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# Comparative Studies between *in vivo* and *in vitro* Spermatogenesis of Japanese Eel (*Anguilla japonica*)

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**ABSTRACT**—In order to check the quality of *in vitro* spermatogenesis of Japanese eel, *in vitro* 11-ketotestosterone (11-KT) induced spermatogenesis was compared with *in vivo* spermatogenesis induced by a single injection of human chorionic gonadotropin (hCG) in detail. DNA contents of germ cells from *in vitro* and *in vivo* testicular fragments were compared using flow cytometry. Since the *in vitro* result of flow cytometry showed prominent 1C peak including spermatozoa and spermatids, the reduction of DNA by meiosis was assumed to progress normally, (i.e., haploid spermatozoa were produced in this *in vitro* system). In the testes of *in vitro* culture, however, spermatozoa were not released into lumen. Furthermore, the number of mitotic divisions of the *in vitro* experiment (6 divisions) was fewer than that of *in vivo* (10 divisions). In electron microscopy observations, both of *in vivo* and *in vitro* spermatozoon had a crescent-shaped nucleus with a flagellum, and a single large spherical mitochondrion. However, the elongation of the sperm head was not sufficient and the mitochondrion was not always located at the anterior end as is observed for the spermatozoa obtained from hCG injected eels. Eel spermatogenesis related substance-11 (eSRS11) is homologue of histone H1 which is up-regulated during spermatogenesis. Using this probe, *in vitro* spermatogenesis was also evaluated in molecular levels. In Northern blot analysis, eSRS11 mRNA was detected in both *in vivo* and *in vitro* testes. However, the expression of *in vitro* was much weaker than that of *in vivo*. These differences indicate that the stimulation of 11-KT is not sufficient, and another factors are needed to induce complete spermatogenesis *in vitro*.

**Key word:** spermatogenesis, Japanese eel, testicular organculture, 11-ketotestosterone, histone H1

## INTRODUCTION

Even though spermatogenesis is the same in both vertebrates and invertebrates, its control mechanisms are not well understood. Spermatogenesis is a complex process that can be divided into three major phases; spermatogonial proliferation, meiosis, and spermiogenesis. In brief, spermatogonia proliferate by mitosis, and then develop into spermatocytes that undergo meiosis to produce haploid spermatids which differentiate into spermatozoa released into the lumen (Grier, 1981; Loir *et al.*, 1995; Pudney, 1995; Miura and Miura, 2001). Many kinds of sequentially acting hormones and unknown factors appear to be involved in the control of the spermatogenetic process (Steinberger, 1971; Hansson *et al.*, 1976; Callard *et al.*, 1978; Billard *et al.*, 1982; Cooke *et al.*, 1998).

Spermatogenesis of fish appears to proceed in a similar fashion. Spermatogonia enter meiosis after a fixed number of mitotic divisions (Courot *et al.*, 1970) and develop into the haploid spermatids which differentiate into spermatozoa. It is well established that gonadotropin (GTH) is the primary hormones regulating gametogenesis in vertebrates including fish. However, it appears that GTHs do not act directly but rather work through the gonadal biosynthesis of steroid hormones (Nagahama, 1994). 11-ketotestosterone (11-KT) was first identified as a major androgenic steroid in male sockeye salmon *Oncorhynchus nerka* (Idler *et al.*, 1961).

In the Japanese eel *Anguilla japonica*, insufficient gonadotropin in the pituitary is attributed to the immature state of the testis under culture conditions (Nagahama and Yamamoto, 1973). A single injection of human chorionic gonadotropin (hCG) can induce all stages of spermatogenesis in the Japanese eel, and this induction is achieved via gonadotropin stimulation of Leydig cells to produce 11-KT (Miura *et al.*, 1991b). In turn, 11-KT induces complete spermatogenesis from premitotic spermatogonia to spermatozoa through

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the actions of Sertoli cells (Miura *et al.*, 1991c). Testicular cells secrete peptide factors, i.e., activin B (Nagahama, 1994; Miura *et al.*, 1994), and insulin-like growth factors (IGFs) (Loir *et al.*, 1994) to regulate the functions of germ cells. Recent several evidences show that growth factors appear to mediate the autocrine/paracrine interactions between testicular cells (Fostier *et al.*, 1994; Giordano *et al.*, 1991).

In order to investigate the individual effect of these hormones, an *in vitro* culture system where testicular fragments contain only non-proliferated spermatogonia is necessary. Under culture conditions, male Japanese eel has immature testes containing only premitotic spermatogonia together with inactive testicular somatic cells such as Leydig and Sertoli cells (Miura *et al.*, 1991a). A single injection of hCG can induce complete spermatogenesis in the Japanese eel (Miura *et al.*, 1991a; 1997). Hormonal induction of spermatogenesis is mediated by gonadotropin stimulation of Leydig cells to produce 11-KT, a major androgen in male eels (Miura *et al.*, 1991a). A single injection of hCG (*in vivo*) stimulated spermatogonia proliferation after 3 days, meiosis after 12 days and spermiogenesis after 18 days. In other work, it was already demonstrated that *in vitro*, 10 ng/ml of 11-KT in the presence of 1 µg/ml of bovine insulin (blns) is optimal for the induction of all stages of spermatogenesis in the Japanese eel after a 36 day culture period (Miura *et al.*, 1991c). Nine days after the start of the *in vitro* culture with 11-KT, spermatogonia began mitotic division, producing late type B spermatogonia. Zygotene spermatocytes of the meiotic prophase occurred in testes cultured for 18 days. After 21 days, spermatids and spermatozoa were observed for the first time. These spermatids had small, round, and heterogeneous nuclei. Each spermatozoon possessed a crescent-shaped nucleus. On the caudal end of the base of the nucleus, a flagellum with a 9+0 axonemal structure was attached. After 36 days, all stages of germ cells were present (Miura *et al.*, 1991c). To our knowledge, the Japanese eel is the only animal for which complete spermatogenesis has been induced by hormonal treatments *in vitro* using an organ culture system and a germ-somatic coculture system (Miura *et al.*, 1991b; 1991c; 1996).

It was reported that the testes cultivated in an organ culture system had several differences in comparison with the testes of eels injected with hCG. The period (24 days) required for the progression of premitotic spermatogonia to spermatozoa in eel testes cultured *in vitro* is longer than that of *in vivo* (18 days). This difference appears to be due to the difference in the period required for the initiation of spermatogonial proliferation (3 versus 9 days) (Miura *et al.*, 1991c). Furthermore, the lobular structures of the cultured testes were not clear, and the elongation of the nuclei of the sperm was not sufficient (Miura *et al.*, 1991b; 1991c). In order to understand these differences, it is imperative to compare the spermatogenesis of Japanese eel induced by hCG injection with that induced by 11-KT *in vitro*. In this study, therefore, we compared the eel spermatogenesis between *in vivo* and *in vitro* in detail.

## MATERIALS AND METHODS

### Animals

Male cultivated eels were purchased from a commercial supplier, and they were kept in circulating fresh-water tanks with a capacity of 500 liters at 23°C. To some eels, a single injection of human chorionic gonadotropin (hCG) dissolved in saline (150 mM NaCl) was administered intramuscularly at a dose of 5 IU per gram body weight. They were sacrificed on the 18th day after hCG injection and one part of the testes was collected for the extraction of RNA and another part for flow cytometry or morphological studies.

### Testicular organ culture techniques

Testicular organ culture was carried out as described in Miura *et al.* (1991 b; 1991c) with minor modifications. Freshly removed eel testes were cut into 1×1×0.5 mm pieces, and then placed on floats of elder pith covered with a nitrocellulose membrane. They were then cultured in glass shale dishes 9 cm in diameter, containing 20 ml of the basal culture medium with or without 10 ng/ml of 11-KT for 24 days at 20°C in humidified air. The basal culture medium consisted of Leibovitz L-15 supplemented with 1.7 mM proline, 0.1 mM aspartic acid, 0.1 mM glutamic acid, 0.5% bovine serum albumin (BSA) fraction V, bovine insulin (blns) at 1 mg/l, and 10 mM Hepes, adjusted to pH 7.4.

### Morphological studies

For the light and electron microscopy, testes from eels after 18 days of the single hCG injection, and testicular fragments after 24 days of *in vitro* culture were fixed in 1% paraformaldehyde and 1% glutaraldehyde in 0.1% cacodylate buffer at pH 7.4 overnight, then transferred to 10% sucrose in the same buffer for a minimum of 20 min. These samples were then postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour and embedded in epoxy resin according to standard procedures.

One micrometer sections were stained with toluidine blue for light microscopic examination. Ultrathin sections were stained with uranyl acetate and lead citrate for electron microscopic examination.

Testes from eels after 18 days of the single hCG injection, and cultured testicular fragments of eel were also fixed in Bouin's solution. They were then dehydrated by ethanol and lemosol, and embedded in paraffin according to standard procedures. Five micrometer sections were cut and stained with Masson's trichrome using hematoxyline, ponceau xylydine, and aniline blue (Masson, 1929) for light microscopy. Spermatocytes in the testes of the *in vitro* experiment in 30 cysts were recognized and counted according to the classification of stages of germ cells reported by Miura *et al.* (1991a).

### Flow cytometry

Samples were prepared according to Miura *et al.* (1996) and Block *et al.* (1987) with minor modifications. hCG injected fish and cultured testes were minced with scissors and dissociated into single cells by incubation for 2 hr in 10 ml eel physiological saline solution (Miura *et al.*, 1991a) containing 0.2% collagenase (236 U/mg), 0.6 units dispase and 0.002% DNase I. The cell suspension was successively filtered through three meshes of decreasing pore size (76, 42 and 25 µm) and centrifuged at 900 ×g for 10 min in 13.8% Nycodenz (Nycomed, Oslo, Norway) to remove erythrocytes. Cells remaining in the supernatant (1×10<sup>6</sup>/ml) were plated in plastic culture dishes and cultured at 20°C in humidified air. After overnight culture, fibroblasts and interstitial cells adhered firmly to the bottom of the dish, whereas germ cells and Sertoli cells did not. Culture medium including the cell culture was separated into 1 ml aliquots in 15 ml test tubes, and centrifuged at 900 ×g for 10 min. Supernatant was decanted and the pellet was resuspended in phosphate

buffered saline (PBS) at 4°C for washing. Cells were washed twice, centrifuged and decanted. Cells were fixed in 10 ml cold 70% ethanol (stored at -20°C) with gentle vortexing. The cell suspension was kept overnight in a refrigerator (at 4°C). Cells were washed twice with PBS, centrifuged and decanted. The pellet was resuspended with 1 ml PBS including 0.25 mg RNase and the suspension was transferred to 1.5 ml test tube, and then incubated at 37°C for 1 hr. Propidium iodide (PI) solution was added to the samples as to adjust 50 µg of the final concentration of PI and stained at 4°C in the dark for a minimum of 30 min. Prior to flow-cytometric analysis, the cell suspension was filtered through 40 µm mesh to remove debris and cell clumps.

Prepared samples were analyzed by excitation with an argon ion laser set at 488 nm wave length in a EPICS ELITE flow cytometer (Colter, USA). DNA fluorescence emitted by each nucleus after excitation was detected through a multichannel analyzer and quantified.

#### Cloning of marker cDNA for eel spermatogenesis

The differential mRNA display technique was carried out as described in Miura *et al.* (1999). In this study, we isolated the fingerprint products detected only the testes at 15 and 18-day after hCG injection. Reamplified cDNA fragments were ligated into the pCR<sup>TM</sup>II vector using the TA cloning kit (In vitrogen, San Diego, CA, USA) and used as a probe for northern blot analysis to detect differentially expressed genes. Full-length cDNA clones for differentially expressed genes were isolated by screening a cDNA library constructed from eel testes 18 days after hCG injection. Total RNA taken from the testes on day 18 was reverse transcribed to cDNA by Superscript Reverse Transcriptase II (Gibco BRL, Gaithersburg, MD, USA). The cDNA was size-fractionated on Sepharose CL4B column with a lower cut off value of 500 bp and inserted into λzap II arms following the addition of *Eco*R I adapters (Amersham International Plc., UK).

About 10<sup>4</sup> plaques were blotted onto two nylon membranes (Hybond-N+, Amersham) and hybridized with 1×10<sup>7</sup> c.p.m. of [ $\alpha$ -<sup>32</sup>P] dCTP-labeled cDNA probes (Random Primer Plus Extension kit, NEN Research Products Inc., USA) for 18 hr at 65°C in hybridization solution containing 6×SSC (1×SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.2% Ficoll, 0.2% polyvinylpyrrolidone (PVP), 0.1% sodium dodecyl sulfate (SDS), and 100 mg/ml denatured, fragmented salmon sperm DNA. The membranes were washed twice with 1% 1×SSC/1% (W/V) SDS at 65°C for 1hr.

Positive clones were plaque-purified and the longest insert of positive clones was selected and ligated into pBluescriptII KS (+/-). Sequence determination was performed on an ABI PRISM TM 310 Genetic Analyzer using the DNA Sequencing Kit (Applied Biosystems, USA). Sequence analysis and comparison were carried out using the DNASIS software (Hitachi, Japan). Similarity search of the predicted amino acid sequence of the obtained cDNA was carried out using the Swiss Protein Sequence Data Bank.

#### Northern blot Analysis

Five micrograms of total RNA isolated from the testes of eel at 0, 1, 3, 6, 9, 12, 15 and 18 days after hCG injection and 24 days after *in vitro* culture with or without 11-KT was electrophoresed on a 1% (W/V) agarose gel containing formaldehyde, then transferred onto a nylon membrane (Hybond-N+, Amersham). The membrane was washed briefly, then baked at 80°C for 2 hr. The cDNA fragments were labeled by Random Primer Plus Extension Kit (NEN) with [ $\alpha$ -<sup>32</sup>P] dCTP to serve as probes. The membranes containing total RNA were pre-hybridized at 65°C for 1 hours in 6×SSC containing 0.2% BSA, 0.2% Ficoll, 0.2% PVP and 0.5% SDS, denatured salmon sperm DNA (100 mg/ml). Membranes were then hybridized with 1×10<sup>6</sup> c.p.m. of the radio-labeled probe in the hybridization solution at 65°C for 18 hr and washed with 1×SSC/0.01% SDS solution at 65°C for 2 hr. The membrane was exposed

to an imaging plate for 24 hr and analyzed by a BAS 2000 Bio-Image Analyzer (Fujifilm, Japan).

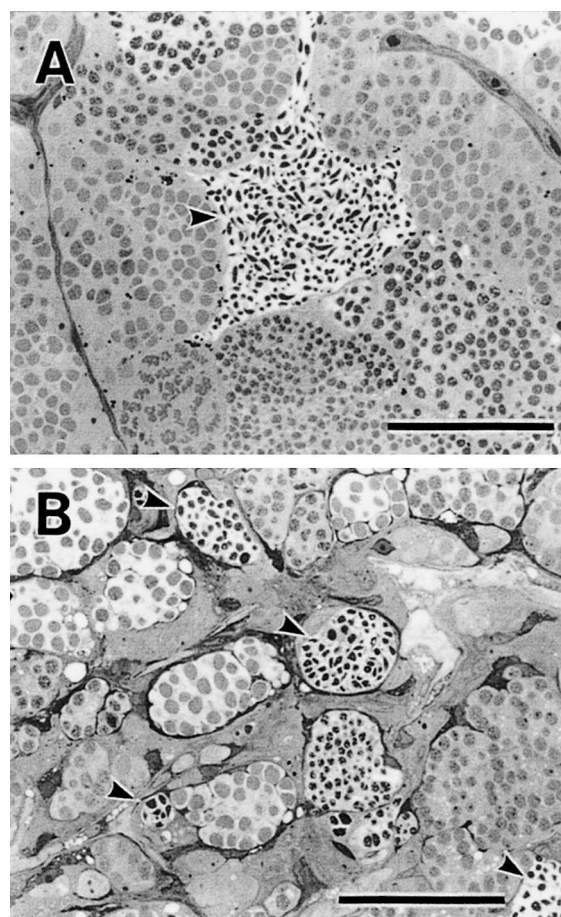
#### *In situ* hybridization

Testes from eel after 18 days of hCG injection and from 24 days after *in vitro* culture fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C overnight. Tissue samples were dehydrated through a graded ethanol series, embedded in paraffin, and serial sections of 5 µm were mounted on glass slides. Approximately 200 bps cDNA of eSRS11 was subcloned into pBluescript II KS<sup>-</sup>. Sense and anti-sense RNA probes were transcribed *in vitro* using digoxigenin (DIG)-labeled UTP (Boehringer Mannheim, Germany) and T3 or T7 RNA polymerase (Gibco BRL, USA). *In situ* hybridization was performed as described by Miura *et al.* (1999).

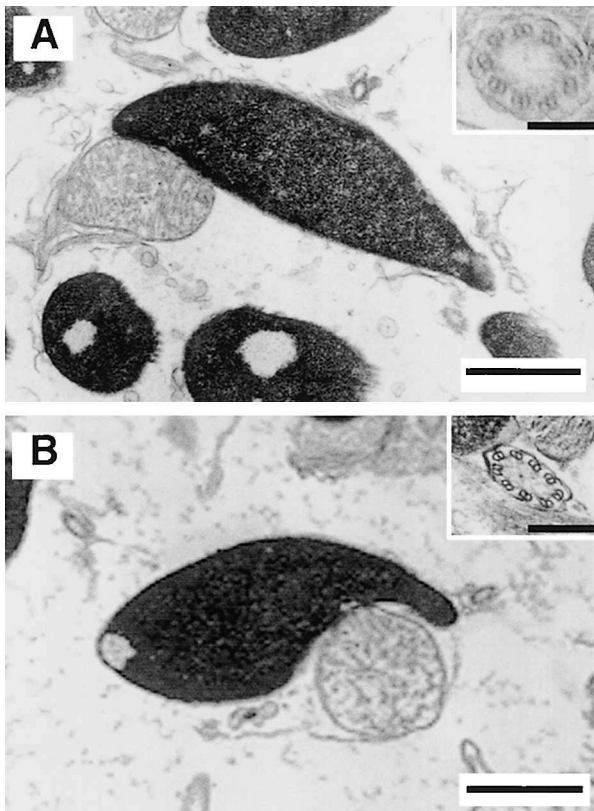
## RESULTS

#### Morphological studies

Testes collected from eels 18 days after hCG injection *in vivo*, testes cultivated with 11-KT for 24 days *in vitro*, were compared, using light (Fig. 1) and electron microscopy (Fig. 2). In both testes of *in vivo* and *in vitro*, all stages of spermatogenesis, i.e., from spermatogonia to spermatozoa, were observed. Whereas, in the testes of the *in vitro* culture,



**Fig. 1.** Light micrographs of the testis of 18 days after human chorionic gonadotropin (hCG) injection (A) and 24 days after *in vitro* culture with 11-ketotestosterone (B). Arrowheads indicate spermatozoa. Bar, 50 µm.



**Fig. 2.** Electron micrographs of spermatozoa showing a mitochondrion and 9 + 0 flagellum (insert). (A) 18-day after hCG injection and (B) 24-day after *in vitro* culture with 11-ketotestosterone. Bar, 1  $\mu\text{m}$  (insert, 0.1  $\mu\text{m}$ )

lumen, characteristic structures of lobular type, was not observed, and the cysts including spermatozoa were observed. Furthermore, the number of spermatogonia in the testes of *in vitro* was smaller than that of *in vivo*. Electron microscopy observations revealed that both of *in vivo* and *in vitro* spermatozoon had crescent-shaped nuclei, one

large mitochondrion and flagella with 9+0 axonemal structure. In *in vitro* spermatozoa, however, the elongation of the sperm head was not sufficient and the mitochondrion was not always located at the anterior end as is observed for the spermatozoa obtained from hCG injected eels.

#### Flow cytometry

The flow-cytometric histogram representing DNA contents of testes obtained *in vivo* is shown in Fig. 3A. The flow cytometry showed prominent 1C peak including spermatozoa and spermatids, 2C peak including spermatogonia and somatic cells at the G1 phase and secondary spermatocytes, and 4C peak including primary spermatocytes and somatic cells at the G2 and M phases. DNA content analysis revealed that complete stages of germ cells in spermatogenesis were observed.

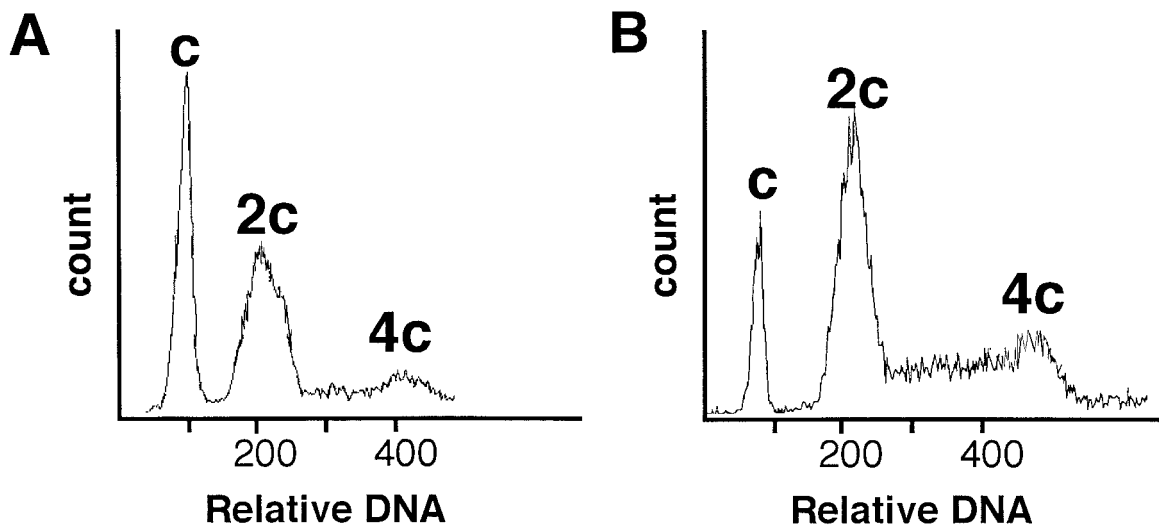
The histogram of the *in vitro* samples showed the same three distinct peak patterns compared with the hCG injection histogram (Fig. 3B). This result showed that the DNA reduction of the *in vitro* system progressed normally. i.e., haploid spermatozoa were produced.

#### The number of mitotic divisions

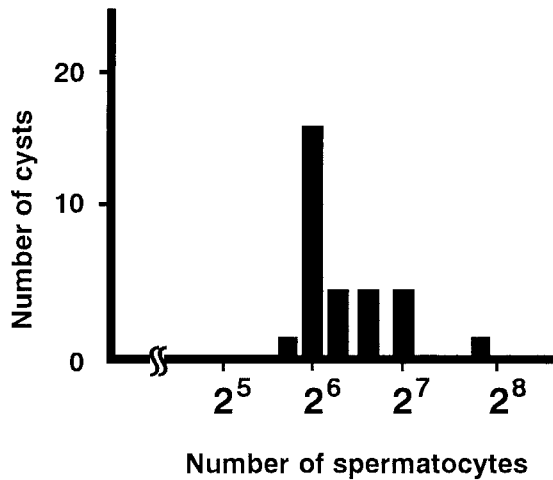
The spermatocytes in one cyst of the testes cultured *in vitro* for 24 days with 11-KT were found to be concentrated at  $2^6$  cells though a few cyst had  $2^7$  or  $2^8$  (Fig. 4). The number of mitotic divisions of the *in vitro* experiment (6 divisions) was smaller than that of *in vivo* (10 divisions).

#### Characterization of differentially expressed mRNA

In order to characterize the *in vitro* spermatogenesis not only at the morphological level, but also at the molecular level, the cloning of the marker gene with changing expression during spermatogenesis *in vivo*, was attempted. To detect such DNA clones, the differential mRNA display technique was carried out using total RNA extract from testes



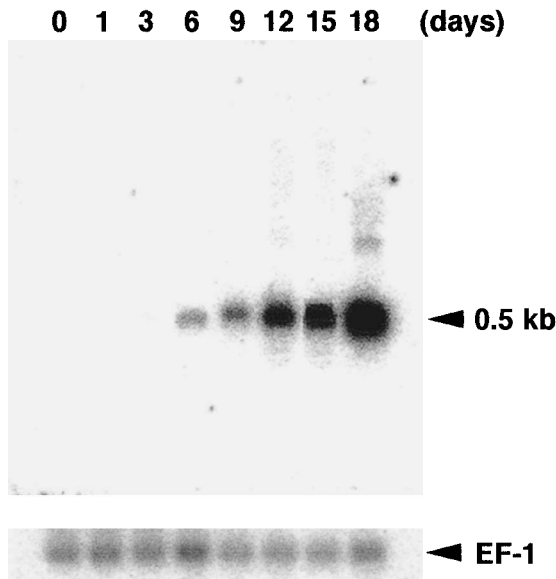
**Fig. 3.** Flow-cytometric histograms of testes of (A) 18-day after hCG injection and (B) 24-day after *in vitro* culture with 11-ketotestosterone. c; Peaks of haploid cells. 2c; Peaks of diploid cells. 4c; Peaks of tetraploid cells.



**Fig. 4.** The number of cysts containing spermatocytes in each 30 cysts of *in vitro* testes cultivated for 24 days with 11-ketotestosterone.

0, 1, 3, 6, 12, 15 and 18 days after hCG injection, and many fingerprint products with changing expression during spermatogenesis were obtained.

After reamplification and cloning, northern blot analysis was performed to evaluate sequential changes during spermatogenesis. As a result, the differentially expressed clone termed eel spermatogenesis relating substance-11 (eSRS11) fragment was obtained. The eSRS11 mRNA was not detected in the testes of untreated eels or in testes collected from eels 1 day to 3 days after hCG injection, while a transcript of approximately 0.5 kb was detected in the testes collected from eels 6–18 days after hCG injection (Fig. 5). The signal was weak on day 6, became gradually stronger, and



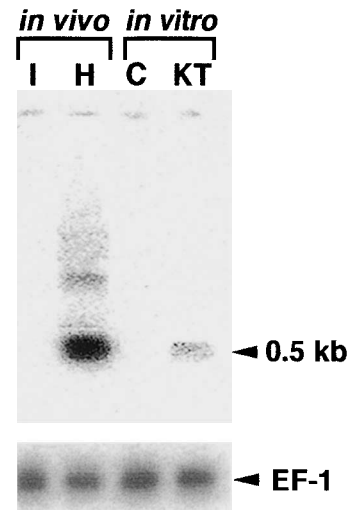
**Fig. 5.** eSRS11 mRNA expression in developing testes. Northern blot analysis was performed using total RNA extracted from testes 0, 1, 3, 6, 9, 12, 15 and 18 days after hCG treatment. Lower panel represent elongation factor-1 (EF-1) control.

reached a maximum on days 12–18, showing an up-regulated expression by hCG injection.

As control, the same blots were probed with labeled elongation factor-1 (EF-1) cDNA. The level of EF-1 tran-

eSRS11	1	MARKKAAARP	MKTCCSTTNV	AALIVEAVSG	30
human-H1	22	K.A...GGT.	R.A--GPP.	SE..TK..AA	49
mouse-H1	22	KR.G.KPGMA	TARKPRGFS.	SK..P..LM	51
chicken-H1	21	KKP...GGA	KPRKPAGFS.	TE..TK...A	50
r-trout-H1	15	APK....K.	K---AGFS.	GE..K...A	41
b-trout-H1	15	APK....K.	K---GPA.	GE.AGK..AA	41
eSRS11	31	SKEKSGVSM	ALQKALTAGG	YDVAHNVS	60
human-H1	50	...R...L.	..K...A.A.	...EK.N..I	79
mouse-H1	52	.Q.RA.M.L.	..K...A.A.	...EK.N..I	81
chicken-H1	51	...RK.L.L.	..K...A...	...EK.N..I	80
r-trout-H1	42	...R...L.	..K.S.A...	...EK.N...	71
b-trout-H1	42	...R...L.	..K.S.A...	...EK.N...	71
eSRS11	61	KQAVKSLVSN	GTLQSKGPG	AV-CFILNAG	89
human-H1	80	.LGL.....S	..V.T..T.	.SGS.K..KK	109
mouse-H1	82	.L.L.R..NK	.V.V.T..T.	.SGS.K.SKK	111
chicken-H1	81	.LGL.....K	..V.T..T.	.SGS.K..KK	110
r-trout-H1	72	.I.....TK	..V.T..T.	.SGS.K..KK	101
b-trout-H1	72	.I.....TK	..VET..T.	.SGS.K..KK	101
eSRS11	90	AGSPSPKTK	KTTPRKAKRS	SSQSPKTRA	119
human-H1	110	KKAGGT.PK.	PVGAA.KPKK	AASGEA.PKV	139
mouse-H1	112	KK.A.A.AK.	LGLS.ASRSP	AASGND.GKG	141
chicken-H1	111	KKPAAKP.KP	AAKPA.AAK	PGETKA.ATK	140
r-trout-H1	102	.AA.KA..VA	AKK.AA..K-	AVEAKKPAKK	130
b-trout-H1	102	.AA.KA..VA	AKK.AA..KP	AVEAKKPAKK	131
eSRS11	120	PRKATRSRPRK	AKSARPKKAK	TARRKK	145
human-H1	142	AAGGATPKKS	..KTPK.AK.	P.AATV	165
mouse-H1	142	KSSK.KVVK.	P.AT.T.GSG	SR.KT.	167
chicken-H1	141	KP.KAAAVK.	SPKKAK.P.A	A.TK.A	166
r-trout-H1	131	.K.VAAKKAV	.AKKS....	KPATP.	156
b-trout-H1	132	KKV.AKKA	..KSPK.AK.	P.TP..	157

**Fig. 6.** Amino acid sequence comparison of eSRS11 with histone H1 of human, mouse, chicken, rainbow trout (r-trout) and brown trout (b-trout). Dots indicate conserved positions throughout sequences. Hyphens indicate gaps.



**Fig. 7.** Differences of eSRS11 mRNA expression between *in vivo* and *in vitro*. Northern blot analysis was performed using total RNA extracted from testes 0 (I) and 18 (H) days after hCG treatment and from testes fragments cultivated *in vitro* for 24 days without (C) or with (KT) 11-ketotestosterone. Lower panel represents elongation factor-1 (EF-1) control.

scripts was constant in every lanes, confirming that the observed changes in eSRS11 mRNA expression were not artificial.

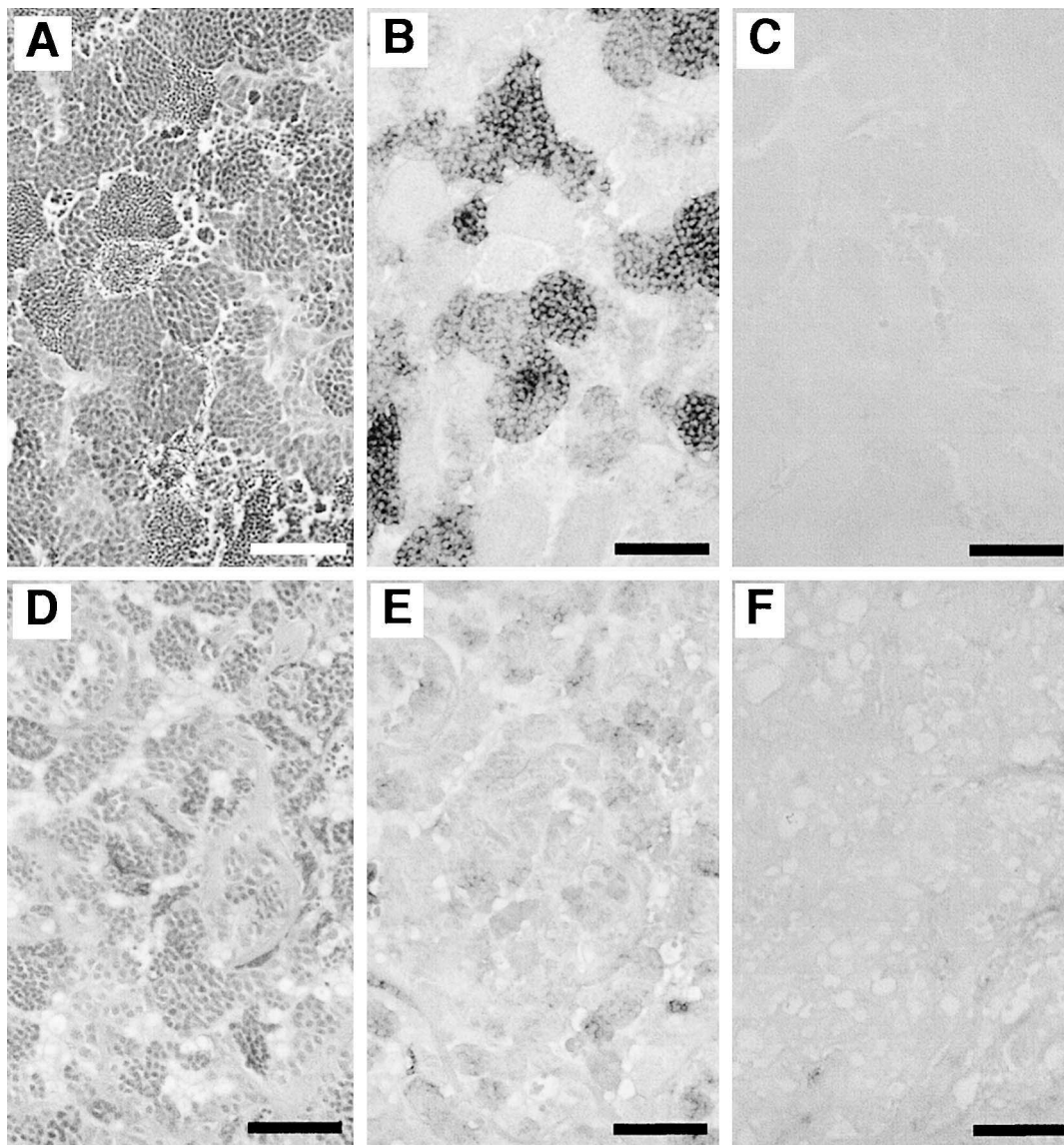
#### Screening and nucleotide sequence

Using eSRS11 cDNA as a probe, the cDNA library of the testes of eel 18 days postinjection was screened and 30 positive clones were obtained. The longest cDNA clone was chosen and sequenced. A complete nucleotide sequence and a deduced amino acid sequence of the 497-bp of eSRS11 cDNA insert are presented (DDBJ/EMBL/Gene Bank accession number: AB073744). The sequence contained a long open reading frame of 335 bp nucleotide encoding 145 amino acids and showed high similarity to histone H1 (Fig. 6). At the amino acid level, eSRS11 was 49%

homologous to the histone H1 of rainbow trout (Mezquita *et al.*,1985), 46% to that of brown trout (Mcleod *et al.*,1977), 46% to that of chicken (Sugarman *et al.*,1983), 43% to that of mouse (Cheng *et al.*,1989; Yang *et al.*,1987) and 46% to that of human (Eick *et al.*,1989; Ohe *et al.*,1989). Furthermore, in this nucleotide sequence, the polyadenylation signal was not recognized. Therefore, it was concluded that eSRS11 encoded the substance which resembled histone H1.

#### Comparison of the expression of eSRS11 between *in vivo* and *in vitro*

Northern blot analysis was performed to compare the *in vivo* and *in vitro* spermatogenesis, based on the expression of eSRS11. The eSRS11 mRNA was not detected in the



**Fig. 8.** Cellular localization of eSRS11 mRNA in testes collected from eels after 18 days of hCG injection (A, B, C) and testes cultured with 11-ketotestosterone for 24days (D, E, F). *In situ* hybridization was performed using digoxigenin-labeled anti-sense (B, E) and sense (C, F) probes. Panel A and D show testicular serial sections stained with hematoxylin and eosin. Bar, 50 µm.

testes of initial fish or in testes fragments cultured without 11-KT for 24 days (control). A signal of the same size as *in vivo* (0.5 kb) was detected in the testes collected from eels 18 days after hCG injection. The same signal was also detected in the *in vitro* testes cultured with 11-KT for 24 days, however, the expression was much weaker than that of *in vivo* (Fig. 7).

As control, the same blots were probed with labeled elongation factor-1 (EF-1) cDNA. The level of EF-1 transcripts was constant in every lanes, confirming that the observed changes in eSRS11 mRNA expression were not artificial.

### ***in situ* hybridization**

To determine the location of the expression of eSRS11, testes collected from eels after 18 days of hCG injection and testes cultured with 11-KT for 24 days, including all stages of spermatogenesis, were fixed, sectioned and hybridized with sense and anti-sense DIG-labeled RNA probes specific to eSRS11 (Fig.8). Anti-sense probes of eSRS11 were detected in late type B spermatogonia and spermatocytes both *in vivo* and *in vitro*. The expression of *in vitro* samples, however, was weaker than that of *in vivo*.

The number of late type B spermatogonia with eSRS11 signals was counted in the testes of *in vivo* and *in vitro*. In the *in vivo* testes, the number of late type B spermatogonia ranged from  $2^6$  to  $2^9$ . Strong eSRS11 signals were observed in late type B spermatogonia at the 9 mitotic divisions and spermatocytes. The signal was neither detectable in the cysts with less than  $2^5$  spermatogonia, i.e., type A and early type B spermatogonia nor in other testicular cell types including spermatids and spermatozoa and somatic cells. On the other hand, in the *in vitro* testes, late type B spermatogonia were only observed at the 5th mitotic division. The eSRS11 signals were detected in late type B spermatogonia and spermatocytes. The signal was neither detectable in cysts with less than  $2^4$  spermatogonia nor in other testicular cell types including spermatids and spermatozoa and somatic cells. Sense probe for negative control did not hybridized in any cells.

## **DISCUSSION**

In agreement with previous studies (Miura *et al.*, 1991b; 1991c), 11-KT was successful in producing spermatozoa in Japanese eel testicular fragments cultured *in vitro*. Spermatogenesis induced *in vitro* by 11-KT included all stages of spermatogenesis, i.e., proliferation of spermatogonia, meiosis and spermiogenesis. These results indicate that spermatogenesis of the Japanese eel can be induced by 11-KT.

In this study, the flow-cytometric histogram of the *in vitro* culture showed 1C peak including haploid cells, i.e., meiosis and the reduction of DNA progressed normally in the testes cultured *in vitro*. This study is the first report showing that meiosis progressed normally morphologically and qualitatively, in the testes cultivated with 11-KT.

However, spermatogenesis of the *in vitro* experiment had several differences in comparison with the *in vivo*. Firstly, in the testes of *in vitro* with advanced spermatogenesis, characteristic structures of lobular type were not observed. In teleosts, two types of testes have been identified: 1) the tubular type; and 2) the lobular type (Grier, 1981). In the tubular type, spermatocytes develop and move internally through the testis as they advance through spermatogenesis to release spermatozoa in the efferent ductulus. In the lobular type, spermatogonia are present along the wall of the tubule which has a patent lumen and the cysts are in contact with the basement membrane only on one side (Loir *et al.*, 1995). At the time of spermiation, the cysts burst to release spermatozoa into the lumen. The testes of most species of teleosts belong to the lobular type. It is known that the testes of Japanese eel belong to the lobular type (Miura *et al.*, 1991a). In the testes of *in vitro*, however, the lobular structure was not formed. As a result, spermatozoa were not released into the lumen, and remained in the cysts.

Secondly, in the *in vitro* culture, the number of mitotic divisions of spermatogonia before entry into meiosis is smaller than that of *in vivo*. Generally, spermatogonia enter meiosis after a fixed number of mitotic divisions (Courot *et al.*, 1970). In teleosts, the number of mitotic divisions has been described in several species and was found to be 8 in the medaka (*Oryzias latipes*), 6 in Sakhalin taimen (*Hucho perryi*), 8 in masu salmon (*Oncorhynchus masou*), 8 in goldfish (*Carassius auratus*) (Ando *et al.*, 2000), 14 in the guppy *Poecilia reticulata* (Billard, 1986) and 5 to 6 in the zebrafish *Danio rerio* (Ewing, 1972). In Japanese eel, it was also reported that the number of mitotic divisions was found to be 10 (Miura *et al.*, 1991a). However, it was estimated by the observation of light microscopy that the size of cysts including spermatogonia of *in vitro* was smaller than that of *in vivo*. Furthermore, the number of spermatocytes in 30 cysts was counted. As a result, it was clear that most cysts of spermatocytes of *in vitro* had  $2^6$  cells. Since the number of spermatocytes was concentrated at  $2^1$  and that germ cells in one cyst were at the same stage of development in the cell cycle, indicated that spermatogonia divided synchronously. These results show that spermatogonia in the *in vitro* culture system entered meiosis after 6 divisions.

Thirdly, the shape of spermatozoa of *in vitro* was different when compared to that of *in vivo*. From electron microscopy observations, spermatozoa of *in vivo* had crescent-shaped nuclei, becoming more round posteriorly with one mitochondrion located at the anterior end and flagella with a 9+0 axonemal structure located at the caudal end of the base of the nuclei (Todd, 1976; Miura *et al.*, 1991a; Gwo *et al.*, 1992). Nevertheless the spermatozoa of *in vitro* also had crescent-shaped nuclei and flagella with a 9+0 axonemal structure located at the caudal end of the base of the nuclei, the elongation of the sperm head was insufficient and the mitochondrion was not always located at the anterior end.

As mentioned above, there were several morphological



differences between the *in vivo* and *in vitro* spermatogenic cycle. Therefore, in the next step, comparison between spermatogenesis of *in vitro* and *in vivo* was attempted at the molecular level. Attention focused on the eSRS11 gene, since it displayed changing expression during spermatogenesis. The expression is up-regulated by hCG, i.e., the signal appeared on day 6, became gradually stronger, and reached a maximum on days 12–18 after hCG treatment. And eSRS11 encodes the substance which resembles histone H1. The expression of eSRS11 was compared between *in vivo* and *in vitro* using northern blot analysis and *in situ* hybridization.

Northern blot analysis showed that eSRS11 expression of *in vitro* was much weaker than that of *in vivo*. In order to detect the localization of eSRS11 expression, *in situ* hybridization was performed. As a result, eSRS11 was expressed in 2<sup>6</sup> to 2<sup>9</sup> late type B spermatogonia and spermatocytes in the testes of *in vivo*. Especially, strong expression was detected in 2<sup>9</sup> late type B spermatogonia and spermatocytes. Whereas, in the *in vitro* testes, the signal was observed in 2<sup>5</sup> spermatogonia and spermatocytes. The signal was much weaker than that of *in vivo*. These results suggested that the expression of eSRS11 was related to the number of mitotic divisions. The eSRS11 encoded some substance with similarity to histone H1. Histone H1 is necessary for the condensation of nucleosome chains into higher order structures (Doenecke and Drabent, 1995). These results indicated that eSRS11 might affect the structure of sperm nuclei. Furthermore, this implied that the number of mitotic divisions of spermatogonia might be related to the structure of the sperm nuclei.

Using northern blot analysis and *in situ* hybridization, it was clear that eSRS11 is less expressed *in vitro* than *in vivo*. However, it is reported that activin B, the substance that induces mitotic division, is expressed in the *in vitro* testes, at the same levels as *in vivo* (Miura *et al.*, 1995a,b). These results allude to the presence of other substances than 11-KT involved in the regulation of eSRS11 expression. The detection of these substances is important to better analyze the control mechanisms of spermatogenesis.

From the results mentioned above, this study showed that spermatogenesis of *in vitro* differed from that of *in vivo*, not only at the morphological level, but also at the molecular level. These observations of the *in vitro* spermatogenesis indicate that spermiogenesis is subject to some mechanism regulated by some factor except 11-KT. However, spermatocytes of medaka *Oryzias latipes* cultured *in vitro* without any hormones divided and progressed to fertile spermatozoa (Saiki *et al.*, 1997). This suggested that spermiogenesis did not necessarily require factors to induce this process. Spermiogenesis is the process where the shapes of germ cells change drastically, and the mechanisms controlling this process are very interesting to analyze.

This study also showed that meiosis, the reduction of DNA, could be induced perfectly by only 11-KT. However, structures of testes of lobular type, number of mitotic divi-

sions of spermatogonia and spermiogenesis could appear to require other substances than 11-KT. Therefore, some substances or factors except 11-KT are needed to induce the complete spermatogenesis. Morphological observations obtained from the testes cultured with hCG are the same as the results obtained from the *in vitro* experiment with 11-KT (Miura *et al.*, 1991b). Therefore, hCG does not seem to be the substance required for the induction of complete spermatogenesis *in vitro*. In the future, in order to use this *in vitro* culture system to obtain fertile sperm, substances or factors which are absent from this *in vitro* culture must be detected and analyzed.

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