Laminin in the Male Germ Cells of *Drosophila*

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Abstract. To study genes that may be crucial for the male germ cell development of *Drosophila* we screened a cDNA expression library with a polyclonal antiserum against testis proteins of *Drosophila hydei*. We identified a cDNA fragment that exhibited a complete sequence similarity with the cDNA of the laminin B2 chain, an important component of the extracellular matrix. Transcripts of laminin B2 were detected in the RNA of male germ cells with the polymerase chain reaction and by in situ hybridization. We studied the reaction of different polyclonal antibodies including those against a *Drosophila* laminin B2-lac fusion protein, the entire *Drosophila* laminin complex, or against the mouse laminin complex and against laminin A and B1 chains with specific structures in developing male germ cells of *Drosophila*. Antigenic sites against laminin B2 were found in the lampbrush loops in primary spermatocyte nuclei, in nuclei of spermatids, and in heads of spermatozoa. The axonemes of elongating spermatids react with antibodies against the *Drosophila* laminin B1, B2 and laminin A chains. The possible biological functions of the laminin in the male germ cells of *Drosophila* are discussed.

LAMININ is a large glycoprotein complex representing a major component of the extracellular matrix (ECM) in both vertebrates and invertebrates. As a protein complex with multiple structural domains, laminin appears to fulfill diverse biological functions such as mediating muscle and epithelial cell differentiation, or directing the outgrowth of neurites (Kleinman et al., 1985; Lander et al., 1985; Montell and Goodman, 1988; Timpl 1989). Like its vertebrate counterpart, *Drosophila* laminin also consists of three covalently linked subunits, the laminin A, B1, and B2 chains with apparent molecular masses of 400, 215, and 185 kD, respectively (Fessler and Fessler, 1989). Both Southern blot analysis of *Drosophila melanogaster* genomic DNA done in our laboratory and the in situ hybridization of larvae polytene chromosome with laminin probes confirmed that laminin is a single copy gene in the genome as was reported by other authors before (Montell and Goodman, 1988).

Previous research revealed a differential expression pattern of laminin in the developing tissues of *Drosophila*. In brief, *Drosophila* laminin is transcribed from 6–8 h of development onwards, i.e., from the beginning of the germ band extension stage; the cellular laminin mRNA content increases rapidly to reach the peak value at 9 h, followed by a dramatic decrease to a level below detection. In contrast, the storage of laminin protein in the developing embryo increases continuously towards a steady state value in larvae, pupae, and adult flies (Garzino et al., 1989). Immunostaining of embryos with antibodies against laminin locates the glycoprotein to those tissues covered by a basement membrane, like gut, nerve cord, and brain.

The adult testis of *Drosophila* is in its major part ensheathed by two-layered epithelial cells. Inside the testis tube, where spermatogenesis takes place, germ cells at different developmental stages (e.g., spermatogonia, primary and secondary spermatocytes, young spermatids, and mature spermatozoa) are aligned sequentially in *D. hydei* (Hennig and Kremer, 1990) while in *D. melanogaster* the different cell types are distributed in groups along the testis tube (Lindsley and Tokuyasu, 1980). In our current investigations, we have concentrated on laminin B2, which is expressed in the testis of *Drosophila*. It is localized not only along the epithelial wall of the testis tube (where the basement membrane is deposited), but also in the nuclei of primary spermatocytes and young spermatids, and in the heads and axonemes of elongated spermatids. In the axoneme, also laminin A and B1 can be detected. Our results clearly showed that laminin is an intracellular constituent of *Drosophila* male germ cells. To our knowledge, this is the first report of laminin as an intranuclear constituent.

Materials and Methods

The cDNA library constructed from adult *D. melanogaster* mRNA in the λgt11 expression vector was provided by Dr. H. Saumweber (Universität Köln, Köln, Germany), the antiserum raised against *D. hydei* testis proteins was prepared in our laboratory. We used wild type strains of *D. melanogaster* Canton S and *D. hydei* from our lab collection. Mutant stocks used to produce Y chromosome aneuploids were kindly provided by Dr. D. L. Lindsley (University of California, San Diego, La Jolla, CA), and Dr. G. Reuter (Martin-Luther-Universität, Halle-Wittenberg, Germany).

Screening the cDNA Library

The λgt11 cDNA library was screened with a polyclonal antiserum against

D. hydei testis proteins by the plaque screening methods according to the procedures of Huynh et al. (1985) and Mierendorf et al. (1987) except that PBS (0.137 M NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, and 7 mM Na_2HPO_4, pH 7.4) was used instead of TBS. NBT and BCIP were purchased from Sigma Chemical Co. (St. Louis, MO) and Serva, and alkaline phosphatase conjugated goat-anti-rabbit IgG was from Jackson Laboratories Inc. Immuno Research (Avondale, PA).

DNA Sequencing

The original λgt11 cDNA insert DmLk11-44 was first subcloned into the plasmid vector pGEM™-4 (Promega Biotec, Madison, WI), resulting in a recombinant plasmid denominated as pDmLk11-44. The insert was then subcloned into M13mp18. The cDNA obtained by PCR amplification was first double digested with ClaI and HincII, and then cloned into M13mp18. Single-stranded templates were isolated following the protocols published by Amersham (1984). Sequencing was performed by the dideoxy chain termination method of Sanger et al. (1977). Homology analysis of the sequences to the *D. melanogaster* laminin B2 subunit was accomplished by the computer program FASTA (Devereux et al., 1987).

Hybridization Techniques

For Northern analysis, RNA was extracted from frozen tissues, electrophoresed on formaldehyde agarose gels, and blotted onto Hybond-N (Amersham International, Amersham, UK) according to Jowett (1986). Blots were hybridized with ^{32}P-labeled probes at 42°C in 50% formamide, 5× SSC, 5× Denhardt's solution, 1% SDS for at least 16 h, and afterwards washed under highly stringent conditions (0.1× SSC at 68°C). As marker, a BRL RNA ladder was run on the same gel, blotted and stained with methylene blue for molecular weight determinations.

For Southern analysis, cDNA products from PCR amplification were extracted with chloroform, electrophoresed on normal agarose gels, and blotted onto Hybond-N membranes according to Sambrook et al. (1989). The hybridization of the blots was done according to the Amersham protocols (1985) with normal washing conditions (2× SSC and at 68°C).

In both Southern and Northern hybridizations, nick-translated DmLk11-44 was used as a probe.

PCR Amplification of Laminin B2 cDNA Fragments

RNA used for reverse transcription was extracted from embryos, testes, and male germ cells, respectively, with the hot-phenol method according to Jowett (1986). Before the PCR reaction, DNase I treatment of the RNA has been carried out to remove contaminations with genomic DNA.

cDNA synthesis was carried out according to Sambrook et al. (1989). For amplification, the cDNA was first extended at 72°C for 40 min, followed by 40 cycles of amplification using a step program (94°C, 2 min; 50°C, 2 min; 72°C, 3 min) and a 15-min final extension at 72°C. To exclude that amplification was from contaminating genomic DNA, PCR amplification was also carried out without previous reverse transcription using the same batch of RNA. In this case, no amplification products were found.

Generation and Purification of Polyclonal Antiserum

Fusion protein composed of bacterial β-galactosidase and the COOH-terminal portion of *Drosophila* laminin B2 was produced in *E. coli* strain Y1089 and isolated according to the procedures described previously (Mierendorf et al., 1987). Purification of the fusion protein was carried out on preparative SDS-polyacrylamide gels as described by Laemmli (1970). After electrophoresis, the gel region containing the fusion protein was excised, lyophilized, and ground into fine powder for injection (Harlow and Lane, 1988). The antiserum was raised in rabbits according to Harlow and Lane (1988). The antiserum, denominated as K44, has been affinity purified with the laminin B2-fusion protein blotted on the PVDF membrane (Millipore Corp., Bedford, MA).

Western Blotting

Protein samples from *Drosophila* testes or carcasses were prepared as described before (Hulsebos et al., 1984). To obtain embryonic proteins, 0–15-h embryos were collected on yeast, rinsed with tap water and 0.9% NaCl solution, and homogenized in the sample buffer (Laemmli, 1970). After 10 min of denaturation in boiling water, the protein mixture was fractionated by SDS-PAGE. Proteins were electro-transferred onto PVDF membrane following the procedures from Gueltekin and Heermann (1988) with small modifications (using transfer buffer without methanol). To isolate the spermatocytes of male flies, testes from young males (1–3 d) were dissected and their envelopes were punctured with a forceps. The outflowing germ cells were collected with a siliconized Pasteur capillary pipette under microscopic control. These germ cells were treated as the other protein samples with Laemmli sample buffer. The separation of the proteins from germ cells was carried out on ultrathin, covalently glass-bound SDS-polyacrylamide gels (Boxberg et al., 1990). After electrophoresis, proteins in the gel were blotted onto nitrocellulose membrane by capillary diffusion (Boxberg et al., 1990).

For immunostaining, the blots were preincubated with 5% BSA in PBS solution at 37°C for 1 h, and then incubated at room temperature with K44 diluted in PBS containing 0.05% Tween-80 for 2 h. Alkaline phosphatase conjugated goat-anti-rabbit IgG was 1:5,000 diluted in the same buffer and used for detecting the first antibodies bound on the Western blots. After 1 h of incubation at room temperature, the blots were developed in NBT/BCIP solution (Blake et al., 1984).

Immunofluorescence and Immuno-alkaline Phosphatase Staining

For testis tissues, procedures from Hulsebos et al. (1984) were followed with some modifications. Briefly, testes from 2- to 3-d-old males were collected in a drop of testis buffer on a microscopic slide. The tissues were gently squashed under the cover slip and frozen immediately in liquid nitrogen. After the cover slip is removed, the slide can be stored in 96% ethanol until used. The tissues were fixed in 3% paraformaldehyde/PBS solution at room temperature for 10 min, and afterwards extensively washed with PBS. The preparations were first incubated with antilaminin antiserum at 4°C overnight, washed in several changes of PBS, and then incubated with fluorescein-labeled or alkaline phosphatase-labeled goat-anti-rabbit IgG (1:40 diluted) at room temperature for 1 h in the dark. Alkaline phosphatase was detected after developing in NBT/BCIP solutions as above. The slides were studied under a Zeiss Photomicroscope III with epifluorescence equipment or in phase contrast as described previously (Hulsebos et al., 1984). Photographs were taken on Agfapan 400 Professional film.

The preparation of fixed whole embryos was according to Mitchison and Sedat (1983) with some modifications. After fixation, the devitellinized embryos were collected from the 100% methanol and rehydrated in a series of decreasing alcohol steps. For whole mount staining, the first and the second antibody incubation were carried out in tissue culture wells. The other conditions are the same as for the immunostaining of testis squashes.

For immunoreactions, we used (i) rabbit-anti-*Drosophila* laminin B2 antiserum (K44) obtained against the fusion protein; (ii) rabbit-anti-*Drosophila* laminin complex antiserum from Fessler et al. (1987); (iii) rat-anti-*Drosophila* laminin A antiserum from C. Henchcliffe (University of California, Berkeley, Berkeley, CA); (iv) rat-anti-*Drosophila* laminin B1 antiserum from Dr. L. Fessler (Los Angeles), (v) rabbit-anti-mouse laminin antiserum from Klein et al. (1988), as well as (vi-vii) two different rabbit-anti-mouse laminin antisera from Sigma Chemical Co. (St. Louis, MO) (L 9393) and BRL. For the detection of rabbit-anti-*Drosophila* laminin complex antiserum we used an alkaline phosphatase-conjugated secondary antibody, the other antisera were detected with FITC-labeled secondary antisera (Jackson Immuno Research Laboratories Inc.).

Immunogold Electronmicroscopy

Testes of *D. melanogaster* (1–7-days old) were isolated in testis isolation medium (Hennig, 1967), washed in PBS and fixed in 2% glutaraldehyde or 3% paraformaldehyde or a 2% mixture of both in PBS overnight on ice. After dehydration with ethanol the testes were infiltrated with Lowicryl K4M (Roth) according to the user's protocol, and transferred in gelatine cups at −18°C. The resin was polymerized by UV irradiation. Ultrathin sections were made on a LKB 8800 Ultrotome and collected on formvar-coated nickel grids (200 mesh). For the immunological detection of antigens all steps of the procedure were carried out on a 30-μl drop of the respective solutions. The first antibodies were used with the following concentrations: Rabbit-anti-mouse laminin serum (Klein et al., 1988), rat anti-*Drosophila* laminin A antiserum and mouse anti-*Drosophila* laminin B1 antiserum: dilution 1:100 in PBS1; K44 serum, i.e. rabbit anti-*Drosophila* laminin B2 antiserum and rabbit anti-*Drosophila* laminin complex antiserum: dilution 1:1,000 in PBS1.

The incubations were carried out for 1 h in a moist chamber after floating the grids for 5 min on PBS and PBS1 (= PBS + 1% BSA + 0.1% Tween 20). After two washes with PBS1 and two washes with PBS2 (PBS + 0.1% BSA + 0.1% Tween 20), each for 10 min, the second antibody reaction was

carried out for 1 h in a moist chamber. The second antibody was 10-nm gold-labeled goat-anti-rabbit IgG (Amersham International, Amersham, UK), goat-anti-rat (Jackson Immuno Research Laboratories) or goat-anti-mouse IgG (Amersham International), respectively, diluted 1:10 in PBS2. Grids were rinsed with PBS2, floated twice for 10 min on PBS2, and three times for 5 min on distilled water.

The tissue sections were contrasted with uranyl acetate for 4–5 min, rinsed with distilled water for 15 s, and contrasted with lead citrate for 45 s. After a last rinse with distilled water for 15 s, they were dried and studied with a Philips EM201 electron microscope.

In Situ Hybridization

In situ hybridization was carried out on whole mount testis or testis squashes. Squashes were prepared as described for the immunofluorescence studies. All incubations except those indicated were carried out at room temperature. We designed the whole mount in situ hybridization protocol on the basis of the methods of Hemmati-Brivanlou et al. (1990) and Tautz and Pfeifle (1989).

To label the probes, we used the DIG-RNA labeling mixture from Boehringer Mannheim (Mannheim, Germany) in combination with the T3/T7 RNA polymerase transcription kits from Stratagene (Palo Alto, CA). The two complementary RNA strands were synthesized from the appropriate pBluescript II KS+ inserts of various cDNA clones from laminin B2 gene. In all experiments the DIG-labeled probes corresponding to the anti-mRNA strand or to the mRNA were used separately for hybridization.

Results

Laminin Is Expressed in Drosophila Testes

To study genes coding for proteins important for the development of the male germ cells of *Drosophila*, we screened a cDNA library, constructed from adult *D. melanogaster* mRNA in the λgt11 expression vector, with a polyclonal antiserum prepared against testis proteins of *D. hydei*. One of the cDNA clones recovered (designated as DmLk11-44) attracted our particular interest as sequencing revealed a complete homology of the cloned insert to a 3′ region of the cDNA encoding laminin B2 subunit of *D. melanogaster* (see Fig. 2) (Chi and Hui, 1988). We studied the expression of this gene in testis with the methods summarized in Table I.

Presence of Laminin mRNA in Testes: Northern Blots and PCR Reactions. DmLk11-44 was used to probe the Northern blots extracted from *D. melanogaster* embryos (0–15 h) and from testes of young male flies. But after hybridization, laminin B2 transcripts were only detected in the embryo RNA pool (Fig. 1 *A*). The size of the transcript is 5.8 kb, which is in accordance with the molecular mass of laminin B2 chain (M_r 185,000). Previously, the expression of all the three subunits of laminin gene during the embryonic development of *Drosophila* has been investigated by Northern hybridization (Montell and Goodman, 1988). According to that report, the major increase of the mRNA for laminin occurs after gastrulation, with a maximum expression between 6–9 h. From 15 h onwards, the transcriptional expression of laminin in embryos decreases drastically and no evidence for laminin transcription in adult flies was obtained by these early studies. Thus, we assumed that the amount of mRNA for laminin in testes as well as in other adult tissues is too small to permit detection by Northern blot assays.

As a more sensitive approach to detect transcripts, the PCR was applied. The two primers were chosen according to the published cDNA sequence of *D. melanogaster* embryonic laminin B2 chain (Chi and Hui, 1988): 5′ amp (5′-GCCATCGATTTGCAGCT-3′) and 3′ amp (5′-GTCAGGTTGACGCGATT-3′) (Fig. 2). Supposing that this isoform of the laminin B2 subunit from embryos is also present in testes of flies, we expected to see a specific cDNA fragment 1.0 kb in length after amplification. The PCR products were exam-

Table I. Overview of the Experiments Done to Confirm Laminin Expression in Male Germ Cells

Antiserum	Origin	Reaction
Immunology		
Drosophila		
Laminin complex	Dr. L. Fessler	Axoneme, nuclei
Laminin B1	Dr. L. Fessler	Axoneme
Laminin A	Dr. C. Henchcliff	Axoneme
Laminin B2, fusion protein	University of Nijmegen	Axoneme, nuclei
Mouse		
Laminin complex	Dr. G. Klein (Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Tübingen, Germany)	Axoneme, nuclei
Laminin complex	Sigma Biochemicals	Axoneme, nuclei
Laminin complex	BRL	Axoneme, nuclei
Western blots		
Drosophila laminin B2 fusion protein	University of Nijmegen	185-kD band in hand-isolated germ cell extracts
Nucleic acids		
PCR amplification of laminin B2 mRNA	University of Nijmegen	with RNA from hand-isolated spermatocytes (sequenced)
In situ hybridization with cDNA probes of laminin B2	University of Nijmegen	
(anti-sense strand)		in male germ cells up to individualization
(sense strand)		no reaction

In this table, only experiments with isolated germ cells are summarized. Experiments with total testis RNA or protein are not indicated. Antisera are all polyclonal. In situ hybridization: Only anti-mRNA strand reacts in the cytoplasm.

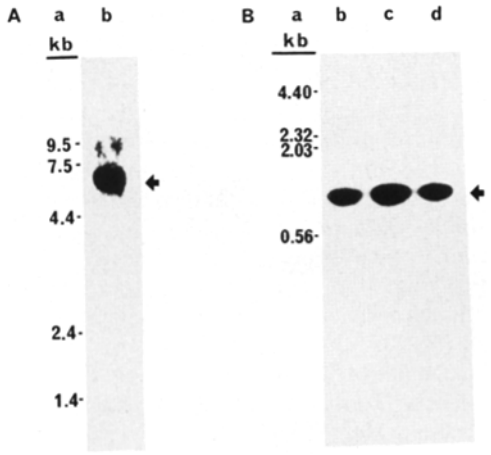

Figure 1. Transcription of the laminin B2 gene in *Drosophila* embryos and testis of adults. (*A*) Northern hybridization of total RNA isolated from 0–15-h *D. melanogaster* embryos with ^{32}P-labeled DmLkl1-44 as probe (lane *b*). The arrow indicates the 5.8-kb mRNA for laminin B2. RNA size markers (from BRL) are at the left side (lane *a*). (*B*) Southern analysis of the PCR amplification product from *D. melanogaster* embryo (lane *b*), testis (lane *c*), and male germ cell RNA (lane *d*). For hybridization, the same probe is used as in *A*. The 1.0-kb fragment expected from the amplification is indicated by the arrow. Lane *a* represents DNA size markers.

ined by Southern blot analysis using DmLkl1-44 as a probe for hybridization. The result showed that the expected 1.0-kb cDNA fragment was amplified from both embryo RNA and from adult testis RNA (Fig. 1 *B*). This was verified by sequencing of this fragment after cloning. Controls to exclude DNA contaminants were carried out as described in Materials and Methods.

PCR Reactions with RNA of Manually Isolated Germ Cells. In the next step, we addressed the question whether the male germ cells express laminin or whether laminin transcripts are restricted to only the somatic epithelial cells of the testis wall. Thus, RNA was isolated from spermatocytes, collected manually under microscopic control, and analyzed with the PCR procedure and the same primers as described before. The results showed that in male germ cells of *D. melanogaster*, laminin was transcribed since a PCR product of the expected length was found (Fig. 1 *B*). The PCR product was cloned and sequenced. Sequencing data confirmed the complete identity between the embryonic laminin B2 mRNA and its counterpart in the male germ cells, at least within this selected region (positions 3960–4984).

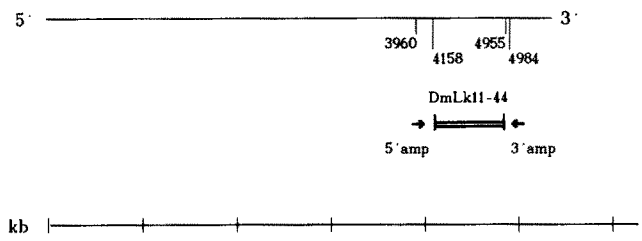

Figure 2. The relative position of the two PCR primers within the laminin B2 mRNA. Both 5′ amp and 3′ amp primers are 17 nucleotides in length (positions 3960–3976 and 4968–4984, respectively), and flank the cDNA clone DmLkl1-44. The direction of the arrow indicates the direction of cDNA extension from the primer during the polymerase chain reactions.

Localization of Laminin mRNA by In Situ Hybridization. To further confirm the expression of laminin B2 in male germ cells and to determine the cellular stages of its expression we carried out in situ hybridization experiments either with whole mount testes or with testis squashes. As DIG-labeled probes we used T7 and T3 RNA polymerase transcripts of cDNA fragments cloned in pBluescript II KS+. Both antisense and sense probes were hybridized separately to whole mount testes or squash preparations. The results obtained are identical in both squashed testes and whole mounts. Hybrids from the antisense probes were found in spermatogonia, primary spermatocytes and spermatids up to individualization with the highest signal in spermatocytes. In elongating spermatids the signal is very faint and in the cystic bulge only a low hybridization signal is observed indicating the presence of some residual mRNA, which is removed during the individualization process. In Fig. 3 *a* we show hybridization with spermatocytes that were hybridized with the anti-mRNA probe. A strong signal occurs in the cytoplasm which is not obtained with the mRNA-like probe (Fig. 3 *b*). Fig. 3 *c* demonstrates the low signal from the anti-mRNA probe in the cystic bulge, which removes residual cytoplasm and membranes from the elongated spermatids. These observations show that the laminin B2 gene becomes active in spermatogonia, but has its highest level of expression in primary spermatocytes. The label in postmeiotic cells must be due to stabilized laminin B2 mRNA since no transcription occurs after meiosis (Hennig, 1967). In addition, label is detected in the testis envelope as expected from the presence of a basement membrane.

The results of this section are summarized in Table I.

Laminin B2 Is Detected in Drosophila Testis by Immunoreaction on Western Blots

Since the transcripts of laminin B2 were found in *Drosophila* developing male germ cells, the laminin B2 peptide also is likely to be present. Antiserum against the fusion protein consisting of a truncated bacterial β-galactosidase and of a COOH-terminal fragment of *Drosophila* laminin B2 (encoded by DmLkl1-44) (see Fig. 2) was raised in rabbits. The specificity of the antiserum, denominated as K44, was controlled by two kinds of experiments: (*a*) K44 was checked on a Western blot of protein extracts from the embryos where laminin is highly expressed. The result showed a specific reaction between the antibody and one protein species with an electrophoretic mobility of M_r 185,000. According to the previous reports (Fessler et al., 1987; Montell et al., 1988), this protein fraction represents the B2 subunit of laminin and is translated from the 5.8-kb transcript described before (Fig. 4 *A*). (*b*) If embryos, which had developed for 11–12 h, were immunostained with K44 and a secondary antibody conjugated to FITC, many tissues characteristically covered by a basement membrane, like nerve cord, brain, and gut, etc., gave strong signals in the fluorescence microscope. Fig. 5 *b* shows one of the examples. This result is in agreement with the immunostaining patterns of *Drosophila* embryos with the antilaminin antibodies described by Fessler and Fessler (1989). Thus, K44 can be used as a serum specifically detecting the *Drosophila* laminin B2 subunit.

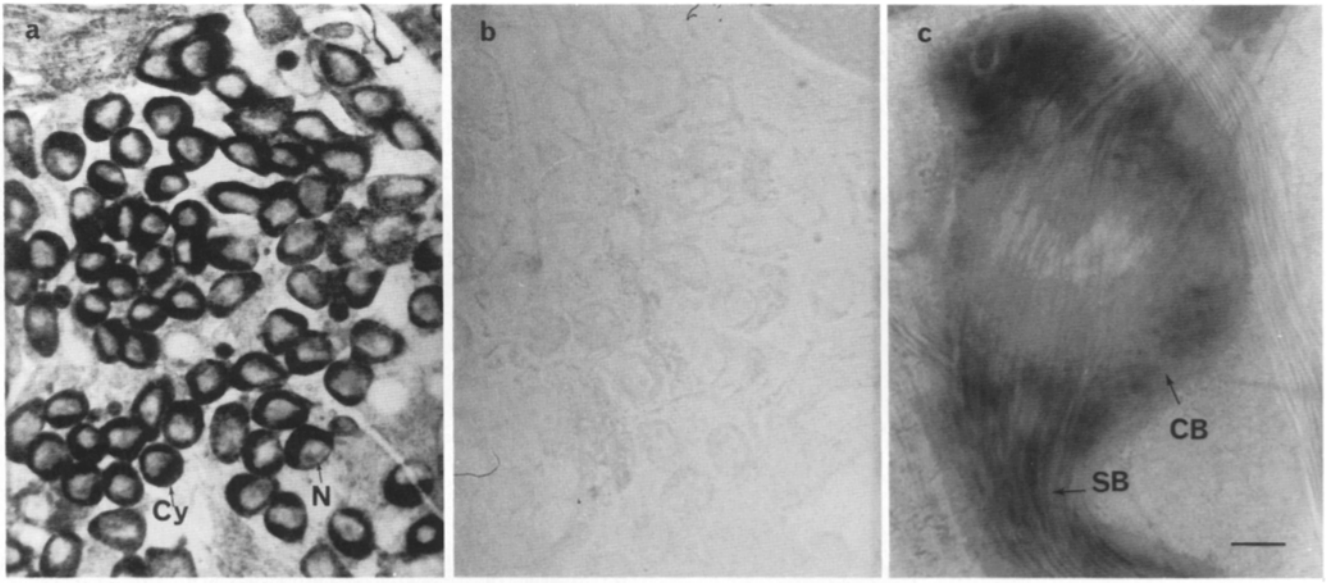

Figure 3. In situ hybridization of testis squashes with DIG-labeled laminin B2 cDNA probe. When the anti-sense probe is used, the hybridization to transcripts is obtained in the cytoplasm of primary spermatocytes (*a*) and all subsequent developmental stages up to the individualization. The cystic bulge (*c*) removes residual RNA molecules. In parallel experiments with a sense-strand probe these signals are not obtained (*b*). Hybrids are detected with anti-DIG-antibody AP conjugates and an alkaline phosphatase reaction with NBT/BCIP. Cy, cytoplasm; N, nucleus of a primary spermatocyte; CB, cystic bulge; SB, spermatiden bundle. Bar, 20 μm.

We applied the antiserum K44 to immunostaining the Western blots of protein extracts from both testes and carcasses of flies. In both cases, a protein fraction in size corresponding to laminin B2 was detected by the serum (Fig. 4 *A*, lanes *c* and *d*). Parallel blots stained with preimmune serum from the same rabbit only gave weak and nonspecific background (Fig. 4 *A*, lanes *e* and *f*).

To further confirm the location of the polypeptide chain in developing germ cells, spermatocytes from young males were manually collected under microscopic control in the lysis buffer (Laemmli, 1970) and loaded on a SDS-polyacrylamide gel. The Western blots of the samples were immunostained with K44. As shown in Fig. 4 *B*, in this way the presence of laminin B2 in the male germ cells of *Drosophila* is also demonstrated.

These conclusions on the expression of laminin B2 in male germ cells were further confirmed by the indirect immunofluorescence assays, as well as by immunoelectronmicroscopy as described below.

The results of this section are summarized in Table I.

Intracellular Localization of Laminin in Drosophila Male Germ Cells

The cellular localization of laminin in testis of *Drosophila* can be revealed by the indirect immunofluorescence or immuno-alkaline phosphatase staining assays on testis squashes. Our experiments were carried out by using several kinds of polyclonal antisera: (*a*) K44 raised against the *Drosophila* laminin B2 COOH-terminal fragment; (*b*) antiserum against *Drosophila* laminin complex (Fessler et al., 1987); (*c*) anti-*Drosophila* laminin B1 antibody (Fessler et al., 1987); (*d*) anti-*Drosophila* laminin A antibody (obtained from C. Hentchcliff, University of California at Berkeley, see Montell and Goodman, 1988); (*e*) antisera against the mouse laminin complex from Klein et al. (1988); or (*f* and *g*) from two commercial sources (Sigma Chemical Co. and BRL).

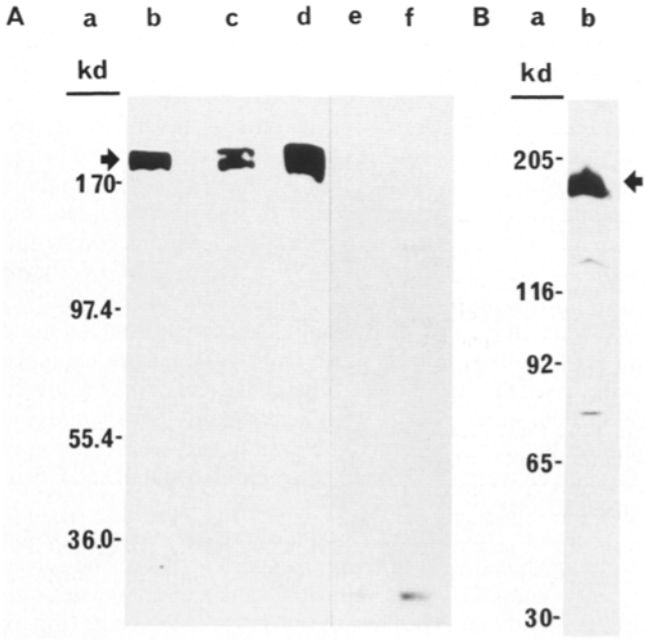

Figure 4. Western blots stained with antibodies specific for the laminin B2 subunit. (*A*) Protein extracts from *D. melanogaster* 0–15-h embryos (lane *b*), adult testis (lanes *c* and *e*), and adult carcasses (lanes *d* and *f*) are separated on a 3-mm SDS-polyacrylamide gel and transferred to PVDF membrane. The blot is stained with purified K44 serum (lanes *b–d*) or preimmune serum (lanes *e* and *f*). (*B*) Protein extracts from the male germ cells are fractionated on an ultrathin (0.1 mm) SDS–polyacrylamide gel and blotted to nitrocellulose filter. The blot was immunostained with the same antiserum as in *A*. In all sample lanes, the M_r 185,000 fraction (see *arrows*) is decorated by the antibodies. In both *A* and *B*, the protein size marker is presented in lane *a*.

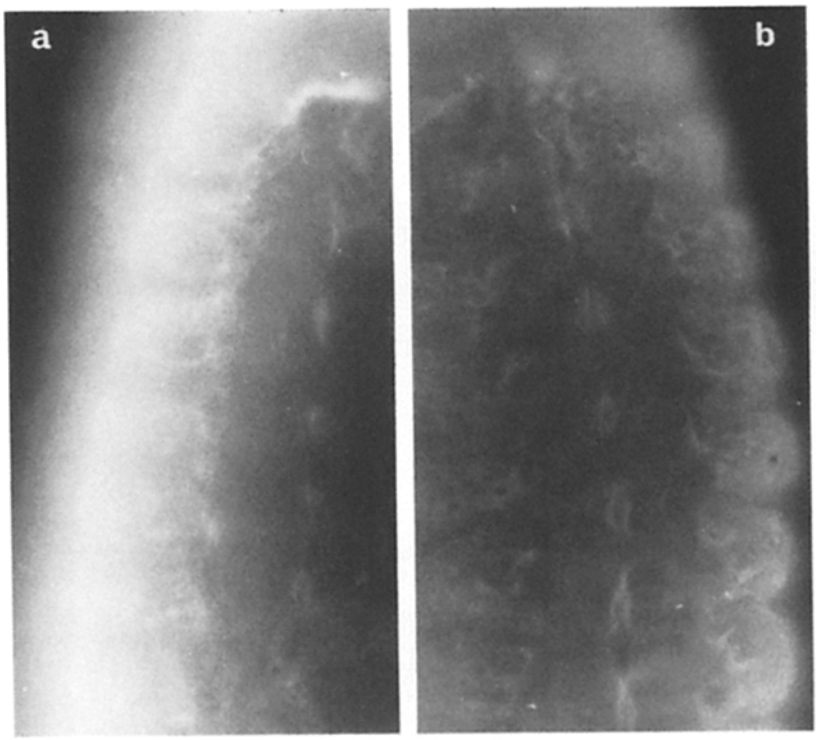

Figure 5. Whole embryos stained with antilaminin antibodies. 11–12-h embryos were immunostained first with antimouse laminin antibodies (*a*), and anti-*Drosophila* laminin B2 antibodies (*b*), respectively, and then with FITC-conjugated secondary antibodies. In both cases, the focal plane is approximately at the interface of the nerve cord.

All antisera against laminin B2 and against the whole laminin complex gave essentially the same reactions. The properties of K44 have already been described. The specificity of the reaction with *Drosophila* laminin of the antisera raised against the murine laminin complex, has been verified by immunoprecipitation which showed that *Drosophila* laminin can be specifically precipitated by the antisera (data not shown). The same antisera have also been checked by immunofluorescence experiments where *Drosophila* embryos (11–12 h whole mount) were stained. One of the results is presented in Fig. 5 *a*. The specificity of the antisera is similar to K44 as they stained those embryonic tissues equipped with basement membranes. The consistent results achieved with both kinds of antisera, those against *Drosophila* and mouse laminin, confirmed the evolutionary conservation of laminin between murids and *Drosophila* (Montell and Goodman, 1988).

To locate laminin in *Drosophila* testis, squashes of testes were incubated with either of the above antisera, and subsequently with FITC- or alkaline phosphatase–conjugated secondary antibodies. Besides the envelope of the testis (Fig. 6, *g* and *h*), which contains a basement membrane and is expected to be stained by the antisera, developing male germ cells were specifically decorated as well. The nuclei of primary spermatocytes (Fig. 6, *a* and *b*) and the head regions of bundles of elongated spermatids (Fig. 6, *e* and *f*) reacted with the antibodies against laminin B2 (i), as well as with the antibodies against the laminin complex (ii and iii). Within the nuclei of primary spermatocytes, the signal is not uniformly distributed, but distinct structures were stained. In the tails of elongating and mature sperm, a weak fluorescence was noticed which was just above the background. In contrast, the preimmune sera only gave very weak and unlocalized background (Fig. 6, *c* and *d*). Autofluorescence of testis tissue has never been observed.

Reactions were also carried out with antisera against the laminin A and B1 chains. With these antisera only a weak fluorescence was obtained on the tails of elongating spermatids and sperm, but not in spermatocyte or spermatid nuclei.

It is well known that in *D. melanogaster* primary spermatocytes, the activity of Y chromosome is accompanied by the formation of lampbrush loops (Meyer et al., 1961). Three loops have been defined and denominated as loop A, B, and C (Bonaccorsi et al., 1988). Although the biological implication of Y chromosomal loops is still open to speculation (Hennig, 1988; Hennig and Kremer, 1990), findings that those loops contain relatively large amounts of protein suggest that the loop-forming DNA and its associated transcripts may provide a structural framework for a compartmentalized accumulation of proteins involved in the developmental processes during or after meiosis (Bonaccorsi et al., 1988; Grond, 1984; Hennig, 1985, 1988; Hennig and Kremer, 1990; Hulsebos et al., 1984). The distribution of the antigens recognized by the antibodies towards laminin in primary spermatocyte nuclei strongly suggested an association with one or several of the lampbrush loops (see Fig. 6, *a* and *b*). This result induced an analysis of some mutants with deletions for one or several of the loop-forming regions on the Y chromosome (Fig. 7). Although males carrying such Y deficiencies are sterile, their spermatogenesis proceeds far into the postmeiotic development and is normally defective before or during the individualization of the elongated spermatids. Our observations showed that developmental abnormalities resulting from deficiencies in the Y chromosome are reflected in modified reactions of male germ cells with the laminin antisera at various stages. We noticed that in wild type primary spermatocytes, loop A is the prominent loop recognized by the antibodies. But in some mutants with Y deficiencies, including the loop A-forming region, the anti-

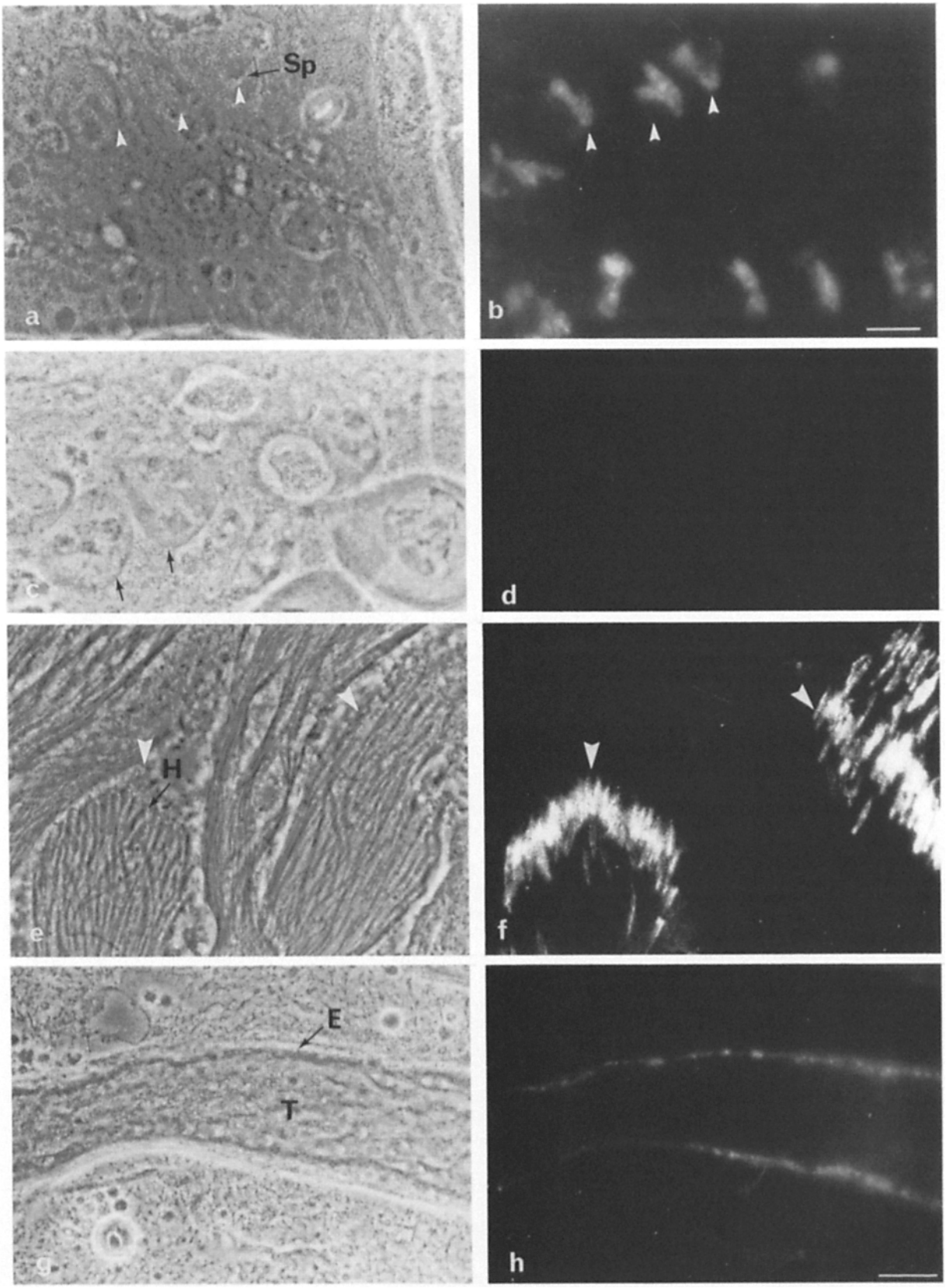

Figure 6. Localization of laminin in the male germ cells of *D. melanogaster* as visualized by indirect immunostaining. Testis squashes from young males are first incubated with the antilaminin antibodies (*a*, *b*, *e*, *h*) or preimmune serum (*c* and *d*), and subsequently with FITC-labeled goat-anti-rabbit IgG. Spermatocyte nuclei (*a* and *b*) and the head regions of elongating spermatids (*e* and *f*), display reactions with laminin antiserum. (*a*, *c*, *e*, *g*) Phase contrast; (*b*, *d*, *f*, *h*) fluorescence photographs. Arrowheads indicate identical positions in the phase-contrast and fluorescence pictures Arrowheads in *a* and *b* point to the lampbrush loops within spermatocyte nuclei. Arrowheads in *e* and *f* point to spermatid heads of the bundles. Sp, primary spermatocyte nuclei; H, heads of spermatids; T, testis tube; E, epithelium of testis tube. Bar, 10 μm.

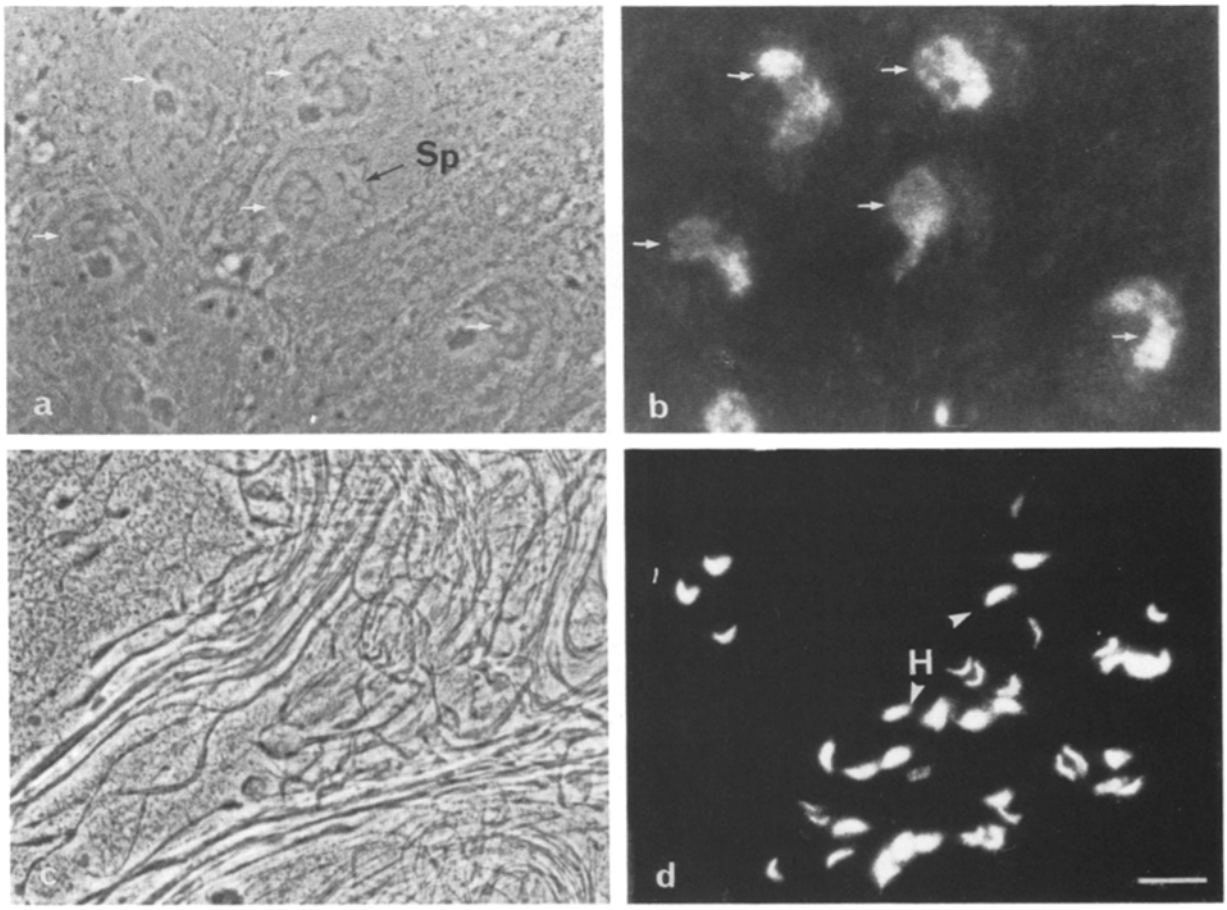

Figure 7. Distribution of laminin in the germ cells from two of the mutants with Y deficiencies. All the mutated tissues are treated in the same way as described for Fig. 6. *a* and *c* are phase-contrast photographs; *b* and *d* are corresponding immunofluorescence micrographs. (*a* and *b*) Spermatocyte nuclei of X/Df(Y)S11 males (see Gatti and Pimpinelli [1983] for the details of their genotype) which indicates a possible association of laminin with other loop-forming region(s) on the Y chromosome when loop A is deficient. (*c* and *d*) Spermatids with deformed heads from X/Df(Y)S7 males (see also Gatti and Pimpinelli [1983] for detail). The heads reacted strongly with the anti-laminin IgG. Arrows identify identical positions in *a* and *b*. Sp, primary spermatocytes; H, heads of spermatid. Bar, 10 μm.

bodies can decorate other lampbrush loops in the primary spermatocytic nuclei although at lower intensities (e.g., Fig. 7, *a* and *b*; see Fig. 6, *a* and *b*). The influence of Y chromosome deficiencies on male germ cell development was also displayed by the strong reaction of deformed sperm heads with the antilaminin antibodies (e.g., Fig. 7, *c* and *d*). As has been observed for other antisera (Bonaccorsi et al., 1988; Hulsebos et al., 1984), in XO spermatocytes, a diffuse reaction can be observed on the nuclei, indicating a nonlocalized presence of some antigen (Fig. 8, *a* and *b*) which cannot be deposited on its nuclear target sites.

The results of this and the following section are summarized in Table I.

Ultrastructural Localization of Laminin in Male Germ Cells by Immunoelectronmicroscopy

The reaction of the laminin antisera in *Drosophila* testis was also surveyed at an ultrastructural level. In concert with the immunofluorescence pictures from light microscopy, we found reactions of the antibodies against laminin B2 and against the whole laminin complex in primary spermatocyte nuclei, in the nuclei and cytoplasm of young spermatids and the epithelial cell layers of the testis wall (see Fig. 10 *f*) in a pattern that corresponds to that obtained in immunofluorescence and immuno-alkaline phosphatase staining reactions. An overview is given in Fig. 10. As an additional location, a central region in the axoneme of elongating spermatids was decorated by all the antisera described above (*a–e*). Antibodies against laminin A and B1 react only in the axoneme, but not in meiotic or postmeiotic nuclei.

In all experiments control reactions have been carried out with pre-immune serum (as far as available), and reactions with only the secondary antibody. The reaction with the antiserum against the fusion protein was also competed with an excess of purified laminin of *Drosophila*, which was kindly provided by Drs. L. and J. Fessler (Molecular Biology Institute, University of California at Los Angeles, Los Angeles, CA). In the presence of the laminin competitor, no reactions are observed. Parallel sections incubated with the preimmune sera gave hardly any background and no reactions are seen if only secondary antibodies are used. The background in all reactions is very low.

From the reactions of the antisera at the ultrastructural level, the weak tail reactions observed in immunofluorescence can be confirmed as a specific antibody reaction and more precisely located. The immunogold-label is restricted

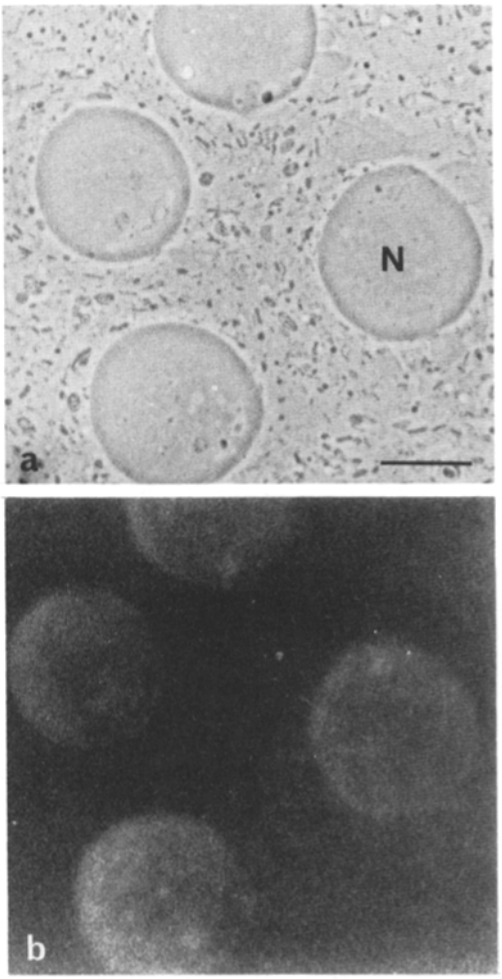

Figure 8. Localization of laminin in XO male germ cell nuclei of *D. melanogaster* as visualized by indirect immunostaining. Testis squashes from young males are first incubated with the antilaminin antibodies and subsequently with FITC-labeled goat-anti-rabbit IgG. Note the diffuse signal on the nuclei (compare with Fig. 7 *a* and *b*). N, nuclei (*a*) Phase contrast; (*b*) FITC signal. Bar, 10 μm.

to a central region in the axoneme of elongating spermatids (Figs. 9 *b* and 10, *a–e*).

The axoneme is one of the two main components of the sperm tail. It grows out of the centriole and contains the 9 + 2 microtubule structure which is characteristic for all eukaryotic cilia and flagellae. In the space between a double membranous sheath (centriolar sheath) and the microtubules, proteinaceous accessory material is deposited (for detail, see Lindsley and Tokuyasu, 1980; Grond, 1984; Hennig and Kremer, 1990). The nine doublet microtubules are connected to the two central microtubules with radial spokes, called Afzelius spokes. The antigen is colocalized with the head region of the radial Afzelius spokes. It is remarkable that in all cases the label is confined to one to three of the spokes while the others are free of label. This labeling pattern finds its equivalent in longitudinal section (Fig. 10, *d* and *e*), where periodically in equal distances gold grains are located on central parts of the axoneme. Both the patterns obtained in cross sections and in longitudinal sections can be explained by an asymmetric positioning of the laminin complex.

The deposition of laminin in the axoneme of elongated spermatids deserves particular interest. To see whether this kind of specific localization of the antigen is common among various eukaryotic cilia and flagellae, which contain 9 + 2 microtubule structure, the unicellular alga *Chlamydomonas* bearing two flagellae, as well as sperm from *Platynereis* and humans were analyzed with the same antibodies by immunoelectronmicroscopy. In all the samples examined, a similar immunoreaction was found in the axonemes (unpublished data of M. Hanske, F. Wang, and W. Hennig).

Discussion

In our approach to identify molecular constituents of the male germ cells of *Drosophila*, we have found that these cells contain laminin. This observation is unexpected as laminin has so far been considered exclusively as a component of the extracellular matrix. Therefore, we have tried to obtain independent evidence for the presence of laminin in male germ cells in several independent ways (Table I). The following experiments were used to confirm the presence of laminin in developing male germ cells: (*a*) demonstration of laminin B2 transcripts in the RNA of manually collected male germ cells with the PCR and by in situ hybridization (Table I); (*b*) staining of the laminin B2 fraction by the antiserum raised against a *Drosophila* laminin B2 COOH-terminal fragment (K44 serum) on a Western blot of protein extracted from manually collected male germ cells (Table I); and (*c*) decoration of specific structures in the male germ cells by four different antisera against *Drosophila* and three against murine laminin complex or against single laminin chains with indirect immunostaining and immunoelectronmicroscopy (Table I). All experiments are fully consistent and leave no doubt that laminin is expressed in germ cells.

The Distribution of Laminin in Developing Germ Cells

We observed in adult testis of *D. melanogaster* that laminin first appears in the nuclei of primary spermatocytes (Fig. 6, *a* and *b*). In the nuclei of primary spermatocytes, the signals from the antibodies were specifically localized on some of the Y chromosomal lampbrush loops.

Because the first and second meiotic divisions in *D. melanogaster* proceed very fast, it is difficult to analyze germ cells at these stages. Cytological data indicate that during meiosis, laminin seems to be connected with the parafusorial membranes surrounding the nuclear compartment.

After meiosis, laminin is detected in both nuclei and the cytoplasm of young spermatids. When spermatids start elongation, the antigen is found in the axoneme, as well as in the head (Figs. 6, *e* and *f*, 9, and 10, *a–e*). The experiments with antisera against different chains of laminin show that antigens of all three chains of the laminin complex, A, B1, and B2, can be detected in the axoneme, while the nuclear reactions in all stages are restricted to laminin B2. Similar reaction patterns in axonemes are obtained throughout the animal kingdom. We have observed the same reactions in the flagellae of *Chlamydomonas*, and in the sperm of *Platynereis* and humans.

What Is the Function of Laminin in Developing Germ Cells?

Laminin is well known to be active in biological events

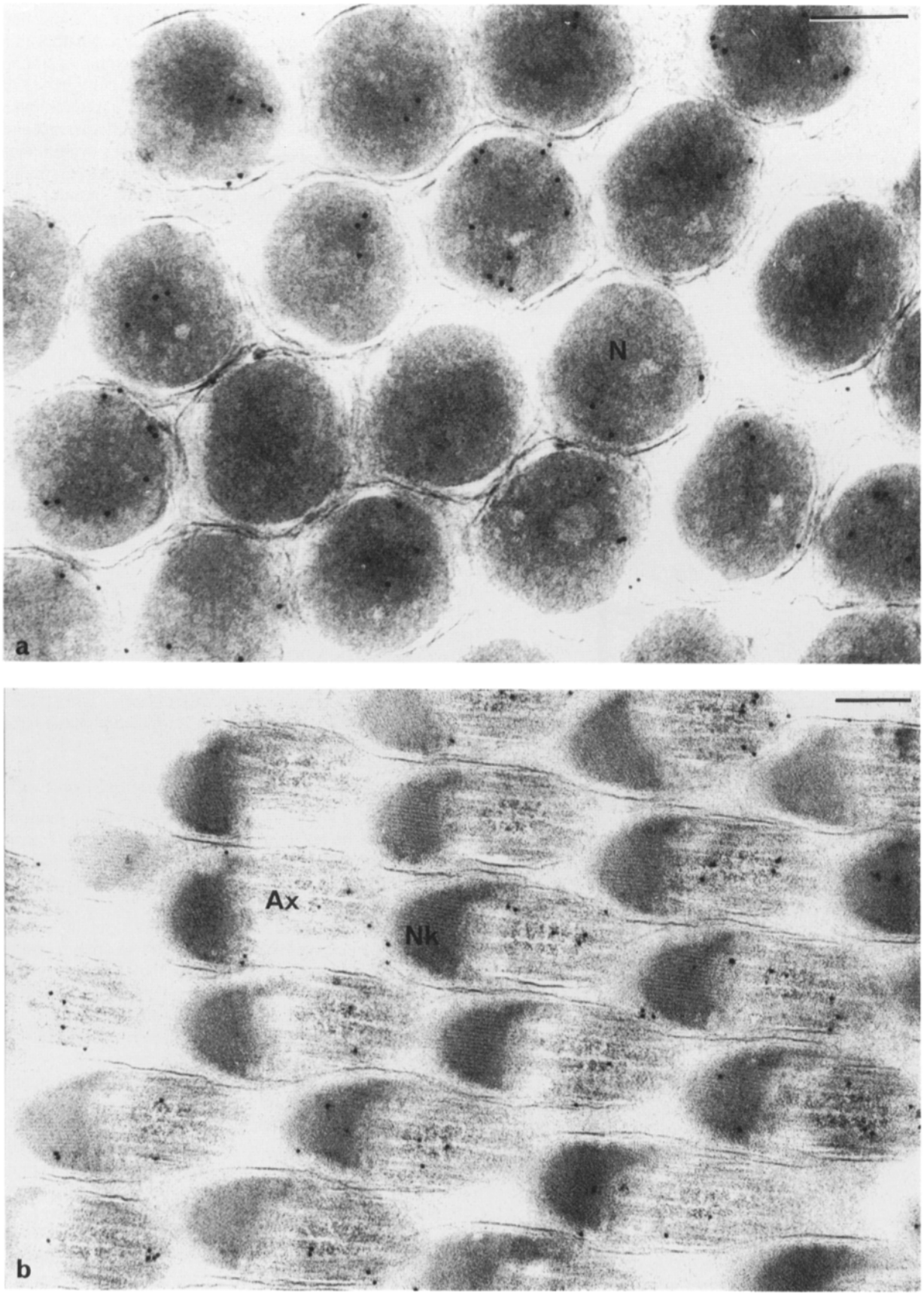

Figure 9. Overview of cross sections through spermatid heads (*a*) and tails (*b*) after immunolabeling with antilaminin B2 antiserum. The sections through the tails are oblique, but show the concentration of grains in the inner part of the axoneme. In spermatid heads, label is observed within the nuclei (*a*). Note that in both preparations hardly any background is observed. Bars, 200 nm.

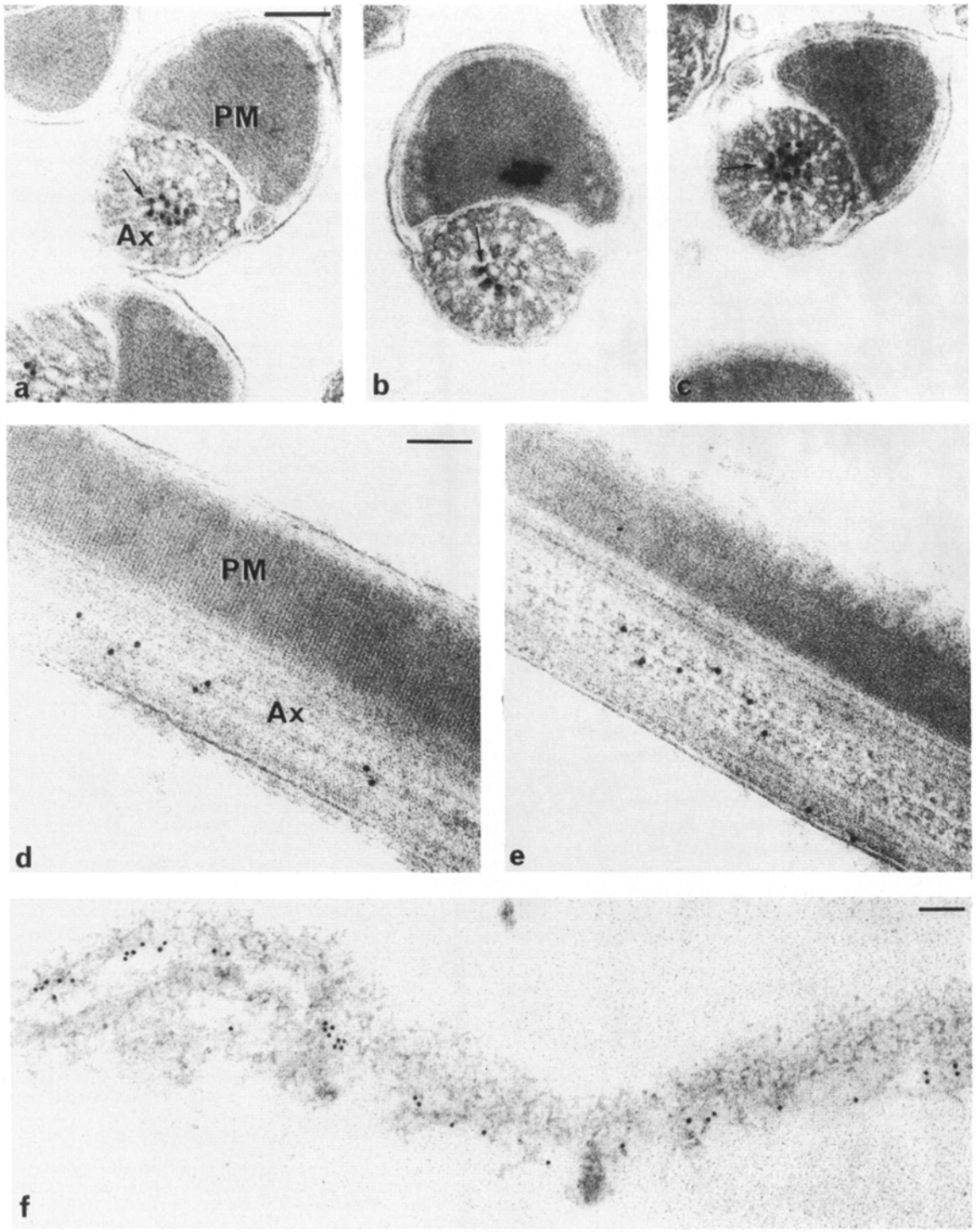

Figure 10. Immunoelectronmicroscopy of *D. melanogaster* testis. (*a–c*) Cross sections of sperm tails. The gold labeling is localized at some of the spoke heads in the axoneme (*arrows*) (see *c*); (*d* and *e*) longitudinal sections of sperm tails (see *b*). The gold labeling is restricted to the inner part of the axoneme. The asymmetric distribution suggests a spiral arrangement of the antigen within the axoneme; *f*, part of the testis wall (basement membrane) with gold labeling. *a–c* are incubated with K44; *d–f* are incubated with the antiserum against mouse laminin. PM, paracrystalline material; Ax, axoneme. Bars, 100 nm.

related to cell–cell and cell–matrix interactions. Taking such a function as the particular property of this molecular complex, one has to consider potentially related intracellular functions during the male germ cell development. Several processes during sperm morphogenesis display a relationship to the suggested functions of laminin complex in the extracellular matrix, e.g., the outgrowth of the flagellum, the interaction of intracellular membranes after meiosis, the attachment and penetration of the sperm into the egg, the anchoring events of spermatid heads within the head cyst cell, etc. These various possibilities are neither necessarily exclusive to each other nor exhaustive. The function of laminin in *Drosophila* developing male germ cells remains an open question. It is also connected with many other questions. For

example, are there receptors mediating the function of laminin in the male germ cells (there is evidence that in the cytoplasm of some vertebrate cells laminin receptors, or their homologues, can be detected [Mecham, 1991]); and are there functional correlations among those antigens detected in the nuclei of spermatocytes, in the cytoplasm of young spermatids, in the axoneme and head region of elongated spermatids?

Finally, it is necessary to point out that the above discussions are based on knowledge of functions assigned to the molecular complex of all three subunits of laminin (A, B1, and B2) which, however, do not necessarily have similar temporal and spatial expression patterns. According to Montell and Goodman (1988), in *Drosophila* embryos all the three subunits are synchronously expressed. However, uncoordinate expression of laminin subunits has clearly been demonstrated for adult murine tissues (Kleinman et al., 1987; Laurie et al., 1989), and it now seems clear that laminins lacking the A chain are common in the mouse embryos as well (Cooper and MacQueen, 1983; Ekblom et al., 1990; Klein et al., 1988). According to the antisera reactions, we must assume that all three laminin chains are present in the axoneme, but possibly not in the nuclei.

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