

## THE CHARACTERIZATION OF HUMAN MALE-SPECIFIC 5.7 kb DNA(pKY-1) AND ITS APPLICATION TO FETAL SEX-DETERMINATION PROBE

KOZO NAGAI,<sup>1</sup> Satoru KŌIKE,<sup>2,\*</sup> Ryo KOMINAMI,<sup>2,\*\*</sup>  
Masami MURAMATSU,<sup>2</sup> Yutaka NAKAHORI,<sup>3</sup> Yasuo NAKAGOME,<sup>3</sup>  
Hiroaki SOMA,<sup>4</sup> and Isamu YANAGISAWA<sup>1</sup>

*Departments of <sup>1</sup>Biochemistry, <sup>4</sup>Obstetrics and Gynecology, Tokyo Medical College,  
Shinjuku-ku, Tokyo 160, Japan*

*<sup>2</sup>Department of Biochemistry, University of Tokyo School of Medicine,  
Bunkyo-ku, Tokyo 113, Japan*

*<sup>3</sup>Department of Congenital Abnormalities Research, National Children's Medical  
Research Center, National Children's Hospital, Setagaya-ku, Tokyo 154, Japan*

**Summary** A male-specific 5.7 kb DNA(pKY-1) was cloned by screening human genomic libraries. Subsequent to structural characterization, a possibility of its application for prenatal diagnosis probe was tested. The results obtained were as follows: 1) The pKY-1 was specifically hybridized with human Y-chromosome specific repetitive 3.5 kb, but not with female DNA and 2.1 kb, known to be another human Y-specific repetitive-DNA. 2) *Hae*III, *Eco*RI and their double digestion did not produce the Y-specific 3.5 kb fragments from pKY-1. 3) Although the digestion of pKY-1 with *Bam*HI liberated 0.55 kb, *Cla*I clove remained 5.10 kb into 2.40 and 2.7 kb fragments, while their hybridizabilities with the 3.5 kb DNA were identically conserved.

These data suggest that 5.7 kb fragment (pKY-1) should be proposed as a member of Y-chromosome-specific repetitive DNA family in human genome. Thus, this cloned DNA will be not only a useful probe for human fetal sex-determination but also an effective tool for analysis of Y-chromosome with its structure and repetitive DNA.

**Key Words** male-specific 5.7 kb DNA, Y-specific repetitive DNA, Y-chromosome, fetal sex determination, prenatal diagnosis

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\* Present address: Department of Biochemistry 1, Niigata University School of Medicine, 757 Ichibancho, Asahimachi-Dori, Niigata 951.

\*\* Present address: Department of Microbiology, the Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113.

Abbreviations: kb, 10<sup>3</sup> bases; SSC, 0.15 M sodium chloride-0.015 M sodium citrate; SDSa, sodium *N*-lauroylsarcosinate; EDTA, ethylenediaminetetraacetic acid sodium salts.

## INTRODUCTION

Recently, several investigations have been concentrated on antenatal diagnosis using DNA analysis of sex determination (Gosden *et al.*, 1984a, b; Lau *et al.*, 1984; Nagai *et al.*, 1984, 1986). Such an antenatal diagnostic method for sex-linked disorders such as Duchenne muscular dystrophy, hemophilia, and Lesch-Nyhan syndrome is important, because if fetal sex is determined a female, further testing is unnecessary. However, Y-chromosome-specific DNA probe have been being applied for not only fetal sex determination but also for the studies on structural analysis and clinical diagnosis of Y-chromosome and its variants (Cooke *et al.*, 1982; Gänshirt and Pawlowitzki, 1984; Lau, 1985; Nakahori and Nakagome, 1986). In addition, the use of Y-specific DNA-probes for fetal sex determination can provide several advantages over conventional karyotypic analysis. In order to determine fetal sex as early as possible, cytogenetic techniques by culturing amniotic fluid cells usually will consume more time and contamination with maternal tissue sometimes gives little or no effective results. Fetal sex determination method by the use of Y-specific DNA probes shortens the time required for analysis, and DNA extraction can be avoided if dot hybridization is applied. In this study, fetal sex determination using a male-specific new DNA probe was identified, thereby uncultured samples such as amniotic fluid cells, placenta, and blood (or lymphocytes) also can be applied.

## MATERIALS AND METHODS

*Enzymes and chemicals.* Restriction enzymes were purchased from Takara Shuzo Co. and Nippongene Co. (Japan) and were used under conditions recommended by the suppliers. Radiochemicals were obtained from Amersham or New England Nuclear (England).

*DNA cloning.* The 3.5 kb fragments obtained from *Hae*III-digested human genomic-DNA of a male was separated on a 0.8% agarose gel, and was recovered by electroelution (Maniatis *et al.*, 1982). Isolated DNA was labeled with [ $\alpha$ -<sup>32</sup>P]-dCTP by nick-translation (Kelly *et al.*, 1970) and was used as a probe for screening human genomic libraries (constructed with  $\lambda$ -Charon 4A and *Eco*RI fragments by T. Maniatis). Although three positive plaques could be isolated from about four thousands ones, one of them had 5.65 kb *Hae*III fragment which could specifically hybridize with human Y-specific 3.5 kb repetitive-DNA. The 5.65 kb DNA was subcloned by ligating into *Sma*I site of plasmid pUC19. As a result, it was designated as 5.7 kb pKY-1. The previously cloned Y-specific 3.5 kb DNA (pHY-10) (Nakahori and Nakagome, 1986) was also used for human Y-specific reference-DNA-probe.

*DNA preparation from fetal specimens.* DNA from placenta was extracted after isolation of nuclei by a modified method of Gross-Bellard *et al.* (1973). DNA

isolated from chorionic villi, lymphocyte or whole blood can be prepared by essentially similar method as described above and plasmid was prepared by alkaline procedure (Birnoboim and Doly, 1979).

*Restriction enzyme digestion and agarose slab-gel electrophoresis.* The isolated human genomic DNAs were digested to completion with a variety of restriction endonucleases using appropriate buffers (Takara Inc., Japan). In most reactions, 1 to 2 units of enzymes per  $\mu\text{g}$  of DNA were used and digestion was carried out at a concentration of less than 200  $\mu\text{g}/\text{ml}$  for 1 to 10 hr at 37°C.

After extraction with phenol, restriction products were subjected to electrophoresis on 0.8% (w/v) agarose slab gel with a  $\lambda$ -HindIII marker. After electrophoresis, restriction patterns of DNA were visualized by staining with ethidium bromide (0.5  $\mu\text{g}/\text{ml}$ ). The migration during electrophoresis of  $\lambda$ -HindIII DNA was measured as molecular weight reference.

*Blotting and hybridization procedures.* Fragments of DNA were transferred from the agarose gel to nitrocellulose sheets and hybridized to nick-translated restriction fragments according to the methods of Southern (1975). Prior to hybridization, the filters were successively treated at 65°C with 10 ml of  $3\times\text{SSC}$  for 30 min, Denhard's solution [0.02% (w/v) bovine serum albumin, 0.02% (w/v) polyvinylpyrrolidone and 0.02% (w/v) Ficoll (Pharmacia Fine Chemicals)] in  $3\times\text{SSC}$  for 60 min, and were rinsed with 50 ml of the Denhard's in NEST buffer (1 M NaCl, 10 mM EDTA, 0.1% SDSa, 50 mM Tris-HCl, pH 7.6). After pretreatment, hybridization was carried out in NEST buffer containing denatured herring sperm, human female DNA (10  $\mu\text{g}/\text{ml}$ ) and radioactive probe ( $10^7$  to  $10^8$  cts/min per  $\mu\text{g}$  DNA) at 65°C for 10 hr. Then, the filters were washed twice with  $0.3\times\text{SSC}$ -containing 0.1% SDSa for 30 min at 65°C.

Autoradiography was performed on Fuji-RX film with Dupont Lightning Plus intensifier for 1 to 24 hr.

## RESULTS

### *DNA cloning*

Three positive plaques were found in about 4 thousands ones by screening human genomic library. One of positive plaques had 5.65 kb *Hae*III fragments which were specifically hybridized with Y-chromosome specific-3.5 kb repetitive-DNA. Since it was subcloned by plasmid pUC19, this was designated as 5.7 kb pKY-1. Thus, cloned pKY-1 DNA which was analyzed on its restriction site map and was also applied for fetal sex-determination probe as described in following sections.

### *Analysis for restriction site map of pKY-1*

A restriction site map of pKY-1 was generated by single and double digests of the DNA with various restriction enzymes. The digests were analyzed by Southern hybridization after 0.8% agarose slab gel electrophoresis. After hybridiza-

tion by employing both probes of pKY-1 and pHY-10, completely identical patterns were demonstrated (Fig. 1). As shown in Fig. 1, digestion of pKY-1 with a variety of the enzymes clarified that the cloned phage DNA had 16.4 kb fragment as an insert containing the sites for the following enzymes: one *EcoRI*, two *BamHI*, and two *HaeIII*. Human Y-specific 3.5 kb DNA was specifically hybridized only with the 5.7 kb fragments (pKY-1) isolated by *HaeIII* digestion of insert from the phage DNA. Furthermore, the 5.7 kb pKY-1 DNA was cloven into 0.55, 2.40 and 2.70 kb fragments by double digestion with *BamHI* and *ClaI* (Nagai *et al.*, unpublished). The restriction site map was consequently summarized from above described results (Fig. 2). The 3.5 kb repeated units however, were not liberated from pKY-1 even by *EcoRI* and *HaeIII* as well as their double

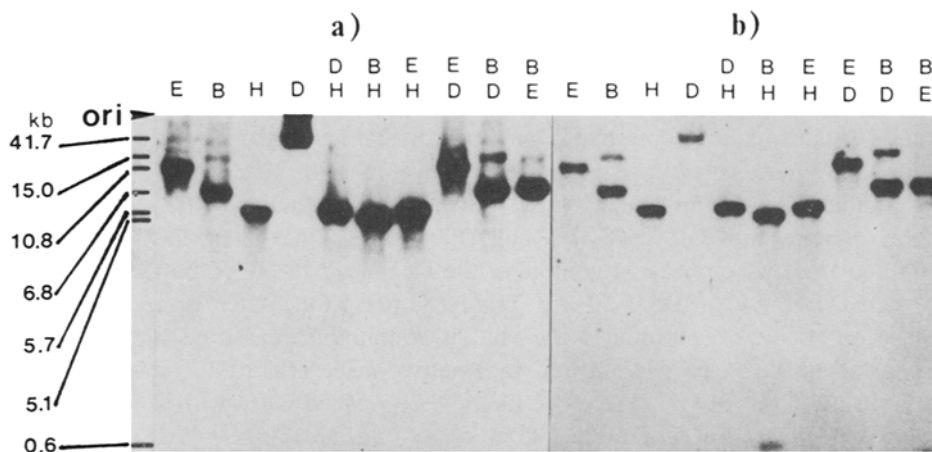


Fig. 1. Restriction endonuclease digestion of newly cloned DNA. Five  $\mu\text{g}$  of phage DNA were digested by a variety of restriction endonucleases and were analyzed on 0.8% agarose slab gel-electrophoresis. Southern hybridizations were carried out as described in MATERIALS AND METHODS. a) 5.7 kb (pKY-1) probe. b) 3.5 kb (pHY-10) probe. Abbreviations of restriction endonucleases: E, *EcoRI*; B, *BamHI*; D, *HindIII*; H, *HaeIII*.

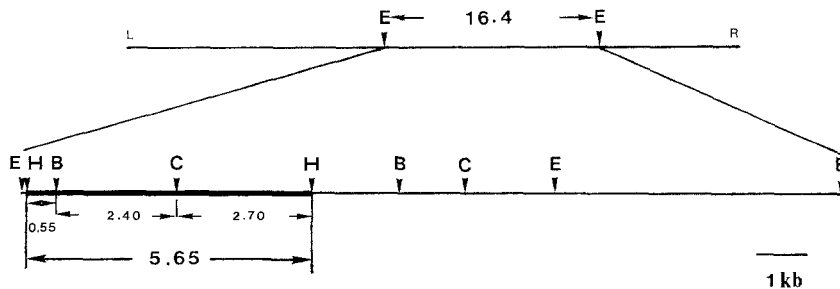


Fig. 2. The restriction site map of newly cloned DNA. The restriction sites of enzymes were indicated by similar abbreviations as described in Fig. 1, except *ClaI* was shown by C.

digestion. To confirm the hybridizability of pKY-1 with Y-specific 3.5 kb DNA and subsequent applicability for fetal diagnosis probe, following experiments were undertaken.

*Fetal sex determination by employing 5.7 kb DNA probe*

For fetal sex determination, 5  $\mu$ g human fetal DNA-specimens (A, B and C) were analyzed with reference to human male genomic DNA by Southern blot hybridization (Fig. 3). The hybridizabilities of pKY-1 as a DNA probe with Y-specific 3.5 kb DNA observed in the specimen of A and male reference DNA were demonstrated in essentially identical patterns as that of pHY-10. Since no positive signals were detected in both DNA specimens of B and C, they were identified as females. In addition, pKY-1 did not hybridize with another human Y-specific 2.1

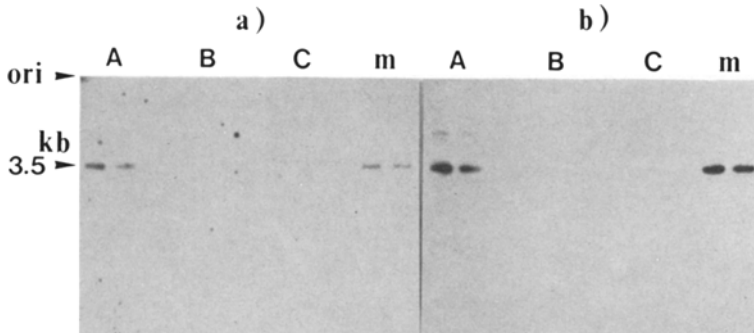


Fig. 3. The fetal sex determination by Southern hybridization. Five  $\mu$ g human placental DNA was digested completely with *Hae*III and were analyzed by Southern hybridization as described in MATERIALS AND METHODS. A, B, and C are placental DNA specimens. m, a male reference-DNA. a) 5.7 kb (pKY-1) probe. b) 3.5 kb (pHY-10) probe.

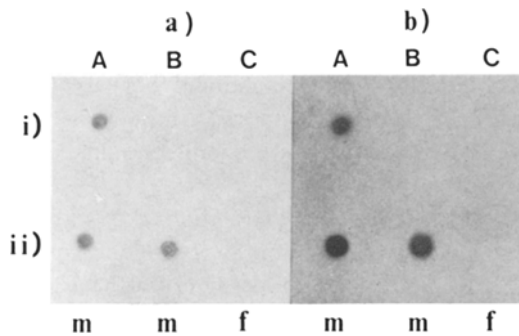


Fig. 4. The fetal sex determination by dot blot hybridization. Two  $\mu$ g placental DNA treated with 0.2 N NaOH in 0.6 M NaCl were blotted on to nitrocellulose filters, and were neutralized with 0.5 M Tris-HCl (pH 7.6)-0.6 M NaCl and 2  $\times$  SSC. The hybridizations were carried out as described in Fig. 3. i) A, B and C are fetal DNA specimens as arranged in Fig. 3. ii) Reference DNA: m, male; f, female. a) 5.7 kb (pKY-1) probe. b) 3.5 kb (pHY-10) probe.

kb DNA known to be unable to cross-hybridize with the 3.5 kb DNA. These results indicate that a male specific signal in human genomic DNA can be discriminated by employing a more rapid test. Similar DNA specimens as arranged in Fig. 3 were applicable to sex determination by dot blot hybridization (Fig. 4). The hybridizabilities for both DNA probes of pKY-1 and pHY-10 were distinctly demonstrated as identical male specific signals. This was also confirmed by using tissue homogenates specimens without extracting DNA (Nagai *et al.*, unpublished data). From the autoradiograms obtained from Southern and dot hybridization using  $^{32}\text{P}$ -labeled 5.7 kb DNA as a probe, its copy number in human genome was deduced as about a dozen or more. However, good hybridization and its specificity with Y-chromosome-specific 3.5 kb repetitive-DNA will be effective for human sex determination. In addition, we could confirm the human gene specificity by evidence that the pKY-1 DNA probe didn't show cross-hybridization with male and female genomic DNA of other eukaryotes such as monkey, rat, mice, chicken, rana and masou (Nagai *et al.*, manuscript in preparation).

#### DISCUSSION

In this study, the human male-specific 5.7 kb DNA (pKY-1) was specifically hybridized with Y-specific 3.5 kb repetitive-DNA. From this result, the pKY-1 must be a member of Y-chromosome-specific repetitive-DNA family. This was supported by  $\text{Ag}^+\text{Cs}_2\text{SO}_4$  equilibrium centrifugation that both buoyant density of pKY-1 and 3.5 kb DNA had relatively coincided at  $1.535 \text{ g/cm}^3$  (Nagai *et al.*, unpublished data). These results suggest that both DNA has relatively common base composition and/or the sequences in their fragments. Therefore, by labeling the pKY-1 with  $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ , we have tested a possibility to apply a sex determination probe for fetal derived DNA materials. Although the pKY-1 gave reasonable results as DNA probe for human sex determination, it took a slightly longer exposure time since its copy number was lower than those of 3.5 kb DNA. On the other hand, good male specificity of the pKY-1 probe simplifies the test procedure and offers several advantages over conventional karyotypic analysis as those of other probes (Gosden *et al.*, 1984a, b; and Lau *et al.*, 1984). If dot hybridization is applied to avoid DNA-extraction, only small amounts of fetal DNA materials are required.

However, during the cloning, characterization of 5.7 kb DNA and subsequent its application for fetal sex determination probe, a few interesting problems have arisen that *Hae*III, *Eco*RI and their double digestion have produced no Y-specific 3.5 kb fragments. On the other hand, *Bam*HI-digestion liberated the 0.55 and 5.10 kb from pKY-1 and latter fragments was cloven into 2.40 and 2.70 kb by *Cla*I-digestion. All these fragments however, had essentially similar hybridizability with Y-specific 3.5 kb DNA (Nagai *et al.*, unpublished data). In addition, human gene specificity was confirmed by evidence that pKY-1 did not cross-hybridize with other animal genomic DNA. This suggests that the sequence and its repetition

of pKY-1 is essentially different from other eukaryotic genome-DNA such as monkey, rats, mice, chicken, rana and masou. The origin, biological role of pKY-1 and its relationship with previously cloned repetitive 3.5 kb DNA from human Y-chromosome should be further clarified. These results confirm that a male specific 5.7 kb DNA (pKY-1) could be provided as a new probe not only for human fetal sex-determination but also for structural study of Y-chromosome with its variants and specific repetitive DNA.

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