Flagellar mitochondrial association of the male-specific Don Juan protein in
*Drosophila* spermatozoa

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**Summary**

The *Drosophila don juan* gene encodes a basic protein (Don Juan protein), which is solely expressed postmeiotically during spermiogenesis in elongated spermatids and in mature sperm. Transgenic expression of a GFP-tagged Don Juan protein (DJ-GFP) in the male germ line showed an association of the fusion protein with the sperm tail. Detailed examination of DJ-GFP localization revealed novel insights into its distinct temporal and spatial distribution along the sperm tail during the last phase of spermatid maturation. Co-localization of DJ-GFP with actin-labeled cysts demonstrated its emergence in elongated spermatids during individualization. Additionally, the endogenous Don Juan protein was detected with epitope-specific antibodies in finally elongated nuclei of spermatids. After completion of nuclear shaping Don Juan is no longer detectable in the sperm heads with the onset of individualization. Mislocalization of the DJ-GFP protein in flagella of a mutant with defective mitochondrial differentiation provides evidence of mitochondrial association of the fusion protein with flagellar mitochondrial arrays. Ectopically expressed DJ-GFP in premeiotic germ cells as well as salivary gland cells confirmed the capability of the fusion protein to associate with mitochondria. Therefore we suppose that Don Juan is a nuclear-encoded, germ-cell specifically expressed mitochondrial protein, which might be involved in the final steps of mitochondrial differentiation within the flagellum.

Key words: Spermiogenesis, *Drosophila melanogaster*, Nuclear shaping, Mitochondrial protein, Chromatin

**Introduction**

*Drosophila* spermatogenesis provides a useful model system to study several general biological aspects like stem cell determination and renewal, regulation of mitotic and meiotic cell cycle, cytokinesis and cellular and subcellular differentiation processes on the genetic, molecular and cytological level (for a review, see Fuller, 1993). In the past years much progress has been made towards elucidating meiotic cell cycle regulation (for reviews, see Maines and Wasserman, 1998; White-Cooper et al., 1998). In contrast, less is known about molecular processes taking place postmeiotically that govern the morphogenesis of the highly specialized sperm cell. The intensive ultrastructural analysis of postmeiotic spermatid differentiation (so-called spermiogenesis phase) presented by Tokuyasu and coworkers provided an excellent overview about subcellular morphogenetic processes (Tokuyasu et al., 1972a,b; Tokuyasu, 1974a,b, 1975a,b; summarized in Lindsley and Tokuyasu, 1980). In *Drosophila melanogaster*, during a timespan of about 134 hours after meiosis, mature sperm, characterized by their extremely long flagella (about 1.8 mm in *D. melanogaster*), are generated from small round spermatid cells (Lindsley and Tokuyasu, 1980). During this period of postmeiotic male germ cell differentiation, certain morphogenetic processes occur at the subcellular level including, for example, the arrangement and differentiation of the axoneme along with the mitochondrial derivatives to establish the flagellar structure of the sperm tail as well as the formation of the needle-shaped sperm head.

The two mitochondrial derivatives stem from the Nebenkern organelle of early spermatids (onion-stage spermatids), which consist of two large, fused, innerwrapped mitochondrial structures. Recently, the functional role of a cell-type specific mitochondria-associated GTPase (encoded by the *fuzzy onions* gene) in controlling the initial step of postmeiotic mitochondrial fusion has been discovered (Hales and Fuller, 1997). During axonemal growth the two mitochondrial derivatives elongate along the entire length of the flagellum. Then the mitochondrial derivatives undergo several distinct modifications and processes, giving rise to the typical mitochondrial architecture along each flagellum (Tokuyasu, 1974a). Thus action of additional tissue-specific gene products for controlling or participating in the remodeling of this organelle has to be requested. So far neither tissue-specific, nuclear-encoded gene products nor gene loci involved in mitochondrial shaping during late spermiogenesis have been identified.
Intensive studies demonstrating tissue-specific microtubule function led to a better understanding of the assembly of the flagellar axoneme (summarized in Fuller, 1993; Raff et al., 1997; Hutchens et al., 1997 and references therein). In this case, only defined tubulin isoforms such as the testis-specifically expressed β2 tubulin, for example, possess the ability for assembly and function of cell-type specific microtubular arrays (Kaltenschmidt et al., 1991; Raff et al., 1997; Hutchens et al., 1997). Furthermore, it has been shown that axonal microtubules finally undergo post-transitional modifications at the end of sperm cell maturation (Bressac et al., 1995; Bré et al., 1996).

The reorganization of the chromatin into a compact, transcriptionally inactive form accompanied by nuclear shaping provides an additional example of a characteristic postmeiotic differentiation event. In particular, investigation of chromatin reorganization in mammals showed that postmeiotic chromatin packaging is accomplished by replacement of nucleosomal proteins (histones) with either sperm-specific histone variants (e.g. H1t; Doenecke, 1988) or a certain set of specialized basic proteins (transition proteins and protamines; Hecht, 1989; Wouters-Tyrou et al., 1998). For Drosophila this event has only been described cytologically at the cellular and subcellular levels (Tokuyasu, 1974b; Kremer et al., 1986; Cenci et al., 1994; Hennig, 1996; Akhmanova et al., 1997), and Drosophila-specific gene products participating in this process are not known so far. Due to the distinct histone distribution within the male germ line of Drosophila similar processes have also been postulated for Drosophila (Hennig, 1996; Akhmanova et al., 1997).

Recently, we have described the identification of a novel male germ-line-specific protein, Don Juan (DJ) in Drosophila melanogaster (Santel et al., 1997). This 29 kDa protein is characterized by its high lysine content and striking repetitive hexapeptide-motif DPCKKK. Expression analysis of reporter genes revealed a protein distribution confined to postmeiotic germ cells. However, mRNA is already premeiotically detectable, suggesting a negative translational control mechanism that represents a typical regulatory mechanism in spermatogenesis (for a review see Schäfer et al., 1995).

Previous localization of the don juan gene product by expression of a GFP-tagged DJ protein allowed the visualization of the fusion protein along the entire length of elongated spermatids as well as spermatozoa, demonstrating its excellent property as a marker in living flagella. In order to investigate the functional role of the DJ protein during postmeiotic germ cell differentiation and spermatid maturation we extended the analysis of proper temporal and spatial distribution of this gene product in postmeiotic germ cells. Our analysis implies that the DJ protein is a characteristic component of Drosophila spermatids/spERM cells that is associated with the mitochondrial derivatives in flagella of spermatids from the individualization stage onwards; this suggests a specific role for DJ in mitochondrial differentiation during final sperm cell maturation. Additionally, antibody staining localizes the DJ protein in the final shaped nuclei of elongated spermatids.

**MATERIALS AND METHODS**

**Drosophila strains**

The w*, sn* strain was used as wild type for spermatogenesis. The DJ-GFP strain was initially described in Santel et al. (1997). The following strains were used for targeted gene expression experiments: UAS-GFP (established by M. Gonzalez-Gaitan, Göttingen); nanos-GAL4-VP16 (Van Doren et al., 1998), Sgs4-GAL4 (A. Hofmann, unpublished) and UAS-DJ-GFP (this paper; see below). DJ-GFP expression was tested in the male sterile mutant fzo-1 (Hales and Fuller, 1997) and TW-nos(3)2033 (P. Wilson and M. T. Fuller, unpublished).

**Antibodies, immunofluorescence staining and microscopy**

Four independently raised antibodies (made in rabbit by Eurogentec) against selected peptide sequences from the deduced DJ primary structure were used in immunofluorescence stainings. Two independent antisera raised against an oligopeptide derived from the N-terminal part of the DJ primary sequence (KEGNQDELENNMKNEC) are denoted anti-DJ-NI and anti-DJ-NII; two antisera against the repetitive motif from the C terminus (DPCKKKDPCKKKKDPC) are designated as anti-DJ-CI and CII (see Fig. 3A); ‘anti-DJ-C’-labeled stainings refer to the anti-C1 antibody. Immunoglobulins of the IgG-class were recovered from all antisera by protein A-Sepharose (Pharmacia) purification as described by Sambrook et al. (1989). The antibodies were tested by immunoblotting with protein extracts from wild-type adult Drosophila testes (or, as already shown for the anti-DJ-NI in Santel et al., 1997, demonstrating absence of DJ protein in the male sterile mutant boule in contrast to wild type) and partially purified recombinant DJ protein (Fig. 3B). Immunofluorescence stainings of flattened testes contents were carried out essentially as described in Hime et al. (1996). The anti-DJ primary antibodies were incubated in a 1:15 dilution (anti-DJ-NI and NII; 12 mg IgG/ml) and 1:20-1:30 dilution (anti-DJ-CI: 37 mg IgG/ml; anti-DJ-CII: 20 mg IgG/ml, respectively).

**Microscopy, fluorescence analysis of DJ-GFP strains and mitochondrial staining**

Immunofluorescence and GFP samples were examined using a Zeiss Axioshot microscope or a BioRad MRC confocal imaging system connected Zeiss Axioskop microscope (by courtesy of Matthew Scott). Images were individually recorded and processed with Adobe Photoshop 3.0 or pictures were taken using Fuji Sensia color reversal film 400 and subsequently processed after scanning using Adobe Photoshop 3.0. Mitochondria were visualized using Mitotracker Green or Rhodamine123 dye (Johnson et al., 1980). Mitotracker Red CXTos (Molecular Probes) was used for co-localization with DJ-GFP. Staining was performed by incubating freshly prepared tissues with 0.05 μM of reagent in PBS for 30 minutes at 37°C. Actin was visualized by counterstaining fixed testes squashes of the DJ-GFP fly strain with TRITC-Phalloidin (Sigma) according to Fabrizio et al. (1998).

**Western blot analysis**

Western blot analysis was carried out with recombinant DJ protein as well as testes extracts as previously described (Santel et al., 1997). DJ-GFP expression of two independent transgenic fly strains was demonstrated in western blots using a polyclonal anti-GFP antibody (made in rabbit; Clontech; not shown) or anti-DJ antibodies (made in rabbit).

**Construction of UAS-don juan-GFP and ectopic gene expression**

UAS-dj-GFP was generated by inserting the dj-GFP fusion gene as described in Santel et al. (1997) into a BamHI-Xbal opened pUAST (Brand and Perrimon, 1993). Constructs were transformed using P-element-mediated transformation into flies of w-mutant background and independent lines were established. Ectopic expression of UAS-con structs was guided by a nanos-GAL4-VP16 (for expression in early premeiotic male germ cells; kindly provided by Mark Van Doren; Van Doren et al., 1998) and Sgs4-GAL4 (for driving expression in salivary glands; A. Hofmann, unpublished). Tissue-specific GAL4 driving gene expression was examined by using the
UAS-GFP reporter strain prior to ectopic dj-GFP gene expression. Crosses were kept at 18°C and 24°C, respectively.

RESULTS

Germline-specific expression of the DJ-GFP fusion protein labels late spermatids and spermatozoa: analysis of late spermatid maturation

Exact localization of a novel gene product is a prerequisite for elucidating protein function within the appropriate tissue. We have previously examined the DJ-GFP fusion protein expression under the control of its natural regulatory sequence in order to determine the temporal expression of the male germ line-specific DJ protein (Santel et al., 1997). This analysis showed that the dj-mRNA is translationally repressed in premeiotic as well as early postmeiotic cells. We extended the analysis of DJ-GFP expression in these transgenic strains to obtain a better insight into its cellular distribution. Germ line-specific expression of a GFP-tagged DJ-protein, under the control of the same dj-promoter region, demonstrates the restricted labeling of elongated spermatids (Fig. 1A). Furthermore, DJ-GFP is still detectable in the mature sperm cells (see filled seminal vesicles; Fig. 1A, arrow).

We analyzed the subcellular distribution of the DJ-GFP protein in elongated spermatids in greater detail. Fig. 1B is a close-up view of single parallel-arranged flagella of DJ-GFP-expressing spermatids. Here we used confocal microscopy for better resolution of subcellular structures. As reported earlier (Santel et al., 1997), the DJ-GFP protein appears to be lined up like single ‘pearls’ along a ‘fine thread’. The confocal picture clearly demonstrates that distinct structures are labeled by the fluorescent fusion protein. The pearl structure is arranged in a fairly constant pattern in connection with the GFP-labeled ‘thread structure’. Labeling of spermatid chromatin by staining testes with Hoechst 33258 or propidium iodide after fixation (Fig. 1C, red) identifies the spermatid stage and clarifies that DJ-GFP (green) is clearly absent from sperm heads (Fig. 1C). Therefore Fig. 1C illustrates that DJ-GFP is exclusively expressed in its characteristic pattern in elongated spermatids when nuclear shaping has already been completed (see below; Fig. 2).

Fig. 1. Dynamic distribution of the DJ-GFP protein during spermatid maturation. GFP-fluorescence was directly examined in living tissue using either epifluorescence microscopy (A,F,G) or confocal microscopy (B-E). (A) dj-driven DJ-GFP expression definitely begins in elongated spermatids and is stably incorporated into sperm flagella located in the seminal vesicle (small arrow). Distinct distribution of DJ-GFP exhibits a ‘pearl’- and ‘thread’-like structure (B). (C) The fusion protein is clearly absent from elongated spermatid heads (red, chromatin). (D) DJ-GFP arranges in rows in the middle of the cystic bulge of individualizing spermatids (arrow). (E) DJ-GFP is present in motile sperm (double-headed arrow). The ‘pearl-structure’ can only be found in flagella of elongated spermatids (arrow) and is absent from mature sperm flagella (double-headed arrow). (F) DJ-GFP labeled sperm are also detectable in the female reproductive organ where the sperm are stored in the receptaculum seminis (RS) and the two spermathecae (Sp). (G) DJ-GFP misdistribution in the male sterile mutant TW-ms(3)2033. (H) Phase contrast micrographs of spermatid flagella (arrows).
After completion of the post-elongation phase, the syncytial spermatid cyst is separated into individual sperm cells by insertion of new plasma membranes. This process is reflected at the cellular level by the emergence of a bubble-like structure called the cystic bulge (Tokuyasu et al., 1972a; Lindsley and Tokuyasu, 1980). This structure (Fig. 1D) migrates from the spermatid heads onwards caudally along the flagella of the cyst, collecting excessive organelles and cytoplasm and generating new separating membranes (see below for more details; Fig. 2). The DJ-GFP protein aligns in a few rows at the most expanded site of the cystic bulge where individualization takes place (Fig. 1D, arrow; Tokuyasu et al., 1972a). At the distal as well as apical end of the cystic bulge, DJ-GFP distribution seems to be unaffected. In addition, the thread structure also remains fluorescent during cystic bulge migration. Hence the DJ-GFP has to be incorporated into certain flagellar structures and is not withdrawn from excessive cytoplasm during individualization. However, as shown in Fig. 1E, the flagellum of mature sperm (double-headed arrow) does not exhibit the previously described 'pearl structure' that is observed for elongated and individualizing spermatids (arrow). Therefore we conclude that the dot-like pattern indicates an intrinsic structure of elongated spermatids during individualization, which is lacking in the flagellum of mature sperm. However, it is still unclear when and how the dotted pattern will be withdrawn from the flagella. Thus, we suppose that after individualization an additional process is responsible for removal of this structure.

The spermatozoa contain DJ-GFP and these sperm are still capable of fertilization. Thus GFP-labeled sperm are still located in the receptaculum seminis as well as spermathecae of inseminated females, where sperm cells are usually stored prior to fertilization (Fig. 1F). In addition it is possible to detect the fluorescent sperm tail arranged in its characteristic three-dimensional pattern (Karr, 1991) in the fertilized egg, as recently shown with the DJ-GFP-expressing fly strain in the course of paternal effect gene analysis by Fitch and Wakimoto (1998). Furthermore, incorporation of the DJ-GFP-protein has no effect on motility, male fertility as measured by fertility tests (not shown) or embryonic development.

To underline the marker function of the DJ-GFP strain, we analyzed DJ-GFP expression and distribution in the homozygous background of certain male sterile mutants, which show no obvious defects in flagellar elongation and differentiation on the cellular level. For example, the uncharacterized male sterile mutant TW-ms(3)2033 (P. Wilson and M. T. Fuller, unpublished) exhibits flagella with no obvious differences in length and shape as compared with wild-type flagella (Fig. 1H). In contrast to DJ-GFP distribution in the wild type, DJ-GFP is localized in local aggregations of enlarged 'pearl-structures' in the TW-ms(3)2033 mutant (Fig. 1G).

The DJ-GFP protein expression starts at the onset of spermatid individualization

As shown in Fig. 1, expression of the DJ-GFP fusion protein in transgenic animals begins in elongated spermatids. Spermatid elongation is followed by a phase for final spermatid maturation (post-elongation) and sperm individualization lasting approximately 50 hours (Lindsley and Tokuyasu, 1980), during which spermatids are morphologically indistinguishable at the light microscopic level. Detailed analysis of DJ-GFP expression in elongated spermatids revealed that reporter-expressing cysts as well as reporter-lacking cysts are present in testes of those transgenic fly strains. Therefore we aimed to determine the exact time of expression of DJ at the end of spermiogenesis by using the DJ-GFP strain. In order to determine the germ cell differentiation stage, DJ-
GFP expression was firstly assessed by examining the nuclear shape in elongated spermatids. Chromatin was visualized by counterstaining testes squashes with the dye Hoechst 33258. Fig. 2 shows different cysts with spermatids that are already fully elongated, but which differ in their nuclear shape. While the cyst carrying nearly completely elongated nuclei (Fig. 2A, B; arrow) does not exhibit DJ-GFP expression along the flagella, elongated spermatids with needle-shaped nuclei (double-headed arrows) clearly show the characteristic DJ-GFP protein distribution (green), which is depicted in a higher magnification in Fig. 2B. Hence DJ-GFP expression appears solely in cysts with their nuclei finally shaped, which are at the end of the post-elongation phase. After nuclei have reached their final shape, investment cones originate in the head region where excess nuclear envelope and cytoplasm are displaced caudally, a process that characterizes the beginning of individualization. These investment cones contain numerous microfilaments and demark the trailing edge of the cystic bulge (Lindsley and Tokuyasu, 1980). Actin staining with TRITC-Phalloidin was performed in order to recognize cysts of elongated spermatids at the individualization stage. As recently reported, actin is a component of the individualization complex (Fig. 2C) that is responsible for resolving the syncytial cyst to individual sperm cells after the post-elongation phase (see above; Tokuyasu et al., 1972b; Lindsley and Tokuyasu, 1980; Fabrizio et al., 1998). Fig. 2C shows on the left side a cyst in which the IC is labeled in the spermatid head region (starting IC formation), whereas the right cyst exhibits an IC transversing along the flagella (compare with corresponding Hoechst-staining; Fig. 2D). Comparison of DJ-GFP expression (Fig. 2E) with the appearance of actin-labeled individualization complexes revealed that DJ-GFP expression is first observed in individualizing cysts when the ICs are located at the spermatid head region (Fig. 2C). Hence we conclude that DJ-GFP (and DJ; see below) expression starts in elongated spermatids with the onset of the individualization phase. Therefore DJ-GFP serves as additional marker for elongated spermatids of the individualization stage.

**Immunofluorescence shows DJ localization along the flagella as well as in spermatid heads**

A computer-based analysis of the DJ primary structure for potential subcellular localization predicts a high chance (88%) of locating this protein in the mitochondrial matrix (PSORT, Nakai and Kanehisa, 1992; Fig. 3A, blue letters). We employed anti-DJ-antibodies to ascertain the localization of the GFP-tagged DJ protein in the transgenic assay and to exclude artificial localization due to the GFP-tag. The properties of the antibodies raised against two selected domains (N-peptide, C-peptide) from the DJ primary structure are shown in Fig. 3 (see Materials and methods for details). In western blots the generated antibodies recognize the recombinant DJ protein as well as the DJ-GFP fusion protein in testes extracts from transgenic animals (Fig. 3B, shown with anti-DJ-NI as an example). Subsequently the antibodies were used to carry out immunofluorescence studies on squashed adult testes. In elongated spermatids two structures are predominantly decorated. On the one hand the flagella are decorated in the way

![Fig. 3. Detection of DJ in the flagellum and sperm heads with immunofluorescence.](image-url)

(A) The primary structure of the DJ-protein bears an internal mitochondrial localization site (blue letters) located adjacent to a single predicted protein cleavage site (arrow). The entire lysine-rich C-terminal half of the protein carries several nuclear localization signals (underlined in green; the DJ-characteristic hexapeptide motif is shown in red). Antibodies were raised against two selected peptides derived from the N- and C-terminal parts of the protein (blue underlined). (B) The specificity of the anti-DJ antibodies used was tested in western blots (here shown with anti-DJ-NI). The anti-DJ-antibodies specifically recognize the recombinant protein (left panel) as well as the DJ-GFP protein in testes extracts derived from DJ-GFP-expressing transgenic animals in comparison to equal amounts of extracts from wild-type flies (right panel, double-headed arrow; endogenous DJ in testes-extracts, arrows; asterisk indicates unspecific cross reaction; all antibodies give identical results in western blots). (C, D) Confocal pictures of immunofluorescence analysis with different anti-DJ-antibodies revealed localization of the DJ protein in the flagella (C; characteristic dot structure indicated by arrows) and additionally in the heads (D) of elongated spermatids.
observed for the DJ-GFP distribution in live testes (Fig. 3C), on the other hand the sperm heads were labeled (Fig. 3D). While the antibody raised against the repetitive hexapeptide motif of the C terminus recognizes both the head and tail structures of elongated spermatids (Fig. 3C), the antibodies against the N-terminal peptide preferentially recognize epitopes of the sperm head (Fig. 3D). Further immunofluorescence analysis revealed that the DJ protein is localized in a fairly dynamic manner during the final phase of spermatid maturation, when the sperm head undergoes its ultimate shaping. Sperm head elongation is accompanied by nuclear transformation: the spermatid nucleus finally gets its compact, needle-shaped form. The DJ protein co-localizes with spermatid chromatin at the end of post-elongation when nuclear transformation occurs (Fig. 4A-C). In contrast, flagellar structures are faintly stained in this stage. After finishing nuclear transformation the spermatid chromatin is characterized by its needle-like structure (compare Fig. 4B with E). The DJ protein is undetectable in the nucleus of this spermatid stage (Fig. 4D-F) and therefore no longer co-localizes with the chromatin (Fig. 4F). However, we cannot rule out that we fail to detect DJ protein in the nuclei of further stages due to accession problems of the antibodies.

Interestingly, the DJ protein now appears in its known pattern along the flagella, as observed with the GFP-tagged DJ (Fig. 4D). Therefore we suppose that the DJ protein is only used in the final phase of nuclear transformation before expression in the flagella. In summary, the localization of the DJ protein as well as DJ-GFP revealed a distinct subcellular distribution of this protein during the final spermatid maturation phase, at the transition from post-elongation to individualization stage.

**DJ-GFP distribution is affected in the male sterile mutant fuzzy onions, which has defects in postmeiotic mitochondrial differentiation**

The stable incorporation of the DJ-GFP fusion protein in the flagellum implies that the DJ protein might be a component of either the axoneme or the mitochondrial derivatives, which are the two major structures in the mature flagellum. To investigate subcellular association with one of those two major flagellar structures, we looked at DJ-GFP expression and distribution in certain male sterile mutants exhibiting distinct postmeiotic defects. Initial experiments with mutants bearing defects in axonemal arrangement revealed no differences in DJ-GFP expression and distribution compared to the wild type, suggesting a potential association of the DJ-GFP fusion protein with mitochondrial derivatives. Considering the predicted mitochondrial localization (see above) we focused on DJ-GFP expression analysis in mutants with defects in the assembly or differentiation of the mitochondrial derivatives. Recently, the male sterile mutant *fuzzy onions* (*fzo*) has been described with defects in postmeiotic mitochondrial differentiation at the ultrastructural level (Hales and Fuller, 1997). Examination of DJ-GFP distribution in *fzo* mutants (Fig. 5B) compared with wild type (Fig. 5A) clearly demonstrates severe mislocalization of the DJ-GFP protein along the elongated flagella. Contrary to the distinct arrangement of the DJ-GFP protein in wild type (Fig. 5A), the fusion protein is localized in a dispersed manner.

Since aberration in the correct distribution of the DJ-GFP protein in the flagellum is a feature of male sterile mutants with known distinct defects in mitochondrial morphogenesis (rather than of mutants with a defective axoneme), we conclude that DJ-GFP is mislocalized in the *fzo* male sterile mutant, which shows characterized defects in mitochondrial morphogenesis. The characteristic dotted distribution of the GFP-tagged DJ protein (A) is disrupted in the *fzo*-mutant (B), where its distribution is dispersed.
DJ-GFP is localized in the mitochondrial derivatives of the flagellum.

**Ectopic DJ-GFP expression in premeiotic germ cells reveals association of the fusion protein with mitochondria**

The localization experiments described above suggest that there is a differential distribution of the DJ protein between the two cellular compartments, nucleus and mitochondria. Considering the observed mislocalization of the DJ-GFP fusion protein in the fzo-mutant and its predicted mitochondrial localization, we focused on this possibility in the flagellum. To assess the capability of this protein to reside in mitochondria we performed targeted ectopic DJ expression studies using the GAL4-UAS-system (Brand and Perrimon, 1993). For this purpose we established transgenic lines carrying the dj-GFP reporter fusion gene fused to the GAL4 inducible UAS-hsp70-promoter (constructs UAS-dj-GFP). In order to evaluate the effect of premature DJ expression during spermatogenesis, we aimed to direct dj-GFP gene expression in premeiotic germ cells.

Therefore a GAL4-driver line was established which directs premeiotic GAL4-expression in spermatocyte phase (A. Santel and R. Renkawitz-Pohl, unpublished results). However, while GAL4 expression was switched on, no premeiotic gene expression was observed after crossing the GAL4-driver line with appropriate reporter strains (using UAS-lacZ or UAS-GFP; data not shown). Alternatively, we used the nanos-GAL4-VP16 fly strain (Van Doren et al., 1998) driving transcription factor GAL4-VP16 expression in a nanos-corresponding manner. Besides its expression during oogenesis and embryogenesis (Van Doren et al., 1998), the nanos promoter also directs gene expression during spermatogenesis, as revealed by looking at nanos-controlled GFP-reporter expression (UAS-GFP). As shown in Fig. 6A, GFP distribution is restricted to a subset of early premeiotic germ cells such as stem cells and spermatogonia, and is faintly visible in early primary spermatocytes. In these cells, the GFP protein is

![Fig. 6. Premeiotic DJ-GFP expression revealed an association of the protein with mitochondria.](image)

(A,B) DJ-GFP was premeiotically expressed using the nanos-GAL4-VP16 driver and directly monitored in living testes (right, GFP reporter fluorescence; left, corresponding phase contrast view). Nos-GAL4-VP16 is active during spermatogenesis in a subset of early germ cells, as visualized by using a UAS-GFP reporter strain (A) in comparison to ectopically expressed DJ-GFP distribution in premeiotic germ cells (B). (C) Subcellular localization of the DJ-GFP protein shows an association with mitochondrial structures in polar primary spermatocytes (arrows) and growing primary spermatocytes (double-headed arrows). (D,E) Spread testis contents showing that the DJ-GFP remains stably associated with mitochondrial structures of postmeiotic germ cells like the Nebenkern in onion-stage spermatids. Some prematurely expressing DJ-GFP spermatids of the onion stage appear misshapen in cell size and Nebenkern formation (D, double-headed arrows; compare with spermatids without any obvious defects, arrows; black sphere. Nebenkern; white sphere, nucleus). (E) Single Nebenkern spermatids are shown close up.
uniformly distributed. In comparison to premeiotic GFP-expression, the corresponding nanos-GAL4-VP16 driven DJ-GFP expression reveals that the fusion protein is exclusively located in the cytoplasm. Additionally, the DJ-GFP protein appears to be stably incorporated into cellular structures due to the persistence in later cell stages of spermatogenesis (primary spermatocyte growth phase as shown in Fig. 6B), when nanos-GAL4-VP16 expression has already been stopped (Fig. 6A).

The incorporation into subcellular structures is clearly supported by the following observation presented in Fig. 6B. After premeiotic expression the DJ-GFP fusion protein is specifically targeted to mitochondrial structures within the differentiating germ cells. In young primary spermatocytes mitochondria aggregate in a distinct fashion at the opposite side of the nucleus giving rise to dark spherical structures (polar primary spermatocytes; Fuller, 1993). Fig. 6C clearly demonstrates the association of the fusion protein with the cluster of aggregated mitochondria in the polar primary spermatocyte stage (Fig. 6C, arrows). During the following growth phase of the primary spermatocytes the mitochondrial aggregation disintegrates (Fuller, 1993), which can be followed by the distinct labeling of the mitochondrial structure with the DJ-GFP protein (Fig. 6B). Since the DJ-GFP protein was associated with mitochondria, the fusion protein is still detectable postmeiotically. Therefore characteristic mitochondrial structures like the Nebenkern of onion-stage spermatids are definitively decorated (Fig. 6D,E; arrows). However, the fusion protein was never observed in the nucleus of any cell stage. Consequently it seems that premeiotic DJ-GFP distribution reflects the same subcellular localization as proposed from the postmeiotic DJ-GFP expression analysis resembling the restriction to mitochondrial compartments (see above). We assume that DJ-GFP is preferentially located in mitochondrial structures, whereas it is absent from nuclear arrays, thus confirming our hypothesis.

Then we looked at possible effects on cell fate and differentiation assigned to premeiotic DJ-GFP expression. Males expressing DJ-GFP premeiotically produce motile sperm and are still fertile (not shown). Only a few germ-cell cysts exhibit minor aberrations in cell shape differentiation (see Fig. 6D, double-headed arrows, compare to normally shaped cells, arrows) and degenerated material in the testis tube was observed, too.

**Ectopically expressed DJ-GFP in salivary glands is associated with mitochondria**

Pre- and postmeiotically expressed DJ-GFP fusion protein is proposed to associate exclusively with mitochondria. To determine whether DJ-GFP can associate with mitochondria as well as nuclear structures (presumably with chromatin, as predicted from the immunofluorescence analysis of DJ), we examined ectopically expressed DJ-GFP in salivary gland cells. Salivary gland cells have some advantages for assaying subcellular protein localization and possible chromatin association, due to their large cytoplasm and polytene chromosomes. To induce tissue-specific DJ-GFP expression in salivary glands we used the Sgs4-GAL4 driver line (A. Hofmann; unpublished). GFP-fluorescence in unsquashed salivary glands after ectopic expression is shown in Fig. 7. Using the UAS-GFP reporter strain the cytoplasm and the nucleus are uniformly labeled (Fig. 7A). In comparison to that, the DJ-GFP distribution pattern appears to be more dynamic. The fusion protein is spread in a reticulated pattern in the cytoplasm with an obvious concentration around the nucleus (Fig. 7B). The Sgs4-promoter drives a high expression of DJ-GFP, so it is not clear whether the occasional DJ-GFP appearance in the nucleus is due to this extraordinary expression level, as nuclear localization is also observed with UAS-GFP lines. The import into mitochondria, however, has always been observed. Ectopic expression of the
The dot-like appearance of the fusion protein in the cytoplasm resembles the pattern of dispersed mitochondria, as observed after staining salivary glands with specific mitochondria dyes (Mitotracker; Rhodamine 123, not shown). To confirm this assumption we counterstained DJ-GFP-expressing salivary glands with the Mitotracker Red dye (Molecular Probes), specifically labeling mitochondria. Double labeling clearly shows co-localization (Fig. 7E, yellow) of the DJ-GFP (Fig. 7C, green) with mitochondria (Fig. 7D, red). These results suggest that DJ-GFP is also capable of associating with mitochondria outside the male germ-line, indicating that DJ represents a nuclear-encoded, tissue-specifically expressed mitochondrial protein.

DISCUSSION

Several morphogenetic processes are necessary during differentiation and establishment of certain structures within a mature spermatozoon. However, besides some structural components of the axoneme (e.g. β-tubulin, dynein; Fuller, 1993), tissue-specific gene products which might be involved in those processes are not known in Drosophila. The differential subcellular localization of the testis-specifically expressed DJ protein implies that this protein is probably engaged in maturation of elongated spermatids during spermiogenesis in Drosophila. Using two independent approaches for subcellular protein localization, we were able to show a preferential localization of the DJ protein in mitochondria besides its association with spermatid heads at the end of the post-elongation phase.

DJ is a nuclear-encoded mitochondrial protein specifically expressed in the male germ-line

Several lines of evidence indicate that the location of the DJ-GFP protein along the spermatid/sperm flagellum is due to its localization in the mitochondrial derivatives. Firstly, GFP-tagged DJ protein was found to be mislocalized along the flagella of male sterile mutants with postmeiotic mitochondrial differentiation defects. Secondly, misexpression of the DJ-GFP protein in premeiotic germ cells obviously displays its association with mitochondria and thirdly, co-localization with mitochondria is seen after ectopic expression in salivary gland cells. Thus, we propose that the DJ-GFP protein is capable of localization to mitochondria. These observations are consistent with the predicted computer protein localization (PSORT, Nakai and Kanehisa, 1992), which indicates a putative localization of the DJ protein to the inner mitochondrial space. Interestingly, the first nine N-terminal amino acid residues resemble a mitochondrial signal peptide sequence that has recently been identified in trypanosomes (Häusler et al., 1997). Mitochondrial localization potential is supported by the prediction that the putative internal mitochondrial targeting signal resides directly contiguous to a potential protein cleavage site. This observation suggests that the first 17 residues are probably a mitochondrial leader peptide. Regarding the proposed mitochondrial localization of the DJ protein, the observed dynamic changes in DJ-GFP distribution during the final steps of post elongation and individualization phases might reflect alterations in mitochondrial structures. In other words, the presence of the ‘pearl structure’ in conjunction with the ‘thread’ structure visualized by the DJ-GFP protein in elongated spermatids might be a result of the two types of mitochondrial derivatives (minor and major mitochondrial derivatives), that undergo final shaping during the individualization phase, accompanied by the reduction of the minor mitochondrial derivative (implied by the disappearance of the dotted structure in mature spermatozoa; Tokuyasu et al., 1972a). In conclusion, the DJ-GFP represents in general a useful marker for labeling flagellar mitochondrial derivatives as well as mitochondrial arrays in living cells of other tissues outside the male germ line. Concerning any speculated DJ protein function specific to final mitochondrial stages, DJ-GFP has no obvious effect on mitochondrial differentiation fate in other cells, but partners necessary for the proposed DJ function might not be available. Further analysis will focus on potential interactions with other (preferably mitochondrial) proteins.

Possible roles of DJ in the nucleus of elongated spermatids

In contrast to the localization of the DJ-GFP protein in the flagellum and its absence from the nucleus of elongated spermatids, DJ-specific antibodies detect the DJ protein in both structures. There are several possible reasons why the DJ-GFP is not found in the nucleus of elongated spermatids. It is, for example, plausible that the fused GFP-tag at the C terminus of the protein masks specific protein domains preventing the tissue-specific import into the nucleus. In particular, the lysine-rich C-terminal domain consists of several predicted nuclear localization signals (Fig. 3A). Therefore it is possible that the DJ protein, besides its mitochondrial association, also resides in the nucleus. In addition the DJ primary structure shares certain structural features characteristic of linker histones. Computer analysis revealed that, for example, the N-terminal part of the primary structure contains some peptide stretches that are capable of forming α-helices and, consequently, a globular domain on the tertiary structural level. The N terminus is followed by the lysine-rich C terminus. A similar general structure can be found in linker histone variants (for a review see Wolffe et al., 1997) like the histone H1 protein or, more obviously, the avian histone H5 variant (Thomas and Wilson, 1986). This idea of the DJ participation in nuclear functions would also explain the initial finding of the DJ protein as a DNA-binding protein (Santel et al., 1997). Taking into account the time-restricted localization of DJ in elongating sperm heads, it can be speculated that DJ probably acts as a transition protein during the last phase of spermatid chromatin condensation, as it has been shown in mammals (Hecht, 1986; Wouters-Tyrou et al., 1998). For Drosophila no chromatin proteins equivalent to mammalian transition proteins and protamines have so far been described. In addition, it has been reported that a sea urchin histone H1 has been found along the flagella of spermatozoa, with a distribution resembling that of DJ-GFP (Multigner et al., 1992).

Dual subcellular localization and dual functional role of proteins

Consistent with the computer prediction for a nuclear and mitochondrial DJ localization we were able to show the DJ association with both subcellular structures in differentiating
spermatids. The mitochondrial location was also confirmed by ectopically expressed DJ-GFP in salivary glands. Thus the DJ-GFP can be imported into mitochondria of somatic cells, probably due to presence of the predicted import signals. However, it may not fulfill its function, since interacting partners of the specialized mitochondria in spermatids are presumably missing. Therefore, its localization suggests that DJ may perform several functions during spermatid maturation. Differential subcellular localization of certain gene products has recently been shown for the basic zinc-finger protein, basonuclin (Yang et al., 1997). This protein has unexpectedly been found in different subcellular structures of differentiating spermatocytes/spermatids like the centrosome, mitochondria and acrosome in mouse (Yang et al., 1997). A germ cell-specific hexokinase has also been reported to reside in mitochondria as well as in the head and fibrous sheath of murine spermatozoa (Travis et al., 1998). A possible function for Don Juan as a specific chromatin protein has been discussed above. Concerning its mitochondrial localization, a similar function can be considered. It is known that nuclear-encoded DNA-binding and chromatin-associated proteins are imported into mitochondria. Outside Drosophila, nuclear-encoded histone H1- and HMG-like proteins in trypanosomatids and yeast have been reported as ‘mitochondrial chromatin’ components or mitochondrial transcription factors, respectively (Paris and Clayton, 1991; Megraw and Chae, 1993, Xu et al., 1996). Interestingly, the testis-specific human isoform of the mitochondrial transcription factor A (h-mtTFa) has been speculated to be involved in regulating the mtDNA copy number in male germ-cells (Larsson et al., 1997). Similar functional roles can also be attributed to the DJ protein. Regulation of mitochondrial morphogenesis within the flagellum is presumably controlled by communication between the nucleus and the differentiating mitochondrial derivatives, therefore the participation of nuclear encoded proteins in this regulation has to be requested. Future analysis will provide insight into the properties of DJ as a putative mitochondrial and DNA-binding protein, basonuclin (Yang et al., 1997). This protein has been speculated to be involved in regulating the mtDNA maturation. Differential subcellular localization of certain gene products or mitochondrial transcription factors, histone H1- and HMG-like proteins in trypanosomatids and yeast have been reported as ‘mitochondrial chromatin’ components or mitochondrial transcription factors, respectively (Paris and Clayton, 1991; Megraw and Chae, 1993, Xu et al., 1996). Interestingly, the testis-specific human isoform of the mitochondrial transcription factor A (h-mtTFa) has been speculated to be involved in regulating the mtDNA copy number in male germ-cells (Larsson et al., 1997). Similar functional roles can also be attributed to the DJ protein. Regulation of mitochondrial morphogenesis within the flagellum is presumably controlled by communication between the nucleus and the differentiating mitochondrial derivatives, therefore the participation of nuclear encoded proteins in this regulation has to be requested. Future analysis will provide insight into the properties of DJ as a putative mitochondrial and nuclear chromatin protein and its possible contribution to mitochondrial morphogenesis during spermatogenesis.

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