TABLE 1 Lod scores for linkage between D5S39 and CPSMA

Family	Number affected	Geographic origin	Recombination fraction (θ) 0	0.05	0.1	0.2
Type II						
2	2	Algeria	0.30	0.23	0.18	0.09
5	2	France	0.24	0.21	0.18	0.11
6	2	France	0.72	0.61	0.50	0.30
7	2	France	0.30	0.23	0.18	0.09
8	2	France	0.24	0.21	0.18	0.11
10	2*	France	0.30	0.25	0.21	0.13
11	2	France	0.30	0.25	0.21	0.13
13	2	France	0.42	0.32	0.22	0.08
14	2	Spain	0.60	0.51	0.42	0.26
18	2	France	0.30	0.25	0.21	0.13
24	2	France	0.30	0.21	0.14	0.05
Subtotal			4.06	3.35	2.69	1.53
Type III						
16	2*	Morocco	0.42	0.37	0.31	0.20
17	2	Belgium	0.30	0.25	0.21	0.13
19	2	Germany	0.30	0.25	0.21	0.13
21	2	France	0.30	0.25	0.21	0.13
22	2	Lebanon	0.48	0.41	0.34	0.22
23	2*	Tunisia	0.72	0.61	0.50	0.30
Subtotal			2.54	2.18	1.82	1.13
Total			6.60	5.53	4.51	2.66

The asterisk indicates the presence in the pedigree of inbred affecteds. Seven non-informative families are not shown (5 type II, 2 type III).

Assuming that these diseases result from a single-gene defect, we generated an exclusion map for >60% of the genome. Greater than 80% of chromosomes 3, 9, 10, 18 and 19, 40% of chromosomes 1, 2, 11, 15, 16, 17 and 21, and ~25% of chromosomes 5, 8, 12 and 14 were excluded (data available on request).

Linkage was demonstrated eventually between the mutation causing the disease and the anonymous probe D5S39 (plasmid p 105-153 Ra) located on the long arm of chromosome 5 (5q12-q14). Using the enzyme *Msp*I, this probe detects a restriction fragment length polymorphism (RFLP) of 5 kilobases (kb) and 8 kb, having frequencies of 60% and 40% respectively[6]. We assessed the potential genetic linkage of D5S39 to CP-SMA for all families using the computer program MLINK (version 4.9) of the LINKAGE package[7]. The results of this analysis are presented as lod scores in Table 1. The maximal lod score between CP-SMA and the locus D5S39 for combined sexes is +6.6 at a recombination fraction $\theta = 0.00$ (0-0.05 1-lod-unit confidence interval). No recombinants were observed between the CP-SMA locus and this probe. Subdivision of the families into types II and III, as previously described, resulted in lod scores of 4.06 and 2.54 respectively at $\theta = 0.00$.

These linkage data, derived from geographically isolated families and meeting strict diagnostic criteria, clearly establish that the defect causing types II and III SMA is located on the long arm of chromosome 5. In addition, our data show no evidence of heterogeneity between type II and type III as no recombinants were observed with D5S39 locus, suggesting that the two types are allelic disorders. Augmenting the number of informative meioses should establish if recombinations exist with this marker in each subgroup. At this point the use of flanking markers and multipoint analysis will become valuable.

It is possible and important to investigate the D5S39 locus in families with the type I form of the disease. Here, pedigrees are often very small because of the severe course of the disease, making primary linkage analysis difficult. Furthermore, continued insight into childhood SMA may now be gained from the Wobbler mutant mouse. This animal model displays a degenerative hereditary lower motor neuron disease similar to SMA[8] and has previously been studied at the molecular level[9]. Given the existence of conserved homologous regions within mammalian species, linkage of a marker in the Wobbler mouse chromosomal region analogous to human chromosome 5q12-q14 would prove extremely valuable.

Finally, for affected families, prenatal diagnosis will be shortly forthcoming, and the goal of gene characterization is now feasible. □

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Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification

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OVER 200 recessive X chromosome-linked diseases, typically affecting only hemizygous males, have been identified. In many of these, prenatal diagnosis is possible by chorion villus sampling (CVS) or amniocentesis, followed by cytogenetic, biochemical or molecular analysis of the cells recovered from the conceptus. In others, the only alternative is to determine the sex of the fetus. If the fetus is affected by the defect or is male, abortion can be offered. Diagnosis of genetic defects in preimplantation embryos would allow those unaffected to be identified and transferred to the uterus[1]. Here we report the first established pregnancies using this procedure, in two couples known to be at risk of transmitting adrenoleukodystrophy and X-linked mental retardation. Two female embryos were transferred after *in vitro* fertilization (IVF), biopsy of a single cell at the six- to eight-cell stage, and sexing by DNA amplification of a Y chromosome-specific repeat sequence. Both women are confirmed as carrying normal female twins.

Five couples at risk of transmitting recessive X-linked diseases, including X-linked mental retardation, adrenoleukodystrophy, Lesch-Nyhan syndrome and Duchenne muscular dystrophy (DMD) were counselled about the possibility of having preimplantation embryos sexed after IVF and those identified as females replaced in the uterus. All of the women had previous terminations of affected fetuses but most had had difficulties in conceiving; each couple expressed a preference for this approach even though, in most cases, a specific diagnosis capable of distinguishing normal males and carrier females would be possible later in pregnancy. Over 6 months, 10 treatment cycles using superovulation after pituitary suppression[2] were performed (Table 1). Three couples had two treatments, and the other two couples, one and three treatments, respectively. The couples abstained from sexual intercourse during treatment cycles.

The number of oocytes collected and normally fertilized, 11.2 and 6.3 per treatment cycle, were consistent and similar to those we have previously reported[2]. A high proportion of normally

TABLE 1 Number and sex of normally fertilized human embryos biopsied at the eight-cell stage

Patient/treatment	No. of oocytes	No. fertilized*	No. biopsied	Male	Female	Not sexed	No. transferred
A1	8	2	2	1	1	—	1
A2	9	6	6	3	3	—	2
B1	8	7	7	2	3	2	2
B2	8	4	3	1	2	—	1
B3†, ‡	11	5	5	3	2	—	2
C1	12	8	5	2	3	—	2
C2	21	11	6	3	3		1
D1	14	10	7	4	2	1	2
E1	11	3	3	1	1	1	1
E2†	10	7	6	3	3	—	2
Total	112	63	50	23	23	4	17
(Range)	(8–21)	(2–11)	(2–7)	(1–4)	(1–3)		
Mean	11.2	6.3	5.0	2.3	2.3	0.4	1.7

* Normally fertilized with two pronuclei visible 16–18 h post insemination.
† Two eight-cell stage cells biopsied.
‡ Samples also reamplified with nested primers.

fertilized embryos (79%) developed to the six- to ten-cell stages on day 3 *in vitro* and were biopsied as early as possible. This allowed the biopsied embryos to be transferred later on the same day, after identification of the sex by DNA amplification (which took 6–8 h). For biopsy, a hole was drilled through the zona pellucida with acid Tyrodes (pH 2.4) applied locally from a fine micropipette. A larger micropipette was then introduced through this hole, and eight-cell stage cells aspirated for DNA analysis. In two treatment cycles, two cells were biopsied from each embryo for duplicate amplification (Table 1, B3, E2). Apart from the reduction in cellular mass, the development of human embryos to the blastocyst stage *in vitro* is unaffected by biopsy and removal of one or two cells at the eight-cell stage[3]. After uterine transfer, human cleavage stage embryos with reduced numbers of cells, for example after freezing and thawing[4], can implant and develop normally.

Cell biopsies were examined for nuclei using interference contrast microscopy or vital labelling of DNA with a polynucleotide-specific fluorochrome and fluorescence microscopy. Labelling with the fluorochrome and brief exposure to ultraviolet light does not interfere with DNA amplification (E.H.K., K.H. & A.H.H., manuscript in preparation). In one case, this revealed that a cell biopsy was an anucleate cytoplasmic fragment and the corresponding embryo, therefore, could not be analysed (Table 1, E1). Despite lysis of some cell biopsies after removal, the nucleus remained intact and DNA amplification was unaffected.

Amplification of a short fragment of a Y-specific repeat sequence[5] was consistently detected with dilutions of male DNA down to the equivalent amount in a single cell (2 pg), with no detectable background contamination (Fig. 1, male DNA). Amplification from single male cell biopsies was easily detectable and in some cases exceeded the levels achieved with a similar amount of male DNA (Fig. 1, biopsy 2). With other cells, no amplification was detectable and these were classified as female. Weak hybridization between the ends of the Y-specific primers results in amplification of a prominent 'primer-dimer' fragment (Fig. 1, bottom arrow) and provided a crude control for amplification failure. Even when background contamination increased, quantitative differences were sufficient for identification of the sex of the biopsied embryos. In a few cases, a regular ladder of amplified fragments, possibly arising from multimerization of the primers, prevented analysis (Table 1, B1, D1). Duplicate amplification from two cell biopsies with or without reamplification using nested primers (Table 1, B3, E2) did not significantly add to the discrimination of male and female embryos.

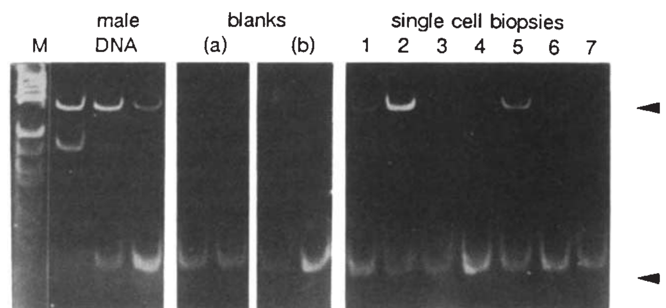

FIG. 1 Amplification of Y chromosome-specific DNA. Polymerase chain reaction (PCR) products using oligonucleotide primers for a 149 base pair (bp) fragment[5,6] of a 3.4 kilobase (kb) sequence (DYZ1)[8] repeated 500 to 8,000 times on the Y chromosome were analysed by polyacrylamide gel electrophoresis and stained with ethidium bromide. DNA size markers (M); serial dilutions of male DNA: 20 ng, 200 pg and 2 pg; wash drop buffer (a) and water (b) blanks; single cell biopsies from seven cleavage stage embryos (Table 1, D1). All of the samples were amplified and run together. Y-specific fragment (top arrow) (a smaller nonspecific fragment was amplified with 20 ng male DNA); 'primer-dimer' (bottom arrow). Among the single cell biopsies, the amplified fragment was clearly detectable with four cells (1, 2, 5, 6), in a fifth, several fragments of smaller sizes were visible which prevented analysis (4) and in the remaining two cells (3, 7) no amplification was detected.

METHODS. Sample preparation and PCR were as described previously[6] with some modifications. Single cell biopsies were washed twice in PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin) transferred to 10 μl water in 0.5 ml Eppendorf tubes, using a finely drawn pipette under a dissecting microscope to observe the cell entering the tube, and frozen to −20 °C. Samples were thawed, overlaid with 100 μl mineral oil and heat-denatured at 95 °C for 35 min. PCR mix (90 μl) was added to each sample (1 μg each oligonucleotide primer, 2.5 U Taq polymerase and 200 μm each dNTP in PCR buffer), the DNA denatured for 4 min at 94 °C, followed by 40 cycles at 62–65 °C (90 s) and 94 °C (30 s), and a final extension at 72 °C (15 min) using an automated hot block (Perkin-Elmer Cetus). For reamplification, 2 μl original amplification mix was removed after 15 cycles and added to 100 μl fresh PCR mix containing nested primers (5'-ATTACACTACATTCCCTTCCA-3'; 5'-AGTGAAATTGTATGCAGTAGA-3') and amplified for an additional 40 cycles. Amplification product (10 μl) was run on a 12% polyacrylamide minigel, stained with ethidium bromide and examined on an ultraviolet transilluminator. White cells separated from whole blood samples from both partners were tested in each case for the presence and absence of the repeat normally located on the long arm of the Y chromosome[8]. In rare cases, this region is deleted in males[9] and also it can be translocated to autosomes and carried by women[10].

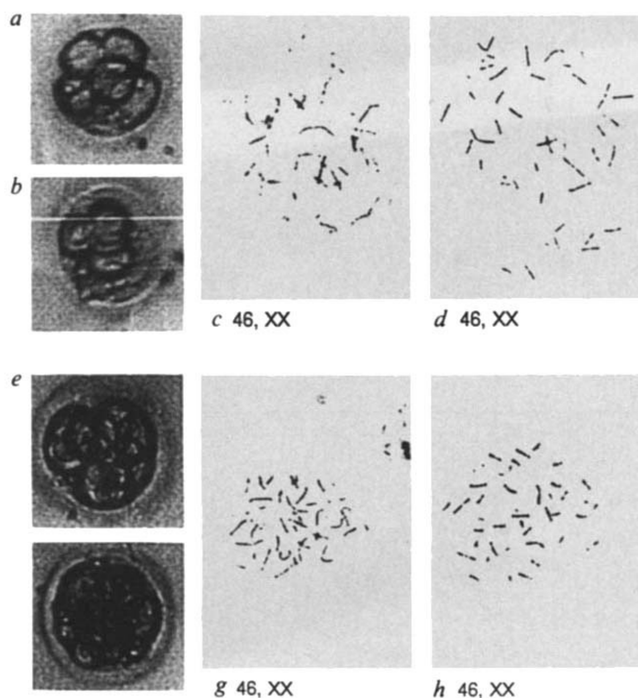

FIG. 2 The two pairs of biopsied embryos identified as female and transferred to the two women pregnant with twins. Patient/treatment cycle as follows. D1: *a*, seven-cell embryo (one cell removed and another damaged); *b*, seven-cell embryo (one cell removed and two others damaged). A2: *e*, six-cell embryo (one cell removed); *f*, eight-cell embryo (one cell removed). (Photographs of video image.) Metaphase spreads of banded chromosomes from cultured chorion villus cells recovered from each conceptus at ~10 weeks (D1: *c*, *d*; A2: *g*, *h*).

With the consent of the patients, the sex of some biopsied embryos (not selected for transfer) was later checked by amplifying the DNA of the embryo itself. With ten embryos (six male and four female), the predicted sex was confirmed. We have previously demonstrated the accuracy of sexing of normally fertilized embryos independently by *in situ* hybridization and fluorescent labelling of metaphase chromosomes[6].

The sex ratio among the 46 biopsied embryos, which were classified, was exactly 50:50 but there was variation between batches of embryos. Although the probability of an embryo being female is only 0.5, in most treatment cycles, there were at least two female biopsied embryos for transfer. We consider this to be the maximum safe to transfer. CVS samples can be obtained from each twin with only a small risk of miscarriage whereas screening greater numbers of fetuses would be difficult.

Chorionic gonadoptrophin (hCG) levels were assayed from 12 days after transfer; raised levels indicated implantation and early development in five cases. In two of these cases (Table 1, A1, B1), hCG levels were not maintained, indicating a 'biochemical' pregnancy. In a third case (Table 1, B3), serum hCG has continued to rise but clinical pregnancy is not yet confirmed. In each of the two remaining cases (Table 1, A2, D1), ultrasonography confirmed the presence of fetal sacs at 3 weeks and two fetal hearts at 4 weeks. These results suggest that pregnancy rates after transfer of biopsied six- to eight-cell embryos might equal those for intact embryos transferred to infertile patients. Within a 6 month period, pregnancies have been established in two (and possibly three) of the five women treated and after only one or two treatment cycles in each case.

CVS was performed in both sets of twins at 10 weeks and cytogenetic analysis of banded metaphase chromosomes confirmed that each of the fetuses has a normal female karyotype (Fig. 2). Both pregnancies are being carefully monitored by ultrasonography and all fetuses are normal on detailed examination at 22 and 20 weeks. Both sets of twins appear on ultrasound to be dizygotic presumably arising from the implantation and development of both the transferred biopsied embryos. This will need to be confirmed at birth by DNA fingerprinting, but if both sets are dizygotic, the successful development of four biopsied embryos out of 17 transferred (24%) indicates that preimplantation diagnosis will be a viable option for many families carrying genetic defects. Unique sequences closely linked to the cystic fibrosis gene and an exon of the dystrophin gene frequently deleted in DMD have been amplified from single embryonic cells[7], and the prospects for these families are promising. □

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Arterial dilations in response to calcitonin gene-related peptide involve activation of K^+ channels

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CALCITONIN gene-related peptide (CGRP) is a 37-amino-acid peptide produced by alternative processing of messenger RNA from the calcitonin gene[1,2]. CGRP is one of the most potent vasodilators known[3]. It occurs in and is released from perivascular nerves[1,4] and has been detected in the blood stream[5–7], suggesting that it is important in the control of blood flow[8,9]. The mechanism by which it dilates arteries is not known. Here, we report that arterial dilations in response to CGRP are partially reversed by blockers of the ATP-sensitive potassium channel (K_{ATP}), glibenclamide[10–12] and barium[10,13]. We also show that CGRP hyperpolarizes arterial smooth muscle and that blockers of K_{ATP} channels reverse this hyperpolarization. Finally, we show that CGRP opens single K^+ channels in patches on single smooth muscle cells from the same arteries. We propose that activation of K_{ATP} channels underlies a substantial part of the relaxation produced by CGRP.

Rabbit mesenteric arteries contracted by noradrenaline (10 μM) were relaxed by CGRP (Fig. 1), with 1.2 nM causing half relaxation. We tested the effects of potassium-channel blockers on CGRP-induced dilations. Glibenclamide (1 μM), a sulphonylurea that inhibits K_{ATP} channels in mesenteric arteries[10], cardiac muscle, and pancreatic β-cells, and which is specific for this channel type[11,14], reversed part of the dilation to CGRP (Fig. 1*a*). Glibenclamide (1 μM), which had no effect