Dazl protein expression in adult rat testis is up-regulated at meiosis and not hormonally regulated

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Summary

The Y-chromosomal *DAZ* (deleted in azoospermia) gene and the autosomal *Dazl* (deleted in azoospermia-like) gene are two crucial factors for the achievement and maintenance of spermatogenesis. Whereas Y-chromosomal *DAZ* is present in certain primates, it is lacking in rodents and other species. We have investigated the expression of *Dazl* protein during spermatogenesis in the adult rat testis using immunohistochemistry. *Dazl* immunoreactivity was found predominantly in the cytosol of primary pachytene spermatocytes. A weaker but clearly detectable signal was present in intermediate and B spermatogonia and in early spermatocytes from preleptotene to zygotene. The highest expression patterns were observed between stages IV and VIII during the spermatogenic cycle when spermatocytes prepare for the first meiotic division. Specific staining could also be observed in step 11–19 elongating spermatids in the acrosome region.

Treatment for 42 days with a potent GnRH-antagonist abolished gonadotrophin secretion and led to a regressed testis, lacking most of the advanced germ cell types such as spermatids but still bearing spermatogonia and spermatocytes. No difference in staining pattern for *Dazl* protein was observed in GnRH antagonist-treated rats despite the lack of gonadotrophins and substantial impairment of the spermatogenic process, indicating that *Dazl* expression is clearly hormone-independent.

The localization and level of *Dazl* expression suggests an important role in the regulation of the first meiotic stages of spermatogenesis. The hormone independent onset of expression points to an autonomous cell-cycle event in which *Dazl* seems to be essential for the entry into meiosis. The presence of *Dazl* in the acrosome region of elongating spermatids might reflect an unknown role of *Dazl* as a morphogenetic factor during spermiogenesis.

Keywords: *Dazl*, meiosis, RNA-binding protein, spermatogenesis

Introduction

Deletions of the azoospermia factor region on the Y-chromosome result in spermatogenic failure and infertility. A putative fertility factor is the recently cloned DAZ (deleted in azoospermia) gene (Reijo *et al.*, 1995). Autosomal homologues of the Y-chromosomal DAZ gene have been cloned from a variety of species in which they appear to be expressed exclusively in the germ cells. DAZ homologues

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have been identified for humans (*DAZL*) (Yen *et al*., 1996), monkey (cyn*DAZL*A) (Carani *et al*., 1997), mouse (Cooke *et al*., 1996), fruitfly (Eberhart *et al*., 1996) and recently in *Xenopus* (Houston *et al*., 1998). Common to all homologues is the presence of two RNA-binding motifs forming the RNA-binding domain and a single DAZ repeat (Saxena *et al*., 1996). The overall homology of the different *Dazl* genes ranging from fruitflies to humans is high, suggesting an essential function during gametogenesis. Interestingly, Y-chromosomal DAZ is present only in old-world monkeys, great apes and humans, whereas it is lacking in all other species (Shan *et al*., 1996; Gromoll *et al*., 1998). According to the current hypothesis on the evolution of the DAZ gene family, human DAZ arose from the autosomal ancestor *Dazl* via a series of structural transformations (Saxena *et al*., 1996; Gromoll *et al*., 1999).

The essential role of autosomal *Dazl* for spermatogenesis has been shown by studies in flies and mice. In *Drosophila* the loss of *boule*, the *Dazl* homologue, results in a meiotic arrest and azoospermia (Eberhart *et al*., 1996). Similar to the fruitfly, disruption of *Dazl* in mice leads to a loss in the number of germ cells and complete absence of spermatogenesis (Ruggiu *et al*., 1997). In the mouse testis, *Dazl* is expressed in the early stages of spermatogenesis, with highest levels in pachytene spermatocytes (Niederberger *et al*., 1997; Ruggiu *et al*., 1997).

Although *Dazl* and *DAZ* encode RNA-binding proteins involved in RNA storage and splicing events, a specific target mRNA has not been described yet. Studying the pattern of expression during spermatogenesis can, therefore, be useful for better understanding of the crucial role of *Dazl* for spermatogenesis. The aim of this study was to evaluate the expression and localization of *Dazl* protein in rat germ cells during the different stages of spermatogenesis. Because the quantitative formation and development of these germ cells in the rat is influenced by the pituitary factors luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Weinbauer & Nieschlag, 1998; for review), we also investigated whether *Dazl* protein expression is under the control of gonadotrophic hormones.

Materials and methods

Animals and experimentation

Adult male Sprague–Dawley rats (Charles River, Sulzfeld, Germany), weighing 220–280 g, were maintained under standard conditions with free access to rat chow and tap water. To evaluate the effects of gonadotrophin withdrawal, animals were treated with the potent GnRH antagonist Cetrorelix (ASTA Medica, Frankfurt, Germany) according to a protocol described previously (Gromoll *et al*., 1997).

Cetrorelix was administered at a daily dose of 112.5 μg/kg, while control animals received the vehicle only (5.25% glucose in saline) for 42 days of treatment ($n = 5$ per group). This experimental paradigm was chosen to eliminate the influence of reproductive hormones and to lower the number of germ cells produced. Testis pieces were fixed by immersion in Bouin's solution for histological evaluation.

The experimental studies were conducted in accordance with the German Federal Law on the Care and Use of Laboratory Animals (animal licence number 72/92).

Preparation of anti-Dazl antibody

Antibodies were prepared in rabbits (Research Genetics, Inc., Huntsville, AL, USA) from a sequence (EVDPGA-DILPNECSVHD) chosen to be specific for the mouse autosomal *Dazl* protein (Reijo *et al*., 1996). Twelve to 20 weeks after the initial injection of peptides, serum antibodies were purified on protein A columns according to manufacturer's instructions (Hi-Trap, Pharmacia Inc., Uppsala, Sweden) and fractions were eluted in 0.1 M sodium citrate, pH 4, and neutralized with Tris-Cl, pH 8.5. Antibodies were pre-absorbed on mouse liver acetone powder to remove non-specific binding activity before use on paraffin-embedded tissues. For assessment of method specificity the primary antibody was replaced by 5% bovine serum albumin (BSA). Antibody specificity was evaluated by pre-incubation of the primary antibody overnight at 4 °C with the synthetic antigenic peptide. For the experiments, antibody dilution was kept constant at 1:400 and a 0.1% peptide (1 mg/mL) solution was added at volumes ranging from 1:1, 2:1, 5:1 and 8:1 (peptide/antibody). On the next day, following washing steps, the immunohistochemical protocol was applied as described above, using the peptide/antibody mixture as primary antibody solution.

Immunocytochemistry

Bouin's fixed specimens were dehydrated, embedded in paraffin, and sectioned at 5 μm for the localization of *Dazl* protein. After deparaffinization and rehydration, the sections were incubated with a rabbit polyclonal antibody at 1:400 dilution for 60 min at room temperature. A second antibody (monoclonal mouse antirabbit Ig, 1:50 dilution) (Dako, Hamburg, Germany) and a third antibody (goat antimouse Ig, 1:50 dilution) (Sigma Chemical Co., St. Louis, MO, USA), followed by incubation with extravidin–peroxidase complex (Sigma Chemical Co.) and diaminobenzidine (DAB) were used for visualization of bound primary antibody.

Sections were rinsed three times, 5 min each, in TBS (0.05 M Tris, 0.15 M NaCl, pH 7.6) between the incubation steps with the antibodies. Antibody buffers contained 0.1% (w/v) BSA. Non-specific binding was blocked with 5% normal goat serum.

Sections were counterstained for 30 sec with Mayer's haematoxylin and mounted. For control studies primary antibody was replaced with 0.1% BSA.

Histological analysis

Spermatogenic stages were classified as described by Leblond & Clermont (1952) from haematoxylin stained sections.

Results

Using *Dazl* antibodies, strong specific immunostaining was detected in the cytoplasm of primary spermatocytes (Fig. 1a,b,d). Thereafter, the signal was absent in diplotene spermatocytes. At higher magnification, *Dazl* immunoreactivity could be detected earliest in the cytoplasm of intermediate and B spermatogonia but not in A spermatogonia (Fig. 1a,b,d). Weak but specific *Dazl* staining was also observed in primary spermatocytes during preleptotene to zygotene stages. Furthermore, weak but specific *Dazl* staining was found frequently in the acrosome region and around elongating spermatids (Fig. 1c). *Dazl* immunoreactivity was always confined to the cytoplasm of the different types of germ cells, whereas Sertoli cells were negative (Fig. 1d). The staining was stage-specific as evidenced by the loss of staining following omission of primary antibody (Fig. 1h) and after pre-incubation of the antibody with the antigen (Fig. 1g). In qualitative terms, staining intensity was highest in stages IV–VIII, declined thereafter, and became undetectable in stages XIII–XIV (Fig. 2).

We have further analyzed tissue sections from GnRH antagonist treated rats in order to see if the depletion of gonadotrophin action changes the expression of *Dazl*. GnRH antagonist treatment abolished reproductive hormone secretion and significantly lowered testicular weights. By day 42 of GnRH antagonist exposure, the seminiferous epithelium consisted mainly of spermatogonia and spermatocytes. More advanced stages were lacking due to the lack of gonadotrophins. *Dazl* expression in treated rats appeared unchanged and was evident in intermediate, B-type spermatogonia and primary spermatocytes (Fig. 1e,f). The level of expression was obviously maintained when compared to control rats (Fig. 1a–d).

Discussion

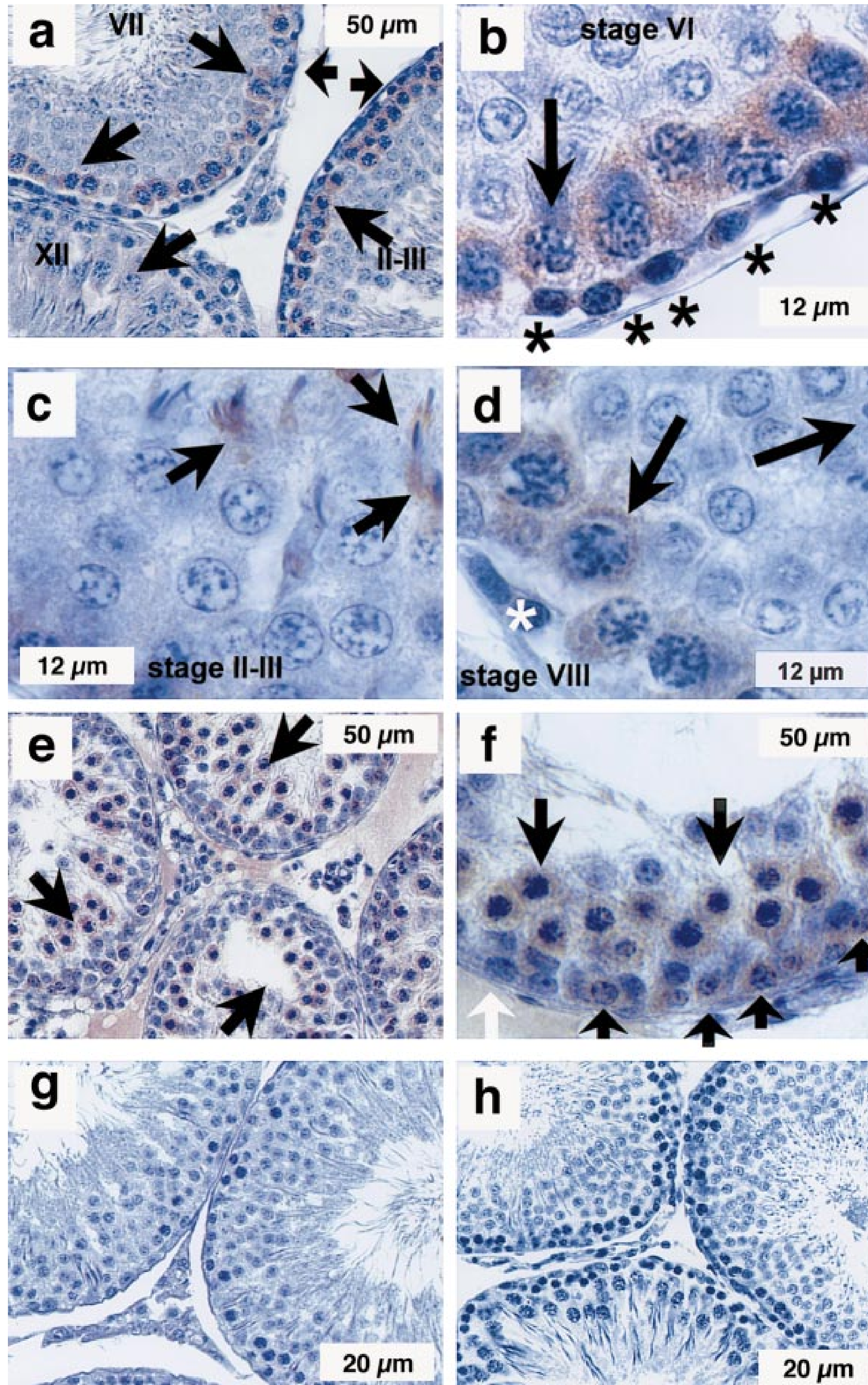
In the current study we investigated *Dazl* expression in the adult rat testis during spermatogenesis. Highest expression of *Dazl* could be localized to the cytoplasm of pachytene spermatocytes where the first meiotic division is initiated. During the pachytene stage a fully formed synaptonemal complex exists which keeps the homologous chromosomes together bivalently and closely tied. The pachytene stage generally persists for approximately 10 days, until desynapsis begins at the diplotene stage, in which the chiasmata are first seen. These events exactly resemble *Dazl* expression levels which are maximal in pachytene stages, when the synaptonemal complex is formed, and absent in diplotene, when desynapsis takes place.

From studies of the *Dazl* homologue (*boule*) in the fruitfly it became evident that loss of function of this gene leads to meiotic arrest (Eberhart *et al*., 1996). Presumably *boule* regulates the processing, stability or translation of factors which are important for the G2/M transition of meiosis I. The *Dazl* expression pattern obtained in the rat (this study) and mouse (Ruggiu *et al*., 1997), in which highest expression was detected in pachytene spermatocytes, would support this hypothesis, indicating a putative role of *Dazl* during the recombination process in vertebrates as well.

Dazl belongs to the group of RNA-binding proteins which are involved in mRNA transcription splicing and storage events. Some of these proteins such as RBM are expressed in the nuclei of spermatogonia and spermatids, suggesting a role in pre-mRNA splicing, polyadenylation or nascent RNA (Elliott *et al*., 1997). The apparent cytoplasmic localization of *Dazl* found in the rat during meiosis, however, indicates a different site of action. In the cytoplasm of meiotic cells *Dazl* might be involved in the regulation of translation by storing, packaging or localizing mRNA. Indeed, it has been shown recently that *boule*, a Drosophila orthologue of *Dazl,* controls the translation of *twine,* a Cdc 25-type phosphatase, which is involved in the transition from G2 to M phase during meiosis (Maines & Wasserman, 1999).

Since no mRNA expression of *Dazl* could be detected by in situ hybridisation in spermatids of the mouse (Niederberger *et al*., 1997), the observed *Dazl* protein expression in the rat, in the acrosome region, might be reminiscent of *Dazl* mRNA expression during the pachytene stages. As the cytoplasm in elongating spermatids is drastically reduced, the level of expression is difficult to determine. Nevertheless, the presence of *Dazl* in spermatids might point to an additional role of *Dazl* during spermiogenesis. From heterozygous *Dazl* knockout mice it is known that one copy of *Dazl* is sufficient to maintain spermatogenesis but causes severe malformations of mature spermatozoa, such as head abnormalities and abnormal tail formation (Ruggiu *et al*., 1997). This indicates a quantitative requirement for *Dazl*, suggesting a role as a morphogenetic factor. A putative new function of *Dazl* could have strong implications for the process of fertilization in which a normal spermatozoon is a prerequisite for successful fertilisation. Taken together, these observations suggest that *Dazl* is crucial for meiosis and also perhaps for spermiogenesis.

From studies in the mouse, it is clear that loss of *Dazl* may cause a reduction in spermatogonial cell number (Ruggiu *et al*., 1997). This indicates a role of *Dazl* in the early development of male germ cells. Indeed, *Dazl* expression can be detected in primordial germ cells and prospermatogonia (Seligman & Page, 1998), when only mitotic divisions occur. This implies that *Dazl* may determine spermatogonia cell number. In our study we did not observe any immunostaining for *Dazl* in type A



spermatogonia. Onset of expression was noted at the time of differentiation of type B spermatogonia. This indicates a shift of *Dazl* function during germ cell maturation from a factor involved in mitotic steps during early germ cell development to an essential factor for the prophase of meiosis I in spermatogenesis, and finally acting as a morphogenetic factor in elongating spermatids during spermiogenesis.

The expression of *Dazl* protein in B-type spermatogonia prompted us to manipulate the gonadotrophin status in the rat. It is known that gonadotrophins are capable of stimulating spermatogonial proliferation in the developing

Figure 1. Micrographs depicting the expression of *Dazl* protein as revealed by immunocytochemistry in the intact rat testis (a-d), and after gonadotrophin withdrawal (e,f) and following control staining (g,h). Sections were counterstained with haematoxylin. The stages of the cycle of seminiferous tubules are marked in Roman numerals. (a) Expression is seen in spermatogonia (short arrows) and spermatocytes (long arrows). Note that *Dazl* expression is absent in pachytene spermatocytes in a stage XII tubule. (b) Pachytene spermatocytes in stage VI are stained (large arrow). Asterisks highlight intermediate to B spermatogonia expressing *Dazl*. (c) *Dazl* expression seen around elongating spermatid heads (arrows). (d) Specific staining in the cytoplasm of spermatocytes (arrow) of a stage VIII tubule. Asterisk is next to an A-type spermatogonium lacking *Dazl* expression. (e) 42 days of GnRH antagonist treatment: spermatogenesis is severely impaired (mainly arrest at spermatocyte level) but *Dazl* expression is retained (arrows). (f) High power magnification from testis exposed to GnRH antagonist for 42 days. Spermatocytes retain the *Dazl* signal (large arrows) as do intermediate to B spermatogonia (small arrows). A-type spermatogonia are negative (white arrow). (g) Pre-incubation of the primary antibody with antigen abolished the staining. (h) Omission of the primary antiserum eliminated the stain.

rat testis (Meachem *et al.*, 1998). This is mainly mediated through the steel factor secreted by the Sertoli cell and acting on the c-kit receptor, which is located on spermatogonial cells (Loveland & Schlatt, 1997). To evaluate whether *Dazl* expression could be regulated by gonadotrophins, rats were rendered hypogonadotrophic by treatment with a potent GnRH antagonist. Although the effects of the treatment are drastic, e.g. a dramatic reduction of tubule diameter, loss of advanced germ cell stages, etc., the staining pattern of *Dazl* was unchanged. *Dazl* expression could be located to the B-spermatogonia and was highest in pachytene spermatocytes (Fig. 1d,f). In further experiments we selectively reduced testosterone production by treatment of rats with ethane-dimethane sulfonate (EDS), a toxic Leydig cell-specific compound, but again the expression pattern was unchanged (data not shown).

The unchanged expression pattern implies that *Dazl* is hormone-independent. Notwithstanding the compelling evidence of the gonadotrophin control of germ cell formation and development, it must be borne in mind that during spermatogenesis gonadotrophic hormones maintain or increase germ cell viability in the rat (Bartlett *et al.*, 1989; El Shennawy *et al.*, 1998). Hence, even after prolonged periods of gonadotrophin deficiency, meiotic cells are present and even some haploid germ cells are occasionally encountered (Clermont & Morgentaler, 1955; Chandolia *et al.*, 1991). The pattern of *Dazl* expression, that is, low expression just prior to and in early meiotic cells and high expression in mid-pachytene stages, suggests that *Dazl* may be necessary for the transition of spermatogonia into the meiotic phases.

In summary, our results show that *Dazl* is up-regulated at meiosis during rat spermatogenesis. Onset of expression starts in B spermatogonia reaching the highest levels during pachytene stages. The expression of *Dazl* is not gonadotrophin-dependent. The presence of *Dazl* in the acrosome region of elongating spermatids points to an unknown role in spermiogenesis, in addition to its strong implications as an essential factor for meiosis.

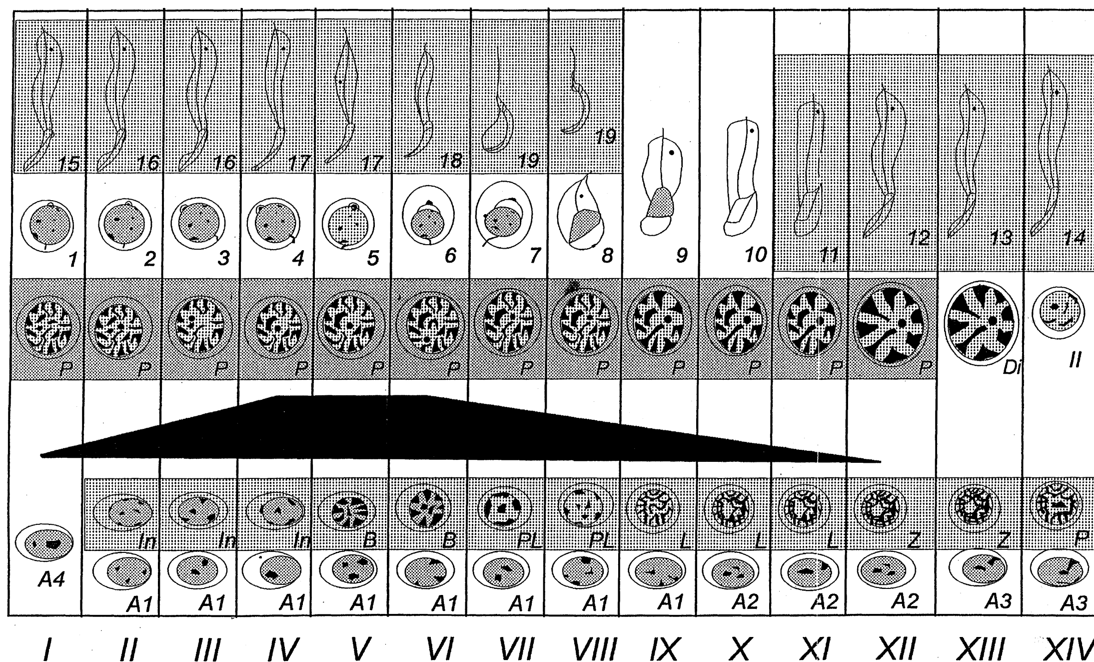


Figure 2. Synopsis of *Dazl* expression in the rat during the different spermatogenic stages showing cellular localization and expression levels. Shaded boxes indicate the germ cell types in which expression was observed. Qualitative intensity of expression levels in pachytene spermatocytes is indicated by the black box.

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