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Stage-Dependent Levels of Specific mRNA Transcripts in Sertoli Cells[1]

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ABSTRACT

Localization and stage-dependent levels of transferrin and sulfated glycoprotein-2 (SGP-2) mRNAs were examined in rat testes by in situ and soluble hybridization of mRNA with a single-stranded RNA probe prepared with the SP65 vector. Biotinylated RNA probes were identified in testicular tissue by using a biotinylated glucose oxidase-avidin system followed by a treatment with an appropriate electron carrier and a tetrazolium salt. This procedure demonstrated that the anatomical site of transferrin and SGP-2 gene expression was the Sertoli cells. Tritium-labeled RNA probes were visualized by radioautography. Negative and positive controls as well as in situ hybridization in Sertoli and myoid cells in culture indicated again that the cytoplasm of Sertoli cells was the anatomical site of transferrin and SGP-2 expression. Quantitative radioautography revealed cyclic variations in the level of both transferrin and SGP-2 mRNAs. The level of transferrin mRNA was relatively high from Stage I to Stage VIII. At Stage IX, the level decreased acutely and remained low in Stage X. The level of transcripts increased dramatically at Stage XIII, remaining high until Stage XIV. In the case of SGP-2 mRNAs, levels of transcripts were similar in most stages except at Stages VII and VIII, where higher levels were observed. These data were substantiated by similar results obtained by solution hybridization of both recombinant cRNAs with mRNAs from selected seminiferous tubules staged by transillumination. Thus, our results demonstrated a stage-specific regulation of transferrin and SGP-2 mRNA levels in Sertoli cells.

INTRODUCTION

Sertoli cells have been shown to be responsible for the biosynthesis and release of several proteins found in the lumen of seminiferous tubules (Koskimies and Kormano, 1973; Hagenas et al., 1975; Wright et al., 1983; Sylvester and Griswold, 1984). These proteins presumably play important roles in the regulation and maintenance of spermatogenesis. One of these proteins, transferrin, was purified from spent Sertoli cell culture medium by hydrophobic and immunoaffinity chromatography, and was found to constitute 7–15% of the total secreted proteins (Skinner and Griswold, 1980; Sylvester and Griswold, 1984).

Testicular transferrin has an iron-binding capacity, a molecular weight, and a polypeptide composition similar to those of rat hepatic transferrin (Skinner and Griswold, 1982; Skinner et al., 1984). The synthesis of transferrin by cultured Sertoli cells was examined by radioimmunoassay and by hybridization of mRNA to a nick-translated cloned transferrin cDNA probe constructed from purified mRNA (Skinner and Griswold, 1982; Huggenvik et al., 1984). Testicular transferrin appears to play an important role in the transport of iron from Sertoli cells to the spermatocytes and spermatids present in the adluminal compartment of the seminiferous epithelium (Huggenvik et al., 1984).

Another major polypeptide secreted by Sertoli cells, sulfated glycoprotein-2 (SGP-2), previously designated dimeric acidic glycoprotein (DAG), constitutes approximately 30–50% of the total mass of secreted proteins (Griswold et al., 1984; Sylvester et al., 1984). SGP-2 was also purified from spent Sertoli cell culture medium by column chromatography and by high-performance liquid chromatography (HPLC; Sylvester et al., 1984; Griswold et al., 1986b). Although immunocytochemical studies revealed that secreted SGP-2 was localized in the lumen of the seminiferous tubules and on the surface of the released spermatozoa (Griswold et al., 1984; Sylvester and Griswold, 1984), the function of this protein is still unknown. Recently, immunopurified

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mRNA was used to generate a pUC13 cDNA library enriched for SGP-2 sequences, and a clone containing SGP-2 cDNA was identified by hybridization-release translation (Collard and Griswold, 1985; Griswold et al., 1986a; Collard and Griswold, unpublished results).

The use of recombinant cDNA probes has made it possible to identify specific mRNAs in various testicular populations (Kleene et al., 1983; Distel et al., 1984; Huggenvik et al., 1984; Ponzetto and Wolgemuth, 1985) and therefore to examine the regulation of gene expression of testis-specific and stage-specific transcripts (Gizang-Ginsberg and Wolgemuth, 1985).

The present investigation deals with the anatomical localization of SGP-2 and transferrin gene expression in testicular tissue and with the determination of the relative level of transcripts of these two proteins throughout the 14 stages of the cycle of the seminiferous epithelium. Thus, the combination of solution and in situ hybridization of mRNA with a single-stranded RNA probe showed a stage-specific transferrin and SGP-2 mRNA distribution in Sertoli cells.

MATERIALS AND METHODS

Preparation of Tissues for In Situ Hybridization

Mature Sprague-Dawley rats (300–400 g), obtained from the Laboratory Animal Resource Center at Washington State University, were anesthetized with sodium pentobarbital, and the testes, liver, and kidneys were fixed by perfusion through the abdominal aorta with 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.5). After perfusion, the tissues were removed and placed in the same fixative for 1 h at 4°C and then dehydrated and embedded in paraffin blocks by standard techniques. Sections (5-μm thick) were collected on polylysine-coated slides and heated for several hours at 40°C prior to hybridization.

Cell Culture

Sertoli cell cultures were prepared according to the method of Dorrington and Fritz (1975), as modified by Wilson and Griswold (1979). This modification consisted in using only 230 μg/ml of collagenase type III (Sigma Chemical Co., St. Louis, MO), and the digestion of the seminiferous tubules in collagenase was limited to 15 min. Ham's F-12 medium (Gibco Laboratories, Grand Island, NY) was buffered with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.45) and was supplemented with 0.215 g/l of glutamine and antibiotics (fungizone, 0.625 μg/ml; penicillin, 50 μg/ml; streptomycin, 50 μg/ml). Cell cultures prepared in this manner consist of at least 90% Sertoli cells (Fritz et al., 1975; Tung et al., 1975).

In some cases, myoid cells were collected from the supernatant obtained from the collagenase digestion of preparations of Sertoli cells (Wilson and Griswold, 1979). The supernatant from the collagenase digestion was centrifuged at 800 × *g* for 3 min and the pellet was resuspended in Hanks' Balanced Salt Solution (HBSS) and plated. Myoid cells were grown in Ham's F-12 medium containing 1% fetal calf serum and supplemented with glutamine and antibiotics (Wilson and Griswold, 1979).

For *in situ* hybridization, cells were seeded onto 8-well glass chamber slides (Lab-Tek, Miles Scientific, Naperville, IL). On Day 4 of culture, the chamber slides were immersed in 4% paraformaldehyde and 0.5% glutaraldehyde in phosphate-buffered saline (PBS, pH 7.5) for 1 h at 4°C. Cultured cells were then dehydrated in 95% ethanol, air-dried, and stored at −20°C until further use.

Isolation of Nucleic Acids from Seminiferous Tubules

Seminiferous tubules were staged by transillumination and dissected into defined groups of stages (Parvinen and Vanha-Perttula, 1972). The seminiferous tubules were grouped in the following manner: Group 1, Stages II–VI; Group 2, Stages VII–VIII; Group 3, Stages IX–XII; Group 4, Stages XIII–I. The length of the different segments of seminiferous tubules was measured by using a 1-mm mesh placed beneath the Petri dish (Parvinen and Ruokonen, 1982). The groups of staged seminiferous tubules were homogenized in buffer (150 mM NaCl, 10 mM Tris(hydroxymethylaminomethane [Tris, pH 7.5], 0.75% sodiumdodecyl sulfate [SDS], 5 mM ethylenediaminetetraacetate [EDTA] plus 1×10^4 cpm ^{3}H-lambda cRNA for 1 min, using a biohomogenizer (Biospec Products, Bartlesville, OK). ^{3}H-Labeled lambda cRNA was synthetized from 1 μg of control template (Promega Biotec, Madison, WI). Synthesis was carried out in transcription buffer: 40 mM Tris (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 0.5 mM each adenosine 5′-triphosphate (ATP) and

cytidine 5′-triphosphate (CTP), 0.1 mM uridine 5′-triphosphate (UTP), 8 μC ^{3}H-UTP (32.2 Ci/mmol), 10 mM dithiotreitol, with the addition of 50 units RNasin (Promega Biotec) and 10 units SP6 RNA polymerase (Promega Biotec). The reaction was carried out at 37°C for 1 h and cRNA was then extracted from the unincorporated nucleotides by successive precipitations at −20°C in the presence of ammonium acetate and ethanol. The homogenate was treated with proteinase K (Sigma), 50 μg/ml for 45 min at 45°C and extracted with phenol:chloroform (1:1). The nucleic acid in the aqueous phase was precipitated with 2.2 volumes of ethanol at −20°C, and the pellet was resuspended in H_2O. An equal volume of 4 M LiCl was added to the nucleic acids and precipitated at 0°C. RNA concentration was determined from the absorbance at 260 nm wavelength. An aliquot of RNA was removed and used to determine the percentage of ^{3}H-lambda cRNA recovered. From this, the original amount of RNA in each sample and the ng RNA per mm tubule was calculated.

Recombinant DNA Probes

The initial isolation of the mRNA and the confirmation of the cDNA probes were possible because of specific antibodies to purified rat transferrin and SGP-2 (Skinner and Griswold, 1980; Sylvester et al., 1984).

Purified mRNA for transferrin was obtained from polysomes immunoprecipitated from rat liver homogenates (Huggenvik et al., 1984). The cDNA was prepared from this enriched mRNA and cloned initially into the PstI site of pBR322. The identity of the inserted cDNA was established by differential screening, hybridization release-translation, and nucleotide sequencing. The inserted transferrin cDNA consisted of a 1-kilobase (kb) sequence, which coded for the carboxy terminal one-half of the protein (Huggenvik et al., 1986). A 688 base-pairs fragment was generated by digestion of the cDNA with the restriction enzymes PstI and HincII, and was subcloned into the HincII and PstI sites of the SP65 and SP64 plasmids. In the SP64 and SP65 plasmids, the subcloned fragments are in opposite orientation with respect to the SP6 promoter. Thus, while transcription originating from the promoter of the SP65 plasmid produces a cRNA strand complimentary to transferrin and SGP-2 mRNAs, transcription originating from the SP6 promoter of the SP64 plasmid produces a cRNA strand homologous to transferrin and SGP-2 mRNAs. These transcripts were used to construct a standard curve for liquid hybridization (Idzerda et al., 1986).

Purified SGP-2 mRNA was obtained from testis homogenates by immunoprecipitation of polysomes with the SGP-2 immunoglobulin G (IgG). The cDNA was prepared from this enriched mRNA and cloned into the PstI site of pUC-13. The identity of the inserted cDNA was established by differential screening, hybridization release-translation, and nucleotide sequencing (Collard and Griswold, 1985; Griswold et al., 1986a; Collard and Griswold, unpublished results). The original, cloned SGP-2 cDNA represented a full-length cDNA that contained the coding region for a precursor to SGP-2, which is processed to form the disulfide-linked subunits of the mature protein. SGP-2 was purified by HPLC, as described by Griswold et al. (1986b), and N-termini of the subunits were partially sequenced by gas-phase protein sequencing. The partial amino acid sequence of the subunits (24 and 18 amino acids from each N-terminal) agreed with the sequence predicted from the mRNA (Collard and Griswold, unpublished results). The cloned SGP-2 cDNA was digested with the restriction enzymes XbaI and PvuII to generate an 829-bp fragment, which was subcloned into the XbaI and SmaI sites of the SP65 and SP64 plasmids.

Northern Blot Analysis

Poly(A)+RNA (1 μg) isolated from cultured Sertoli cells from 20-day-old rats was incubated in 6% formaldehyde, 20 mM HEPES (pH 7.8), 1 mM EDTA, and 50% formamide for 5 min at 68°C. Samples were quick-cooled and electrophoresed in a 1.2% agarose gel for 15 h at 20 volts. The RNA was transferred to nitrocellulose and hybridized for 20 h at 45°C using a ^{35}S-labeled in vitro-transcribed cRNA probe (5×10^7) cpm/μg of SP6 transferrin and SP6 SGP-2 cRNA). RNA probes complimentary to their specific mRNAs were synthesized from 1 μg of linearized plasmid in transcription buffer: 40 mM Tris (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 0.5 mM each ATP, CTP, and guanosine 5′-triphosphate (GTP), 0.1 mM UTP, 25 μCi ^{35}S UTP (1145 Ci/mol), 0.2 M dithiotreitol, with the addition of 50 units RNasin and 10 units SP6 RNA polymerase per reaction.

Solution Hybridization

RNA (2-5 μg) from staged seminiferous tubules

was hybridized to 2×10^4 cpm of the appropriate ^{35}S-labeled RNA transcript in 20 mM Tris (pH 7.5), 0.6 M NaCl, 5 mM EDTA, 0.2% SDS in 20 μl during 16 h at 75°C (Idzerda et al., 1986). After hybridization, the samples were diluted with 1 ml of 0.3 M NaCl, 10 mM Tris (pH 7.5), 5 mM EDTA, and treated with 200 units/ml of RNase T1 and 25 μg/ml of RNase A during 30 min at 37°C to digest the unhybridized RNA. The hybrid RNA was precipitated with the addition of 100 μl of 100% w/v trichloroacetic acid and 10 μg/ml yeast tRNA. The precipitated RNA was collected on glass fiber filters and the radioactivity determined in a liquid scintillation counter. The specific mRNA in each sample was estimated by comparison to a standard curve generated by hybridization of the ^{35}S-labeled RNA probe to the respective complimentary cRNA strand. The cRNA strands complimentary to the ^{35}S-labeled cRNA probes were transcribed from SP64 plasmids containing the transferrin and SGP-2 cDNAs. To determine the copies of transferrin and SGP-2 mRNAs per Sertoli cell, the number of copies of transferrin and SGP-2 probes per mm of seminiferous tubules was first determined. Since the number of Sertoli cells per unit length of seminiferous tubule was constant (Wing and Christensen, 1982), and Sertoli cells were the only testicular cells to express transferrin and SGP-2 mRNA, the copies of transferrin and SGP-2 could then be calculated.

In Situ Hybridization

Both the deparaffinized sections and the cells cultured on chamber slides were pretreated with 0.2 N HCl for 20 min at room temperature, followed by 2 μg/ml of proteinase K in 20 mM Tris-HCl and 2 mM $CaCl_2$ (pH 7.5) for 20 min at 37°C. The sections were then dehydrated in a graded series of alcohols and air-dried.

Single-stranded RNA probes were prepared as described above, except that biotinylated UTP (1 mM Bio-11-UTP) or 8 μCi of ^{3}H-UTP (32.2 Ci/mmol) was used to label the cRNA. The biotinylated UTP had an 11-carbon spacer arm between the biotin and the pyrimidine ring. *In situ* hybridization was performed according to a modification of the procedure of Guelin et al. (1985). Briefly, the modification consisted in the pretreatment of the sections with a prehybridization mixture containing yeast tRNA (Sigma) and denatured salmon sperm DNA (Sigma). The cRNA concentration was approximately 0.35 ng/μl) for SGP-2 and 0.75 ng/μl for transferrin; the sp. act. of the ^{3}H-labeled RNA probes were 2.7×10^6 cpm/μg and 6×10^6 cpm/μg, respectively. All hybridization and wash buffers contained 4X STE (STE: 150 mM NaCl; 2.5 mM Tris-HCl; 0.25 mM EDTA; pH 7.4) and 50% formamide. After the final wash, the tissue sections and cell preparations were dehydrated in alcohol and air-dried. For specificity controls, some tissue sections and Sertoli cell preparations were digested with ribonuclease A or incubated with the same probes at 4°C.

Visualization of the Hybridized mRNA

Tissue sections and cell preparations were coated with Kodak NTB-2 nuclear emulsion, and the ^{3}H-cRNA:mRNA hybrids were visualized by radioautography (Kopriwa and Leblond, 1962). After 2 days' or 7 days' exposure, the slides were developed with a Kodak D-170 developer and post-stained in hematoxylin and eosin. For the identification of the biotinylated probes, a biotinylated glucose oxidase-avidin system (Vectastain ABC-GO; Vector Labs., Burlingame, CA) was used, followed by a treatment with phenazine methosulfate as an intermediate electron carrier and tetranitroblue tetrazolium, which upon reduction formed a black-colored, insoluble formazan in the site of the reaction (Campbell and Bhatnagar, 1976).

Quantitative Analysis of In Situ Hybridization

One paraffin block of the testicular tissue was obtained from each of the four animals. Radioautographs of sections from these blocks were examined under the light microscope to select and stage seminiferous tubules (Leblond and Clermont, 1952). The total number of silver grains per cross section of seminiferous tubules was counted in those tubules that were cut as transverse sections.

Statistical Analysis

Silver grain counts from light microscopic radioautographs were submitted to the following statistical analysis: 1) least square analysis of variance, to determine if counts of silver grains varied significantly among the different testes hybridized *in situ* with each of the probes used in this investigation, and also to determine if there were statistically significant variations between the 14 stages of the cycle; 2) least

significant difference test, to analyze variations from the mean and to determine whether such variations were statistically significant. Grain counts and information about each of the different experimental procedures were stored in computer files of the Statistical Services of Washington State University, Pullman, WA, and with a statistical package, SAS/GLM (SAS Institute Inc., SAS User's Guide).

RESULTS

It has previously been shown that nick-translated cDNA from the transferrin sequence cDNA in pBR-322 plasmid or from the SGP-2 sequences in pUC-13 plasmids recognized a single band of mRNA in Northern blots of Sertoli cell mRNA. The transferrin cDNA recognized RNA of 2.7—3.0 kb (depending on the standards used) and the SGP-2 RNA recognized a smaller (2.1—2.4 kb) mRNA (Griswold et al., 1986). When Northern blots of Sertoli cell RNA were probed with the cRNA products obtained after subcloning of transferrin and SGP-2 cDNA into SP6 plasmids, the cRNA from these recombinant probes each hybridized with a single mRNA species corresponding to transferrin and SGP-2 mRNAs (Fig. 1).

Thus cRNA from the recombinant SP6 plasmids was labeled with biotinylated UTP, hybridized *in situ* and visualized with the avidin-glucose oxidase reaction. Both the biotinylated transferrin cRNA and biotinylated SGP-2 cRNA hybridized to sequences localized exclusively in Sertoli cells in the testes (Figs. 2a, 2c). Treatment of testis sections with RNase A or *in situ* hybridization at 4°C prevented any hybridization and subsequent color development (Figs. 2b, 2d).

Liver is the major source of serum transferrin and was used as a positive control for the transferrin *in situ* hybridization experiments. The biotinylated cRNA hybridized to hepatocytes but not to hepatic connective tissue-derived cells (Fig. 3c). The SGP-2 probe did not hybridize to any of the different types of liver cells (Fig. 3d).

The biotinylated cRNA probes allow for the precise anatomical localization of specific mRNAs, and they provide a high level of spatial resolution throughout the tissue section. However, analysis of the mRNA levels required a more sensitive and quantifiable technique, such as that provided by ^{3}H-cRNA and autoradiography.

The ^{3}H-cRNA probes were detected after *in situ* hybridization by light microscope radioautography. On testicular tissues, both transferrin and SGP-2 ^{3}H-RNA probes gave rise to a considerable number of silver grains (Figs. 4a, 4c), but the reaction was negligible or absent on kidney tissues (Figs. 3d, 3e). As expected, the silver grain distribution over the testicular tissues presented a scattered appearance. However, the high proportion of silver grains appeared to overlay those areas where the cytoplasm of Sertoli cells was present (Figs. 4a, 4c). Similarly to the biotinylated probes, the ^{3}H-labeled transferrin cRNA gave rise to silver grains on hepatic cells, whereas the ^{3}H-labeled SGP-2 probe did not show hybridization (Figs. 3a, 3b). Prior treatment of tissue sections with RNase or *in situ* hybridization at 0°C eliminated radioautographic reaction in either tissue (Figs. 4b, 4d). The number of grains in experiments using the SGP-2 probe cannot be directly compared to those using the transferrin probes, since different sp. act. probes, different number of copies per cell, and different lengths of time for radioautography

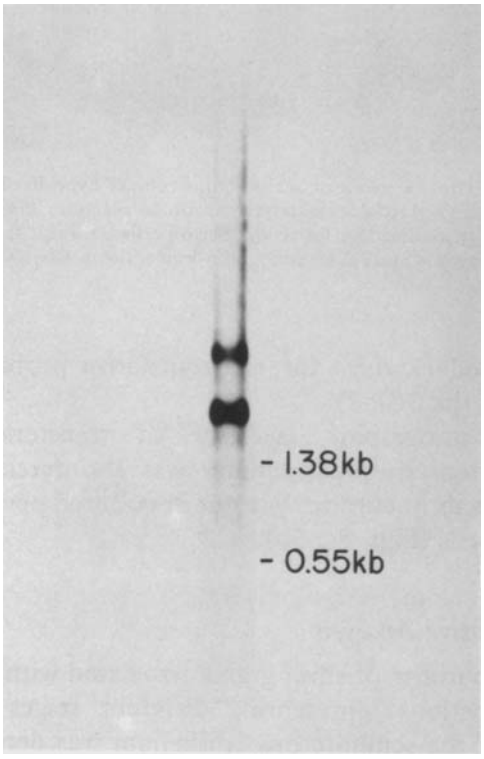

FIG. 1. Northern blot analysis of Sertoli cell mRNA probed with ^{35}S-cRNA transcribed from the SP6 plasmid containing transferrin or SGP-2 cDNA. The mRNA was isolated from cultured Sertoli cells from 20-day-old rats. Approximately 1 μg of mRNA was subjected to agarose gel electrophoresis, transferred to nitrocellulose, and probed with the transferrin or SGP-2 cRNA independently. The *upper band* is the mRNA recognized by the transferrin cRNA, and the *lower band* is detected by the SGP-2 cRNA.

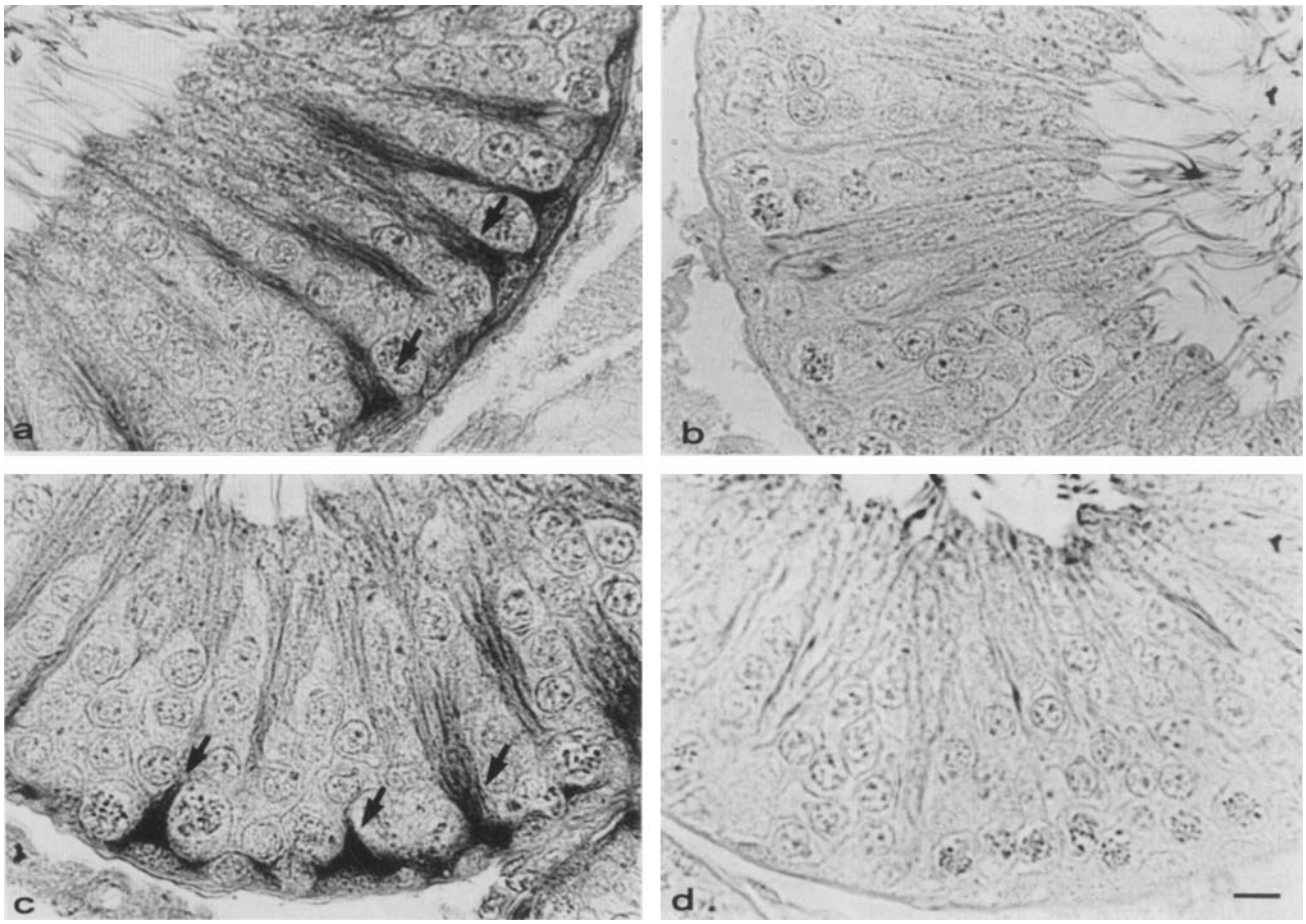

FIG. 2. Cross sections of seminiferous tubules hybridized *in situ* with biotinylated cRNA probes transcribed from SP6 plasmids, and visualized with the avidin-glucose oxidase/tetranitroblue reaction. *Black deposit* of insoluble formazan indicates that both transferrin cRNA (*a*) and SGP-2 cRNA (*c*) are localized exclusively in Sertoli cells (*arrows*). Treatment of testes sections with RNase A (*b*) or *in situ* hybridization at 4°C (*d*) prevents any hybridization and subsequent color development. *Bar*, 10 μm.

were used (7 days for the transferrin probe and 2 days for the SGP-2).

Radioautographic evidence of transferrin and SGP-2 transcriptional activity was also detectable in Sertoli cells in culture, but not in cultured peritubular myoid cells (Figs. 5a, 5b).

Quantitative Analysis

The number of silver grains associated with tubular cross sections representing different stages of the cycle of the seminiferous epithelium was determined for both the transferrin and SGP-2 probes (Figs. 6, 7). In the case of the transferrin probe, the statistical analysis showed that there were differences in the number of silver grains per cross section of seminiferous tubule between the 14 stages of the cycle (least square analysis of variance, $p \leqslant 0.0001$). However, no statistically significant differences were found between the 4 rats investigated. Grain counts showed that the level of transferrin mRNA per cross section of seminiferous tubules increased from Stage X to reach the highest level during Stage XIII (434 ± 45.40 SD). The level of transcripts decreased moderately during Stages XIV and I, and remained relatively high until Stage VIII. The lowest level of transferrin mRNA was registered at Stage IX (202 ± 28.77 SD). Statistical analysis verified that the low value of Stage IX was different from the other 13 stages of the cycle (least significant difference, $p \leqslant 0.05$). Similarly, the highest value registered at Stage XIII was different from 12 of the stages of the cycle (least significant difference, $p < 0.05$), but not from Stage XIV (Fig. 6).

When the SGP-2 ^{3}H-cRNA probe was used for hybridization, statistically significant differences were

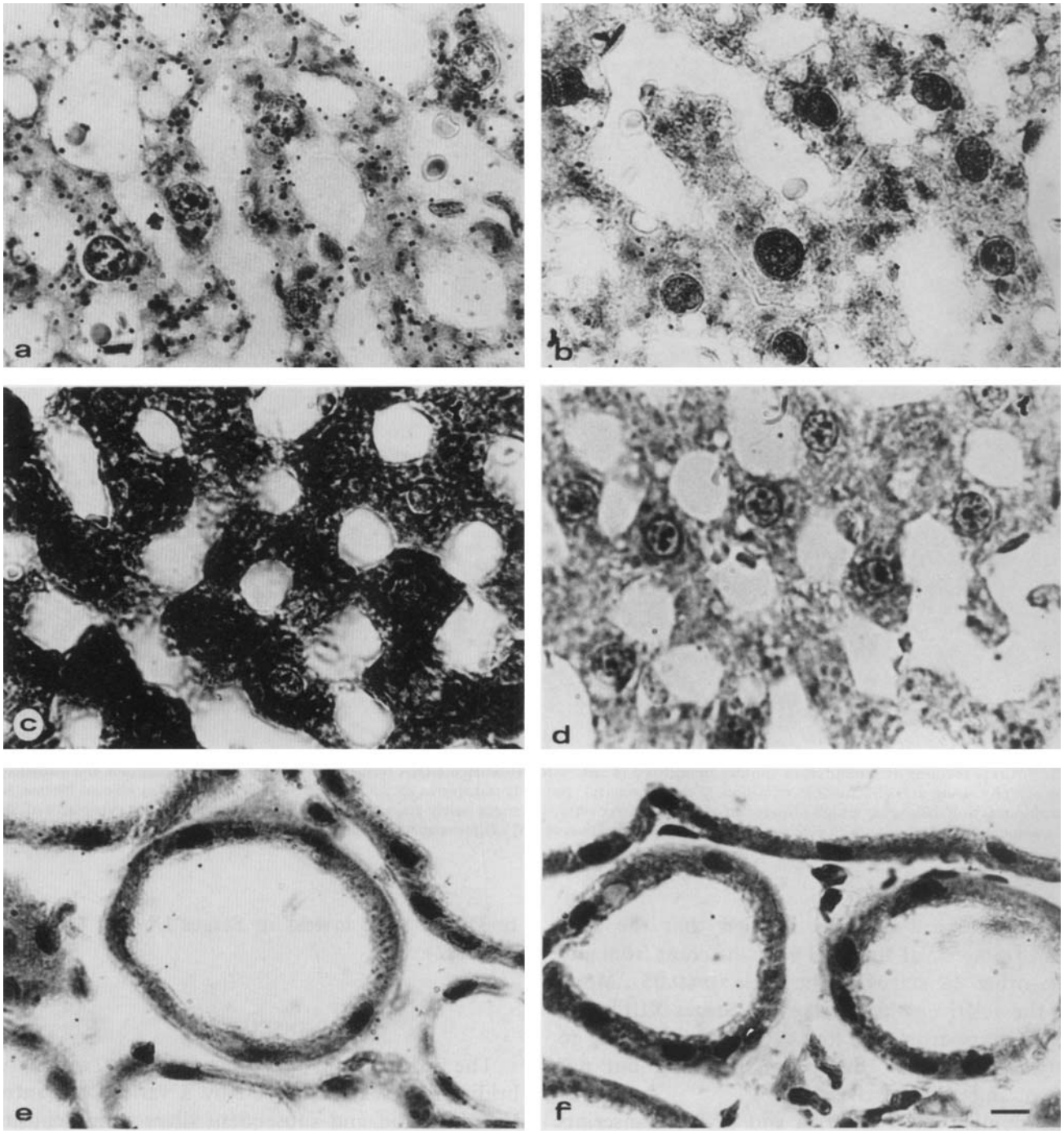

FIG. 3. Liver and kidney sections hybridized *in situ* with biotin and ^{3}H-labeled cRNA probes. Both ^{3}H-labeled (*a*) and biotinylated (*c*) probes complimentary to transferrin mRNA produce a positive reaction in the cytoplasm of hepatocytes. On the other hand, ^{3}H-labeled (*b*) and biotinylated (*d*) probes complimentary to SGP-2 mRNA do not react with the liver cells. Note the absence of radioautographic reaction in kidney sections hybridized *in situ* with either ^{3}H-transferrin (*e*) or ^{3}H-SGP cRNA probes (*f*). *Bar*, 10 μm.

found in the number of silver grains between the 14 stages of the cycle (least square analysis of variance, $p \leqslant 0.0179$). For SGP-2 mRNA there were also no statistically significant differences between the 4 rat testes. Two distinct levels of SGP-2 transcripts per cross section of seminiferous tubules were detected (Fig. 7). The highest level was from Stage XIII–XIV to Stage VIII and the lowest level was from Stage IX to stage XII. Stage VII (257 ± 18.80 SD) and Stage VIII (292 ± 23.40 SD) had the highest levels of SGP-2

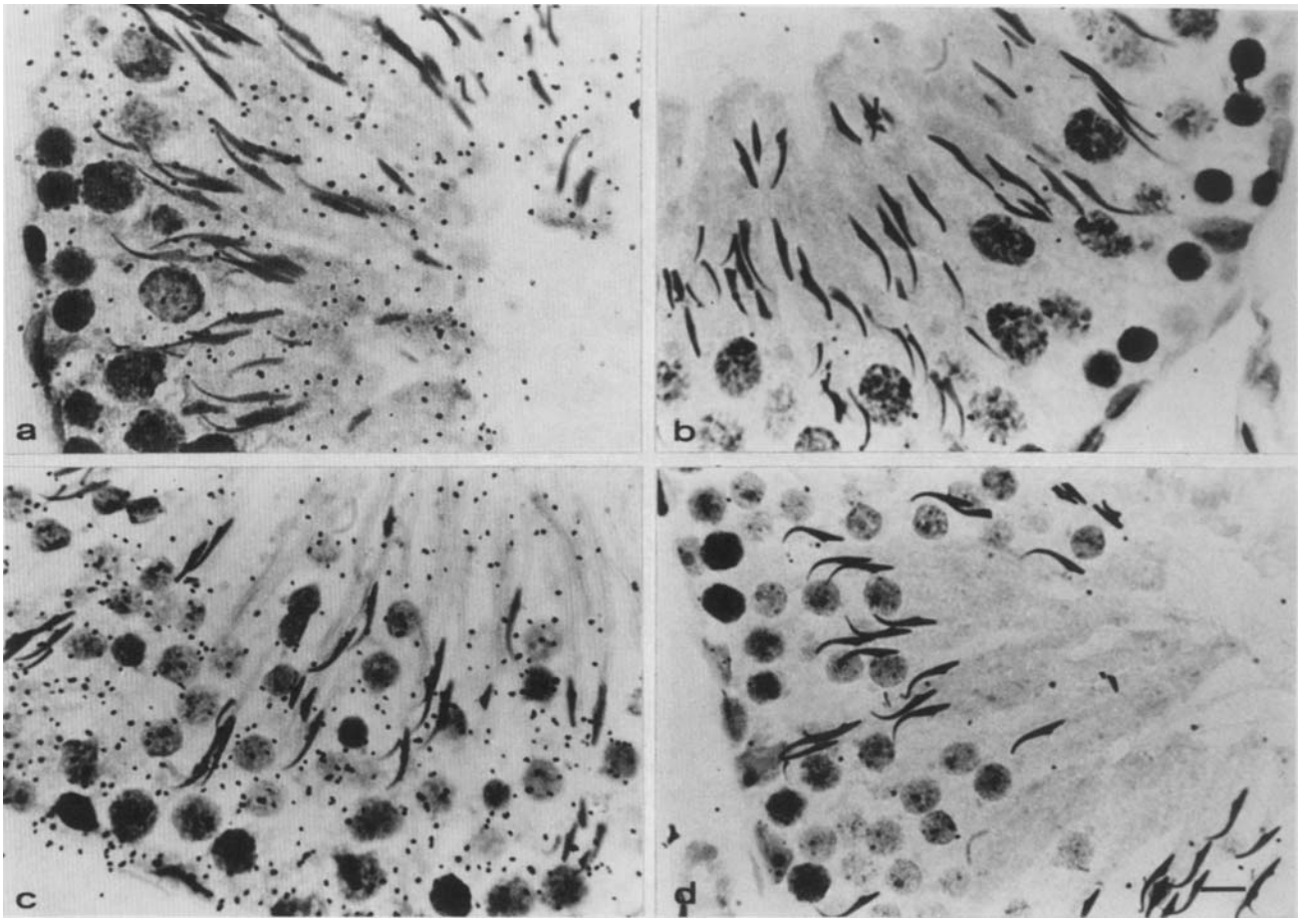

FIG. 4. Cross sections of seminiferous tubules hybridized *in situ* with ^{3}H-labeled cRNA probes transcribed from SP6 plasmids and visualized by radioautography using a NTB2 nuclear emulsion (7-day exposure). Both ^{3}H-transferrin cRNA (*a*) and ^{3}H-SGP-2 cRNA (*c*) give an intense radioautographic reaction. Note that a high proportion of silver grains overlay the more palely stained areas, which correspond to the cytoplasm of Sertoli cells. Treatment of the sections with RNase (*b*) or *in situ* hybridization at 4°C (*d*) prevents the radioautographic reaction. *Bar*, 10 μm.

mRNA. Statistical analysis verified that the high values of Stages VII and VIII were different from any of the other 12 stages of the cycle ($p \leqslant 0.05$). Moreover, the relatively high values of Stages XIII to VI were different from the low values of Stages IX to XII (least significant difference, $p \leqslant 0.05$), but not different among themselves.

The number of transferrin and SGP-2 transcripts per Sertoli cell from seminiferous tubules at Stages II–VI, VII–VIII, IX–XII, and XIII–I were determined by solution hybridization (Fig. 8). The results showed that there were stage-specific differences in the number of transferrin and SGP-2 transcripts per Sertoli cell. The copies of transferrin transcripts per Sertoli cell were highest in Stages XIII–I (1054 ± 95 SD, n=4) and were lowest in Stages VII–VIII (390 ± 35 SD, n=4). The copies of SGP-2 mRNA per Sertoli cell were highest in Stages VII–VIII (2368 ± 118 SD, n=3) and were lowest in Stages IX–XII (1941 ± 97 SD, n=2).

DISCUSSION

The validity of our procedures for *in situ* hybridization was established by a variety of controls. Hybridization and subsequent silver grain deposition did not occur when the tissues were pretreated with ribonuclease, when the hybridization was carried out at 5°C, and when tissues that do not express the sequence were used. In addition, control tissues that express the sequence (for example, transferrin in hepatocytes) showed positive hybridization. The *in situ* hybridization and radioautography confirmed previous studies which showed that Sertoli cells express transferrin and SGP-2 mRNA proteins (Skinner and Griswold, 1980; Huggenvik et al., 1984;

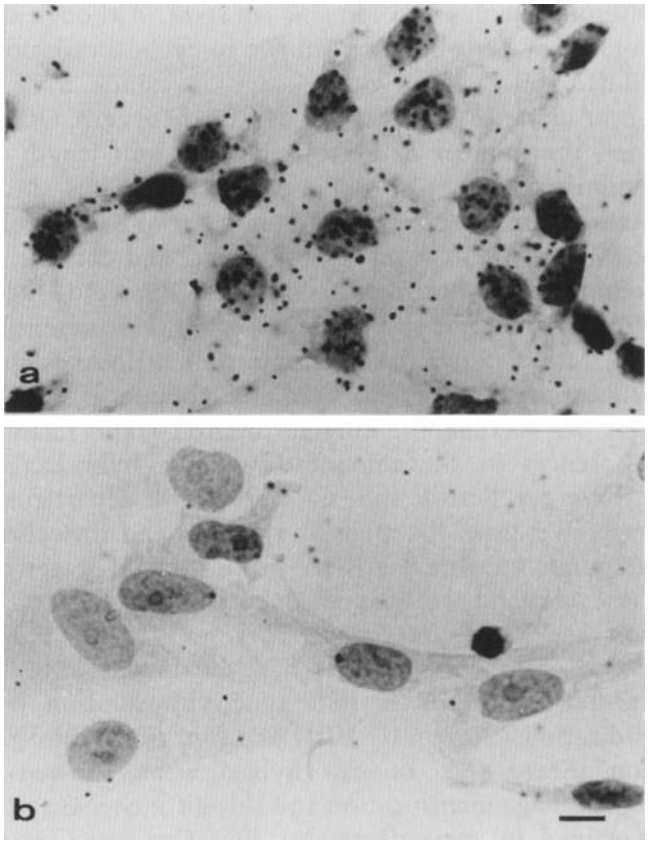

FIG. 5. Sertoli and myoid cells in culture hybridized *in situ* with ^{3}H-labeled transferrin cRNA. The radioautographic reaction demonstrates transferrin transcriptional activity in Sertoli cells in culture (*a*). Note the absence of radioautographic reaction in cultured myoid cells (*b*). *Bar*, 10 μm.

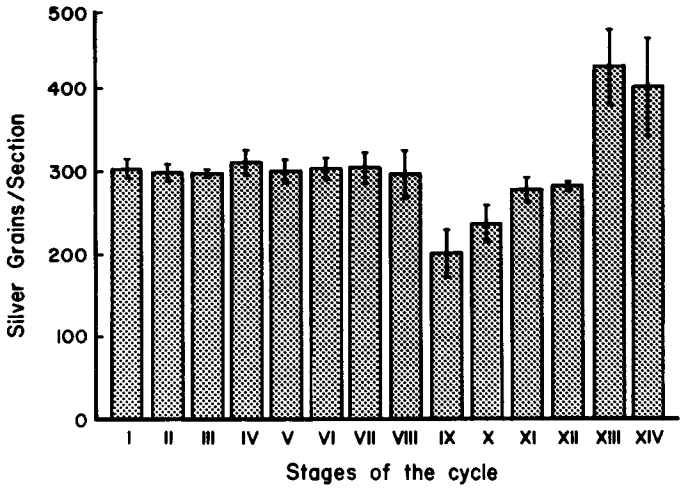

FIG. 6. Variations in the level of transferrin transcripts per cross section of seminiferous tubules at the 14 (I to XIV) stages of the cycle. Each column of the histogram represents the mean (±SD) of grain number counted in 4 rats. The level of transcripts varied markedly during the cycle. From Stage I to VIII, the level remains relatively constant. After a sharp drop at Stage IX, the level increases and reaches a peak at Stage XIII, remaining high until Stage XIV. Statistical analysis verified that the high levels of Stage XIII and XIV and the low level of Stage IX are significantly different from the rest of the stages of the cycle (least significant difference $p<0.05$).

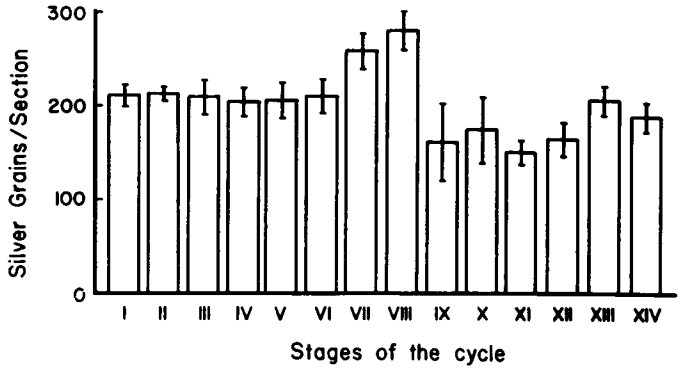

FIG. 7. Variations in the level of SGP-2 transcripts per cross section of seminiferous tubules at the 14 stages of the cycle. Each column of the histogram represents the mean (±SD) of grain number counted in 4 rats. The plot shows two distinct levels of SGP-2 mRNA. A high level from Stage XIII–XIV to Stage VIII and a low level from Stage IX to Stage XII. Stages VII and VIII presents the highest levels of SGP-2 transcripts. Statistical analysis verified that the high values of Stages VII and VIII are statistically significant (least significant difference $p<0.05$).

Sylvester et al., 1984; Griswold et al., 1986a). Both the biotinylated and the tritiated hybridization probes demonstrated that the Sertoli cells were the exclusive site for the expression of these sequences in testicular tissue. The biotinylated probes were much less sensitive in our hands, but they allowed the visualization of the presence of mRNA in the main body of the Sertoli cell throughout the 5-μm tissue

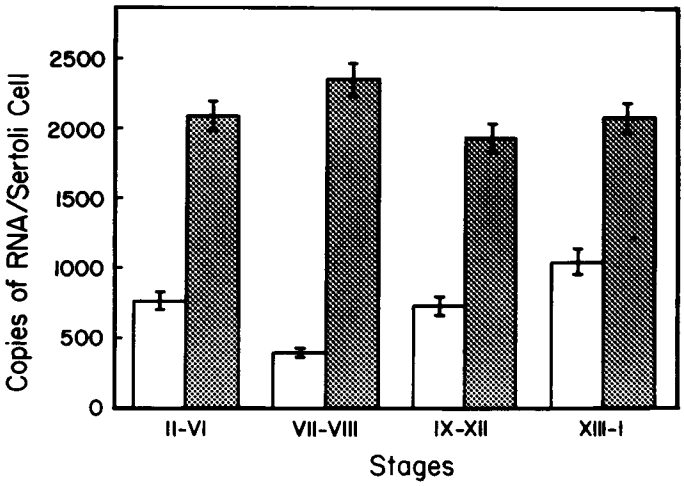

FIG. 8. Number of transferrin and SGP-2 transcripts per Sertoli cell (*white* and *stippled columns*, respectively), determined by solution hybridization. Each *column* of the histogram represents the mean number of copies per cell (±SD) from isolated and staged seminiferous tubules of 2–4 rats. The number of transferrin transcripts were highest in stages XIII–I and were lowest in Stages VII–VIII. The number of SGP-2 transcripts were highest in Stages VII–VIII and were lowest in Stages IX–XII.

section. The use of the tritiated probes and subsequent radioautography provided a very sensitive determination of the hybridizable mRNA, which was associated with the tissue that was in contact with the emulsion. The numerous cytoplasmic extensions of Sertoli cells that interact with the germinal cells contain hybridizable mRNA as well as the main body of the cell. The presence of hybridizable transferrin mRNA in Sertoli cells in whole testis sections does not support the contention of Shabinowitz and Kierszenbaum (1986) that no synthesis of transferrin occurs within the testis.

In situ hybridization techniques have been probed to be very specific for the detection of mRNAs. Recently, Lum et al. (1986) detected the localization of transferrin mRNA in inducer T lymphocytes, using *in situ* hybridization and a ^{3}H-labeled cDNA probe complimentary to human transferrin mRNA. Similarly, *in situ* hybridization, using a transferrin ^{32}P-labeled cDNA probe (1.4-kb-long) revealed specifically the presence of mRNA in oligodendrocyte of the rat brain (Bloch et al., 1985).

The combination of *in situ* hybridization and quantitative radioautography for the examination of mRNA expression in Sertoli cells in relation to the different stages of the seminiferous epithelium offers a powerful alternative to other methods currently in use. The determination of potential variable Sertoli cell functions during the cycle of the seminiferous epithelium has required the manual dissection of tubules containing different stages (Parvinen, 1982). These procedures have been used to show that FSH binding, total secreted protein, as well as the secretion of several specific proteins by Sertoli cells vary according to the stages of the cycle (Parvinen, 1982; Wright et al., 1983). This dissection procedure is laborious and subject to dissection error; it is limited by the amount of tissue available, and the results can be difficult to interpret because of the many cell types present in the testis. Meaningful results from both the dissection technique and the combination of *in situ* hybridization and radioautography procedures are dependent on the observation of Wing and Christensen (1982) that the number of Sertoli cells per unit length of seminiferous tubule remains constant throughout the 14 stages of the cycle. These data were consistent with those presented by Bustos-Obregon (1970) from Sertoli cell counts in whole amounts of seminiferous tubules at Stages II, IV, V and VII. Therefore, the number of Sertoli cells per cross section in perfect transverse sections of seminiferous tubules also remains constant. Although the volume of Sertoli cells is subject to cyclical variations (Buffe and Ploen, 1986), changes in volume have to occur along the basal-apical axis of these cells. Otherwise, the number of Sertoli cells per unit length of seminiferous tubule will be affected. Thus, in a perfect transverse section, the number of Sertoli cells remains constant from stage to stage, and changes in volume are proportional to the area (Elias and Hyde, 1980) of the cytoplasm of the Sertoli cells present in the section. Since the distribution of mRNAs is also proportional to the number and volume of Sertoli cells per section, the number of silver grains reflects differences in the amount of specific hybridizable mRNA per Sertoli cell associated with a particular stage. We used the transillumination and dissection technique to obtain mRNA from grouped stages of the cycle, and we quantified by solution hybridization and radioautography. There was a small difference in the stages that had the lowest level of transferrin mRNA if determined by solution hybridization (Stage VII–VIII) or by *in situ* hybridization (Stage IX). *In situ* hybridization allowed a precise stage identification and this difference may be explained in some degree by dissection error introduced during tubule isolation for solution hybridization. However, the data from both methods correlated quite well, but *in situ* hybridization allowed for more detailed results with less labor.

Grain counts in seminiferous tubules hybridized with the transferrin RNA probe showed that the highest level of transferrin mRNA was registered at Stages XIII–XIV. The high values of transferrin mRNA in Stages XIII–XIV coincided with the two meiotic divisions of spermatocytes. An immediate consequence of the meiotic divisions is a significant increase in the numerical ratio of germ cells to Sertoli cell in the adluminal compartment. Conversely, the lowest level of transferrin transcripts registered at Stage IX coincided with a reduction in the numerical ratio of germ cells to Sertoli cell due to the release of the late generation of spermatids (spermiation) during the preceding Stage VIII. Wright et al. (1983), using the transillumination and dissection technique, concluded that transferrin is secreted maximally at Stages XIII–XIV and minimally at Stages VII–VIII.

Recently, immunocytochemical and biochemical studies demonstrated that both spermatocytes and spermatids expressed high affinity-binding sites characteristic of receptors for testicular transferrin (Holmes et al., 1983; Sylvester and Griswold, 1984;

Brown, 1985). When a monoclonal antibody was used to localize the transferrin receptor on germinal cells, it was found that both mitotically and meiotically active cells expressed relatively high levels of the transferrin receptor (Brown, 1985). Sylvester and Griswold (1984) showed by indirect methods that germinal cells from primary spermatocytes through spermatids display receptors, but testicular transferrin was found bound to early spermatids.

All of the available data show that transferrin is apparently made in Sertoli cells throughout all stages of the cycle of the seminiferous epithelium, but it also implies a special role for transferrin during the meiotic divisions. Transferrin has been shown to be important because of its actions in stimulation of the proliferation and differentiation of many types of cells (Barnes and Sato, 1980; Ekblom et al., 1983; Larrick and Creswell, 1979). Transferrin was shown to be the main serum component responsible for kidney differentiation, and its primary function was to stimulate cell proliferation (Larrick and Creswell, 1979). Even though the Fe^{+3} requirements for meiosis and mitosis might be different, it is possible that the increased synthesis of transferrin in Stages XIII and XIV of the cycle of the spermatogenic epithelium is stimulatory to the meiotically dividing cells and thus could be considered a major regulatory factor in spermatogenesis.

The radioautographic analysis of seminiferous tubules hybridized *in situ* with the SGP-2 RNA probe showed two distinct levels of transcripts in relation to the stages of the cycle. The highest number of transcripts was registered at Stage VIII of the cycle. Although it is well known that SGP-2 is one of the major polypeptides secreted by Sertoli cells (Sylvester et al., 1984), the function of this protein is still unknown. Immunocytochemical evidences have recently revealed that secreted SGP-2 becomes a component of sperm cell membranes (Sylvester et al., 1984). In coincidence with the highest level of SGP-2 mRNAs, the most intense immunostaining was observed on the heads, tails, and residual bodies of the late spermatids at Stage VIII of the cycle (Sylvester et al., 1984).

Morphological, histochemical, and biochemical observations strongly suggest the existence of a functional cycle on Sertoli cells (Leblond and Clermont, 1952; Niemi and Kormano, 1965; Kerr and deKretser, 1975; Hilscher et al., 1979; Parvinen, 1982; Morales et al., 1986). Quantitative differences in the expression of transferrin and SGP-2 transcripts in Sertoli cells support this notion irrespective of the exact mechanism by which transcription is regulated. Although several factors may influence directly or indirectly the cyclic expression of transferrin and SGP-2 (Griswold et al., 1984; Huggenvik et al., 1984), it is entirely plausible that specific germ cells may signal the Sertoli cells to produce molecular changes that affect either the transcriptional activity or the life span of this transcript.

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