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Genetics

Enzymatic amplification of a Y chromosome repeat in a single blastomere allows identification of the sex of preimplantation mouse embryos

(polymerase chain reaction/prenatal diagnosis)

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ABSTRACT **The polymerase chain reaction (PCR) technique has been adapted to identify the sex of preimplantation mouse embryos rapidly. PCR was used to amplify a specific repeated DNA sequence on the Y chromosome from a single isolated blastomere in under 12 hr. The remainder of the biopsied embryo was then transferred to a pseudopregnant female and carried to term. Using this technique, 72% of embryos can be classed as potentially either male or female. Transfers of such embryos have produced pregnancies with 8/8 fetuses (100%) being of the predicted sex. Variations of the technique have demonstrated certain limitations to the present procedure as well as indicated possible strategies for improvement of the assay. The PCR technique may have wide application in the genetic analysis of preimplantation embryos.**

Numerous attempts have been made to identify genetic traits of preimplantation embryos. One trait that has received much attention is sex. The sex of an organism may be of interest for medical or economic reasons, depending on the species. In humans, males suffer from sex-linked inherited disorders, such as hemophilia A, Lesch–Nyhan syndrome, and Duchenne muscular dystrophy. If, after *in vitro* fertilization, the sex of human embryos could be identified before transfer, those with sex-linked genetic diseases could be excluded from transfer, thus eliminating the need for more hazardous and traumatic pregnancy termination procedures performed after implantation. In cattle, superovulation and embryo transfer allows many calves to be obtained from a single mating of a valuable pair of animals. For purposes of herd improvement, an excess of female or male progeny may be desired.

Several approaches have been developed to identify the sex of preimplantation mammalian embryos. Biopsies of embryos have been cultured and karyotyped to identify the sex chromosomes (1). A male-specific antigen has been detected on embryos (2), and antibodies directed against this antigen have been used either for complement-mediated lysis (3) or immunofluorescent staining (4) of male embryos. The relative activity of X chromosome-linked and autosomal enzymes has also been used to differentiate between XX and XY embryos. This strategy exploits the fact that the second X chromosome of XX embryos is not inactivated until the blastocyst stage (5) and, thus, that XX cells have twice the activity of X chromosome-linked enzymes as do XY cells (6, 7). A technique using a Y chromosome-specific DNA probe in a slot blot (8) is sensitive at the level of two cells, but it takes 8 days to complete the analysis. All of these techniques have proved partially successful but have fallen short of the ideal sex determination protocol. Often, the procedures are sufficiently time consuming that embryo freezing, often an undesirable procedure, becomes necessary. In other cases, the assay is <100% accurate, or it requires that biopsied blastomeres remain intact so that X chromosome-linked gene products can be accurately measured.

We have developed a technique that can be applied to a single blastomere, which requires <12 hr, and does not depend on expression of genes in embryos. This method uses polymerase chain reaction (PCR) (9) to amplify a selected DNA sequence. Many copies of this sequence are found on the mouse Y chromosome. Embryos analyzed by this method have been transferred and fetuses of the expected sex have been obtained. With refinements, this technique may identify the sex of embryos from any species with accuracy approaching 100%.

MATERIALS AND METHODS

Mice. B6D2F$_1$/J mice were obtained from The Jackson Laboratory. CD-1 mice were obtained from Charles River Breeding Laboratories. Mice were housed on a 14:10 light/dark cycle. Prepuberal females were superovulated with 5 international units of pregnant mares' serum gonadotropin (Gestyl, Diosynth, Chicago) followed 48 hr later with 5 international units of human chorionic gonadotropin (hCG) (Pregnyl, Diosynth). After the hCG injection, the females were placed with males of their own strain overnight. Mating was confirmed by the presence of a vaginal plug the following morning. Randomly cycling mature females (CD-1) were mated to vasectomized males to induce pseudopregnancy.

Selection and Analysis of a Repeated Element from the Mouse Y Chromosome. A plasmid clone of a mouse Y chromosome repeated sequence pY353/B (10) was provided by C. Bishop. This plasmid contains a 1.5-kilobase (kb) *Eco*RI fragment of mouse Y-chromosomal DNA. This fragment was subcloned into the plasmid pGEM4 (Promega) to produce a plasmid designated pYREP19 and was used in Southern (11) hybridizations of *Eco*RI-digested XX and XY mouse DNA. Fifteen micrograms each of male and female mouse DNA was digested with *Eco*RI and separated by electrophoresis on a 1% agarose gel. Serial dilutions of a sample of *Eco*RI-digested pYREP19 were also present on the gel, representing 1–800 DNA copies per diploid genome. After transfer to nitrocellulose, the DNA was hybridized to pYREP19 labeled with [^{32}P]dCTP by random priming according to instructions supplied with a kit from Amersham. Hybridizations were carried out according to the method of Wahl *et al.* (12).

DNA Sequencing and Oligonucleotide Preparation. To prepare Y chromosome-specific oligonucleotide primers for

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Abbreviation: PCR, polymerase chain reaction.

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PCR, the 1.5-kb insert of pYREP19 was partially sequenced by the Sanger dideoxynucleotide chain-termination method (13) with the reagents and protocol from the GEM Seq K/RT kit (Promega). Sequencing reactions were resolved on denaturing 6% polyacrylamide gels. The insert was sequenced from both ends using the T7 and SP6 primers, resulting in ≈360 base pairs (bp) of sequence information. From the sequencing data, a 102-bp region of the insert was chosen for amplification. Two oligonucleotide primers corresponding to the first and last 23 bases of the 102-bp region were then synthesized on an Applied Biosystems synthesizer using cyanoethyl phosphoramidite chemistry. In most experiments, the oligonucleotides were further purified on purification cartridges obtained from Applied Biosystems (no. 400771).

Embryo Biopsy. Two days after mating, females were sacrificed by cervical dislocation. The oviducts were removed and embryos at the eight-cell stage were flushed into M16 medium (14) with a blunt 30-gauge needle. The embryos were transferred to fresh medium and the zonae pellucidae were removed by pipetting a stream of acidified Tyrode's solution (15) over them. After washing, the embryos were placed in modified Dulbecco's phosphate-buffered saline containing 106 mM NaCl/2.7 mM KCl/1.5 mM KH_2PO_4/8.1 mM Na_2HPO_4/5.6 mM glucose/25 mM sodium lactate/0.33 mM sodium pyruvate/3 mg of bovine serum albumin per ml/2 mM EDTA. This chelating medium was also lacking in Ca^{2+} and Mg^{2+}. Embryos were kept in chelating medium for 10 min at room temperature and then gently and repeatedly aspirated with a pipette with a diameter slightly smaller than that of the embryo, until a blastomere detached. A second pipette with a large diameter was used to transfer the blastomere to a 0.6-ml microcentrifuge tube (Robbins Scientific, Mountain View, CA) containing 20 μl of distilled water. The remainder of the embryo was transferred to a microdrop of M16 medium under oil. Embryos were then incubated overnight at 37°C in 5% CO_2/95% air. Embryos for biopsy were from $B6D2F_1$ females crossed to $B6D2F_1$ males. Carrier embryos were from CD-1 females mated to CD-1 males. Therefore, all experimental embryos were pigmented while carriers were albino. Carrier embryos were flushed and had their zonae removed before culture overnight in microdrops. Tubes with blastomeres were analyzed by PCR.

PCR. Tubes with blastomeres were placed in a Perkin–Elmer/Cetus thermal cycler and heated to 94°C for a minimum of 3 min. Samples were then cooled to room temperature and briefly centrifuged to collect condensate. Eighty microliters of 1.25× reaction mixture was then added to each tube. The reaction mixture was prepared according to directions from reagents in the Cetus Gene Amp kit and consisted of 50 mM KCl; 10 mM Tris·HCl (pH 8.3); 1.5 mM $MgCl_2$; 0.01% gelatin; 200 μM each dATP, dCTP, dGTP, and TTP; 100 pM each oligonucleotide primer (final concentration, 1 μM); 5 μCi of [α-^{32}P]dCTP (1 Ci = 37 GBq); 1 ng of bacteriophage λ DNA; and 2.5 units of *Thermus aquaticus* DNA polymerase (*Taq* polymerase). Samples were overlaid with 100 μl of mineral oil. Control tubes contained DNA from mouse lymphocytes prepared by the boiling method (16), equivalent to either 1 or 2.5 cells. The thermal cycler was programmed to heat the samples to 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min. After 30 cycles, the 72°C step was lengthened by 5 sec every cycle. At the end of 15 of these longer cycles (45 total), the samples were held at 72°C for 7 min and then cooled. Samples were then either analyzed immediately by acrylamide gel electrophoresis or frozen at −20°C overnight and analyzed the following day.

Gel Electrophoresis. Ten microliters of loading buffer containing glycerol, bromphenol blue, and xylene cyanol was added to each sample before loading on the gel. Electrophoresis was carried out in 8% polyacrylamide gels with Tris borate/EDTA buffer (17). Thirty-microliter samples were loaded onto the gel along with molecular weight markers consisting of *Hin*fI-digested pBR322, which was end-labeled using T4 DNA polymerase and [α-^{32}P]dCTP. After electrophoresis at 18 mA for 90–120 min, the gel was dried and autoradiographed for 2–4 hr. The sex of the embryos was determined by the intensity of the 102-bp band amplified by PCR. From the time of embryo biopsy to examination of the x-ray film, the procedure required 8–11 hr; when samples were frozen prior to electrophoresis, 18–22 hr elapsed before results were obtained.

Embryo Transfer and Sexing of Fetuses. Embryos were transferred at the early blastocyst stage, ≈30 hr after flushing from the oviducts. Embryos identified as males were transferred to one uterine horn of a pseudopregnant CD-1 female. Presumed female embryos were transferred to a different pseudopregnant female. In both cases, carrier embryos were transferred to the contralateral horn.

Fourteen to 16 days after transfer, the recipients were sacrificed and the fetuses were recovered for dissection. Experimental fetuses were identified by the pigmentation of the eye. Viewed with a dissecting microscope, males were identified by the presence of testes in the lower abdominal cavity, and females were recognized by the presence of ovaries and a uterus. Confirmation of the phenotype was obtained by removal and dissection of one gonad, with larger size and the presence of seminiferous cords providing unequivocal indications of maleness. For two of the fetuses, sex identification was performed by DNA extraction (17) followed by the same PCR assay as was used for sexing the blastomeres. For this assay, 1 ng of purified DNA was used.

RESULTS

Southern Blot Analysis of pY353/B. Fig. 1 presents results of Southern hybridization of *Eco*RI-digested XX and XY DNA probed with pYREP19, which contains the 1.5-kb *Eco*RI insert of pY353/B. Quantities of purified plasmid ranging in amount from 1 to 800 copies per cell genome were included as controls. As demonstrated, XY DNA contains >800 copies of the 1.5-kb fragment. A lower level of hybridization is seen in the size range of 3.6 and 6.0 kb. The 1.5-kb fragment is absent from XX DNA; however, a low level of homology is seen at the 3.6- and 6.0-kb size ranges. We therefore conclude that pY353/B is a Y chromosome-specific

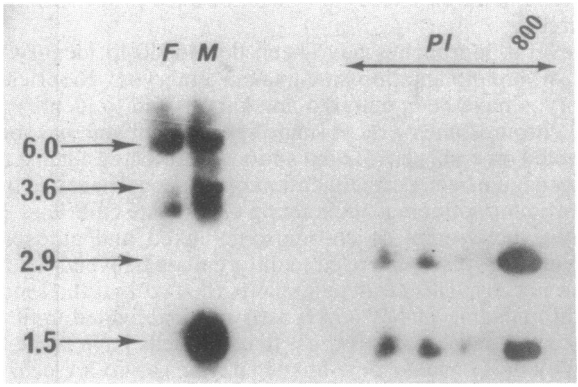

FIG. 1. Southern blot hybridization of *Eco*RI-digested female (F) and male (M) DNA (15 μg each), loaded with increasing quantities of a recombinant plasmid containing pY353/B cloned into pGEM4. Lane 800 represents 800 copies of the plasmid per cell. The 1.5-kb fragment of the plasmid is pY353/B, and the 2.9-kb fragment is pGEM4. As shown, the 1.5-kb element is present >800 times on the DBA Y chromosome. Lower levels of hybridization are seen in the 3.6-kb and 6.0-kb regions of the mouse genome, with lower homology in female than in male DNA.

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5'-------------->3'

GAA TTC ATA TAT ATG ACA GAG GCA ACA GCA TTG TGT CTG GTT TAA ACG GGC
CTT AAG TAT ATA TAC TGT CTC CGT TGT CGT AAC ACA GAC CAA ATT TGC CCG

AAG CAT CTT CAG TCC CAG GCA ATG AGC AAG TAT GAT ATT TGA AGG GAA TGG
TTC GTA GAA GTC AGG GTC CGT TAC TCG TTC ATA CTA TAA ACT TCC CTT ACC
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FIG. 2. Sequence of pY353/B used for oligonucleotide synthesis and PCR. The two oligonucleotides are highlighted with a line above and below the respective sequences. The 5' end of the sequence is arbitrarily designated, and the 5' → 3' direction is indicated.

repeated element, but that similar DNA sequences are present either on the X chromosome or one or more autosomes.

Synthetic Oligonucleotides Derived from the pY353/B Sequence Can Distinguish XX and XY DNA After PCR. Because it was not possible to search the entire pY353/B insert for sequences not represented in the XX genome, sequencing data were used to choose an arbitrary 102-bp region of pY353/B for PCR. Oligonucleotides corresponding to opposite strands of the first and last 23 bases of this region were synthesized and tested. Fig. 2 presents the sequence used, with the highlighted regions representing the oligonucleotides.

To determine whether enzymatic amplification of this 102-base region could distinguish XY from XX DNA at the level of a single cell in our rapid assay, preliminary experiments were carried out on DNA of mouse lymphocytes. Blood was obtained from male and female mice and was boiled to liberate the DNA (16). Serial dilutions were made and amounts of DNA equivalent to 2500, 250, 25, and 2.5 cells were amplified for 45 cycles by PCR. The results are shown in Fig. 3. Although there is some amplification of the 102-bp segment when female DNA is used as a template, the male DNA shows a much greater amplification. This result indicated that the region selected for PCR is probably present in XX DNA, but that the degree of repetitiveness in XY DNA is far higher, and that the relative intensity of the 102-bp amplified region was sufficient to distinguish XY from XX cells.

Rapid Sexing of Preimplantation Embryos by PCR. The above results led us to attempt sexing of single blastomeres by embryo biopsy and PCR. Fig. 4 shows the results of such an experiment. As illustrated, the blastomeres fell into two major categories with respect to the intensity of the 102-bp amplified band. Those with high intensity were scored as males and the others were scored as females. A third smaller group of blastomeres exhibited intermediate levels of amplification (e.g., samples labeled "?" in Fig. 4). Their sexes were considered undetermined.

To learn whether these differences in PCR were due to the presence or absence of a Y chromosome in the tested blastomeres, embryos were transferred into the uterus and examined as late fetuses or, in two instances, evaluated at day 9 of gestation by the PCR for the presence of Y chromosomal DNA. Table 1 shows the results of this evaluation. Of the embryos that developed, 100% (8/8) were of the sex predicted by PCR. In the two examples in which males were confirmed by PCR (Exp. 8 in Table 1), the high quantity of starting material used in the reaction allowed visualization of Y chromosome-specific bands by ethidium bromide staining. In both fetuses, a clear band corresponding to the 102-bp male-specific fragment was seen, although this band was more intense in one sample than in the other. Both the female control and a control consisting of a PCR containing all components except template DNA were negative. The differences in intensity of the 102-bp amplified band in fetal DNAs may be attributable to the fact the fetuses were in the process of resorption at the time of retrieval. To determine whether embryos with intermediate amplification were indeed a mixture of the two sexes, members of this group were also transferred. Fetuses that developed from this one successful experiment were of both sexes, results which demonstrate uncertainty in the sexing assay for 28% of embryos (Exp. 5 in Table 1).

The Sexing Assay Is Sensitive to Oligonucleotide Selection and Genetic Background of the Embryo. As part of our efforts to characterize and improve the PCR protocol, oligonucleotides were varied. In one series of experiments, the oligonucleotide at the 5' end of the sequenced fragment was moved 1 base in the 3' direction by removing the 5' G and adding an A at the 3' end (see Fig. 2). When PCR was conducted, a markedly different result was obtained. Although the ex-

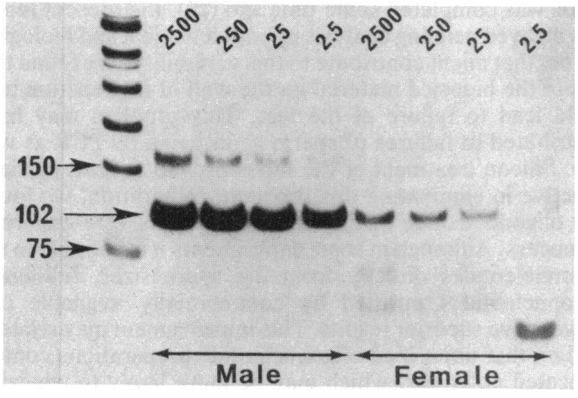

FIG. 3. Purified DNA from XY (male) and XX (female) DNA included in amounts equivalent to 2500, 250, 25, and 2.5 cells, respectively, in the PCR. The reactions were subjected to 45 rounds of amplification. DNA size markers (bp) (left lane) consist of *Hin*fI-digested pBR322 end-labeled with T4 polymerase in the presence of [α-^{32}P]dCTP.

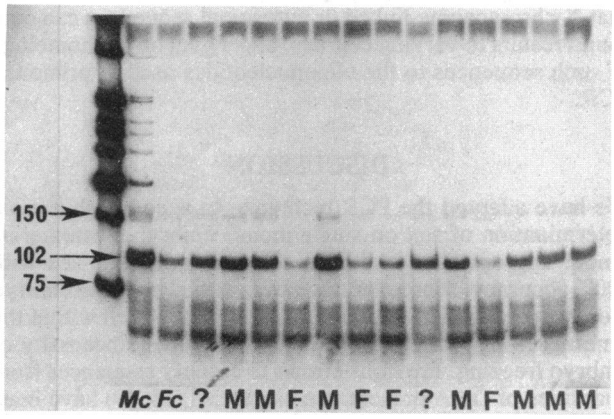

FIG. 4. Results of PCR on 13 single blastomeres, shown with purified XY (lane Mc) and XX (lane Fc) DNAs included in amounts equivalent to 2.5 cells. Large arrow shows the 102-bp band amplified in the reaction. The intensity of this band was used to score the embryos, with strong amplification considered to be indicative of an XY blastomere. Left lane contains DNA size markers (bp). A small amount of marker DNA contaminates the Mc lane.

Table 1. Data from PCR of biopsied embryos

Exp.	Predicted sex	Transferred*	Implanted†	Sex‡
1	4 M	4	0	—
	4 F	0	—	—
	5 ??	0	—	—
2	6 M	6	0	—
	5 F	5	0	—
	2 ??	0	—	—
3	7 M	4	2	2 M
	4 F	0	—	—
	2 ??	0	—	—
4	7 M	7	0	—
	4 F	3	0	—
	4 ??	0	—	—
5	4 M	4	0	—
	4 F	4	0	—
	7 ??	7	2	1 M, 1 F
6	6 M	6	0	—
	6 F	6	0	—
	3 ??	0	—	—
7	7 M	7	3	3 M
	6 F	6	1	1 F
	6 ??	0	—	—
8	7 M	7	2	2 M§
	5 F	0	—	—
	4 ??	0	—	—

M, male; F, female; ??, unknown after PCR. Note that in all eight cases in which the sex could be diagnosed and in which embryos developed, the sex was predicted correctly.
*Number of experimental embryos transferred to a single recipient.
†Number of fetuses with pigmented eyes found. Carriers have unpigmented eyes.
‡Sex of fetuses as determined by gonadal morphology.
§Sex determined by PCR analysis of implant DNA.

pected 101-bp intervening region was amplified, it was far lower in intensity than a new, 150-bp band (Fig. 5). Moreover, the intensity of the larger band did not vary substantially between embryos. We conclude from this experiment that sequences in female DNA that are related to pY353/B can be more readily primed for amplification by the new oligonucleotides. We have also observed spurious amplification of higher molecular weight when embryos derived from CD-1 females crossed with B6D2F$_1$ males are used (data not shown). Although the Y chromosome in these experiments was from the same inbred strain as in previous tests (DBA), larger bands were prominent. These data indicate that X chromosome-linked or autosomal sequences can confound results to varying degrees, depending on the homology of such sequences to the oligonucleotides used to prime the PCR.

DISCUSSION

We have adapted the PCR technique to accomplish a rapid determination of sex on single mouse embryo blastomeres. Under appropriate conditions, this assay predicts sex with 100% accuracy. Moreover, the time interval between embryo biopsy and completion of the test is <12 hr—well within the time necessary for diagnosing sex without the necessity of embryo freezing. Previous efforts to amplify sequences from quantities of DNA present in a single cell (18, 19) have been complicated by the annealing of oligonucleotides to spurious sites within the target DNA, with resultant amplification of material other than that of the gene of interest (18). This problem has been solved by conducting filter hybridizations with radiolabeled probes specific for the target gene (18, 19). Even with filter hybridization, it is not always possible to discriminate changes in unique sequences from single whole

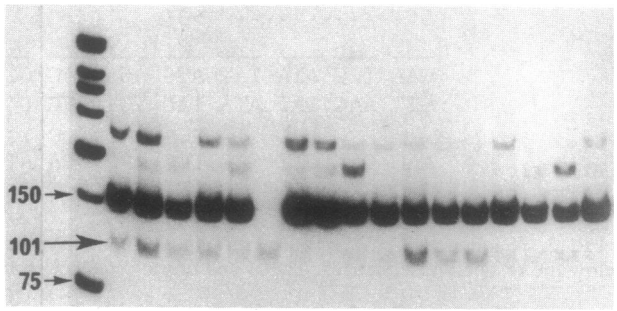

FIG. 5. Results of PCR on single blastomeres after changing the 5′ oligonucleotide by moving it one base 3′. The expected band of 101 bp (large arrow) is very faint relative to a new major band at ≈150 bp. The intensity of the latter band did not correlate with embryo sex. Left lane, DNA size markers (bp).

cells (19). In the present study, we sought to avoid a hybridization procedure because of the desire to complete the assay in under 24 hr. This goal was achieved by adding [^{32}P]dCTP directly to the PCR mixture. While some spurious priming was seen, it was still possible to identify embryo sex in 72% of cases. Success is probably attributable to the fact that the target for PCR in this case was repeated >800 times per diploid genome, while previous studies with single cells or their DNA equivalents evaluated single-copy genes.

It is highly unlikely that the sex of embryos was successfully predicted by chance. If it is assumed that 50% of embryos are males, the probability of 8 consecutive correct guesses is $(1/2)^8$, or 0.0039. Thus, the chance is low that random assignment of sex accounted for observed success. In these experiments, 28% of the reactions gave ambiguous results. We assume that less-stringent criteria for assigning sex would reduce the number of embryos in the undiagnosed group but, correspondingly, would be associated with an increased error rate. However, it is important that under many circumstances it is not required that all embryos be correctly diagnosed; rather, it is imperative that the diagnosis be certain. Thus, even in its present state of sophistication, this assay has a number of potential applications.

Variability in our assay indicates that further improvements are necessary before PCR as used in these experiments can be routinely applied to genetic diagnosis of sex. Both oligonucleotide selection (Fig. 5) and genetic background can influence results. In addition, some variability between experiments was observed, such that in some instances, a greater percentage of reactions gave ambiguous results (e.g., Exp. 5 in Table 1). Although successful sexing of 6/6 embryos was completed some time ago (20), the present report was delayed pending analysis of both technical and biological factors that might contribute to this variability. We found that loss of the biopsied material on the wall of the reaction tube could lead to failure of the test. This problem may have contributed to failures of sperm genotyping by PCR as well (19). Silicon treatment of the tubes proved at least partially effective in eliminating this problem. In addition, we found that oligonucleotide quality and preparation were important to success. Although in some experiments it is possible to use oligonucleotides directly from the synthesizer, full-length oligonucleotides purified by commercially available cartridges gave superior results. This improvement may relate to the fact that unpurified oligonucleotide preparations contain truncated polymers, which may be more likely to anneal at sites in the genome related to the desired target but not located on the Y chromosome. Obviously, oligonucleotides spanning regions of DNA that are completely Y chromosome specific would also be important. Here it is important to recognize, however, that sequences that appear to be completely Y chromosome specific by Southern blotting may not

be so after PCR, which amplifies short regions of DNA. The probe we used in this study was originally thought to be entirely Y chromosome specific (10). Another important factor is prevention of contamination. After performing the PCR several times in the laboratory, contamination by the amplified material in subsequent tests becomes more likely. Therefore, it is essential that the highest standards of quality control be maintained and that negative controls consisting of female DNA and the reaction mixture with no added DNA are included in every reaction. At the time we had successfully sexed 6/6 mouse embryos (20), Handyside *et al.* (21) reported results of a strategically identical experiment with human embryos available from an *in vitro* fertilization program. In their experiments, sex genotype was confirmed by cytological testing for a Y chromosome, as embryo transfers could not be performed. They also noted the danger of contamination, and, like ourselves, were apparently unable to obtain results in every case: although 38 embryos were initially evaluated, results were obtained for 14 (21). A variety of technical parameters are under investigation for improving assay reliability and consistency. These include varying oligonucleotide length, the temperature of annealing, and the time allotted to the polymerization reaction. Inclusion of carrier DNA can also improve results. After several experiments, we determined that the present assay is significantly improved by addition of 1 ng of λ DNA, while addition of human or bovine carrier DNA was not helpful. Thus, a variety of conditions can be modified to increase the specificity of the reaction.

The data reported here indicate that in principle it is possible to identify the sex of all mammalian embryos by PCR. This capability would impact significantly on the animal sciences, and perhaps on medicine as well. Furthermore, the data imply that under appropriate conditions it may be possible to evaluate unique sequences in cleaving embryos. The present test does not depend for its success on expression of genes; it requires only that a sequence be present in the cell. While we are clearly far from the goal of evaluating allelic differences at single loci with this technique, the data demonstrate that such evaluation may be possible. As optimal conditions for PCR have become better defined, steady progress toward direct evaluation of single genes from individual cells has been achieved (19). While special conditions may be required for analysis of genes in cells as large as blastomeres, results with cultured cells (22) indicate that such tests are feasible. Attainment of this goal would constitute a further significant advance in prenatal diagnosis.

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