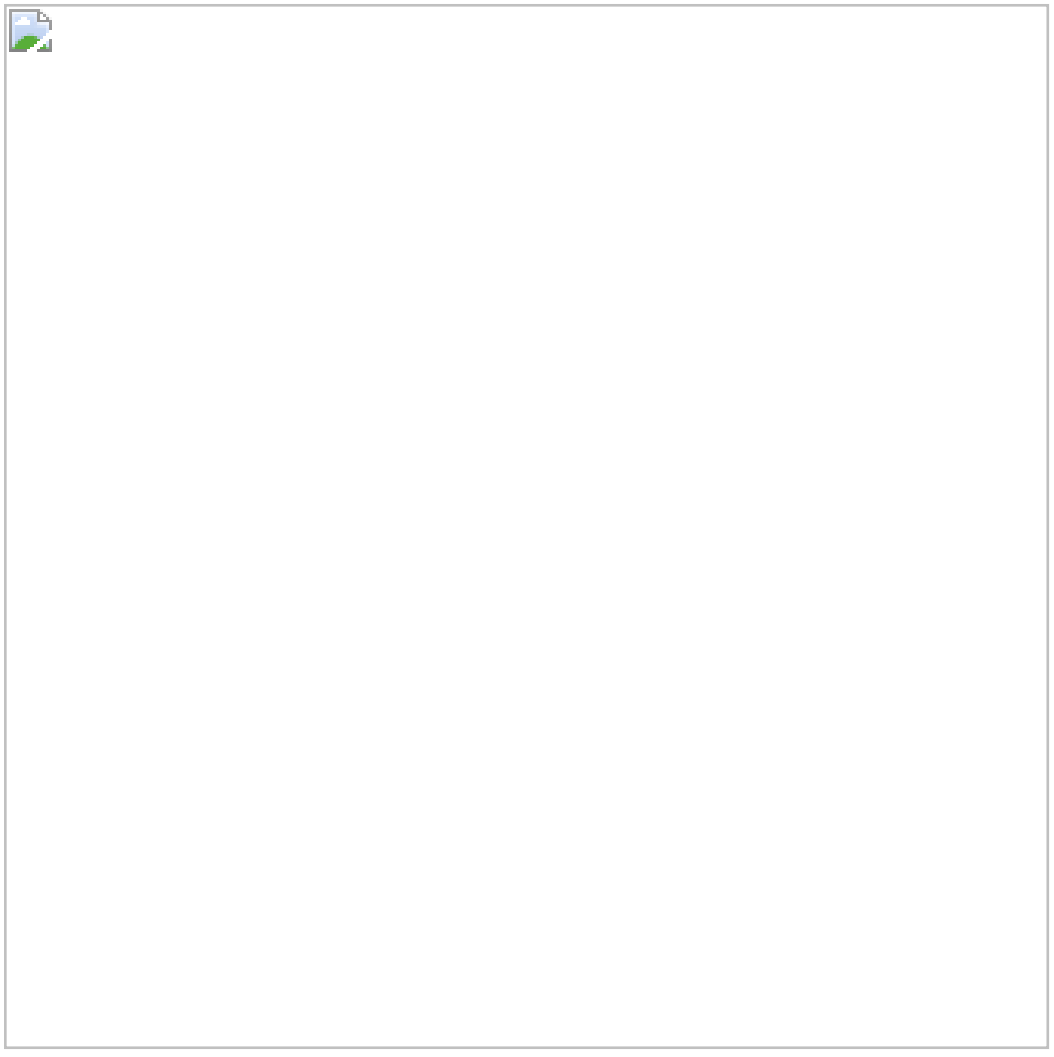

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Authors: Miura, Chiemi, Miura, Takeshi, and Yamashita, Masakane

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PCNA Protein Expression during Spermatogenesis of the Japanese Eel (*Anguilla japonica)*

Chiemi Miura[1]*, Takeshi Miura[2] and Masakane Yamashita[3]

[1]*Division of Marine Biosciences, Graduate School of Fisheries Science, Hokkaido University, Hakodate 041-8611, Japan*
[2]*Field Science Center for Northern Biosphere, Hokkaido University, Hakodate 041-8611, Japan*
[3]*Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan.*

ABSTRACT—Spermatogenesis can be initiated by a single injection of human chorionic gonadotropin (hCG) into the cultivated Japanese eel, which produces only spermatogonia in the testis. To isolate the genes responsible for regulating spermatogenesis, we performed a differential mRNA display using poly $(A)^+$ RNA extracted from the testes at different time points after hCG injection. Among several cDNA clones, the expression of which was initiated before the onset of meiosis, one clone has high homology with the proliferating cell nuclear antigen (PCNA). In this study, we investigated the protein expression of eel PCNA and found for the first time in any species that two forms (32-kDa and 36-kDa) of PCNA are present in the testis. Although the 36-kDa form existed in both the testis and spleen, the 32-kDa form was specifically expressed in the testis. In contrast to the appearance of 36-kDa PCNA 1 day after the hCG treatment, the 32-kDa PCNA appeared only 9 days after the hCG treatment, at which time active spermatogonial proliferation occurred in the testis. Both the 32- and 36-kDa forms were recognized by antibodies raised against different epitopes of PCNA, and their N-terminal amino acid sequences were identical. The 36-kDa form, but not the 32-kDa form, was recognized by antibodies against phosphoamino acids. These results suggest that the two PCNA proteins are the same molecule with different chemical modifications, including phosphorylation. We discuss the roles of these two forms of PCNA in the spermatogenesis of the Japanese eel.

Key words: spermatogenesis, Japanese eel, PCNA, spermatogonia

INTRODUCTION

Spermatogenesis is a complex developmental process in which numerous factors are involved. Spermatogenesis consists of the renewal of spermatogonial stem cells, the mitotic proliferation of spermatogonia, the entrance into and the progress of meiosis, and the spermiogenesis, during which haploid spermatids are converted into spermatozoa. In order to understand the molecular and cellular mechanisms of spermatogenesis, the Japanese eel (*Anguilla japonica*) provides an excellent experimental system. Under cultivated conditions, the male Japanese eel has immature testes containing only a few number of quiescent spermatogonia. Spermatogenesis can be induced both *in vivo* by injecting human chorionic gonadotropin (hCG) into the eel and *in vitro* by treating the testis with hCG or 11-ketotestosterone (11-KT), the major androgen in the male eel, in organ culture or germ-somatic cell coculture (Miura *et al*., 1991a, b, c, 1996). Using the advantages of this model, we have investigated the genes controlling spermatogenesis of the Japanese eel by differential mRNA display and obtained several cDNA clones that appear only after the induction of spermatogenesis with hCG. The entire base sequence of one of these clones shows high homology with the proliferating cell nuclear antigen (PCNA) (Miura *et al*., 1999a).

PCNA was first discovered as a nuclear protein, the appearance of which correlates with the proliferation of the cell (Miyachi *et al*., 1978). It is an acidic, non-histone nuclear protein with an apparent isoelectric point of 4.8-4.9 and molecular mass of 33-36 kDa (Bravo *et al.,*1981; Mathews *et al.,*1984; Takasaki *et al*., 1984). PCNA was finally identified as an auxiliary protein for DNA polymerase δ (Tan *et al*., 1986; Bravo *et al*., 1987; Prelich *et al.,* 1987). PCNA is

* Corresponding authour: Tel. 81-138-40-5546;
FAX.81-138-40-5545.
E-mail: chiemi@fish.hokudai.ac.jp

associated with the D-type cyclin, the cyclin-dependent kinase (cdk) and the cdk inhibitor p21 protein, among these proteins, D-type cyclin might be responsible for adjusting the function of PCNA (Xiong *et al*., 1992). PCNA principally plays a key role in the replication and repairing of DNA during the mitotic division of somatic cells under the control of cdks and cdk inhibitors (Waga and Stillman, 1994). However, thus for there has been no report concerning the protein expression of PCNA in germ cells, nor has there been a discussion of the changes in expression patterns of PCNA during gametogenesis. Therefore, the roles played by PCNA in germ cells and gametogenesis remain to be elucidated.

In this study, we investigated the protein expression of PCNA during hCG-induced eel spermatogenesis. We provide for the first time evidence for the presence of two forms (32 and 36 kDa) of PCNA, which might reflect different phosphorylation states of the same molecule. The difference in the expression patterns of two PCNA proteins suggests that they play different roles in spermatogenesis.

MATERIALS AND METHODS

Animals and hormone injections

Males of the cultivated Japanese eel *Anguilla japonica* (body weight, 180–200 g) were used. Fish were kept in circulating freshwater tanks with a capacity of 500 liters at 23°C. A single injection of hCG dissolved in saline (150 mM NaCl) was given intramuscularly at a dose of 5 IU per g body weight. Fish were sampled after 0, 1, 3, 6, 9, 12, 15 and 18 days of hCG injection.

Protein extraction

Testes were homogenized with eel Ringer (3.0 mM NaCl, 3.0 mM KCl, 3.5 mM $MgCl_2$, 5.0 mM $CaCl_2$, 10 mM HEPES, pH 7.4). The homogenate was mixed with an equal volume of SDS sample buffer (125 mM Tris-HCl, 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.05% bromophenol blue, pH 6.8), boiled at 100°C for 5 min, and clarified by centrifugation at 9000 g for 5 min. Protein concentration was determined by Bio-Rad Protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Antibodies and western blotting

Polyclonal and monoclonal antibodies that recognize different epitopes of PCNA were used in this study. Polyclonal antibodies were raised against the following three peptides corresponding to the amino acid sequences of eel PCNA: amino acids 100-120, ALVFETLNQEKVSDYEMKL; amino acids 180–197, VKSQTSNVDEEDDAVTI; amino acids 242–260 (the C-terminal sequence), ADMGHVKYYLAPKIDEEAS. Each peptide was coupled to bovine serum albumin and injected into rabbits and mice to produce polyclonal antibodies. Monoclonal antibodies against rat recombinant PCNA were purchased from Cosmo Bio (PC10; epitope, amino acids 112–121), Wako (epitope, amino acids 181–195) and DAKO (epitope unknown).

Proteins (100 μg/lane) were separated by SDS-PAGE using a 10% polyacrylamide gel and were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were treated for 30 min with 5% skim milk in 20 mM Tris-HCl, pH 7.5, then incubated overnight at 4°C with one of the primary antibodies against PCNA. After washing with TBS containing 0.025% Tween 20 (T-TBS) and with TBS, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody diluted to 1:1000 in TBS for 2 hr. After washing, HRP activity was visualized using a freshly prepared solution of 0.06% 4-chloro-1-napthol in TBS containing 0.06% H_20_2.

Immunoprecipitation

Frozen samples (100 mg) were homogenized with an equal volume of 0.25 M sucrose including 10 μg/ml leupeptin and 100 mM phenylmethanesulfonyl fluoride. After centrifugation at 9000 g for 10 min, the supernatant was incubated with 10 volumes of immunoprecipitation buffer (10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 1% Nonidet P-40, pH 7.5) containing 50 μl of anti-PCNA PC10 antibody. After overnight incubation at 4°C, the immunocomplex was absorbed with 50 μl of protein A-Sepharose (Pharmacia, Tokyo, Japan) for 1 hr at 37°C. The beads were washed with T-TBS by centrifugation at 1000 g for 10 min and treated with 5-fold concentrated SDS sample buffer. Proteins were separated by a 10% acrylamide gel and blotted onto PVDF membrane. A part of the membrane was immunostained with anti-PCNA PC10 antibody or rabbit polyclonal antibodies against phosphorylated amino acids (a mixture of anti-phosphoserine, anti-phosphothreonine and anti-phosphotyrosine; Cosmo Bio), and the remainder was stained with Coomassie brilliant blue R-250 (CBB).

Two-dimensional gel electrophoresis

Testicular fragments removed from the eels after 15 days of hCG injection and were homogenized with 0.25 M sucrose and treated with a sample buffer consisting of 9.5 M urea, 2% Triton X-100, 5% 2-mercaptoethanol, and 5% Ammpholine (Pharmacia). The samples were separated by isoelectric focusing, followed by SDS-PAGE using a 10% gel. Proteins resolved by two-dimensional gel electrophoresis were transferred onto PVDF membranes. The membranes were incubated in TBS containing 5% skim milk and anti-PCNA antibody PC10 (diluted to 1:100) overnight at 4°C.

Amino acid sequence analysis

PCNA proteins in testis homogenate obtained from the eels 15 days after hCG injection were precipitated with anti-PCNA PC10 antibody, as described above. The immunoprecipitates were separated by SDS-PAGE, blotted onto PVDF membrane, and stained with CBB. Bands of 36- and 32-kDa PCNA proteins were cut out of the membrane and subjected to N-terminal protein sequence analysis using an Applied Biosystems 494 sequencer.

Organ culture

The organ culture was performed as previously described (Miura *et al*., 1991a, b, c). Briefly, testes were removed and cut into small pieces (1×1×0.5 mm), then placed on floats of elder pith covered with a nitrocellulose membrane in 6-well culture dishes (Iwaki, Chiba, Japan). They were then cultured in Leibovitz L-15 culture medium with or without 10 ng/ml of 11-ketotestosterone (11-KT) or 100 pg/ml of Estradiol-17β (E2) for 15 days in humidified air at 20°C. After the culture, testicular fragments were collected and a Western blot analysis was performed on the samples.

RESULTS

Expression of eel PCNA protein during spermatogenesis

Eel PCNA proteins were immunologically detected to evaluate changes in their expression patterns during spermatogenesis. Polyclonal antibody raised against the peptide corresponding to the C-terminal amino acid sequence of eel PCNA detected 32- and 36-kDa bands in the Western blot analysis of the extract from hCG-treated testes (Fig. 1). When the homogenate of hCG-treated testis was immunoprecipitated by the same antibody, 36- and 32-kDa bands were also detected in the precipitates. Since these two bands were also recognized by other antibodies against eel

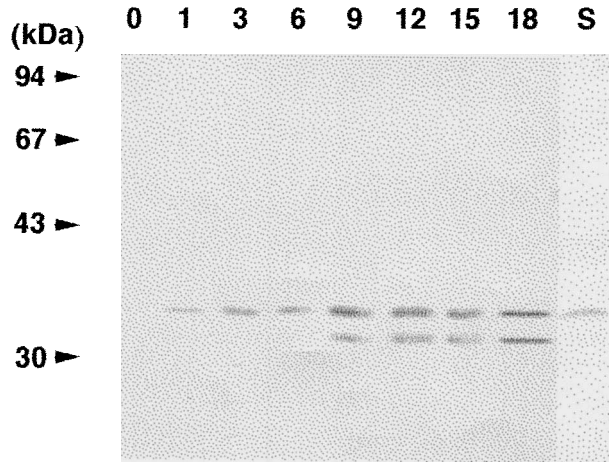

Fig. 1. Western blot analysis of eel PCNA protein expression during hCG-induced spermatogenesis. Samples were obtained from the testis after 0, 1, 3, 6, 9, 12, 15, and 18 days of hCG injection and from the spleen (S), and were immunoblotted with the antibody raised against the C-terminal amino acid sequence of eel PCNA.

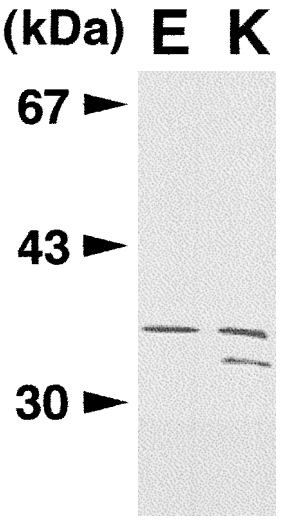

Fig. 2. Western blot analysis of eel PCNA proteins of testicular fragments cultured *in vitro* for 15 days in the presence of estradiol-17β (E) or 11-ketotestosterone (K). PCNA proteins were stained with anti-PCNA PC10 antibody.

and rat recombinant PCNA proteins (see "Characterization of two PCNA proteins") and also because they were not detected in a negative control without the primary antibody (data not shown), we concluded that these bands were the eel PCNA.

In eel spermatogenesis, proliferation of spermatogonia, meiosis and spermiogenesis occur at definite times, namely, 3, 12, and 18 days after hCG injection, respectively. The testes of hCG-untreated eels expresed neither 32- nor 36-kDa PCNA. The 36-kDa PCNA was detected in the testes collected 1-18 days after hCG injection, with a gradual increase in the PCNA protein content that corresponded with the progression of spermatogenesis (Fig. 1). The 32-kDa PCNA was not detected 0-6 days after hCG-injection but appeared 9 days after the injection. Its expression level was maintained thereafter (Fig. 1). The expression pattern of PCNA proteins is consistent with that of PCNA mRNA shown in our previous research (Miura *et al*., 1999a). In contrast to the presence of two forms of PCNA in the testis, other organs, including the head kidney, metanephros, muscle, and intestine did not express any PCNA proteins with the exception of the spleen, in which only the 36-kDa PCNA was expressed (Fig. 1).

Expression of eel PCNA protein during *in vitro* spermatogenesis

In organ cultures of eel testes, 11-KT-treatment induces spermatogenesis but E2-treatment does not, although E2 induces the renewal of spermatogonial stem cells (Miura *et al*., 1991, 1999b). The 32-kDa PCNA was detected strongly in the 11-KT-treated testis but not in the E2-treated testis, although the 36-kDa PCNA was detected in both groups (Fig. 2).

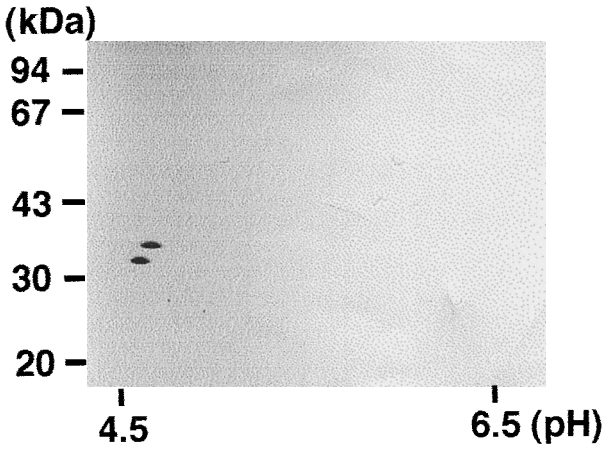

Fig. 3. Two-dimensional gel electrophoresis of eel PCNA proteins obtained from the testis 15 days after hCG injection. Two PCNA proteins were visualized by anti-PCNA PC10 immunoblotting.

Two-dimensional gel electrophoresis of PCNA proteins

Two-dimensional gel electrophoresis of testis 15 days after hCG injection showed that the 32- and 36-kDa PCNAs exhibited apparent isoelectric points of 4.6 and 4.7, respectively (Fig. 3).

Characterization of two PCNA proteins

Although our previous Northern blot analysis suggested the presence of a single species of PCNA mRNA in eel testis (Miura *et al*., 1999a), the two PCNA proteins might be derived from different mRNAs produced by alternative splicings. To examine this possibility, we performed reverse transcription-polymerase chain reaction (RT-PCR) analysis using poly $(A)^+$ RNA extracted from the testis 12 days after hCG injection. RT-PCR with two primers that amplified the

entire open reading frame of the PCNA yielded a single band (data not shown), indicating that only one species of mRNA was responsible for the production of the two PCNA proteins.

To further characterize these two PCNA proteins, we used antibodies that recognized different epitopes of eel PCNA (amino acids 100-120, 180-197, and 242-260) and rat recombinant PCNA (amino acids 112-121 and 181-195). All of the antibodies used in this study recognized the same two bands (36- and 32-kDa) in the testis 15 days after hCG injection (data not shown). In particular, it is of note that two bands were recognized by the antibody against the C-terminal amino acid sequence (residues 242-260) of eel PCNA (Fig. 1). This result excludes the possibility that the difference in apparent molecular mass of the two proteins is not due to the truncation of the C-terminal amino acid sequence.

To investigate whether the truncation of the N-terminus is responsible for the difference in the apparent molecular mass of the two PCNAs, we carried out an N-terminal amino acid sequence analysis of the 36- and 32-kDa PCNA proteins that were immunologically purified from the testis 15 days after hCG treatment using anti-PCNA PC 10 antibody. The N-terminal amino acid sequences of 36- and 32-kDa PCNA exhibited the same sequence, MFEARLVOGSIL, excluding the possibility of truncation at the N-terminus.

Taken together, the results obtained from RT-PCR and the analyses of the PCNA antibodies and amino acid sequences suggest that the two forms of PCNA are indeed the same molecule with different chemical modifications.

Phosphorylation states of PCNA

It is very likely that the difference in the apparent molecular mass of two PCNA proteins is due to different phosphorylation states of the proteins. To verify this, we used antibodies that recognize phosphorylated amino acids (phosphoserine, phosphothreonine, and phosphotyrosine). The 36-kDa PCNA protein, but not the 32-kDa protein, was stained by the antibodies (Fig. 4). These results suggest that the 36- and 32-kDa PCNA proteins are phosphorylated and dephosphorylated forms, respectively. We tried to confirm phosphorylation-induced differences in the apparent molecular mass by conversion of the 36-kDa form to the 32-kDa form by phosphatase treatment. We purified the 36-kDa-PCNA by immunoprecipitation with anti-PCNA PC10 antibody and treated the protein with alkaline and acid phosphatases. However, the treatment did not cause any electrophoretic mobility shift from 36- to 32-kDa (data not shown).

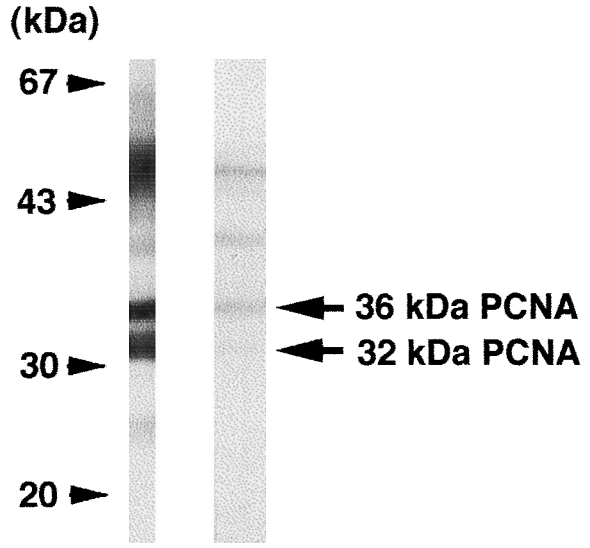

Fig. 4. Phosphorylation states of 32- and 36-kDa PCNA proteins. Anti-PC10 immunoprecipitates from the testis 15 days after hCG treatment were immunoblotted with the same antibody (left lane) and polyclonal rabbit antibodies against phosphoamino acids (right lane).

DISCUSSION

We show here that eel PCNA exhibits two forms with different molecular masses and isoelectric points (pI); one of these forms is a 32-kDa protein with a pI value of 4.6 and the other is a 36-kDa protein with a pI of 4.7. To our knowledge, there has been no report of two forms to data of PCNA in any species.

RT-PCR analysis using testis poly $(A)^+$ RNA and primers corresponding to the 5' and the 3' end of the open reading frame rules out the possibility that the two forms of PCNA are derived from different mRNAs produced by alternative splicing processes. The two proteins were equally recognized by antibodies raised against different epitopes, and were identical in the N-terminal amino acid sequence. Anti-phosphoamino acid antibodies recognized the 36-kDa form of PCNA but not the 32-kDa one. These results suggest that the two PCNA proteins are the same molecule with different chemical modifications, including phosphorylation (the 36-kDa form would be phosphorylated and the 32-kDa form unphosphorylated). However, the pI values of the two proteins are inconsistent with the phosphorylation states suggested above; the tentatively unphosphorylated 32-kDa protein would be more acidic than the tentatively phosphorylated 36-kDa protein. In addition, treatment of the 36-kDa PCNA with alkaline and acid phosphatases failed to induce a change in its apparent molecular mass from 36- to 32-kDa. Although we cannot exclude the possibilities that the ordinary phosphatases cannot remove phosphates present on specific sites of the 36-kDa PCNA, phosphorylation might not be the sole reason for the difference between the two PCNA proteins. Further studies including verification of chemical modifications other than phosphorylation are necessary for the full-characterization of the two PCNA proteins.

The expression patterns of the two PCNA proteins during spermatogenesis was observed as being definitively different. The expression of 36-kDa PCNA was initiated 1 day after hCG-injection and its expression level increased according to the progression of spermatogenesis. In contrast, 32-kDa PCNA was not detected during the early phase

of spermatogenesis, but its expression did occur 9 days after hCG injection, and expression was maintained thereafter. Nine days after hCG injection, the testis underwent active spermatogonial proliferation and a number of germ cells entered meiosis 12 days after hCG injection (Miura *et al.,* 1991a). Although the 36-kDa form of PCNA was also present in other organs such as the spleen, the 32-kDa PCNA was not. These results indicate that the 32-kDa form of PCNA is specifically expressed in germ cells undergoing mitotic and meiotic divisions.

Spermatogonial mitosis can be categorized as one of two types; spermatogonial stem cell renewal and spermatogonial proliferation toward meiosis (Clermont, 1972). Treatment of eel testis with 11-KT can induce the entire process of spermatogenesis, including spermatogonial proliferation, meiosis, and spermiogenesis (Miura *et al.,* 1991c). On the other hand, E2-treatment induces spermatogonial stem cell renewal but does not induce spermatogonial proliferation, indicating that E2 is involved in the regulation of spermatogonial stem cell renewal but not in spermatogonial proliferation in the eel (Miura *et al.,* 1999b). We showed in the present study that 32-kDa PCNA is expressed strongly in testes treated with 11-KT, but not in those treated with E2, although strong expression of 36-kDa PCNA is induced by both 11-KT and E2. These data raise the possibility that 36-kDa PCNA is involved in mitosis and spermatogonial stem cell renewal, while 32-kDa PCNA is involved in the spermatogonial proliferation toward meiosis in Japanese eel spermatogenesis.

Recently, Cobb *et al.* (1999) have demonstrated that the meiotic competence of spermatocytes is acquired after homologous chromosome pairing is established, being coincident with the appearance of testis-specific histone H1t and CDC25C protein phosphatase in spermatocytes. It is thus likely that protein dephosphorylation plays a key role in the control of the meiotic competence of spermatocytes. Consistent with this notion, we have demonstrated here that the appearance of the putatively unphosphorylated 32-kDa PCNA coincides with the acquisition of meiotic competence during Japanese eel spermatogenesis. Further studies will be required for an understanding of the actual roles played by the two different forms of PCNA in eel spermatogenesis.

In summary, we have shown in the present study that two forms of PCNA are expressed in the Japanese eel testis. The expression patterns of the two forms differ : the expression of (unphosphorylated) 32-kDa PCNA is initiated at the period of spermatogonial proliferation before entering meiosis, whereas that of (phosphorylated) 36-kDa form begins soon after the induction of spermatogenesis with hCG or 11-KT. These results suggest that the two forms of PCNA play different roles in regulating spermatogenesis in the Japanese eel.

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REFERENCES

Bravo R, Fey SJ, Bellatin J, Larsen PM, Arevalo J, Celis JE (1981) Identification of a nuclear and of a cytoplasmic polypeptide whose relative proportions are sensitive to changes in the rate of cell proliferation. Exp Cell Res 136: 311–319

Bravo R, Frank R, Blundell PA, Macdonald-Bravo H (1987) Cyclin/PCNA is the auxiliary protein of DNA polymerase-δ. Nature 326: 515–517

Clemont Y (1972) Kinetics of spermatogenesis in mammals. Seminiferous epithelium cycle and spermatogonial renewal. Int Rev Cytol 70: 27–100

Cobb J, Cargile B, Handel MA (1999) Acquisition of competence to condensed metaphase I chromosomes during spermatogenesis. Dev Biol 205: 49–64

Mathews MB, Bernstein RM, Franza BR, Garrel JI (1984) Identity of the proliferating cell nuclear antigen and cyclin. Nature 309: 374–376

Miura C, Miura T, Yamashita M, Yamauchi K, Nagahama Y (1996) Hormonal induction of all stages of spermatogenesis in germ-somatic cell coculture from immature Japanese eel testis. Dev Growth Differ 38: 257–262

Miura C, Miura T, Kudo N, Yamashita M, Yamauchi K (1999a) cDNA cloning of a stage-specific gene expressed during HCG-injected spermatogenesis in the Japanese eel. Dev Growth Differ 41: 463–471

Miura T, Miura C, Ohta T, Nader MR, Todo T, Yamauchi K (1999b) Estradiol-17β stimulates the renewal of spermatogonial stem cells in males. Biochem Biophys Res Commun 264: 230–234

Miura T, Yamauchi K, Nagahama Y, Takahashi H (1991a) Induction of spermatogenesis in male Japanese eel, *Anguilla japonica*, by a single injection of human chorionic gonadotropin. Zool Sci 8: 63–73

Miura T, Yamauchi K, Takahashi H, Nagahama Y (1991b) Human chorionic gonadotropin induces all stages of spermatogenesis *in vitro* in the male Japanese eel (*Anguilla japonica*). Dev Biol 146: 258–262

Miura T, Yamauchi K, Takahashi H, Nagahama Y (1991c) Hormonal induction of all stages of spermatogenesis *in vitro* in the male Japanese eel (*Anguilla japonica*). Proc Natl Acad Sci USA 88: 5774–5778

Miyachi K, Frizler MJ, Tan EM (1978) Autoantibody to a nuclear antigen in proliferating cells. J Immunol 121: 2228–2234

Prelich G, Tan C-K, Kostura M, Mathews MB, So AG, Downey KM, Stillman B (1987) Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxiliary protein. Nature 326: 517–520

Takasaki Y, Fishwild D, Tan EM (1984) Characterization of proliferating cell nuclear antigen recognized by autoantibodies in lupus sera. J Exp Med 159: 981–992

Tan CK, Castillo C, So A, Downey KM (1986) An auxiliary protein for DNA Polymerase-δ from fetal calf thymus. J Biol Chem 261: 12: 310–316

Waga S, Stillman B (1994) Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication *in vitro*. Nature 369: 207–212

Xiong Y, Zhang H, Beach D (1992) D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell 71: 505–514

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