Changes in Nuclear Proteins of Rat Testis Cells Separated by Velocity Sedimentation*

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ROBERT D. PLATZ, SIDNEY R. GRIMES, MARVIN L. MEISTRICH, AND LUBOMIR S. HNILICA

From the Department of Biochemistry and the Department of Experimental Radiotherapy, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas 77025

SUMMARY

The technique of velocity sedimentation at unit gravity has been used to separate rat testis cell suspensions into fractions enriched in particular cell types. Changes in nuclear proteins from the various fractions have been characterized by polyacrylamide gel electrophoresis, and correlated with the changing morphology of the nucleus during spermatogenesis. The most striking alterations in both protein composition and nuclear morphology occur during spermatid maturation as both histone and non-histone proteins are replaced by highly basic, low molecular weight, spermatidal proteins. This replacement process is accompanied by a quantitative reduction in both histone and non-histone proteins.

The synthesis of at least three basic proteins has been identified with late stage spermatids. One of these proteins is a highly basic sperm-specific protein containing high levels of cyst(e)ine and arginine. A second protein synthesized in late stage spermatids is lysine rich, while the third protein contains cyst(e)ine and co-migrates with histone F2aI on acid-urea polyacrylamide gels.

The changes in protein composition of rat testis nuclei after irradiation or hypophysectomy reflect the resulting changes in the cellular composition of the testis. After selective elimination of the germinal cells by irradiation, the electrophoretic pattern of acid-soluble proteins from the testis is very similar to that of somatic tissue. Thus, the cellular specificity of nuclear proteins demonstrated here using cell separation techniques is also apparent following treatments which selectively alter the cellular composition of the testis.

Spermatogenesis involves a progressive differentiation of the cells of the seminiferous epithelium toward a terminal stage represented by the mature spermatozoan (1). The process begins with a period of proliferation in which spermatogonial cells divide several times to augment the number of potential germ cells. Most of these cells enter a period of growth as spermatocytes, and each spermatocyte undergoes two meiotic divisions to produce haploid spermatids. This process concludes with a period of spermatid maturation, during which the nuclear material condenses to form the headpiece of a compact, motile spermatozoan, and most of the cytoplasm is shed as a residual body.

During these processes of proliferation, growth, and maturation, significant changes occur in the kinds and quantities of macromolecules synthesized by the cells (1, 2). Of primary interest to us are mechanisms underlying the replacement of nuclear proteins by sperm-specific proteins, such as those described for trout by Dixon and co-workers (3). Arginine-rich proteins isolated from sperm of several mammalian species have been characterized (4-6). With the exception of mouse (6), all are rich in arginine and cyst(e)ine, and they contain 2 tyrosine residues, and have alanine as the NH*-terminal amino acid. A similar arginine-rich protein has been isolated from rat epididymal sperm and characterized by Kistler et al. (7). In addition, another low molecular weight basic protein, similar to the protein isolated by Lam and Bruce (6) from mouse, was found in rat testis. This protein contains both lysine and arginine, but not cyst(e)ine, and may be replaced by the cyst(e)ine-containing, arginine-rich protein during the later stages of spermatid maturation (7, 8).

In addition to the process of nuclear protein replacement by specialized basic proteins, we are interested in the changes, properties, and functions of the non-histone nuclear proteins in spermatogenic cells. These proteins are of special interest because of their proposed role in the regulation of gene transcription (9). The hormone-dependent changes in the synthesis and phosphorylation of acidic chromatin proteins during maturation of the rat testis (10) are consistent with the proposed function of these proteins in the regulation of gene expression.

Since the adult mammalian testis contains a population of cells in the process of differentiating toward a specific cell type, spermatogenesis may be viewed as a model system for studying changes in nuclear proteins associated with cellular differentiation. The feasibility of using the mammalian testis as a source of differentiating cells for biochemical studies has been greatly enhanced by the development of techniques for the separation of testis cells into relatively homogeneous populations (11). The separation of testicular cells by velocity sedimentation at unit gravity provides fractions enriched in specific cell types, which can be identified with particular stages of spermatogenesis (11-14). Further purification of specific stages may be achieved by a
second Staput separation of the nuclei isolated from selected fractions (15).

The data presented here focus on changes in the nuclear proteins of rat testis cells during the process of spermatogenesis. Quantitative and qualitative changes in these proteins are demonstrated to correlate with cells in particular stages of spermatogenesis. The most dramatic changes occur during spermatid maturation with the replacement of histone and nonhistone proteins by low molecular weight, arginine-rich proteins. Our results indicate that at least three acid-soluble proteins are actively synthesized in late stage spermatids.

MATERIALS AND METHODS

Animals Used and Treatments—Male Sprague-Dawley rats (250 g) were used throughout this study. Hypophysectomized rats were obtained from Hormone Assay, Chicago, and maintained for 28 or 55 days before use. For some experiments, the testes of normal rats were locally irradiated using cobalt 60, with two exposures of 1300 rads each, given 1 week apart.

Preparation of Cell Suspensions—The EDTA-trypsin method described previously (16) for obtaining high yields of spermatogonia and young primary spermatocytes from various age groups was used with some modifications. Suspensions were prepared using two minced testes in 40 ml of calcium-magnesium-free phosphate-buffered saline containing 5 mM EDTA and 0.1% glucose at pH 7.4. After 10 min of incubation at 37°C, purified trypsin (Worthington Code TIK) dissolved in saline was added (0.1% final concentration), and the sample was incubated for another 10 min without stirring. MgCl₂ and crude DNAse (Sigma, beef pancreatin, DN-25) were then added to make final concentrations of 10 mM MgCl₂ and 17 μg/ml of DNAse. After stirring for another 10 min, fetal calf serum was added to a concentration of 7.7%. Undispersed tubules were removed by settling and filtration (80-μm screen), and the cells were centrifuged at 500 X g for 15 min. The cells were resuspended by gentle homogenization of seminiferous tubules in 0.31 M sucrose containing 3 mM MgCl₂, 5 mM sodium bisulfite, 10 mM potassium phosphate, pH 6.0, and 0.05% Triton X-100. After centrifugation for 10 min, the crude nuclear pellet was suspended in 5 mM MgCl₂, 5 mM sodium bisulfite, 5 mM potassium phosphate, pH 6.8, containing 0.025% Triton X-100 and 0.0025% soybean trypsin inhibitor, and treated as described for Staput fractions under “Isolation of Nuclear Proteins.” Purified sperm heads from testis or epididymides were prepared by sonication as described elsewhere.

Staput Separation—The Staput method of velocity sedimentation at unit gravity was used for separation of testicular cells or nuclei. A large chamber (28.1-cm diameter) was used for preparative scale separations (17), while a small chamber (12.5-cm diameter) was used for analytical scale separations of cells or for further purification of nuclei. Samples were introduced into the large or small chambers in volumes of 100-ml or 20-ml, respectively.

Cell separations were performed in 1 to 470 gradients of bovine serum albumin by suspending in PBS and centrifuging 10 min at 400 X g. After aspirating the supernatant, the cells were lysed by suspending in MPB buffer containing 0.25% Triton X-100 was added to a concentration of 0.1% before collecting the nuclei by centrifugation at 500 X g for 15 min. The addition of Triton aids in the formation of a compact pellet and results in higher recovery of nuclei. The nuclei were resuspended in MPB buffer and processed as described below.

Isolation of Nuclear Proteins—All procedures were carried out at 4°C. Cells from each Staput fraction were washed free of bovine serum albumin by suspending in PBS and centrifuging 10 min at 400 X g. After aspirating the supernatant, the cells were lysed by suspending in MPB buffer containing 0.25% Triton X-100 and 0.025% soybean trypsin inhibitor. If subsequent Staput separation of the nuclei was planned, this suspension was taken up in a syringe, forced through a 25-gauge needle twice, and loaded directly on the second Staput. If no Staput separation of nuclei was planned, the suspension was centrifuged, the supernatant aspirated, and the pellets resuspended in 6 ml of MPB using a Pasteur pipette. This suspension was taken up in a syringe and forced through a 25-gauge needle twice. A solution of 2.4 mM sucrose made up in MPB and containing 0.05% Triton X-100 was added to bring the final sucrose concentration to 2.0 M. Nuclei were recovered by centrifugation in an SW 27 rotor for 60 min at 24,000 rpm. The pellets were suspended in MPB, and aliquots were taken for microscopic analysis and quantitation. After centrifugation, the nuclear pellets were extracted twice with homogenization in 0.25 N HCl followed by centrifugation. The combined supernatants containing the acid-soluble proteins were neutralized with NaOH before dialysis overnight against cold distilled water using cellulose hollow fibers (Bio-Fiber 50, Bio-Rad, nominal molecular weight cutoff 5000). If any insoluble residue formed during dialysis, it was washed once with cold distilled water and suspended in freshly made loading solution, composed of 0.125 M Tris-HCl, pH 6.8, 4.0% sodium dodecyl sulfate, 20% glycerol, 10% β-mercaptoethanol, and 0.0003% bromphenol blue. Solubilization was accomplished by heating samples at 100°C for 5 to 10 min. If necessary, the DNA was digested by homogenization or sonication. In some cases, the DNA was removed by centrifugation prior to electrophoresis.

Electrophoresis—Acid-soluble proteins were separated by electrophoresis in 15% polyacrylamide gels containing 2.5% urea (20). Gels were stained in 0.1% Amido black and destained by transverse electrophoresis in 40% ethanol/35% acetic acid. The molecular weights of acid-soluble proteins were determined in 12% sodium dodecyl sulfate-polyacrylamide slab gels using the procedure of Lasemlli (21) and an apparatus described by Reid and Bieleski (22), which is commercially available from Glenco, Houston, Tex. Gels were stained overnight in 0.05% Coomassie blue, 10% acetic acid, and 25% isopropyl alcohol, and were destained by washing in 10% acetic acid/10% isopropanol (23). Estimation of the molecular weights of individual protein bands was based on the plots of mobility versus molecular weight for proteins of known molecular weight. Standards used were cytochrome c (12,000), myoglobin (17,000), chymotrypsinogen (25,700), ovalbumin (45,000), catalase (60,000), and bovine serum albumin (68,000).

Radioisotopes—[3H]Arginine (specific activity 20 to 40 Ci/mmol or 750 to 200 mCi/mmol), and [14C]leucine (specific activity 200 mCi/mmol, 250 μCi/testis) was administered by intratesticular injection in volumes of 100 to 50 μl at 24 hours before excision of the testis. For some experiments, 250...
The percentages of rat testis cell types were determined in fractions obtained after 3 hours of sedimentation at unit gravity. The counts were performed on random areas of air-dried smears, fixed in Bouins, and stained with periodic acid-Schiff-hematoxylin, as described previously for the mouse (14). Identification of cells was made using similar criteria as in the mouse with the following exceptions. (a) The dark-staining centromeric heterochromatin was not apparent in round spermatids of the rat, making it more difficult to distinguish between round spermatids and secondary spermatocytes. (b) The stages of spermatid nuclear elongation are identified according to the description of Leblond and Clermont (27), and on the basis of our observation that the most marked decrease in hematoxylin staining of the spermatid nucleus occurs between Steps 15 and 16 of development. Multinucleate cells were counted as single cells. At least 500 cells were counted in each fraction. The predominant cell type in each fraction is enclosed in a box.

| Steps | D | C | D | E | F | G | H | I |
|-------|---|---|---|---|---|---|---|---|
| 1-8   | 13 | 24 | 43 | 71 | 67 | 9 | 0 | 0 |
| 9-10  | 2  | 1.8 | 2 | 5 | 3 | 6 | 3 | 0.2 |
| 11-15 | 7 | 10 | 7 | 5 | 7 | 15 | 20 | 21 |
| 16-19 | 0.4 | 1.2 | 0.4 | 0.7 | 0.4 | 2 | 4 | 0.8 |
| Unknown and degenerating | 3 | 4 | 5 | 2 | 3 | 11 | 6 | 0.4 |
| Nongerminal | 3 | 4 | 2 | 2 | 3 | 5 | 0 | 0 |
| Residual Bodies | 5 | 6 | 8 | 5 | 6 | 38 | 59 | 50 |

\( ^a \) Includes preleptotene spermatocytes which are indistinguishable from type B spermatogonia.  
\( ^b \) Includes leptotene, zygotene, and early pachytene spermatocytes.  
\( ^c \) Includes secondary spermatids.  
\( ^d \) Includes Sertoli cells, Leydig cells, and macrophages.  
Erythrocytes were not counted.  
*Residual bodies are defined as cytoplasmic fragments which contain ribonucleoprotein aggregates. Other cytoplasmic fragments were not scored.

\( \mu Ci \) of \( [3H] \) thymidine (specific activity 5 Ci/mm) were injected intraperitoneally.

Incorporation of \( [3H] \) arginine into acid-soluble proteins was determined following electrophoresis in cylindrical gels. Gels were fractionated at 1 mm intervals using a Gilson Aliquogel fractionator. Gel fractions were dried in an oven and solubilized in 0.2 ml of 30% hydrogen peroxide. After incubating capped vials for 3 to 5 hours at 80°C, 0.2 ml of water and 5 ml of a Triton-toluene mixture (1 part Triton X-100 (Rohm and Haas), 2 parts toluene, 4 g/liter of Omnifluor (New England Nuclear)) were added to each vial. Radioactivity was measured using a Packard model 3375 liquid scintillation spectrometer.

**Analytical Procedures**—DNA was determined by the diphenylamine reaction, as modified by Burton (24), using calf thymus DNA as a standard. Protein was assayed according to the Lowry procedure (25), or, in some cases, by measuring turbidity at 400 nm, 20 min after precipitation with trichloroacetic acid at a final concentration of 25% (26).

**RESULTS**

Fractions from the Staput were checked microscopically and pooled on the basis of composition into eight major fractions, designated D to I. The cellular compositions of these fractions are shown in Table I, with the predominant cell type in each fraction indicated by the boxes. Highly enriched preparations of pachytene spermatocytes (Fraction B), round spermatids (E and F), and late spermatids (H and I) were routinely obtained. The relative purity of these fractions is demonstrated by comparing the photomicrographs of cells in Fractions C, F, and H with a photomicrograph of a total testis cell suspension before separation (Fig. 1). Nuclei isolated from each fraction were examined by electron microscopy, and judged to be free of visible cytoplasmic contamination. Nuclei from fractions enriched in late spermatids, however, are generally contaminated by the acrosome, as well as by the ribonucleoprotein aggregates derived from the residual bodies, and by the flagella of spermatids. These contaminants can be eliminated from late stage spermatids by sonication. As a further check for possible cytoplasmic contamination, \( [3H] \) arginine-labeled nuclei were isolated in the presence of cytoplasm (10,000 x g supernatant) from \( [3H] \) arginine-labeled testis and vice versa. The cross-contamination of nuclear proteins by cytoplasmic proteins in these experiments was less than 2%. To determine the extent of non-chromatin-associated proteins remaining in the nuclei, chromatin was prepared from nuclei isolated by the described procedure, and the electrophoretic profile of chromatin non-histone proteins was compared with that of whole nuclei (Fig. 2). The close similarity in protein composition indicates that any contamination of nuclei by non-chromatin proteins does not contribute significantly to the protein pattern observed in polyacrylamide gels.

Acid extraction of nuclei from the various Staput fractions removes histone and some non-histone proteins. The electrophoretic patterns of the acid-soluble proteins from fractions representing different cell types are remarkably different (Fig. 3). For example, the spermatid proteins (Fig. 3, Bands 3 and 4), which are major components of the fraction enriched in late spermatids (Fig. 3g), are virtually absent from fractions repre-
FIG. 1. Photomicrographs of rat testis cell suspensions and Staput fractions prepared as described in Table I. a, unseparated cell suspension; b, Staput Fraction C enriched in late pachytene spermatocytes. Also shown are two Sertoli cells, a Leydig cell, a tetranucleate spermatid, a binucleate secondary spermatocyte, and a cell in metaphase of meiosis I. c, Staput Fraction F, enriched in round spermatids. Also shown are elongating spermatids in Steps 11 to 15, leptotene spermatocytes, and residual bodies. d, Staput Fraction H, enriched in elongating spermatids in Steps 11 to 15, cytoplasmic fragments detached from these spermatids, and residual bodies. Magnification, × 640.
togenesis. When Fractions H and I were pooled and the isolated
F2al histone is selectively synthesized in later stages of spermatogenesis, occurring in the rapidly migrating proteins, while a small but enriched in later stage spermatids, most of the incorporation of arginine is observed in the region of histone F2al. In Fraction I, which is enriched in late spermatids, major incorporation of [3H]arginine occurs in late pachytene spermatocytes. In Fraction H (Fig. 4B), enriched in late spermatids, a differential synthesis of the histones in different Staput fractions (Fig. 4B) demonstrates that a cyst(e)ine-containing protein which migrates with histone F2al on acid-urea polyacrylamide gels (20) is rapidly synthesized in late stage spermatids, and comprises a major protein component of sonication-resistant sperm heads. Although not apparent from the data shown, two closely migrating bands are evident in the F2al region of these gels.

Fig. 6 also demonstrates the presence of the two rapidly migrating spermatidal proteins observed in Fig. 3. The slower migrating protein has a high arginine and cyst(e)ine content, while the faster migrating protein is high in lysine and arginine. The data in Fig. 5 and Table II suggest that synthesis of these spermatidal proteins is localized in the late spermatid cells. The close correlation between the presence of these proteins and the distribution of elongated spermatids (Steps 16 to 19) is shown in Fig. 7.

The amino acid composition of the two spermatidal proteins from sonication-resistant testicular sperm heads is shown in Table III. Allowing for slight contamination of these proteins by other proteins, the analyses are reasonably close to those reported by Kistler et al. (7) for a testis-specific protein, TP (Band 4), and a sperm-specific protein, S1 (Band 3).

The non-histone proteins (acid-insoluble residue) of rat testis nuclei are a complex and heterogeneous group, ranging in molecular weight (determined by sodium dodecyl sulfate-acrylamide gel electrophoresis) from 10,000 to 120,000 (Fig. 8). For purposes of discussion, the gel is divided into five regions. Against a background of proteins which are present in every cell fraction, many quantitative changes are consistently observed. For example, several protein bands which are present in Region I of Fraction C which are enriched in pachytene spermatocytes, are greatly reduced or absent in Fractions H and I, enriched in elongating spermatids. During spermatid maturation, proteins migrating in Regions II and III undergo quantitative shifts and a progressive reduction in heterogeneity (Figures 8, F, H, and I). Several protein bands in Region IV disappear after the round spermatid stage of spermatogenesis (compare Fractions D and F with H and I), while at least two major proteins are present in all fractions. In Region V, one non-histone protein, which is absent or reduced in the early stages of spermatogenesis, appears as a major component of elongating spermatids (Fractions H and I). In general, the over-all heterogeneity is significantly lower in late

Fig. 2. Electrophoretic patterns of non-histone nuclear proteins from rat testis separated on sodium dodecyl sulfate-acrylamide slab gels (21). The acid-insoluble residue after extraction of nuclei with 0.25m HCl was dissolved in loading solution as described under "Materials and Methods." Chromatin was then washed twice in 0.08 M NaCl, 0.02 M EDTA (pH 6.3), followed by three washes in 1.5M NaCl, 0.15 mM sodium citrate (pH 7.0) (28). The chromatin was then extracted twice with 0.25m HCl. (1, 2) Non-histone proteins from nuclei, 50 μl, 100 μl. (3, 4) Non-histone proteins from chromatin 100 μl, 100 μl. (5) Chromatin proteins from rat testis chromatin showing major histone components, 50 μl. Note that the non-histone patterns from nuclei and chromatin are virtually identical.
which is similar to, but slower migrating than, histone F2b, and F2b region, presumably resulting from the absence of a protein irradiation. Second, a change is observed in the density of the migrating of the 2 lysine-rich histones (Band 1) is absent after normal control pattern in at least three ways. First, the slower proteins from the irradiated testes (Fig. 10) is different from the of germinal cells. The electrophoretic pattern of the acid-soluble composition of the testis (31-33). Seventy days after local irradia-
tion of the testes with cobalt 60, cytological examination revealed
information regarding the differential synthesis of nuclear proteins by specific cell types. Our data indicate that both histone and non-histone proteins are progressively replaced by rapidly migrating basic proteins in the later stages of spermatid maturation. The synthesis and accumulation of these basic proteins which is unique to the testis (34). Third, the spermatidal protein(s) are absent after irradiation. As a result of these changes, the histone pattern after irradiation is very similar to that of liver, or any other somatic tissue. The pattern changes after hypophysectomy are somewhat less striking, and may reflect the presence of early stage germinal cells which remain in the testis after hypophysectomy (31).

**DISCUSSION**

The results presented here demonstrate several changes in the nuclear proteins of rat testis cells during spermatogenesis. We have used cell separation techniques to correlate these changes with cells in specific stages of spermatogenesis, and to obtain information regarding the differential synthesis of nuclear proteins by specific cell types. Our data indicate that both histone and non-histone proteins are progressively replaced by rapidly migrating basic proteins in the later stages of spermatid maturation. The synthesis and accumulation of these basic proteins which is unique to the testis (34). Third, the spermatidal protein(s) are absent after irradiation. As a result of these changes, the histone pattern after irradiation is very similar to that of liver, or any other somatic tissue. The pattern changes after hypophysectomy are somewhat less striking, and may reflect the presence of early stage germinal cells which remain in the testis after hypophysectomy (31).
Rat testis cells were separated by Staput after labeling in vivo for 24 hours with $[^3]H$-arginine or $[^3]H$-thymidine (see under "Materials and Methods"). Nuclei were isolated, and the specific activities of the basic proteins were estimated after separation on polyacrylamide gels (20) containing 2.5 M urea. The amount of protein in each band was estimated from densitometric scans by multiplying the weight of each peak as a fraction of total scan weight times the total micrograms of protein loaded on the gel. The specific activity of the DNA in nuclei from each fraction was determined directly.

### TABLE II

**Specific activities of basic proteins and DNA from Staput fractions**

| Staput fractions | B   | C   | D   | E   | F   | G   | H   | I   |
|------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Basic proteins (cpm/μg) |     |     |     |     |     |     |     |     |
| F2b region       | 16  | ND  | 28  | 38  | 29  | 26  | 22  | 28  |
| F2al             | 14  | ND  | 13  | 26  | 26  | 52  | 75  | 50  |
| Spermatidal proteins | Trace | ND | Trace | Trace | 105 | 110 | 144 | 93  |
| DNA (cpm/μg)     | 2.3 | 1.7 | 4.0 | 7.8 | 11.0| 24.8| 6.7 | 1.5 |

a This region of the gel includes F3, F2b, F2a2, and two additional acid-soluble protein bands unique to testis cells.

b ND, not determined.
c Insufficient protein or counts to allow an accurate estimate.

correlate closely with the appearance of elongated spermatid nuclei in Steps 16 to 19. The changes in protein pattern after irradiation and hypophysectomy complement and support the results obtained using cell separation techniques. That is, the proteins identified with germinal cells using cell separation techniques are absent after treatments which alter the cellular composition of the testis.

Considerable effort was made in this study to minimize the possibility of cytoplasmic contamination. The effectiveness of our isolation procedures in providing nuclei free of cytoplasmic membranes and attached ribosomes was monitored by electron microscopy. The main source of contamination present in our nuclear preparations was the ribonucleoprotein aggregates derived from residual bodies (35). Residual bodies are the cytoplasmic fragments which are sloughed off during the last steps of spermatid maturation. By using a combination of sonication followed by Staput separation of the resistant nuclei, we were able to reduce the contamination of spermatid nuclei by ribonucleoprotein aggregates from 50% to <4%. In other experiments, cross-contamination of nuclear fractions by nuclei from different cell types was reduced by running two Staput separations in sequence. After the separation of testis cells on the first Staput, the isolated nuclei from selected fractions were purified.
on a second Staput using a sucrose gradient (15). This technique was used to obtain spermatid nuclei free from contamination by spermatogonial nuclei. Obtaining purified spermatid fractions was considered to be essential. Since histone synthesis in mammalian cells is generally thought to be coupled with DNA synthesis (36), even slight contamination by S-phase cells (spermatogonia or preleptotene primary spermatocytes) (37) could mask the determination of spermatid histone synthesis. After labeling with [3H]arginine, therefore, the contribution of spermatogonia to the specific activity of the spermatid histones could be considerable.

Our data indicate that at least three acid-soluble nuclear proteins are actively synthesized in late stage spermatids (Steps 12 to 19). Two of these proteins are identified as the testis-specific protein, TP, and the sperm-specific protein, Sl, described by Kistler et al. (7). The synthesis of TP and Sl by late spermatids, as demonstrated here for the rat, is consistent with the findings of Louie and Dixon (2), for trout, Monesi (38), and Lam and Bruce (6) for mouse, and Loir (39) for the ram. In addition, our results demonstrate the synthesis of an additional protein(s) in late spermatids, which has the mobility of histone F2a in acid-urea polyacrylamide gel (20), and contains both cyst(e)ine and arginine. The evidence of two bands in the F2a region leads us to entertain the possibility that more than one additional protein may be present and perhaps synthesized in late spermatids.

The possibility that the cyst(e)ine-containing protein is a dimer of the Sl protein appears unlikely, since its specific activity increases when spermatids are purified, while the specific activity of TP and Sl decreases (see Fig. 5 legend). Further analysis of this cyst(e)ine-containing spermatidal protein is in progress.

In contrast to the well-characterized histones (40), the non-histone (acid-insoluble) proteins are highly heterogeneous and poorly characterized. Within the limitations of the techniques used in this study to separate cells and to fractionate proteins, some tentative identification of non-histone protein bands with specific cell types can be made. For example, protein bands in Region I having a molecular weight greater than 84,000 are primarily associated with the pachytene spermatocyte stage of spermatogenesis. This result is consistent with Kadokama and Turckington's observation (10) that the high molecular weight non-histone proteins do not appear during development of the rat testis until spermatogonia and spermatocytes have differentiated. A protein band at 53,000 molecular weight appears to be a component of developing germ cells up through the early spermatid stage, but is absent after about Step 8. The presence of a band at 31,000 molecular weight is associated with spermatocytes and early spermatids (Steps 1 to 8). A band at 48,000 molecular weight is predominantly associated with elongating spermatids (Steps 11 to 15), and bands at 44,000 and 20,000 molecular weight appear to be major components of late stage spermatids (Steps 16 to 19).

The high molecular weight non-histone proteins are found predominantly in fractions enriched in metabolically active cell types. The absence of these proteins correlates with late stage spermatids in the final stages of differentiation into a cell type which is inactive in RNA synthesis. These observations suggest that in rat testis cells, as in a wide variety of other systems (9),

### Rat testis spermatids

### Amino acid composition of basic proteins from rat testis spermatids

| Amino acid | Band 3 | Band 4 |
|------------|--------|--------|
| Lysine     | 5.6    | 15.6   |
| Histidine  | 0.4    | 4.2    |
| Arginine   | 50.8   | 25.3   |
| Aspartic acid | 1.2   | 6.1    |
| Threonine  | 1.6    | 3.0    |
| Serine     | 8.4    | 12.6   |
| Glutamic acid | 0.7 | 1.0    |
| Proline    | Trace  | 1.9    |
| Glycine    | 0.5    | 8.9    |
| Alanine    | 1.6    | 3.7    |
| Cysteine²  | 13.2   | 3.2    |
| Valine     | Trace  | 1.6    |
| Methionine | Trace  | 2.7    |
| Isoleucine | Trace  | 0.6    |
| Leucine    | 0.5    | 4.7    |
| Tyrosine   | 6.5    | 4.0    |
| Phenylalanine | 2.0  | 0.5    |

* No correction was made for hydrolysis of serine or threonine.

² Using separate samples, cysteine was determined as cysteic acid after performic acid oxidation (30).
and a correlation exists between the amount of non-histone protein associated with DNA and the level of RNA synthesis.

Careful fractionation of the non-histones, combined with further studies on their synthesis and modification in rat testis cells, should help in identifying and characterizing individual proteins. It should then be possible to formulate and test meaningful hypotheses regarding the function of specific nuclear proteins in modifying the structure of chromosomes and in regulating the transcription of RNA.

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Fig. 8 (left). Non-histone nuclear proteins from Staput fractions. After extraction of purified nuclei with 0.25 M HCl, the acid-insoluble residue was solubilized in a solution of 4.0% sodium dodecyl sulfate/10% a-mercaptoethanol prior to electrophoresis on a sodium dodecyl sulfate-polyacrylamide slab gel (21). The cellular composition of Fractions B through I before isolation of nuclei is given in Table I. A, C, D, F, G, H, and I are Staput fractions from which non-histone nuclear proteins were isolated. T, trypsin control: whole testis cell suspension prepared with trypsin for Staput separation and held at 4°C for 4 hours to simulate conditions used for Staput fractionation. b, Mechanical control: whole testis cell suspension prepared without trypsin and after trypsin under conditions designed to minimize protein degradation (see under "Materials and Methods"). Note that the non-histone proteins of Z' and H' are Staput fractions from spermatid fraction after being purified on Staput. Composition is 96% spermatids, 4% residual bodies, 0.3% round nuclei. Position of major protein bands is indicated. Densitometric scans of the acid-soluble proteins extracted from these fractions are shown in Fig. 5.

Fig. 10 (right). Electrophoretic patterns of acid-soluble nuclear proteins from rat testes after treatments to alter the cellular composition. Proteins were separated on polyacrylamide gels containing 2.5 M urea (20). RL, rat liver; IR, proteins isolated 70 days after local irradiation of the testis with cobalt 60 to destroy all germinal cells; N, normal rat testis control; HX, proteins isolated 55 days after hypophysectomy. Note that after irradiation (IR) the pattern from testis resembles the somatic histone pattern represented by rat liver (RL). After irradiation and hypophysectomy, the spermatidial proteins are absent.
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