Am. J. Hum. Genet. 62:533–541, 1998

A Human Homologue of the *Drosophila melanogaster diaphanous* Gene Is Disrupted in a Patient with Premature Ovarian Failure: Evidence for Conserved Function in Oogenesis and Implications for Human Sterility

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Summary

Premature ovarian failure (POF) is a defect of ovarian development and is characterized by primary or secondary amenorrhea, with elevated levels of serum gonadotropins, or by early menopause. The disorder has been attributed to various causes, including rearrangements of a large "critical region" in the long arm of the X chromosome. Here we report identification, in a family with POF, of a gene that is disrupted by a breakpoint. The gene is the human homologue of the *Drosophila melanogaster diaphanous* gene; mutated alleles of this gene affect spermatogenesis or oogenesis and lead to sterility. The protein (DIA) encoded by the human gene (*DIA*) is the first human member of the growing FH1/FH2 protein family. Members of this protein family affect cytokinesis and other actin-mediated morphogenetic processes that are required in early steps of development. We propose that the human *DIA* gene is one of the genes responsible for POF and that it affects the cell divisions that lead to ovarian follicle formation.

Introduction

Disorders of ovulation are very common in humans, and they account for ~15% of all infertility problems. The terms "premature ovarian failure" (POF [MIM 311360]), "hypergonadotropic ovarian failure," and "hypergonadotropic ovarian dysgenesis" have been used to indicate a group of disorders in which amenorrhea is associated with elevated levels of serum gonadotropins (Coulam 1982); that occur long before the age of 40 years. The terms are descriptive and do not indicate causal mechanisms, most of which are unknown. The relative frequency of cases that are associated with abnormalities of the sex chromosomes has suggested a genetic component (Therman et al. 1990). Complete absence of one of the two X chromosomes (Turner syndrome) and a variety of X-chromosome rearrangements have been observed in patients affected with POF. Familial cases with a normal karyotype have also been described. In Finnish families, a mutation in the FSH-receptor gene was described; until now, this is the only gene that has been shown to be responsible for POF (Aittomaki et al. 1995).

Cytogenetic studies of X-chromosome aberrations have suggested that it is mainly the long arm of the X chromosome that is involved in defects of ovulation. These studies have defined a large region, from Xq13 to Xq26, as the "critical region" for normal ovarian function (Therman et al. 1990). We have reported fine mapping, to a 15-Mb YAC contig, of balanced X-autosome translocations that are associated with POF (Sala et al. 1997). Our data and the few comparable data from the literature (Powell et al. 1994; van der Maarel et al. 1995) have confirmed that the great majority of the breakpoints are spread along a large chromosomal segment that corresponds to the whole Xq21 region and that flanks regions in Xq13 and Xq22, from DXS233 to DXS1171. The few remaining breakpoints have been mapped to distal Xq (authors' unpublished data). On the basis of the extension of the region, we have tentatively excluded the possibility that a single gene is involved, and we have suggested that several genes for ovary development and/or oogenesis may be present

Received October 9, 1997; accepted for publication January 13, 1998; electronically published February 23, 1998.

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0002-9297/98/6203-0006$02.00

along the critical region and that they may be interrupted by the balanced translocations.

Evidence for the presence of the first such gene came from sequence comparison between human expressed sequence tags (ESTs) and genes that cause mutant phenotypes in *Drosophila melanogaster* (Banfi et al. 1996). The DRES25 clone, a human EST that shows significant homology with a *Drosophila* gene, *diaphanous* (*dia*) (Castrillon and Wasserman 1994), was mapped, by FISH, to Xq22. Mutant alleles of *dia* are responsible for sterility in male and female fruit flies. The localization of the human cDNA and the peculiar phenotype determined by mutations in the *Drosophila dia* gene suggested that the human homologue of *dia* could be involved in human ovary development. We now report characterization of the human homologue of the *Drosophila dia* gene, and we demonstrate that this gene is interrupted by a breakpoint associated with a familial case of POF (patient BC, reported in Philippe et al. [1993] and Sala et al. [1997]). We propose that *DIA* is one of the genes that are essential for normal human ovarian development and function.

Material and Methods

cDNA Isolation

The cDNAs were obtained either from the Image Consortium (http://www-bio.llhl.gov/bbrp/image/image.html) or by hybridization screening of human ovary, testis, and teratocarcinoma and mouse brain cDNA libraries (Stratagene, Clontech).

Isolation of Genomic Clones

The CEPH MegaYAC and the P1 artificial chromosome (PAC) library were screened, at the YAC Screening Centre (YSC) (http://www.spr.it/iger), by PCR, with primer pairs 12C-f/12C-r and 156-f/156-r (table 1). Cosmids, from the Lawrence Livermore chromosome X–specific cosmid library, were isolated by hybridization, with clone DRES25 as probe.

Nucleotide Sequencing

cDNA and cosmid clones were sequenced, by use of vector and internal primers, as described elsewhere (Bione et al. 1994).

FISH Mapping

FISH was performed on metaphase preparations, with YAC and PAC DNA, as described elsewhere (Rossi et al. 1994). DNA was labeled by nick-translation, with biotin-16 dUTP (Boehringer Mannheim).

Table 1

Sequence of PCR Primers

Primer	Sequence
D-f2	5′-GATAATCTTCTAGAAGCCCTACAATCAG-3′
156-r	5′-TATTACAAATGCACACTACTTCATCC-3′
12C-r	5′-GTCATGTTGTACCATCACCCAATCCTG-3′
D-f1	5′-TTCTTTGGTGATCTCAACAACTTC-3′
12C-f	5′-GTGGTAAATCATCCCTGTGCAACAAGGG-3′
156-f	5′-TCTCATCTAAGTGATTCCTGATGC-3′
1A3	5′-TAATTGCCTAGAATCCCTCAGGGTTTC-3′
D-r7	5′-TCTGCTTCATTCCATAAGGTAGATTAAG-3′
mD-1	5′-CTTCTGGTTGGAAACTACATGAACTA-3′
mD-4	5′-ACATATTGCTGTGCATGGTGGACAG-3′
Hprt-f	5′-CCTGCTGGATTACATTAAAGCAC-3′
Hprt-r	5′-GTCAAGGGCATATCCAACAACAAAC-3′

Reverse Transcriptase–PCR (RT-PCR)

Total RNA from various human tissues was either purchased from Clontech or extracted from lymphoblastoid cell lines or mouse tissues that were dissected at the indicated developmental time. RNA was extracted from Mouse tissues by means of the RNeasy Mini Kit (Qiagen). Reverse transcription of 1 μg of total RNA was performed as described elsewhere (Bione et al. 1994). PCR amplification was performed in 50-μl reactions for 40 cycles, unless otherwise indicated, in 0.2 mM dNTPs, 0.5 μM primer, 1.5 mM Mg^{++}, and 1.25 U *Taq* polymerase (Promega). PCR was performed for 30 s at 94°C, 30 s at 58°C–64°C, and 40 s at 72°C, with 7 min of final elongation. "Hot start" was performed for 5 min at 94°C.

Northern Blot Analysis

Human multiple-tissue northern blots (Clontech) were hybridized at 65°C, as described elsewhere (Bione et al. 1993). The exon 156–specific probe was a 429-bp fragment, obtained by *Rsa*I digestion of the PCR product Df-2/T7, amplified from DRES25. The exon 12C–specific probe was a 260-bp fragment, obtained by *Hin*fI digestion of the PCR product DF-2, amplified from the cDNA HT12C.

Pulse-Field Gel Electrophoresis (PFGE) Analysis

DNA of the YACs 933D12 and 796E9 was digested by the restriction enzymes indicated above and was fractionated by PFGE, at 170 V, in 1.5% agarose gels, for 24 h at 14°C. Pulse intervals were 60–150 s. The gels were blotted and hybridized by use of the probes indicated above.

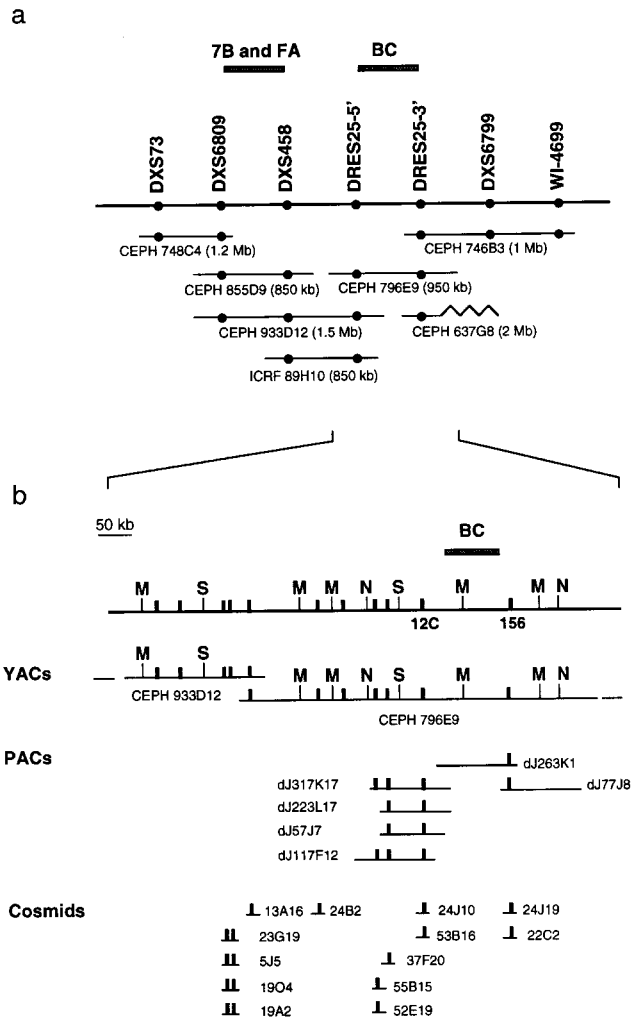

Figure 1 *a,* YAC contig in the region of the BC breakpoint. STSs and probes are indicated by dots. Positions of the breakpoints in POF patients are indicated by rectangles above the map. *b,* Rare cutter-restriction map of the 3′ end of the *DIA* gene. Exons are indicated by bars. YACs 933D12 and 796E9, PACs, and cosmids are shown below the map. Rare cutter-restriction enzymes are indicated by the letters "M" (*Mlu*I), "S" (*Sac*II), and "N" (*Nru*I).

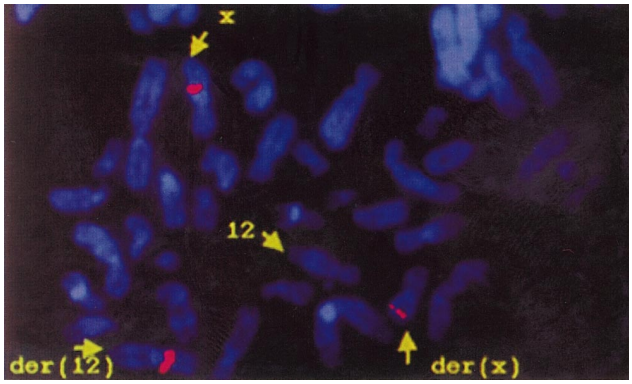

Figure 2 FISH on metaphase preparations of patient BC. The probe used was PAC dJ263K1. Normal and derivative chromosomes are indicated by arrows. Chromosomes were stained with 4,6-diamidino-2-phenylindole.

Results

The Human DIA *Gene Is Localized in Distal Xq21 and Is Disrupted by the Breakpoint of an X;12 Translocation, Which Causes POF*

The DRES25 EST (Banfi et al. 1996) was sequenced, and it was shown to contain an open reading frame (ORF) of 1,001 bp. The 361 amino acids encoded by the ORF showed high similarity (41.5% identity, 64.8% similarity) with the C-terminal portion of the product of the *Drosophila dia* gene (amino acids 714–1091).

By means of hybridization to the YACs of the POF critical region (Sala et al. 1997), the DRES25 clone was mapped to the distal portion of the contig, in Xq21.3/Xq22. Representative YACs of the region are shown in figure 1*a*. The 500 nt at the 5′ of the DRES25 cDNA (DRES25-5′) hybridized to YACs 933D12, 89H10, and 796E9, whereas the rest of the cDNA (DRES25-3′) hybridized to 796E9 and to the more telomeric YACs, 637G8 and 746B3.

The breakpoint of the balanced X;12 translocation (46,X,t[X;12][q21;p1.3]) in the family of patient BC was localized, by FISH, to the same genomic region (Sala et al. 1997). Patient BC had secondary amenorrhea, with no other associated features, at the age of 17 years. Her mother carried the same chromosomal rearrangement and was diagnosed with premature menopause at the age of 32 years. At diagnosis, both mother and daughter had high gonadotropin levels and inactivation of the normal X chromosome (Philippe et al. 1993). The breakpoint was mapped, by FISH, to the rearranged YAC 2C11 (Sala et al. 1997) and, subsequently, to YAC 796E9 (data not shown).

To better define the breakpoint, a rare cutter-restriction map of the two YACs, 933D12 and 796E9, was constructed, with DRES25, newly isolated cDNAs, and sequence-tagged sites (STSs) of the region as probes. Part of the map is shown in figure 1*b*. Cosmids and PACs were isolated and are also shown in figure 1*b*. Cosmids sequenced from cDNA internal primers demonstrated that the whole DRES25 sequence was contained in eight exons that were localized to restriction fragments of the YAC map (fig. 1*b*). The last intron of the gene was cloned, in overlapping PACs, and its length resulted of ~200 kb. PAC clones dJ317K17 and dJ263K1 were used in FISH; clone dJ263K1 spanned the BC breakpoint (fig. 2), which was therefore mapped to a region of ~100 kb, in the last 200-kb intron of the gene.

The Human DIA *cDNAs*

The DRES25 clone and cDNA fragments that extended toward the 5′ end of the gene were used to isolate full-length cDNA. Overlapping clones were identified from different libraries and were sequenced from both strands. Most of the cDNAs were identical, and the established consensus sequence was 4,040 bp (European Molecular Biology Laboratory [EMBL] accession nos. Y15908 and Y15909). It contained an ATG, at position 351, preceded by in-frame stop codons. An ORF of 3,306 bp encoded a 1,101–amino acid protein that was highly similar to the *Drosophila* dia protein (fig. 3).

In most clones, a stop codon was localized at position 3653 and was followed by a 3′ UTR. A poly A tail was present in two of the cDNAs isolated, but no canonical consensus sequence for polyadenylation was observed. The 6-kb sequence of cosmid DNA downstream of the DRES25 3′ end identified five canonical consensus sequences for polyadenylation. Results of RT-PCR and 3′ rapid amplification of cDNA ends experiments (data not shown), together with the finding of two ESTs, in GenBank, that contain a poly A tail (Image Consortium clone identification nos. 133877 and 148120), confirmed that two of the sites were used. The first site, which was 1.3 kb from the stop codon, would produce a transcript of 4.9 kb, whereas the second site, which was 5.7 kb downstream, would produce a transcript of 9.3 kb.

Two clones from a testis library contained a 3′ end that diverged from the consensus sequence at nt 3590. The divergent sequence encoded a 48-nt ORF and a short 3′ UTR. A probe that was specific for the alternative sequence hybridized to YACs 796E9 and 637G8; to PACs dJ317K17, dJ223L17, dJ57J7, and dJ117F12; and to two X chromosome–specific cosmids, 53B16 and 24J10 (fig. 1*b*). This result mapped the alternative sequence between the two last exons of the gene, proximal to the breakpoint in the family of patient BC. Results of sequencing cosmids 53B16 and 24J10 from cDNA internal primers demonstrated the presence of splice junctions and suggested that the alternative sequence was an alternative last exon. The two exons indicated were exon 156 and exon 12C (fig. 1*b*). The 5′ end of the *DIA* cDNA (1–201 bp) was mapped to the restriction map of YAC 933D12 (data not shown); the human *DIA* gene was determined to be ~1 Mb.

The Human DIA Proteins

The alignment of the human DIA and *Drosophila* dia proteins indicates that they are highly similar (fig. 3). Humans and *Drosophila* have 39.3% identical and 66.2% conserved residues, along the entire length of 1,101 and 1,096 amino acids, respectively. Of the two alternative last exons of the human gene, only exon 156 encodes 21 amino acids that are significantly similar to those of the fruit fly protein; exon 12C encodes a sequence that is quite different from that of *Drosophila*.

The human DIA possesses the two conserved domains FH1 and FH2 (formin homology 1 and 2, boxed in fig. 3) as well as characteristics of the FH1/FH2 protein family that have been described in a large number of distant organisms (Maas et al 1990; Woychik et al. 1990; Emmons et al. 1995; Petersen et al. 1995; Chang et al. 1997; Evangelista et al. 1997; Harris et al. 1997; Imamura et al. 1997). Other conserved features include the distance between the FH1 and FH2 domains and the presence of two coiled-coil domains (boxed in fig. 3) that flank the FH1 and the FH2 domains.

Computational analysis with PSORT predicted a nuclear localization signal (NLS) near the C-terminal of the protein. An NLS is also found, in different localizations, in formins (Chan and Leder 1996).

Alternative Forms of the Transcript and Expression Analysis

The DRES25 was hybridized to northern blots that contained poly A^+ RNA from various human adult and fetal tissues (fig. 4). Three major bands, of ~9.3, ~4.9, and ~4.4 kb (forms a–c), were visible and were ubiquitously expressed. In adult testis, a specific fourth band, of 2.7 kb, was also present (form d). The two major bands appeared to be slightly (~3–4 times) more abundant in testis, ovary, and small intestine (compared with mRNA quantity normalized by β-actin control hybridization). Forms a and b were absent in spleen and leukocytes and were very rare in thymus and colon.

To determine whether the four bands corresponded to different 3′ ends, probes that were specific for exons 12C and 156 were hybridized to the northern blots: bands a and b contained exon 156, and bands c and d contained exon 12C. Thus, the four different transcripts of the *DIA* gene differed in their 3′ end. The 2.7-kb testis-specific transcript contained at least part of the DRES25 sequences and the 12C exon, but it was too small to encode the whole ORF. It was not characterized further.

To verify that transcripts a and b were interrupted by the BC breakpoint, we looked for specific transcripts, in total RNA of lymphoblastoid cell lines of patient BC, by RT-PCR amplification, from primers in the coding region and in exon 12C or exon 156 (fig. 5*a*). Only primers from exon 12C could synthesize cDNA from lymphoblasts of patient BC (fig. 5*b*); no product that was specific for exon 156 was detected (fig. 5*b* and *c*).

Lymphoblastoid cell lines, from POF patients 7B, FA, and WD, that carried X-autosome translocations with breakpoints centromeric to that in patient BC (Sala et al. 1997) were analyzed, by RT-PCR, in the same ex-

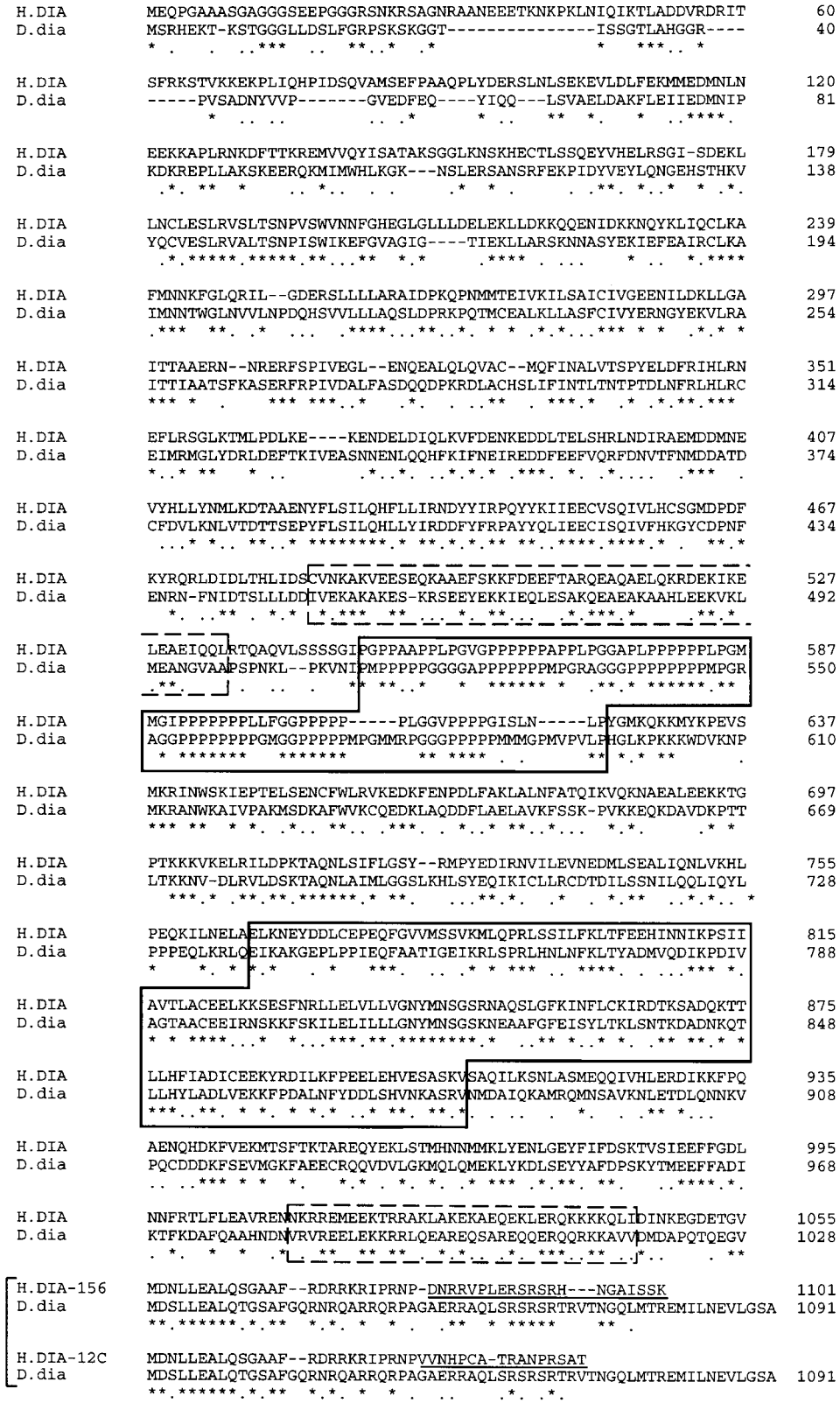

Figure 3 Alignment of human DIA and *Drosophila* dia proteins, as determined by CLUSTALV. The FH1 (amino acids 549–623) and the FH2 (amino acids 766–907) domains are boxed with continuous lines. The coiled-coil domains are boxed with broken lines. The two C-termini of the human DIA protein (exons 156 and 12C) are underlined. Identical residues are indicated by asterisks, and conserved residues are indicated by dots.

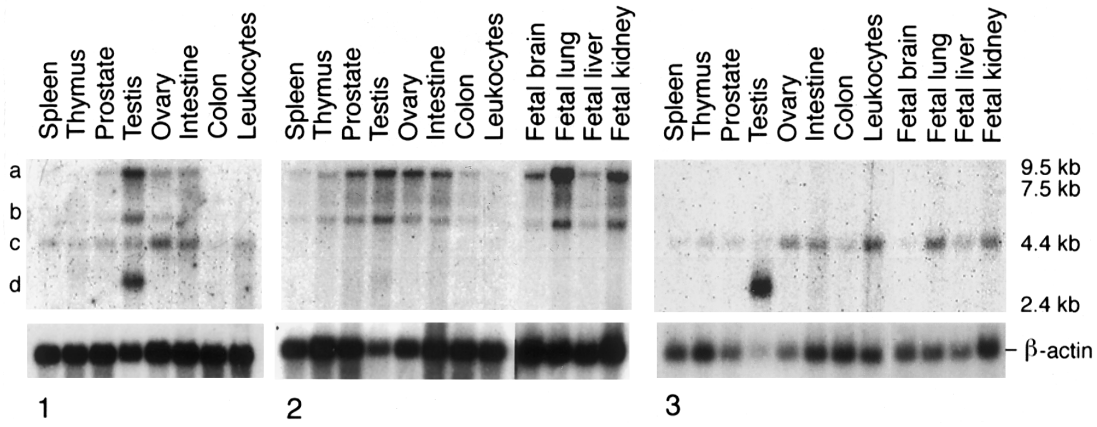

Figure 4 Northern blot analysis of the *DIA* 3′ end. Two micrograms of poly A^+ RNA from various human adult and fetal tissues were hybridized with (1) the DRES25 clone insert, (2) a probe specific for the 156 last exon, or (3) a probe specific for the 12C last exon. Letters ("a–d") on the left indicate the four *DIA* transcripts. Molecular size markers are on the right. Control hybridization was performed with human *β*-actin (Clontech).

periment. The *DIA* transcript was synthesized in all three patients (fig. 5*c*).

The DIA *Gene Is Expressed in Developing Ovaries*

The human *DIA* gene is ubiquitously expressed, in rather low amounts, in human adult and fetal tissues. To determine whether it is also expressed early in embryogenesis, during gonad development, we isolated and sequenced a mouse-cDNA fragment that corresponds to the distal portion of the human gene (amino acids 752–1101). The mouse-cDNA fragment (EMBL accession no. Y15910) encodes 349 amino acids, 85.6% of which are identical to those in the corresponding region of the human protein. Primers were designed to amplify, by RT-PCR, a 396-bp fragment from RNA of various mouse tissues at various stages of development. RT-PCR was performed with liver, heart, kidney, ovary, and testis tissues dissected from mouse at developmental stages E16, P6, and P16. The mouse *Dia* gene was expressed in both ovary and testis, as well as in all other tissues from the E16 stage (fig. 6).

Discussion

We have characterized a human homologue of the *Drosophila dia* gene; mutated alleles of this gene affect spermatogenesis and oogenesis (Castrillon and Wasserman 1994). We propose that the human *DIA* gene described in this article is involved in oogenesis and that it is one of several genetic loci that are responsible for POF. *DIA* was mapped to the POF critical region (Therman et al. 1990) and was disrupted by the breakpoint of a balanced X;12 translocation that was identified in the family of patient BC (Philippe et al. 1993; Sala et al. 1997), in which the same chromosomal rearrangement cosegregated with POF. The breakpoint was mapped to the last intron of the gene. As a consequence of the translocation, the *DIA* gene on the derivative X chromosome could be fused to sequences from chromosome 12, and an altered protein might be synthesized. Alternatively, in the absence of the last coding exon and of the 3′ UTR, the truncated transcripts might be both unstable and likely to degrade soon after transcription. In either case, a very long 3′ UTR, common to a and b mRNAs, would be missing. The 3′ UTR was AT-rich and contained several motifs (AUUU[U]A and AUUUU[U]AUU) that have been implicated, because of their effects on poly A tail length and mRNA stability, in translational control of mRNAs (Vassalli and Stutz 1995). This control mechanism is especially relevant in oocytes and early embryos, in which many mRNAs can remain untranslated for long periods of time and can be recruited for translation at specific developmental stages. DIA expression in ovaries may therefore be subjected to such posttranscriptional control, and the lack of the regulatory motif may alter the pattern of events that lead to oocyte and ovary maturation. Of the remaining *DIA* transcripts, the 2.7-kb transcript d is testis-specific, and the 4.4-kb transcript c is the more ubiquitous. The latter transcript may have different properties because of the presence of the alternative 3′ coding exon and 3′ UTR.

DIA is the human homologue of the *Drosophila dia* gene; along the whole sequence, >39% of amino acids are identical, and >66% are conserved. DIA is a member of the FH1/FH2 family of proteins. The *Drosophila* genes *dia* (Castrillon and Wasserman 1994) and *cappuccino* (Emmons et al. 1995); the mouse formins (Woychik et al. 1990; Maas et al.); the yeast genes *fus1* (Pe-

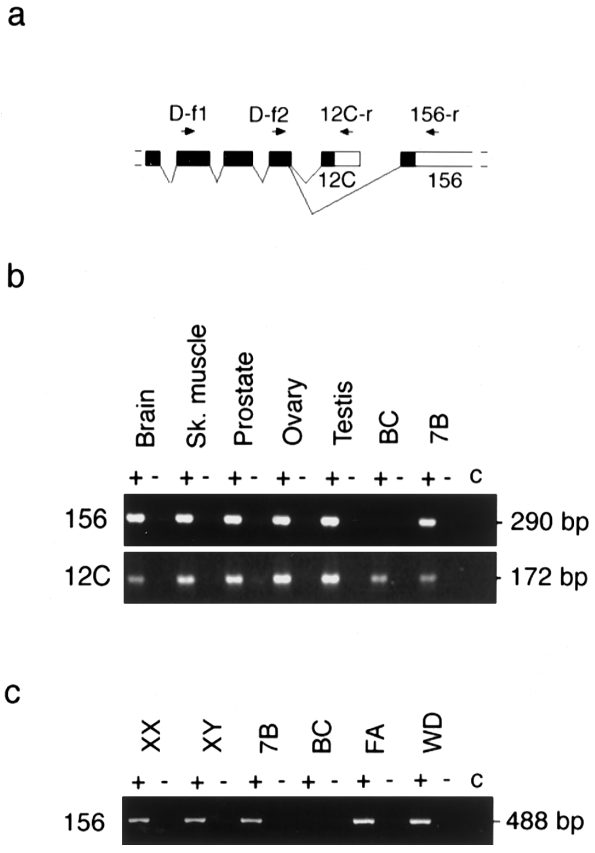

Figure 5 RT-PCR analysis of the *DIA* transcripts. *a,* Schematic representation of the 3′ end of the gene and of the two alternative last exons. Positions of primers (table 1) are indicated by arrows. *b,* RT-PCR products synthesized from RNA of the human tissues indicated and from lymphoblastoid cell lines of POF patients BC and 7B. PCR was performed with primers D-f2 and 156-r or 12C-r. *c,* RT-PCR products from RNA of lymphoblastoid cell lines, from normal female (XX), from normal male (XY), and from POF patients 7B, BC, FA, and WD, amplified from primers D-f1 and 156-r. The RT reactions were carried out with (+) or without (−) reverse transcriptase. *c,* PCR reactions without DNA template. RT-PCR products were fractionated on 3% agarose gels and stained with ethidium bromide. Molecular weights are indicated on the right.

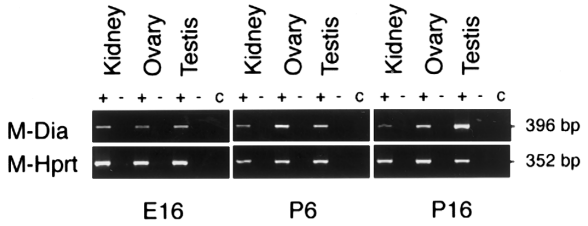

Figure 6 RT-PCR products from total RNA extracted from the indicated mouse tissues at developmental stages E16, P6, and P16. PCR of mouse *Dia* cDNA was from primers mD-1 and mD-4; control mouse *Hprt* cDNA was amplified from primers Hprt-f and Hprt-r. The RT reactions were carried out with (+) or without (−) reverse transcriptase. *c,* PCR reactions without DNA template. The sequences of the primers are displayed in table 1. The amplification products were fractionated on 3% agarose gels and stained with ethidium bromide. Molecular weights are indicated on the right.

tersen et al. 1995), *cdc12* (Chang et al. 1997), *Bni1p* (Evangelista et al. 1997), and *Bnr1p* (Imamura et al. 1997); and the *Aspergillus nidulans* gene *sepA* (Harris et al. 1997) have been shown to participate in the establishment of cell polarity and cytokinesis. They have been implicated in reorganization of the actin cytoskeleton through interaction with profilin (Inamura et al. 1997) and, possibly, with other actin-binding proteins. Accordingly, Bni1p, Bnr1p, and *p140mDia* (a mouse homologue of *DIA* that is different from the one we have isolated) have been shown to be targets for the small G protein Rho (Kohno et al. 1996; Inamura et al. 1997; Watanabe et al. 1997), which has been localized to the cytoskeleton and is also involved in actin-mediated morphogenetic processes (Takai et al. 1995).

The *dia* locus was identified, in *Drosophila*, as a result of a screen for male-sterility mutations, and it was shown to be required for cytokinesis (Castrillon and Wasserman 1994). Like human *DIA*, *Drosophila dia* is ubiquitously expressed. Alterations in cytokinesis that are associated with mutated *dia* alleles have been described in several different tissues, but the main phenotype produces sterility. Null mutations result in early pupal lethality; this finding is consistent with the suggestion that *dia* is an essential mitotic gene. Our results strongly indicate that human *DIA* has conserved the *Drosophila dia* gene's role in cell divisions that lead to gonad development. Accordingly, *DIA* transcripts are present in ovary and testis at early stages of development (E16) as well as at stages during which the ovarian follicles undergo differentiation (P6–P16).

Mutation analysis of a large number of patients may demonstrate that *DIA* has a definite role in ovary development. Such analyses may also determine the frequency of mutations in the *DIA* gene, among POF patients. Like *Drosophila* dia, human DIA is expressed in the testis, where it presents a common and a testis-specific form. Mutation analysis of sterile human males could show whether *DIA* is responsible for sterility in humans, just as *dia* is responsible for sterility in *Drosophila*.

While this article was undergoing revision, Lynch et al. (1997) reported a second homologue of the *Drosophila dia* gene, localized to chromosome 5q31, that was responsible for DFNA1, an autosomal dominant sensorineural progressive hearing loss. They also suggested that the Xq22-linked gene was a candidate for the nonsyndromic X-linked deafness, DFN2, mapped to Xq22. However, in the family of patient BC, the two females affected with POF were not reported to have

hearing problems. Since the chromosome 5 *DIA* homologue did not affect fertility (Lynch et al. 1997), it appears that the various members of the *DIA* family that have evolved from an ancestral unique *dia* gene have maintained a ubiquitous role, but they have acquired specific functions in highly specialized cell types, such as the follicular cells of the ovary and the hair cells of the inner ear.

POF is a very heterogeneous disorder, but the molecular mapping of breakpoints of X-autosome translocations suggests that POF may be ascribed to disruption of a limited number of Xq21 genes involved in ovary development (Sala et al. 1997). Our previous work could not exclude the possibility that mispairing at meiosis, due to chromosome rearrangement, may cause POF; however, we tentatively excluded long-range position effects, since genes in the region apparently were not affected by the presence of the breakpoints. The best example was the choroideremia (*CHM*) gene: disruption of *CHM* in females was responsible for POF and CHM, but POF without CHM was reported in patients who carry breakpoints outside the *CHM* gene. We also proposed that gene-dosage effects were not a likely explanation for POF that is associated with balanced translocations in Xq21, since large deletions that remove the whole critical region for POF, in Xq21, apparently were not associated with ovarian failure (Merry et al. 1989). The identification of *DIA* further supports our hypothesis that several Xq21 genes are involved in POF. Moreover, since DIA expression was not affected by breakpoints, in Xq21, outside the *DIA* gene itself and since DIA was expressed only by the active X chromosome (fig. *5b* and *c*; authors' unpublished data), our results suggest that other loci or mechanisms may account for the remaining cases of the disorder.

Acknowledgments

We thank Massimiliano Agnelli, of the YSC, Milan, for screening the yeast artificial chromosome library; Margherita Gatti, for technical assistance; and L. Ferretti, for help with the figures. This work was funded by European Community grants Gene CT930088 and BMHA CT961134 and by Téléthon-Italy grants E297 and 837

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