

## Follicle-Stimulating Hormone Induction of Steel Factor (SLF) mRNA in Mouse Sertoli Cells and Stimulation of DNA Synthesis in Spermatogonia by Soluble SLF

PELLEGRINO ROSSI, SUSANNA DOLCI, CRISTINA ALBANESI, PAOLA GRIMALDI, ROSSANA RICCA, AND RAFFAELE GEREMIA

Department of Public Health and Cell Biology, University of Rome "Tor Vergata," Via Orazio Raimondo 8, 00173 Rome, Italy

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Follicle-stimulating hormone (FSH) and its intracellular mediator, cAMP, increase the mRNA levels for the Steel factor (SLF, the c-kit ligand) in cultured primary mouse Sertoli cells. The inductive effect of cAMP is more evident in cultures from 13-day-old animals than in cultures from 18-day-old animals. Analysis through the polymerase chain reaction (PCR) indicates that (Bu)<sub>2</sub>cAMP or FSH treatment increases the levels of the mRNAs for both the potentially soluble form and the transmembrane form of SLF in cultured Sertoli cells. The ratio between mRNAs encoding the potentially soluble form and the transmembrane form of SLF increases during postnatal testis development, and it is higher in cultured Sertoli cells with respect to total testis, suggesting that, under the *in vitro* conditions, SLF could be produced by Sertoli cells mainly as a soluble factor. Soluble recombinant SLF stimulates, in a dose-dependent fashion, thymidine incorporation in cultures of isolated germ cell populations enriched in the mitotic stages (spermatogonia), independently of the presence of serum, whereas cAMP analogs have no effect. Autoradiographic analysis shows that SLF selectively stimulates DNA synthesis in type A spermatogonia. © 1993 Academic Press, Inc.

### INTRODUCTION

Mutations at either the W or the Sl locus in mice, which imply defects in the genes encoding the c-kit proto-oncogene (a transmembrane tyrosine kinase receptor) (Chabot *et al.*, 1988; Geissler *et al.*, 1988) and its ligand (SLF, Steel factor) (see, as reviews, Witte, 1990; Besmer, 1991), respectively, are associated with impairment of gametogenesis, together with anemia and defects of pigmentation (Russel, 1979). In the W mutation the defect is intrinsic to the affected cell lineage, whereas in the Sl mutation the defect lies in the microenvironment in which stem cells grow, migrate, and differentiate. We have previously shown, in agreement with genetic analysis of the W and Sl mutants, that the c-kit proto-oncogene mRNA is expressed in the male

germ cell line (Sorrentino *et al.*, 1991), whereas Sertoli cells are the site of expression of the c-kit ligand (SLF) mRNA (Rossi *et al.*, 1991). We have also shown that SLF mRNA expression is enhanced, in primary Sertoli cell cultures from 18-day-old mice, by treatment with cAMP analogs (Rossi *et al.*, 1991). Since cAMP analogs are known to mimic many effects of follicle-stimulating hormone (FSH) on Sertoli cells (Rossi *et al.*, 1990), this finding suggested a potential hormonal regulation of SLF expression in the male gonad.

Alternatively spliced SLF mRNAs encode for two transmembrane forms (Anderson *et al.*, 1990; Flanagan *et al.*, 1991), which, at different rates, can be cleaved in the extracellular domain and release a soluble form of the factor (Huang *et al.*, 1992). We have recently provided evidence that the transmembrane form of SLF is able to support the survival but not the proliferation of primordial germ cells (PGCs) *in vitro* (Dolci *et al.*, 1991). The soluble form, which had been previously shown to stimulate mast cell proliferation (Anderson *et al.*, 1990), has also been shown to support survival of PGCs in culture (Godin *et al.*, 1991) and, under particular culture conditions, to stimulate a slight increase in DNA synthesis in these cells (Matsui *et al.*, 1991).

Biologically active SLF seems to be produced by cultured Sertoli cells, since Sertoli cell cultures from *W<sup>o</sup>/W<sup>o</sup>* but not *Sl<sup>d</sup>/Sl<sup>d</sup>* mice are able to support the growth of cocultured mast cells (Tajima *et al.*, 1991).

In this paper we show that (1) FSH, the physiological regulator of Sertoli cell function, stimulates an increase in the mRNA levels for the c-kit ligand in cultured primary mouse Sertoli cells; (2) the transcripts for both the potentially soluble and the transmembrane forms of SLF are stimulated by FSH and/or cAMP of Sertoli cell monolayers; (3) the ratio of the levels of transcripts for the potentially soluble and the transmembrane form of SLF, both in total testis and in cultured Sertoli cells, increase with the age of the animals; and (4) the soluble

form of SLF is able to promote DNA synthesis in type A spermatogonia *in vitro*.

#### MATERIALS AND METHODS

**Biological materials.** Male Swiss-CD1 mice at the indicated ages were obtained from Charles River Italia (Como, Italy). Germ cell suspensions enriched in the mitotic stages were obtained by sequential collagenase-hyaluronidase-trypsin treatment of freshly withdrawn testes from 7- to 8-day-old mice, as previously described (Bucci *et al.*, 1986). Animals at this age were chosen to avoid contamination with DNA-synthesizing meiotic germ cells, namely, preleptotene spermatocytes, which first appear in mouse seminiferous tubules around Day 10 after birth (Kong Sung *et al.*, 1986). At Day 7 or 8 after birth type A and B spermatogonia are the prevailing germ cell types detectable in seminiferous tubules. To remove somatic cell contaminants, cell suspensions were preplated for 3–4 hr on 10-cm-diameter tissue culture dishes, taking advantage of the observation that somatic cells rapidly adhere to plastic, whereas germ cells do not. Preplating was performed in Eagle's minimum essential medium (MEM) supplemented with glutamine, nonessential amino acids, gentamycin, streptomycin, and penicillin (GIBCO, Grand Island, NY), 2 mM sodium lactate, 1 mM sodium pyruvate, and 10% fetal calf serum (FCS) in a 5% CO<sub>2</sub> atmosphere at 32°C. After this preincubation step, floating mitotic germ cells were collected, washed twice to remove serum, and resuspended in the same medium without FCS for further experimental procedures. Primary Sertoli cell-enriched cultures from 13- or 18-day-old mice were prepared as previously described (Galdieri *et al.*, 1984). Briefly, seminiferous tubules were prepared by sequential trypsin and collagenase treatment of dealbuginized testes. Tissue explants were allowed to adhere and to form confluent monolayers on 6-cm-diameter tissue culture dishes for 3 days in serum-free MEM supplemented with glutamine, nonessential amino acids, gentamycin, streptomycin, and penicillin (GIBCO) in a 5% CO<sub>2</sub> atmosphere at 32°C. Cultures were treated on the third day with hypotonic solution (3 min with 20 mM Tris-HCl, pH 7.5) to lyse remaining germ cells. Twenty-four hours later stimulating agents or equal volumes of solvent medium were added to the dishes. The next day cells were harvested for RNA extraction.

**RNA preparation and analysis.** RNA was extracted with guanidine isothiocyanate and purified on CsCl gradients (Chirgwin *et al.*, 1979) or, alternatively, prepared with a modified guanidine isothiocyanate-acid phenol protocol (Stallcup and Washington, 1983). Poly(A)<sup>+</sup> RNA was prepared by oligo(dT) cellulose (Boehringer-Mannheim, Mannheim, Germany) affinity chromatography (Aviv and Leder, 1972).

For Northern blot experiments, RNA samples were fractionated on 1.1% agarose-formaldehyde gels and transferred onto Hybond-N membranes (Amersham, UK) by capillarity. Blots were hybridized and washed under high-stringency conditions (Maniatis *et al.*, 1982) with a [<sup>32</sup>P]DNA probe labeled by random priming (Feinberg and Vogelstein, 1983). The probes used were a 0.5-kb *EcoRI-HindIII* fragment isolated from a plasmid containing a mouse SLF cDNA (a gift from Peter Besmer, Sloan Kettering Cancer Center, New York) and a 1.7-kb *PstI* insert from a plasmid containing a chicken  $\beta$ -actin cDNA (a gift from Bruce Paterson, NIH, Bethesda, MD). Plasmid DNA was prepared by double banding on CsCl gradients (Maniatis *et al.*, 1982).

For RT-PCR analysis (Wang *et al.*, 1989) 1  $\mu$ g of total RNA or 0.5  $\mu$ g of poly(A)<sup>+</sup> RNA was reverse-transcribed using random hexamers as primers, using a Perkin-Elmer RNA PCR kit, following the instructions of manufacturers. Amplifications of cDNAs were performed in a final volume of 100  $\mu$ l, with a Perkin-Elmer/Cetus DNA thermal cycler (38 step cycles: 95°C for 40 sec, 60°C for 45 sec, 72°C for 15 sec), using synthetic oligonucleotides as 5' primer (primer A: GAGCTCCAGAACAGCTAAACGGAGTCG) and 3' primers (primer C: CGTCCACAA-TTACACCTCTTGAAATTCTCTCTC), immediately upstream and downstream from the open reading frame encoding SLF, deduced from the published cDNA sequence (Flanagan *et al.*, 1991). Alternatively, a synthetic oligonucleotide (primer B: GAATCTCCGAAGAGGCCAGAACTAGATCCTTT), corresponding to the cDNA sequence encoding amino acids 100–110 of the SLF open reading frame, was used as 5' primer together with primer C; in this case 32 step cycles were performed during PCR amplification. After extraction with 150  $\mu$ l of TE-saturated chloroform, 15  $\mu$ l of each sample was analyzed on 0.9–1.2% agarose gel electrophoresis. Gels were stained with ethidium bromide and photographed with an UV transilluminator. As an internal control for the amount of RNA used, in some PCR reactions we added oligonucleotides for amplification of hypoxanthine phosphoribosyltransferase mRNA (HPRT primers, expected cDNA product: 570 bp).

**[<sup>3</sup>H]Thymidine incorporation.** Mitotic germ cell-enriched suspensions were plated at a density of 10<sup>4</sup> cells in 200  $\mu$ l of medium into microtiter wells. SLF was added in the concentration range 1–400 ng/ml. After 24 hr the cultures were pulsed for 4 hr with 1  $\mu$ Ci [<sup>3</sup>H]-thymidine. Cells were collected on fiberglass filters with an automated collector, and incorporated [<sup>3</sup>H]thymidine was counted by liquid scintillation spectrometry. For autoradiographic analysis, cells were pulsed with [<sup>3</sup>H]-thymidine for 2 hr. Part of the cell suspensions was fixed with 3:1 methanol-acetic acid. After further treatment with 45% acetic acid to remove cytoplasm, nuclei

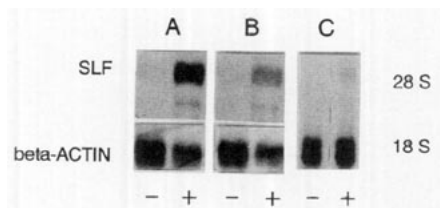


FIG. 1. FSH and  $(\text{Bu})_2\text{cAMP}$  increase mRNA levels for SLF in primary Sertoli cell cultures from 13-day-old mice. (A, C) Northern hybridization of 20  $\mu\text{g}$  total RNA from homogeneous Sertoli cell cultures that had been treated for 24 hr (+) with 1 mM  $(\text{Bu})_2\text{cAMP}$  (A) or 150 ng/ml FSH (C), or with the same volume of solvent medium (-). (B) Northern hybridization of 20  $\mu\text{g}$  total RNA from mixed cultures of testicular cells that had been treated for 24 hr (+) with 1 mM  $(\text{Bu})_2\text{cAMP}$  or with the same volume of solvent medium (-). Mixed cultures were obtained by reaggregation of 3 vol of cell pellet from the supernatant of collagenase-treated seminiferous tubules with 1 vol of Sertoli cell-enriched tissue explants. In (A) and (B) blots were hybridized with the SLF cDNA probe (3-day exposure) and then stripped and rehybridized with the  $\beta$ -actin cDNA probe (12-hr exposure). In (C) the blot was directly hybridized with a mixture of both SLF and  $\beta$ -actin cDNA probes (12-hr exposure).

were spreaded onto glass slides and processed for autoradiography. After 3 days of exposure, slides were developed and stained with Giemsa. Assignment of mitotic germ cells on the basis of nuclear morphology was assessed according to Meistrich *et al.* (1973).

**Growth factors and other reagents.** Ovine FSH (NIDDK-oFSH-17) was a gift from the NIH National Hormone and Pituitary Program. Recombinant Steel factor was a generous gift from D. E. Williams (Immunex Co., Seattle, WA) and was produced as previously described (Williams *et al.*, 1990).  $(\text{Bu})_2\text{cAMP}$  was purchased from Sigma Chemical Company (St. Louis, MO; Catalog No. D0260).

## RESULTS

### *Stimulation by cAMP Analogs and FSH of SLF mRNA Expression in Sertoli Cells*

Treatment with 1 mM  $(\text{Bu})_2\text{cAMP}$  of homogeneous cultures of Sertoli cells from 13-day-old mice results in a dramatic increase in SLF mRNA expression (Fig. 1A). The inductive effect is much more evident than that previously observed in cultures from older animals (Rossi *et al.*, 1991). Figure 1B shows that in a culture of Sertoli cells mixed with peritubular cells, myoid cells, and germ cells (i.e., cells present in the supernatant obtained after collagenase treatment of seminiferous tubules prior to plating Sertoli cell-enriched tissue explants), induction of the SLF signal after  $(\text{Bu})_2\text{cAMP}$  stimulation is less evident. These results might be the consequence either of the dilution of SLF mRNA or of a possible negative influence on the induction of SLF mRNA in Sertoli cells

by the contaminating cell types. In both cases, these data indicate that Sertoli cells are the major site of SLF mRNA expression within seminiferous tubules.

FSH (150 ng/ml) treatment stimulates an increase in SLF mRNA levels in homogeneous Sertoli cell cultures from 13-day-old mice, as shown in Fig. 1C (note that the exposure time of the Northern blot shown in this figure was sixfold shorter than that in lanes in A and B). FSH induction of SLF mRNA levels was confirmed through RT-PCR analysis (Fig. 2A, lane 2; see below).

### *Alternative Transcripts for SLF in Mouse Sertoli Cells*

SLF can exist in both a soluble form and a transmembrane form, encoded by alternatively spliced mRNAs (Flanagan *et al.*, 1991). These mRNAs differ only for 84 nucleotides. The shorter transcript lacks the sequence encoding a stretch of 28 amino acids (amino acids 149–176), corresponding to a potential proteolytic cleavage signal in the extracellular domain of the SLF polypeptide. A cDNA clone, MGF-94, has been isolated by Anderson *et al.* (1990) from a library prepared by a mouse bone marrow cell line: the corresponding transcript would lack 48 nucleotides, encoding a stretch of 16 amino acids (amino acids 149–164 of the SLF open reading frame). After reverse transcription, the different mRNA species can be identified through analysis with the polymerase chain reaction method (RT-PCR).

**RT-PCR with primers A and C.** Using as primers the oligonucleotides A and C indicated under Materials and Methods, one would expect cDNA-amplified products of 900 bp for the SLF transcript encoding the potentially soluble form, and of 816 bp for the SLF transcript encoding the transmembrane form of the c-kit ligand described by Flanagan *et al.* (1991). A cDNA amplification product of 852 bp would represent an mRNA corresponding to cDNA clone MGF-94 described by Anderson *et al.* (1990). Figure 2A shows that, using RNAs from Sertoli cell monolayers from 13-day-old mice, both FSH (lane 2) and  $(\text{Bu})_2\text{cAMP}$  (lane 3) treatments increase the levels of the mRNAs for SLF. Two cDNAs, which seem to correspond to the 900- and 816-bp amplification products, rather than to a 852-bp cDNA, appear to be induced by both treatments at comparable levels.

SLF cDNA-amplified signals are absent in RNA preparations from germ cells (Fig. 2B, lane 1), and quite low in RNA preparations from brain of adult mice (Fig. 2B, lane 5). With Northern blot analysis Matsui *et al.* (1990) reported higher levels of SLF mRNA in brain from newborn mice than in testis; however, in the same report it was evident that dramatic changes in SLF mRNA levels occurred with age in other organs, such as kidney.

The ratio between cDNAs, which should correspond to mRNAs encoding the potentially soluble form and the

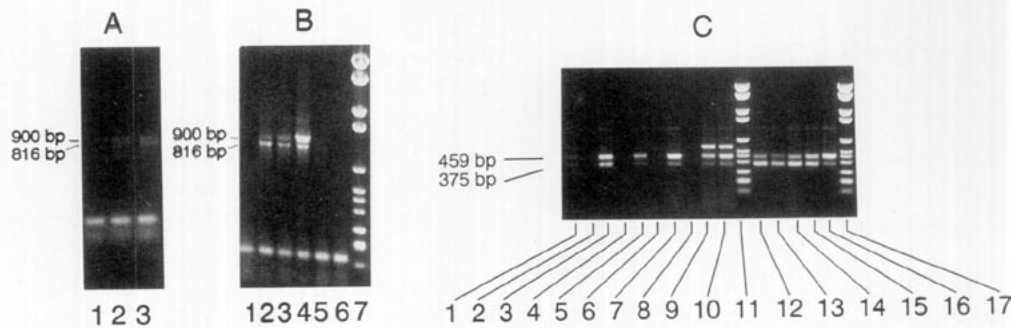


FIG. 2. FSH and  $(\text{Bu})_2\text{cAMP}$  induce the transcripts for both the potentially soluble (900 bp) and the transmembrane (816 bp) forms of SLF in Sertoli cell cultures. (A) RT-PCR amplification of  $1 \mu\text{g}$  total RNA from primary Sertoli cell cultures from 13-day-old mice, using primers A and C described under Materials and Methods: (1) unstimulated monolayers, (2) monolayers treated for 24 hr with  $150 \text{ ng/ml}$  FSH, (3) monolayers treated for 24 hr with  $1 \text{ mM}$   $(\text{Bu})_2\text{cAMP}$ . Electrophoretic analysis was performed with a 0.9% agarose gel. (B) RT-PCR amplification, using primers A and C indicated under Materials and Methods, of  $0.5 \mu\text{g}$  poly(A)<sup>+</sup> RNA from (1) germ cell suspension from adult mice, (2) total testis from immature (8-day-old) mice, (3) total testis from adult (60-day-old) mice, (4)  $(\text{Bu})_2\text{cAMP}$ -treated Sertoli cell monolayers from 18-day-old mice, and (5) mouse brain. Lanes 6 and 7 represent, respectively, a control reaction with  $3 \mu\text{g}$  tRNA and DNA molecular weight markers (mixture of fragments from cleavage of pBR328 DNA with *Bgl*I and of the same DNA cleaved with *Hinf*I). Sizes of the markers, in decreasing order, are 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220, and 154 bp. Electrophoretic analysis was performed with a 0.9% agarose gel. (C) RT-PCR amplification, using primers B and C indicated under Materials and Methods, of  $1 \mu\text{g}$  total RNA (lanes 1–10) or  $0.5 \mu\text{g}$  poly(A)<sup>+</sup> RNA (lanes 12–16). In lanes 2, 4, 6, and 8 reverse transcriptase was omitted from the reactions. RNA sources were unstimulated Sertoli cells from 13-day-old mice (1, 2);  $(\text{Bu})_2\text{cAMP}$ -treated Sertoli cells from 13-day-old mice (3, 4); unstimulated Sertoli cells from 18-day-old mice (5, 6, 9);  $(\text{Bu})_2\text{cAMP}$ -treated Sertoli cells from 18-day-old mice (7, 8, 10, 16); total testis from 8-day (12)-, 13-day (13)-, 15-day (14)-, and 60-day (15)-old mice. In samples 9 and 10 HPRT primers were included in the amplification step. Lanes 11 and 17 represent DNA molecular weight markers described in the legend to (B). The band above SLF-specific signals in lanes 9 and 10, whose position is intermediate between those of the 653- and 517-bp markers, corresponds to the predicted 570-bp cDNA amplification product of mouse HPRT mRNA. Electrophoretic analysis was performed with a 1.2% agarose gel. See Materials and Methods for experimental details.

transmembrane form of SLF, appears to change during postnatal testis development. It is higher in total testis from 60-day-old mice (Fig. 2B, lane 3) than in total testis from 8-day-old animals (Fig. 2B, lane 2). In  $(\text{Bu})_2\text{cAMP}$ -treated Sertoli cell monolayers from 18-day-old mice (Fig. 2B, lane 4), the cDNA probably corresponding to the mRNA encoding the potentially soluble form of SLF is by far the prevailing detectable form. A faint band of about 1200 bp is visible in poly(A)<sup>+</sup> mRNA-derived samples in Fig. 2B.

**RT-PCR with primers B and C.** To rule out the possibility that one of the two major observed cDNA species could represent the 852-bp amplification product predicted for a mRNA corresponding to clone MGF-94 described by Anderson *et al.* (1990), we performed additional RT-PCR experiments using different couples of oligonucleotide as primers. Using as primers oligonucleotides B and C indicated under Materials and Methods, one would expect cDNA-amplified products of 459 and 375 bp, respectively, for transcripts corresponding to the potentially soluble form and the transmembrane form of SLF, as described by Flanagan *et al.* (1991). A cDNA amplification product of 411 bp would represent a mRNA corresponding to cDNA clone MGF-94 described by Anderson *et al.* (1990). Figure 2C shows that only cDNA products of 459 and 375 bp are amplified from mouse testis and Sertoli cell mRNAs, and no trace of the

transcript corresponding to cDNA clone MGF-94 is detectable. A faint band of about 800 bp is detectable in some samples: this band is proportionally shorter than the faint 1200-bp band observed using primers A and C (Fig. 2B), suggesting that it could correspond to SLF cDNA sequences derived from incompletely processed RNAs. Again, a progressive increase was observed in the ratio between transcripts encoding the potentially soluble form (459 bp) and the transmembrane form (375 bp) of SLF during postnatal testis development (Fig. 2C, lanes 12–15). Analogously, a clear increase in the ratio between the two transcripts is detectable in cultured Sertoli cells from 18-day-old mice (Fig. 2C, lanes 5 and 9), with respect to cultures from 13-day-old animals (Fig. 2C, lane 1).

Treatment with cAMP analogs appears to increase the levels of the two transcripts in cultures from both 13-day-old (Fig. 2C, lane 3), and 18-day-old (Fig. 2C, lanes 7 and 10) mice. The ratio between the two cDNA-amplified products in  $(\text{Bu})_2\text{cAMP}$ -stimulated cells is similar using as a source either total (Fig. 2C, lanes 7 and 10) or poly(A)<sup>+</sup>-selected mRNAs (Fig. 2C, lane 16), suggesting that the observed differences do not reflect changes in the degree of polyadenylation of the two alternative transcripts. We can conclude that, at least under the culture conditions we use, SLF might be produced by Sertoli cells mainly as a soluble factor.

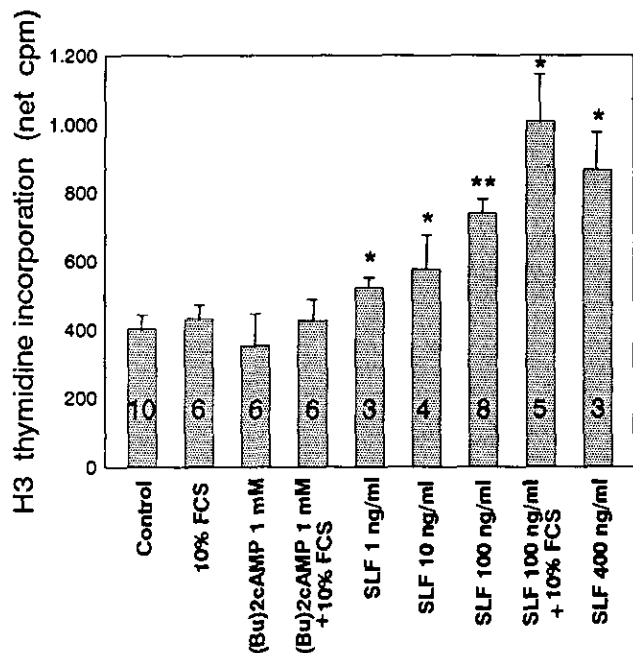


FIG. 3. The soluble form of SLF stimulates DNA synthesis in dividing germ cell populations from 7- to 8-day-old mice. Mitotic germ cell-enriched suspensions were plated at a density of  $10^4$  cells in  $200 \mu\text{l}$  of medium into microtiter wells. After 24 hr of treatment with the indicated agents, the cultures were pulsed for 4 hr with  $1 \mu\text{Ci}$  [ $^3\text{H}$ ]thymidine and processed as described under Materials and Methods. Data represent means  $\pm$  SD of  $n$  independent determinations ( $n$  is indicated inside each bar). Net cpm were calculated after background subtraction ( $262 \pm 75$ ). A two-tailed nonparametric Mann-Whitney test was used for statistical analysis of differences in [ $^3\text{H}$ ]thymidine incorporation among different treatments. A single asterisk indicates a significant difference ( $P < 0.05$ ), and two asterisks indicate a highly significant difference ( $P < 0.01$ ) with respect to controls.

RT-PCR products are derived from RNA, since no signals were present in reactions in which reverse transcriptase was not added (Fig. 2C, lanes 2, 4, 6, and 8).

#### *Soluble SLF Stimulates DNA Synthesis of Type A Spermatogonia in Culture*

In preliminary experiments we had observed that Sertoli cell cultures that had been pretreated with (Bu)<sub>2</sub>cAMP stimulated DNA synthesis in mitotic germ cell (spermatogonia) populations, following reaggregation of these cells to Sertoli cell monolayers (data not shown). No stimulatory effect by (Bu)<sub>2</sub>cAMP by itself was detected on DNA synthesis in isolated cultures of mitotic germ cells (Fig. 3). Since RNA analysis showed that FSH and (Bu)<sub>2</sub>cAMP increase SLF mRNA levels in Sertoli cell cultures, and that the mRNA for the potentially soluble form of SLF is the prevailing transcript under the *in vitro* conditions we use, we hypothesized that the soluble form of SLF could be released by these

cultures after hormonal stimulation. Release of soluble SLF would account for stimulation of DNA synthesis in germ cells reaggregated to (Bu)<sub>2</sub>cAMP-treated Sertoli cell monolayers. In agreement with this hypothesis, we found that soluble recombinant SLF stimulated thymidine incorporation in isolated cultures of mitotic germ cells in a dose-dependent fashion, up to twofold, independently of the presence of serum (Fig. 3). Autoradiographic analysis (Fig. 4) showed that type A spermatogonia are the mitotic germ cell subtype in which DNA synthesis is selectively stimulated, between two- and threefold, by SLF treatment, whereas no stimulation of [ $^3\text{H}$ ]thymidine incorporation is detectable in type B spermatogonia (see Table 1).

#### DISCUSSION

The differentiative process of spermatogenesis is strictly regulated by hormones, such as FSH and andro-

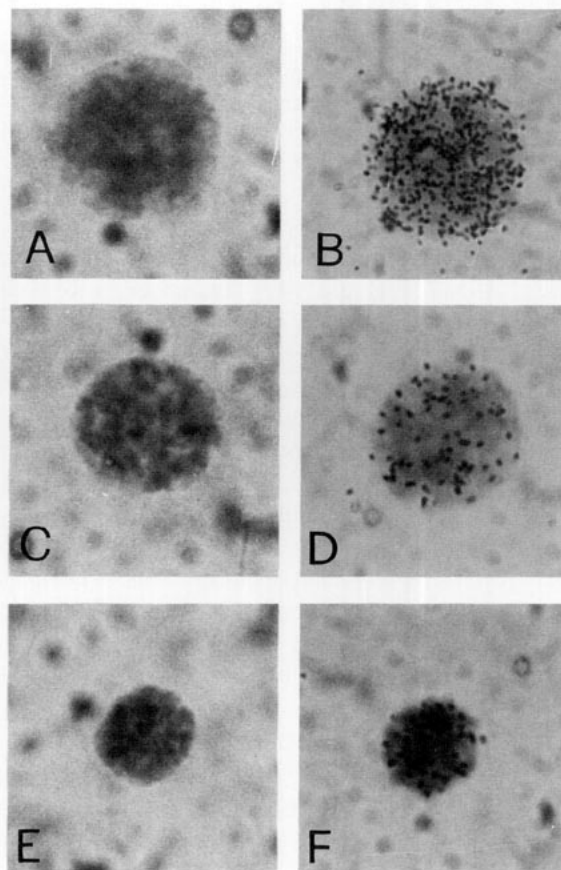


FIG. 4. Autoradiographic images of stained nuclei in mitotic germ cell suspensions from 7- to 8-day-old mice. Representative pictures are shown of unlabeled (A, C, E) and labeled (B, D, F) nuclei from type A spermatogonia (A, B: nucleus with larger size; C, D: nucleus with smaller size) and type B spermatogonia (E, F). Enriched mitotic germ cell suspensions were pulsed for 2 hr with [ $^3\text{H}$ ]thymidine. Autoradiographic analysis and staining were carried out as described under Materials and Methods.  $\times 2200$ .

TABLE 1  
 SOLUBLE FORM OF SLF (100 ng/ml) STIMULATES PROLIFERATION  
 SELECTIVELY IN TYPE A SPERMATOGONIA<sup>a</sup>

	Experiment 1		Experiment 2	
	Control	SLF	Control	SLF
Labeled B spermatogonia	9.3	10.3	8.5	10.0
Labeled A spermatogonia	11.5	21.5	8.0	26.0

<sup>a</sup> The reported values represent the percentages of labeled nuclei observed out of 250 nuclei of the corresponding morphological type.

gens. The hormonal targets in the seminiferous tubules are Sertoli cells (as a review, see Stefanini *et al.*, 1984), which mediate hormone action on germ cells through still unknown mechanisms. These mechanisms could be the release of soluble factors, or membrane-membrane interactions, or the control of the microenvironment in which the differentiative process occurs.

A major breakthrough in the understanding of the mechanisms of Sertoli cell-mediated control of spermatogenesis might be represented by the c-kit/SLF system. Several reports have shown the presence of c-kit transcripts in spermatogonia (Manova *et al.*, 1990; Sorrentino *et al.*, 1991; Motro *et al.*, 1991) and of SLF transcripts in Sertoli cells (Rossi *et al.*, 1991; Motro *et al.*, 1991). We have previously shown that high levels of expression of the proto-oncogene c-kit mRNA are detectable in type A spermatogonia, suggesting a role for this receptor in the control of the onset of spermatogenesis (Sorrentino *et al.*, 1991). We have also shown that SLF mRNA expression is enhanced in primary Sertoli cell cultures from 18-day-old mice by treatment with cAMP analogs (Rossi *et al.*, 1991).

We now report that cAMP induction of SLF mRNA levels is even more evident in pure Sertoli cell cultures from younger mice, since the basal levels of SLF mRNA expression in Sertoli cell cultures from 13-day-old mice are much lower. Our previous suggestion (Rossi *et al.*, 1991) that the Sertoli cell is the main cell type expressing SLF mRNA within seminiferous tubules has been now substantiated by two observations: (1) contamination of Sertoli cell monolayers with other testicular cell types present in seminiferous tubules results in a much lower induction of the SLF signal after stimulation with cAMP analogs; (2) more importantly, FSH, the physiological regulator of Sertoli cell function, stimulates an increase in the mRNA levels for the c-kit ligand in cultured primary mouse Sertoli cells.

RT-PCR analysis yielded amplification products that actually correspond to the SLF cDNA sequence, since they were obtained with different couples of specific SLF primers (Fig. 2). Messenger RNAs for both the po-

tentially soluble form and the transmembrane form of SLF (Flanagan *et al.*, 1991) are stimulated by FSH or cAMP treatment of Sertoli cell monolayers from 13-day-old mice. The ratio between transcripts for the potentially soluble and transmembrane forms of SLF in total testis appears to increase slightly with the age of the animal. The increase in this ratio with the age of the animal is much more evident in cultured Sertoli cells, since, under the culture conditions we use, the mRNA for the potentially soluble form is by far the prevailing transcript in Sertoli cells from 18-day-old mice with respect to what is observed in cultures from 13-day-old animals. This finding might reflect the shift with age in the SLF RNA splicing pattern in Sertoli cells, which would be more evident under the culture conditions we use. It is in fact well known that Sertoli cell function varies according to the stage of the seminiferous tubule (Parvinen, 1982), and it is possible that we obtained a functional "synchronization" in the *in vitro* system.

The transmembrane form of SLF is able to support the survival but not the proliferation of PGCs (Dolci *et al.*, 1991). On the contrary, the soluble form of SLF, which is known to stimulate mast cell proliferation (Anderson *et al.*, 1990), has been reported to slightly stimulate proliferation of PGCs, under particular culture conditions (Matsui *et al.*, 1991). We now show that the soluble form of SLF is able to promote DNA synthesis in isolated mitotic germ cell populations from postnatal testis cultured *in vitro*, in the absence of other cofactors. Autoradiographic analysis indicates that SLF stimulates DNA synthesis selectively in type A spermatogonia. This observation suggests the presence of functional c-kit receptors on this cell type, which we have previously indicated as the main site of expression of c-kit mRNA in the adult testis (Sorrentino *et al.*, 1991). Our data are in agreement with the observation that *in vivo* injection of monoclonal antibodies directed against the c-kit receptor depletes seminiferous tubules of adult mice of proliferating type A spermatogonia (Yoshinaga *et al.*, 1991).

FSH-regulated SLF production in Sertoli cells could play a major role in the regulation of spermatogenesis: our findings that FSH increases mRNA levels for c-kit ligand in Sertoli cells and that at least the soluble form of SLF stimulates DNA synthesis in type A spermatogonia represent, to our knowledge, the first example of a direct correlation between gonadotropin action on the somatic compartment of seminiferous tubules and release of a growth factor acting on the germ cell compartment.

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