY13 and 2 intense bands in CY15. These hybrids contained various combinations of chromosomes 20, 21, and 22 (Table 1) as their predominant Satellite III DNA containing chromosomes. The band intensity of their TaqI fragment patterns was very varied. Hybrid CY4, which contains only chromosome 13 as its Satellite III source showed limited hybridization to the probe 228S with only a few fragments greater than about 4 kb (Fig. 4).

The DNAs from two human cell lines, PK14 and T85-43, which were used to generate CY14 and CY15 respectively were also digested with TaqI and the results compared with their respective hybrids (Fig. 4). Hybrid CY14 has 17 out of 23 fragments greater than about 4 kb in common with its parent PK 14. However it appears that one fragment, at about 13 kb, and possibly another at about 6 kb, were unique to the hybrid itself. This cannot be immediately explained. Hybrid CY15 has 6 (2 intense and 4 minor) out of 17 fragments greater than about 4 kb in common with its parent T85-43. One band in this hybrid, at about 10 kb, was markedly more intense than the same fragment in its parent, as were a number of other, much smaller, poorly resolved fragments.

Digestion of hybrid HORL I with KpnI and Southern analysis using probe 228S showed 1.8 and 3.6 kb KpnI fragments, confirming them to originate from chromosome 15 (Fig. 5). No like fragments were detected in KpnI digests of the other hybrids (Table 1), including hybrid 64063 (Fig. 5). The exception was hybrid CY14 (Fig. 5). The presence of 1.8 and 3.6 kb KpnI fragments in this hybrid was surprising in that distamycin/DAPI staining had failed to identify whole or part of chromosome 15 in it. Furthermore, the band intensity of these fragments would suggest that this should have been easily identified. The presence of two TaqI hybrid-specific fragments in CY14 (see above) suggests some de novo alteration of Satellite III sequences in this hybrid. Alternatively this KpnI/Satellite III tandem repeat may exist in chromosomes other than 15 in some individuals (see Discussion).

Sequence homogeneity of the TDPS. The Satellite III TDPS are generally resistant to double digestion with a range of other restriction enzymes. For example, double digestion of the DNA from one individual firstly with TaqI and then either HaeIII, AluI or RsaI little altered the polymorphic pattern of this person (Fig. 6a), though it did appear to alter the relative intensity of some TaqI fragments. This may in part have been due to losses resulting from the



more extensive manipulations required in preparing double digests. Double digestion with RsaI showed the greatest effect with, for example, the disappearance of a band at about 27 kb and the appearance of a band at about 13 kb. Stripping the membrane of probe 228S and reprobing with 216S clearly confirmed that the additional digestions had actually taken place (Fig. 6b).

Similar double digest experiments were made using TaqI with either PstI, KpnI, EcoRI or MboI (results not shown). The TDPS were similarly resistant to digestion with these enzymes. The greatest alterations in TaqI patterns were seen when either EcoRI or MboI were used, the results being similar to those seen with TaqI + RsaI (Fig. 6a). Double digestion with TaqI + KpnI little altered the polymorphic patterns, confirming that the K-domain Kpn 3.6 and 1.8 kb fragments are not themselves part of the TDPS.

Discussion

Satellite III like sequences represent the dramatic expansion of the pentameric repeat TTCCA (Frommer et al. 1982; Prosser et al. 1986), mostly in the centric and pericentric regions of some autosomes and the long arm of the Y chromosome (Fig. 1). The amplification of this sequence is recent in evolutionary time. Satellite III sequences are common to humans and higher primates only, while the related Satellite II repeat is apparently unique to humans (Mitchell et al. 1981).

Genomic location of the TDPS in human Satellite III DNA

The in situ distribution of genomic sequences homologous to the 228S probe (Fig. 1) is similar to that of a cloned Satellite III sequence specific to a chromosome 22 library (Jeanpierre et al. 1985). There is less similiarity with a cloned Satellite III sequence near specific for 15p (Higgins et al. 1985), and still less with an uncloned satellite III sub-fraction (Yq 3.4) isolated by gel electrophoresis (Burk et al. 1985).

The results show that the TDPS detected by the probe 228S do not arise from either of the two currently recognised Satellite III domains, namely autosomal K-domains (Burk et al. 1985) or Yq (Nakahori et al. 1986). Both these regions, though relatively pure in TTCCA repeats, have many TaqI sites in their repeat units and thus would not contribute to the polymorphism. Rather, the TDPS arise from other Satellite III regions characterised not only by their few TaqI sites per unit length, but also by a more general lack of restriction endonuclease sites, other than HinfI (Prosser et al. 1986).

The primary locations of these TaqI-deficient domains and hence the TDPS are probably the heterochromatin of

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Fig. 6. **a** TaqI and TaqI + other double digestion of DNA from one individual (no. 1) compared with TaqI digestion of three other unrelated individuals (nos. 2, 3, 4). FIGE (88 miniramps), and Southern analysis using (^{35}S)-labelled 228S. *Lane 1* lambda molecular size standard; *lane 2* TaqI control no. 1; *lane 3* TaqI + HaeIII no. 1; *lane 4* TaqI + AluI no. 1; *lane 5* TaqI + RsaI no. 1. Compare with single TaqI digests in lane 6, no. 2; lane 7, no. 3, lane 8, no. 4. About 2.5 µg each lane; film exposure 60 h. **b** Membrane in **a** stripped and reprobed with ^{35}S-labelled 216S to confirm equivalence of all TaqI digestions and to confirm TaqI + other double digestions. Film exposure 24 h

chromosome 20, and the short arms of chromosomes 21 and 22. Limited support for this is the similarity of the situ distribution using 228S and that from the Satellite III sequence specific for the chromosome 22 library (Jeanpierre et al. 1985). Other minor contributory regions are the pericentric heterochromatin of chromosome 9 and the short arms of chromosomes 15 and perhaps 13 and 14. There may well be marked inter-individual variation in the proportion of the contribution from each of these.

Given the near specificity of the biotinylated probe 228S for region 9qh at high stringency and low probe concentration (Fig. 2) it was expected this region would be the predominant source of the TDPS. This appears not to be so, since this region contributed relatively few bands to the TDPS. The 9qh-specific in situ hybridization result may thus be explained by either the biotinylated 228S probe preferentially locating this major block of Satellite III sequence at limiting probe concentration, or else the sequence 228S is most complementary to a region of Satellite III of relatively high TaqI frequency.

The TDPS represent only a minor proportion of total genomic Satellite III and it is not known if they are contiguous or are in any way separate from the bulk of Satellite III sequences which have a far greater TaqI frequency per unit length. Cytogenetic evidence using high resolution in situ techniques indicates that there are separate regions of Satellite III within individual chromosomes, e.g. within region 9qh and separate regions localized to the centromere and terminal knobs of all Satellite III-containing acrocentric chromosomes (Frommer et al. 1988). It is confirmed that K-domain fragments segregate from chromosome 15. However, not all the Satellite III DNA in this chromosome is K-domain (normally well digested with TaqI – Fig. 3) because hybrid HORL I also contained some moderately large TaqI-deficient fragments (lane 7, Fig. 4). This distinction in Satellite III/TaqI fragment size may eventually be found to correlate with their separate locations on chromosome 15 (Frommer et al. 1988).

These results also show that some 1.8/3.6 kb KpnI Satellite III fragments could arise from chromosomes other than 15 (Fig. 5). It is possible that such regions of localized amplification are individual specific. Alternatively, the results may be evidence for the instability of such sequences, particularly in hybrids. Reported examples of the instability of 15 p is its translocation to chromosome 22 (Buhler and Malik 1988) and to chromosomes X, 3 and 9 followed by expansion of the 1.8 kb KpnI Satellite III unit by unequal sister chromatid exchange (Holden et al. 1987).

Homogeneity of the TDPS

The general inertness of TDPS to restriction enzymes such as HaeIII, AluI, PstI, KpnI, and to a lesser degree EcoRI, MboI and RsaI is unusual compared with the frequency of restriction by these enzymes in other genomic sequences. This inertness appears to be greater the fewer the number of TaqI sites per unit length of Satellite III. The restriction map of Yq, for example, includes 64 TaqI sites and 5 RsaI sites within 3.5 kb (Nakahori et al. 1986) but one TDPS fragment apparently contained only a single RsaI site in 27 kb (Fig. 6a). The TDPS therefore appear featureless and difficult to map by conventional methods.

Two conclusions can be made. First, the TDPS are only rarely and intermittently interrupted by either non-satellite III sequences or regions of Satellite III which have a low frequency of 'rare' restriction sites. The extensive regions of apparently homogeneous Satellite III sequences which comprise the TDPS have not themselves accumulated by random drift any marked degeneracy in sequence and therefore probably represent examples of very recent TTCCA amplification events in the genome. Previous evidence has shown the Satellite III polymorphism to be TaqI specific and unlikely to have arisen from non-reciprocal recombination events (Fowler et al. 1988a). It is therefore more likely caused by DNA amplification, with post amplification TaqI point mutation or perhaps pre-existing TaqI sites being caught up in an amplification event(s).

Second, this restriction enzyme immunity suggests a very low level of transposon invasion, e.g. by Alu or LI, into such regions, either because of environmental unsuitability or because of their very recent amplification. These observations are broadly consistent with the reported minimal hybridization of Alu and LI sequences to heterochromatin (Korenberg and Rykowski 1988) and consistent with induction of C-banding in human chromosomes by restriction enzymes (Babu et al. 1988).

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References

Babu A, Agarwal AK, Verma RS (1988) A new approach in recognition of heterochromatic regions of human chromosomes by means of restriction endonucleases. Am J Hum Genet 42:60–65

Buhler EM, Malik NJ (1988) DA/DAPI heteromorphisms in acrocentric chromosomes other than 15. Cytogenet Cell Genet 47:104–105

Burk RD, Szabo P, O'Brein S, Nash WG, Yu L, Smith KD (1985) Organization and chromosomal specificity of autosomal homologs of human Y chromosome repeated DNA. Chromosoma 92:225–233

Burns J, Chan VTW, Jonasson JA, Fleming KA, Taylor S, McGee JOD (1985) Sensitive system for visualising biotinylated DNA probes hybridized in situ: rapid sex determination of intact cells. J Clin Pathol 38:1085–1092

Callen DF (1986) A mouse-human hybrid cell panel for mapping human chromosome 16. Ann Genet (Paris) 29:235–239

Callen DF, Baker E, Simmers RN, Seshadri R, Roninson IB (1987) Localization of the human multiple drug resistance gene, MDR1, to 7q21.1. Hum Genet 77:142–144

Cooke HJ, Hindley J (1979) Cloning of human satellite III DNA: different components are on different chromosomes. Nucleic Acids Res 10:3177–3197

Fowler JC, Drinkwater RD, Burgoyne LA, Skinner JD (1987) Hypervariable lengths of human DNA associated with a human satellite III sequence found in the 3.4 kb Y-specific fragment. Nucleic Acids Res 15:3239

Fowler JC, Drinkwater RD, Skinner JD, Burgoyne LA (1988a) Human satellite III DNA: an example of a "macrosatellite" polymorphism. Hum Genet 79:265–272

Fowler JC, Skinner JD, Burgoyne LA, McInnes JL (1988b) Improved separation of multi-locus DNA restriction fragments by field inversion gel electrophoresis and fragment detection using a biotinylated probe. Appl Theor Electrophoresis 1:23–28

Frommer M, Prosser J, Tkachuk D, Reisner A, Vincent PC (1982) Simple repeated sequences in human satellite DNA. Nucleic Acids Res 10:547–563

Frommer M, Paul C, Vincent PC (1988) Localization of satellite DNA sequences in human metaphase chromosomes using bromodeoxyuridine-labelled probes. Chromosoma 97:11–18

Higgins MJ, Wang H, Shtromas I, Haliotis T, Roder JC, Holden

JJA, White BN (1985) Organization of a repetitive human 1.8 kb KpnI sequence localized in the heterochromatin of chromosome 15. Chromosoma 93:77–86

Holden JJA, Hough MR, Reimer DL, White BN (1987) Evidence for unequal crossing-over as the mechanism for amplification of some homogeneously staining regions. Cancer Genet Cytogenet 29:139–149

Hollis M, Hindley J (1988) Satellite II DNA of human lymphocytes: tandem repeats of a simple sequence element. Nucleic Acids Res 16:363

Jeanpierre M, Weil D, Gallano P, Creau-Goldberg N, Junien C (1985) The organization of two related subfamilies of a human tandemly repeated DNA is chromosome specific. Hum Genet 70:302–310

Korenberg JR, Rykowski MC (1988) Human genome organization: Alu, Lines, and the molecular structure of metaphase chromosome bands. Cell 53:391–400

McInnes JL, Dalton S, Vize PD, Robins AJ (1987) Non-radioactive photobiotin-labelled probes detect single copy genes and low abundance mRNA. Biotechnology 5:269–272

Mitchell AR, Gosden JR, Ryder OA (1981) Satellite DNA relationships in man and the primates. Nucleic Acids Res 9:3235–3249

Moyzis RK, Albright KL, Bartholdi MF, Cram LS, Deaven LL, Hildebrand CE, Joste NE, Longmire JL, Schwarzacher-Robertson T, Meyne J (1987) Human chromosome-specific repetitive DNA sequences: Novel markers for genetic analysis. Chromosoma 95:375–386

Nakahori Y, Mitani K, Yamada M, Nakagome Y (1986) A human Y-chromosome repeated DNA family (DYZ1) consists of a tandem array of pentanucleotides. Nucleic Acids Res 14:7569–7581

Prosser J, Frommer M, Paul C, Vincent PC (1986) Sequence relationships of three human satellite DNAs. J Mol Biol 187:145–155

Simmers RN, Stupans I, Sutherland GR (1986) Localization of the human haptoglobin genes distal to the fragile site at 16q22 using in situ hybridization. Cytogenet Cell Genet 41:38–41

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Letter to the Editor

Satellite DNA and Higher-Primate Phylogeny

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The major human satellite sequences are tandemly repeating DNA sequences generally associated with centric and pericentric heterochromatic regions (Singer 1982). In humans, the major forms are satellites I, II, III, IV, and alphoid. Satellites I–IV were originally isolated by gradient centrifugation (Singer 1982), each fraction being of similar DNA composition—and thus of similar buoyancy—but not necessarily being of similar sequence. For example, satellites II and III are cobuoyant with some alphoid sequences (Frommer et al. 1982; Prosser et al. 1986). The consensus base sequence of each of the predominant components of these human satellites is well defined [satellites I–IV by Frommer et al. (1982) and Prosser et al. (1986), satellite II by Hollis and Hindley (1988), and satellite alphoid by Waye and Willard (1987)].

Examples of such sequences have here been used to investigate higher-primate phylogeny. The DNA from one human, two chimpanzee, one gorilla, and one orangutan (all males) was digested with the following three different restriction enzymes: *Hae*III, *Eco*RI, and *Kpn*I. The fragments were examined by Southern analysis (for methods, see Fowler et al. 1988). The same membrane was analyzed three times with satellite DNA probes of known sequence, by probing, stripping, and then reprobing the membrane (for details, see legend to fig. 1.).

Satellite III (fig. 1a)

The human/*Hae*III digest shows a Y chromosome-specific fragment (Cooke et al. 1983) at 3.5 kb (lane 6). The human/*Kpn*I digest shows two fragments at ~1.8 and ~3.6 kb (lane 16) that are believed to segregate from chromosome 15 (Higgins et al. 1985). These fragments appear unique to humans, being absent in similar digests of nonhuman-higher-primate DNA.

Digestion of nonhuman-primate DNA with either *Hae*III (lanes 2–5) or *Eco*RI (lanes 7–10) and probing with human satellite III shows extensive hybridization. There are extensive interspecies differences in the restriction-fragment patterns. There is also interindividual variation in the fine-band structure of the DNA from the two male chimpanzees (*Hae*III, lanes 2 and 3; *Eco*RI, lanes 7 and 8). This is quite unlike similar digests of human DNA, which normally show little or no interindividual variation with *Hae*III and *Eco*RI digests (Fowler et al. 1988).

Satellite II (fig. 1b)

The human/*Eco*RI digest (lane 11) shows an orderly series of satellite II–related fragments at 1,340, 1,770, 2,190 and 2,410 bp (Drinkwater et al. 1986) that is coincident with a similar human/*Eco*RI fragment series—1,350, 1,770, and 2,200 bp—obtained with a probe of a satellite II mixture prepared by gradient centrifugation (Mitchell et al. 1981). The results (fig. 1b) suggest that human satellite II is virtually human

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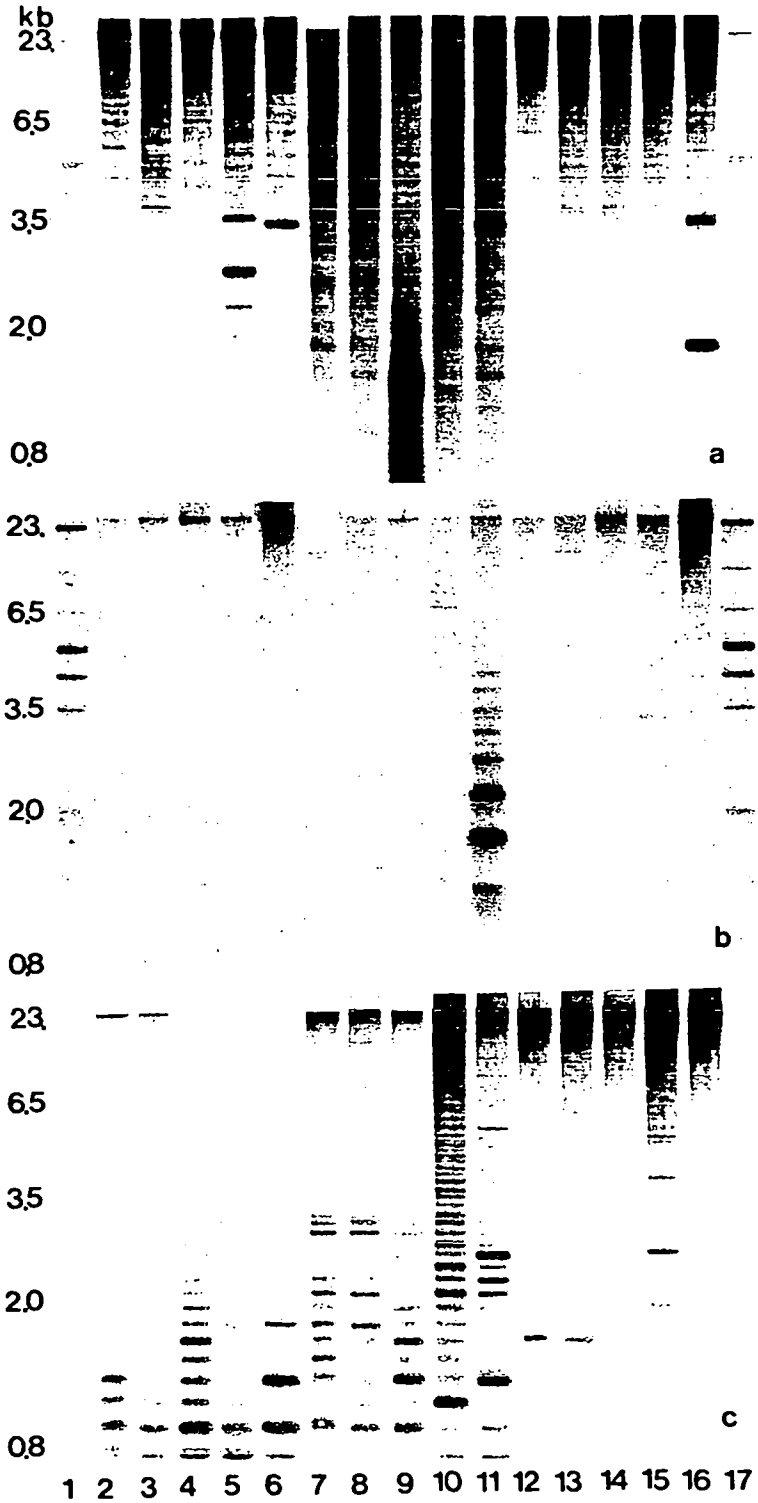
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specific. This finding is in accord with that of Mitchell et al. (1981) but not with that of Gosden et al. (1977). The only strong evidence of human satellite II hybridization to nonhuman primates is a single band at ~3.3 kb in the *Kpn*I digest of orangutan DNA (lane 15).

Satellite Alphoid (fig. 1c)

The *Hae*III (lanes 2–6) and *Eco*RI (lanes 7–11) digests of human and nonhuman-primate DNA show an orderly series of restriction fragments in each species, these being based on 170- and 340-bp alphoid repeat units (Waye and Willard 1987). However, the differing extent of the laddering and the varying band intensities suggest that the alphoid sequences are moderately diverged in sequence in these species. This is confirmed by the *Kpn*I/alphoid results (lanes 12–16), in which the nonhuman primates may be distinguished from one another by their fragment patterns.

The two chimpanzee samples show similar results (with minor band intensity variations) in each of their *Hae*III (lanes 2 and 3), *Eco*RI (lanes 7 and 8), and *Kpn*I (lane 12 and 13) digestions. These results act as internal controls for the results above, as they confirm that the satellite III interindividual differences seen in these two samples (fig. 1a) are not technical artifacts.

These results may be compared with those of other investigators. In the past, when human-derived satellite sequences have been used to examine DNA from nonhuman higher primates, the probes used were generally prepared by gradient centrifugation (e.g., see Gosden et al. 1977; Mitchell et al. 1977, 1981). Such probes were heterogenous mixtures of satellite DNAs, and, as noted by Singer (1982), this rendered the results more confusing than if clones of known sequence and property had been used. For example, the results of Mitchell et al. (1981) and Gosden et al. (1977) are conflicting. Mitchell et al. (1981) found human satellite II DNA to be absent from chimpanzees, gorillas, and orangutans, but Gosden et al. (1977) found it to be present in gorillas and orangutans but absent from chimpanzees. Gosden et al. (1977) also reported that human satellite IV was present in chimpanzees, gorillas, and, in the Y chromosome only, orangutans. Holmquist et al. (1988), in their review, incorrectly cite the satellite II and IV results of Gosden et al. (1977).

A summary of our results and those of previous investigators would suggest the following: First, human satellite III is present in humans, chimpanzees, gorillas, and orangutan (Mitchell et al. 1977, 1981). This is likely to be true for human satellite IV as well, since the evidence of Frommer et al. (1982) and Prosser et al. (1986) suggests that the major sequences and properties of satellites III and IV are the same. Second, satellite alphoid is present in humans, chimpanzees, gorilla, and orangutan. This is established from the results of Miller et al. (1988) and Lund Jorgensen et al. (1987). The results of Mitchell et al. (1981) are also in accord with this. However, the probe used by Mitchell et al. (1981) was probably a mixture of satellite III and alphoid sequences, giving for *Hae*III-digested human male DNA a typical alphoid

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FIG. 1.—Southern analysis of primate and human DNA (1% agarose). The membrane was probed first (panel a) with sequence 228S-satellite III (Fowler et al. 1988), then stripped and reprobed (panel b) with sequence 227S-satellite II (Drinkwater et al. 1986), then stripped and reprobed (panel c) with 216S-satellite alphoid (Fowler et al. 1988). Final membrane wash for each probing was 0.1 × SSC (20 × SSC = 3 M NaCl, 0.3 M trisodium citrate) at 55°C. Samples (1 μg from male animals)/digest enzymes were as follows: lane 1, lambda molecular-size standard; lane 2, chimpanzee 1/*Hae*III; lane 3, chimpanzee 2/*Hae*III; lane 4, gorilla/*Hae*III; lane 5, orangutan/*Hae*III; lane 6, human/*Hae*III; lane 7, chimpanzee 1/*Eco*RI; lane 8, chimpanzee 2/*Eco*RI; lane 9, gorilla/*Eco*RI; lane 10, orangutan/*Eco*RI; lane 11, human/*Eco*RI; lane 12, chimpanzee 1/*Kpn*I; lane 13, chimpanzee 2/*Kpn*I; lane 14, gorilla/*Kpn*I; lane 15, orangutan/*Kpn*I; lane 16, human/*Kpn*I; lane 17, lambda molecular-size standard. Probes were labeled with [^{35}S] dATP (Fowler et al. 1988). Exposure times were as follows: panel a, 20 h; panel b, 96 h; panel c, 14 h.

ladder as well as a satellite III–associated 3.5-kb Y-specific fragment (as would a composite of fig. 1a and fig. 1c). Last, the collective evidence of Mitchell et al. (1981) and Miller et al. (1988) is that human satellite III/IV and satellite alphoid are found in higher primates only, whereas satellite II, on the basis of our results and those of Mitchell et al. (1981), is close to human specific.

These results show no compelling evidence supporting any phylogeny of the higher primates. They do show, however, that, while satellite III is apparent across all higher primates, it is evolving rapidly—and probably more rapidly than speciation of the higher primates, as indicated by the extent of the interspecies variation as compared with the interindividual variation in satellite III patterns. Furthermore, the differences between the two chimpanzee individuals is much greater than what would be expected between two humans. If significant, this would imply that the chimpanzee, as a species, is more ancient than the present-day human species. The radiation of the human race would thus have occurred in orders of magnitude less time than the speciation of the higher primates. These conclusions assume satellite III to have diverged at a relatively fast but constant rate.

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LITERATURE CITED

COOKE, H. J., J. FANTES, and D. GREEN. 1983. Structure and evolution of human Y chromosome DNA. Differentiation **23** (Suppl): S48–S55.

DRINKWATER, R. D., L. A. BURGOYNE, and J. D. SKINNER. 1986. Two human repetitive elements: a new interspersed repeat found in the factor IX gene, and a satellite II tandem repeat sequence. Nucleic Acids Res. **14**:9541.

FOWLER, J. C., R. D. DRINKWATER, J. D. SKINNER, and L. A. BURGOYNE. 1988. Human satellite-II DNA: an example of a "macrosatellite" polymorphism. Hum. Genet. **79**:265–272.

FROMMER, M., J. PROSSER, D. TKACHUK, A. H. REISNER, and P. C. VINCENT. 1982. Simple repeated sequences in human satellite DNA. Nucleic Acids Res. **10**:547–563.

GOSDEN, J. R., A. R. MITCHELL, H. N. SEUANEZ, and C. M. GOSDEN. 1977. The distribution of sequences complementary to human satellite DNAs I, II and IV in the chromosomes of chimpanzee (*Pan troglodytes*), gorilla (*Gorilla gorilla*) and orangutan (*Pongo pygmaeus*). Chromosoma **63**:253–271.

HIGGINS, M. J., H. WANG, I. SHTROMAS, T. HALIOTIS, J. C. RODER, J. J. A. HOLDEN, and B. N. WHITE. 1985. Organization of a repetitive human 1.8 kb KpnI sequence localized in the heterochromatin in chromosome 15. Chromosoma **93**:77–86.

HOLLIS, M., and J. HINDLEY. 1988. Satellite II DNA of human lymphocytes: tandem repeats of a simple sequence element. Nucleic Acids Res. **16**:363.

HOLMQUIST, R., M. M. MIYAMOTO, and M. GOODMAN. 1988. Higher-primate phylogeny—why can't we decide. Mol. Biol. Evol. **5**:201–216.

LUND JORGENSEN, A., C. JONES, C. J. BOSTOCK, and A. LETH BAK. 1987. Different subfamilies of alphoid repetitive DNA are present on the human and chimpanzee homologous chromosomes 21 and 22. EMBO J. **6**:1691–1696.

MILLER, D. A., V. SHARMA, and A. R. MITCHELL. 1988. A human-derived probe, p82H, hybridizes to the centromeres of gorilla, chimpanzee, and orangutan. Chromosoma **96**:270–274.

MITCHELL, A. R., J. R. GOSDEN, and O. A. RYDER. 1981. Satellite DNA relationships in man and the primates. Nucleic Acids Res. **9**:3235–3249.

MITCHELL, A. R., H. N. SEUANEZ, S. S. LAWRIE, D. E. MARTIN, and J. R. GOSDEN. 1977. The location of DNA homologous to human satellite III DNA in the chromosomes of chimpanzees (*Pan troglodytes*), gorilla (*Gorilla gorilla*) and orangutan (*Pongo pygmaeus*). Chromosoma **61**:345–358.

PROSSER, J., M. FROMMER, C. PAUL, and P. C. VINCENT. 1986. Sequence relationships of three human satellite DNAs. J. Mol. Biol. **187**:145–155.

SINGER, M. F. 1982. Highly repeated sequences in mammalian genomes. Int. Rev. Cytol. **76**: 67–112.

WAYE, J. S., and H. F. WILLARD. 1987. Nucleotide sequence heterogeneity of alpha satellite repetitive DNA: a survey of alphoid sequences from different human chromosomes. Nucleic Acids Res. **15**:7549–7580.

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