THE INTERACTION OF TESTICULAR ANDROGEN-RECEPTOR COMPLEX WITH RAT GERM CELL AND SERTOLI CELL CHROMATIN

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SUMMARY: Chromatin acceptor sites for testicular androgen-receptor complex were studied in purified preparations from Sertoli cells, testicular germ cells, thymus, and liver. Sertoli cell nuclei did not sediment through sucrose solutions more concentrated than 1.8M. The combination of cell culture and discontinuous sucrose gradient centrifugation yielded a preparation of Sertoli cell nuclei with greater than 95% purity. Cytoplasmic androgen-receptor complex was prepared from the testes of hypophysectomized rats by *in vitro* labeling, ammonium sulfate precipitation, and Sephadex G-25 chromatography. The purified chromatin from Sertoli cells and germ cells bound 3 to 5 times as much receptor complex as did that from thymus or liver. The binding of increasing aliquots of complex to a fixed amount of chromatin DNA revealed the presence of at least two classes of binding sites. These data support previous suggestions that Sertoli cells and germ cells are primary targets of androgen action in the testis.

It is generally accepted that a steroid hormone, upon entry into a target cell, binds to a specific cytoplasmic receptor. This hormone-receptor complex is "activated" and then transferred into the cell nucleus. In the nucleus, this "activated" complex binds to the target cell genome at specific acceptor sites in the chromatin. By a yet undefined process, the target cell responds with increased RNA synthesis followed by increased protein synthesis (1,2,3,4).

The nature of the nuclear acceptor site has not been firmly established. Steroid receptor complexes have been reported in various steroid responsive tissues to bind to DNA (5,6,7), to histones (8), to a class of nuclear acidic nonhistone proteins termed AP_3 (9,10,11), to a fraction of tightly bound nuclear nonhistone proteins termed NP, (12) and to nuclear basic nonhistone proteins (13,14).

Binding of receptor-estrogen complex and receptor-androgen complex to isolated chromatin has been demonstrated to be tissue specific (9,12,15), but the cell types involved have not been delineated. This paper describes the interaction of testicular androgen-receptor complex with nuclear acceptor sites on the chromatin of isolated germ and Sertoli cells.

MATERIALS AND METHODS

Materials: Sprague-Dawley male rats 30 or 31 days of age were used as the source of thymus, liver, germ cells and Sertoli cells. Male Sprague-Dawley rats hypophysectomized at 65 days of age were obtained from Hormone Assay Laboratory (Chicago, Ill.) and were used for the preparation of receptor-testosterone complex 14-21 days after surgery. Testosterone [1α, 2α-^{3}H(N)] (59 Ci/mmole) and testosterone [1,2,6,7-^{3}H (N)] (89 Ci/mmole) were purchased from New England Nuclear Corp. and were judged to be >95% pure by thin layer chromatography.

Isolation of nuclei: Rat thymus or liver were minced and homogenized in 10 volumes of 0.25 M sucrose-TKM.[a] After filtration through 6 layers of cheese cloth, the homogenates were centrifuged (1,000 x g, 10 min). The pellets were resuspended in 3 volumes of 0.25 M sucrose-TKM-0.3% Triton X-100. After standing in ice for 10 min, 2.2M sucrose-TKM was added to the suspension to a final concentration of 1.6M sucrose and the resulting suspension was centrifuged at 105,000 x g for 1 hr. The pellets were considered as purified rat thymus or liver nuclei.

Sertoli cells were isolated from the testes of 20 rats (30 days of age) and cultured for two days as described elsewhere (16). The cells were collected by scraping the dishes with 0.85% NaCl-10mM Tris pH 7.4 (Tris-saline), centrifuged (800 x g, 10 min), washed once with Tris-saline, and then lysed in 50 ml of 5 mM $MgCl_2$ followed

[a] TKM = 50mM Tris-HCl pH 7.4, 25 mM KCl, 5mM $MgCl_2$

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by addition of 10 ml of 1.5M sucrose-TKM and centrifugation (1000 x g, 10 min). The pellet was resuspended in 0.25M sucrose-TKM-0.3% Triton X-100, allowed to stand in ice for 5 min and then centrifuged at 1000 x g for 10 min. The pellet was resuspended in 40 ml of 0.25M sucrose-TKM and overlayed in aliquots of 7 ml per tube on top of 27 ml of a discontinuous sucrose gradients formed in a SW 27 centrifuge tube. The gradient was comprised of 9 ml of 2.2M sucrose-TKM, 9 ml of 1.8M sucrose-TKM and 9 ml of 1.6M sucrose-TKM. After 2 hrs. of centrifugation (120,000 x g), the Sertoli cell nuclei were collected from the interphases of 2.2M-1.8M sucrose and 1.8M-1.6M sucrose. After adjusting the sucrose concentration to 1.6M, the purified Sertoli cell nuclei were collected by centrifugation (140,000 x g, 1 hr).

The germ cells were obtained from seminiferous tubules of 31 day old Sprague-Dawley rats as described previously (17). Germ cells were homogenized in 0.25M sucrose-10mM Tris-5mM $MgCl_2$, and centrifugated at 1000 x g for 10 min. The pellet was resuspended in 0.25M sucrose-TKM-0.3% Triton X-100. After standing in ice for 15 min, 2.2M sucrose-TKM was added to a final concentration of 1.6M sucrose and the suspensions were centrifuged (105,000 x g, 1 hr). The resulting pellet was composed of purified germ cell nuclei.

Preparation of chromatin: The purified nuclei were gently homogenized by hand in 0.08M NaCl-0.02M EDTA pH 6.3 and centrifuged at 1500 x g for 10 min as described by Spelsberg *et al.* (18). After this procedure was repeated twice more, the nuclear pellets were homogenized by hand in 0.3M NaCl and centrifuged (10,000 x g, 10 min) to remove the loosely bound proteins (18). The pellets were rehomogenized gently in 0.01XSSC[b] and centrifuged (15,000 x g, 10 min). The process was repeated two times. The washed chromatin preparations from Sertoli cells and germ cells were further purified by centrifugation through 1.7M sucrose-10mM Tris pH 8.0 for 2 hr at 120,000 x g (19), followed by 2 more washes in 0.01XSSC.

Preparation of ^{3}H-testosterone-receptor complex: Testes removed from 5 hypophysectomized adult rats were immediately perfused via the testicular artery with Tris-saline and the tunica albuginea was carefully removed. Following homogenization in 0.32 M sucrose-10mM Tris-1mM EDTA-12mM thioglycerol-10% glycerol pH 7.4, the homogenate was centrifuged at 1,000 x g for 10 min. The supernatant was incubated with 10mM testosterone-[1α, 2α-^{3}H(N)] or testosterone-[1,2,6,7-^{3}H (N)] at 4° C for 30 min and further centrifuged at 140,000 x g for 90 min. The labeled receptor complex was precipitated by slow addition of 100% saturated ammonium sulfate solution (pH 7.4), first to a final concentration of 15% (first precipitation) and then to 30% (second precipitation). After gentle shaking for 20 min, the suspension was centrifuged at 15,000 x g for 20 min. The material precipitating between 15 and 30% ammonium sulfate saturation was redissolved in 0.7 to 0.9 ml of buffer (50mM Tris-HCl pH7.4, 1mM EDTA, 12mM thioglycerol-10% glycerol) and the ^{3}H-testosterone-receptor complex separated from free ^{3}H-testosterone by filtration through Sephadex G-25 (1 x 10 cm column).

The binding of ^{3}H-testosterone-receptor complex to isolated chromatin preparations in vitro: A 1 to 2 ml incubation mixture containing various chromatin preparations (100-200 μg DNA) in 0.15M NaCl, 5mM Tris-HCl, pH 7.4 and 0.5mM EDTA (10) with a fixed amount or increasing amount of ^{3}H-testosterone-receptor complex was incubated at 26°C for 1 hr (17). At the end of 1 hr, 6 ml of cold 0.15M NaCl-10mM $MgCl_2$ were added to terminate further interaction. This was followed by centrifugation at 15,000 x g for 20 min. The chromatin preparations were washed twice more with the same solution and centrifuged as above. The chromatin-associated ^{3}H-testosterone was quantitatively extracted with ether. After evaporation, the radioactivity was counted in Scintiprep 1-toluene (Fisher) at 46% efficiency.

Chemical analysis of chromatin: Standard DNA (calf thymus. DNA Type I, Sigma) solutions and aliquots of chromatin preparations were hydrolyzed in 0.5M perchloric acid. Burton's method (20) was employed to determine the concentration of DNA in the hydrolyzates.

Hydrochloric acid (2.75N) was added to aliquots of chromatin to a final concentration of 0.25N. The suspensions were agitated for 30 min at 0°C and the acid-soluble material was separated by centrifugation (15,000 x g, 10 min). The pellets were re-extracted with half volume (of original chromatin aliquots) of 0.25N HCl. The combined acid extracts were neutralized with 5N NaOH. This fraction is referred to as "acid-soluble chromatin proteins". To the acid-extracted pellets, 1-2 volumes of 0.1N NaOH was added to dissolve the remaining proteins. After standing at room temperature overnight, the alkali extracts were clarified by centrifugation (15,000 x g, 10 min). The resulting supernatants were termed "acid-insoluble chromatin proteins". Protein was determined by the method of Lowry *et al.* (21) using unfractionated rat thymus histones as standard for acid-soluble protein and serum albumin for acid-insoluble protein.

RESULTS

The preparation and properties of chromatin: The majority of testicular cell nuclei sedimented through 2.2M sucrose-TKM. Upon examination after staining with 1% methylene blue, the sediment was found to consist primarily of germ cell nuclei. On the other hand, Sertoli cell nuclei, characterized by the presence of the tripartitate nucleolus,

[b] 0.01XSSC = 1.5mM NaCl, 0.15mM Na-citrate, pH 7.0

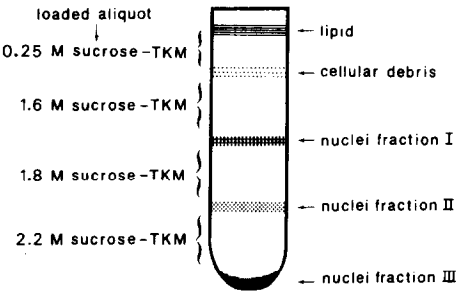

Fig. 1: The sedimentation scheme of cultured Sertoli cell nuclei on a discontinuous sucrose gradient. For details see text.

did not sediment to the bottom of the centrifuge tube. Fig. 1 shows a schematic representation of the nuclear sedimentation pattern on the discountinuous sucrose gradient. Nuclear fraction I and II contained predominantly Sertoli cell nuclei, while fraction III consisted primarily of germ cell nuclei.

The sedimentation pattern and purity of the Sertoli cell nuclei varied as a function of culture age. When a nuclear preparation of freshly isolated Sertoli cells was sedimented through the gradient, nuclei containing > 85% Sertoli cell nuclei accumulated primarily in fraction I and a thick pellet of germ cell nuclei was found at the bottom of the gradient as fraction III. When the isolated Sertoli cells were cultured for 1 day, approximately 2/3 of the Sertoli cell nuclei (> 90% purity) sedimented as fraction I, 1/3 as fraction II, and a substantially diminished quantity of germ cell nuclei settled as fraction III. After the Sertoli cells were cultured for 2 days, only 1/3 of the Sertoli cell nuclei (> 95% purity) sedimented as fraction I, 2/3 settled as fraction II, and a barely discernable pellet of germ cell nuclei settled as fraction III. If Sertoli cells were cultured for 3 to 5 days, only traces of nuclei were observed in fractions I and III; the majority of the Sertoli cell nuclei of greater than 95% purity accumulated in fraction II. The purity of Sertoli cell nuclei was estimated as the percentage of nuclei which could be recognized as Sertoli cell nuclei at 625-1000 times magnification.

The chromatin preparations isolated from rat thymus, liver, and total testis exhibited an acid-soluble protein to DNA ratio close to 1 (Table I). In order to achieve an acid-soluble protein to DNA ratio close to 1 for chromatin of germ cells and 2 day-cultured Sertoli cells, an additional purification step through 1.7M sucrose was required.

Thymus chromatin exhibited an acid-insoluble protein to DNA ratio of considerably less than 1, while the ratio in the chromatin from germ cells and 2 day cultured Sertoli cells exceeded 2. Further purification of the two chromatin preparations through 1.7M sucrose did not reduce the acid-insoluble protein to DNA ratio.

The interaction of testicular androgen-receptor complex with chromatin acceptor sites: When ^{3}H-testosterone-receptor complex was electrophoresed on agarose-polyacrylamide gels, the radioactive macromolecule moved slower than androgen binding protein (ABP). Incubation of the ^{3}H-testosterone-receptor

TABLE I

CHEMICAL COMPOSITION OF CHROMATIN PREPARATIONS RELATIVE TO DNA CONTENT

Chromatin Source	DNA	Acid-Soluble Protein	Acid Insoluble Protein
Thymus	1	0.98	0.3
Liver	1	1.0	1.2
Total Testis	1	1.0	1.1
Freshly isolated Sertoli cells[a]	1	1.0	1.4
Germ cells[b]	1	1.1	2.2
2-day cultured Sertoli cells[b]	1	1.2	2.2

a. Chromatin did not pass through 1.7M sucrose

b. Chromatin sedimented through 1.7M sucrose

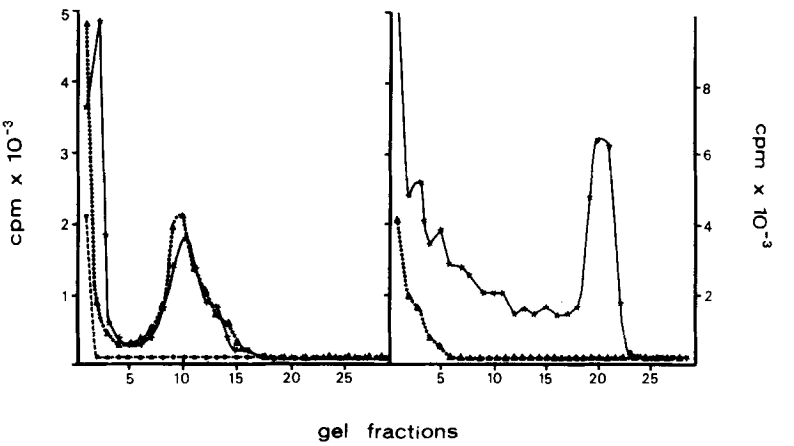

Fig. 2: The electrophoretic patterns of ^{3}H-testosterone-receptor complex and ^{3}H-testosterone-ABP on 0.5% agarose-3.3% polyacrylamide gels (22,23). The left panel shows the pattern of ^{3}H-testosterone-receptor complex: (★—★) ^{3}H-testosterone-receptor complex, (▲....▲) ^{3}H-testosterone-receptor complex incubated with 100 x excess testosterone at 4°C for 1 hr, (●---●) ^{3}H-testosterone-receptor complex after heating at 50°C for 30 min and followed by centrifugation at 20,000 x g for 10 min. The right panel shows the pattern of rat epididymal ^{3}H-testosterone-ABP on the gels: (★—★) ^{3}H-testosterone-ABP, (▲....▲) ^{3}H-testosterone-ABP after incubation with 100 x excess of testosterone at 4°C for 1 hr. Gels were electrophoresed for 2 hr at 0° and 1.5 mA/gel, sliced, and counted in a toluene-based fluor.

complex at 50°C for 30 min abolished the receptor peak but not the ABP peak (Fig. 2). These data indicate that the androgen receptor-complex preparation was free of contamination with ABP (22).

Fig. 3 demonstrates that Sertoli cell and germ cell chromatin bound 3 to 5 times as much testosterone receptor complex as did thymus or liver chromatin. Fig. 4a also shows that chromatin of 5 day cultured Sertoli

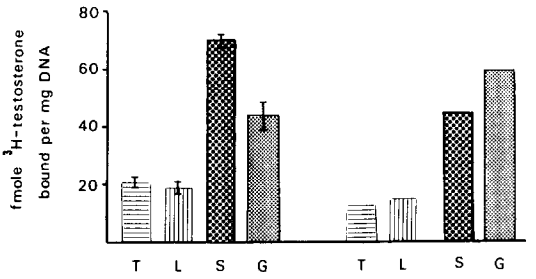

Fig. 3: The incubation conditions were as described in Methods. T = thymus chromatin, L = liver chromatin, S = Sertoli cell chromatin and G = germ cell chromatin. The left set of experiments were performed in 1.5 ml of reaction media containing 200 μg DNA of chromatin and 67 fmole of testosterone-[1,2,6,7,-^{3}H (N)]-receptor complex. The germ cells were isolated from 33 day old Long-Evans rats while all the other tissues were from 30-31 day old Sprague-Dawley rats. The right set was performed in 1 ml media containing 100 μg DNA and 30 fmole of testosterone [1α, 2α-^{3}H (N)]-receptor complex.

cells bound to 4 times as much receptor complex as did thymus chromatin for the ^{3}H-testosterone-receptor complex. The Scatchard plots derived from the data in Fig. 4a (Fig. 4b) indicate that there are at least two binding components in the chromatin for testicular androgen receptor complex. The component with higher binding affinity has a Kd of approximately 10^{-11}M. The data in Fig. 4a and b suggest that both thymus or liver chromatin also contained a small but detectable number of acceptor sites for the androgen-receptor complex.

DISCUSSION

It has been demonstrated in our laboratory (23) that cytosol prepared from cultured Sertoli cells contains an androgen-receptor distinguishable from androgen-binding protein (ABP) on the basis of size, electrophoretic mobility and heat stability. In addition, direct labeling and nuclear exchange techniques have also demonstrated specific androgen retention in nuclei of both Sertoli cells and germ cells (17,23). The present observation of the binding of ^{3}H-testosterone-receptor complex to both Sertoli cell and germ cell chromatin constitute further evidence to support the suggestion that these cells represent testosterone-responsive target cells.

Although results from previous studies (12) suggest that a tightly bound, nonhistone chromosomal protein fraction, NP, is responsible for the interaction with androgen-receptor complex in rat testis, it is important to point out that the Sertoli cell chromatin was probably not included in the chromatin preparation made by these authors. The present study has demonstrated (Table I) that the purification of testicular nuclei by centrifugation through 2.2 M sucrose completely excludes Sertoli cell nuclei from the sedimented nulcear pellet. Sertoli cell nuclei did not sediment in any sucrose solution more dense than 1.8 M, even when total testis was used as the source. The reason for the decrease in the number of germ cell nuclei in fraction I, II, and III (Fig. 1) with respect to days of culture could be the fact that germ cells do not attach to the surface of the culture dishes (24) and are rinsed off from the attached Sertoli cells.

Prostate and whole testis chromatin were shown by Kleyzsejko-Stefanowicz *et al.* (12) to bind twice the amount of androgen-receptor complex as that bound by chromatin prepared from pancreas or liver chromatin.

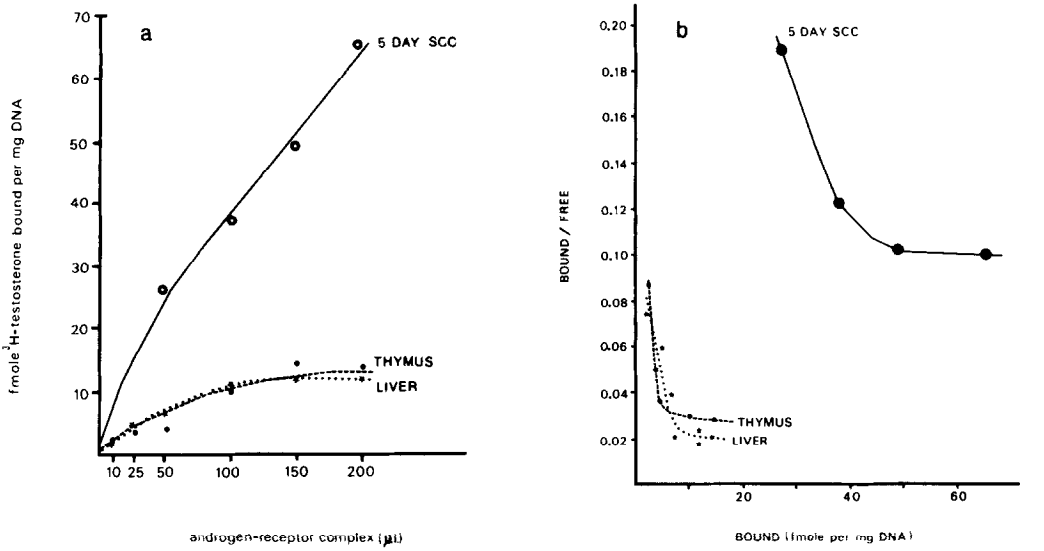

Fig. 4: (a) Sertoli cells were cultured for 5 days. The medium was changed after 2-3 days, Sertoli cells, thymus and liver were obtained from the same animals (11 day posthypophysectomy, operated at 65 day of age). The binding activity was assayed in 1.5 ml incubation media containing 100 μg DNA. Every 100 μl of androgen-receptor complex contained 35.3 fmole of testosterone-[1α, 2α - ^{3}H (N)]. (b) Scatchard plates of Fig. 4a.

The present study shows that germ cell and Sertoli cell chromatin preparations exhibit even higher binding for testicular androgen-receptor complex, 3-5 times that of chromatin from thymus or liver (Fig. 3a). This may reflect the enhanced enrichment of target cells in the preparations.

The accumulation of labeled androgen in Sertoli cell nuclei exhibited a Kd of 2.5 x 10^{-9} M and n in the range of 0.1-0.3 pmole per mg DNA (23). The Scatchard analyses of several experiments in addition to that shown in Fig. 4b give approximate Kd and n values in the range of 10^{-11}-10^{-10} M and 0.04-0.15 pmoles per mg DNA respectively. However, a series of more vigorously controlled experiments at constant total protein concentration (25) is in progress in order to calculate the binding characteristics with greater precision.

The data in figure 4a and 4b suggest that both androgen-responsive cells and non-responsive cells contain acceptor sites for the androgen receptor complex but in different proportions. These observations are in agreement with the findings in other systems (26).

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