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Meiosis-specific protein selectively associated with sex chromosomes of rat pachytene spermatocytes

(sex vesicle/XY body/meiotic prophase/cell nucleus/spermatogenesis)

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ABSTRACT **During the first meiotic prophase of mammalian spermatogenesis, the sex chromosomes X and Y show a characteristic allocyclic behavior with respect to the autosomes. This is particularly evident during pachytene stage when sex chromosomes form the so-called sex vesicle. This structure is characterized by the condensed state of chromatin, transcriptional inactivity, and the limited extension of chromosome pairing, which is usually restricted to a short segment of sex chromosome axial elements. The molecular basis and functional significance of sex vesicle formation during mammalian spermatogenesis remain obscure. Here we report on the identification of a meiosis-specific sex vesicle protein we called XY40. Immunocytochemical localization on rat testis cryosections with a XY40-specific monoclonal antibody revealed that the labeling is confined to the axial elements of sex chromosomes. Biochemical characterization showed that protein XY40 (40 kDa; pI 5.7–5.8) can be extracted from rat pachytene spermatocytes and recovered in particles of 9.5 S with a native molecular mass of ≈152 kDa. We speculate that protein XY40 may be involved in the allocyclic behavior of sex chromosomes during male meiotic prophase.**

During male meiotic prophase in mammals, the sex chromosomes X and Y show a characteristic allocyclic behavior; i.e., they show differences in condensation with respect to the autosomes. Light microscopic observations of mammalian pachytene spermatocytes reveal the sex chromosomes forming a mass of condensed chromatin attached to the nuclear envelope: the so-called sex vesicle (1) or XY body (2). This contrasts with the situation in the oogenesis where the condensation degree and behavior of both X chromosomes is indistinguishable from that of autosomes. Ultrastructural examination of sex vesicles during pachytene of most eutherian mammals reveals that the differentially condensed chromatin fibers are attached to two axial elements of different lengths, corresponding to the X and Y chromosomes. The axial elements remain largely unpaired during pachytene with the exception of a common end that exhibits a short synaptonemal complex. Both the paired and the free ends of the axial elements are attached to the nuclear envelope (ref. 3; for reviews, see refs. 2 and 4). In correlation with their heteropyknotic state, sex chromosomes are thought to be, for the most part, metabolically inactive during pachytene, as judged by their inability to incorporate radioactive precursors. This is also in clear contrast to the situation of autosomes, which are transcriptionally active during pachytene (refs. 5 and 6; for review, see ref. 7). The molecular basis and the biological significance of allocyclic behavior of sex chromosomes during mammalian spermatogenesis are poorly understood. Although it has been proposed that X chromosome inactivation is an essential event for proper progression of spermatogenesis (8), to our knowledge, this has not been demonstrated (for review, see ref. 9).

To understand the mechanisms underlying the unique sex chromosome structure and behavior during male meiotic prophase, it is essential to understand the macromolecular composition of the sex vesicle. However, very little is known about the composition of the XY body. Cytochemical methods have indicated that the sex vesicle is composed of DNA but not of RNA (1, 10). In addition, the presence of basic proteins was shown in the chromatin and especially in the axial elements (for review, see ref. 2). More recently, immunocytochemical data showed that the axial elements of the XY body stain with a monoclonal antibody specific for 30/33-kDa proteins, which have been located (11) in the lateral elements of synaptonemal complexes.

Here we describe a monoclonal antibody, SV4a4, that enabled the identification of a meiosis-specific protein that is selectively associated with sex chromosome axial elements of rat spermatocytes.

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MATERIALS AND METHODS

Animals and Cells. Wistar rats and BALB/c mice were obtained from Charles River Breeding Laboratories. Rat pachytene spermatocytes and round spermatids were isolated by centrifugal elutriation as described (12–14).

Monoclonal Antibodies and Immunolocalization. Monoclonal antibody SV4a4 (IgM) was obtained after immunizing BALB/c mice with 2×10^6 whole rat pachytene cells that had been boiled in 50 μl of sample buffer (15) and diluted with 200 μl of PBS (140 mM NaCl/2.7 mM KCl/7 mM Na_2HPO_4/1.5 mM KH_2PO_4). Mice were immunized intraperitoneally with 250 μl of complete Freund's adjuvant plus the pachytene cell extract above followed by five booster injections with incomplete adjuvant containing the pachytene cell extract over a period of 3 months. The last booster injection was without adjuvant. Fusion was according to standard protocols (see also ref. 14). Monoclonal antibody SV4a4 was used in the form of hybridoma medium supernatant. Monoclonal antibody SC14f10 was described elsewhere (14).

Cryostat sections (5–10 μm) of shock-frozen rat tissues were fixed in acetone (−20°C; 10 min), and immunofluorescence was as described (14, 16). In some cases cryosections were processed for double-label immunofluorescence using monoclonal antibodies SV4a4 and SC14f10.

Cell Fractionation. Purified pachytene spermatocytes and round spermatids were homogenized in PBS or in 10 mM Tris·HCl (pH 7.4) containing 1% Triton X-100 and centrifuged at 1200 × g (14). In some cases the first supernatant of such a preparation was subsequently centrifuged at 100,000 × g for 1 h. Pellets and supernatants were then processed for polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis (14).

Sucrose Gradient and Gel Chromatography. The 100,000 × g supernatant of 2–3 × 10^7 rat pachytene spermatocytes was loaded onto a 5–30% sucrose gradient. After centrifugation at

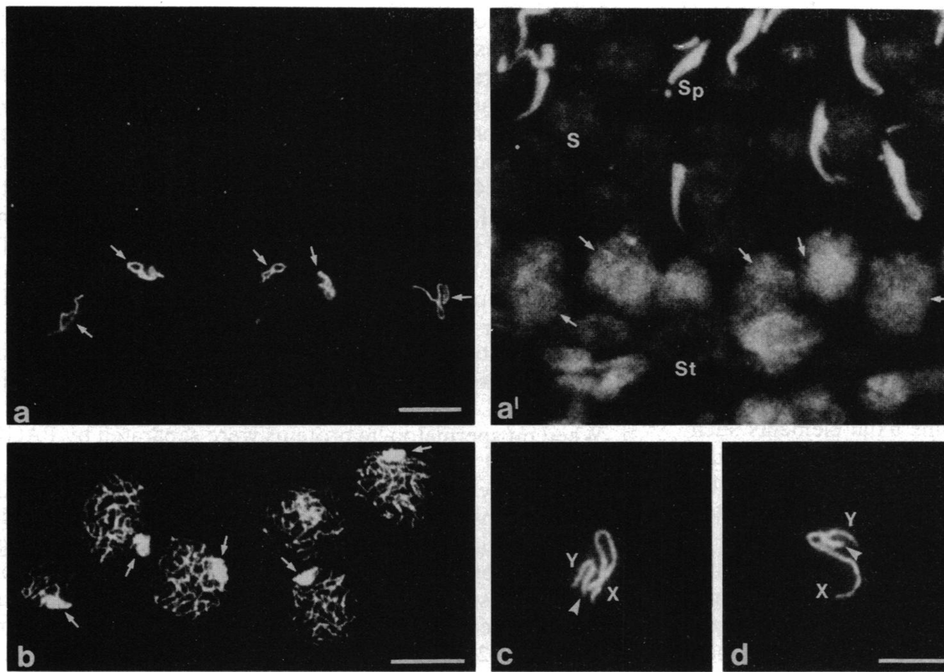

FIG. 1. Immunofluorescence microscopy of cryosections through rat testis. Monoclonal antibody SV4a4 alone (*a*, *c*, and *d*) or plus synaptonemal complex antibody SC14f10 (*b*) were used. (*a*) Antibody SV4a4 reacts with thread-like structures located within spermatocyte nuclei (arrows). (*a'*) The same section was stained with the DNA-specific fluorochrome Hoechst 33258. (*b*) The staining of synaptonemal complexes is shown with the structures recognized by antibody SV4a4 (arrows). (*c* and *d*) Thread-like structures as in *a* are shown at higher magnification. St, Sertoli cells; S, spermatids; Sp, sperms; X, X chromosome axial element; Y, Y chromosome axial element. (Bars: *a* and *b*, 10 μm; *c* and *d*, 5 μm.)

35,000 × *g* in an SW41 rotor (Beckman) for 18 h, 0.5-ml fractions were collected. For S-value estimation, reference proteins (bovine serum albumin, 4.3 S; IgG, 6.5 S; catalase, 11.3 S; thyroglobulin, 16.5 S) were centrifuged in parallel (for details, see ref. 17). For gel chromatography, 100,000 × *g* supernatants were loaded onto a 78 cm × 1.5 cm Sephadex G-200 column (Pharmacia). Proteins were eluted at a flow rate of 4 ml/h, and 2.4-ml fractions were collected (for details, see ref. 17). The following proteins served as standards: bovine serum albumin (68 kDa), IgG (160 kDa), catalase (243 kDa), and thyroglobulin (670 kDa). Native protein molecular mass determination and particle shape estimation were as described (18, 19). Protein fractions obtained from sucrose gradients and gel chromatography were examined by PAGE and immunoblot analysis.

PAGE and Immunoblot Analysis. One-dimensional PAGE was performed according to Laemmli (15) using 10% or 5–20% polyacrylamide gels. Two-dimensional PAGE (isoelectrical focusing in the first dimension) was as described (20). Immunoblot analysis was according to Kyhse-Andersen (21). Nitrocellulose sheets were blocked with 10% (wt/vol) nonfat dry milk in TBST (150 mM NaCl/0.1% Tween 20/50 mM Tris·HCl, pH 7.4) before incubation with SV4a4 hybridoma supernatant. The following steps were as described (14).

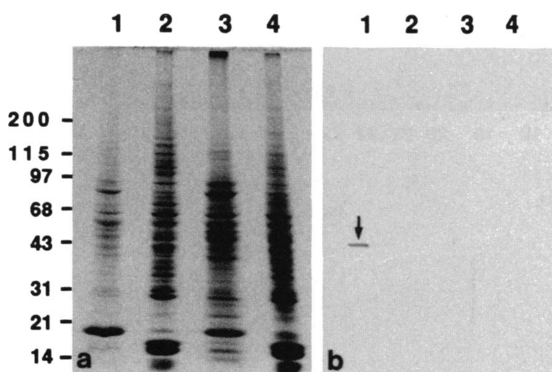

FIG. 2. Identification of the antigen recognized by monoclonal antibody SV4a4. (*a*) Gradient gel (5–20% polyacrylamide) stained with Coomassie blue. Lanes: 1, polypeptides present in a 1200 × *g* supernatant of Triton X-100-treated pachytene spermatocytes (see ref. 14); 2, polypeptides of the corresponding pellet; 3, polypeptides present in the supernatant of round spermatids prepared as described for spermatocytes; 4, polypeptides of the corresponding pellet. The equivalent of 5 × 10^6 cells was loaded for spermatocytes and the equivalent of 10^7 cells was loaded for spermatids. (*b*) In the corresponding immunoblot, antibody SV4a4 reacts exclusively with a 40-kDa polypeptide in pachytene spermatocyte supernatants (lane 1). Molecular masses of reference proteins are indicated in kDa.

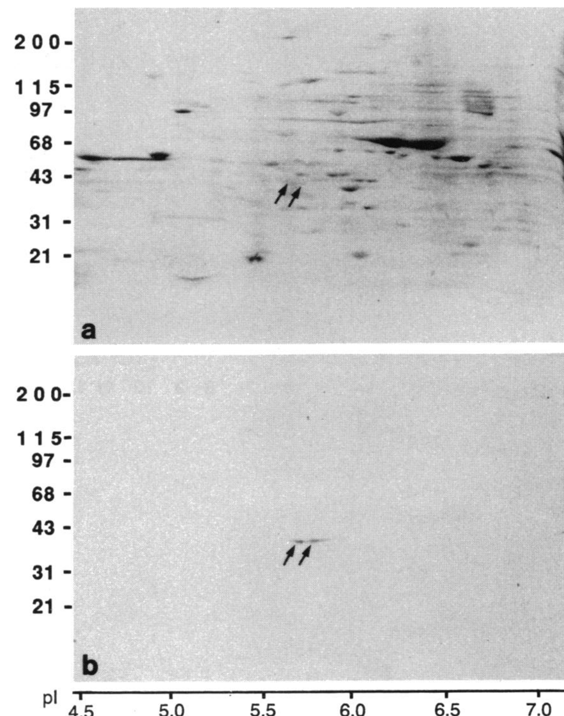

FIG. 3. Characterization of the antigen recognized by monoclonal antibody SV4a4 after two-dimensional PAGE (isoelectric focusing in the first dimension and a 10% polyacrylamide gel in the second dimension). (*a*) Polypeptides present in the 1200 × *g* supernatant of Triton X-100-treated pachytene spermatocytes stained with Coomassie blue. (*b*) In the corresponding immunoblot, the protein of 40 kDa is resolved into two isoelectric variants of pI 5.7 and 5.8. Molecular masses of reference proteins are shown in kDa.

RESULTS

As shown in Fig. 1 *a* and *a'*, monoclonal antibody SV4a4 labeled thread-like coiled structures located at the periphery of nuclei in certain cells of rat seminiferous tubules. These cells were identified as pachytene spermatocytes by their size, abundance, and association with other cell types (22). This was corroborated by double-label immunofluorescence microscopy using monoclonal antibody SV4a4 and the synaptonemal complex antibody SC14f10 (Fig. 1*b*). By immunoelectron microscopy, it could be shown that the thread-like structures labeled with antibody SV4a4 correspond to the axial elements of sex chromosomes (data not shown).

In favorable sections of pachytene spermatocytes, immunofluorescence microscopy demonstrated various types of associations between sex chromosome axial elements such as: (*i*) termino-terminal association (Fig. 1*c*, arrowhead) and (*ii*) pairing via the common end (Fig. 1*d*, arrowhead). Similar configurations have been described in spread silver-stained preparations of rat pachytene spermatocytes (see figure 9 of ref. 23). Furthermore, our results in Fig. 1 are compatible with published three-dimensional data of rodent sex chromosome axial elements obtained by electron microscopic analysis (3, 24). In meiotic prophase stages other than pachytene (e.g., lepto-zygotene and diplotene), spermatocytes were weakly labeled with our antibody (data not shown) whereas postmeiotic stages were negative (Fig. 1*a*).

Immunofluorescence microscopy on frozen sections of several tissues (brain, liver, gut, and muscle) from both male and female rats was negative with monoclonal antibody SV4a4. Remarkably, the antibody also showed no reaction when tested on frozen sections of rat fetal ovaries containing pachytene oocytes (21-day fetuses). In contrast, the control antibody SC14f10 showed the expected positive reaction with synaptonemal complexes (data not shown). Monoclonal antibody SV4a4 showed a rather restricted cross-reactivity with species other than rat. It stained mouse spermatocytes but was negative on hamster and bull testes (data not shown). In summary, our immunofluorescence data are in full agreement with observations on the assembly–disassembly process of sex chromosome axial elements during male meiotic prophase (2).

When rat spermatocyte proteins were separated by PAGE and analyzed on an immunoblot using antibody SV4a4, a 40-kDa polypeptide band was specifically recognized (Fig. 2). In two-dimensional immunoblots, the 40-kDa polypeptide was resolved into two spots with pI values of 5.7 and 5.8 (Fig. 3). Expression of the 40-kDa polypeptide appears to be restricted to spermatocytes. Consistent with data of immunofluorescence microscopy, other cell types such as spermatids (Fig. 2) and hepatocytes were negative in immunoblots when tested with antibody SV4a4. Thus because of its mobility in gels and location in the sex vesicle, this polypeptide was termed XY40.

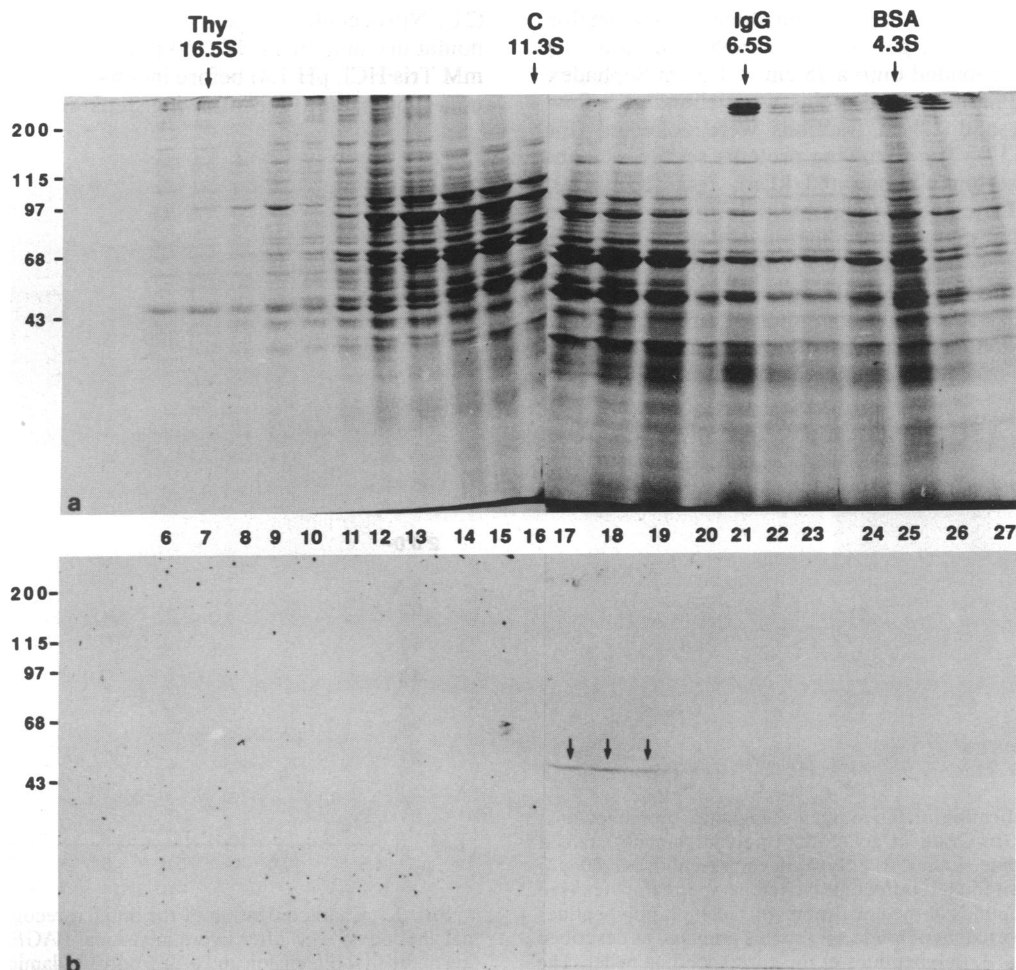

FIG. 4. Sucrose gradient (5–30%) of pachytene spermatocyte proteins present in the 100,000 × *g* supernatant. (*a*) Polypeptides of collected fractions were separated by SDS/PAGE (10% polyacrylamide) and stained with Coomassie blue. (*b*) Corresponding immunoblot after incubation with antibody SV4a4. Fraction numbers are indicated. Fate of protein XY40 is shown by arrows. Position and S value of reference proteins in a parallel gradient are indicated. Molecular masses are shown in kDa. BSA, bovine serum albumin; C, catalase; Thy, thyroglobulin.

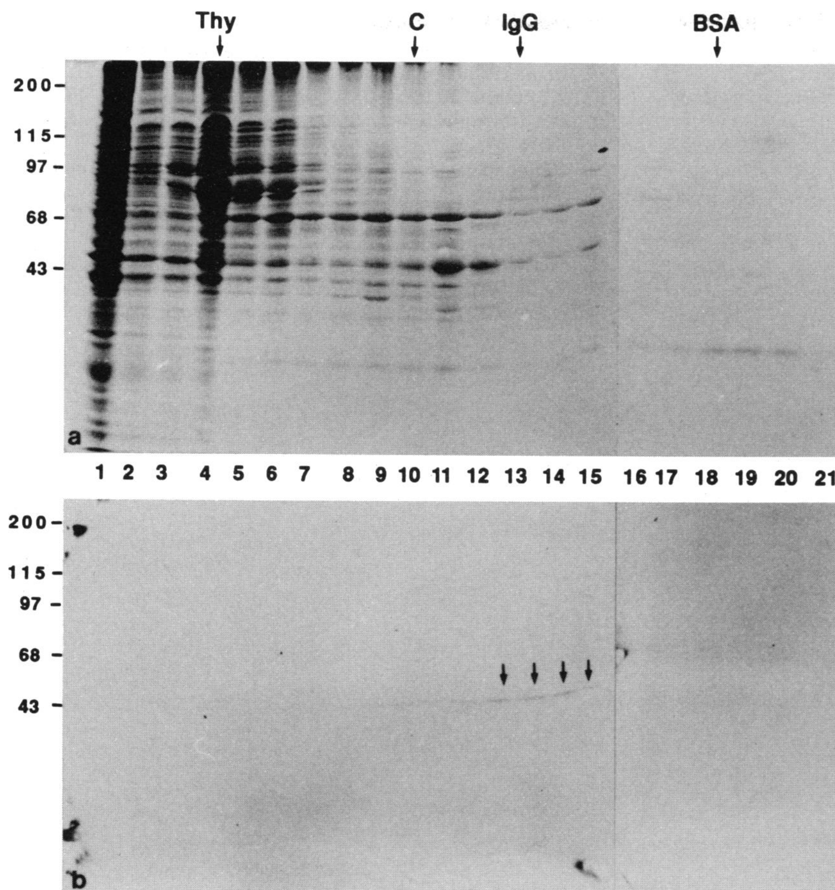

FIG. 5. Gel chromatography of proteins present in the $100{,}000 \times g$ supernatant of pachytene spermatocytes. (*a*) Polypeptides of the collected fractions were separated by SDS/PAGE (10% polyacrylamide gel) and stained with Coomassie blue. (*b*) Corresponding immunoblot after incubation with antibody SV4a4. Fraction numbers are indicated. Fate of protein XY40 is shown by arrows. Reference proteins from the same column for determination of native molecular masses are indicated. Molecular masses are shown in kDa. BSA, bovine serum albumin; C, catalase; Thy, thyroglobulin.

In cell fractionation experiments, protein XY40 was easily extracted. When pachytene spermatocytes were homogenized in a low salt buffer containing nonionic detergents, protein XY40 was found in the supernatant after centrifugation at $1200 \times g$ (Fig. 2). Similar results were obtained if homogenization of spermatocytes was in PBS. Even after centrifugations at $100{,}000 \times g$, virtually all protein XY40 was found in the supernatant. To characterize this protein further, $100{,}000 \times g$ supernatants of rat pachytene spermatocytes were analyzed by sucrose gradient centrifugation. Under these conditions protein XY40 had a sedimentation coefficient of ≈9.5 S (Fig. 4). Gel filtration revealed an apparent molecular mass of ≈147 kDa (Fig. 5). According to these data protein XY40 is contained in a globular particle with a native molecular mass of ≈152 kDa (see refs. 18 and 19).

DISCUSSION

We report the identification and biochemical characterization of a protein, XY40, that is selectively associated with axial elements of rat sex vesicles. Furthermore, our immunocytochemical and immunochemical data strongly suggest that protein XY40 is exclusively expressed during meiotic stages of spermatogenesis. To our knowledge, no protein has been described with the subcellular localization and expression pattern we observed for XY40. Thus, the immunological approach used here has proved to be a promising strategy for analyzing sex vesicle composition.

We have presently no direct evidence as to the function of protein XY40. It is tempting to speculate that, because of its selective association with sex chromosome axial elements, protein XY40 may play a role in sex vesicle chromatin organization. Future experiments should elucidate the primary structure of protein XY40 and provide clues on its function.

The availability of monoclonal antibodies such as SV4a4 is also relevant from a methodological point of view. By applying well-characterized monoclonal antibodies to sectioned testis material, it should be possible to molecularly define certain nuclear domains within spermatocytes and to study changes in temporal–spatial organization of these domains at the light microscopic level (see also refs. 14 and 25). In addition, our results indicate that immunofluorescence microscopy on sectioned testis material reveals at least part of the three-dimensional details previously achieved only after tedious reconstructions at the electron microscopic level. We predict that immunocytochemistry, because of the availability of various monoclonal antibodies, will become a method of choice for studying nuclear organization and dynamics during meiosis.

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