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Use of a Probe for the Putative Sex Determining Gene, Zinc Finger Y, in the Study of Patients With Ambiguous Genitalia and XY Gonadal Dysgenesis

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Using reverse genetics, a candidate for the sex determining gene from the Y chromosome has recently been cloned. We have used a DNA probe from this gene to assess the presence of this crucial region of the Y chromosome in patients with sexual ambiguity or gonadal dysgenesis. The DNA from 3 cases of gonadal dysgenesis, one complicated by somatic anomalies and mental retardation, reacted normally with this putative sex determining gene. A patient having a small phallus and pseudovaginal, perineoscrotal hypospadias (PPSH) also had normal Y chromosomal DNA. We hypothesize that the defect in sex determination in all 4 cases is most probably subsequent to the primary sex determining switch.

KEY WORDS: gonadal dysgenesis, sex determination, Y chromosome

INTRODUCTION

In a number of patients it is important to know whether their genome includes an intact Y chromosome. A region on the proximal long arm, perhaps involved in spermatogenesis and/or H-Y expression [Tiepolo and Zuffardi, 1976; Simpson et al., 1987; Burgoyne et al., 1986], has been implicated in the development of gonadoblastoma with intra-abdominal gonads [Schellhas, 1974; McDonough et al., 1986; Page, 1987]. The presence of the sex determining region on the distal short arm of the Y is a prerequisite for normal male external development, even in XX males [de la Chapelle, 1986; Stalvey and Erickson, 1988], while male development in its absence is incomplete [Waibel et al., 1987]. In the past, patients with anomalies of sexual development could be studied with probes near to, but not definitive for, this critical region [Ostrer et al., 1987; Stalvey et al., 1988]. The recent cloning of a candidate sex determining gene [Page et al., 1987] has allowed for more complete studies of such patients.

CLINICAL REPORTS

Patient 1

The mother was a 25-year-old primiparous white woman who had multiple congenital anomalies. They included imperforate anus, bilateral hydroureteronephrosis, bicornuate uterus, abnormally implanted urethral meatus into the anterior vaginal wall, dextroconvex thoracolumbar scoliosis, and S1 spina bifida occulta. At 10 weeks of gestation, a large multicystic mass (seen by ultrasonography and located cephalad to the left uterine horn) was aspirated. Cytology and culture studies were negative. A cervical cerclage was placed, and an oral glucose tolerance test was normal. Nitrofurantoin was given during pregnancy for recurrent urinary tract infections. Later in pregnancy, the gestational sac was found in the left uterine horn, and the fetal presentation was breech. The infant was delivered at 39 weeks of gestation by cesarean section. Apgar scores were 9 at 1 and 5 minutes. Birthweight was 3.46 kg (75th centile), head circumference (OFC) 35 cm (50th centile), and length 52.5 cm (75–90th centile). The abnormalities on physical examination were limited to the genitalia and included a 1.5 cm phallus with a ventral urethral opening, unfused labioscrotal folds, no palpable gonads, and an anteriorly placed anus. Serum electrolytes and 17-hydroxyprogesterone were normal. Abdominal ultrasound examination showed normal bladder, kidneys, and rectum. A genitogram demonstrated the presence of a vagina and probably a uterus. Chromosomes were normal (46,XY). Basal serum testosterone and dihydrotestosterone were 0.87 and 37 ng/dl, respectively. After 3 days of hCG stimulation they rose to 4.83 and 140 ng/dl, respectively. At age 3 weeks the infant developed a right inguinal hernia. Three weeks later clitoroplasty, vaginoplasty, and bilateral inguinal herniorrhaphy were done. An ovotestis with an epididymis and a fallopian tube present on the right side

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were excised. The left gonad was an immature ovary. The patient will be raised as a female.

Patient 2

The patient was the product of a pregnancy complicated by premature rupture of membranes for 1 month, without evidence of chorioamnionitis, and delivered by emergency C-section for fetal distress and nuchal cord. At birth, she was noted to have a cleft palate and apparently low-set ears. She required O_2 by hood for shallow breathing and gavage feedings for a weak suck. Presently she has a prominent nasal root; large, but well-formed, posteriorly angulated ears; and poor growth. She is severely retarded. Chromosome study showed 46,X+ marker. Studies with a Y centromeric probe showed that the marker was a Y chromosome derivative and that DNA hybridizing to the short arm probe p75/79 was present [Stalvey et al., 1988]. External genitalia were normal female, and surgical exploration showed normal uterus and fallopian tubes with streak gonads (which were removed).

Patient 3

This Arabic patient presented at age 27 years because of primary amenorrhea. She had developed some breast tissue at puberty but no menstrual periods. Hormone injections produced some menstrual spotting lasting 4–7 days. She failed to conceive in a total of 4 years of relationships to two previously fertile men. Physical examination showed Tanner stage 1 breasts and a small uterus with a cervix present. Chromosome study showed 46,XY.

Patient 4

This patient presented at age 19 years because of primary amenorrhea. Some breast budding was noted at age 13 and 1 month's cycle of estrogen and progesterone resulted in light bleeding for a week. Physical examination demonstrated Tanner stage 3 breasts and a visible cervix. Ultrasound showed a very small uterus measuring 1 × 2 × 3 cm. A small left ovary, but no right ovary, was identified. Chromosomes were 46,XY. She underwent laporatomy and subsequent salpingo-oophorectomy. One ovary contained gonadoblastoma as a major component with early dysgerminoma also present.

MATERIALS AND METHODS

Fresh heparinized or EDTA-treated blood samples were obtained from the patients. DNA was extracted according to previously published methods [Stalvey et al., 1988]. Restriction digests were performed using 5–10 μg of DNA with 5 units of restriction enzyme per μg of DNA, according to each manufacturer's instructions. Agarose gel electrophoresis was performed using 0.8–1% agarose gels and Tris Borate EDTA (TBE) running buffer at 55V for 16–20 hours. After electrophoresis, gels were photographed and placed in 1.5 M NaCl, 0.5 M NaOH for 20 minutes prior to transfer to a nylon membrane, Gene Screen (Dupont) using the same solution. Southern transfers were allowed to proceed for 16 hours. Filters were neutralized in 1 M ammonium acetate; 0.02 M NaOH for 5 minutes, air dried, and baked at 80°C for 30 minutes. The DNA was UV cross-linked to the filter using a 330 nm light source for 1.5 minutes.

The following probes have been used in this study: 1) A 300 bp *Hind*III-*Eco*RI fragment, isolated from the Y-specific clone, p27a, which maps within 100 kb of the ZFY locus [Verga and Erickson, 1989]; 2) a 2.7 kb *Eco*RI fragment from the clone p47z (DSXY5), which maps on the centromeric side of ZFY on Yp and also detects homologous sequences on the long arm of the X chromosome [Geldwerth et al., 1985]; 3) a 1.3 kb *Hind*III fragment from the human ZFY clone (pDP1007) [Page et al., 1987]; 4) a 5.5 kb *Eco*RI insert from cosmid clone Y97 [Wolfe et al., 1985].

The DNA probes p27a, p47z, pDP1007, and Y97 were all labeled by the random priming method [Feinberg and Vogelstein, 1984] to a specific activity of 1×10^9 cpm/μg. Hybridizations were carried out in 0.5 M Na_2HPO_4, pH 7.2, 7% SDS, and 1 mM EDTA according to the method of Church and Gilbert [1984] for 16 hours. Post-hybridization washes were performed in 0.4 M Na_2HPO_4, 0.5% SDS, and 1 mM EDTA at room temperature for 15 minutes and then at 65°C for three 15 minute intervals. Filters were exposed to Kodak XAR5 film with intensifying screens for 1–7 days. Filters were stripped in 1 × 10 mM Tris HCl, pH 8.0, 1 mM EDTA, and 1% SDS at 85°C and then washed at 65°C for at least 30 minutes before rehybridization. DNA fragment sizes were determined using λ, *Hind*III size markers (BRL).

RESULTS

The DNA samples from the patients were studied with the Zinc finger Y probe (ZFY) and other Y short arm or centromeric probes. As summarized in Table I, all the patients gave male (i.e., Y) -specific patterns of hybridization. As seen in Figure 1, the 2.8 kb Y-specific

TABLE I. Presence of Y Sequences in Patient's DNA

Patient	pDP1007 zinc finger Y	27a [Pritchard, et al., 1987]	47z [Geldwerth, et al., 1985]	Y97 centromeric alphoid repeat
1	2.8 kb *Taq*I	2.2 kb *Taq*I Yp 1.95 kb *Taq*I Yq	4.3 kb *Taq*I	5.5 kb *Eco*RI
2	2.8 kb *Taq*I	2.2 kb *Taq*I Yp	4.3 kb *Taq*I	5.5 kb *Eco*RI
3	2.8 kb *Taq*I	2.2 kb *Taq*I Yp 1.95 kb *Taq*I Yq	4.3 kb *Taq*I	—
4	2.8 kb *Taq*I	2.2 kb *Taq*I Yp 1.95 kb *Taq*I Yq	4.3 kb *Taq*I	5.5 kb *Eco*RI

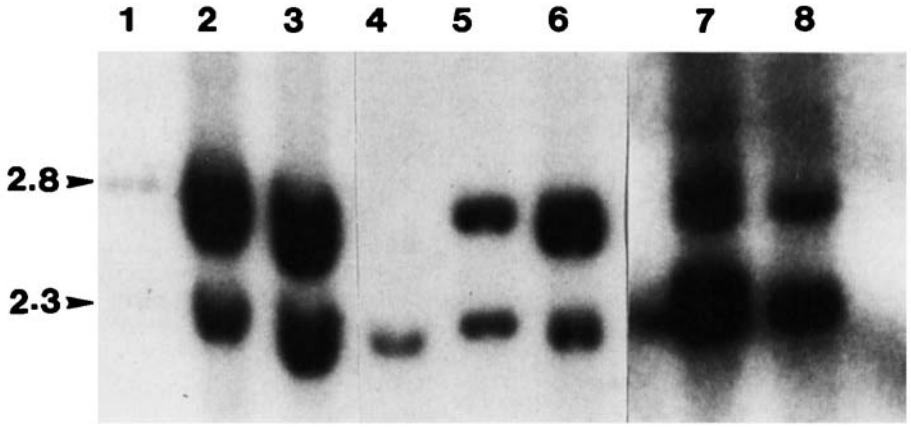

Fig. 1.(pDP1007). Autoradiograph of ZFY (pDP1007) hybridized to *Taq*I-digested human DNA showing patient 3 (**lane 1**) 1 (**lane 2**), an XX male (**lane 3**), normal female (**lane 4**), XX male (**lane 5**), normal male (**lane 6**), patient 2 (**lane 7**), and patient 4 (**lane 8**). The 2.8 kb fragment is Y specific, and the 2.3 kb fragment is X-specific.

band was found in all the patients and in a XX control male and a normal male, but not in the normal female. The 2.3 kb X-derived band is shared by all the samples (but is poorly visible in patient 4 because of a low DNA concentration). Similarly both the short arm (2.2 kb; Pritchard et al. [1987]; and long arm 1.95 kb; our unpublished data) specific bands of hybridization were found with p27a, which was isolated from within 100 kb of Zinc finger Y (ZFY) [Verga and Erickson, 1989] in patients 1, 3, and 4, but only the short arm fragment was present in patient 2. Two of the patients and various controls are illustrated in Figure 2. The more proximal 47z and the Y centromeric probe were not studied in all the patients but confirmed the normalcy of the Y chromosomes by these criteria in those patients in which they were used.

DISCUSSION

It is generally agreed that sex differentiation is complex and that many genes must be involved. However, genes mediating events of sexual development subsequent to the Y-initiated, prime event cannot be sorted out until the status of the Y gene for sex determination is known; i.e., is a defect in the Y-chromosomal gene present? For some years, studies on H-Y antigen have been used to assess the Y chromosomal gene's activity. The results have been controversial [Silver et al., 1982], and it is clear that the accurately measurable, transplantation H-Y does not map to the sex-determining region of the Y-chromosome [Simpson et al., 1987]. Recent progress in cloning portions of the Y chromosome have provided more definitive ways to assess the presence or absence of subportions of this chromosome [Ostrer et al., 1987; Stalvey et al., 1988]. The availability of a probe for the putative sex determining gene, Zinc finger Y, allows further refinement in assessing Y chromosomal DNA.

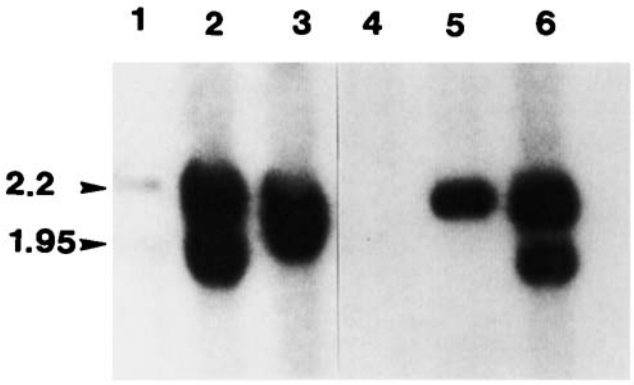

Fig. 2.(27a). Autoradiograph of Y-specific sequence, p27a, hybridized to *Taq*I-digested human DNA. The 2.2 kb and 1.95 kb fragments are present in patient 4 (**lane 1**), 1 (**lane 2**), and normal male (**lane 6**). The 2.2 kb, (but not the 1.95 kb fragment) is present in two XX males (**lanes 3, 5**). There is no hybridization to the normal female (**lane 4**).

Our first patient illustrates the importance of assessing the normalcy of the Y sex determining region before invoking other possible causes of her ambiguous genitalia. The patient's mother had a "caudal dysplasia sequence" but was able to reproduce after corrective surgery. Dominant inheritance of caudal dysplasia has been described previously [Pouzet, 1938; Robert et al., 1974; Say and Coldwell, 1975; Welch and Aterman, 1984], but our patient has anterior, rather than posterior, defects possibly related to mesodermal deficiency. Although a true hermaphrodite with a predominance of ovarian tissue, she had enough testicular tissue to masculinize significantly in utero and to show a rise in serum testosterone and dihydrotestosterone after hCG stimulation. Thus, we predicted that the cytogenetically normal Y chromosome would hybridize to all the Y probes tested, including pDP1007, and the prediction was confirmed. Sequencing this patient's Zinc finger Y gene will be required to prove that ZFY is normal; the normal restriction fragments suggest that there is no deletion or rearrangement of the gene. Since the chance occurrence of the mother's caudal dysplasia and the patient's hermaphroditism are unlikely, we think that it is possible that a single, dominantly inherited gene influenced the familial occurrence of these defects of lower trunk development.

The second patient provides another conundrum in which the knowledge of the status of the sex determining region was important. Her condition had led to two prior cytogenetic interpretations of the marker as an autosomal fragment. We previously reported that this marker was identifiably of Y-origin by in situ analysis with the Y centromeric, alphoid repeat [Stalvey et al., 1988]. In the interval, laporatomy confirmed the presence of completely normal female internal genitalia, and histopathology demonstrated streak gonads. Thus, the patient has no detectable evidence of male sex determination, yet our studies with pDP1007 show an apparently structurally normal Zinc finger Y gene. Again, with the proviso that only sequencing can confirm its normalcy, it seems more likely that some underlying developmental defect has prevented a response to the sex determining gene as well as causing significant dysmorphology and retardation. Her cleft palate, lack of cutaneous syndactyly and her good survival makes Smith-Lemli-Opitz syndrome, type II unlikely [Curry et al., 1987]. Genito-palato-cardiac syndrome [Greenberg et al., 1987] may be a variant of Smith-Lemli-Opitz, type II but our patient lacks the cardiac problems prominent in this possibly separate disorder.

Our last two patients are examples of 46,XY gonadal dysgenesis [Swyer, 1955; Simpson et al., 1971]. They are sexually immature, amenorrheic individuals who were discovered to have a normal male chromosome constitution. Previous studies have shown that only a small portion of XY females are missing Y sequences [Müller et al., 1986], but a probe for Zinc finger Y was not yet available. Our current results show that these two patients have a normal Y sex determining gene to the extent that it can be determined by Southern analysis with this probe. Thus, the familial aggregation that sometimes occurs with this disorder [Simpson et al., 1971] is better understood in terms of autosomal or X-linked [German et al., 1978] defects.

In conclusion, the zinc finger Y probe, pDP1007, provides a useful marker for studying Y DNA in patients with sexual ambiguity or gonadal dysgenesis. Our results with this probe are compatible with the hypothesis that ZFY is a primary sex determining gene.

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