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Contrasting levels of transferrin gene activity in cultured rat Sertoli cells and intact seminiferous tubules

(testis/secretory proteins/cDNA hybridization/gel electrophoresis)

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ABSTRACT **Two-dimensional polyacrylamide gel electrophoresis of proteins and transfer blot hybridization of RNA have been used to study the activity and expression of the rat transferrin gene in cultured Sertoli cells and in whole testis and isolated seminiferous tubules of sexually immature and mature rats. Although the transferrin gene in cultured Sertoli cells is actively engaged in the transcription of mRNA and the mRNA is translated into a secretory product, little transferrin mRNA and transferrin protein are present in whole testes and isolated seminiferous tubules. Sertoli cells upon culturing show a time-dependent transferrin gene activation, and abundant transferrin mRNA can be detected 6 hr after plating. A similar study using intact seminiferous tubular segments from the same rats failed to show a comparable temporal activation of the transferrin gene. Results of this study, together with previous experimental data, suggest that Sertoli cells *in vivo* are most likely not actively engaged in the synthesis of a testicular transferrin but, instead, rely mainly on plasma transferrin contributed by the liver. *In vitro*, Sertoli cells, released from the physiological constraints that operate *in vivo*, rapidly activate the transferrin gene, resulting in abundant newly synthesized Sertoli cell transferrin product.**

Rat transferrin (Tf) is a major serum glycoprotein synthesized primarily by the liver and consisting of a single polypeptide chain (M_r 76,500; pI 5.85–5.65) (1). Each molecule of Tf has two globular domains, each containing a binding site for one trivalent atom of iron (2, 3). Serum Tf mediates the transport of iron from the absorptive intestinal epithelium, placenta, and storage sites to actively dividing and specialized nondividing cells (4–6). Iron plays a relevant role in crucial biological energetic reactions catalyzed by iron-containing enzymes (7). The growth-stimulating effects of Tf are dependent on the presence of its receptor (8, 9) for delivering iron intracellularly through the receptor-mediated endocytosis pathway. Tf is an obligatory growth factor for cells grown in serum-free medium (10).

It has been proposed that, in rat seminiferous tubules, Sertoli cells are involved in the delivery of Tf-bound iron to developing spermatids (11). Tf receptors have been reported in Sertoli cells and subclasses of meiotic prophase spermatocytes (12). Spermatocytes and spermatids are excluded from direct access to plasma-borne molecules by a physiological barrier (13) whose major components are Sertoli and seminiferous peritubular cells (14). Cultured rat Sertoli cells synthesize and secrete a protein designated "testicular Tf" that is essentially identical to serum Tf (15). Testicular Tf of cultured Sertoli cells and serum Tf are possibly products of the same gene expressed in the testis and the liver (15). However, the testicular synthesis of Tf has not been explored in detail using *in vivo*-like experimental conditions.

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In a study designed for the identification of newly synthesized proteins in fluids collected from the rat seminiferous inter- and intratubular compartments, we noticed that the amount of radiolabeled Tf was very low following *in vitro* intratesticular administration of [^{35}S]methionine. However, Tf was heavily radiolabeled after *in vivo* intratesticular labeling (16). These results suggested that testicular synthesis and secretion of Tf were not as conspicuous as in cultured rat Sertoli cells.

An important question then arises: Are Sertoli cells *in vivo* involved in the synthesis and secretion of Tf as they are in culture? Because of the possible biological role of Tf in spermatogenesis (11, 12, 15), it was of interest to determine whether Tf has a testicular origin in addition to the widely accepted liver source.

In this paper, we have used (*i*) two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to resolve [^{35}S]methionine-labeled proteins accumulated in the medium of cultured rat Sertoli cells and incubated seminiferous tubules and (*ii*) a rat Tf cDNA probe in hybridization experiments with total RNA extracted from liver, whole testis, isolated seminiferous tubules, and cultured Sertoli cells. We demonstrate that although the Tf gene in Sertoli cells is activated gradually upon culturing, Tf gene activity in whole testes and isolated seminiferous tubules is significantly low.

MATERIALS AND METHODS

Isolation and Culture of Rat Sertoli Cells. Sertoli cell cultures were prepared from sexually immature rats (23–25 days old, Charles River Breeding Laboratories) as described (17, 18). Cultures, containing about 30% spermatogenic cells, were plated and maintained in Eagle's minimum essential medium supplemented with hormones and growth factors (18). Sertoli cell cultures free of spermatogenic cells were also used to determine the possible contribution of spermatogenic cells to the experimental results. Holmes *et al.* (12) reported that media from incubated spermatogenic cells or cultured intestitial and peritubular cells did not contain Tf.

Isolation of Seminiferous Tubules. Seminiferous tubules from 25-day-old rats were teased apart in Hanks' balanced salt solution under sterile conditions and examined under a stereomicroscope to verify removal of intertubular tissue. Pools of seminiferous tubular segments (0.5–1 cm in length) were used for [^{35}S]methionine-labeling and for incubation and total RNA extraction as described below.

[^{35}S]Methionine Labeling of Cultured Sertoli Cells and Isolated Seminiferous Tubules. Radiolabeling was carried out in serum-free, hormone/growth factor-supplemented medium containing 1/10 the usual concentration of methionine. [^{35}S]Methionine (1100 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) was added to a final concentration of 250 μCi/ml. Cultured cells and isolated seminiferous tubular segments

Abbreviations: Tf, transferrin; 2D, two-dimensional.

were incubated for 12–16 hr at 32°C in a humidified atmosphere containing 5% CO_2 in air. The radioactive medium was aspirated, centrifuged (15,600 × *g*, 5 min at room temperature), frozen (−20°C), lyophilized, and resuspended in lysis buffer (19) for 2D-PAGE and autoradiography (19–21).

2D-PAGE and Autoradiography. Radiolabeled samples were fractionated by 2D-PAGE as reported (21). Isoelectric focusing gels contained ampholytes (pH 5–7: 1.6%; pH 3–5: 0.4%; Serva Fine Biochemicals, Garden City Park, NY). The second dimension was run in a 5–15% gradient slab gel. Standards of known molecular weight and pI were resolved by one- and 2D-PAGE in the same gels containing radiolabeled protein samples. Following Coomassie blue staining, gels were processed for autoradiography as reported (21).

Preparation of Labeled Rat Tf cDNA. The pBR322 plasmid carrying a 1400-base-pair rat Tf cDNA insert at the *Pst* I site (4D11; a gift from Axel Kahn) was labeled with ^{32}P by nick-translation to a radiospecific activity of about 10^8 cpm/μg (22) in the presence of [α-^{32}P]dATP (600 Ci/mmol, New England Nuclear) as a labeled nucleotide.

Isolation of RNA and Transfer Blot Hybridization. Tissues and cells were either homogenized or spun in a Vortex in 6 M guanidium isothiocyanate and RNA was pelleted by centrifugation through a cushion of 5.7 M CsCl in 0.1 M EDTA for 22 hr in a Beckman 65 rotor at 40,000 rpm as described by Chirgwin *et al.* (23). Concentration of RNA was determined by reading absorbance at 260 nm. RNA samples (4–10 μg) were denatured in formaldehyde and formamide and separated by electrophoresis through a 1% agarose gel in formaldehyde according to the method of Lehrach *et al.* (24). The pBR322 DNA cut with various restriction endonucleases was denatured and electrophoresed on the same gel as size markers. After electrophoresis, RNA was transferred from the gel to a nitrocellulose filter and the filter was hybridized for 16 hr with the labeled rat Tf cDNA plasmid (1–2 × 10^6 cpm/ml) in 50% formamide, 0.75 M NaCl/75 mM sodium citrate, Denhardt's reagent (25), 100 μg of *Escherichia coli* DNA per ml, and 20 mM sodium phosphate (pH 6.8) at 42°C (26). The filter was washed with 0.3 M NaCl/30 mM sodium citrate at room temperature and with 15 mM NaCl/1.5 mM sodium citrate/0.1% sodium dodecyl sulfate at 55°C and exposed to Kodak XAR film at −70°C in the presence of a DuPont intensifying screen.

RESULTS

2D-PAGE Analysis of [^{35}S]Methionine-Labeled Tf in Cultured Sertoli Cells and Isolated Seminiferous Tubules. We have compared the patterns of [^{35}S]methionine-labeled proteins accumulated in the medium of cultured Sertoli cells and isolated seminiferous tubules from 25-day-old rats. Fig. 1 shows that (*i*) Sertoli cell-specific secretory proteins S70, S45, and S35 (21) are present in media of Sertoli cells and seminiferous tubules and (*ii*) Tf is more abundant in the medium of cultured Sertoli cells than that of incubated seminiferous tubules. Similar results were observed after incubation with a ^{14}C-labeled amino acid mixture (27). Together with previous results of *in vivo* and *in vitro* testicular radiolabeling experiments (16), these autoradiographic results suggested that the Tf gene is active and preferentially expressed when Sertoli cells are released from the constraints of the seminiferous tubule.

Transfer Blot Hybridization of Total Cellular RNA Extracted from Liver and Whole Testis of Sexually Mature Rats with ^{32}P-Labeled Rat Tf cDNA. Our next approach was to evaluate the activity of the Tf gene in liver and whole testis of sexually mature rats (70–100 days old). Total cellular RNA was isolated in the presence of 6 M guanidium isothiocyanate, which inhibits nucleolytic degradation of RNA (23), and separated by gel electrophoresis under denaturing conditions. After transfer of RNA from the resolving gel to a nitrocellulose filter, Tf mRNA was identified by hybridization with a ^{32}P-labeled rat Tf cDNA plasmid. Fig. 2 shows the presence of an intense band of 2400 nucleotides (nt) in the lane containing rat liver RNA. The 2400-nt band correlates

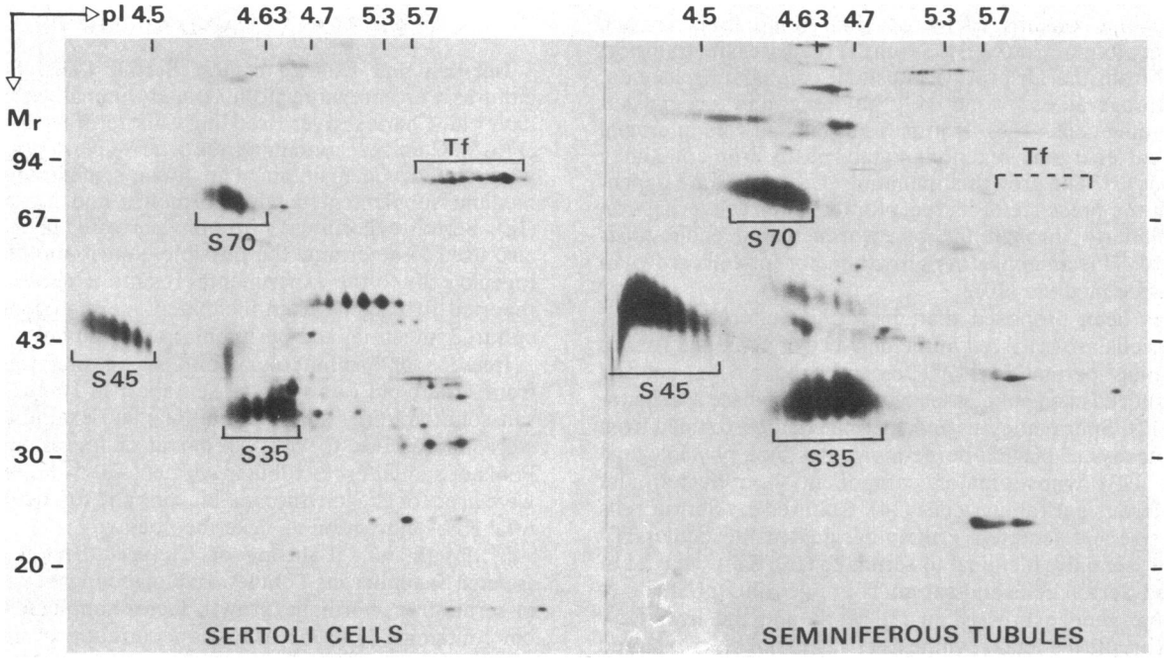

FIG. 1. Autoradiograms of [^{35}S]methionine-labeled proteins accumulated in the incubation medium of cultured Sertoli cells and isolated seminiferous tubules from 25-day-old rats. S70, S45, and S35 are Sertoli cell-specific secretory proteins (21). The location and expected position of Tf [previously determined in electrophoretic transfer blots (16)] are indicated in Sertoli and seminiferous tubular samples, respectively. Numbers at the top are pI; numbers at the left are size standards in kilodaltons. Number of cpm loaded per sample: 300,000 cpm (Sertoli cells) and 200,000 cpm (seminiferous tubules). Exposure times: 5 days (Sertoli cells) and 7 days (seminiferous tubules).

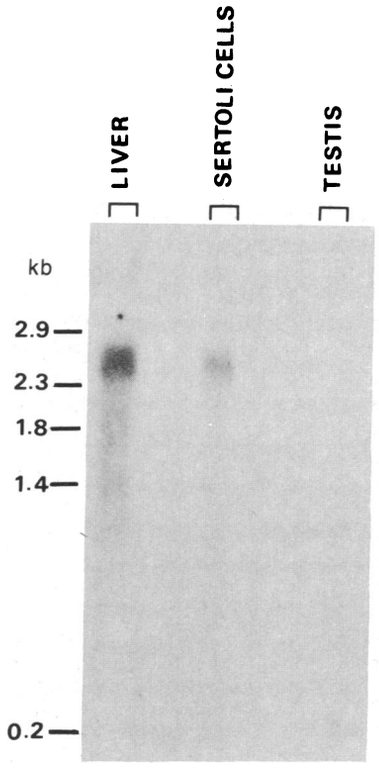

FIG. 2. Transfer blot hybridization of the RNAs from liver, testis, and cultured Sertoli cells. Total RNA was extracted from liver and testis from sexually mature rats and from cultured Sertoli cells (prepared from 25-day-old rat testes). Equal amounts of RNA (6 μg) were denatured in formamide and formaldehyde and fractionated by electrophoresis through a 1% agarose gel in formaldehyde. The RNA was transferred to a nitrocellulose filter and the filter was hybridized with a ^{32}P-labeled rat Tf cDNA plasmid (10^6 cpm/ml). Numbers at the left are size markers in kilobases (Kb). Exposure time: 4.5 hr.

with the reported size of rat (28) and human (29, 30) liver Tf mRNA. A minor band observed at 4000 nt (not clearly visible in Fig. 2) could be attributed to a Tf precursor RNA. Although whole testes from 70- to 100-day-old rats contain some Tf mRNA (observed in overexposed autoradiograms, not shown), cultured Sertoli cells (prepared from 23- to 25-day-old rats) contain an amount of Tf mRNA that was about one-third the amount present in the liver (as determined by densitometric tracing of several different exposures of an autoradiogram). Isolated seminiferous tubules from 70- to 100-day-old rats showed traces of Tf mRNA in overexposed autoradiograms (not shown). These results suggest that the Tf gene is predominantly active in liver and cultured Sertoli cells (in the presence or absence of spermatogenic cells) but significantly less active in whole testis or intact seminiferous tubules. Because of technical limitations, we have not examined whether the Tf gene is active in cultured Sertoli cells prepared from sexually mature rats.

Time-Course Activation of Tf Gene in Cultured Sertoli Cells. It was of interest to determine the time-course appearance of Tf mRNA in cultured Sertoli cells prepared from sexually immature rats (23–25 days old). Fig. 3 shows the progressive activation of the Tf gene. Although at time 0 (post-collagenase sample, 90 min after testicular excision) it is possible to visualize traces of Tf mRNA, the intensity of the Tf mRNA band is remarkably enhanced between 6 and 48 hr after Sertoli cell plating. It is possible that the trace amount of Tf mRNA in the zero time sample is due to transcription of Tf gene during the 90 min of time elapsed during the preparation of Sertoli cell cultures. These results suggest that (*i*) the Tf gene was not significantly active in Sertoli cells at the time that the testis was removed for enzymatic cell dissociation and (*ii*) the Tf gene became activated as Sertoli cells gradually adapted to *in vitro* conditions.

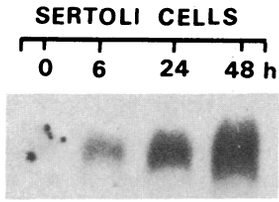

FIG. 3. Activation of the Tf gene in Sertoli cells upon culturing. RNA was extracted from Sertoli cells at various times. Cultures were prepared from 25-day-old rats, and equal amounts of RNA (4 μg) were applied on a 1% agarose gel in formaldehyde for electrophoresis. Blot hybridization with ^{32}P-labeled rat Tf cDNA plasmid was carried out as described in the legend to Fig. 2. Exposure time: 8 hr.

Transfer Blot Hybridization of Total Cellular RNA Extracted from Liver, Whole Testis, and Isolated Seminiferous Tubules of Sexually Immature Rats with Radiolabeled Rat Tf cDNA. Our next approach was to determine whether the preceding observations could be attributed to the sexual maturation stage of the animal. Specimens were collected from sexually immature rats (25 days old) to establish a comparison with results obtained from Sertoli cell cultures (Fig. 3). Fig. 4 shows that although an intense rat Tf mRNA band is visualized in liver samples, the whole testis and isolated seminiferous tubules (incubated from 0 to 24 hr) displayed extremely low gene activation. When compared with the time-course results of cultured Sertoli cells prepared from the same rats (Fig. 3) and the [^{35}S]methionine labeling of secreted Tf in the medium of cultured Sertoli cells prepared from rats of the same age (Fig. 1), it can be concluded that the Tf gene is less active in seminiferous tubules isolated from

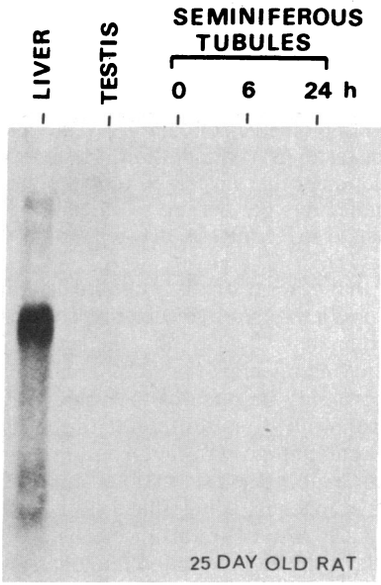

FIG. 4. Tf mRNA in whole testes and seminiferous tubules isolated from sexually immature rats (25 days old). Seminiferous tubular segments from 25-day-old rats were collected (time 0) or incubated for the indicated periods of time in the same medium used for cultured Sertoli cells; RNA was extracted. RNA was also extracted from liver and whole testis from the same rat. Equal amounts of RNA (6.5 μg) were fractionated by electrophoresis. Gel electrophoresis and blot hybridization with radiolabeled Tf DNA were carried out under the conditions described in the legend to Fig. 2. Exposure time: 8 hr.

sexually immature (25 days old) and mature (70–100 days old) rats.

DISCUSSION

We have combined 2D-PAGE and a filter hybridization technique to determine whether Sertoli cells under *in vivo* and *in vitro* conditions synthesize Tf mRNA. In agreement with published data from another laboratory (11), we have found a significant amount of Tf mRNA in cultured rat Sertoli cells, about one-third of the Tf mRNA found in the liver. However, although little Tf mRNA was detected in Sertoli cells at the time of plating, the amount of Tf mRNA increased with time in culture. These results suggest that the Tf gene is activated in Sertoli cells soon after these cells are removed from the seminiferous tubular environment. These findings, together with (*i*) the reduced amount of [^{35}S]methionine-labeled Tf accumulated in the incubation medium of intact seminiferous tubules (as compared to abundant radiolabeled Tf in the medium of cultured Sertoli cells) and (*ii*) results of a previous report showing [^{35}S]methionine-labeled Tf in testicular intertubular and intratubular fluids during *in vivo* radiolabeling experiments, but not *in vitro* (16), support the interpretation that a large amount of Tf found in the testis is of extragonadal origin, presumably from the liver.

It can be argued that the amount of Tf mRNA contributed by Sertoli cells to total RNA extracted from whole testis or isolated seminiferous tubules can be proportionally reduced due to cell heterogeneity. This possibility was considered unlikely because Sertoli cells, at the time of plating, contain very little Tf mRNA, and the amount of Tf mRNA increases with time in culture. Also, in contrast, the synthesis and secretion of Sertoli cell-specific secretory proteins S70, S45, and S35 by incubated seminiferous tubules are still significant (Fig. 1).

It was reported that rat liver contains about 6500 molecules of Tf mRNA per cell, far exceeding that in whole testis (114 molecules per cell; <2% the amount found in liver) (31). We have estimated that cultured Sertoli cells contain about one-third of the amount of Tf mRNA found in liver (representing about 2166 molecules of Tf mRNA per Sertoli cell). This amount of Tf mRNA per cultured Sertoli cell largely exceeds the 114 molecules of Tf mRNA per cell reported for the whole rat testis (31). In addition, the liver of rats raised on an iron-deficient diet increased the amount of Tf mRNA from 6500 molecules per cell up to 15,600 (2.4-fold higher), while no change in Tf mRNA per cell was observed in the whole testis (31).

It is likely that other testicular cells are capable of expressing the Tf gene. *In situ* cytohybridization studies in the human testis using human Tf cDNA show the labeling of a subpopulation of spermatogenic cells (32). This is a finding that merits further study because of the role of Tf as a growth factor for proliferating and differentiating cells (6). Tf is an important circulating growth factor required for cell proliferation during embryogenesis (33). A muscle trophic factor required for myogenesis is identical to Tf (34, 35). The Tf gene, particularly active in fetal muscle, decreases its activity during postnatal development and remains almost undetectable during adult life (28).

Rat seminiferous tubules, deprived of their peritubular cell wall by enzymatic treatment and incubated for 16 hr in a medium containing [^{35}S]methionine, synthesize and secrete a protein tentatively identified as Tf (36). The conditions used for the preparation and incubation of wall-free seminiferous tubules mimic very closely the procedure for establishing primary Sertoli cell cultures. In fact, we have shown that the Tf gene in cultured Sertoli cells is clearly activated 6 hr after plating (Fig. 3).

The low transcription of the Tf gene in the intact seminiferous tubule (Fig. 4) is a striking difference from the gradual increase in Tf mRNA in cultured Sertoli cells (Fig. 3). It is likely that upon culturing, rat Sertoli cells activate the Tf gene (and presumably other genes) by a mechanism that reflects a reprograming of the Sertoli cell genome as these cells adapt to *in vitro* conditions (37). A possible activation or enhancement of selective genes by rat Sertoli cells upon culturing has a precedence: Sertoli cells acquire β-adrenergic responsiveness upon culturing (38).

In vivo, Sertoli cells and peritubular cells are part of a physiological barrier that renders meiotic and postmeiotic spermatogenic cells relatively inaccessible to plasma proteins (13). Whether Sertoli cells *in vivo* find it necessary to produce abundant testicular Tf in addition to plasma Tf for iron delivery to adjacent spermatogenic cells (11) should be regarded as a hypothesis. Results presented in this paper and in a previous report (16) favor an alternate mechanism by which the rat testis *in vivo* may be dependent on plasma Tf for the uptake and delivery of this protein to target testicular cells. The mechanism that regulates the expression of the Tf gene in the testis and the physiological role of Tf during the spermatogenic process are presently unknown.

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