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Original Article

Genesis of newt sperm axial fiber: cDNA cloning and expression of a 29 kDa protein, a major component of the axial fiber, during spermatogenesis

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ABSTRACT Newt sperm has a unique structure: the tail consists of axial fiber, undulating membrane and flagellum. The genesis and chemical composition of the axial fiber remain unknown. The axial fiber consists of about 10 major components, as evidenced by SDS-polyacrylamide gel electrophoresis. In order to clarify the biochemical properties of the components of the axial fiber and study the mechanism of axial fiber formation, we focused our attention on a 29 kDa protein, the major constituent of the axial fiber. Immunofluorescent antibody technique showed that the 29 kDa protein was first expressed in the cytoplasm of early round spermatids but was expressed on fibers in the periphery of the cyst in late round spermatids. Double staining with tubulin antibody and 29 kDa antibody showed that the fibers around the cysts in early round spermatids were flagella alone but those in late round spermatids consisted of flagella and 29 kDa protein. These results indicated that 29 kDa proteins are synthesized in the cytoplasm of round spermatids and enter the preformed flagella in late round and elongated spermatids. A cDNA clone for 29 kDa protein was isolated. A database search could not find any homologous clones, indicating that the 29 kDa protein is a new one. Northern blot with the cDNA showed that mRNA for 29 kDa protein was highly expressed in round spermatids but barely in primary spermatocytes, indicating that the mRNA for 29 kDa protein is haploid-expressed.

KEY WORDS: *newt sperm, axial fiber, 29 kDa protein, immunostaining, haploid expression*

Introduction

Mature sperm of the Urodeles is gigantic, it is 250-1000 μm long and has a unique structure; the head portion comprises the nucleus and the acrosome with the perforatorium. The tail portion consists of the axial fiber, undulating membrane and flagellum; the undulating membrane is attached to one side of the axial fiber and the flagellum runs along the edge of the undulating membrane (Fawcett, 1970; Picheral, 1979).

The axial fiber consists of cortex and medulla. The former is very resistant to treatment with detergents or enzymes, while the latter quickly disappears (Picheral, 1972, 1979). However, the molecular nature of the constituents of the axial fiber have remained to be elucidated. Also, the genesis of axial fiber and tail of newt sperm has not yet been analyzed.

In order to clarify the biochemical properties of the components of the axial fiber and study the mechanism of axial fiber formation, we focused our attention on the 29 kDa protein, the major constituent of the axial fiber, based on the SDS-polyacrylamide gel electrophoresis. By immunofluorescent antibody technique we show that the 29 kDa protein is first expressed in round spermatids; it is expressed in the cytoplasm in early round spermatids but is expressed on fibers in the periphery of the cyst as well as in the cytoplasm of late round spermatids. Based on the comparison of the formation of flagella with 29 kDa expression, the mechanism of axial fiber formation is discussed. Nucleotide sequence of the cDNA clone for 29 kDa protein indicates that 29 kDa protein is a new protein. Northern blotting with a cDNA probe for 29 kDa protein shows that 29 kDa mRNA is expressed post-meiotically.

Abbreviations used in this paper: SDS-PAGE, sodium lauryl sulfate polyacrylamide gel electrophoresis; IgG, immunoglobulin G; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate.

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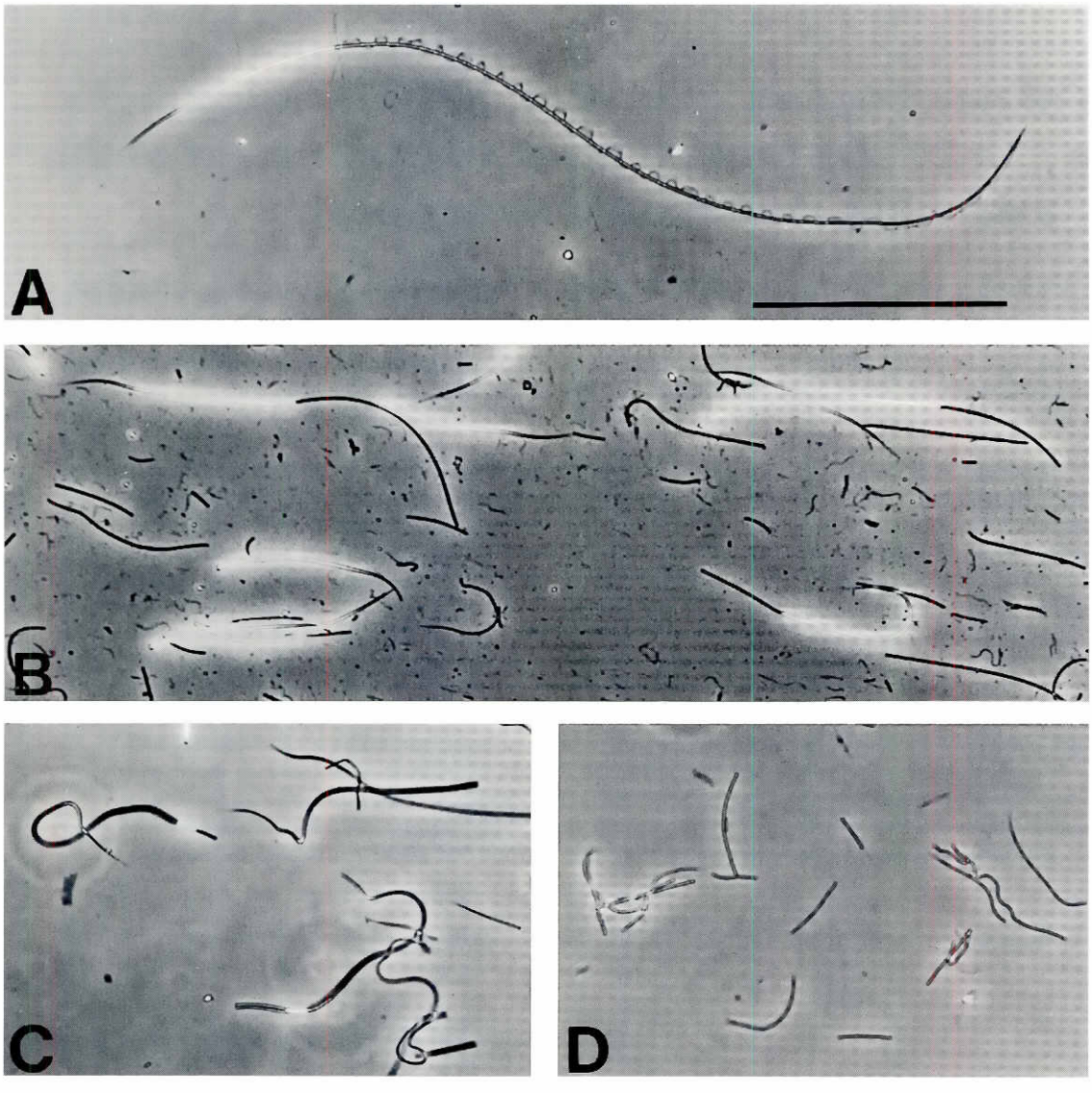

Fig. 1. Fractionation of sonicated newt sperm. **(A)** *Intact sperm.* **(B)** *Sonicated sperm. Some heads were attached to short axial fibers.* **(C)** *Fractionated head parts which swelled to some extent due to centrifugation in CsCl.* **(D)** *Fractionated axial fibers which were severed into various lengths by sonication. Bar, 100 μm.*

Results

Separation of sperm components

When newt sperm (Fig. 1A) was sonicated, axial fibers were cut into several pieces, undulating membranes were disrupted and flagella were severed into small pieces (Fig. 1B). However, heads were very resistant to sonication and the original shape was almost preserved. Frequently short axial fibers were attached to head parts, because the junction between the head and the axial fiber was not always cut off (Fig. 1B). Contaminated erythrocytes were destroyed completely by sonication.

Axial fiber fraction seemed to consist of a very pure population of short pieces of axial fibers alone (Fig. 1D). However, the head fraction comprised population of heads which frequently remained attached to short axial fibers as mentioned above (Fig. 1C). Major protein components in head parts are very probably protamines, which reached the front of the gel (Fig. 2S): the rest of the proteins in head parts should be small in amount, compared those in the axial fibers. Therefore, it is reasonable that SDS-PAGE profile of the axial fiber fraction (Fig. 2A) resembled that of the sperm (Fig. 2S) except in some bands at 40-60 kDa which seemed to be the constituents specific to the head part. Major bands in the axial fiber fraction common to those of the sperm seemed to be mostly the constituents of the axial fibers. Since the 29 kDa band was most abundant among them, we decided to raise an antibody against 29 kDa protein in order to analyze how it is expressed.

Expression of 29 kDa protein by Western blotting

When the testes fragments in various stages such as spermatogonia (SG), primary spermatocytes (PC), round spermatids (RT), elongated spermatids (ET) and mature sperm (MS) were run separately on SDS-PAGE and stained by CBB, the 29 kDa band appeared in elongated spermatid- and mature sperm-stages (Fig. 3). Other major bands observed in the axial fiber fraction (Fig. 2A) also seemed to appear in the elongated spermatid-stage. Western blotting of the antibody against 29 kDa clearly

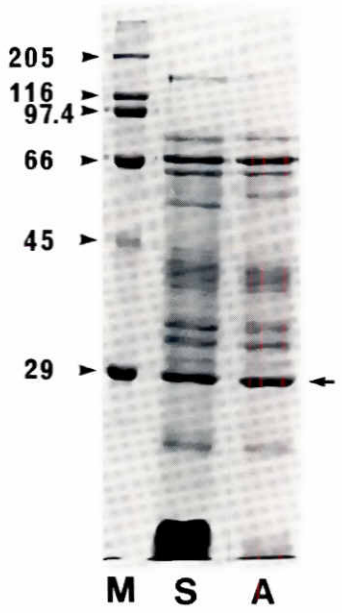

Fig. 2. SDS-PAGE analysis of sperm and fractionated axial fibers. *M, molecular weight markers; S, sperm. A, fractionated axial fibers. Arrow shows the 29 kDa protein band which is a major component of axial fibers.*

detected the 29 kDa protein in elongated spermatid- and mature sperm-stages (arrowhead; Fig. 3). This result indicates that the 29 kDa protein was expressed by the elongated spermatid-stage.

The expression of the 29 kDa protein was examined in several tissues other than testes (brain, lung, stomach, liver, heart, intestine, spleen; data not shown). SDS-PAGE and Western blotting did not detect 29 kDa proteins in any tissues except in the testes. This result indicates that the 29 kDa protein is specifically expressed in the testes.

Expression of the 29 kDa protein by immunofluorescent antibody

When mature sperm was stained by the antibody against the 29 kDa protein and FITC-conjugated second antibody against mouse IgG, only the axial fiber was positive, whereas the rest of the sperm such as head, undulating membrane and flagella, was negative (Fig. 4). This indicates that the 29 kDa protein is localized in the axial fiber of mature sperm.

In order to examine in what stage and how 29 kDa proteins are expressed during newt spermatogenesis, the testes sections were examined by immunofluorescent antibody technique (Fig. 5). The cysts in primary spermatocyte-stage (PC) were negative (Fig. 5A and a) except pericystic cells, which were non-specifically stained (arrows), but those in round spermatid-stage were weakly stained (Fig. 5B and b). In the cysts in late round spermatid-stage (RT), which were located adjacent to those in the elongated spermatid-stage (ET), a strong reaction was observed in a localized area at the periphery of the cysts (arrows) in addition to a significant reaction in the cytoplasm of spermatids (Fig. 5C and c). In the cysts in the elongated spermatid-stage (ET), a positive reaction was clearly detected in the cytoplasm of the elongated spermatids as well as in localized areas at the periphery of the cysts (arrowheads; Fig. 5C and c). And finally in the mature sperm-stage, only the axial fibers were positively stained but the rest was negative (Fig. 5D and d). These results indicate that 29 kDa proteins are expressed first in the early round spermatid-stage and are localized to form axial fibers in the late round spermatid-stage.

To study the expression of the 29 kDa protein in more detail, the testes sections were double-stained by the antibody against 29 kDa with the secondary antibody conjugated with FITC as well as by the antibody against tubulin with the secondary antibody conjugated with TRITC (Fig. 6).

In early round spermatids (Fig. 6A and a), the cytoplasm of primary spermatocytes and round spermatids as well as the fibers around the spermatid cysts (arrows) were stained red, indicating that the fibers are flagella. But in late round spermatids (Fig. 6B and b), the fibers around the cysts (arrowheads) were stained yellow. In elongated spermatids the fibers (double arrowheads) were strongly stained yellow. This result indicates that 29 kDa proteins are expressed in round spermatids, lagging behind the formation of flagella, and that 29 kDa proteins are expressed in the same region, probably in the same fibers as in flagella.

To precisely check whether the 29 kDa protein and tubulin are expressed in the same fibers, namely in flagella, dissociated spermatids were double-stained by 29 kDa protein antibody and tubulin antibody. In round spermatids, the flagellum was stained for the entire length by the tubulin antibody (Fig. 6c) but only the proximal part of the flagellum was stained by the 29 kDa antibody (Fig. 6C). In contrast, whole flagellum was stained by both antibodies in an elongated spermatid (Fig. 6D and d). The proximal half of the flagellum was apparently thicker than the caudal half. These results indicated that 29 kDa proteins are synthesized in the cytoplasm of round spermatids and enter the preformed flagella in late round and elongated spermatids.

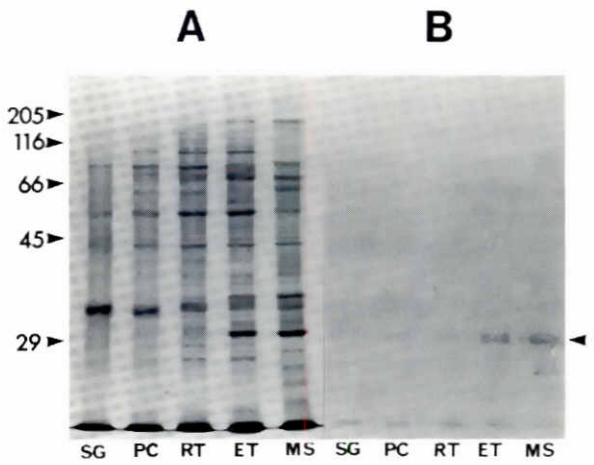

Fig. 3. Expression of the 29 kDa protein during newt spermatogenesis. **(A)** *SDS-PAGE of the testes in stages of secondary spermatogonia (SG), primary spermatocytes (PC), round spermatids (RT), elongated spermatids (ET) and mature sperm (MS).* **(B)** *Western blot using antibody against 29 kDa protein. Arrowhead shows the 29 kDa protein.*

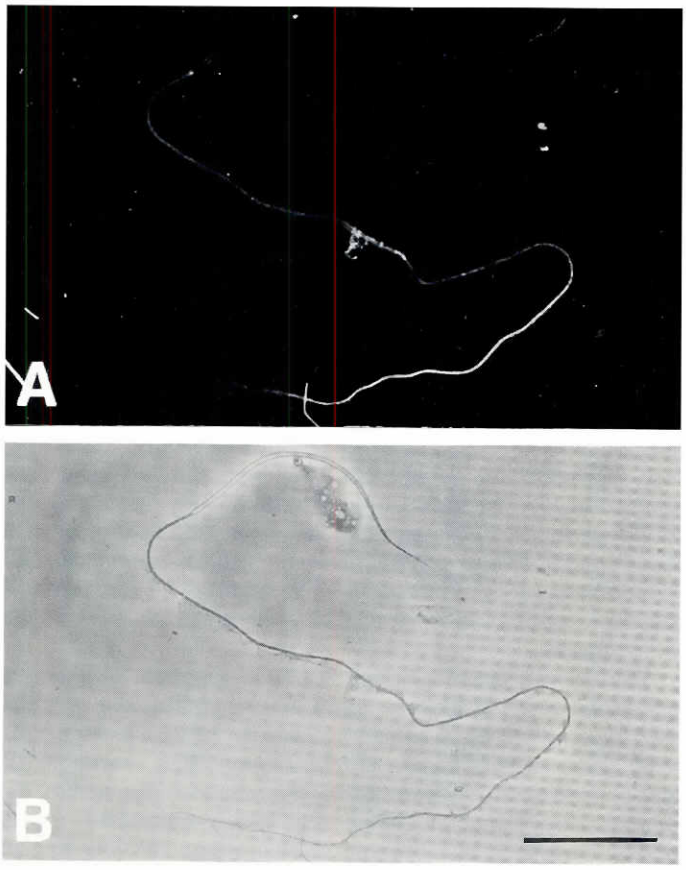

Fig. 4. Expression of the 29 kDa protein on mature sperm. (A) *Immunofluorescent staining of mature sperm using antibody against the 29 kDa protein and FITC-conjugated second antibody.* **(B)** *Phase contrast photomicrograph of the same sperm as shown in* **(A)**. *Bar, 50 μm.*

Cloning of a cDNA for 29 kDa protein

In order to study the gene expression of 29 kDa protein during newt spermatogenesis, we tried to isolate cDNA clones for 29 kDa protein. When λgt11 cDNA expression library was screened by the antibody against the 29 kDa protein, 3 positive clones out of $5x10^4$ pfu were obtained. One of the clones was 933bp in length and contained an open reading frame of 714bp in length, on the assumption that ATG, which appeared first, was the translation initiation codon (Fig. 7). This open reading frame predicted a protein consisting of 238 amino acids and the molecular weight to be 27 kDa, which was close to the value measured by SDS-electrophoresis (29 kDa). The amino acid sequence of the peptide fragments which were obtained by partial digestion of the 29 kDa protein by V8 protease and fractionation by HPLC were in total agreement with the amino acid sequence predicted from the nucleotide sequence of the cDNA (the two boxed regions in Fig. 7). This confirmed the possibility that the cDNA obtained was surely encoded for the 29 kDa protein. A database search could not find any homologous clones, indicating that 29 kDa is a new protein. The amino acid composition deduced from the nucleotide sequence showed that the percentage of hydrophobic amino acids (47.1%) is higher than that of hydrophilic amino acids (30.7%) or neutral ones (22.3%).

Expression of the 29 kDa mRNA during spermatogenesis

RNA was extracted from fractionated primary spermatocytes and round spermatids, electrophoresed, blotted to nylon membranes, and hybridized with the 29 kDa cDNA probe. Northern blot showed that 29 kDa mRNA, approximately 1 kb in length, was barely present in primary spermatocytes but was abundant in round spermatids (Fig. 8). This result indicates that 29 kDa mRNA is transcribed in haploid cells.

Discussion

Urodele sperm has complex and unusual structures besides the acrosome, head and axoneme which are common to the sperm in most species. The additional components are the axial fiber, marginal filament and undulating membrane. The axial fiber extends from the neck down, where in most other species axonemes should be running, while the axoneme in urodele sperm runs in the edge of the undulating membrane which extends from the axial fiber for its entire length toward the lateral direction. It remains to be elucidated how many components the axial fiber consists of and what those components are.

The present study aimed to analyze the identity and expression of the 29 kDa protein, one of the major components of axial fiber of newt sperm during spermatogenesis. The axial fiber of Japanese newt sperm consists of at least 10 major components and several minor components, based on the SDS-PAGE profile of purely fractionated axial fibers. The 29 kDa protein is most abundant among the major components of the axial fiber of newt sperm. Immunofluorescent studies using antibody against 29 kDa protein confirmed that the 29 kDa protein is localized in the axial fiber but not in the head part or in flagella.

According to ultrastructural studies by Picheral (1979), axial fiber of newt sperm consists of medulla and cortex. When the axial fiber is tracked down, the medulla extends from the anterior end to the posterior end of the axial fiber, while the cortex is thicker in the anterior part than in the posterior part and it disappears before it reaches the posterior end of the axial fiber. Since the entire length of the axial fiber is positive for the antibody against the 29 kDa protein, the 29 kDa protein is very probably localized in the medulla of the axial fibers.

When an axial fiber was treated with protease and observed by electron microscopy, some fibers expressing a periodical pattern were observed (Werner *et al.*, 1972; our unpublished observation). It remains to be clarified what components contribute to the formation of the periodical pattern in the axial fiber or whether the 29 kDa protein resides in the fibers with the periodical pattern. In order to analyze the fine structure of the axial fiber, we will have to isolate cDNAs for other major components of the axial fiber and raise antibodies against their products.

The 29 kDa protein is not expressed in any tissues other than testes among the several newt tissues examined. The nucleotide sequence of the cDNA for 29 kDa protein showed no similarity to any clones obtained so far, indicating that the 29 kDa is a new protein which has no special motifs. One characteristic is that hydrophobic amino acids dominate hydrophilic ones.

Fawcett (1970) and Picheral (1979) posed some homologies between urodele and mammalian sperm tail by comparative ultrastructural study. Based on the density, differentiation into cortical and medullary zones and relation to the connecting piece, they

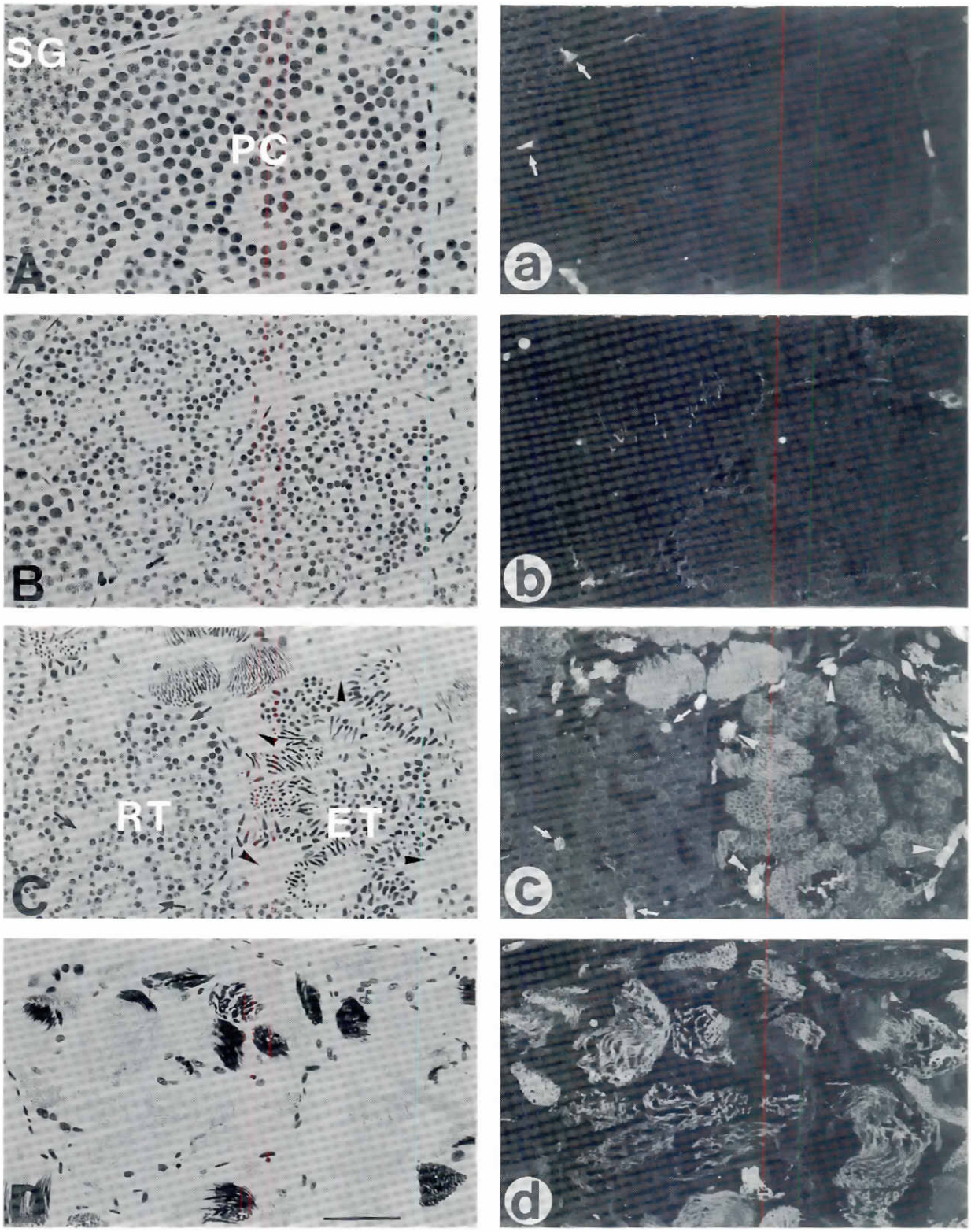

Fig. 5. Expression of the 29 kDa protein during newt spermatogenesis. (A-D) *Histological sections stained by hematoxylin and eosin.* **(a-d)** *Immunofluorescent staining of tissue sections just adjacent to the left one using antibody against the 29 kDa protein and FITC-conjugated second antibody.* **(A and a)** *Lobules in stages of secondary spermatogonia (SG) and primary spermatocytes (PC). In* **(a)** *pericystic cells are non-specifically stained (arrows).* **(B and b)** *Early round spermatid-stage.* **(C and c)** *Late round spermatid-stage (RT) and elongated spermatid-stage (ET).* **(D and d)** *Almost mature sperm-stage. Bar, 100 μm.*

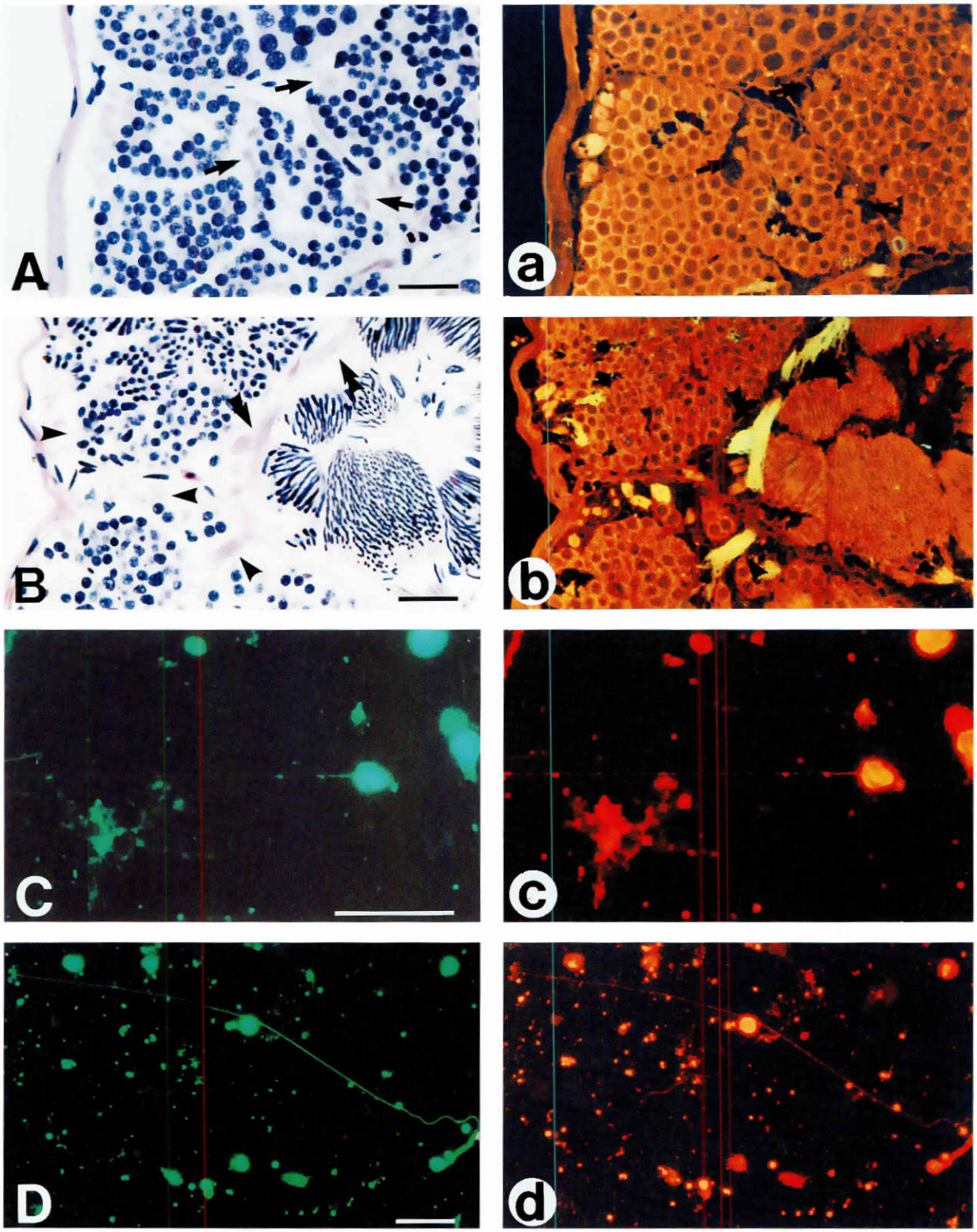

Fig. 6. Expression of the 29 kDa protein during spermatogenesis. (A and B) *Histological sections stained by hematoxylin and eosin.* **(a and b)** *Double immunostaining of the sections just adjacent to the left ones using antibody against tubulin with rhodamine-conjugated second antibody and antibody against the 29 kDa protein with FITC-conjugated second antibody. Sections were first photographed with NIBA filter for FITC staining and then photographed with WIG filter for TRITC staining. The fibers consisting of flagella alone are stained red, while the fibers consisting of flagella and 29 kDa proteins are stained yellow.* **(C and c, D and d)** *Dissociated round spermatid* **(C and c)** *and elongated spermatid* **(D and d)** *were doubly stained and observed through NIBA filter* **(C and D)** *or through WIG filter* **(c and d)**. *Bar, 50 μm.*

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GCCGCAGCACCCGTAGCCAGGAGCAGAGAGACGGTGCCCCCCGGCCGGACATGGAGCTAC   60
TGCAGAGCCATGAGGTGGAGTCCCTCCGCTGCGAGAACGTGCGGCCTGCTGACGGAGAAC  120
         M  R  W  S  P  S  A  A  R  T  C  G  L  L  T  E  N      17
GGCGAGCTGCGCAAGATGGTGGGCCTGATGCAGGAGAACTGGAGCTGCGCTGCACCCTGC  180
G  E  L  R  K  M  V  G  L  M  Q  E  N  W  S  C  A  A  P  C     37
GTCGACCACGAGAGCCGCGTGCGCACCCTCAGCCCCCCTGCAAGGAGCACAAGGACAAGG  240
V  D  H  E  S  R  V  R  T  L  S  P  P  A  R  S  T  R  T  R     57
AGACCCACTGCAGCAAGGATGGCAAAAAGGACCCGTCCACGCCTCGCCTCCCACGCACCC  300
R  P  T  A  A  R  M  A  K  R  T  R  P  R  L  A  S  H  A  P     77
CCCTGCAGAGACCCCAAGCAGCTTCAGCGCTGCCAGAGAGTGGTAGGAGAGATCGCCTTC  360
P  C  R  D  P  K  Q  L  Q  R  C  Q  R  V  V  G  E  I  A  F     97
CAGCTGGACCGGAGGATTCTCTCATCCATCTTCTTGGAACAAAGCAGATTATATGGTTTT  420
Q  L  D  R  R  I  L  S  S  I  F  L  E  Q  S  R  L  Y  G  F    117
ACGGTATCTAATGTGCAGGAAAAAATTATACAGGCGACAACTTGTCCATTGAACATCAAG  480
T  V  S  N  V  Q  E  K  I  I  Q  A  T  T  C  P  L  N  I  K    137
GTGGACGAGGCTCAACGCTGTGAGATGAACCGGCGATACCAGGACATGATGTGCCGTCTT  540
V  D  E  A  Q  R  C  E  M  N  R  R  Y  Q  D  M  M  C  R  L    157
AAGAAGTACGGCTACGACCCACGGGTGCACCCCCTCTTCTCAGAGTATCTGGTTAATACG  600
K  K  Y  G  Y  D  P  R  V  H  P  L  F  S  E  Y  L  V  N  T    177
TATGGCATCATGAAGGACCGGCCCCTGCCAGGCAGTGGAGACGTGGCGTCCTTTAGTGAC  660
Y  G  I  M  K  D  R  P  L  P  G  S  G  D  V  A  S  F  S  D    197
CCGGAGGTTCTGCGAAAGATGGCAGCCGAGGCCATGCCCGCAGATGTGCTGAAAGATGTG  720
P  E  V  L  R  K  M  A  A  E  A  M  P  A  D  V  L  K  D  V    217
CATCTCTTACTTAACTGCTTGGTACAGCTGGCGAAGGAAGACGGGAAACCCCTCTTCATC  780
H  L  L  L  N  C  L  V  Q  L  A  K  E  D  G  K  P  L  F  I    237
TGGTAGAACAAGAGAAAGAAAGAAACTCTTGACTGGTAGAACATTTCCAGACCCATGAAT  840
W  *                                                           238
CCCCCATTAAAGTTCATTCAAGAAAACCATCACTACTGATCAAATCTGGTTCTGCCTGTG  900
GCACCCAGATAGAAAATCATTAAAATACCTCTG                              933
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Fig. 7. Nucleotide sequence of a cDNA clone encoding the 29 kDa protein and its predicted amino acid sequence. *Star shows the stop codon. Square boxes show the regions whose amino acid sequence coincides with those of the peptide fragments fractionated by HPLC.*

thought that the axial fiber in urodele sperm corresponded to one of the outer dense fibers, probably number 3. Vera *et al.* (1984) showed that rat sperm outer dense fibers consist of 6 major components (87,000, 30,400, 26,000, 18,400, 13,000, 11,500) and indicated that components 30,400 and 26,000 had a close structural relationship with each other. Morales *et al.* (1994) isolated a cDNA for 27 kDa protein of rat sperm outer dense fibers which was identical to the cDNA isolated by Burfeind and Hoyer-Fender (1991), who found a high proportion of a repetitive motif, Cys-Gly-Pro, at the carboxy-terminal end. Interestingly, some 30 and 40 kDa proteins were detected over the entire tail of elongated spermatids, the so-called satellites, on the outside of the axoneme in *Drosophila* sperm (Kuhn *et al.*, 1988). The cDNAs encode a protein with a high proportion of the repetitive motif Cys-Gly-Pro (Schäfer *et al.*, 1993). From these results, it was proposed that regions of outer dense fiber genes and their products are evolutionarily conserved (Morales *et al.*, 1994). On the other hand, the 29 kDa protein, the major component of newt sperm axial fiber, has no repetitive motif of Cys-Gly-Pro. In order to explore the relation of newt sperm axial fiber to the accessory structure in mammalian and *Drosophila* sperm tail, we will have to isolate cDNAs for the other major components of newt sperm axial fibers.

It was revealed that the 29 kDa protein and its mRNA are expressed in round spermatids. According to SDS-PAGE analysis of the proteins expressed during spermatogenesis, most of the major components of the axial fibers may be expressed in haploid cells. In the newt, only one precedent of haploid-expressed mRNA has been reported (Yoshinobu *et al.*, 1996): protamine mRNAs, which are also known as haploid-expressed genes in mammals (Hecht, 1989) and fishes (Seniuk *et al.*, 1991). Protamine genes in various species are translationally regulated, i.e., the translation of the genes occurs, lagging their transcription behind. Genes for outer dense fibers of rat sperm are also known to be haploid-expressed and translationally-regulated (Burfeind and Hoyer-Fender, 1991; Morales *et al.*, 1994). It remains to be clarified whether the gene for the 29 kDa protein is translationally regulated like protamine.

Since the 29 kDa protein is one of the major components in the axial fiber of newt sperm, it seems possible to know how the axial fiber is formed during spermatogenesis by comparing the time and place of 29 kDa protein expression to those of the formation of the flagella. Flagella are formed in round spermatids several hours following second meiotic divisions *in vitro* (Abé, 1988). The present study also shows that in early round spermatids flagella are formed in the periphery of the cysts. On the other hand, 29 kDa protein is expressed in some fibers in the periphery of the cysts in late round spermatids, while it is first detected in the cytoplasm of early round spermatids. Detailed observation by double-staining with antibodies against tubulin and 29 kDa protein clearly shows that the fibers containing the 29 kDa protein also contain tubulin. In elongated spermatids well-developed fibers in the periphery of the cysts contain the 29 kDa protein as well as tubulin. These results indicate that 29 kDa proteins are present within the same tube as the flagella are in.

The followings are some possibilities on the mechanism of tail formation in newt sperm; first, flagella and axial fiber are separately extruded from the cells and later fused by the undulating membrane. Second, flagella and axial fiber are formed within a tube and later spatially separated though connected by the undulating membrane. The present immunofluorescent study indicates that the second possibility is probable. Dissociated primary spermatocytes can proceed through two meiotic divisions to give rise to round spermatids which then grow flagella to a length as long as several hundred microns *in vitro* (Abé, 1988; Uno and Abé, 1990). When observed by phase contrast microscopy, the flagella were thin when they were short, but the longer the flagella grew, the thicker they became in the proximal part as compared to the distal part. The present immunofluorescent study showed that thinner flagella contain tubulin but not the 29 kDa protein, whereas the thicker part of the flagella contains the 29 kDa protein as well as tubulin. This indicates that the 29 kDa protein is synthesized in the cytoplasm of spermatids: they enter the tube within which flagella were formed and gradually advance to the distal direction.

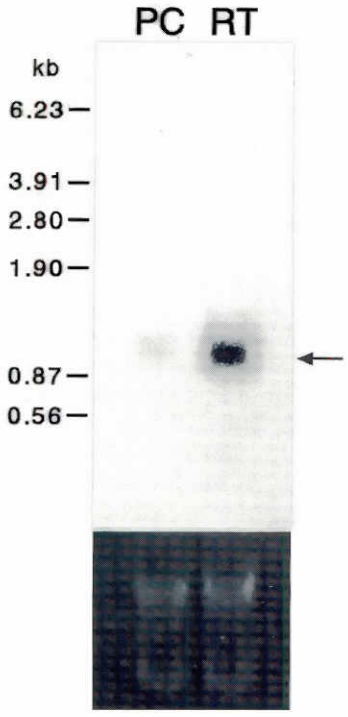

Fig. 8. Northern blot of total RNA *extracted from the testes in stages of primary spermatocytes (PC) and round spermatids (RT) using 29 kDa cDNA clone which was labeled with [^{32}P]dCTP. In each lane 15 μg of RNA was run on 1% formaldehyde agarose gels. Arrow shows 29 kDa mRNA. Lower part shows ribosomal RNA as a control.*

If the latter is the case, how then are the flagella and the axial fiber separated by the undulating membrane? To address this question, it would be helpful to establish a culture system in which the mature sperm are formed. We have recently succeeded in organ culture of testes fragments in which primary spermatocytes underwent two meiotic divisions to give rise to elongated spermatids in FSH-supplemented medium (Ji and Abé, 1994). We are now trying to improve the culture conditions to proceed the differentiation of the spermatids further to mature sperm.

Materials and Methods

Separation of sperm components

Mature parts of newt testes (about 10 newts) containing mature sperm were cut into small pieces by scissors and filtered through gauze. Filtrate was washed three times in OR-2 (balanced salt solution; Wallace *et al.*, 1973) by centrifugation at 3,200g for 5 min at 4°C. Sperm was disrupted by sonication three times each for 10 sec (Branson Sonifier Cell Disruptor 185). Suspension (5 ml) of the disrupted sperm were layered on 30% Percoll (Pharmacia) in OR-2 (5 ml) which was underlayered by 50% (5 ml) and 70% (5 ml) Percoll, and centrifuged at 5,000g for 20 min at 4°C. At the interface between OR-2 and 30% Percoll, membrane and cell debris were found. At the interface between 30% and 50% Percoll, flagella were observed. At the interface between 50% and 70% Percoll, axial fibers were found. At the bottom of the tube there were sperm head and axial fibers entangled with each other.

This pellet was suspended in OR-2 (5 ml) and dispersed by sonication. The suspension was layered on 3 M CsCl (10 ml) and centrifuged at 5,000g for 20 min at 4°C. The pellet was recovered as the head fraction. Axial fibers were recovered from the interface between OR-2 and 3 M CsCl. This fraction was combined with the axial fiber fraction recovered from the interface between 50% and 70% Percoll. Head fraction and axial fiber fraction were washed in OR-2.

Preparation of spermatogenic cells

Dissociation of testes and separation of the spermatogenic cells were done as described previously (Maekawa *et al.*, 1995). Immature parts of the testes were collected and minced in L-15 medium, followed by treatment with collagenase at 22°C for 2 h. The dissociated spermatogenic cells were layered onto a 4-10% continuous Metrizamide gradient (Nyegaard & Co) in OR-2 and centrifuged at 10,000g for 20 min. Secondary spermatogonia, primary spermatocytes and round spermatids were recovered from the fraction of Metrizamide gradient.

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide slab gel electrophoresis was performed according to the methods of Laemmli (1970) using a 12.5% acrylamide gel (pH 8.8) with a 4.5% stacking gel (pH 6.8). Gels were run at constant voltage of 150 V.

Generation of antibody against 29 kDa protein

Testes rich in mature sperm were dissolved in SDS-PAGE sample buffer and run on SDS-PAGE without comb. After staining with Coomassie brilliant blue (CBB), the 29 kDa band was spliced out. The gel was destained, washed in PBS, homogenized and emulsified with Freund's complete adjuvant or incomplete adjuvant. First challenge was performed intraperitoneally with Freund's complete adjuvant to 6 week-old mice. Additional challenge was performed two or three times with incomplete adjuvant until the antibody titer was significantly elevated. The titer was checked by Western blotting.

Western blotting

Proteins were transferred from acrylamide gels to nitrocellulose filters (Immobilon, Millipore Inc., Bedford, MA, USA) electrophoretically at constant voltage of 40 V for 1.5 h. Membranes were incubated for 30 min in TBS containing 0.1% Tween 20 and 10% bovine serum albumin (BSA) (TTBS). After washing with TTBS (3x5 min), membranes were incubated for 1 h with mouse anti-29 kDa antibody (1:1000 dilution in TTBS). The membranes were washed in TTBS (3x5 min), followed by incubation for 1 h with peroxidase-conjugated goat anti-mouse IgG (1:1500; Bio-Rad). Membranes were again washed in TTBS (3x5 min). The procedure was performed with a DAB substrate kit (Funakoshi Inc., Tokyo, Japan).

Fluorescent antibody of mature sperm

Sperm suspension was put onto coverslips which had been washed by 1% HCl in 70% ethanol and coated with 1 mg/ml poly L-lysine (PLL; M.W. 22,400; Sigma Chemical Comp., USA). After a couple of hours the coverslips were washed in OR-2 and fixed for 30 min by 100% methanol (-30°C). After washing in PBS (5x5 min), the coverslips were incubated with 3% BSA for 30 min to block non-specific reactivity. Mouse anti-29 kDa antibody (1:20 dilution in PBS containing 3% BSA) was incubated for 1 h at room temperature, followed by washing in PBS (5x5 min). Then fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody (1:20 dilution; Organon Teknika Corp., West Chester, PA, USA) was incubated for 1 h at room temperature in a dark chamber. After washing in PBS (5x5 min), the coverslips were put on slideglasses with 10% polyvinyl alcohol (Sigma).

Fluorescent antibody of tissue sections

Testes fixed by Bouin solution and embedded in paraffin (Paraplast embedding medium; Monoject, USA) were sectioned at 5 μm in thickness according to conventional histological procedures. Sections were washed in PBS (3x5 min) and incubated in 3% BSA for 30 min to block non-specific reactivity. Then mouse anti-29 kDa antibody (1:20 dilution) and FITC-conjugated goat anti-mouse IgG antibody (1:50 dilution) were incubated as mentioned above. In order to examine the stage of spermatogenic cells, sections adjacent to those stained by antibodies were stained by hematoxylin and eosin.

Double staining by anti-29 kDa protein and anti-tubulin

Cell suspensions derived from the testes rich in spermatids which had been minced by scissors were put on PLL-coated coverslips and fixed by 100% methanol. Testes fixed by Bouin solution and embedded in paraffin were sectioned at 5 μm in thickness.

After blocking with 2% BSA in PBS for 30 min, rabbit anti-chicken brain tubulin polyclonal antibody (1:50 dilution; Chemicon International Inc., Temecula, CA, USA) and mouse anti-newt 29 kDa protein (1:50 dilution) were simultaneously incubated on coverslips with dissociated spermatids. After washing in PBS (3x5 min), tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG antibody (1:50 dilution; Southern Biotechnology Associates, Inc., Birmingham, AL, USA) and FITC-conjugated goat anti-mouse IgG antibody were simultaneously incubated on the coverslips. To the sections, primary antibodies were both diluted to 1:20 and the second antibodies were both diluted to 1:20.

For observation, a reflected light fluorescence microscope (BX60, Olympus, Tokyo, Japan) was used with NIBA filter for FITC and WIG filter for TRITC.

Construction of cDNA library from newt testes

Total RNA was prepared from testes containing primary spermatocytes and round spermatids by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Poly $(A)^+$-RNA was fractionated from the total RNA by chromatography on oligo(dT)-cellulose (Pharmacia; type 7). Double-stranded cDNA was synthesized from the poly$(A)^+$-RNA by oligo(dT) priming with a cDNA synthesis kit (Amersham Corp.) and cloned into λgt11 vector using a λgt11 cDNA cloning kit (Amersham Corp.).

Immunoscreening of cDNA library using antisera against 29 kDa protein

For immunoscreening, the newt testes λgt11 cDNA library was plated at a density of $1x10^4$ plaque forming units/dish with *Escherichia coli* Y1090 as host. After incubation at 37°C and induction with IPTG, nitrocellulose filters were incubated for 1 h with a 1:1000 dilution of the mouse antiserum in Tris-buffered saline (TBS, pH 7.5) against 29 kDa protein. After washing in TBS the filters were incubated for 30 min with a 1:500 dilution of goat anti-mouse IgG coupled with horseradish peroxidase (BioRad) in TBS. Then the filters were washed in TBS, followed by visualization with DAB substrate kit (Vector Laboratories).

DNA sequencing

cDNA fragments were subcloned into the EcoRI site of Bluescript II SK(-) (U.S. and Biochemical Corp.) and sequenced by the dideoxy-chain termination method (Sanger *et al.*, 1977) with Sequenase Ver. 2 DNA sequencing kit (U.S. and Biochemical Corp.). All sequence data were obtained for both strands.

Analysis of amino acid sequence

Testes rich in mature sperm were dissolved in SDS-PAGE sample buffer and run on SDS-PAGE without comb. After staining with cold 1 M KCl, 29 kDa band was spliced out. The gel band was dialyzed electrophoretically against SDS-free electrophoresis buffer (40 V x 2.5 h). The solution in a dialyzed tube was mixed with equal volume of 2 x sample buffer, boiled for 10 min and centrifuged (12,000g x 5 min).

The supernatant was purified by reversed-phase HPLC (model 305, GILSON) equipped with a Synchropak PR-P (Synchrom; 250x4.1 mm) equilibrated with aqueous 0.05% trifluoroacetic acid as described by Yokota *et al.* (1991). The elution was performed with a linear gradient of acetonitrile containing 0.05% trifluoroacetic acid at a flow rate of 1 ml/min. Protein and peptides were monitored by measuring the absorbance at 220 nm.

The purified 29 kDa protein was digested by 0.08 mg/ml V8 protease (STRATAGENE) in SDS-PAGE sample buffer for 30 min at 37°C. The digests and V8 protease were run on SDS-PAGE, blotted to nitrocellulose filter and stained with CBB. The bands specific for 29 kDa protein were spliced out, destained by 60% methanol, put into 100% methanol and dried in air.

Determination of amino acid sequence of the digests was performed on a model 376A protein-peptide sequencer system (Applied Biosystems Inc.) by the pulsed-liquid Edman degradation method.

Northern blot analysis

Total RNA was prepared from the pure population of spermatogenic cells. Fifteen micrograms of total RNA were electrophoresed in a 1% formaldehyde agarose gel and blotted to a nylon-membrane, Hybond(N)+ (Amersham Corp.). Membranes were prehybridized at 42°C for 2 h in the hybridization buffer (5xSSPE, 50% formamide, 0.5% SDS, 5xDenhart's, and 20 mg/ml salmon sperm DNA). cDNA probe labeled with [α-^{32}P]dCTP by Megaprime DNA labeling system (Amersham Corp.) was added to the hybridization buffer and hybridized at 42°C for 16 h. After hybridization, membranes were washed successively in 2xSSPE, 0.1% SDS at 65°C for 30 min, 1xSSPE, 0.1% SDS at 65°C for 30 min and 0.1xSSPE, 0.1% SDS at 65°C for 20 min.

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