The \textit{kl-3} Loop of the \textit{Y} Chromosome of \textit{Drosophila melanogaster} Binds a Tektin-Like Protein

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Manuscript received September 10, 1992
Accepted for publication October 30, 1992

ABSTRACT

Primary spermatocyte nuclei of \textit{Drosophila melanogaster} exhibit three giant lampbrush-like loops formed by the \textit{kl-5}, \textit{kl-3} and \textit{ks-1} \textit{Y}-chromosome fertility factors. These structures contain and abundantly transcribe highly repetitive, simple sequence DNAs and accumulate large amounts of non-\textit{Y}-encoded proteins. By immunizing mice with the 53-kD fraction (enriched in \(\beta\)-tubulin) excised from a sodium dodecyl sulfate-polyacrylamide gel loaded with \textit{Drosophila} testis proteins we raised a polyclonal antibody, designated as T53-1, which decorates the \textit{kl-3} loop and the sperm flagellum. Two dimensional immunoblot analysis showed that the T53-1 antibody reacts with a single protein of about 53 kD, different from the tubulins and present both in \textit{X/Y} and \textit{X/O} males. Moreover, the antigen recognized by the T53-1 antibody proved to be testis-specific because it was detected in testes and seminal vesicles but not in other male tissues or in females. The characteristics of the protein recognized by the T53-1 antibody suggested that it might be a member of a class of axonemal proteins, the tektins, known to form Sarkosyl-urea insoluble filaments in the wall of flagellar microtubules. Purification of the Sarkosyl-urea insoluble fraction of \textit{D. melanogaster} sperm revealed that it contains four polypeptides having molecular masses ranging from 51 to 57 kD. One of these polypeptides reacts strongly with the T53-1 antibody but none of them reacts with antitubulin antibodies. These results indicate that the \textit{kl-3} loop binds a non-\textit{Y} encoded, testis-specific, tektin-like protein which is a constituent of the sperm flagellum. This finding supports the hypothesis that the \textit{Y} loops fulfill a protein-binding function required for the proper assembly of the axoneme components.
and do not affect the unfolding of the kl-5, kl-3 and ks-1 loops (Bonaccorsi et al. 1988).

In situ hybridization studies have shown that each of the three large fertility factors contains a characteristic combination of highly repetitive simple sequence satellite DNAs (Bonaccorsi and Lohe 1991) (see Figure 1). The satellite DNAs contained in the loop-forming sites are specifically and abundantly transcribed on the loop structures of primary spermatocytes, whereas no satellite DNA transcription has been detected in the other cell types of D. melanogaster spermatogenesis (Bonaccorsi et al. 1990; S. Bonaccorsi, C. Pisano and M. Gatti, unpublished results).

Although the Drosophila Y loops have been the subject of extensive experimental work for 25 years, the biological functions of these structures, and hence of the genes that produce them, have remained a mystery (for reviews see Hennig 1985; Lifschytz 1987; Bonaccorsi et al. 1988; Gatti and Pimpinelli 1992). The presence of Y-dependent formations in primary spermatocyte nuclei has been observed in many Drosophila species (Hess 1967), but the bulk of information on these structures comes from studies in Drosophila hydei and D. melanogaster. Remarkably, the Y loops of these relatively distant species, despite their different morphologies, have comparable organizations and properties (Hennig 1985; Lifschytz 1987; Bonaccorsi et al. 1988, 1990; Gatti and Pimpinelli 1992). In both species the Y loops begin to form in young spermatocytes, grow throughout spermatocyte development, reach their maximum size in mature spermatocytes, and disintegrate during the first meiotic prophase. Moreover, cytochemical studies indicate that loops of both species consist of a DNA axis associated with transcribed RNA which, in turn, is associated with large amounts of proteins. The loop-associated transcripts of D. hydei come from middle repetitive DNAs which lack open reading frames (Lifschytz 1979; Vogt, Hennig and Siegmund 1982; Lifschytz et al. 1983; Hareven, Zuckerman and Lifschytz 1986; Vogt and Hennig 1986a,b; Hujiser and Hennig 1987; Trapatitz, Włascher and Bunemann 1988; Hujiser et al. 1988; Brand and Hennig 1989), while those of D. melanogaster are homologous to simple sequence, noncoding satellite DNAs (Bonaccorsi et al. 1990). The D. melanogaster transcripts and at least some transcripts of D. hydei do not migrate to the cytoplasm and are degraded along with the loops prior to the first meiotic division (Lifschytz et al. 1983; Bonaccorsi et al. 1990).

Taken together, the features of the Y loops have suggested the hypothesis that these structures fulfill a protein-binding function (Glatzer 1984; Hennig 1985; Bonaccorsi et al. 1988, 1990). Moreover, the fact that the Y loops are present only in the genus Drosophila, which exhibits enormously long spermt tails (2 mm in D. melanogaster and 10 mm in D. hydei), led us to envisage that the proteins accumulated on the loop structures may be involved in spermio genesis. To substantiate this hypothesis we have addressed our experimental efforts to the identification and functional characterization of the proteins bound to the loops. One of our approaches to the identification of these proteins was the production of antibodies against testis-specific proteins to see whether they reacted with antigens associated with the loops. This paper reports a successful application of this approach. We have obtained an antibody which reacts with a

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**Figure 1.**—Cytogenetic map of the D. melanogaster Y chromosome, showing the localization, organization and molecular composition of the fertility factors. The diagram is a schematic representation of the mitotic Y chromosome stained with Hoechst-33258, showing its division into 25 regions (Gatti and Pimpinelli 1983). Filled segments indicate bright fluorescence, hatched segments indicate dull fluorescence and open segments indicate no fluorescence. C, centromere. The lines immediately above the diagram indicate the location of the fertility factors (kl-5, kl-3, kl-2, kl-1, ks-1 and ks-2). The thick lines corresponding to kl-5, kl-3 and ks-1 indicate their minimum physical size, as defined by noncomplementing sterile breakpoints. Cytogenetic data permitted the localization of kl-2, kl-1 and ks-2, but not an estimation of their size. The lines in the upper row correspond to the loop-forming regions of kl-5 (A), kl-3 (B) and ks-1 (C) (Bonaccorsi et al. 1988). The bars below the diagram indicate the localization of the AAGAG, AAGAC and AATAT satellite repeats as defined by in situ hybridization experiments on mitotic chromosomes (Bonaccorsi and Lohe 1991).
Y Chromosome Loop Proteins

non-Y-encoded, testis-specific protein present on the kl-3 loop and in the sperm flagellum. The characterization of this antibody indicates that it is directed against a tektin-like protein.

MATERIALS AND METHODS

Drosophila stocks: With the exception of TX(Y)R17, the genetics and cytology of the Y chromosome rearrangements used here have been described in detail in previous papers (KENNISON 1981; HARDY et al. 1984; GATT and PIMPINELLI 1983; BONACCORSI et al. 1988). TX(Y)R17 is a translocation induced on a B3/Yr chromosome carrying a deficiency in YS (KENNISON 1981); the breakpoints are in the centric heterochromatin of the X and in region h4 of the Y chromosome. We determined by Hoechst-33258 banding that the Y deficiency spans region h21-h25 of the Y chromosome which contains the ks-1 and ks-2 genes. To compensate for this deficiency KENNISON (1981) attached the YS element of F18K to the XL tip of R17 by recombination. The Xp element of R17 used here was obtained by removing YS by recombination; it carries the kl-3, kl-2 and kl-1 fertility genes and develops only the kl-3 loop.

The wild-type strain used in cytological and biochemical analyses is an Oregon-R stock which has been maintained in our laboratory for about 25 years. The X/O males were generated by crossing Oregon-R males with XX/O females. All stocks and crosses were maintained at 25 ± 1°C.

Preparation of the antisera: Drosophila testis proteins were fractionated on preparative sodium dodecyl sulfate-polyacrylamide gels (PAGE) (see below) and briefly stained with Coomassie blue. The more rapidly migrating component of the β-tubulin fraction was excised from the gel, homogenized in phosphate-buffered saline (PBS) (10 mM Na-phosphate, 150 mM NaCl, pH 7.4) and used for immunization of female BALB/c mice. Each mouse was immunized with protein fractions obtained from about 1000 testes. This material was homogenized in PBS up to a final volume of 1 ml and subdivided into five aliquots which were injected intraperitoneally at 1-week intervals. Four days after the last injection mice were sacrificed and bled.

Indirect immunofluorescence: To prepare testis material for indirect immunofluorescence we developed a new method-anal-acetone fixation procedure. Larval or adult testes were frozen in liquid nitrogen and, after removal of the coverslip and briefly stained with Coomassie blue, the more rapidly migrating component of the β-tubulin fraction was excised from the gel, homogenized in phosphate-buffered saline (PBS) (10 mM Na-phosphate, 150 mM NaCl, pH 7.4) and used for immunization of female BALB/c mice. Each mouse was immunized with protein fractions obtained from about 1000 testes. This material was homogenized in PBS up to a final volume of 1 ml and subdivided into five aliquots which were injected intraperitoneally at 1-week intervals. Four days after the last injection mice were sacrificed and bled.

Peroxidase activity was detected in 50 mM Tris-HCl (pH 6.8), 0.1% SDS (Bio-Rad), 0.07% tetramethyl-ethylene diamine (TEMED) and 0.08% ammonium persulfate (APS). The separation gel contains 9% acrylamide, 0.16% N,N′-methylene-bis-acrylamide, 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.12% TEMED and 0.08% APS. Electrode buffer was the same of LAEMMLI (1970) but electrophoresis was performed at 20 mA for the first 30 min and at 30 mA for the rest of the run. Gels were fixed in 15% acetic acid and 40% methanol for 15 min, stained with 0.5% Coomassie blue, 20% acetic acid and 40% methanol for 1 hr and destained in 10% acetic acid and 40% methanol.

Two-dimensional gel electrophoresis was carried out as described by O’FARRELL (1975) and WARING, ALLIS and MAHOWALD (1978). Testes were homogenized in 9.5 M urea, 2% Nonidet P-40 (NP-40), 5% 2-mercaptoethanol and 2% Amphotiles (1.6% pH 7-9 and 0.4% pH 3.5-10, both from LKB). Isoelectric focusing was performed on slab gels containing 3.8% acrylamide, 0.2% N,N′-methylene-bis-acrylamide, 9 M urea, 0.2% NP-40, 5% Amphotiles (4% pH 7-9 and 1% pH 3.5-10), 0.07% TEMED and 0.1% APS. Electrode buffers, pre-run and run conditions were the same as those used by O’FARRELL (1975). The second dimension gel was the same as that previously described for one-dimensional SDS-PAGE. Protein spots were visualized by silver staining using the Bio-Rad kit.

Proteins separated by one-dimensional or two-dimensional electrophoresis were transferred to nitrocellulose sheets (S & S, BA 85, 0.45 μm) following the procedure of TOWBIN, STAHELIN and GORDON (1979). The nitrocellulose strips containing transferred proteins were preincubated for 2 hr in PBS (10 mM Na-phosphate, 150 mM NaCl, pH 7.4) containing 3% bovine serum albumin (BSA) and then incubated for 16-18 hr with the primary antibody diluted in PBS containing 3% BSA. The T53-1 and T53-2 antisera were diluted 1:5000; the anti-α tubulin (Amersham) and anti-β tubulin (Amersham) monoclonal antibodies, designated as A-Am and B-Am, respectively, were diluted 1:10000. After incubation with the primary antibody the strips were washed two times for 30 min with PBS containing 3% BSA and 0.1% Triton X-100; they were then incubated for 3 hr with peroxidase-conjugated sheep IgG against mouse IgG (Amersham) diluted 1:1000 in PBS containing 3% BSA and washed with 50 mM Tris-HCl (pH 6.8). Peroxidase activity was detected in 50 mM Tris-HCl (pH 7.4).
6.8), 0.014% H$_2$O$_2$ and 0.075% 4-chloronaphthol; the reaction was stopped in distilled H$_2$O.

**Purification of the Sarkosyl-urea insoluble fraction:** To prepare Drosophila axonemes we used a procedure similar to that developed by Gibbons and Fronck (1972) for sea urchin and modified by Linck and Stephens (1987). For each preparation we used at least 1000 seminal vesicles dissected from males which had been kept separated from females for at least 10 days. These vesicles, which are enormously distended and filled with mature sperm, were washed in PBS (10 mM Na-phosphate, 150 mM NaCl, pH 7.4) and centrifuged at 2500 × g for 5 min. The pellet was resuspended in the extraction solution containing 10 mM Tris-HCl (pH 8.3), 150 mM KCl, 5 mM MgSO$_4$, 0.5 mM EDTA, 1 mM dithiothreitol and 1% Triton X-100, and gently homogenized to break the vesicles and free the sperm. This suspension was kept on ice for 30 min and then centrifuged at 2500 × g for 5 min. The supernatant was saved, and the pellet, resuspended again in the extraction solution, was centrifuged at 2500 × g for 5 min. The first and second supernatants were mixed and centrifuged at 10,000 × g for 5 min. The resulting pellet was resuspended in the extraction solution without Triton and centrifuged again at 10,000 × g for 5 min. This step was repeated twice and the final pellet was resuspended in a solution containing 1 mM Tris-HCl (pH 8.3), 0.1 mM EDTA and 0.5 mM dithiothreitol; the suspension was dialyzed against 100 volumes of the same buffer for 24 hr. Microscopic observation of this suspension, after DNA staining with Hoechst33258 and tubulin immunostaining, revealed that it consists mostly of sperm tails and contains only a few sperm heads (data not shown). Sarkosyl-urea extraction was performed according to Linck and Stephens (1987). One volume of purified axonemes was mixed with 9 volumes of 0.5% Sarkosyl (sodium dodecyl sarsosinate), 2.5% urea, 50 mM Tris-HCl (pH 8.3), 50 mM lysine and 1 mM EDTA; this mixture was kept at 4°C for 1 hr and then centrifuged at 100,000 × g for 90 min. The supernatant was discarded and the insoluble pellet was dissolved in Laemmli’s sample buffer for SDS-PAGE or rinsed with distilled H$_2$O and saved frozen at -20°C. The insoluble residue was also analyzed by electron microscopy after negative staining and shown to consist of aggregates of filamentous material (data not shown).

**RESULTS**

**Production of the T53-1 and T53-2 antisera:** To isolate the testis proteins to be used as immunogens we ran SDS-polyacrylamide gels loaded with testis, larval brain and embryonic extracts, and compared the patterns of electrophoretic bands. Those bands which were specifically present, or particularly enriched in testes as compared to other tissues were excised from the gel and used for immunization.

One of the bands chosen for immunization was the protein fraction enriched in β-tubulin which, in our electrophoretic system, has an apparent molecular mass of 53 kD. This fraction is much more abundant in testes than in the other tissues. Moreover, it is known to contain a testis-specific form of β-tubulin (β$_2$-tubulin) that is one of the major structural components of the meiotic spindle and the sperm-tail axoneme (Kemphues et al. 1982). The 53-kD protein fraction was injected into three mice as described in **MATERIALS AND METHODS.** Resulting sera were then tested by immunoblotting for reaction with the 53-kD protein fraction. One of these sera, like the preimmune serum, did not recognize any of the testis proteins; the other two sera reacted strongly with a single electrophoretic band located at the same level as the 53-kD fraction (data not shown; cf. Figure 5). These two antisera, designated as T53-1 and T53-2, gave identical results in all the experiments described below. Thus, henceforth we will refer only to the T53-1 antisera, implying that T53-2 behaves in the same way.

The T53-1 antisera specifically reacts with the kl-3 loop and the sperm flagellum: Indirect immunofluorescence experiments performed on methanol/acetic fixed larval or adult testes (see **MATERIALS AND METHODS**) revealed that the T53-1 antibody decorates the kl-3 loop of primary spermatocytes and the sperm flagellum (Figures 2–4). Examination of primary spermatocytes at various growth stages showed that the T53-1 antigen is present on the kl-3 loop throughout all its developmental phases (i.e., in young small loops, in fully grown loops and in disintegrating loops; see Figure 2). Similarly, the T53-1 antigen is consistently associated with sperm flagella at all times after the beginning of the elongation process. The other cell types of *D. melanogaster* spermatogenesis (i.e., spermatogonia, secondary spermatocytes and young spermatids) exhibit a diffuse, weak fluorescence and do not display intensely immunostained nuclear or cytoplasmic structures (data not shown). Moreover, meiotic spindles and spindle poles are not decorated by the T53-1 antibody.

To ascertain the intranuclear localization of the T53-1 antigen in primary spermatocytes and analyze its behavior in the absence of the kl-3 loop, we examined spermatogenesis in males deficient for different Y chromosome fertility factors, which thus bear different loop combinations. As shown in Figure 4 primary spermatocytes containing the kl-3 and the kl-5 loops, the kl-3 and the kl-1 loops, or only the kl-3 loop, exhibit a strong signal associated with the kl-3 filamentous material, whereas those containing the kl-5 and ks-1 loops, or no loops at all (X/O), do not display fluorescent intranuclear structures. Interestingly, the sperm tails of males of the latter genetic constitutions, like those of males bearing the kl-3 loop, are intensely immunostained by the T53-1 antibody. These observations confirm that the T53-1 antigen is specifically bound to the kl-3 loop. They also indicate that the accumulation of this antigen on the kl-3 loop does not require the presence of the other loops, and that, in the absence of the kl-3 loop, it does not aggregate onto the kl-5 and the ks-1 loops. Moreover, they show that the kl-3 loop is not required for the incorporation of the T53-1 protein into sperm flagella.
FIGURE 2.—Indirect immunofluorescence with the T53-1 antiserum plus fluorescein-labeled goat anti-mouse IgG. (a, c and e) Spermatocyte nuclei at different maturation stages photographed in phase contrast; (b, d and f) the same nuclei after immunostaining with the T53-1 antiserum. The spermatocyte nuclei shown in panels a and c exhibit three clusters of filamentous structures designated as A, B and C. The two more compact clusters A and C correspond to the kl-5 and kl-1 loops, respectively; the other group of thinner and less folded threads (B) corresponds to the kl-3 loop (BONACCORSI et al. 1988). In the nucleus shown in panel e the loop material is falling apart into pieces prior to the first meiotic division. Note that the T53-1 antiserum reacts with young (b), fully grown (d) and disintegrating (f) kl-3 loops. Bar = 10 μm.

The T53-1 antibody does not recognize a tubulin isoform: Although in immunoblots the T53-1 antiserum reacts with a single polypeptide band comigrating with the β-tubulins, several results obtained by immunofluorescence experiments indicate that the protein recognized by this antibody is not a tubulin isoform. The T53-1 antibody does not react with either the cytoskeleton or the meiotic spindle, both of which are highly enriched in tubulins. Conversely, these structures are intensely immunostained by anti-α- and anti-β-tubulin monoclonal antibodies which do not react with the kl-3 loop (data not shown). These antibodies, raised against chicken tubulins, are designated here as A-Am and B-Am, respectively. It has been shown that A-Am recognizes most of the α-tubulin isoforms (MATTHEWS, MILLER and KAUFMAN 1989) while B-Am reacts with both β1 and β2 tubulins (KIMBLE, INCARDONA and RAFF 1989).

These inferences from indirect immunofluorescence were confirmed by one-dimensional SDS-PAGE immunoblotting that showed that the T53-1 antibody does not react with purified embryonic tubulins (Figure 5). This analysis also showed that antibody T53-1 recognizes a testis-protein fraction different from those recognized by A-Am but comigrating with the fraction reacting with B-Am (Figure 5).

The final evidence that the T53-1 antibody does not recognize β-tubulin isoforms was obtained by two-dimensional isoelectric focusing/SDS-PAGE immunoblot analysis of testis proteins. These experiments showed that this antibody recognizes a single protein spot having an isoelectric point around 8.3, well separated from the tubulins which migrate to a lower pH (Figure 6). These findings also indicate that T53-1 behaves as a monospecific antibody.

The T53-1 antibody reacts with a testis-specific protein not encoded by the Y chromosome: To characterize the protein recognized by the T53-1 antibody, several Drosophila tissues were examined by immunoblotting for the presence of this antigen. In these experiments we also asked whether the presence of the T53-1 protein in testis-extracts was dependent upon the presence of the Y chromosome. As shown in Figure 7, the T53-1 protein is present in X/Y testes, X/Y seminal vesicles and X/O testes, but is not detected in extracts from embryos, larval brains, salivary
FIGURE 4.—Immunofluorescence staining of primary spermatoocytes of different genetic constitutions with the T53-1 antiserum. (a, c, e and g) Phase contrast; (b, d, f and h) indirect immunofluorescence with the T53-1 antibody plus a fluorescein labeled goat anti-mouse IgG. (a and b) A spermocyte nucleus from males carrying the Y-proximal X-distal element (Y\textsuperscript{+}X\textsuperscript{-}) of T(X;Y)\textsuperscript{V24} and no free Y chromosome. These males lack region h1–h4 of the Y chromosome which contains the kl-3 loop-forming site, and exhibit only the kl-3 (B) and kl-1 (C) loops. Note that the T53-1 antibody specifically decorates the kl-3 loop. (c and d) A spermocyte nucleus from males carrying the X\textsuperscript{+}Y\textsuperscript{-} element of T(X;Y)\textsuperscript{V24} plus the PX\textsuperscript{-} element of T(X;Y;3)\textsuperscript{W27}. These males lack the kl-3 locus and form only the kl-5 (A) and kl-1 (C) loops. Note that no intranuclear structures react with the T53-1 antibody, while a bundle of sperm tails (sp) is intensely immunostained. (e and f) A spermocyte nucleus from males bearing the Y\textsuperscript{+}X\textsuperscript{-} element of T(X;Y)\textsuperscript{R17} (see MATERIALS AND METHODS) and no free Y chromosome; it exhibits only the kl-3 loop which reacts strongly with the antibody. (g and h) A primary spermocyte nucleus from X/O males showing only the X/O granules (arrows) and no loops. Note that the antibody does not recognize any intranuclear structure. Bar = 10 μm.

FIGURE 5.—Antibody binding to testis proteins and purified embryonic tubulin. (A) Electrophoretograms of testis polypeptides (lane 1) and purified embryonic tubulin (lane 2) after staining with Coomassie blue. Three sets of the same samples were subjected to electrophoresis in parallel, transferred to a nitrocellulose sheet by electroblotting and incubated with the T53-1 antibody (B), the anti-α tubulin monoclonal antibody A-Am (C) or the anti-β tubulin monoclonal antibody B-Am (D). Lanes E and F are blots of testis polypeptides obtained from samples run in parallel with sample A1. E was incubated with the T53-1 and A-Am antibodies; F was incubated with T53-1 and B-Am. For all samples antibody binding was revealed by a peroxidase-conjugated secondary sheep anti-mouse antibody. Note that the T53-1 antibody recognizes a 53-kD protein fraction from adult testes but does not react with purified tubulin (lanes B1, 2). The A-Am antibody recognizes multiple α-tubulin isoforms both in testes and in embryonic tubulin (lanes C1, 2). The B-Am antibody reacts with testis-specific β-tubulin and with embryonic-type β-tubulin of slightly higher molecular weight (lanes D1, 2). Simultaneous incubation with T53-1 and A-Am reveals that these antibodies react with different protein fractions (lane E1). Incubation with both T53-1 and B-Am shows that these antibodies recognize protein fractions not separated by one-dimensional electrophoresis (lane F1).

glands, adult male carcasses (male bodies devoid of testes and seminal vesicles) and adult females. Since the seminal vesicles used in these experiments consist of enormously distended storage organs full of mature sperm, these findings, together with the results of the indirect immunofluorescence, indicate that the protein recognized by the T53-1 antibody is a testis-specific constituent of the sperm flagellum. Moreover, the observation that this protein is present also in X/O males demonstrates that it is not encoded by the Y chromosome.

The T53-1 antibody reacts with a Sarkosyl-urea-insoluble tektin-like protein: The characteristics of the protein recognized by the T53-1 antibody and its association with the sperm flagellum suggested that it might be a member of a set of proteins, called tektins, that form longitudinal filaments in the walls of flagellar microtubules [for review see STEFFEN and LINCK (1989)]. These proteins are highly insoluble and are resistant to Sarkosyl-urea extraction. Based on this property we extracted the Sarkosyl-urea-insoluble fraction from D. melanogaster sperm to determine whether it contained a protein recognized by the T53-
by isoelectric focusing (IEF) were subjected to SDS-PAGE in parallel. One of the two resulting gels was used for protein staining the same first-dimensional gel containing testis proteins separated by SDS-PAGE (upper panel) and the other for immunoblotting (lower panel). Note that the T53-1 antibody reacts with a single protein spot located in the basic part of the gel (small arrow); the large arrow points to the tubulins.

![Image of SDS-PAGE and immunoblotting](image)

**FIGURE 6.**—Binding of the T53-1 antibody to a two-dimensional blot of *D. melanogaster* testis proteins. Two identical slices cut from the same first-dimensional gel containing testis proteins separated by isoelectric focusing (IEF) were subjected to SDS-PAGE in parallel. One of the two resulting gels was used for protein staining (upper panel) and the other for immunoblotting (lower panel). Note that the T53-1 antibody reacts with a single protein spot located in the basic part of the gel (small arrow); the large arrow points to the tubulins.

The T53-1 antibody. As shown in Figure 8 this fraction contains four polypeptides having molecular masses ranging from 51 to 57 kD. One of these polypeptides reacts strongly with the T53-1 antibody in immunoblots but none of them reacts with an anti-β-tubulin (B-Am) antibody. These results indicate that the T53-1 antibody is directed to a testis-specific, Sarkosyl-urea-insoluble axonemal protein which might very well be a tektin.

To further characterize the T53-1 antibody we examined by immunoblotting its reaction with sperm extracts and purified tektins from *Strongylocentrotus purpuratus*. These experiments revealed that the antibody does not exhibit any reaction with this material (data not shown). These results are not surprising in that tektins do not appear to have a high degree of evolutionary conservation (Steffen and Linck 1989). Indeed, none of the four monoclonal antibodies directed to *S. purpuratus* tektins isolated by Chang and Pierino (1987) reacts with testicular extracts of *D. melanogaster* (G. Pierino, personal communication).

In contrast, a polyclonal antibody raised against sea urchin tektins crossreacts with Drosophila testis proteins [E. Raff and J. Hutchens, quoted in Linck et al. (1987)]. However, two-dimensional blotting experiments showed that the protein recognized by this antibody is different from the T53 polypeptide (E. Raff, personal communication). Thus, it is quite possible that the T53 protein is a Drosophila tektin that does not share epitopes with the sea urchin tektins.

**Evolutionary conservation of the T53-1 antigen:** To estimate the degree of evolutionary conservation of the protein recognized by the T53-1 antibody we examined by indirect immunofluorescence the spermatogenesis of three additional Drosophila species (*D. hydei*, *D. virilis*, *D. novamexicana*) chosen as representatives of evolutionarily distant groups within the genus. In each of these species primary spermatocytes exhibit intranuclear formations of various morphologies which react with the T53-1 antibody (Figure 9).

The features and behavior of these structures strongly suggest that they correspond to *Y* chromosome loops. This is particularly evident in *D. hydei* where the T53-1 antibody stains well known loop structures such as the *Pseudonucleolus* and the *Cones*. Moreover, in all the species examined the sperm flagella were intensely immunostained. These findings indicate a high level of conservation not only of the T53-1 protein but also of its location in primary spermatocytes and flagella, suggesting the presence of an integrated system of high selective value.

**DISCUSSION**

The **kl-3 loop binds a tektin-like protein:** We have isolated two polyclonal antibodies, T53-1 and T53-2, which behave in the same way and recognize an antigen associated with the *kl-3* loop and the sperm flagellum. Several findings indicate that this antigen consists of a single protein having an apparent molecular mass of 53 kD. The T53-1 antibody reacts with a single electrophoretic band in immunoblots of whole testes or seminal vesicles extracts; it reacts with a single band in blots from the Sarkosyl-urea insoluble fraction of mature sperm; it recognizes a single spot in two-dimensional blots of sperm proteins. Moreover, we have shown that the T53-1 antigen is testis-specific and is not encoded by the *Y* chromosome.

We have suggested that the protein recognized by the T53-1 antibody belongs to a novel group of sperm proteins, the tektins, extensively characterized in sea urchin [for reviews see Linck et al. (1982) and Steffen and Linck (1989)]. These proteins form Sarkosyl-urea insoluble filaments, called tektin filaments, longitudinally arranged along the walls of doublet axonemal microtubules. The function of the tektin filaments is currently unclear. It has been suggested that they may interact directly with tubulin, thereby stabilizing flagellar microtubules and contributing to the proper three-dimensional organization of all the axonemal components.

The most thoroughly characterized tektins are those of the sea urchin *S. purpuratus*. Sarkosyl-urea extraction of *S. purpuratus* sperm axonemes yields only three insoluble polypeptides of 47, 51 and 53 kD (Linck, Amos and Amos 1985). Three tektins of 46, 51-52 and 56-57 kD have been also found in the sea urchin *Lytechinus pictus* (Steffen and Linck 1989). Moreover, antibodies directed to the three tektins of *S. purpuratus* crossreact with as many as five polypeptides of ciliary axonemes from the molluscan *Aequipecten irradians* (Linck et al. 1987). Thus in every organism so far examined there are from 3 to 5
axonemal tektins having apparent molecular masses ranging from 46 to 57 kD.

The Sarkosyl-urea insoluble fraction obtained from D. melanogaster seminal vesicles can be resolved into four polypeptides of molecular masses between 51 and 57 kD. The number and the apparent molecular masses of these polypeptides conform very well with those found in other organisms, suggesting that three of them, or perhaps all of them, are tektins. The D. melanogaster putative tektins have been purified from seminal vesicles of males that have been kept separated from females for about 10 days. Under such circumstances seminal vesicles accumulate large amounts of mature sperm and become enormously distended. Therefore, the extracts from this type of vesicles should be highly enriched in sperm proteins and contain negligible amounts of proteins from other tissues.

Indirect immunofluorescence revealed that the T53-1 antigen is accumulated on the kl-3 loop throughout all its developmental stages and is still present when this structure begins to disintegrate prior to meiosis. In the late meiotic prophase, when the kl-3 loop has completely disappeared, during the meiotic division and in early spermatids (onion stage), the T53-1 antigen is not organized in recognizable structures. It becomes evident again when axoneme elongation begins, marking this structure throughout its development. Taking into account that Drosophila meiosis is a rather rapid process (our unpublished observations suggest that no more than 3.5 hr elapse between the first meiotic metaphase and the formation of onion stage spermatids), the simplest interpretation of these results is that the T53-1 antigen, after the disintegration of the kl-3 loop, becomes diffused in the cytoplasm to be used later in development during axoneme formation. In this context it should be noted that the presence of the kl-3 loop is not essential for the incorporation of the T53-1 antigen into the axoneme, because males lacking the kl-3 loop, or X/O males, produce sperm tails that contain this antigen.

The localization of the T53-1 antigen on both a Y chromosome loop and the sperm flagella is not a feature peculiar to D. melanogaster. We have shown that the putative anti-tektin T53-1 antibody recognizes loop structures and flagella in four relatively distant Drosophila species. Thus there appears to be an important, evolutionarily conserved requirement for the protein recognized by the T53-1 antibody to be bound to a Y loop during Drosophila spermatogenesis.

The kl-3 loop binds multiple proteins: Previous studies have shown that another antiserum, designated as sph-155, also decorates the filamentous structures of the kl-3 loop. The sph-155 antiserum, generated in rabbits by immunization with a major D. hydei testis-protein fraction of 155 kD, reacts with both the Pseudonucleolus loop and the sperm flagellum of D. hydei (Hulseeos, Hackstein and Hennig 1983, 1984). In D. melanogaster this antiserum immunostains the kl-3 loop but not the sperm tails in preparations fixed with formaldehyde (Hulseeos, Hackstein and Hennig1984; Bonaccorsi et al. 1988) or with methanol/acetone (C. Pisano, S. Bonaccorsi and M. Gatti).
electrophoretograms of polypeptides from seminal vesicles 
urea insoluble sperm polypeptides. (A) Coomassie blue stained 
from the Sarkosyl-urea insoluble fraction of flagellar axonemes (2). 
parallel, electroblotted and immunostained with the 
tains four polypeptides having apparent molecular masses of 
the Sarkosyl-urea insoluble fraction of Drosophila axonemes con-
antibody.

METHODS

GATTI, unpublished results; see MATERIALS AND 
METHODS for the fixation procedure). In addition, in 
primary spermatocytes of D. melanogaster X/O males 
fixed with either procedure, the sph-155 antiserum 
recognizes a few compact intranuclear aggregates 
(sph-155 aggregates) which are absent in males 
that exhibit a normal kl-3 loop (BONACCORSI et al. 1988; C. 
PISANO, S. BONACCORSI and M. GATTI, unpublished 
results). The sph-155 polypeptide(s) is thus a non-Y-
coded protein(s) that associates with the kl-3 loop in 
wild type but that forms aggregates in the absence 
of this loop (BONACCORSI et al. 1988).

In D. melanogaster, both in methanol/acetone and 
in formaldehyde fixed preparations, the T53-1 anti-
body immunostains the kl-3 loop and the sperm flaggellum, 
but does not reveal protein aggregates in spermatocyte nuclei of X/O males (Figures 2–4 and data not shown). In D. hydei methanol/acetone-fixed 
testes the T53-1 antibody exhibits the same immuno-
fluorescence pattern as the sph-155 antiserum; it 
reacts with the Pseudonucleolus loop, the outer region 
of the Cones and the sperm flagellum [cf. Figure 9 and 
HULSEBOS, HACKSTEIN and HENNIG (1984)].

These observations pose the question of whether 
the sph-155 and the T53-1 antisera recognize differ-
ent loop-associated antigens. The best way to answer 
this question would have been a direct comparison of 
these reagents by immunoblot analysis. Unfortunately 
we were unable to perform these experiments because 
the sph-155 antiserum is not currently available. How-
ever, the two antisera can be compared based on 
previously published data. In immunoblots of D. hydei 
testis proteins the sph-155 antiserum recognizes sev-
eral polypeptides having apparent molecular masses 
ranging from 80 to 155 kD (HULSEBOS, HACKSTEIN and 
HENNIG 1983, 1984); in D. melanogaster it appears 
to react weakly with a protein of about 80 kD (HUL-
SEBOS, HACKSTEIN and HENNIG 1983). Conversely, 
the T53-1 antibody recognizes bands of similar mo-
lecular masses in both species. In D. melanogaster it 
reacts with a fraction of 53 kD and in D. hydei with 
one of about 51 kD (the data for D. hydei are not 
shown).

Taken together these results suggest that the sph-
155 and the T53-1 antisera react with different anti-
gens. This implies that the kl-3 loop of D. melanogaster 
and the Pseudonucleolus loop of D. hydei, despite their 
different morphologies, fulfill a similar protein-bind-
ing function and accumulate multiple proteins in-
volved in spermiogenesis.

The biological role of the Y chromosome loops: 
Our results indicate that the kl-3 loop binds a non-Y-
encoded, testis-specific, Sarkosyl-urea insoluble pro-
tein, which is probably a tektin. However, the reason 
why a tektin is accumulated on the kl-3 loop is unclear. 
One of the possible interpretations is that the tektin 
bound to the kl-3 loop undergoes some sort of pos-
translational modification necessary for the normal 
development of the sperm axoneme. Alternatively, 
while accumulated on the loop, the tektin may be 
preassembled with other axonemal components. Un-
der both interpretations the Y loops can be viewed as 
complex organelles devoted to the compartmentali-
ization and processing of some proteins involved in 
spermiogenesis. In the absence of the loop function 
spermiogenesis would occur but the loop proteins 
would be incorporated in the sperm in a somewhat 
disorganized manner, leading to a general, although 
subtle, disorganization of the axoneme (for reviews 
see LINDSLEY and TOKUYASU 1980; LIFSCHYTZ 1987). 
Finally, we would like to speculate that the Y chro-
mosome loops have evolved to facilitate the develop-
ment of the giant sperm tails of the genus Drosophila.

An alternative view of the nature of the loop-form-
ing Y chromosome fertility factors comes from bio-
chemical and ultrastructural observations on males 
deficient for these genes. It has been shown that 
deficiencies of the kl-5 and kl-3 genes lead to the 
basis of two different high molecular mass polypep-
tides (M, \( \sim 300,000 \)) which are normally present in testis extracts of wild-type adult males. Moreover, deficiencies of either of these genes result in the absence of the outer dynein arms of the peripheral microtubular doublets of the axoneme (HARDY, TOKUYASU and LINDSLEY 1981; GOLDSTEIN, HARDY and LINDSLEY 1982). Based on these results it has been suggested that the kl-5 and kl-3 loci contain the coding sequences for the axonemal dyneins (GOLDSTEIN, HARDY and LINDSLEY 1982).

The results reported here do not exclude the possibility that the kl-3-dependent high molecular weight polypeptide is encoded either by a segment of the kl-3 loop or by a region just outside the loop. Similarly, the results of GOLDSTEIN and co-workers do not exclude the possibility that, in addition to coding for dyneins, the kl-3 loop fulfills a protein-binding function. However the finding that the kl-3 loop binds a tektin-like protein suggests an alternative interpretation. However, the finding that the kl-3 loop is a segment of the Y chromosome of Drosophila melanogaster supports the theory of heredity. Genetics 120: 1015–1034.

LITERATURE CITED


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FIGURE 9.—Indirect immunofluorescence staining with the T53-1 antibody of primary spermatocytes of different Drosophila species. (a, b and c) Phase contrast; (d, e and f) indirect immunofluorescence. (a and d) D. hydei; (b and e) D. virilis; (c and f) D. novamexicana. Note that the T53-1 antibody decorates the entire loop Pseudonucleolus (ps) and the margin of the Cones (co) of D. hydei. In D. virilis and D. novamexicana the antibody recognizes intranuclear structures resembling the Y loops. Bar = 10 \( \mu \)m.


Communicating editor: V. G. Finney