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Transcriptional Regulation of Sertoli Cell Differentiation (Transferrin Promoter Activation) During Testicular Development

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ABSTRACT Previously testicular peritubular cells have been shown to produce a paracrine factor PModS that promotes Sertoli cell differentiation. This mesenchymal–epithelial cell interaction appears to regulate a number of Sertoli cell differentiated functions including transferrin gene expression. The current study was designed to identify PModS-activated response elements in the transferrin promoter and correlate this with Sertoli cell differentiation that occurs during testis development. The 3-kb transferrin promoter was digested down to approximately 200-bp fragments. Nuclear extracts from Sertoli cells stimulated with PModS were used in gel mobility shift assays. Two promoter regions located at −2.4 kb and −1.9 kb were designated SE1 and SE2. PModS promoted the presence of factors in Sertoli cell nuclear extracts that bind SE1 and SE2. Displacement studies demonstrated that SE1 and SE2 are distinct. A transferrin promoter–reporter construct containing these apparent response elements was activated by PModS, while a minimal transferrin promoter of 600bp excluding SE1 and SE2 was only partially stimulated by PModS. Therefore, PModS appears to in part activate the transferrin promoter through SE1 and/or SE2. Gel shift assays with Sertoli cell nuclear extracts and 20-day-old testis extracts were the same. Interestingly, the nuclear extract from a newborn testis also had a gel shift. Therefore, some of the nuclear factors stimulated by PModS in Sertoli cells and present in mid-pubertal testis were also present at birth upon completion of embryonic development. Previously transferrin expression has been shown to increase significantly at the onset of puberty. Observations indicate that PModS appears to in part promote transferrin expression through two newly identified response elements designated SE1 and SE2 and that the nuclear factors that bind these elements are present after embryonic development and mid-pubertally.

Key words: Sertoli cell, transferrin promoter, mesenchymal–epithelial

INTRODUCTION

The Sertoli cell develops in the embryonic gonad from a mesenchymal–epithelial transition which is one of the initial events in testis determination. Coincident with this transition is the migration of mesonephric cells into the testis that appear to develop into peritubular cells (Buehr *et al*, 1993). Therefore, a peritubular cell–Sertoli cell association begins at the onset of Sertoli cell development and continues throughout adult life. As with most tissues, the mesenchymal cells are postulated to have a role in the induction and maintenance of epithelial cell development and function [Cunha *et al.*, 1983]. Although some Sertoli cell functions, such as SRY and müllerian inhibiting substance (MIS), are expressed early in Sertoli cell development, most functions that are present in adult Sertoli cells are initially expressed at the onset of puberty. An example of such an adult Sertoli cell function is transferrin expression that is postulated to have a role in iron transport to developing germinal cells [Skinner and Griswold 1980; Skinner, 1991]. Although the transferrin promoter has been investigated regarding cell specificity between Sertoli cells and hepatocytes [Schaffer *et al.*, 1993], the regulation of the promoter in Sertoli cells has not previously been investigated. Previously, peritubular cells were found to produce a paracrine factor that can modulate Sertoli cell function, termed PModS [Skinner and Fritz, 1985; Skinner *et al.*, 1988]. PModS appears to promote Sertoli cell differentiation by stimulating the expression of genes such as transferrin. The current study was designed to identify potential response elements in the transferrin promoter that are activated by PModS and make correlations with the induction of Sertoli cell differentiation.

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MATERIALS AND METHODS

Cell Preparation and Culture

Sertoli cells were isolated from the testis of 20-day-old rats by sequential enzymatic digestion [Dorrington *et al.*, 1977] with a modified procedure described by Tung *et al.* [1984]. Decapsulated testis fragments were digested first with trypsin (1.5 mg/ml; Gibco–Bethesda Research Laboratories, Gaithersburg, MD) to remove the interstitial cells and then with collagenase (1 mg/ml type I; Sigma, St. Louis, MO) and hyaluronidase (1 mg/ml; Sigma). Sertoli cells were then plated under serum-free conditions in 24-well Falcon plates at 1×10^6 cells/well. Cells were maintained in a 5% CO_2 atmosphere in Ham's F-12 medium (Gibco–Bethesda Research Laboratories) at 32°C. Sertoli cell cultures were treated as described in Results, after 48 hr of culture, following transfection. Cultures were treated with greater than maximally effective concentrations of PModS (S-300) (25 μg/ml) or PModS (C4) (25 ng/ml).

Peritubular cells were obtained from the collagenase digestion supernatant as described by Skinner *et al.* [1988] after the tubule segments had sedimentated. Peritubular cells were plated in Ham's F-12 medium containing 10% calf serum and grown to confluence. Cells were then subcultured and plated at 25% confluence. When subcultured cells were confluent, they were washed with serum-free medium. The cells were subsequently cultured in serum-free medium for up to 4 weeks with 48- to 72-hr medium collections.

Freshly collected serum-free conditioned medium from the peritubular cells was treated with phenylmethylsulfonylfluoride (25 μM final concentration) and benzamidine (0.1 mM final concentration) and centrifuged at 1,000*g* for 15 min at 4°C to remove cell debris. When necessary, medium was stored at −20°C. Conditioned medium was concentrated 100-fold by ultrafiltration with an Amicon system (Amicon Corp., Lexington, MA) and a 3,000-mol wt exclusion limit membrane.

PModS Preparation

PModS was purified from concentrated peritubular cell-conditioned medium, as previously described [Skinner *et al.*, 1988]. An ammonium sulfate precipitate of concentrated conditioned medium was applied to a size-exclusion Saphacryl S-300 column (Pharmacia, South San Francisco, CA). The active peak of the S-300, determined by bioassay of Sertoli cell transferrin production, was collected and applied to a chromatofocusing column (Pharmacia). The pH 5.7–6.8 pool was 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. The purity of the PModS preparations was determined with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and a silver stain procedure. The partially purified PModS S-300 pool termed PModS (S-300) and highly purified PModS C4 fraction termed PModS (C4) were stored at −70°C before use in the presence of 1 mg/ml bovine serum albumin (BSA).

Reporter Gene Constructs and Transfection

Plasmids were equipped with the chloramphenicol acetyl transferase (CAT) reporter gene. CAT plasmids containing the −581-bp portion or the −3 kb portion of the mouse transferrin gene promoter were generously provided by Dr. G. Stanley McKnight (University of Washington, Seattle, WA). CAT plasmids containing the −2.6-kb CAT plasmid was constructed by ligating −2.0 kb *Hind*III fragment to the 600-bp minimal promoter. The −3.0-kb CAT construct was made by ligating a 3.0-kb *Bam*HI digest of the mouse transferrin upstream region to the *Bam*HI site in PGL2 CAT plasmid (Promega). Sertoli cells, cultured for 48 hr, were transfected with a reporter gene construct by the calcium phosphate method coupled with hyperosmotic shock (10% glycerol) as previously described [Kingston *et al.*, 1993]. Cells were treated and cultured for 48 h before harvesting for determination of CAT activity.

CAT Assay

Assay of CAT activity was performed as follows. Medium was removed from the wells and the cells were washed once with PBS; 100 μl of 1× cell lysis buffer (Promega Corp., Madison, WI) was added to each well and incubated for 15 min at room temperature. The wells were then scraped and the buffer collected in 1.5-ml microfuge tubes. Tubes were heated to 65°C for 10 min to inactivate endogenous acetylases and then spun at 12,000*g* for 10 min at 4°C, to remove cell debris. An aliquot of cell extract (54 μl) was mixed with 65 μl 0.25 M Tris (pH 8.0), 25 μg *n*-butyryl Coenzyme A (5 mg/ml; Sigma) and 0.1 μCi [^{14}C]chloramphenicol (0.1 μCi/μl; ICN, Costa Mesa, CA) and incubated overnight at 37°C. The mixture was extracted once with 300 μl xylene and back-extracted with 100 μl 0.25 M Tris (pH 8.0). A 200-μl aliquot of the organic phase was counted in a scintillation counter to determine the relative amount of CAT activity.

Gel Mobility Shift Assay

Gel shift assays were performed with nuclear extracts of isolated Sertoli cells. The Sertoli cells were isolated as described above and cultured in 137-mm petri dishes (Falcon). The cells were treated after 48-hr in culture with PModS (S-300). After 72 hr, the cells were scrapped off the petri dishes and washed once with phosphate-buffered sulfate (PBS). The nuclear extracts of these cells were then prepared as described by Guillou *et al.* [1991]. The probes used in gel retardation assays were the *Hpa*I restriction fragments SE1 (200 bp) and SE2 (180 bp) of the 3-kb mouse transferrin 5′ flanking region and were first dephosphorylated and subsequently end labeled with (^{32}P) γ-ATP.

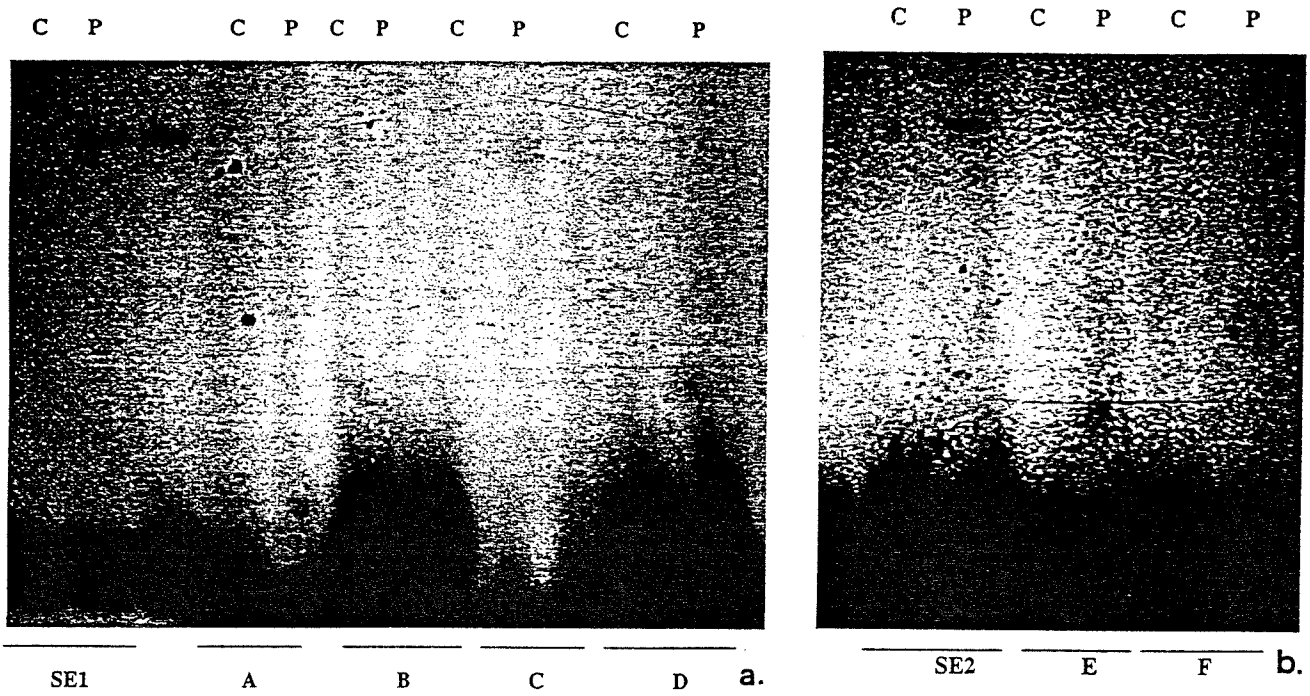

Fig. 1. Gel mobility shift assay with ^{32}P-radiolabeled restriction enzyme digest fragments (~200 bp) (designated SE1, SE2, or lettered A–F) of the 3-kb mouse transferrin promoter. Nuclear extracts from Sertoli cells cultured in the absence (C) or presence of PModS (S-300) (P) were used. The DNA/protein complexes were electrophoretically separated on 5% polyacrylamide gels then dried and autoradiographed.

The gel retardation assay used was a modification of the protocol described by Gardner and Rezvin [1981]. The final reaction volume of 20 μl contained 0.5 ng of 5′ ^{32}P-labeled double-stranded probe, 100 ng sonicated salmon sperm DNA, 2 μg/Poly dI-dC (USB), 20 μg BSA, 20 mM HEPES, pH 8.0, 4 mM Tris, pH 7.9, 50 mM KCl, 600 mM EDTA and EGTA, 500 μM DTT and 5 μg Sertoli cell nuclear proteins. After incubation at room temperature for 20 min, 5 μl of the reaction mixture was electrophoretically separated on a 5% polyacrylamide gel. The gel was dried and autoradiographed. For the competition experiments, 500 times excess of unlabeled oligonucleotide or restriction fragment was added in the binding reaction.

RESULTS

A 3-kb transferrin promoter was digested down to 15 approximately 200-basepair (bp) fragments. The ability of PModS to promote the binding of nuclear proteins to these fragments was examined with nuclear extracts from Sertoli cells stimulated with a PModS (S-300) preparation. The transferrin promoter fragments were radiolabeled, incubated with the Sertoli cell nuclear extracts and then analyzed on a gel mobility shift assay (Fig. 1, and data not shown). Two fragments were identified within the 3-kb transferrin promoter that had the ability to cause a detectable gel shift (Fig. 1). These fragments were designated Sertoli element 1 and 2, SE1 and SE2. The 5′ location in relation to the transcription start site of SE1 is at −2.4 kb and for SE2 at −1.9 kb. No gel shift was observed with SE1 and SE2 control untreated Sertoli cell nuclear extracts. A duplicate lane is shown for SE1 (Fig. 1A). The nucleotide sequence of SE1 or SE2 is currently unknown. None of the other restriction fragments of the 3-kb transferrin promoter, designated A–F and 7 others not shown, had a gel shift with the PModS-stimulated Sertoli cell nuclear extracts (Fig. 1, and data not shown).

To determine whether SE1 and SE2 act as PModS-sensitive response elements to influence transferrin gene expression, a CAT reporter construct was made that contained SE1 and SE2. A 2.6-kb promoter construct was generated that contained both SE1 and SE2. This was compared to a 581-bp reporter construct that has previously been shown to be a minimal transferrin promoter in Sertoli cells and liver [Brunel *et al.*, 1988; Shaeffer *et al.*, 1989, 1993]. PModS (S-300) was found to stimulate the 2.6-kb promoter construct nearly 10-fold and to a lesser degree the 581-bp construct (Fig. 2). This was confirmed with the more highly purified PModS (C4) (data not shown). In contrast, FSH stimulated both the 581-bp and 2.6-kb constructs to the same extent of approximately a threefold increase above control untreated cells (data not shown). Therefore, the 2.6-kb construct containing SE1 and SE2 appears to respond to PModS. Observations suggest that the abil-

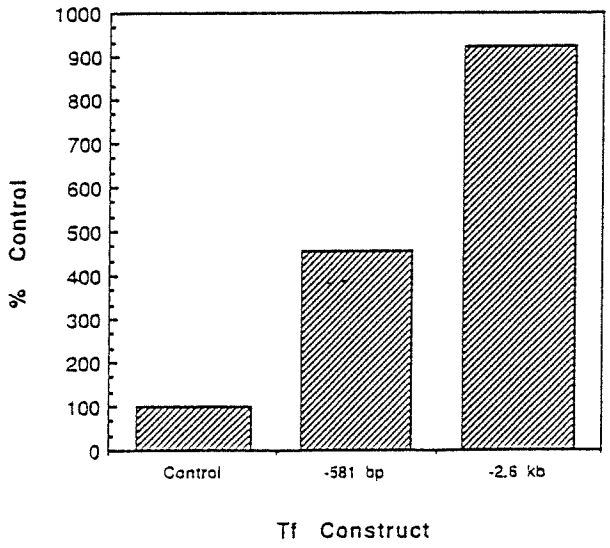

Fig. 2. Regulation of the various transferrin promotor-CAT constructs in Sertoli cells cultured in the presence of PModS (S-300). Data are expressed as percentage of control, nontreated cells, with each construct having a statistically significant difference from control ($P < .01$). Data are representive of a minimum of five different experiments done in replicate.

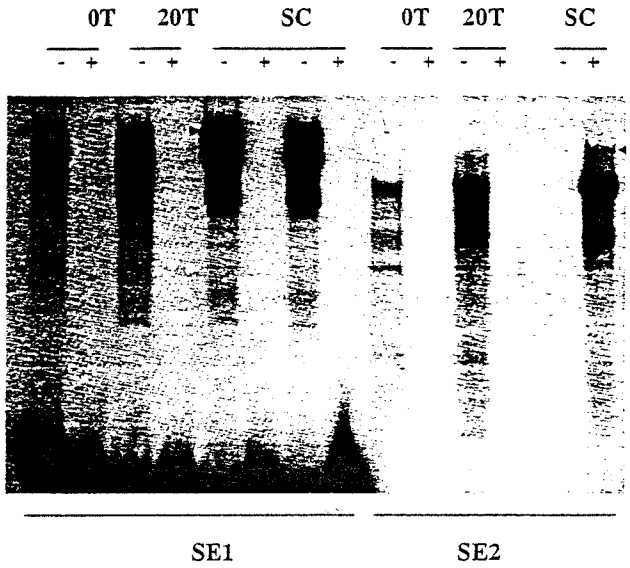

Fig. 3. Gel mobility shift assay with ^{32}P-radiolabeled SE1 and SE2 regions of the transferrin promoter. Nuclear extracts from Sertoli cells cultured in the presence of PModS (S-300) (SC) or 20-day-old testis (20T) or 0-day-old testis (OT) were used. The DNA/protein complexes were electrophoretically separated on 5% polyacrylamide gels then dried and autoradiographed. Incubations with excess unlabeled SE1 or SE2 are indicated (+). Arrowheads denote a unique gel shift not present in the OT. Data are representative of four different experiments.

ity of PModS to influence transferrin gene expression are mediated in part through SE1 and SE2.

To extend these observations, gel mobility shift assays with SE1 and SE2 were performed on Sertoli cell and testis nuclear extracts. Under control conditions, untreated Sertoli cells did not give a gel shift with either SE1 or SE2. PModS did cause a gel shift with both SE1 and SE2, Figure 3. Similar observations were found with either a 4 hr (data not shown) or 72-hr stimulation of Sertoli cells with PModS (S-300). Excess unlabeled SE1 or SE2 displaced binding and the gel shift (Fig. 3). However, SE1 did not displace the SE2 gel shift, nor did SE2 displace the SE1 gel shift (data not shown). Therefore, the two elements appear to be distinct from each other. A nuclear extract from a 20-day-old rat whole testis was found to cause a similar gel shift to that of the purified Sertoli cells treated with PModS. To determine whether the nuclear factors that bind SE1 and SE2 from the pubertal Sertoli cells are present prepubertally, a nuclear extract was prepared from newborn 0-day-old rat whole testis. The nuclear extract from the 0-day-old rat testis also caused a gel shift with SE1 and SE2 (Fig. 3). Several of the band shifts present in Sertoli cells from the 20-day-old testis were not present in the 0-day-old testis. Therefore, some of the nuclear factors promoted by PModS and present in the 20 day-old rat testis are also present prepubertally at the completion of embryonic development.

DISCUSSION

The ability of the testicular paracrine factor PModS to promote Sertoli cell differentiation was used as an initial approach to investigate the transcriptional regulation of Sertoli cell differentiation. Transferrin gene expression has previously been shown to be a useful marker of Sertoli cell differentiation [Skinner, 1991]. Both the mouse and human transferrin promoters have been investigated [Guillou *et al.*, 1991; Schaeffer *et al.*, 1993]. The rat promoter has not yet been cloned or sequenced. Both the mouse and human promoters were found to be regulated differently in Sertoli cells versus hepatocytes. The proximal 600 bp was identified as the minimal promoter and active in Sertoli cells and was iron responsive in hepatocytes [Theisen *et al.*, 1993; Schaffer *et al.*, 1993]. The critical regulatory regions and response elements in this minimal promoter are different between Sertoli cells and liver. In transgenic experiments, the 600-bp promoter directed expression in hepatocytes while expression in Sertoli cells required a 3-kb promoter [Idzerda *et al.*, 1989]. In Sertoli cells, the 600-bp minimal promoter has a higher basal level of activity than that of the 3-kb promoter. Hormonal regulation of the transferrin promoter in Sertoli cells remains to be elucidated.

Since the 3-kb transferrin promoter was required for efficient transgenic expression in Sertoli cells, critical regulatory regions are postulated to be upstream of the 600-bp minimal promoter. PModS induces Sertoli cell differentiation, so potential PModS activated response elements were investigated with restriction digest

fragments of the 3-kb transferrin promoter. The two fragments that had the capacity to bind Sertoli cell nuclear factors in response to PModS stimulation were designated SE1 and SE2. None of the other fragments generated a gel shift. The possibility that a specific response element may have been destroyed by a restriction enzyme digest and thus not cause a gel shift needs to be considered. Therefore, a minimum of two elements appear to be present on the transferrin promoter that can be activated by PModS. A CAT reporter construct containing both SE1 and SE2 was found to be stimulated by PModS. In contrast, the minimal 600-bp transferrin promoter construct was stimulated to a lesser degree by PModS. Therefore, PModS appears in part to activate transferrin gene expression through SE1 and SE2. Whether these two elements synergize or compensate for each other remains to be elucidated.

Gel mobility shift assays demonstrated that PModS induced the presence of Sertoli cell nuclear factors that bind both SE1 and SE2. Displacement experiments with excess unlabeled material demonstrated that SE1 and SE2 appear to be distinct. Further characterization of the nuclear factors that bind these response elements will provide insight into the transcriptional regulation of Sertoli cell differentiation. The same gel shift that was found with PModS stimulated Sertoli cells was also found with 20-day-old rat testis nuclear extracts. Gel shifts with nuclear extracts from 0-day-old newborn testis also promoted a gel shift. However, several of the band shifts observed with the PModS stimulated Sertoli cell and 20-day-old testis were not present with the 0-day-old testis. Therefore, some of the Sertoli cell factors that appear to be involved in the activation of SE1 and SE2 are present prepubertally after embryonic development. Whether the differences observed correlate with the Sertoli cell differentiation that occurs at puberty is currently unknown and can now be investigated. Observations imply that SE1 and SE2 binding proteins are present prepubertally and mid-pubertally. The importance that these elements and binding proteins have in Sertoli cell differentiation remains to be elucidated.

The current study identified two response elements on the transferrin promoter that are activated by the testicular paracrine factor PModS. The ability of PModS to promote Sertoli cell differentiation appears to be induced in part through these elements. When during testis development these elements and binding proteins have a role can now be investigated. Of particular interest is whether SE1 and SE2 may be present in the promoters of other Sertoli cell differentiated markers such as androgen binding protein (ABP). Further investigation of the transcription factors that bind SE1 and SE2, the role these elements have in promoting differentiation and the ontogeny of the activation of these elements will provide insight into the transcriptional regulation of Sertoli cell differentiation during testis development.

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