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A synthetic oligonucleotide probe (5'TTCCA 3')\textsubscript{n} uncovers a male specific hybridization pattern in the human genome

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A pentanucleotide motif 5'TTCCA3', originating from a 3-4 kb repeat fraction (DYZ1), is organized tandemly in the human genome. This motif is abundantly present on the long arm of the human Y (band Yq12) chromosome and is separated by Hae III digestion of male genomic DNA\textsuperscript{1-4}. We have developed a 20 base synthetic oligonucleotide probe, termed OAT20Y, comprising four repeat units of 5'TTCCA3', that uncovers a male-specific hybridization pattern in the human genome. The probe is highly sensitive, since less that 1\mu g of DNA was sufficient to obtain visible signals after hybridization. Our results on discrimination of sex by using OAT20Y with several amniotic fluid samples was in accordance with clinical data. OAT20Y was found to be specific to the human genome as it did not hybridize with DNA of any non-human species. In addition to sexing human embryos in conjunction with severe X-linked genetic diseases, the probe may be useful in ascertaining the origin of tissues or blood samples in forensic cases.

**KEYWORDS:** Synthetic oligonucleotides, DNA probe, human Y chromosome.

INTRODUCTION

Maleness in humans is attributed to a gene mapped to the short arm of the Y chromosome.\textsuperscript{5-7} The long arm of Y is heterochromatic and highly variable within the normal male population. It is endowed with reiterated sequences and does not participate in pairing with other chromosomes during meiosis. The isolation of reiterated sequences from the human Y chromosome (band Yq12) has revealed the existence of two prominent 3-4 kb (DYZ1) and 2.1 kb (DYZ2) DNA fragments discernible with Hae III digestion of male but not female genomic DNA.\textsuperscript{1,2} Sequence analysis of the 3-4 kb (DYZ1) fragment has shown that this fraction is predominantly composed of a pentanucleotide tandem repeat (5'TTCCA3') motif.\textsuperscript{4} Therefore, an oligonucleotide probe of appropriate length composed of 5'TTCCA3' sequences would discriminate male and female DNA. Cloned DNA of the DYZ1 fragment has been used as a probe to detect structural rearrangements involving the Y chromosome.\textsuperscript{8,9}

However, no report is available on the use of a pure synthetic 5'TTCCA3' repeat motif as a DNA probe, or on its hybridization pattern or its evolutionary status. In view of this, we used synthetic oligo probes, comprising 3, 4, 5 and 6 repeat units of 5'TTCCA3' and other motifs with a difference of a single nucleotide in the 5'TTCCA3', for hybridizing to DNA samples from human and several non-human species. We have demonstrated that 5'TTCCA3' sequences when used as probes clearly differentiate between human male and female DNA samples and that these sequences are not evolutionarily conserved.

MATERIALS AND METHODS

Oligonucleotide synthesis and radiolabelling

Oligonucleotide synthesis was carried out on an
automated synthesizer (Pharmacia, Gene Assembler) and purified by polyacrylamide gel electrophoresis using a C18 column (Rainin, USA) as described earlier.\(^{10}\) Approximately 10 pmol of each oligonucleotide was labelled at the 5' terminal hydroxyl group using \([\gamma\sp{32}P]\) ATP (20 pmol, 6000 Ci mmol\(^{-1}\)) and 5-8 u of T\(_7\) polynucleotide kinase in a 10 \(\mu\)l reaction volume containing 67 mM Tris-HCl, pH 8-0, 10 mM magnesium acetate and 10 mM dithiothreitol at 37°C for 45-50 min. The labelled nucleotides were then separated from the \([\gamma\sp{32}P]\) ATP by column chromatography over DE52 cellulose (Whatman) following standard procedures,\(^{10}\) and used for hybridization.

DNA isolation, restriction digestion and agarose gel electrophoresis

For human DNA, a total of 30 peripheral blood samples from unrelated males and females, 12 random amniotic fluid samples (second trimester only) from patients opting for medical termination of pregnancies and sixteen cord blood samples were used in this study. For non-human DNA, blood samples from the rhesus monkey (Macaca mulatta), bonnet monkey (Macaca radiata), cow (Bos taurus), buffalo (Bubalus bubalis), rat (Rattus norvegicus), rabbit (Oryctolagus cuniculus) and langur (Presbytis entellus) were utilized. DNA was isolated following procedures reported earlier\(^{11}\) and 4-5 \(\mu\)g of each sample was digested with Hae III according to the supplier’s specification (New England Biology Labs) and electrophoresed in 0.7% agarose gel (Pharmacia) in TAE buffer (40 mM Tris, 12 mM sodium acetate, 2 mM EDTA, pH 8.3). For human DNA digestion, besides Hae III, the enzymes Mbo I, Alu I and Eco RI were also used. For evaluating the hybridization sensitivity of this probe, serial dilutions of Hae III digested human male and female genomic DNA (4, 2, 1, 0.5, 0.25 and 0.12 \(\mu\)g) were used in agarose gel electrophoresis. For size estimation, lambda DNA predigested with H\(\text{in}\) dill enzyme was included in gel electrophoresis. After electrophoresis, the gels were stained with ethidium bromide, photographed under u.v. and dried under vacuum.

Hybridization Procedure

Oligonucleotide probes used for hybridization are given in Table 1. For hybridizing to DNA within the gel matrix, denaturation and neutralization were carried out as reported earlier.\(^{12}\) Hybridization with OAT15Y and OAT20Y was carried out at 37°C and 50°C, respectively, 5°C below the melting temperature. For the remaining probes, the hybridization temperature was 60°C since the calculated melting temperature was higher than 65°C. All hybridizations were carried out in wide-mouth glass tubes using a hybridization oven for 12-16 h in 5 \(\times\) SSPE (1 \(\times\) SSPE is 180 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 8.1), 0.1% SDS, 5 \(\times\) Denhardt’s solution, 10 \(\mu\)g ml\(^{-1}\) sonicated and denatured (Escherichia coli) DNA and 1 \(\times\) 10\(^6\) cpm ml\(^{-1}\) labelled oligo probes. Post-hybridization washes and autoradiography were carried out as mentioned elsewhere.\(^{12,13}\) For dot-blot hybridization using the OAT20Y probe, approximately 200 ng of DNA samples each from human and several non-human species were used.

RESULTS

Hybridization of Hae III digested human DNA samples

Genomic DNA from normal healthy individual donors, digested with Hae III, was hybridized with the probes shown in Table 1. The probes, OAT15Y, 20Y, 25Y and 30Y, comprising pure repeats of 5'TTCCA3',
revealed discernible signals in high molecular weight range in both male and female DNA samples. However, an additional signal of 3-4 kb was present exclusively in the males (Fig. 1a), which was observed even with less than 1 µg of DNA (Fig. 1b). Probes based on related sequences (see OAT20YM or OAT25YM in Table I) with a single base substitution showed an extremely faint male-specific 3-4 kb band but no signal was seen in the high molecular weight range (not shown). This suggests that variants of the 5'TTCCA3' repeat motifs on the Y chromosome are fewer in number and probably absent completely from the autosomes. The 15 base probe OAT15Y is equally informative but was found to be relatively less stable under highly stringent conditions. The oligos of 20 bases or longer withstand highly stringent hybridization and wash conditions which are important criteria of an ideal DNA probe. Thus, OAT20Y was
finally selected for subsequent studies. The DNA samples obtained from cord blood and amniotic fluid and hybridized with OAT20Y (not shown) corroborated the reliability and specificity of the probe and the results were found to be in accordance with the clinical data. Human DNA samples digested with enzymes other than Hae III did not show male-specific hybridization patterns.

Hybridization of non-human DNA samples

In cross hybridization studies with OAT20Y, Hae III digested DNA from several non-human species together with human samples have shown that these sequences are present exclusively in the human genome (Fig. 2), since no hybridization signal was detected in any of the non-human species. A similar result was obtained with dot-blot hybridization.

DISCUSSION

The maximum length of the 5'TTCCA3' repeat unit in the human genome is not known but it appears that at least 30 residues are present at a stretch, since oligo probes ranging from 15–30 bases have been found to show a consistently discernible male-specific hybridization signal. Oligo probes of 20 bases or longer withstand highly stringent wash conditions, and signals remain unchanged even after 15–20 min wash in 6 × SSC at 60°C. Thus, OAT20Y may be taken as an optimal probe since this would prove to be cost effective compared with those of longer residues. The application of OAT20Y may be extended to severe X-linked genetic disorders (muscular dystrophy, haemophilia, fragile X-linked mental retardation, etc.) in males for antenatal sex determination.

In the human genome, a male-specific hybridization pattern is seen only on digestion of genomic DNA with Hae III. Other enzymes used showed a monomorphic hybridization pattern instead of a sex specific one, indicating that the 3.4 kb fragment is cleaved internally and the male-specific pattern lost. Hae III digested genomic DNA from non-human species, hybridized with the OAT20Y probe, did not show any signal, suggesting that this repeat motif is absent in these species. It may be argued that the absence of signal may be due to the cleavage of internal sites complementary to 5'TTCCA3'. This possibility is ruled out by our dot-blot hybridization studies where undigested DNA samples were used but no signal was detected. Surprisingly, amongst non-humans, 5'TTCCA3' sequences are absent even in closely-related species such as the monkey or langur. This indicates that these sequences have evolved independently and rather recently, probably after diversion of the human from non-human primates. In an analogous observation, a Bam HI repeat element has been reported to be predominantly associated with the neo-Y chromosome of Drosophila miranda but absent in Drosophila melano-
gaster. Thus, it is obvious that some repeat elements have evolved species-specifically.

The fact that 5'TTCCA3' sequences are present exclusively in the human genome makes it an even more attractive candidate as a species-specific genetic marker. Synthetic oligonucleotides used as hybridization probes have several inherent advantages and if these are based on repetitive motifs, less test DNA is required for obtaining signals in dot-blot hybridization. Thus, the OAT20Y probe reported here is useful for Southern as well as for dot-blot hybridization for discriminating human sex and for ascertaining the origin of tissues or blood samples in forensic cases.

A number of short tandem repetitive (STR) motifs, conserved evolutionarily, have been reported, and usually these motifs are polymorphic due to organizational variation in the number of their repeat units. Of the several mutational events, recombinatorial activity has been suggested to be the one responsible for generating variation in the number of STR motifs. The 5'TTCCA3' sequences are not conserved evolutionarily and do not detect polymorphisms (at least with the enzymes we have used), presumably because they are located in the silent domain of the human genome and during the course of evolution have not participated in recombinatorial activities. Currently, we are screening a human genomic library with the OAT20Y probe with the aim of isolating several positive clones and studying the sequences flanking this motif. The function of 5'TTCCA3' sequences, like most repetitive DNA, is not known. Owing to their organizational characteristics in the human genome, they may prove to be a reliable genetic marker useful for biology and forensic science.

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