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Isolation and Characterization of Y Chromosome DNA Probes

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A sorted, cloned Y chromosome phage library was screened for unique Y chromosome sequences. Of the thousands of plaques screened, 13 did not hybridize to radiolabeled 46,XX total chromosomal DNA. Three plaques were characterized further. Clone Y1 hybridized to multiple restriction enzyme fragments in both male and female DNA with more intense bands in male DNA. Clone Y2, also found in female and male DNA, is probably located in the pseudosutosomal region because extra copies of either the X or Y chromosomes increased Y2 restriction enzyme fragment intensity in total cellular DNA. Clone Y5 was male specific in three of four restriction enzyme digests although in the fourth a light hybridizing band was observed in both male and female DNA. Clone Y5 was sublocalized to band Yq 11.22 by hybridization to a panel of cellular DNA from patients with Y chromosome rearrangements. Clone Y5 can be used to test for retention of the proximally long arm Y suggested to cause gonadal cancer in carrier females. The long series of GA repeats in Y5, anticipated to be polymorphic, may provide a sensitive means to follow Y chromosome variation in human populations.

Human sexual dimorphism results from the presence or absence of the Y chromosome. The development of normal and abnormal male and female phenotypes has been correlated with the presence or absence of specific portions of the Y chromosome [c.f. 1]. The "testis determining factor" (TDF) [2, 3, 4] and the H-Y antigen [5, 6] map to different portions of the Y chromosome, and each appears to be associated with male differentiation. Recently, the sex reversal-Y gene (SRY) has been reported to induce testis differentiation and subsequent male development in chromosomally female mouse embryos [7]. The number of additional Y chromosome-specific genes actually involved in male sex determination is currently unknown. In addition, some autosomal genes have been implicated in male differentiation [8].

Cytogenetic analyses of abnormal sex chromosomes have increased our understanding of normal sexual differentiation and sublocalized Y chromosome functions. The completely

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homologous pseudoautosomal regions on the terminal Y and X chromosome short arm are interchanged during male meiosis and insure that the X and Y chromosomes segregate into daughter cells with high fidelity [9]. Five megabases proximal to the pseudoautosomal boundary is the X-linked steroid sulfatase (STS) gene which has a homologous STS pseudogene on the Y chromosome [10]. The SRY and "testis determining factor" (TDF) genes map to a Y chromosome short arm region retained in 46,XX phenotypic males [c.f. 11, 12], while the HY antigen gene is not retained in these individuals [5, 6]. A repetitive human Yq chromosome-specific sequence has been characterized [13]. These and other Y chromosome-specific sequences serve as markers to study the evolution of Y chromosome haplotypes [14] and to identify transcriptionally active Y chromosome regions. For instance, retention of a segment on the proximal long arm results in an increased risk of gonadal neoplasia in phenotypic females [15]. Thus, molecular analyses with multiple Y-specific probes improves medical management of these and other patients [16].

Additional mapped Y chromosome-specific probes will facilitate studies of Y chromosome functions and improve patient diagnosis and management. Toward this end we have characterized novel Y chromosome derived probes isolated from a sorted human Y chromosome phage library.

MATERIALS AND METHODS

Cells and Culture Conditions:

Fibroblast cultures were obtained from UCSF patients with abnormal sex chromosome complements or abnormal secondary sexual characteristics. Cultures were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics under 5% CO_2 in air at 37° C.

DNA Isolation:

DNA from both cultured fibroblasts and peripheral blood lymphocytes was isolated as described previously [17]. Fibroblasts were trypsin harvested, lysed with 0.5% sodium dodecyl sulfate (SDS), digested with 100 ug/ml proteinase K, and the DNA phenol extracted and ethanol precipitated. After RNase digestion, and repeated phenol extraction and ethanol precipitation, purified DNA was dissolved in 10 mM Tris-HCl, 1 mM EDTA (pH 8.0), quantified spectrophotometrically, and stored at -20° C.

DNA from 20 ml of peripheral blood lymphocytes was extracted after lysing the red blood cells with an equal volume of red cell lysis buffer (10 mM Tris-HCl, pH 7.6; 5 mM $MgCl_2$; 10 mM NaCl). Samples were mixed by inversion and centrifuged at 2500 rpm for 15 min. The supernatant was carefully removed, and the pellet resuspended and washed in 30 ml red cell lysis buffer until the cell pellet turned white. The lymphocytes were then disrupted by dropwise addition of 10 ml of freshly prepared lysis buffer (7 M urea; 0.3 M NaCl; 10 mM EDTA; 10 mM Tris-HCl, pH 7.5) and 1 ml of 20% SDS. After 10 min at 37° C the DNA was phenol extracted and ethanol precipitated.

Y-Chromosome Library Screening:

The Y-chromosome library obtained from the Lawrence Livermore National Laboratory (Livermore, CA), contained Y chromosome sequences cloned into the Hind III restriction enzyme site of lambda phage Charon 21A [18]. The library was screened as described previously [19]. Briefly, bacterial strain LE 392 was inoculated with the lambda library by maltose shock. Bacteria were grown overnight and harvested by centrifugation (4000g for 10 min at room temperature). The pellet was resuspended in 1 mM $MgSO_4$ and stored at 4° C. The phage stock was diluted to $5x10^4$ plaque forming units (pfu)/ dish with SM buffer (100 mM NaCl, 1 M $MgSO_4$, 50 mM Tris, pH 7.5, 0.01% gelatin). Dishes were incubated at 37° C until plaques were 1 to 1.5 mm (10-12h) and then chilled at 4° C for greater than 1h to allow the agar to harden. Colony/Plaque Screen filters (New England Nuclear/Dupont) were replica plated for 2-3 min., then placed into a pool of 0.5 N NaOH for 2 min with the colony containing side up, washed in 1M Tris, pH 7.5 for 2 min. and allowed to dry at room temperature.

DNA isolated from cells with a 45,X karyotype and DNA from Y chromosome lambda library was amplified in a lawn of bacteria over agar and ^{32}P-labelled by nick-translation [20] or primer extension [21] to a specific activity > 10^8 DPM/µg. Labelled DNA was hybridized to filter-bound phage DNA under high stringency, washed, and autoradiographed. Replicated plaques that did not hybridize to 45,X DNA and were positive when probed with Y-chromosome DNA were isolated and expanded for further characterization. Initial screening with DNA from cells with a 46,XY or 47,XYY karyotype failed to yield discernable Y-chromosome specific clones. Sensitivity was markedly enhanced when Y chromosome-specific DNA was used. From the screened colonies, 13 male specific clones were identified and isolated.

Isolation and Growth of Plaques:

Unique putative Y chromosome-specific plaques were eluted in SM buffer at room temperature for 1h and then stored at 4° C. A 50 µl aliquot of phage was amplified on a lawn of LE 392 bacteria grown in 0.2% maltose. Phage were eluted from the plates with 5 ml of SM buffer at 4° C for 2 h. A second SM elution was pooled with the first, vortexed with 100 µl of chloroform and centrifuged at 4000g for 10 min at 4° C. The supernatant was stored at 4° C in 0.3% chloroform.

Plasmid Constructions:

The Y chromosome sequences isolated from bacteriophage were ligated into the polylinker cloning site in vector pIBI76 (International Biotechnologies Inc.) at 14° C, and amplified in E. coli. Insertion into the polylinker results in inactivation of the ß-galactosidase gene and results in white rather than blue colonies.

Southern Blot Analysis:

Three Y chromosome probes pY-1, pY-2, and pY-5, isolated in this study, were hybridized to DNA from individuals with defined karyotypic abnormalities and compared to Y chromosome probes p3.4 and pDP1007 reported previously. Probe p3.4 is derived from Eco RI male repeat (DYZ1)[13], and detects a 2000-fold repeated Y-specific 3.4 kb fragment. Probe pDP1007 was derived from a 3.0 kb Eco RI fragment in Yp and cross hybridizes to a 1.6 kb Eco RI fragment in Xp21.3-p22.1 [12].

At least 7 µg of DNA was digested with a 5-fold excess of restriction enzyme Eco RI (Bethesda Research Laboratories) according the manufacturer's specifications. DNA was fractionated by electrophoresis on 0.8% agarose gels in TBE buffer (89 mM boric acid; 2 mM EDTA, pH 8.3) for approximately 15 h at 1.2 V/cm, and transferred to nitrocellulose (Millipore) or nylon membranes (Hybond-N, Amersham) by capillary action [19, 22].

DNA probes were labelled by nick-translation [20] with [^{32}P]-dCTP and/or [^{32}P]-dATP(specific activity > 3000 Ci/mmol, Amerhsam). Probes were hybridized at 42° C for 16 h in 50% formamide; 50 mM sodium phosphate, pH 7.0; 2X Denhardt's solution (1X Denhardt's = 0.02% BSA; 0.02% Ficoll; 0.02% polyvinylpyrrolidone); 3 X SSC; 50 µg/ml salmon sperm DNA, and 10% dextran sulfate. Filters were washed once at room temperature with 1X SSC; 1X Denhardt's solution and then once in 0.1X SSC, 0.1% SDS at 56° C, and four times in 1X SSC, 0.1% SDS at 60° C. Bands were visualized autoradiographically on X-ray film.

Y-5 DNA Sequence Analysis:

Y-5 insert was excised from pIBI76 by Hind III digestion and subcloned in M13mp18. The transformed cultures were propagated into 2% XGal 2xyT top agar. Recombinant clones were amplified in DH5alphaF'IQ plus 2xyT. The single strand DNA in the supernatant from an overnight stationary culture was precipitated with 20% polyethylene glycol in 2.5M sodium chloride and collected by centrifugation according to the manufacturer's instructions (BRL, Gaithersburg MD). The DNA was phenol-chloroform extracted, ethanol precipitated, and redissolved in 20 microliters of sterile distilled water. This single stranded DNA was then sequenced using the dideoxy sequencing method of Sanger (Sequenase version 2.0 from USB, Cleveland, Ohio). The ^{35}S-labelled product was separated on a 5% acrylamide, 50% w/v urea, 600 X 200 X 0.4 mm sequencing gel at 2000 volts, 27.5 mA. The dried gel was autoradiographed overnight and developed.

RESULTS

Of the 13 putative Y chromosome derived unique sequence clones isolated from the Y chromosome library, three were characterized by restriction enzyme analysis (Figure 1). Probes

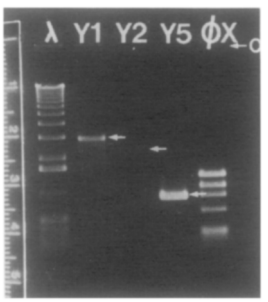
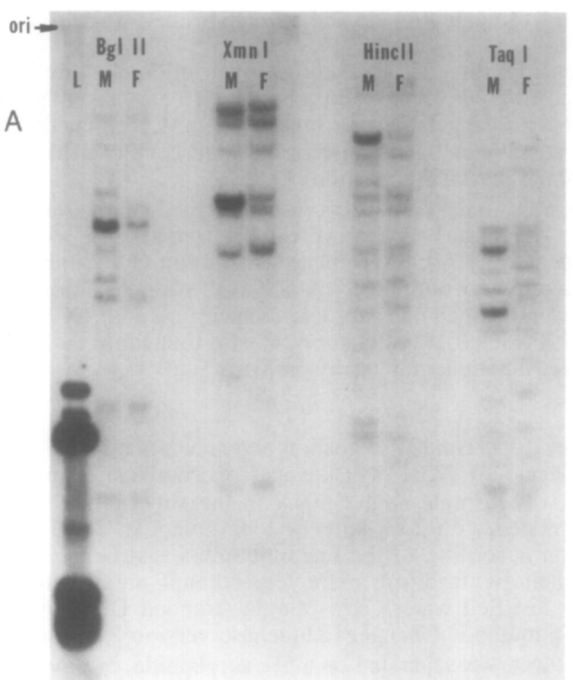


Fig. 1. Sizes of Y chromosome probes. A Hind III digest of Y1, Y2, and Y5 indicate they are 2.9 kb, 2.4 kb, and 0.85 kb, respectively. Lane 1 - one kb lambda ladder, lane 2-Y1, lane 3-Y2, lane 4-Y5, and lane 5-ØX ladder. Arrows indicate probe location.

Y-1 and Y-2, both Y chromosome derived sequences, detected significant homologous sequences in female DNA (Figure 2). Although Y-1 hybridized to both male and female DNA, some bands were Y-specific after digestion with Bgl II, Xmn I, Hinc II, and Taq I (Figure 2A) in these and additional unrelated individuals (not shown). Analysis of the Y-2 probe showed no male specific bands suggesting that the Y-2 probe identifies homologous Eco RI restriction fragments in the pseudoautosomal region of Yp and Xp (Figure 2B), because the bands from digested 49,XXXXY



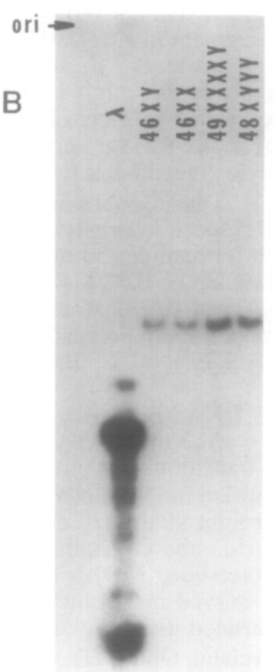


Fig. 2A. Male and female genomic DNA digested with Bgl II, Xmn I, Hinc III and Taq I, respectively, and probed with Y1. Note the extensive homology, although a few Y-specific bands can be identified.

Fig. 2B. Eco RI digest of 46,XY; 46,XX; 49,XXXXY; and 48,XYYY genomic DNAs probed with Y2. Note the increased intensity of the identified fragments as the number of X or Y chromosomes increases.

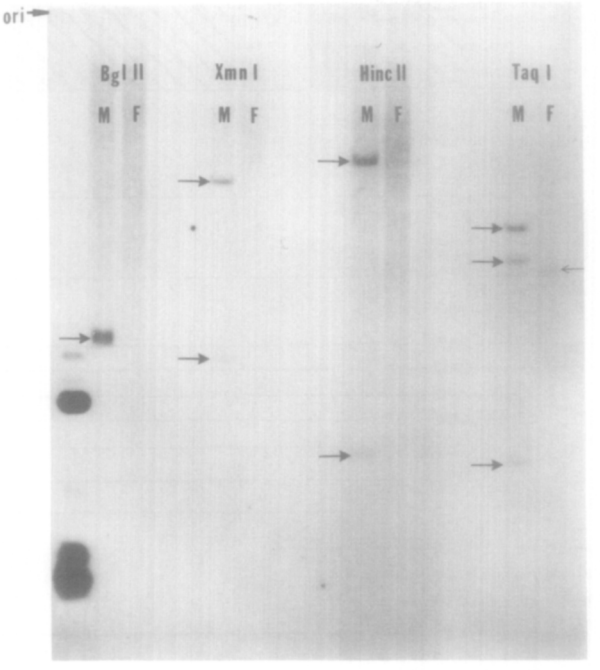


Fig. 3. Male and female genomic DNA digested with Bgl II, Xmm I, Hinc III and Taq I, respectively, and probed with Y5. Note the Y-specific bands (closed arrows) and one light Taq I fragment carried in normal female DNA (open arrow).

and 48,XYYY total DNA hybridized more intensely than equal quantities of 46,XY and 46,XX control DNA.

In contrast, Y-5 probe only hybridized to male DNA but not female DNA following digestion with BgI I Xmn I, and Hinc II (Figure 3). However, Y-5 hybridized weakly to at least one Taq I fragment in normal female DNA. DNA samples of patients with known karyotypes were used to sublocalize Y-5 (Figure 4) to the euchromatic region of Yq11.22 near the bottom of interval 5 of the deletion map of Vergnaud et al. [4]. Analysis of Eco RI digested DNA from a male with a 46,XX karyotype failed to reveal any bands indicating that this probe does not contain male sex determining sequences [22]. Hybridization to Eco RI digested DNA from a 45,X male revealed characteristic 11kb and 2.8kb bands as did DNA from a 46,XY female (Figure 5).

The single stranded Hind III-M13mp18 subcloned Y-5 fragment was sequenced by standard protocols (see Methods). Of the 850 bp Y-5 sequence, 600 were readable by this method. To complete the sequence of the remaining segment, the 850 bp-Hind III double-strand fragment was digested with Pst I and the 400 and 450 bp fragments were subcloned in M13mp18 and M13mp19. Using the same sequencing protocol, we could read the entire sequence of the Hind III-Pst I subcloned fragments in both directions (Fig. 6A). Although this sequence is unique to the Y chromosome, it is remarkable for the number of repetitive sequences it carries including a long series of GA repeats (Fig. 6B). A search of the Socrates sequence database revealed no homologous sequences in other chromosome regions. Analysis of the Y-5 sequence

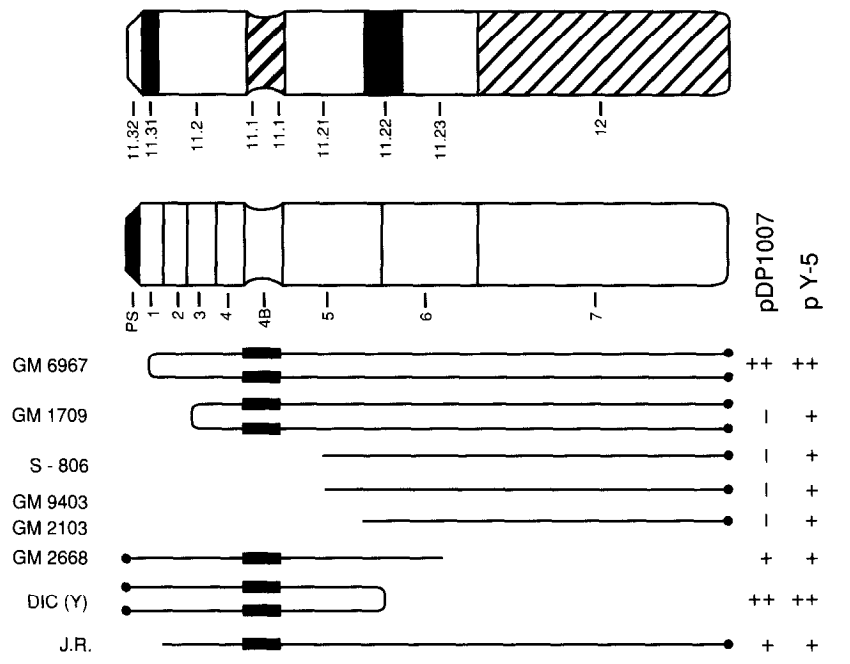


Fig. 4. A series of cell lines with an abnormal Y chromosome had DNA extracted, digested and probed with Y5 for sublocalization, which verified Yq 11.22 as the site of the probe. The figure shows the results obtained for each cell line.
(+) = positive, (++) = double intensity, (-) = negative. The karyotypes utilized were:

Cell Line	Karyotype	Genitalia
GM6967	45,X/46,X,dic(Y) (p11)/47,X,dic(Y) dic(Y)	Amb.
GM1709	46,X,dic(Y) (qter>P11.2: : p11.2<qter)	Fem.
S-806	46,X,-X,+der(X),t(X;Y)(Xqter>Xp22: :Yq11>Yqter)	Fem.
GM9403	46,X,-X,+der(X),t(X;Y) (Xqter>Xp22.3: :Yq11.2>Yqter)	Fem.
GM2103	46,X,-X,+der(X),t(X;Y) (Xpter>Xq11: :Yq11>Yqter)	Fem.
GM2668	45,X/46,X,del(Y) (pter>q11.2)	Amb.
Dic(Y)	46,X,dic(Y) (pter>q11.21: :q11.21>pter)	Mas.
J.R.	46,X,-Y,+der(Y),t(Y;Dor G)(q12 p11)	Mas.

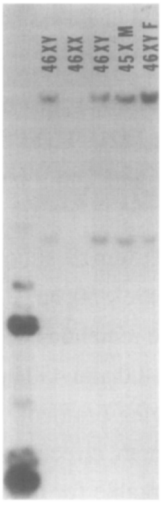


Fig. 5. Eco RI digest of genomic DNAs hybridized with Y5. Lane 1-lambda ladder, lanes 2 and 4-male genomic DNA, lane 3-female genomic DNA, lane 5-45,X male genomic DNA, and lane 6-46,XY female genomic DNA.

A AAGCTT(HindIII)G / CGCAA / CACAG / CAAAA / TCCCA / TGTCT / AATAT / CATAC / CAAAG / CTAGC / TGGGT / GTGGT / GCGCC / ATGGA / TACAA / TCCCA / CGTAC / TTGGG / AGCGT / GATCG / AGGAG / AGTCC / ACTGA / ACCCT / GGGGA / CATGT / TCGAT / GGAAG / AGATC / ACACC / ACTCG / ACTTT / AGACT / TGGCA / CCAGA / GCGAG / ACTCT / GTCTA / AGAAA / TAAAA / ATAAA / ATAAA / ATAAA / AAATG / AGATA / AGAGA / GATGA / GTGAG / AATGC / GTAAC / ACTTG / GTGGG / CATTT / TTGGC / TCATG / CTGGG / AATCC / ATAGT / TTGAG / GGGCT / AAGGT / GGGTG / GATCA / CTTGA / GGACA / GAAGT / TCGAG / TCCAG / CCTGA / GCAAA / CATTG / TAAAC / CTGGT / CTCTA / CTAAG / AATAC / AATTT / AAAAA / AAGCC / AGGAT / CGCTG / TCATA / TGCCTGCAG (PstI)
TT / GCAGT / ACTCA / GGAGG / GTATG / ACTGG / AGAAC / TGCTT / GAACC / CAGGA / TGGGA / GGTTA / CAGTG / AGATG / AGATC / ATGCT / GCTGC / ACTCC / AGCCT / AGGTG / ACAGA / GCAAG / GTTTT / GCCTA / AAAAA / AAAAA / AATCA / AAGAG / AGAAA / CAGAG / ATAGA / GGAAA / AAAAG / AAAGA / AGGAA / GGGAG / GAAGA / AAGGA / AGAGA / AGGAG / GGAGG / GAGGG / AAGGA / AGGAG / GGAGG / GAGGG / TGAGC / AGGCA / GGCAG / GAAGG / AAGGC / AGGAA / GAGAG / AAAGA / GAGAG / GGAGG / GAGGG / AGAGA / AGGAA / GGGAG / AGAGA / GGAAG / GAAGG / AAGGA / AGGAA / GGAAA / GAAGG / AAGGG / AGGAA / AGGGA / GAAAA / GAGAG / AATGA / AAGCG / ATAAA / ACCAA / AAGGA / AATTA / ACAAA / AATAA / AAGCTT (Hind III).

Fig. 6A. The nucleic acid sequence of probe Y5.

Fig. 6B. GA repetitive sequence within Y5. The sequencing autoradiograph demonstrates the highly repeated GA pair.

by the Gene Recognition and Analysis Internet Link exon identification program of the Oak Ridge National Laboratory failed to detect any evidence of an expressed sequence.

DISCUSSION

The high degree of cross-hybridization between labelled total female genomic DNA and the sorted Y chromosome library reflects the small number of Y-chromosome specific sequences. This conclusion is consistent with the finding that testis differentiation and male development in chromosomally female mouse embryos could be induced by a single SRY gene [7]. Furthermore, two of the three clones characterized in this study (Y-1 and Y-2) not only carry Y chromosome sequences but also detect homologous sequences in female DNA (Fig. 1). These data could explain the difficulty investigators have experienced isolating Y chromosome-specific genes and unique sequences. The results leave one to speculate why the Y chromosome carries so much repetitive, non-transcribed DNA. Perhaps the need to increase the fidelity of meiotic sex chromosome segregation without modifying the phenotype led to selection for the unusually large proportion of Y chromosome repetitive sequences.

The genetic map that reflects the recombination rate between different chromosome locations can be derived only for the pseudoautosomal Y chromosome region which recombines with the terminal X chromosome short arm to increase the fidelity of meiotic sex chromosome segregation. Thus, isolated Y chromosome sequences only can be mapped physically to the remaining chromosome regions. As in previous studies, cell lines carrying Y subchromosomal regions were used to map the isolated library fragments.

The full spectrum of clinical syndromes involving Y subchromosomal region abnormalities has yet to be characterized. For instance, retention of the proximal long arm of the Y chromosome has been reported to significantly increase the risk of gonadal cancer so that gonadectomy is indicated for females carrying this chromosome segment. Furthermore, collecting data using all available mapped clones to test all appropriate patients will improve prognostic evaluation in at-risk patients.

The GA-repetitive region in the Y-5 clone suggests that specific PCR primers flanking this region would amplify a very polymorphic Y chromosome region. This would be expected to be quite informative in paternity testing. In addition, population tests of a very polymorphic region could be designed to determine the origin of the father(s) of us all, just as mitochondrial DNA were quite useful in tracing the mother of us all to Africa.

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REFERENCES

1. Schonberg, S. (1989) In: Handbook of Human Growth and Developmental Biology, (E. Meisami, P. Timivas, Eds.), Vol.2, pp.229-237, CRC Press, Boca Raton, FL.
2. Page, D., de la Chapelle, A., and Weissenbach, J. (1985) Nature 315, 224-226.
3. Disteche, C., Casanova, M., Saal, H., Friedman, C., Sybert, V., Graham, J., Thuline, H., and Page, D. (1986) PNAS 83, 7841-7844.
4. Vergnaud, G., Page, D., Simmler, M-C., Brown, L., Rouyer, F., Noel, B., Botstein, D., de la Chapelle, A., and Weissenbach, J. (1986) Am. J. Hum. Genet. 38, 109-124.
5. McLaren, A., Simpson, E., Tomonari, K., Chandler, P., and Hogg, H. (1984) Nature 312, 552-555.
6. Simpson, E., Chandler, P., Goulmy, E., Disteche, C., Ferguson-Smith, M., and Page, D. (1987) Nature 326, 876-878.
7. Koopman, P., Gubbay, J., Vivian N, Goodfellow P, and Lovell-Badge, R. (1991) Nature 351, 117-121.
8. Washburn, L.and Eicher, E. (1983) Nature 303, 338-340.15.
9. Rouyer, F., Simmler, M.C., Johnsson, C., Vergnaud, G. Cooke, H., and Weissenbach, J. (1986) Nature 319, 291-295.
10. Yen, P.H., Marsh, B., Allen, E., Taai, S.P., Ellison, J., Connolly, L., Neiswanger, K, and Shapiro, L.J. (1988) Cell 55, 1123-38.
11. de la Chapelle, A. (1981) Hum. Genet. 58, 105-116.
12. Page, D., Mosher, R., Simpson, E., Fisher, E., Mardon, G., Pollack, J., McGillivray, B., de la Chapelle, A., and Brown, L. (1987) Cell 51, 1091-1104.
13. Lau, Y-F. (1984) In: The Y Chromosome, Part A: Basic Characteristics of the Y Chromosome. (A. Sandberg, Ed.), pp. 177-192. Alan R. Liss, New York.
14. Ngo, K., Vergnaud, G., Johnsson, C., Lucotte, G., and Weissenbach, J. (1986) Am. J. Hum. Genet. 38,407-418.
15. Wolman, S., David, R., and Koo, G. (1985) In: The Y chromosome, Part A: basic characteristics of the Y chromosome. (A. Sandberg, Ed.), pp.477-505, Alan R. Liss, NY.
16. Gemmill, R., Pearce-Birge, L., Bixenman, H., Hecht, B., and Allanson, J. (1987) Am. J. Hum. Genet. 41, 157-167.

17. Goosens, M.and Kan, Y.W. (1981) Method. Enzymol. 76, 805-817.
18. Van Dilla, M.and Deaven, L. (1990) Construction of gene libraries for each human chromosome. Cytometry 11, 208-218.
19. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, 2nd Edition, Cold Spring Harbor Press, NY.
20. Rigby, P., Dieckmann, M., Rhodes, L., and Berg, P. (1977) J.Mol. Biol. 113, 237-251.
21. Feinberg, A.P.and Vogelstein, B. (1984) Anal. Biochem. 137, 266-7.
22. Farah, S., Garmes, H., Cavalcanti, D., Mello, M., Porelli, R., Ramos, C., and Sartorato, E. (1991) Brazilian J. Med. Biol. Res. 24, 149-156.
23. Southern, E. (1975) J. Mol. Biol. 98, 503-517.