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Lampbrush loop-specific protein of *Drosophila hydei*

(chromosomal protein/Y chromosome/indirect immunofluorescence/gene activity)

THEO J. M. HULSEBOS, JOHANNES H. P. HACKSTEIN, AND WOLFGANG HENNIG

Department of Genetics, Katholieke Universiteit Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands

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ABSTRACT **By immunofluorescence techniques and protein blotting experiments we have shown that an antiserum specifically reacts with a M_r 80,000 protein (the "Ps protein") in the lampbrush loop "pseudonucleolus" in spermatocyte nuclei of *Drosophila hydei*. Comparative studies of X/Y and X/0 testes indicate that the gene encoding the Ps protein is not located on the Y chromosome but on an autosome or the X chromosome. The Ps protein is tissue specific. It is likely to be a rather conserved protein since the antigenic determinant recognized by the antiserum could be detected in the spermatocyte nuclei of a number of other *Drosophila* species. For those species with prominent Y chromosomal lampbrush loops, it could be shown that the cross-reaction is, as in *D. hydei*, associated with a specific Y chromosomal loop.**

During the primary spermatocyte stage the Y chromosome of *Drosophila hydei* (and many other *Drosophila* species) develops characteristic lampbrush loops of distinct morphology (1). The loops (2) result from transcription (3) of a subset of the about 16 fertility genes that have been mapped on the Y chromosome of *D. hydei* by cytogenetic methods (4).

Almost nothing is known about the factors determining the differences in morphology of the individual Y chromosomal loops. The specific morphology might be due to differences in DNA organization (5), to loop-specific ribonuclear protein configurations (see refs. 3 and 6), or to loop-specific proteins. Although there is no doubt about the presence of Y chromosome-associated proteins, the association of specific proteins with specific loops has so far not been demonstrated in *Drosophila* (5). In *Triturus*, however, Sommerville and coworkers have shown that certain oocyte nuclear proteins are found only in a limited number of lampbrush loops (reviewed in ref. 7). A less restricted distribution of nonhistone chromosomal proteins has been found in polytene chromosomes by various authors (for example, see refs. 8 and 9).

In *D. hydei*, structurally modified lampbrush loops are autonomously inherited with the affected loop (2 and 4). It has therefore been assumed that the DNA of the particular locus is responsible for the structural modification (10). The situation must however be more complex since X chromosomal (11, 12) and autosomal mutations (unpublished data) interfering with loop morphology have been recovered. These mutations indicate that the loop morphology is also influenced by the protein components, which are at least in part of autosomal origin. This has already been implied by cytological studies. Species hybrids with autosomes of *D. hydei* but a *Drosophila neohydei* Y chromosome display an entirely abnormal loop morphology and tend to be more *D. hydei*-like (6).

During our studies of the synthesis of sperm proteins of *D. hydei* (13, 14), it became apparent that an antiserum directed against one of these proteins, sph155, cross-reacts with a Y chromosomal loop protein. With indirect immunofluorescent staining techniques, we demonstrated that this antiserum specifically reacts with the "pseudonucleolus," probably by reacting with a protein of M_r 80,000 (the "Ps protein"). The antiserum enabled us to detect this Ps protein in a number of mutants with a modified pseudonucleolus or even without a cytologically detectable pseudonucleolus. The Ps protein is tissue specific and evolutionary rather conserved, since it could be detected in the spermatocyte nuclei of a number of other *Drosophila* species. The results obtained strongly indicate that this protein is not encoded on the Y chromosome.

MATERIALS AND METHODS

***Drosophila* Stocks.** *D. hydei*, wild type, *Drosophila neohydei*, wild-type, and *Drosophila melanogaster*, wild-type, were from our laboratory collection. *D. eohydei* was obtained from H. Beck, Geneva. All other *Drosophila* species came from the Drosophila Stock Center (Austin, TX).

The construction of X/0 males of *D. hydei* and *D. melanogaster* as well as of the males deficient for various part of the Y chromosome has been described (4, 14). The following Y chromosomal mutant males of *D. hydei* were used: −X/Df(YL)30 containing a Y chromosomal fragment with the loop-forming sites "nooses," modified "clubs," and modified "tubular ribbons" (complementation groups N–Q); −X/Df(YL)19 containing a Y chromosomal fragment with the loop-forming sites nooses, clubs, and tubular ribbons (complementation groups D–Q); −T(X;Y)37/Df(YL)51 containing the loop-forming sites nooses, pseudonucleolus, and threads (complementation groups A–E and Q); −T(X;Y)-74/0 containing a Y chromosomal fragment with the pseudonucleolus (complementation groups B and C); and −X/ms (Y)C4 male sterile mutation in complementation group C, which "lacks" the pseudonucleolus in phase contrast.

Immunological Procedures. Preparation of the antiserum against sph155 and the immunoblotting procedure have been described (14).

For immunofluorescence staining, spermatocyte regions were collected in a drop of testis preparation buffer (10 mM Tris·HCl, pH 6.8/47 mM NaCl/183 mM KCl) on a microscopic slide. Coverslips were applied, and the preparations were gently squashed and then frozen in liquid nitrogen for 15 s. The coverslips were removed with a surgical knife and the preparations were passed through a series of ethanol washes (90%, 60%, 30%, 10%, 2 min each step). They were washed twice with phosphate-buffered saline (P_i/NaCl), fixed in 3.7% formaldehyde in P_i/NaCl for 10 min, and then incubated for 10 min with 3.7% formaldehyde/45% acetic acid in P_i/NaCl and extensively washed with P_i/NaCl. For immunofluorescence staining of spermatids and spermatozoa, the preparations were permeabilized for 5 min with 1% (wt/vol) Triton X-100 in P_i/NaCl and then washed. This treatment was omitted in cases in which spermatocyte nuclei were examined. The preparations were incubated for 30 min with 50 μl of antiserum or with pre-immune serum (both di-

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Abbreviation: P_i/NaCl, phosphate-buffered saline.

luted 1:100 in P_i/NaCl) at room temperature. After washing, they were incubated for 30 min with fluorescein-labeled sheep anti-rabbit IgG (diluted 1:100; Wellcome Laboratories), washed, and mounted in 50% glycerin in P_i/NaCl.

Immunofluorescence staining of ovaria and neuroblast cells from third instar larvae was carried out as described above. Salivary gland cells with polytene chromosomes were stained as described by Saumweber *et al.* (8) using the acetic acid as well as the formaldehyde fixation procedure.

The slides were studied under a Zeiss photomicroscope III with epifluorescence optics. As filters we used the combination BP 455-490/FT 510/LP 520 (Zeiss). Photographs were taken on Agfapan 100 film.

RESULTS

Specific Immunofluorescence Staining of a Y Chromosomal Loop Protein of *D. hydei*. In a previous communication (14), we reported the generation of an antiserum directed against sph155, a major sperm protein of *D. hydei* of M_r 155,000. By immunofluorescence, it was shown that this antiserum reacts with the elongating spermatids and all subsequent stages of spermiogenesis. Closer examination of the stages preceding meiosis with a refined immunofluorescence methodology revealed that the antiserum, in addition to spermatids and spermatozoa, reacts specifically with the pseudonucleolus in the primary spermatocyte nuclei of *D. hydei* (Fig. 1). The staining partly extends into the "cones," indicating a close relationship between the two structures, as has been postulated from their ultrastructure (15). Often we also found a fluorescent dot next to the nucleolus (Fig. 1). This most likely represents a reaction with the X chromosome (cf. ref. 15). No other nuclear or cytoplasmic structure reacts with the antiserum. Pre-immune serum does not react with any structure in the spermatocytes.

To analyze the immunological specificity of the staining reaction, we made protein blots of the protein content of testes from young wild-type males and probed it with the antiserum. As shown in Fig. 2 (lane A) the antiserum reacts as expected with sph155 (14) and with proteins of lower molecular weight. The latter are most probably degradation products of sph155 formed during the preparation of the sample. These products are manifest in extracts of young testes but almost completely absent in extracts of older testes (cf. ref. 14). In addition, the antiserum reacts with a protein of M_r 80,000. This protein is also present in the testis extracts of males that do not contain sph155—i.e., T(X;Y)37/Df(YL)51 and X/0 males (Fig. 2, lanes B and C, respectively). T(X;Y)37/Df(YL)51 lacks a Y chromosomal region that covers complementation groups F–P. Loci O and P within the deleted region are essential for the accumulation of sph155 in testis (cf. ref. 14). Under the experimental conditions used pre-immune serum shows no reaction at all (data not shown). T(X;Y)37/Df(YL)51 males express the lampbrush loops "threads," pseudonucleolus, and nooses in their spermatocytes. The pseudonucleolus in the spermatocyte nuclei of these flies reacts strongly with the antiserum (Fig. 3*B*). Identical results were obtained with other comparable strains. X/0 nuclei display only the fluorescent dot in the direct neighborhood of the nucleolus and a rather diffuse fluorescence over all the nucleus (Fig. 3*A*). The absence of any reactivity of polytene chromosomes, neuroblast cells, or ovaria with the antiserum indicates a tissue specificity for testes.

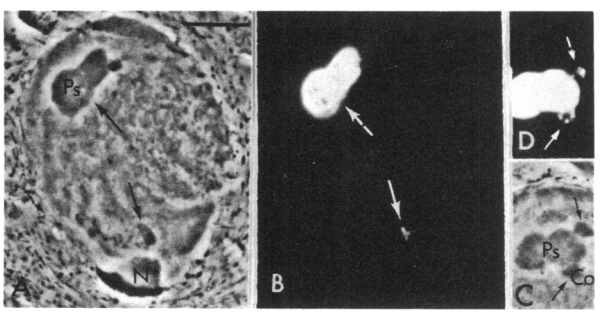

FIG. 1. Spermatocyte nuclei of *D. hydei*, wild-type. (*A*) Phase-contrast image. (*B*) Immunofluorescence pattern after reaction with antiserum. The pseudonucleolus (Ps) and a dot close to the nucleolus (N) are fluorescent. (*C* and *D*) Fluorescence pattern of the pseudonucleolus showing that the cones (Co) are fluorescent only in an outer region. Arrows indicate identical positions in the phase-contrast and fluorescence pictures. (Bar = 10 μm.)

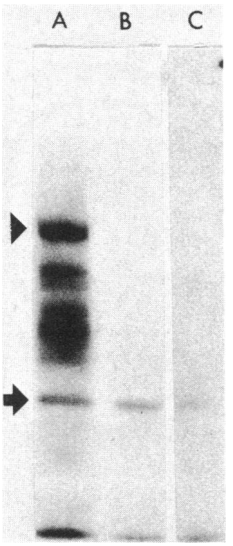

FIG. 2. Presence of the Ps protein in extracts of wild-type and mutant testes of *D. hydei*. The arrow indicates the Ps protein (M_r 80,000) and the arrowhead, the sph155 protein (M_r 155,000). The testes of 10 young wild-type males (lane A) (which have a low sph155 content), of 20 T(X;Y)37/Df(YL)51 males (lane B), and of 20 X/0 males (lane C) were boiled for 4 min in sample buffer (4). The proteins were fractionated on a 6.5% $NaDodSO_4$ gel, transferred to a nitrocellulose filter, probed with the antiserum against sph155, and then treated with ^{125}I-labeled protein A as described (14). Exposure time was 48 hr.

In summary, the antiserum reacts specifically with one Y chromosomal loop—i.e., the pseudonucleolus—by cross-reaction with a loop-specific protein of M_r 80,000. This Ps protein is not encoded by the Y chromosome, since it is found in X/0 nuclei.

Immunofluorescence Staining of Spermatocyte Nuclei of Mutants of *D. hydei*. The specificity of the antiserum for a protein associated with the pseudonucleolus of *D. hydei* enabled us to study mutants in which the pseudonucleolus is cytologically modified or absent.

X/Df(YL)30. By cytogenetic methods, it has been established that Df(YL)30 contains a Y chromosomal fragment with tubular ribbons, clubs, and nooses (4). The threads and pseudonucleolus (with the cones) are absent (Fig. 3*C*). Nevertheless, there is some fluorescence (Fig. 3*C*). Obviously, some pseudonucleolar material remained in the translocated chromosome of this fragment but it cannot be detected cytologically.

X/Df(YL)19. As in X/Df(YL)30, the Y chromosomal fragment of this deficiency lacks the threads and pseudonucleus (with the cones) (Fig. 3*D*). Complementation studies have shown that the Y chromosome is longer than in X/Df(YL)30 (4). In agreement with this, an immunofluorescence pattern emerges that indicates that more pseudonucleolar material is present in this mutant compared with X/Df(YL)30.

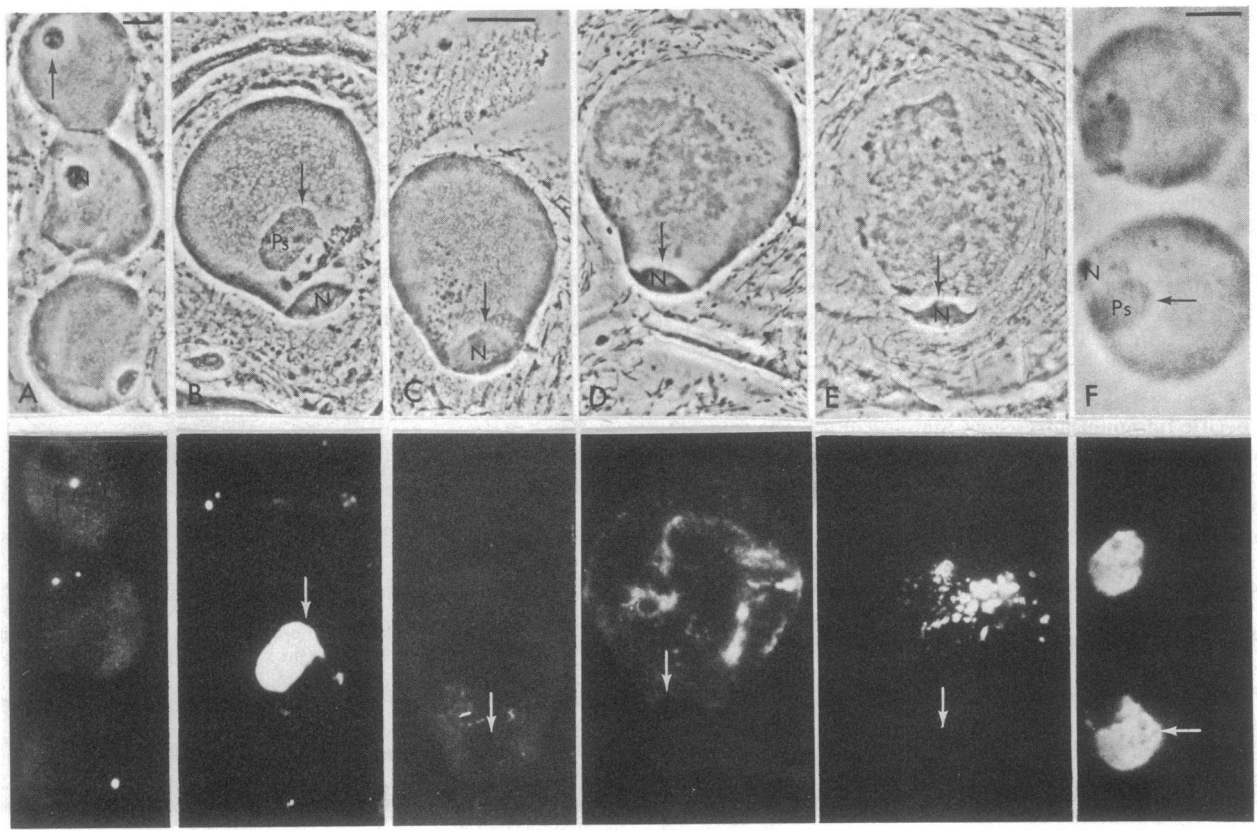

FIG. 3. Spermatocyte nuclei of various mutants of *D. hydei* in phase contrast (*Upper*) and after reaction with antiserum (*Lower*). (*A*) X/0: note the diffuse fluorescence with a concentrated region close to the nucleolus (N). The nucleolus does not react with the antiserum. (*B*) T(X;Y)37/Df(YL)51: the pseudonucleolus reacts strongly. (*C*) X/Df(YL)30, showing a relatively weak reaction in the neighborhood of the nucleolus. (*D*) X/DF(YL)19: the amount of reaction is increased compared with *C*. However, the reacting components do not assemble into a compact structure as in the normal pseudonucleolus (see Figs. 1 and 3*B*). (*E*) X/ms(Y)C4: in this mutant also the pseudonucleolus is not visible as a compact structure in the phase-contrast image. The strong fluorescence indicates its presence in a modified morphology. (*F*) T(X;Y)74/0: only in the phase-contrast image is the pseudonucleolus seen, and it reacts strongly with the antiserum. Arrows indicate identical positions in the phase-contrast and fluorescence pictures. (Bars: *A*, 5 μm; *B–E* and *F*, 10 μm.)

X/ms(Y)C4. Cytologically and genetically, mutant X/ms(Y)C4 was originally characterized as having no pseudonucleolus (Fig. 3*E*). Fig. 3*E* however clearly shows that pseudonucleolar material is present, although in a configuration different from the characteristic morphology of this loop pair (Fig. 1*A*) in wild-type spermatocytes.

T(X,Y)74/0. The Y chromosome fragment in this translocation contains only the pseudonucleolus. All other loop-forming sites are absent (Fig. 3*F*). Nevertheless, the structural integrity of the pseudonucleolus is maintained and wild-type-like, as shown by the immunofluorescence staining of the spermatocyte nuclei (Fig. 3*F*, cf. Fig. 1).

Immunofluorescence Staining of Spermatocyte Nuclei of *D. melanogaster*. We demonstrated previously that sph155 is absent in the spermatids and spermatozoa of *D. melanogaster* (14). However, a cross-reacting protein is present in spermatocyte nuclei. A complex fluorescent structure emerges after incubation with the antiserum (Fig. 4*A*). Autoradiographs of *D. melanogaster* testis protein blots incubated with the antiserum show a weak reaction with a protein of M_r 80,000, suggesting that the same protein is recognized as in *D. hydei* (data not shown). The immunofluorescence pattern in X/0 spermatocyte nuclei is somewhat different from the pattern in wild-type nuclei (Fig. 4*B*). Since the Y chromosomal lampbrush loops in *D. melanogaster* are poorly developed, it cannot be decided whether the difference in the fluorescence patterns is simply due to the absence of the Y chromosome, which might result in a different organization of the nucleus, or whether some of the fluorescent structures are associated with the Y chromosome.

Immunofluorescence Staining of Spermatocyte Nuclei of Other *Drosophila* Species. In the previous section it was reported that distinct structures in the spermatocyte nuclei of *D. melanogaster* specifically react with the antiserum. As this species is only distantly related to *D. hydei*, we studied some other species for their reactions with the antiserum. The results are listed in Table 1. In parallel experiments, we studied the reactions of the spermatids and spermatozoa with the antiserum. In a number of species, especially those more closely related to *D. hydei*, specific structures react with the antiserum. However, for most of them it was impossible to correlate the fluorescent structures with chromosomal lampbrush loops as the cytology of the latter is not known. The fluorescence in the spermatocyte nuclei of some species with characterized Y chromosomal loops—i.e., *D. eohydei*, *D. neohydei*, *D. repleta*, and *D. virilis*—is shown in Fig. 4 *C–F*.

Based on differential Giemsa staining (16) and cytogenetic criteria (17, 18), the "proximal loops" of *D. neohydei* and the pseudonucleolus of *D. eohydei* are considered as homologous to the pseudonucleolus of *D. hydei*. Both assignments are confirmed by the immunofluorescence staining with the antiserum. A protein immunologically related to the Ps protein is associated with these loops (Fig. 4 *C* and *D*).

Most of the members of the repleta group investigated exhibit a bright fluorescence in their spermatocyte nuclei (Table 1). A notable exception is *D. repleta* itself. There is no specific immunofluorescence except for a few tiny dots, one of which is consistently found next to the nucleolus and might represent a reaction with the X chromosome (Fig. 4*E*).

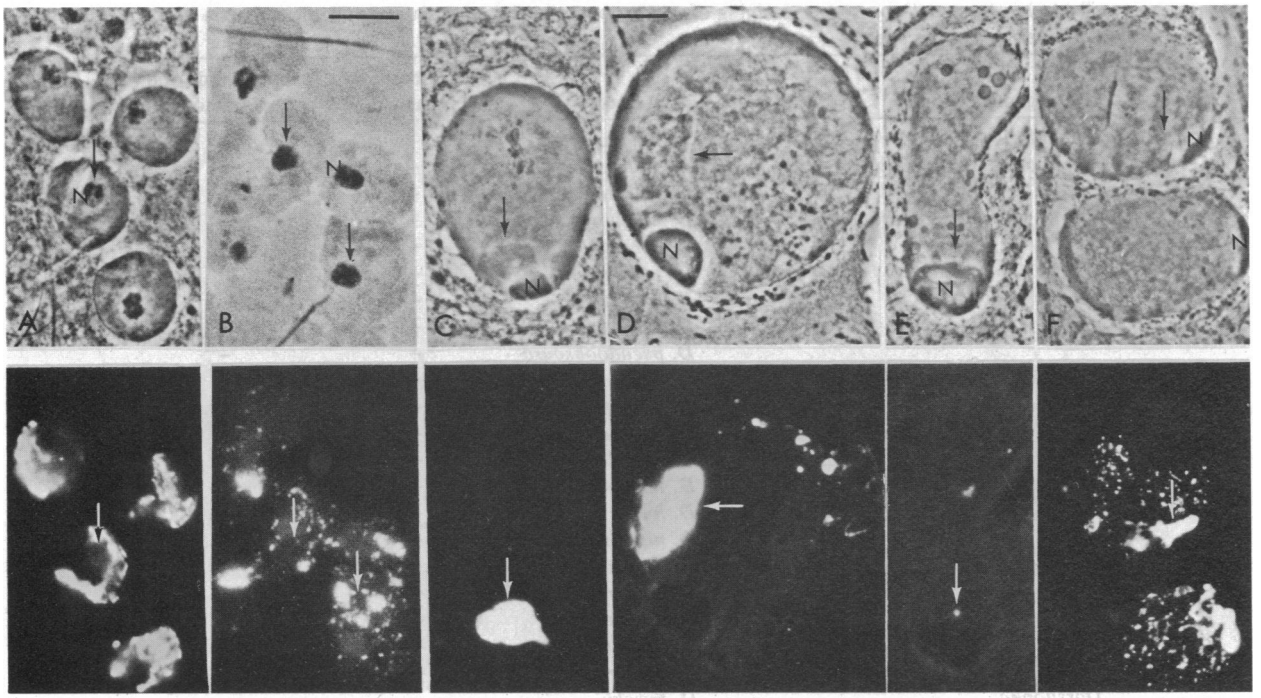

FIG. 4. Spermatocyte nuclei of various *Drosophila* species in phase contrast (*Upper*) and after reaction with the antiserum (*Lower*). (*A*) *D. melanogaster*, wild-type. (*B*) *D. melanogaster*, X/0: note that the distribution of fluorescence is more dispersed than in the wild-type (*A*) but the total amount may be similar. (Note the typical "X/0 crystals" in the phase-contrast picture.) (*C*) *D. eohydei*: the pseudonucleolus reacts strongly. (*D*) *D. neohydei*: the proximal loops react strongly but some other components, not identified in phase contrast, also display a relatively strong reaction. (*E*) *D. repleta*: only minor fluorescent dots are seen, one of which is close to the nucleolus (N). (*F*) *D. virilis*: a more diffuse fluorescence appears, which cannot be assigned to particulate structures in the phase-contrast picture. Arrows indicate identical positions in the phase-contrast and fluorescence pictures. (Bar = 10 μm.)

DISCUSSION

Our antiserum against sph155 most likely recognizes two antigens—the M_r 155,000 protein, a constituent of spermatids and spermatozoa, against which it was originally generated, and a protein of M_r 80,000. The reaction with two different proteins argues that the M_r 80,000 protein is found in testes of T(X;Y)37/Df(YL)51, a mutant in which sph155 does not accumulate (Fig. 2*B*). Also the immunofluorescence studies support this supposition: The pseudonucleolus is specifically stained in mutants that do not have sph155—i.e., T(X;Y)37/Df(YL)51 and T(X,Y)74/0 (Fig. 3). Moreover, specific structures are heavily stained by the antiserum in spermatocyte nuclei of *D. melanogaster*, *D. simulans*, *D. willistoni*, *D. pseudoobscura*, and *D. fulvimacula* but not in spermatids and spermatozoa (Table 1).

The potential of recognizing the same antigenic determinant of two different proteins seems to be an intrinsic property of this particular antiserum. Another polyvalent antiserum and a monoclonal antiserum, both raised against sph155, react with spermatids and spermatozoa, but not at all with the pseudonucleolus (M. Ruiters, personal communication).

However, we cannot definitely rule out the possibility that the Ps protein is a cleavage product of sph155. In this case, one has to suppose that sph155 is rapidly converted into the Ps protein in mutants lacking sph155. In addition, a feedback inhibition on the synthesis of sph155 must be postulated to explain why the amount of Ps protein is not increased compared with the amount in wild-type.

The immunofluorescence pattern of spermatocyte nuclei in Y chromosomal mutants (Fig. 3 *B–F*) and in X/0 males of *D. hydei* (Fig. 3*A*) showed that the Ps protein is synthesized irrespective of the presence of (parts of) the Y chromosome. Together with the observation that the immunofluorescence in wild-type of *D. melanogaster* (Fig. 4*A*) is retained in X/0 nuclei (Fig. 4*B*) this strongly indicates that the gene encoding the Ps protein must be located outside the Y chromosome (at least in these two species).

The presence of the Ps protein in X/0 nuclei raises the question of whether this behavior might be exhibited by other loop-specific proteins. If so, this might explain why Kloetzel *et al.* (5) failed to find loop-specific proteins by examination of the total protein content of wild-type and mutant spermatocyte nuclei of *D. hydei*.

The staining patterns obtained with mutants Df(YL)30 and Df(YL)19 suggest that the Ps protein binds to RNA and argue against a binding to DNA (10): The amount of fluorescence is considerably more than would be expected for binding to DNA, as can be judged by comparison of the intensity of immunofluorescence after reactions with histone antisera (see ref. 19). Also our studies of mutations in the A–C region of the Y chromosome permit the conclusion that the presence of the Ps protein in the pseudonucleolus is related to transcription of this locus.

In our earlier work it was established that mutants ms(Y)C4, Df(YL)30, and Df(YL)19 contain a nonfunctional complementation group C (4). If the Ps protein indeed recognizes transcripts of this locus, then we must conclude that in all three mutants the locus *C* is at least partially transcribed. The incapability of these mutants to complement deficiencies of the pseudonucleolus must therefore be due to a defective final gene product, whether this is a protein or an RNA molecule. The possibility to discriminate between totally inactive and defectively active genetic loci with the aid of this antiserum provides a valuable tool for studying the regulation and function of this locus.

The biological function of the Ps protein is difficult to assess. At present almost nothing is known about the specific functions of chromosomal proteins. Various possibilities have recently been discussed by Risau *et al.* (20). The evolu-

Table 1. Immunofluorescence staining of spermatocyte nuclei, spermatids, and spermatozoa of various *Drosophila* species

Subgenus	Group	Species	Spermatocyte nuclei	Spermatids and spermatozoa
Pholadoris		*D. lebanonensis*	−	−
Sophophora	*saltans*	*D. saltans*	−	−
	willistoni	*D. willistoni*	+	−
	melanogaster	*D. melanogaster*	+	−
		D. simulans	+	−
		D. takahashi	−	−
		D. ananassae	−	−
		D. subobscura	+	−
		D. pseudoobscura	+	−
Drosophila	*virilis*	*D. virilis*	+	+
	funebris	*D. funebris*	+	+
	repleta	*D. repleta*	−*	+
		D. eohydei	+	+
		D. neohydei	+	+
		D. hydei	+	+
		D. nigrohydei	+	+
		D. fulvimacula	+	−
		D. mulleri	−	−
		D. mercatorum	−	−
	melanica	*D. micromelanica*	−	−
	immigrans	*D. immigrans*	−	−
Dorsilopha		*D. busckii*	−	−

+ denotes specific fluorescence (in spermatocyte nuclei irrespective of whether or not associated with cytologically visible structures).
*See Fig. 4*E*.

tionary conservation of the Ps protein suggests a more basic function for this tissue-specific protein.

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