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ISOLATION OF A Y CHROMOSOMAL DNA SEQUENCE AND ITS CLINICAL APPLICATION

Masato TSUKAHARA,[1] Shinya MATSUURA,[1] Fumio KISHI,[1]
Akira YOSHIDA,[2] and Tadashi KAJII[1]

[1]*Department of Pediatrics, Yamaguchi University School of Medicine, Ube 755, Japan*
[2]*Department of Biochemical Genetics, Beckman Research Institute of the City of Hope, Duarte, California 91010*

Summary A 4.6 kb long, Y-specific DNA fragment was isolated from a flow-sorted human Y chromosomal library, and its male specificity was confirmed by Southern blot analysis. The fragment, designated as pY-80, was proven with an *in situ* hybridization experiment to have originated from the Yp11.2-Ypter region. Its 2,808 bp section was sequenced. The polymerase chain reaction proceeded with oligonucleotides flanking a 666 bp *Pst*I-*Eco*RI fragment of the sequence as primers and a male genomic DNA as a template, but not with a female genomic DNA. Preliminary tests of samples of various sources successfully detected the Y-specific fragment in male-derived samples, including mouth wash, single hair roots, urinary epithelial cells, dried blood spots and amniotic fluid cells.

Key Words human Y specific DNA sequences, polymerase chain reaction, clinical application

INTRODUCTION

The human Y chromosome consists mainly of the following three regions: 1) the "pseudoautosomal," X and Y-pairing region at Yp11.3; 2) the pericentromeric, X and Y-non-paring region; and 3) the heterochromatic, non-pairing region at Yq12. Y specific DNA probes from these regions are useful for various purposes including 1) prenatal diagnosis of sex-linked disorders, 2) following up the host-*versus*-graft cells in patients with bone marrow transplants from the opposite sex, and 3) analyses of sex chromosomal disorders such as a Y/autosome translocation, non-fluorescent Y chromosome, Yq isochromosome, and XX males. While a number of Y-specific DNA fragments have been cloned, few of them have been

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sequenced (Cooke *et al.*, 1984; Nakahori *et al.*, 1986; Arnemann *et al.*, 1987; Ellis *et al.*, 1989; Sinclair *et al.*, 1990).

We describe here a Y-specific clone, located on the short arm of the Y chromosome, and its DNA sequence. Its clinical application is also discussed.

MATERIALS AND METHODS

Y chromosome-specific library. A human Y chromosome-specific library was provided by American Type Culture Collection (ATCC No. 57715).

Screening of the human Y chromosome-specific library. The library was screened by the plaque hybridization method with a *Hin*dIII-digested female genomic DNA as a probe. Phage DNAs from the plaques that did not hybridize to the human female genomic DNA were prepared by the plate lysis method.

Isolation of Y-specific DNA. The isolated phage DNAs were digested with *Hin*dIII, electrophoresed on 0.6% agarose gel, and Southern blotting was carried out using *Hin*dIII-cleaved, ^{32}P-labeled human female and male genomic DNAs as a probe. The DNA clones hybridized to the male DNA probe but not to the female probe were further selected, and inserts were separated on 0.8% low melting agarose gel. The selected inserts were subcloned into pUC118.

DNA sequence analysis. Suitable restriction fragments were subcloned into M13mp18 and mp19 vectors and sequenced by the dideoxy method, using an M13 sequencing primer.

Chromosomal assignment. Chromosomal localization of the cloned Y-specific DNA was determined by *in situ* hybridization (Zabel *et al.*, 1983). The DNA probe was labeled with [^{3}H]dCTP, [^{3}H]dATP, and [^{3}H]dTTP to a specific activity of 1.1×10^7 cpm/μg by random primer labeling. Hybridized slides were dipped into Kodak nuclear track emulsion (NTB II), and exposed at 4°C for 10 days. After the autoradiography, the slides were stained using the Hoechst-Giemsa method as described previously (Tsukahara and Kajii, 1985).

DNA extraction from various samples. Peripheral blood DNA was extracted from 6 normal subjects (3 males and 3 females) by the method of Wyman and White (1980). Buccal epithelial cells (3 males and 4 females) were obtained by centrifugation from mouth wash with 15 ml of saline and urinary epithelial cells (4 males and 4 females) from 15 ml of urine each, and washed with 1 ml of Tris-buffered saline. A single hair was plucked each from 6 individuals (3 males and 3 females) and cut with scissors to obtain the hair root. Amniotic fluid cells from 3 pregnant women were collected by centrifugation of amniotic fluid (1.5 ml). The cells thus collected were suspended in 400 μl of lysis buffer (0.32 M sucrose, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100) containing 200 μg of proteinase K and 0.5% sodium dodecyl sulfate. The cell suspension was incubated for 2 hr at 65°C, followed by phenol/chloroform and chloroform extractions. DNA was then precipitated with ethanol and redissolved in 25 μl of sterile water and its 8 μl portion

was used for the polymerase chain reaction (PCR).

Dried blood spots on a filter paper from 5 newborn babies (3 males and 2 females) were obtained. A 1 cm dried blood spot was cut out from each sample and rehydrated by continuously shaking in 2 ml of Tris-buffered saline containing 200 μg of proteinase K for 4 hr. Cells were centrifuged and resuspended in 50 μl of sterile water and boiled for 10 min. Cellular debris were then pelleted by centrifugation for 5 min and the supernatant was used directly for PCR amplification.

Polymerase chain reaction. The oligonucleotides were synthesized on an Applied Biosystem 381A DNA synthesizer and purified by polyacrylamide gel electrophoresis. The reaction mixture consisted of 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin, 25 pmol of each primer, 0.2 mM of each of the four deoxynucleotide triphosphates (dATP, dCTP, dTTP, and dGTP), 2.5 units of *Taq* DNA polymerase and the sample, for a total volume of 25 μl. The amplification reaction was performed in a programmed temperature control system (PC-600, Astec Co, Fukuoka). The samples were subjected to 30 cycles of denaturation at 94°C for 1.5 min, annealing at 50°C for 2 min and extension at 70°C for 3 min. The amplified product was analyzed on 1.5% agarose gel electrophoresis and transferred onto a nylon membrane.

RESULTS

Screening of Y chromosome library and isolation of Y-specific DNA fragments

Of the 3×10^6 recombinant phage particles, 186 plaques that did not hybridize to the human female genomic DNA were obtained. Of the 186 plaques, 9 clones hybridized to male genomic DNA but not to female gemonic DNA (Table 1). Southern blot hybridization of these 9 clones with total female and male DNAs gave a male-specific banding pattern under both low and high stringent washing conditions. Clone pY-80 hybridized with a predominant fragment of 4.6 kb, and in addition with two bands of weaker intensity, 3 and 2 kb, respectively (Fig. 1,

Table 1. Y-specific DNA clones.

Clone	Clone size (kb)	Y-specific *Hin*dIII fragments (kb)	X and/or autosome sequences [a]
pY-80	4.6	4.6; 3.0; 2.0	−
pY-25	4.8	7.2; 4.6	−
pY-48	5.0	5.4; 5.0	+
pY-50	4.4	12.0	+
pY-102	5.2	5.6; 5.1	+
pY-111	5.2	4.6	+
pY-146	4.2	7.0; 3.6	+
pY-148	5.0	6.6	+
pY-153	3.6	3.1	+

[a] X and/or autosome sequences are shown as several bands or smear patterns common to female and male DNA.

a and b). Clone pY-25 hybridized with two Y-specific fragments of 7.2 and 4.6 kb. The other seven clones (pY-48, 50, 102, 111, 146, 148, and 153) each hybridized with Y-specific fragments of various sizes, and in addition, with X and/or autosome sequences as shown as band or smear patterns common to both female and male genomic DNAs (Fig. 1c, Table 1). Of these 9 clones, pY-80 was further characterized.

Restriction map and nucleotide sequence of pY-80

Figure 2 shows the restriction map of pY-80 and its nucleotide sequencing

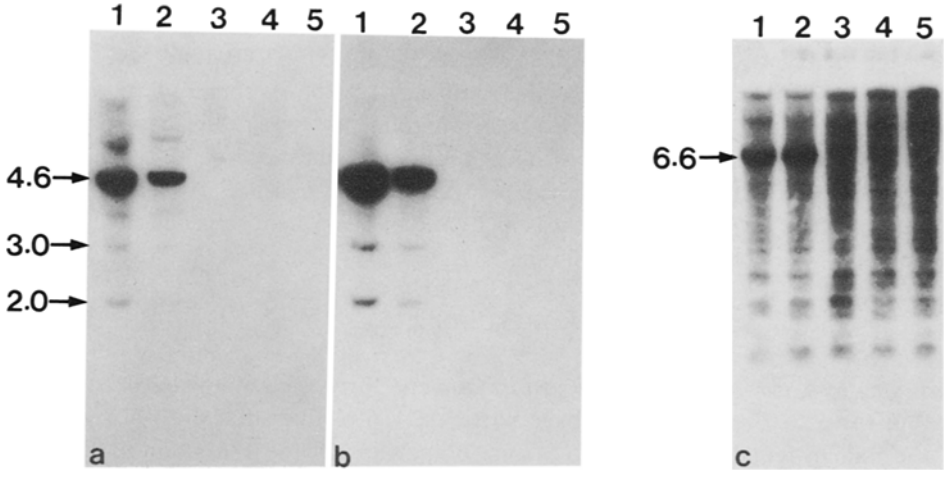


Fig. 1. Hybridization of clones pY-80 and pY-148 to *Hin*dIII-digested DNAs from males (lanes 1, 2) and females (lanes 3–5). Hybridization of clone pY-80 under a) a low-stringent (65°C, 2×SSPE, 0.1% SDS), and b) a high stringent (65°C, 0.1× SSPE, 0.1% SDS) washing conditions. c) Hybridization of clone pY-148. Y-specific bands are marked with arrows.

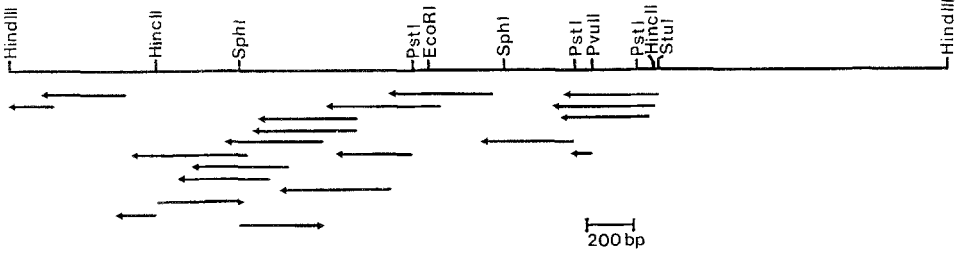
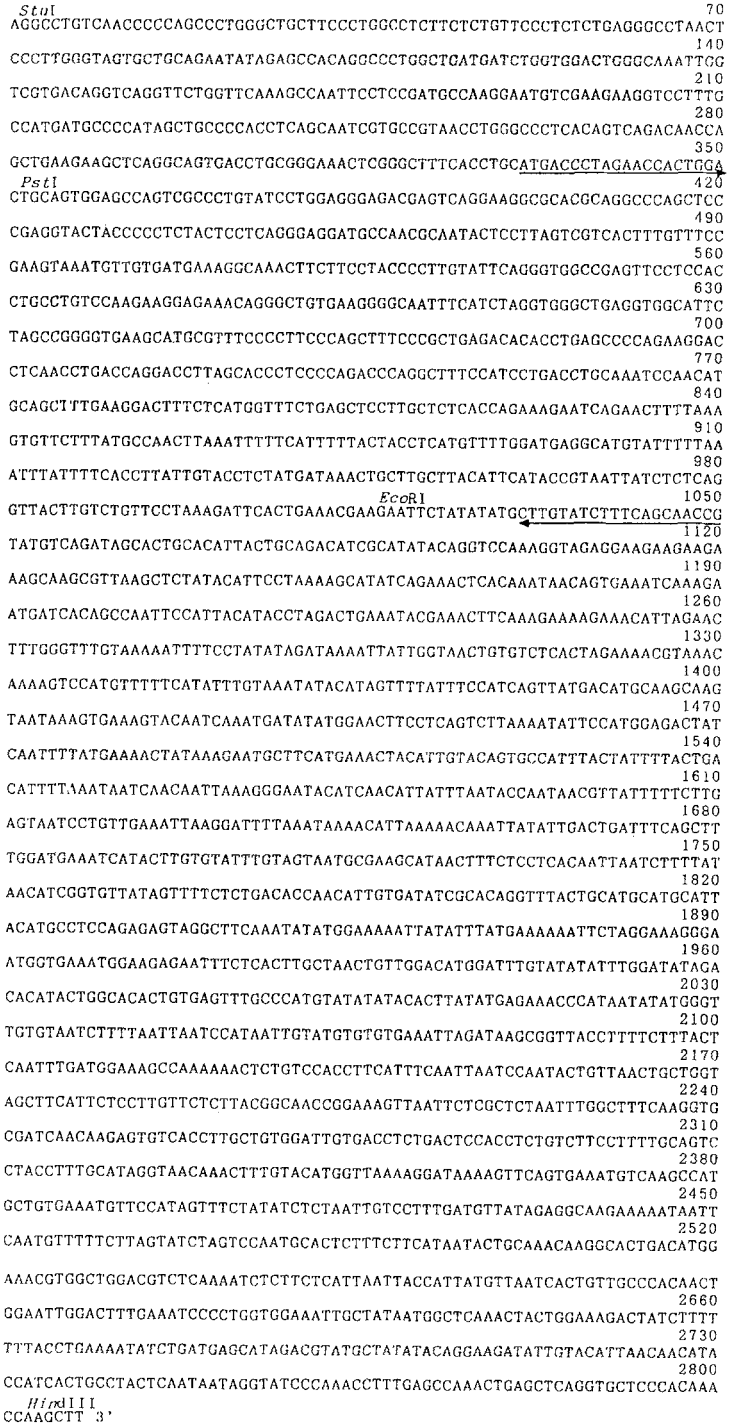


Fig. 2. Restriction map of pY-80 and sequence determination strategy. Horizontal arrows indicate the direction and extent of the sequencing.

Fig. 3. Nucleotide sequence of pY-80. Oligonucleotides used in the polymerase chain reaction are shown by arrows underlying the sequence. The nucleotide sequence has been submitted to the EMBL Data Library with accession number X51582.


StuI
5' AGGCCTGTCAACCCCCAGCCCTGGGCTGCTTCCCTGGCCTCTTCTCTGTTCCCTCTCTGAGGGCCTAACT
CCCTTGGGTAGTGCTGCAGAATATAGAGCCACAGGCCCTGGCTGATGATCTGGTGGACTGGGCAAATTGG
TCGTGACAGGTCAGGTTCTGGTTCAAAGCCAATTCCTCCGATGCCAAGGAATGTCGAAGAAGGTCCTTTG
CCATGATGCCCCATAGCTGCCCCACCTCAGCAATCGTGCCGTAACCTGGGCCCTCACAGTCAGACAACCA
GCTGAAGAAGCTCAGGCAGTGACCTGCGGGAAACTCGGGCTTTCACCTGCATGACCCTAGAACCACTGGA
PstI
CTGCAGTGGAGCCAGTCGCCCTGTATCCTGGAGGGAGACGAGTCAGGAAGGCGCACGCAGGCCCAGCTCC
CGAGGTACTACCCCCTCTACTCCTCAGGGAGGATGCCAACGCAATACTCCTTAGTCGTCACTTTGTTTCC
GAAGTAAATGTTGTGATGAAAGGCAAACTTCTTCCTACCCCTTGTATTCAGGGTGGCCGAGTTCCTCCAC
CTGCCTGTCCAAGAAGGAGAAACAGGGCTGTGAAGGGGCAATTTCATCTAGGTGGGCTGAGGTGGCATTC
TAGCCGGGGTGAAGCATGCGTTTCCCCTTCCCAGCTTTCCCGCTGAGACACACCTGAGCCCCAGAAGGAC
CTCAACCTGACCAGGACCTTAGCACCCTCCCCAGACCCAGGCTTTCCATCCTGACCTGCAAATCCAACAT
GCAGCTTTGAAGGACTTTCTCATGGTTTCTGAGCTCCTTGCTCTCACCAGAAAGAATCAGAACTTTTAAA
GTGTTCTTTATGCCAACTTAAATTTTTCATTTTTACTACCTCATGTTTTGGATGAGGCATGTATTTTTAA
ATTTATTTTCACCTTATTGTACCTCTATGATAAACTGCTTGCTTACATTCATACCGTAATTATCTCTCAG
EcoRI
GTTACTTGTCTGTTCCTAAAGATTCACTGAAACGAAGAATTCTATATATGCTTGTATCTTTCAGCAACCG
TATGTCAGATAGCACTGCACATTACTGCAGACATCGCATATACAGGTCCAAAGGTAGAGGAAGAAGAAGA
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CATTTTAAATAATCAACAATTAAAGGGAATACATCAACATTATTTAATACCAATAACGTTATTTTTCTTG
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TGTGTAATCTTTTAATTAATCCATAATTGTATGTGTGTGAAATTAGATAAGCGGTTACCTTTTCTTTACT
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AGCTTCATTCTCCTTGTTCTCTTACGGCAACCGGAAAGTTAATTCTCGCTCTAATTTGGCTTTCAAGGTG
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CCATCACTGCCTACTCAATAATAGGTATCCCAAACCTTTGAGCCAAACTGAGCTCAGGTGCTCCCACAAA
HindIII
CCAAGCTT 3'

Fig. 3.

strategy. Figure 3 shows the nucleotide sequence of the 2,808 bp section of pY-80 spanning the *Stu*I through *Hin*dIII sites. The GC content of the sequence was 40.2%. The sequence revealed no homology with other Y-specific DNA sequences previously described (Cooke *et al.*, 1984; Nakahori *et al.*, 1986; Arnemann *et al.*, 1987; Ellis *et al.*, 1989; Sinclair *et al.*, 1990).

Chromosomal assignment of pY-80

From a chromosomally normal male, 21 metaphases with well-banded chromosomes were analyzed for *in situ* hybridization (Fig. 4). Overall, 60 grains were observed, of which 29 (48%) were located on the Y chromosome. Of the 29 grains, 25 (86%) were localized at Yp11.2-Ypter.

PCR of pY-80

A flanking site of *Pst*I (nucleotide position, 351)–*Eco*RI (nucleotide position, 1017) fragment of pY-80 was used for the PCR. The oligomers used were Y1; 5′-ATGACCCTAGAACCACTGGA-3′ (upstream primer) and Y2; 5′-CGGTTGC-TGAAAGATACAAG-3′ (downstream primer). The oligomers span a 719 bp section of pY-80 (Fig. 3). Electrophoretic analysis of the PCR products revealed a 0.7 kb band with male specificity (Fig. 5a). Southern blot analysis of the gel was performed with a ^{32}P-labeled *Pst*I–*Eco*RI fragment of pY-80 to confirm the origin of the amplified product. The 719-bp amplified products were hybridized

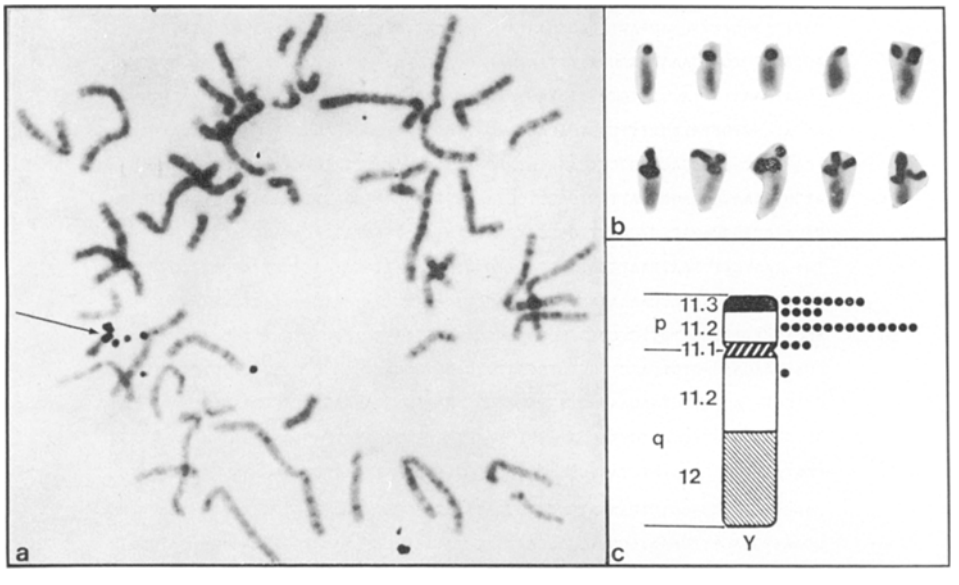

Fig. 4. a: Lebeling of the short arm of the Y chromosome (arrow). b: Representative Y chromosome with labeling. c: Regional distribution of grains on the Y chromosome.

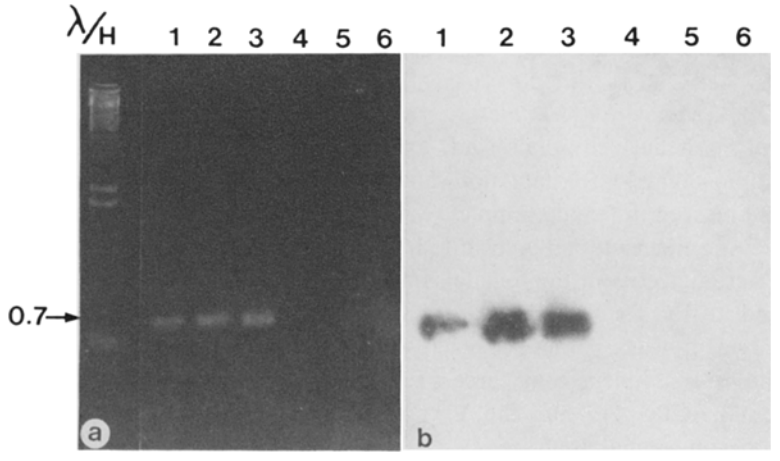

Fig. 5. a: Electrophoretic analysis of PCR products from males (lanes 1–3) and females (lanes 4–6). b: Southern blot analysis of the gel with a ^{32}P-labeled *Pst*I–*Eco*RI fragment probe. The size of the PCR product is marked in kb.

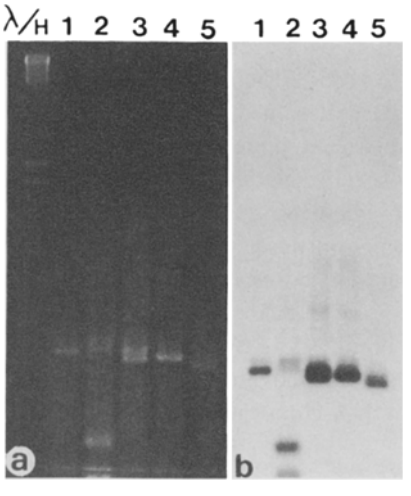

Fig. 6. Restriction mapping of the amplified PCR product (a) and Southern blot analyses (b) from purified peripheral blood. Lane 1, no enzyme added; lane 2, *Sph*I digestion; lane 3, *Eco*RI digestion; lane 4, *Pst*I digestion; lane 5, *Eco*RI and *Pst*I double digestion.

with the probe (Fig. 5b). Physical mapping was further performed to characterize the Y-chromosomal amplified product (Fig. 6). *Sph*I digestion resulted in 404-bp and 316-bp fragments (lane 2); *Eco*RI digestion showed 689-bp and 31-bp fragments (lane 3); *Pst*I gave 697-bp and 23-bp fragments (lane 4); *Eco*RI and *Pst*I double digestion resulted in 666-bp, 31-bp, and 23-bp fragments (lane 5). The restricted fragments were hybridized with the probe (Fig. 6b). Smaller fragments run out

of the current gel shown in Fig. 6. These results were in accord with the complete nucleotide sequence of the target region of pY-80 (Fig. 3).

PCR of DNA from various samples

Buccal epithelial cells, hair root, and urinary epithelial cells were all suitable for Y-chromosomal PCR (data not shown). None of the discrete amplified products could be seen in female samples. All of the 719-bp amplified products derived from male specimens tested hybridized with the probe, whereas no hybridizable products were detected in the female samples. Dried blood spots on a filter paper were stored at 4°C for periods up to 4 weeks and then DNA was extracted. The 719-bp fragment was synthesized exclusively in male DNA after the PCR. Amniotic fluid cell samples from three pregnant women were analyzed with the Y-chromosomal PCR. All had the Y chromosome. These findings were also confirmed by chromosomal analyses.

DISCUSSION

The pY-80 clone described here was derived from the short arm of the Y chromosome, and had no homology with previously sequenced Y specific clones (Cooke, *et al.*, 1984; Nakahori *et al.*, 1986; Arnemann *et al.*, 1987; Ellis *et al.*, 1989; Sinclair *et al.*, 1990). It is yet to be determined whether or not the clone is identical with previously identified, but yet to be sequenced, DNA clones at the short arm of the Y chromosome. Its male specificity was confirmed by Southern blot analysis and the PCR amplification. In view of the shortage of sequenced Y-specific probe, it would be useful for various purposes that include PCR amplification.

The results of our preliminary tests indicated that the clone is useful for sex determination in samples of various sources, including mouth wash products, plucked hair, urine, dried blood spots on a filter paper, and amniotic fluid cells.

Further analysis is being carried out of the clone's localization in relation to other previously identified DNA clones in the short arm of the Y chromosome.

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