

# Regulation of Sertoli Cell Differentiation by the Testicular Paracrine Factor PModS: Potential Role of Immediate-Early Genes

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**Testicular peritubular cells produce a paracrine factor, PModS, under androgen control that modulates Sertoli cell functions that are essential for the process of spermatogenesis. PModS has a more dramatic effect on Sertoli cell differentiated functions *in vitro* than any regulatory agent previously shown to influence the cell, including FSH. Investigation of the actions of PModS on a molecular level have used transferrin expression as a marker of Sertoli cell differentiation. PModS was found to stimulate transferrin gene expression while having no effect on transferrin mRNA stability. The ability of PModS to elevate transferrin mRNA levels was inhibited by cycloheximide. Therefore, the actions of PModS require ongoing protein synthesis and appear to be indirectly mediated through *trans*-acting early event genes. PModS was found to dramatically increase mRNA levels for *c-fos*, but had no effect on *c-jun* mRNA levels. The *c-fos* mRNA levels increased transiently within a few minutes to a maximal level of stimulation at 1 h and returned to basal levels within 6 h. The rise in *c-fos* mRNA preceded the elevation in transferrin mRNA, which started to increase at 2 h to a maximum level between 6–12 h that was maintained at high levels for several days in cell culture. Treatment of Sertoli cells with an antisense *c-fos* oligonucleotide was found to inhibit the actions of PModS on transferrin expression. Combined results support the hypothesis that PModS acts indirectly through transcription factors (e.g. *c-fos*) to induce Sertoli cell differentiated functions (e.g. transferrin expression). Therefore, PModS appears to act as a differentiation-type factor to promote and maintain optimal Sertoli cell function. (Molecular Endocrinology 6: 2018–2026, 1992)**

## INTRODUCTION

The maintenance and control of testicular function and the process of spermatogenesis require cooperative interactions among the various somatic cell types (1). One critical cell type is the Sertoli cell, which helps form the seminiferous tubule and provides both cytoarchitectural and nutritional support for the developing germinal cells. Therefore, regulation of Sertoli cell function and differentiation indirectly effects germinal cell development. Peritubular cells form the outer border of the seminiferous tubules and, in conjunction with Sertoli cells, produce the basement membrane required to maintain normal tubule morphology. The interactions between peritubular cells and Sertoli cells provide an example of a mesenchymal-epithelial cell interaction. Mesenchymal-epithelial cell interactions have previously been shown to modulate the differentiation of the epithelial cell type. The possibility that peritubular cells may regulate Sertoli cell differentiation has been investigated. Peritubular cells under androgen control produce a paracrine factor, termed PModS, that modulates Sertoli cell differentiated functions. PModS has been shown to have a more dramatic effect on Sertoli cell function *in vitro* than any agent previously known to influence the cell, including FSH (2, 3). PModS has been purified and shown to influence a wide variety of Sertoli cell differentiated functions (2). Two forms of PModS have been identified, PModS(A) and PModS(B), in which PModS(A) appears to be a processed form of PModS(B) with identical biological activity. It is anticipated that the combined actions of PModS and FSH will be important in the regulation of Sertoli cell differentiated functions.

Regulatory agents, such as FSH and PModS, bind to specific receptor molecules on the cell membrane and initiate a signal transduction event that alters cellular functions on a molecular level. FSH uses a common signal transduction pathway that involves a specific receptor coupled to adenylate cyclase via a G-protein

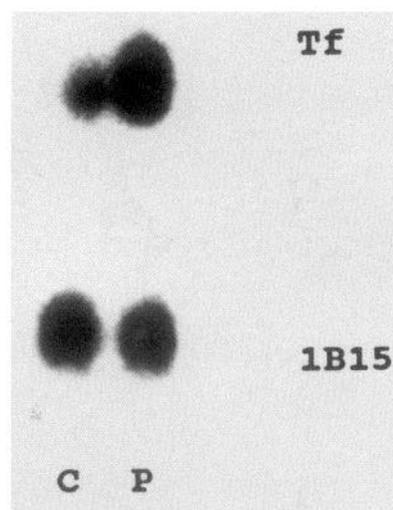
that can elevate cAMP levels (4). An elevation of cAMP levels leads to activation of many genes important for Sertoli cell function, including androgen-binding protein (5) and transferrin (3). Transferrin expression has previously been shown to be a useful marker of Sertoli cell differentiation (1–3) and is used in the current study. FSH can also increase the expression of the immediate-early genes *c-fos* and *jun-B* in Sertoli cells. It has been speculated that immediate-early genes may in part mediate the actions of FSH on Sertoli cell function and differentiation (6). The mechanism of action of P-ModS remains to be elucidated and is investigated in the current study.

Differentiation has been termed an active process that is dependent on the relative concentrations of both positive and negative regulators (7). The induction and maintenance of cellular differentiation, therefore, are dependent on a set of unique transcription factors. FSH can influence Sertoli cell differentiation potentially through increasing *c-fos* and *jun-B* message levels (8). PModS has been shown to have dramatic effects on a variety of Sertoli cell differentiated functions. The current study was designed to examine the actions of PModS on Sertoli cell differentiation and the potential involvement of nuclear transcription factors.

## RESULTS

Investigation of the actions of PModS used transferrin gene expression as a marker of Sertoli cell differentiation. Sertoli cells were cultured for 5 days in the presence of PModS. Total RNA was isolated from these cells and analyzed with a Northern blot procedure. The transferrin cRNA probe used detected a 110-basepair (bp) coding region of the transferrin mRNA that is a single 2.6-kilobase transcript. As detected by increased hybridization of the riboprobe, PModS elevated transferrin mRNA levels in Sertoli cells (Fig. 1). Cyclophilin (1B15) was used as a constitutively expressed gene. There was no detectable difference in 1B15 mRNA levels between control and PModS-treated cells. Expression of the 1-kilobase transcript for 1B15 was not influenced by the regulatory agents used in the current study, so 1B15 expression was used to normalize data. Results indicate that PModS increases transferrin mRNA levels in Sertoli cells, and that the riboprobes used are specific for their respective transcripts. To quantitate mRNA levels, a nuclease protection assay was developed with both the transferrin and 1B15 cRNA probes.

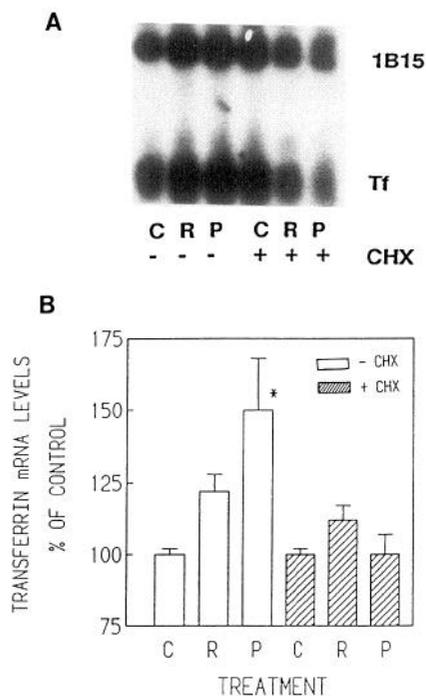
The influence of PModS on Sertoli cell transferrin mRNA levels in the presence or absence of the protein synthesis inhibitor cycloheximide was investigated. In the absence of cycloheximide, the combination of 8-bromo-cAMP, insulin, and retinol (R) increased transferrin mRNA to approximately 125% of control levels. Cyclic AMP was used in these experiments to reduce the variable of protein synthesis required for the FSH



**Fig. 1.** Northern Blot Analysis of Transferrin (Tf) and Cyclophilin (1B15) mRNA Levels in Sertoli Cells

Northern blot analysis of 5  $\mu$ g total RNA from Sertoli cells cultured in the absence (C) or presence of 25 ng/ml PModS A (P) was carried out. Transferrin hybridization is associated with a 2.6-kilobase transcript, and 1B15 hybridization is associated with a 1.0-kilobase transcript. The autoradiograph is representative of three different experiments.

receptor and cyclase activation. A crude preparation of PModS, peritubular cell secreted proteins (PSP), elevated transferrin mRNA levels to 150% of those found in control cells (Fig. 2). In the presence of cycloheximide, PSP did not elevate transferrin mRNA levels in Sertoli cells. Protein synthesis was inhibited more than 97% with the concentration of cycloheximide used (data not shown). Purified PModS was used to confirm the observations obtained with PSP. In the absence of cycloheximide, both PModS(A) and PModS(B) increased transferrin expression to levels similar to that obtained with PSP (Fig. 3). In the presence of cycloheximide, transferrin mRNA levels were not increased by either form of purified PModS. These results suggest that ongoing protein synthesis is required for PModS to elevate transferrin mRNA levels in Sertoli cells. Basal levels of transferrin protein secretion from Sertoli cells of 10-day old animals are lower than those from cells of 20-day old animals. The fold stimulation of transferrin secretion from Sertoli cells of 10-day old animals by PModS is greater than that from cells of older animals due to this lower basal secretion (9). To determine if a similar correlation may be seen with transferrin mRNA levels, the influence of PModS on transferrin mRNA levels in Sertoli cells obtained from 10-day-old animals (early puberty) was examined. PSP stimulated transferrin mRNA to about 135% of control levels (Fig. 4). In contrast, PSP in the presence of cycloheximide did not elevate transferrin mRNA levels. These observations indicate that PModS has similar effects on Sertoli cells from prepubertal and midpubertal rats in the absence or presence of cycloheximide. In addition, these data indicate that the differences observed in protein pro-



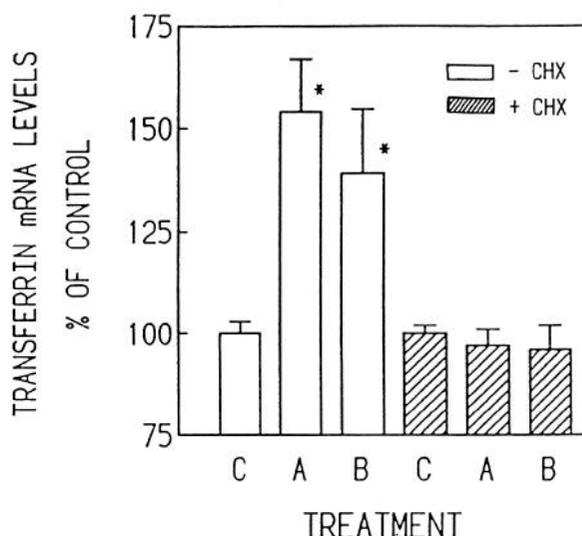
**Fig. 2.** The Effect of Cycloheximide on Transferrin (Tf) mRNA Levels in Sertoli Cells Treated with Hormones and PSP

The nuclease protection assay was carried out on 5  $\mu$ g total RNA from Sertoli cells of 20-day old animals cultured in the absence (C) or presence of 0.1 mM 8-bromo-cAMP, 5  $\mu$ g/ml insulin, 0.35  $\mu$ M retinol (R), and 50  $\mu$ g/ml PSP (P) for 6 h in the presence or absence of 5  $\mu$ g/ml cycloheximide (CHX). An autoradiograph of a representative experiment (*top panel*) and results of quantitation from seven experiments (*bottom panel*) are presented. Data are normalized to 1B15 and are presented as the mean  $\pm$  SEM. \*, Statistically significant difference from the control ( $P < 0.05$ , determined by analysis of variance).

duction do not correlate with mRNA levels, suggesting differences in posttranslational modification or utilization of mRNA in prepubertal vs. midpubertal Sertoli cells.

The influence of PModS on transferrin mRNA stability was examined in cultured Sertoli cells. Transferrin mRNA levels were determined at various times after addition of the transcriptional inhibitor actinomycin-D to the medium. The concentration of actinomycin-D used prevented greater than 92% of transcription, as demonstrated by tritiated uridine incorporation (data not shown). Linear regression was used to determine the half-lives for control and treated cells. The correlation coefficients for control and PModS-treated Sertoli cells are  $-0.95$  and  $-0.96$ , respectively. Transferrin mRNA half-lives were 4.39 and 4.60 h for control and PModS-treated Sertoli cells, respectively (Fig. 5). An analysis of variance procedure demonstrated that the half-lives were not significantly different.

The observations presented in Figs. 1–5 suggest that ongoing protein synthesis is required for PModS to elevate levels of transferrin transcription in Sertoli cells. The potential that nuclear transcription factor(s) may

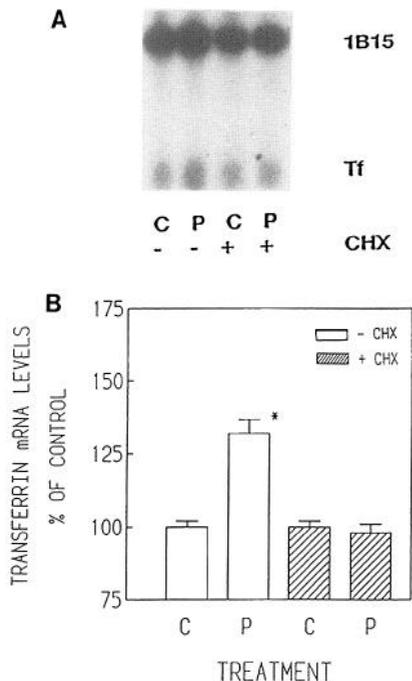


**Fig. 3.** The Effect of Cycloheximide on Transferrin mRNA Levels in Sertoli Cells Treated with PModS

The nuclease protection assay was carried out on 5  $\mu$ g total RNA from Sertoli cells of 20-day-old animals cultured in the absence (C) or presence of 25 ng/ml PModS(A) (A) and 25 ng/ml PModS(B) (B) for 6 h in the presence or absence of 5  $\mu$ g/ml cycloheximide (CHX). Results from four experiments are presented. Data are normalized to 1B15 and are presented as the mean  $\pm$  SEM. \*, Statistically significant difference from the control ( $P < 0.05$ , determined by analysis of variance).

mediate the actions of PModS, therefore, was examined. Sertoli cells were cultured for 48 h in the absence of serum and regulatory agents, then the medium was changed and, after an additional 18 h of incubation, was treated with regulatory agents. After a 1-h treatment, RNA was collected for analysis. Northern blots were hybridized with riboprobes to *c-fos* and *c-jun*. Both PModS(A) and PModS(B) increased *c-fos* mRNA levels in Sertoli cells (Fig. 6). As previously reported, FSH was also found to stimulate *c-fos* mRNA levels (6). Levels of *c-fos* mRNA were low and only slightly above background in control nontreated Sertoli cells. Neither purified PModS nor FSH increased *c-jun* mRNA levels in Sertoli cells (Fig. 6). In addition, PModS and FSH had no effect on the mRNA levels of another nuclear transcription factor, C/EBP (data not shown). C/EBP is associated with differentiation in adipocytes and is expressed in a variety of tissues (10). These observations imply that both PModS and FSH increase mRNA levels for *c-fos* in Sertoli cells, but have no influence on *c-jun* mRNA levels.

The time course for the induction of *c-fos* and transferrin mRNA levels was examined in Sertoli cells treated with PModS. Cells were incubated for different times with FSH and PModS, and the levels of *c-fos* mRNA were determined by a Northern blot procedure. Both FSH and PSP increased *c-fos* mRNA levels in Sertoli cells in a transient fashion, with maximum levels obtained after 1 h (Fig. 7). Quantitation of these data indicated that PModS stimulated *c-fos* mRNA levels

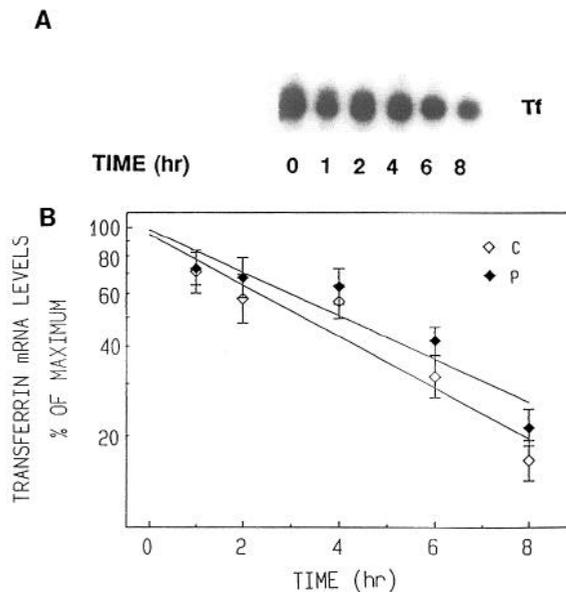


**Fig. 4.** The Effect of Cycloheximide on Transferrin (Tf) mRNA Levels in Sertoli Cells Obtained from 10-Day-Old Animals

The nuclease protection assay was carried out on 10  $\mu$ g total RNA from Sertoli cells of 10-day-old animals cultured in the absence (C) or presence of 50  $\mu$ g/ml PSP (P) for 6 h in the presence or absence of 5  $\mu$ g/ml cycloheximide (CHX). An autoradiograph of a representative experiment (*top panel*) and results of quantitation from four experiments (*bottom panel*) are presented. Data are normalized to 1B15 and are presented as the mean  $\pm$  SEM. \*, Statistically significant difference from the control ( $P < 0.05$ , determined by analysis of variance).

within 15 min to a maximum at 60 min and returned to basal levels between 4–6 h (Fig. 8). Transferrin mRNA levels were also determined in PModS-treated Sertoli cells by a nuclease protection assay. These studies showed an initial increase in transferrin mRNA levels after 2 h of PModS treatment that reached a maximal level of stimulation between 6–12 h (Fig. 8) and was maintained at high levels for up to 5 days (Fig. 1). Results indicate that PModS causes a rise in *c-fos* mRNA levels that precedes the elevation in transferrin mRNA.

Several additional regulatory agents have been shown to influence Sertoli cell differentiated functions (2, 3). The ability of these agents to alter *c-fos* and *c-jun* mRNA levels was evaluated to determine whether this is a unique aspect of PModS's actions. Only FSH and PModS elevated *c-fos* mRNA levels in Sertoli cells after 1 h (Fig. 9). Sertoli cells treated with insulin, retinol, or testosterone were found to have *c-fos* mRNA levels similar to those in control nontreated cells after quantitation by scanning densitometry. No regulatory agent stimulated *c-jun* mRNA levels in cultured Sertoli cells (data not shown). These observations suggest that the actions of FSH and PModS on *c-fos* expression are not



**Fig. 5.** Effect of PSP on Transferrin (Tf) mRNA Stability in Sertoli Cells

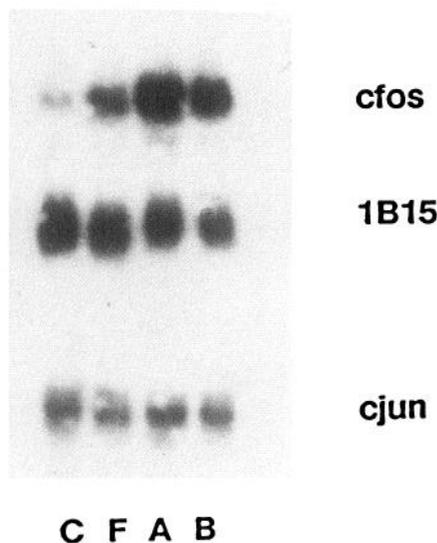
The nuclease protection assay was carried out on 10  $\mu$ g total RNA from Sertoli cells of 20-day-old animals cultured in the absence (C) or presence of 50  $\mu$ g/ml PSP (P) and actinomycin-D. After a 12-h incubation to allow maximum stimulation of transferrin mRNA by PModS, control and treated Sertoli cells were subjected to the transcriptional inhibitor actinomycin-D (5  $\mu$ g/ml). Zero hour of the experiment correlates to the addition of actinomycin-D to the medium. An autoradiograph of a representative experiment (*top panel*) and the results of seven experiments (*bottom panel*) are presented. Data are presented as a percentage of the maximum levels at the zero hour of the experiments (mean  $\pm$  SEM).

a general aspect of all regulatory agents known to influence Sertoli cells.

Sertoli cells were treated with an antisense *c-fos* oligonucleotide to inhibit the translation of *c-fos* and determine the role of *c-fos* in mediating the action of PModS. Sertoli cells were cultured in the absence of serum and regulatory agents for 48 h, then treated for 48 h with PModS, FSH, or FSH, insulin, and retinol in the absence or presence of antisense *c-fos*. Antisense *c-fos* was found to reduce the ability of PModS and FSH to stimulate transferrin production (Fig. 10). Antisense *c-fos* had no effect on transferrin production in control cultures. The antisense *c-fos*, however, did not completely inhibit the actions of PModS with the procedures used (Fig. 10). Control irrelevant oligonucleotides of similar size as the antisense *c-fos* had no effect on the ability of PModS to stimulate transferrin production (data not shown). The results imply that translation of *c-fos* is in part required for PModS and FSH to influence transferrin production by Sertoli cells.

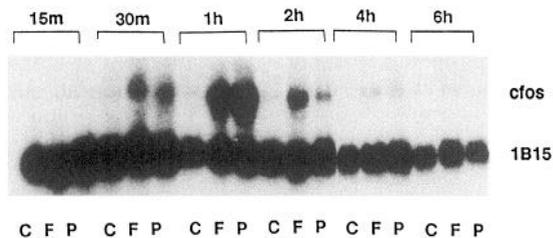
## DISCUSSION

PModS is a testicular paracrine factor postulated to be important in the maintenance and control of Sertoli cell



**Fig. 6.** The Effects of FSH and PModS on *c-fos* and *c-jun* mRNA Levels in Sertoli Cells

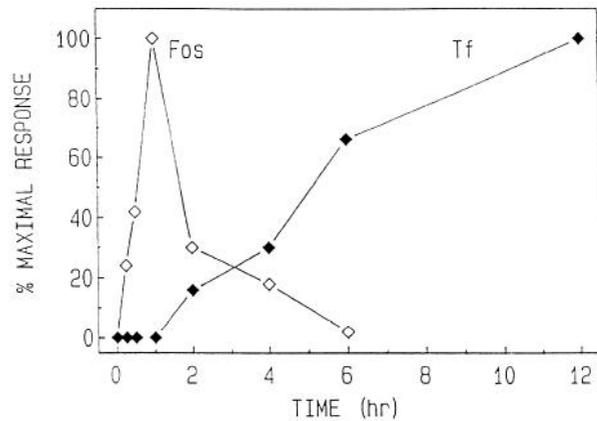
A Northern blot analysis was carried out on 10  $\mu$ g total RNA from Sertoli cells cultured for 60 min in the absence (C) or presence of 100 ng/ml FSH (F), 25 ng/ml PModS(A), and 25 ng/ml PModS(B). The autoradiograph is representative of five experiments.



**Fig. 7.** Time Course of FSH and PSP Stimulation of *c-fos* mRNA Levels in Sertoli Cells

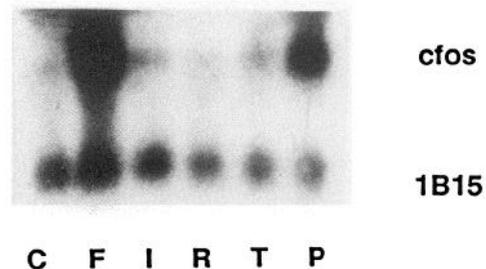
A Northern blot analysis was carried out on 10  $\mu$ g total RNA from Sertoli cells cultured for 15 min through 6 h in the absence (C) or presence of 100 ng/ml FSH (F) and 50  $\mu$ g/ml PSP (P). The autoradiograph is representative of four experiments.

differentiation. Sertoli cell functions influenced by PModS include aromatase activity (11), inhibin production (12), androgen-binding protein secretion, and transferrin secretion (3). Transferrin mRNA levels were used as a marker of Sertoli cell differentiation in the current study. It was postulated that PModS acts as a differentiation factor for Sertoli cells by increasing the expression of specific transcription factors. One way to test this hypothesis is to determine whether the protein synthesis inhibitor cycloheximide blocks the actions of PModS. The current study found that ongoing protein synthesis is required for PModS to elevate transferrin mRNA levels in Sertoli cells. Although several aspects of a signal transduction pathway may require ongoing protein synthesis (13), observations are consistent with the postulate that PModS may influence Sertoli cell



**Fig. 8.** Time Course of PSP-Induced Elevation of *c-fos* and Transferrin (Tf) mRNA Levels in Sertoli Cells

*C-fos* ( $\diamond$ ) and transferrin ( $\blacklozenge$ ) mRNA levels were determined in Sertoli cells cultured from 15 min through 12 h in the presence of 50  $\mu$ g/ml PSP. Data are the mean from four experiments for *c-fos* and six experiments for transferrin.

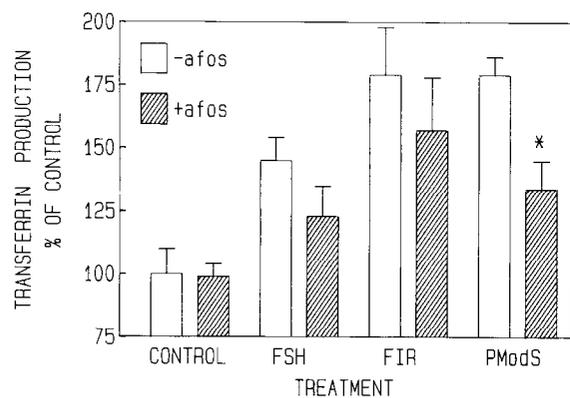


**Fig. 9.** The Effects of Various Regulatory Agents on *c-fos* mRNA Levels in Sertoli Cells

A Northern blot analysis was carried out on 10  $\mu$ g total RNA from Sertoli cells cultured for 60 min in the absence (C) or presence of 100 ng/ml FSH (F), 5  $\mu$ g/ml insulin (I), 0.35  $\mu$ M retinol (R), 1  $\mu$ M testosterone (T), or 50  $\mu$ g/ml PSP (P). The autoradiograph is representative of three experiments.

differentiation through the production of transcription factors.

The actions of PModS on transferrin expression could be at the level of transcription or alterations in mRNA stability. The transcription inhibitor actinomycin-D prevents the production of new transferrin transcripts and was used to determine the stability of transferrin mRNA in Sertoli cells. In both control and PModS-treated Sertoli cells, curvilinear lines were obtained that corresponded to the half-life values for transferrin mRNA levels. Analysis of the combined data with linear regression analysis implies that PModS had no effect on mRNA stability. It is possible, however, that the transcriptional inhibitor did not have optimal influence on cultured Sertoli cells until after 4 h of treatment. Transferrin mRNA half-lives in control and PModS-treated Sertoli cells determined from only the last three time points taken after a 4-h treatment were not statistically different and were 2.7 and 2.8 h, respectively. This corresponds to the half-life for chicken oviduct



**Fig. 10.** The Effect of Antisense *c-fos* Oligomer on the Actions of PModS

Sertoli cells were cultured in the absence (Control) or presence of 50  $\mu\text{g/ml}$  PSP (PModS), 100 ng/ml FSH (FSH), or FSH, 5  $\mu\text{g/ml}$  insulin, and 0.35  $\mu\text{M}$  retinol (FIR) as well as in the absence or presence of an antisense *c-fos* (afos) oligomer (4  $\mu\text{M}$ ) for 48 h, starting on day 2 of culture. The amount of transferrin produced was determined by RIA and normalized for micrograms of Sertoli DNA (nanograms of transferrin per  $\mu\text{g}$  DNA). Data presented are the mean  $\pm$  SEM from three experiments and are presented as a percentage of the control. \*, Statistically significant difference between with afos and without afos within a treatment group ( $P < 0.05$ , determined by analysis of variance).

transferrin (conalbumin) mRNA of 2.9 h (14). Results suggest that PModS does not influence transferrin mRNA levels posttranscriptionally by an increase in message stability. This is in contrast to the transferrin receptor in Sertoli cells. Low iron levels and chronic administration of either testosterone or FSH stabilize transferrin receptor mRNA (15) through protein binding in the 3'-untranslated end of the mRNA (AU-rich sequence) (16, 17). However, Sertoli cell transferrin mRNA levels appear to be elevated by PModS at the level of transcription. Nuclear run-off experiments with isolated nuclei are required to confirm a direct effect of PModS on transcription of the transferrin gene.

The elevation of transferrin mRNA levels in Sertoli cells by PModS was found to require ongoing protein synthesis and may involve the synthesis of transcription factors. The leucine zipper class of transcriptional factors is involved in the growth and differentiation of many cells and include the *c-fos* and *c-jun* immediate-early genes (18). Homodimers of *jun-jun* and heterodimers of *fos-jun* will bind functional genes at an AP-1 DNA response element site and subsequently increase the expression of proximal genes (19, 20). The heterodimer complex has a higher binding affinity than the homodimer (19, 21). This suggests that regulation of only an individual transcription factor may be sufficient to influence the expression of responsive genes. This is supported by the observations that the ratio of *c-fos* to *c-jun* dictates the actual function of a composite glucocorticoid response element (22). Likewise, recent observations suggest that DNA is bent at different angles dependent upon the composition of the transcriptional

complexes that bind to a gene's response element(s). For example, a *fos-jun* heterodimer will induce a DNA flexure at an AP-1 site that is in opposite orientation to that seen from a *jun-jun* homodimer (23, 24). Previous observations have demonstrated that FSH increases *c-fos* mRNA levels in Sertoli cells (6). The current study confirms these observations and demonstrates that PModS elevates *c-fos* mRNA levels in Sertoli cells. In contrast, neither FSH nor PModS appeared to influence *c-jun* mRNA levels. However, the basal level of *c-jun* mRNA may be adequate to allow activity of the transcription factor. Recently, it was shown that an increase in gene expression for *c-jun* is not necessary for transcriptional activation, but that phosphorylation controls the activity of existing protein (25, 26). Future studies should determine whether PModS can influence *c-jun* phosphorylation and if the relative concentrations of *c-fos* and *c-jun* influence the expression of responsive genes.

Both FSH and PModS stimulated *c-fos* mRNA levels in cultured Sertoli cells in a time-dependent manner. The rapid and transient rise in *c-fos* mRNA correlates with previous reports of expression of this immediate-early gene (6, 8, 27). Interestingly, the rise in *c-fos* mRNA preceded the rise in transferrin mRNA levels. This suggests that *c-fos* may be involved in mediating the effects of PModS on various Sertoli cell functions associated with differentiation. An antisense *c-fos* oligonucleotide was found to partially inhibit the actions of PModS. Antisense oligonucleotides to *c-fos* mRNA prevent translation of *c-fos* mRNA into protein. The inability of antisense *c-fos* to completely inhibit the actions of PModS may be associated with the procedures used, which are limited by a relatively rapidly degraded antisense oligonucleotide, and the efficiency to inhibit translation of *c-fos*. In addition, it is likely that other transcription factors are involved that may compensate for a loss in *c-fos*. Observations indicate that *c-fos* is one of the early event genes involved in mediating the actions of PModS.

In a comparison of the actions of maximally effective concentrations of FSH and PModS, FSH increased transferrin secretion to 200% of control levels, whereas PModS stimulated transferrin secretion to 400% of control levels (3). This observation implies that FSH and PModS have different factors involved in their action on Sertoli cell functions. For example, FSH also increases *jun-B* mRNA levels in Sertoli cells (8). *Jun-B* is another transcription factor that will form a heterodimer with *c-fos*. *Jun-B* is suggested to inhibit activity at the AP-1 site when bound as a heterodimer with *c-fos* (28, 29). Whether PModS influences *jun-B* mRNA levels similar to FSH remains to be investigated. PModS and FSH may have differential effects on a wide variety of transcription factors. Several additional regulatory agents known to influence Sertoli cell function include insulin, retinol, and testosterone. These agents did not influence *c-fos* or *c-jun* expression. These observations imply that FSH and PModS may have a more extensive

effect on Sertoli cell functions associated with the differentiation of the cell.

Elevation of *c-fos* mRNA levels by FSH and PModS in Sertoli cells raises important questions regarding the role of immediate-early genes in the promotion of Sertoli cell differentiation. Induction of *c-fos* gene expression by phorbol esters and growth factors mediates the differentiation of various cell lines (30–32). These observations support the possibility that stimulation of *c-fos* mRNA levels by PModS enhances differentiation of Sertoli cells. As a nuclear transcription factor, *c-fos* may subsequently influence the expression of a variety of Sertoli cell genes. It is anticipated that a number of different cell-specific transcription factors will be required to induce and maintain Sertoli cell differentiation. Therefore, the effects of PModS on *c-fos* alone will not likely be the only actions of PModS involved in the induction of Sertoli cell differentiation. The experiments described in the current study support the postulate that PModS acts as a differentiation factor to modulate Sertoli cell functions indirectly through the production of nuclear transcription factors. Further investigations are needed to evaluate a causal role for immediate-early genes in the actions of PModS as well as identification of other transcriptional factors involved in the control of Sertoli cell differentiation.

## MATERIALS AND METHODS

### Cell Preparation and Culture

Sertoli cells were isolated from the testis of 10- and 20-day-old rats by sequential enzymatic digestion (33) with a modified procedure described by Tung *et al.* (34). Decapsulated testis fragments were digested first with trypsin (1.5 mg/ml; Gibco-Bethesda Research Laboratories, Gaithersburg, MD) to remove interstitial cells and then by collagenase digestion (1 mg/ml type I; Sigma, St. Louis, MO) and hyaluronidase digestion (1 mg/ml; Sigma). Sertoli cells were then plated in six-well Falcon plates at about  $2 \times 10^6$  cells/well. Cells were maintained at 32 C in a 5% CO<sub>2</sub> atmosphere in Ham's F-12 medium (Gibco-Bethesda Research Laboratories). Sertoli cell cultures were treated, as described in *Results*, after 48 h of culture, when medium was replenished. Cultures were treated with FSH (100 ng/ml; oFSH-16, National Pituitary Agency), insulin (5 µg/ml), retinol (0.35 µM), and 8-bromo-cAMP (0.1 mM). PSP and PModS were used at greater than maximally effective concentrations. Dose-response curves were determined on each preparation of PSP and PModS. A maximally effective concentration of PSP was 5–10 µg/ml, that of PModS(A) was 5–10 ng/ml, and that of PModS(B) was 7–14 ng/ml. PSP was used at a concentration of 50 µg/ml, and PModS (A) and PModS (B) at a concentration of 25 ng/ml.

Peritubular cells were obtained from the collagenase digestion supernatant after tubule segments had sedimented, as described by Skinner *et al.* (2). Peritubular cells were plated in medium containing 10% calf serum and grown to confluence. Cells were then subcultured and plated at 25% confluence. After 3–4 days of culture, subcultured cells were confluent and were washed with serum-free medium. The cells were then cultured for up to 2 weeks in serum-free medium with 48-h medium collections.

Freshly collected serum-free conditioned medium from peritubular cells was made 25 µM with phenylmethylsulfonylfluoride and 0.1 mM with benzamide and then centrifuged at

1000 × *g* for 15 min at 4 C to remove cell debris. When required, medium was frozen and stored at –20 C. Conditioned medium was concentrated 100-fold by ultrafiltration with an Amicon system (Amicon Corp., Lexington, MA) and a 3000 mol wt exclusion limit membrane.

### PModS Preparation

PModS was purified from concentrated peritubular cell-conditioned medium, as previously described (2). Briefly, an ammonium sulfate precipitate of concentrated conditioned medium was applied to a size-exclusion HPLC apparatus. The active peak, determined by bioassay of Sertoli cell transferrin production, was collected and applied to a 1 × 15-cm heparin-Sepharose affinity column. Eluted proteins were applied to two successive C4 reverse phase columns (Vydac, Hesperia, CA) and eluted with a linear gradient from 25–60% acetonitrile. Active peak fractions obtained from the columns were homogeneous for both PModS(A) and PModS(B). The purity of the PModS preparations was determined with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and a silver stain procedure. Purified PModS was stored at –70 C before use in the presence of 1 mg/ml BSA.

### Molecular Probes

The rat transferrin cRNA probe was obtained from a cDNA fragment that contains a 390-bp coding region (35) (generously provided by Dr. M. D. Griswold, Washington State University, Pullman, WA). The insert was subcloned into the plasmid SP65 in the antisense orientation with regard to the transcription direction of the SP6 promoter. This plasmid was linearized with *Nco*I to generate a 134-bp transferrin cRNA probe. The human *c-fos* cRNA probe was obtained from a full-length cDNA fragment subcloned into the plasmid SP70 under control of the T7 promoter, and the human *c-jun* cRNA probe was obtained from a full-length cDNA fragment subcloned into the plasmid GEM4z under transcription control of the SP6 promoter (36) (generously provided by Dr. J. T. Holt, Vanderbilt University, Nashville, TN). A 700-bp insert of p1B15 (37), a rat cDNA that encodes cyclophilin, was subcloned into plasmid SP65 to produce a cRNA probe. This plasmid was linearized with *Bst*NI to generate a 228-bp probe 1B15 cRNA probe. The p1B15 is a gene that is constitutively expressed and was used for data normalization. The cRNA probes were labeled with [<sup>32</sup>P]UTP, as described by Melton *et al.* (38).

### RNA Isolation and Northern Analysis

Total RNA was extracted from Sertoli cells with a mixture of 5 M guanidine isothiocyanate, 10 mM EDTA, 50 mM Tris at pH 7.5, and 8% (vol/vol) β-mercaptoethanol and passed through a 22-gauge needle. Cellular extracts were either frozen at –20 C or layered over a 5.7-M CsCl<sub>2</sub> cushion for ultracentrifugation at 200,000 × *g* for 3.5 h. Pelleted total RNA was washed with 70% isopropanol-0.3 M sodium acetate and suspended in 0.3 M sodium acetate, followed by the addition of 2.5 vol absolute ethanol and stored at –20 C. Total RNA was separated electrophoretically on 1.0% agarose-formaldehyde gel, transferred to a nylon membrane, and analyzed with a Northern blot procedure, described by Thomas (39). Complementary RNA probes were hybridized at 65 C overnight. Blots were then washed once for 20 min at room temperature in 0.3 M NaCl, 0.03 M sodium citrate, and 0.1% SDS, then washed three times for 30 min each time at 65 C in 0.03 M NaCl, 0.003 M sodium citrate, and 0.1% SDS. Hybridized cRNA probes were detected with autoradiography. Different exposures of the blots were scanned by a densitometer to confirm the range of linearity.

### Nuclease Protection Assay and Antisense Procedure

The levels of transferrin mRNA and 1B15 mRNA were quantitated by a nuclease protection assay, described by Meunier

*et al.* (40). Total RNA was hybridized with cRNA probes overnight at 48°C in a buffer containing 40 mM piperazine-*N,N*-bis[2-ethanesulfonic acid] (pH 6.5), 0.4 M NaCl, and 80% formamide and then digested with S1 nuclease at 42°C for 1 h in a buffer containing 400 mM NaCl, 30 mM Na acetate, and 3 mM ZnCl<sub>2</sub>. Digestion was terminated by the addition of 0.02 vol 0.5 mM EDTA. Samples were precipitated for 2 h at -20°C and separated electrophoretically on a 4% polyacrylamide-8 M urea gel. Appropriate bands were cut from the gel, and radioactivity was determined in a scintillation counter.

A *c-fos* antisense oligonucleotide, 3'TACTACGGTGCA5', was prepared and used as previously described (41, 42). Sertoli cells were treated with antisense oligomer (4 μM) starting on day 2 of culture and retreated every 6–12 h for a total of 48 h before collection of conditioned medium for transferrin analysis with a RIA and normalization of data with Sertoli cell DNA content (2).

### Statistical Analysis

When designated, each data point was converted to percent maximum stimulation or a percentage of the control, and the mean and SEM from multiple experiments were determined as indicated in the figure legends. Data were analyzed using a SPSS statistical package (SPSS, Inc., Chicago, IL), with an analysis of variance and a linear regression procedure for the mRNA stability study.

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