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The *Dazh* Gene Is Expressed in Male and Female Embryonic Gonads before Germ Cell Sex Differentiation

Judith Seligman*,[1] and David C. Page†

**Developmental Biology Laboratory, Department of Obstetrics/Gynecology, Rambam Medical Center, P.O.B. 9602 Haifa 31096, Israel; and †Howard Hughes Medical Institute, Whitehead Institute and Department of Biology, Massachusetts Institute of Technology, 9 Cambridge Center, Cambridge, Massachusetts 02142*

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The autosomal homologs of the human Y-chromosomal DAZ gene (DAZH and Dazh in human and mouse, respectively) are strong candidate for Azoospermia factor and encode a testis specific RNA-binding proteins. We studied the expression pattern of the mouse Dazh during embryonic development by using Northern-blotting of developing gonads. In the mouse, we have detected 3.5kb and 4.5kb transcripts in male and female embryonic gonads at 12.5 dpc (days post coitum). During this period, the only germ cells present in the gonad are primordial germ cells. Dazh transcripts were not detected in embryonic gonads of mice that lack germ cells because of mutation in W gene, suggesting that expression is limited to germ-cells. In females, oogonia enter meiosis at 13.5-14.5 dpc: at this time Dazh transcription levels are similar to those of the male (when prospermatogonia are in the male gonad). Transcription levels decrease steadily after birth as the number of oocytes is depleted and is hardly detectable by puberty. A human DAZH transcript was also detected by Northern-blotting in the human ovary in levels which are of about 100 fold lower than those observed in the human testis. The expression of the Dazh in male and female gonad before germ cell sex differentiation suggests that these genes may act at the first phase of male and female gametogenesis.

Key Words: **meiosis; Dazh; primordial germ cells; embryonic gonad.**

Deletion of the Azoospermia Factor (AZF) region on the long arm of the Y chromosome results in spermatogenic failure and infertility [1, 2]. A strong candidate for AZF is the putative RNA-binding protein DAZ (Deleted in Azoospermia); the only transcription unit reported in this region [2]. Autosomal genes homologous to the Y-chromosomal DAZ gene have been shown to be present in many vertebrates [3] and were characterized in human [4, 5, 6, 7], mice [3, 8] and Drosophila [9] in which they appear to be expressed exclusively in germ-cells [3, 9, 10, 11]. It has been postulated that the human DAZ gene on the Y-chromosome arose from the autosomal ancestor DAZH (DAZ homolog, known also as DAZLA) mapped recently to human chromosome 3 via a series of structural transformations [4].

As gauged by DAZ/DAZH protein sequences and expression patterns, the newly emergent Y gene clusters retained key functional characteristics of its autosomal ancestor [4]. Northern-blotting [3] and In-situ hybridization [10] demonstrate that both, the human DAZ/DAZH and the mouse homolog of DAZ (Dazh, also known as Dazla) are expressed in spermatogonia cells of the adult testis. The specificity of DAZ expression in germ cells in early stages of spermatogenesis strongly suggests that DAZ and its homologous genes function at the first phases of spermatogenic differentiation or earlier, in the maintenance of the spermatogonial stem cell populations [3, 10]. Although no human DAZ point mutants have been reported, recent genetic findings in Drosophila and mouse provide strong support for the hypothesis that DAZ is AZF. In the Drosophila and the mouse, mutants that have lost DAZ homologous gene function (*boule and Dazh*, respectively) were found to be azoospermic, just as with human AZF [9, 12].

High similarity in nucleotide sequences and expression between the human and mouse autosomal genes is found. Within the 82-residue RNA-binding domain, the products of human DAZH and mouse Dazh differ by only one amino acid substitution, while both differ from human Y-encoded DAZ at nine residues. Moreover, some evidence, as demonstrated by reverse-transcriptase PCR (RT-PCR), suggests that the human and mouse autosomal genes are expressed in low levels in the ovaries too [4, 8]. These preliminary evidence on

[1] To whom correspondence should be addressed. Fax: 011-972-4-826 1689. E-mail: j_seligman@rambam.health.gov.il.

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ovarian expression are very interesting and have never been checked thoroughly.

The purpose of our work was to study the ovarian transcription sizes and abundance in the ovaries. We wanted to know whether Dazh expresses in the germ-cells compound of the ovary and how early Dazh is expressed during gonadal development? We found that Dazh expresses in the developing male and female embryonic gonads well before the onset of meiosis. This expression pattern suggests that these genes act at the first phase of male and female gametogenesis.

MATERIAL AND METHODS

Animals and tissue dissections. C57BL/6J and C57BL/6W^V/+ mice were obtained from Jackson laboratories (Bar Harbor, ME). For all embryo dissections it was assumed that mating took place midway through the dark period; therefore midday on the day of appearance of the vaginal plug is approximately 0.5 day post-coitum (dpc). Embryos homozygous for W^V allele were recognized by their pale appearance relative to heterozygous and wild-type littermates. Male and female embryonic gonads were sexed under dissecting microscope and kept frozen for RNA extraction.

RNA extraction and Northern-blot hybridization. RNA samples were prepared using Trizol reagent (Gibco BRL, Grand Island, NY). Gonads were suspended in 1ml Trizol, and 0.2 vol of chloroform was added to each sample. After centrifugation to remove cell debris, RNA in the supernatant was precipitated with isopropanol, rinsed with ethanol, and resuspended in deionized water. Following electrophoresis, gels were stained in Ethidium bromide and photographed to assess loading differences (not shown). Northerns were hybridized with Dazh cDNA pDP1580 [3] or with DAZH cDNA pDP1580 [4]. The gene encoding Alpha-tubulin was used as a reference probe for mouse samples [13] and the gene encoding RPS4X was used as a reference probe for human samples [14] to control the loading of different quantities of RNA. The alpha-tubulin and RPS4X autoradiograms reflect the differences in sample loading observed by Ethidium bromide staining (not shown). All hybridizations were performed for 20 h at 42°C in 50% formamide, 5XSSC, 1× denhardt's reagent, 20mM Na phosphate, pH 6.6, 1mg/ml transfer RNA, 1% sodium dodecyl sulfate, 10% dextran sulfate. The blots were washed three times for 15 min. each at 57°C in 0.1XSSC, 0.1% sodium dodecyl sulfate.

Comparison of Dazh/DAZH expression levels by densitometry. Densitometric profiles of the Dazh/DAZH and alpha-tubulin/RPS4X were performed on the autoradiogrames using the NIH Image 1.61. In order to compare the changes in Dazh/DAZH expression, the densitometry intensity (total area of the peaks) of the alpha-tubulin/RPS4X of one sample was adjusted to the corresponding other samples on the same gel.

RESULTS

Germ cell line is one of the best characterized lineage in mammals. Several different stages, time points, and/or cell types within this lineage have been described. As a result, simple Northern-blotting of RNAs from developing gonads can provide much information as to the developmental stages and cell types in which a germ cell specific gene is expressed. By using this Northern approach, we previously showed that Dazh is expressed in testicular germ cells even before puberty (in prospermatogonia and spermatogonia). To explore further Dazh expression during gonadal development, we sampled male and female embryonic gonads from 11.5 dpc to 18.5 dpc. and used them for Northern blot analysis. As shown in Fig. 1A, a 3.5 kb Dazh transcript and higher less abundant transcript of about 4.5 kb were detected in embryonic gonads at 12.5 dpc. Expression of Dazh in male and female gonads was detected even earlier, at 11.5 dpc by RT-PCR (not shown), when the only germ cells present in the gonad are primordial germ cells (PGCs). The Dazh transcription levels increase and reach similar levels in male and female gonads at 14.5 dpc, when first oogonia enter meiosis. In the female gonad, Dazh transcription level decreases to a half from 14.5 dpc to 16.5 dpc (as calculated by optical density of the 3.5kb bands; Fig. 1A) and remains on that level while oogonia proceed meiosis and arrest in prophase I, shortly before birth. In the male gonad, steady levels of transcription were detected through embryonic development, with a prominent decrease (half level) at 16.5 days (Fig. 1A).

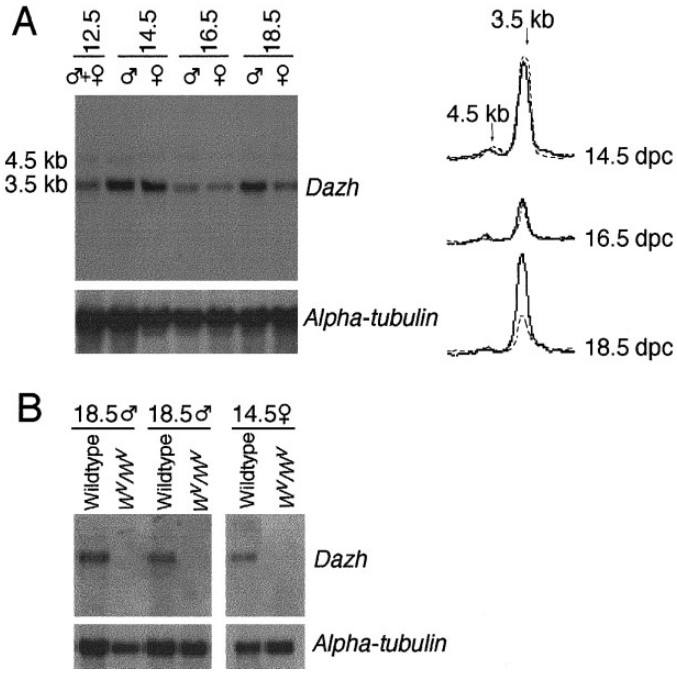

FIG. 1. Dazh expression in male and female gonads during embryonic development from 12.5 to 18.5 dpc. (A) Right, an autoradiogram produced by hybridizing Dazh cDNA pDP1580 to total RNAs (50 μg/lane) from mouse embryos. Hybridization of alpha-tubulin cDNA [13] as control for loading. Left, Densitometry of the Dazh autoradiogram of male (solid line) and female (broken line) embryonic gonads at 14.5 dpc, 16.5 dpc and 18.5 dpc. Densitometric intensities of the corresponding alpha-tubulin were normalized to the same value, so that the levels of the Dazh transcripts at different time point could be compared. (B) Germ cell dependent Dazh expression in embryonic gonads. Autoradiogram produced by hybridizing Dazh cDNA pDP1580 to total RNAs from mouse embryonic gonads W^V/W^V and their littermates wildtypes. Hybridization of alpha-tubulin cDNA [13] as control for loading.

We next asked the question, Is Dazh transcription

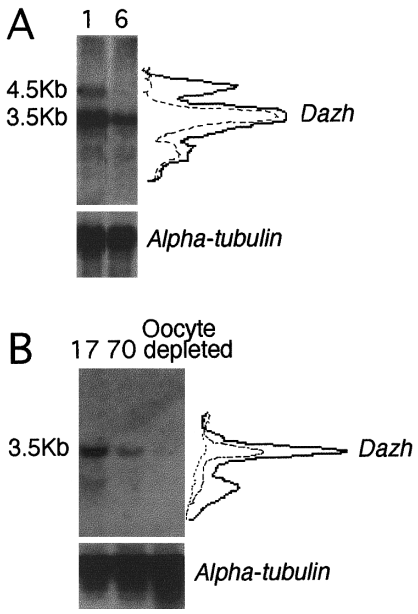

FIG. 2. Correlation between oocyte number and Dazh transcription level in mouse ovaries. Autoradiograms produced by hybridizing Dazh cDNA pDP1580 to total RNAs (50 μg/lane). (A) Ovaries isolated from mouse females at 1 and 6 days after birth. Densitometry of the Dazh autoradiogram of 1 day (solid line) and 6 days (broken line). (B) Ovaries isolated from mouse females at 17 and 70 days after birth and from oocyte depleted ovary obtained from female mutated in Zfx gene [17]. Densitometry of the Dazh autoradiogram of 17 days (solid line), 70 (wide broken line) and oocyte-depleted (dense broken lines) ovaries. Hybridization of alpha-tubulin cDNA as control for loading. Densitometric intensities of the corresponding alpha-tubulin were normalized to the same value, so that the levels of the Dazh transcripts at different time point could be compared.

during embryonic development restricted to germ cells? To distinguish between germ cell and somatic cell expression, we used the W^V (White spotted) mutants which are deficient in germ cells as a result of impairment in proliferation and/or migration of PGCs to the gonads; the somatic element of the gonad differentiate appropriately [15]. We showed previously that Dazh does not transcribe in testis isolated from W^V mutants lucking germ cells [3], indicating that Dazh expression is restricted to germ cells. Consistent with our previous results, we did not detect Dazh transcripts in embryonic gonads isolated from male and female W^V homozygous embryos (Fig. 1B). Our results suggest that Dazh is transcribed in germ cells; in PGCs before sexual differentiation, and after it, in prospermatogonia of the testis, and in oogonia and primary oocytes of the ovary.

Atresia destroys many of the oocytes long before they are fully grown. The decrease in actual number of oocytes is very rapid during the period from birth to puberty [16]. The Dazh transcription levels are correlated with the depletion of oocytes numbers in the ovary. As shown in Fig. 2A, the levels of the two transcripts (4.5 kb and 3.5 kb) were decreased significantly from day one to day 6 after birth and during adulthood. A dramatic decrease of the levels of transcription between 17 to 70 days after birth is demonstrated in Fig. 2B. The Dazh transcription is hardly detected in ovaries depleted oocytes as a result from mutation in Zfx gene [17] (Fig 2B). These results support the observation that Dazh is expressed in the germ cell compound of the ovary, probably in primary oocytes.

In all mammals, male germ cells replicate consistently and maintain their numbers, while female germ cells do not replicate and their numbers are depleted. In the mouse, the number of spermatogonia per testis increased significantly from 0.5×10^5 at birth to about 6×10^5 cells per testis after day 25 [18], while the number of oocytes per ovary decreased significantly from about 0.1×10^5 at birth to about 4500 when females reach puberty [16]. The Dazh transcription levels in the testis and ovary reflect these differences in germ cell numbers in the gonads. The Dazh transcriptions are hardly seen (over-night exposure) in the ovary by puberty, while in the testis a highly expressed 3.5 kb with 4.5 kb and some smaller, less abundant Dazh transcripts were observed (Fig 3A). As expected, a comparison between male and female Dazh densitometry intensities demonstrated that the Dazh transcription levels in the testis are about 100 fold higher than in the ovary (Fig. 3A).

Does human DAZH show a similar expression pattern? Taking into account the fact that expression is limited to primary oocytes, we chose for this study a young human ovary (from a 23 year-old woman) with a significant number of oocytes. As shown in Fig. 3B, a transcript of about 4.5kb was detected in the human ovaries, while 3.5kb transcript is most abundant in the human testis. The ratio between the ovarian and testicular transcription levels is similar to the ratio observed in the mouse (demonstrated by densitometry, Fig 3B). These results, in addition to the observation that DAZH does not transcribe in other human tissues [4] suggest that human DAZH, like the mouse Dazh, is expressed in male and female germ-cells at a similar, if not identical manner.

DISCUSSION

We have previously showed that Dazh is expressed in testicular germ cells long before puberty. In this work, we show that Dazh is expressed in male and female embryonic gonads earlier during development, well before sex-differentiation. We detected Dazh transcripts in the male and female embryonic gonad at 11.5-12.5 days when PGCs are the only germ cells in the gonad.

The PGCs are first recognized in the extraembryonic mesoderm at 7.5 dpc and subsequently migrate to the developing genital ridges at 11.5 dpc. From 8.5 to 13.5 dpc PGCs replicate by mitosis at a uniform rate, with

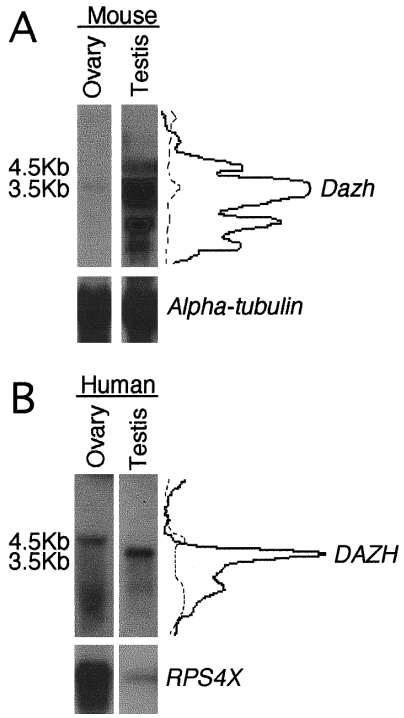

FIG. 3. The Ratio between testicular and ovarian transcriptions of mouse Dazh and human DAZH. Autoradiogram produced by hybridizing DAZH cDNA pDP1648 to total RNAs (50 μg/lane). Densitometry of the Dazh autoradiogram of male (solid line) and female (broken line). Hybridization of alpha-tubulin cDNA as control for mouse tissues loading. Hybridization of RPS4X cDNA [14] as control for human tissues loading. The mouse autoradiogram was exposed for 24 h. The human Autoradiogram was exposed for 6 days. Densitometric intensities of the corresponding alpha-tubulin or RPS4X were normalized to the same value, so that the levels of the Dazh transcripts at different time point could be compared.

a doubling time of about 16 hours [19]. Throughout this period the germ cells develop in a similar manner in males and females. However, once the germ cells initiate sexual differentiation at about 13.5 dpc in the mouse, the subsequent kinetics of germ cell development show a dramatic sexual dimorphism: oogonia enter meiotic prophase, while prospermatogonia continue to divide mitotically until 14.5 dpc and than prospermatogonia remain quiescent until after birth [20]. We found that Dazh transcription levels correlate with germ cell sexual differentiation and dimorphism of the gonads. At 14.5 dpc, when germ cell sexual differentiation just commences, similar levels of Dazh transcriptions in male and female gonads were observed. The level of Dazh decreased in the ovaries through meiosis from 14.5 dpc to birth, while the level of Dazh in the male gonad did not change dramatically from 14.5 dpc to birth, as prospermatogonia remain quiescent.

We have previously demonstrated by Northern blotting that Dazh transcription is restricted to testicular germ cells (prospermatogonia and spermatogonia). Our results suggest that Dazh transcriptions in the male and female embryonic gonads are also restricted to germ cells (most likely PGCs, oogonia, primary oocytes and prospermatogonia). The Dazh gene could belong to a group of markers that have been instrumentaly used in tracking the early period of germ cells development in the mouse such as cell surface alkaline phosphates activity [21, 22] and other surface markers which may not been specific to germ-cells [20]. The Dazh expression pattern is very similar to nuclear antigen (GCNA1) which was shown to be expressed exclusively in germ cells at similar developmental stages [23].

It is most likely that human DAZH and the mouse Dazh proteins perform similar functions. The human and the mouse genes are both expressed predominantly in the testis and in lower levels in ovaries, but not in other tissues [3, 4]. Our results demonstrate similar ratio of transcription levels in testes and ovaries of human and mice and suggest that transcription is limited to germ cells at similar or identical developmental stages. However, Northern-blotting also reveals that human and mouse express different size of transcripts in the ovaries. In the human ovary a 4.5 kb DAZH transcript was observed, while a 3.5kb transcript is observed in the mouse ovary. Such 4.5 kb transcript was observed in low levels in human (not-shown) and mouse testes (See Fig. 2) and in higher levels during early embryonic development (Fig. 1A). Since the DAZ genes are organized as cluster genes [4], it is possible that the two transcripts are expressed from different copies of the genes. Another possibility is that the two different transcripts are from the same origin; RNA processing such as reported previosely in DAZH mRNAs [4] may also account for differences in transcription size. The biological significant and function of the 3.5 kb and 4.5 kb transcription units remain to be studied. These transcription units may possess different functions, such as the Ddc cluster of in Drosophila which express different transcripts, some of which are involved in female fertility and some which do not restrict ovary development [24].

The decision as to whether it is oogenesis or spermatogenesis on which PGCs embark seems to depend on their environment, and not on their own chromosomes [25]. However, the ability of germ cells to enter mieosis seems to be intrinsic to germ-cells [26]. Thus, factors in the germ cells that are involved in sex differentiation and entry into mieosis would be expected to be expressed in germ cells of both sexes (XX and XY gonads) such as the Dazh gene. Our expression results are supported by the recent evidence in the mouse suggests that Dazh is essential for the differentiation of male and female germ cells [12]. As expected, disruption of the Dazh gene leads to loss of germ cells and complete absence of male and female gametes.

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