

Immunohistochemical Localization of Androgen Receptors in the Rat Testis: Evidence for Stage-Dependent Expression and Regulation by Androgens*

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ABSTRACT

Androgens are essential for the maintenance of normal spermatogenesis in the rat. We assessed the sites, developmental pattern, and hormonal control of androgen receptors (AR) in the rat testis. Adult male rats were studied after 1) no treatment; 2) ethane dimethane sulfonate (EDS), which eradicates Leydig cells and endogenous testosterone (T); 3) EDS plus T replacement beginning at the time of EDS administration; or 4) methoxyacetic acid, which leads to the loss of specific germ cell types. Testes were also obtained from normal immature rats (aged 5, 14, 16, 21, 28, 31, 35, 38, and 45 days). After microwave antigen retrieval, immunohistochemistry was performed using a rabbit polyclonal antibody (Novocastra) raised against a peptide unique to the N-terminal region of the AR and detection with biotinylated swine antirabbit immunoglobulin G, avidin-biotin complex/alkaline phosphatase, and nitroblue tetrazolium salt (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP) substrate. In adults, nuclear immunostaining of Sertoli cells (SC) increased progressively in intensity from stages II through VII of the spermatogenic cycle, and then declined precipitously during stage VIII to become barely detectable in stages

IX–XIII. Prominent AR immunostaining was also evident in peritubular myoid cells, arterioles, and interstitial cells; staining in these cells did not vary with the stage of the cycle of the adjacent tubules. EDS caused a severe loss of AR immunostaining in all cell types. Replacement of T in EDS-treated animals resulted in a pattern of AR immunostaining comparable to that in controls, although staining intensity was reduced. Methoxyacetic acid administration did not affect the pattern of AR staining. In immature rats, peritubular myoid cell immunostaining was prominent from day 5; SC staining was detectable on day 5, increased in intensity with age, and became stage dependent between days 21–35.

The following conclusions were reached. 1) Immunohistochemically detectable AR expression in SC occurs predominantly in stages II–VII of the spermatogenic cycle, with highest levels at stage VII. 2) AR immunostaining is also prominent in peritubular myoid cells, arterioles, and Leydig cells (but not in germ cells), but is unrelated to the stage of adjacent tubules. 3) Endogenous T and/or its metabolites control the expression of AR in the testis. 4) AR immunostaining is detectable by day 5 of age and becomes stage specific in SC between days 21–35. (*Endocrinology* 135: 1227–1234, 1994)

ANDROGENS are essential for the maintenance of normal spermatogenesis in the rat (1). Recent work (1, 2) demonstrated that there are marked differences in androgen action on seminiferous tubules at different stages of the spermatogenic cycle. For example, protein production by tubules at stages VI–VIII is much more affected by androgens than production from tubules at other stages (2). Earlier work had shown that changes in cell morphology in response to T deprivation were more pronounced in tubules at stage VII than in tubules at other stages (3, 4). As presumably all tubules in the testis are bathed in the same local concentration of T, it seems likely that tubules at different stages have the inherent capacity to respond differently to the same amount of T. Androgen action in the testis, as in other tissues, requires the androgen receptor (AR) to mediate transcrip-

tional activation (5). Therefore, one potential mechanism for the tubular specificity of response to androgens is differential expression of the AR. In this study, we examined the stage specificity of AR expression in the rat testis.

The molecular cloning of the rat AR (6, 7) allowed deduction of the amino acid sequence of the receptor and synthesis of unique peptide segments of this protein. Such peptides have then been used to generate specific antibodies, which are useful in immunohistochemistry (8, 9). Previous work demonstrated AR localization by immunohistochemistry in Sertoli cells (SC), peritubular myoid cells, and interstitial cells of the rat testis (8, 9). However, these studies have not reported stage specificity of AR expression, perhaps because technical aspects of fixation, tissue preparation, and staining did not allow precise evaluation. Both perfusion-fixation and microwave preparation of tissue, as used in our studies, allow considerably greater accuracy in assessment of AR expression. The results of our work suggest that the stage specificity of the effects of androgen may be due to stage-specific expression of the AR. We have also demonstrated that the stage specificity is acquired during normal development as spermatogenesis matures, and that AR expression is maintained by endogenous testicular androgens.

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Materials and Methods

Animals and treatments

Adult male Sprague-Dawley rats (aged 90–120 days), obtained from our colony (MRC Laboratories, Edinburgh, Scotland), were maintained under standard animal house conditions. Animals ($n = 3\text{--}4/\text{group}$) were studied after 1) no treatment; 2) ethane dimethane sulfonate (EDS; 75 mg/kg, 6 days before death); 3) EDS as above, plus testosterone (T) esters (Sustanon, Organon Laboratories, Cambridge, UK; 25 mg in 0.1 ml arachis oil) at the time of EDS injection and 3 days later; and 4) methoxyacetic acid (MAA; 650 mg/kg, orally, 1, 3, 7, 14, 21, and 28 days before death). EDS at the dose used destroys all Leydig cells within 3 days (10) and reduces serum and testicular T to levels that are undetectable by RIA (4). T at the dose used is known to maintain spermatogenesis quantitatively in EDS-treated rats (11). MAA is a selective germ cell toxicant that leads to selective loss of pachytene and later spermatocytes at all stages of the spermatogenic cycle other than early to midstage VII (12). In MAA-treated rats, spermatogenesis proceeds with normal kinetics, such that at certain times after treatment, specific germ cell types are absent because of maturation depletion (e.g. round spermatids on days 7–14 and elongate spermatids on days 21–28).

Animals were anesthetized with ether, then perfused via the thoracic aorta with physiological saline containing 0.01% heparin until testicular blood vessels cleared, then with Bouin's fixative for 30 min (13). The testes were removed, decapsulated, and cut into 0.5-cm transverse sections. The tissue slices were postfixed in Bouin's for a further 5 h, rinsed, and stored in 70% ethanol before processing into paraffin wax. Immature male Sprague-Dawley rats (aged 5, 14, 16, 21, 23, 28, 31, 35, 38, and 45 days) obtained from the same colony as the adults were killed by carbon dioxide inhalation and cervical dislocation. Testes were removed and immersion fixed in Bouin's solution for 5 h, decapsulated, and cut into 0.5-cm transverse sections. They were then rinsed and stored in 70% ethanol before processing into paraffin wax.

Immunohistochemistry

Tissue was processed on an automatic tissue processor using a standard 20-h cycle (14). Sections (5 μm) were cut and mounted on slides coated with 3-aminopropyl triethoxy-silane (Sigma Chemical Co., St. Louis, MO). Slides were dried overnight at 50 C, then dewaxed with Histoclear and rehydrated in graded ethanol before washing in water. Sections were then microwaved four times (5 min each) at full power (650 watts) under 0.01 M sodium citrate buffer (pH 6.0), after which they were allowed to stand for 20 min in a microwave oven (15). Sections were then washed twice (5 min each) in Tris-buffered saline (TBS; 0.05 M, pH 7.4, and 0.85% NaCl) and blocked (20 min) with normal swine serum diluted 1:5 in TBS. The primary antibody was a rabbit polyclonal (Novocastra, Newcastle-on-Tyne, England) raised against the first 17 amino acids of the N-terminal region of the AR. This antibody was diluted 1:20 in normal swine serum-TBS (see above), and sections were incubated under plastic coverslips overnight at 4 C. Control sections were incubated either with nonimmune rabbit serum, diluted to the same extent as the first antibody, or a 20-fold excess of free peptide.

Coverslips were removed, and the sections were washed in TBS twice (5 min each), then incubated for 30 min with biotinylated swine anti-rabbit immunoglobulin (Dako, Glostrup, Denmark) diluted 1:500 in normal swine serum-TBS (see above), followed by two washes of 5 min each in TBS. Sections were then incubated in a solution of alkaline phosphatase conjugated to avidin-biotin (Dako) for 30 min, washed in TBS buffer (twice, 5 min each), and then washed in Tris buffer [100 mM (pH 9.5), 100 mM NaCl, and 50 mM MgCl₂] for 5 min. Sections were finally incubated in nitro-blue tetrazolium (337.5 $\mu\text{g}/\text{ml}$), 5-bromo-4-chloro-3-indolylphosphate (175 $\mu\text{g}/\text{ml}$), and levamisole (0.001 M) in 10 ml Tris-MgCl buffer for 30–120 min (16). Counterstaining was performed with 0.5% aqueous light green or Harris's hematoxylin. Sections were dehydrated in absolute ethanol and cleared in xylene before coverslipping with Pertex mounting medium (Cell Path, Hemel, Hempstead, UK).

To confirm the specificity of AR localization, some sections were double immunostained for sulfated glycoprotein-1 (SGP-1) and AR. SGP-1 is a known SC cytoplasmic protein (17). After AR was localized

as described above, sections were washed overnight at 4 C in Tris (100 mM, pH 8.0) and EDTA, blocked with normal swine serum as described above, then incubated with rabbit anti-SGP-1 (supplied courtesy of S. Sylvester). Staining was carried out as described above until after the alkaline phosphatase-avidin-biotin complex; sections were washed (twice, for 5 min each) in TBS, then incubated with vector red substrate (Vector Laboratories, Burlingame, CA) for 5–10 min before counterstaining with Harris's hematoxylin. Sections were rehydrated in absolute ethanol, cleared in xylene, and coverslipped.

Quantitation of AR immunostaining

The optical density of SC nuclei was determined using an Olympus BH-2 microscope (Olympus Corp., New Hyde Park, NY) fitted with a constant illuminating light source and a Sony CCD video camera (supplied by Olympus Corp.). Video images were captured using a Quick Capture Frame Grabber card (Data Translation) fitted to a Macintosh IIcx computer with color monitor (Apple Computer Inc., supplied by Scotbyte, Livingston, UK). Quantitation of immunostaining intensity was performed using the uncalibrated optical density function [OD = $10/\log(255/255 - \text{pixel value})$] of NIH Image 1.52 (18). Briefly, the illumination was standardized between slides from the same immunostaining experiment by calibrating the light intensity to a predetermined uncalibrated OD using a part of the slide containing no tissue section. After standardization of the slide illumination, SC nuclei were delineated, and the uncalibrated OD was measured. The background or baseline was determined for each section by calibrating the illumination, as described above, and then measuring, at random, 20 stage VII SC nuclei from a corresponding tissue section incubated with nonimmune serum. Background values were subsequently subtracted from the mean OD values measured for each tubule. For each animal, 5 tubules in each group of the following stages of the spermatogenic cycle were assessed (I–II, III–IV, VII, VIII–IX, and XII). A mean value for each tubule cross-section was derived from the measurement of 10 SC nuclei, and data were computed as the mean \pm SD for 5 tubules. To avoid errors arising from differences between experiments, sections from a single animal in each of the treatment groups were analyzed and compared, as described above. This procedure was repeated using tissue sections from different animals, and comparable results were obtained. Statistical significance of differences in OD values was assessed by analysis of variance with repeated measures.

Results

Normal adults

In the testis, immunohistochemical localization of AR expression was prominent in nuclei of SC, peritubular myoid cells, and interstitial cells presumed to be Leydig cells (Figs. 1 and 2); the nuclei in small arterioles also exhibited prominent immunostaining (not illustrated). In SC, however, a striking finding was that AR localization was specific to tubules in spermatogenic stages II–VII, with occasional weak staining in stages I and VIII–XIV (Figs. 1 and 2). Quantitation of the intensity of SC expression of the receptor in tubules in various stages confirmed the visual impression (Fig. 3). The optical density of SC nuclei in tubules at stage VII was greatest, followed by earlier stages (III–IV), with lowest levels in stage XII. Combined staining of AR and SGP-1 confirmed the SC localization of the AR (Fig. 2b). No clear immunohistochemical detection of AR could be detected in germ cells or residual bodies (Fig. 2c). AR was localized prominently in peritubular myoid cells, interstitial cells, and small arterioles. In these cells, staining did not vary with the stage of the adjacent tubules (Figs. 1 and 2). In testicular sections preincubated with an excess of the immunogen, no staining was evident (Fig. 1d). In the prostate, intense AR staining was

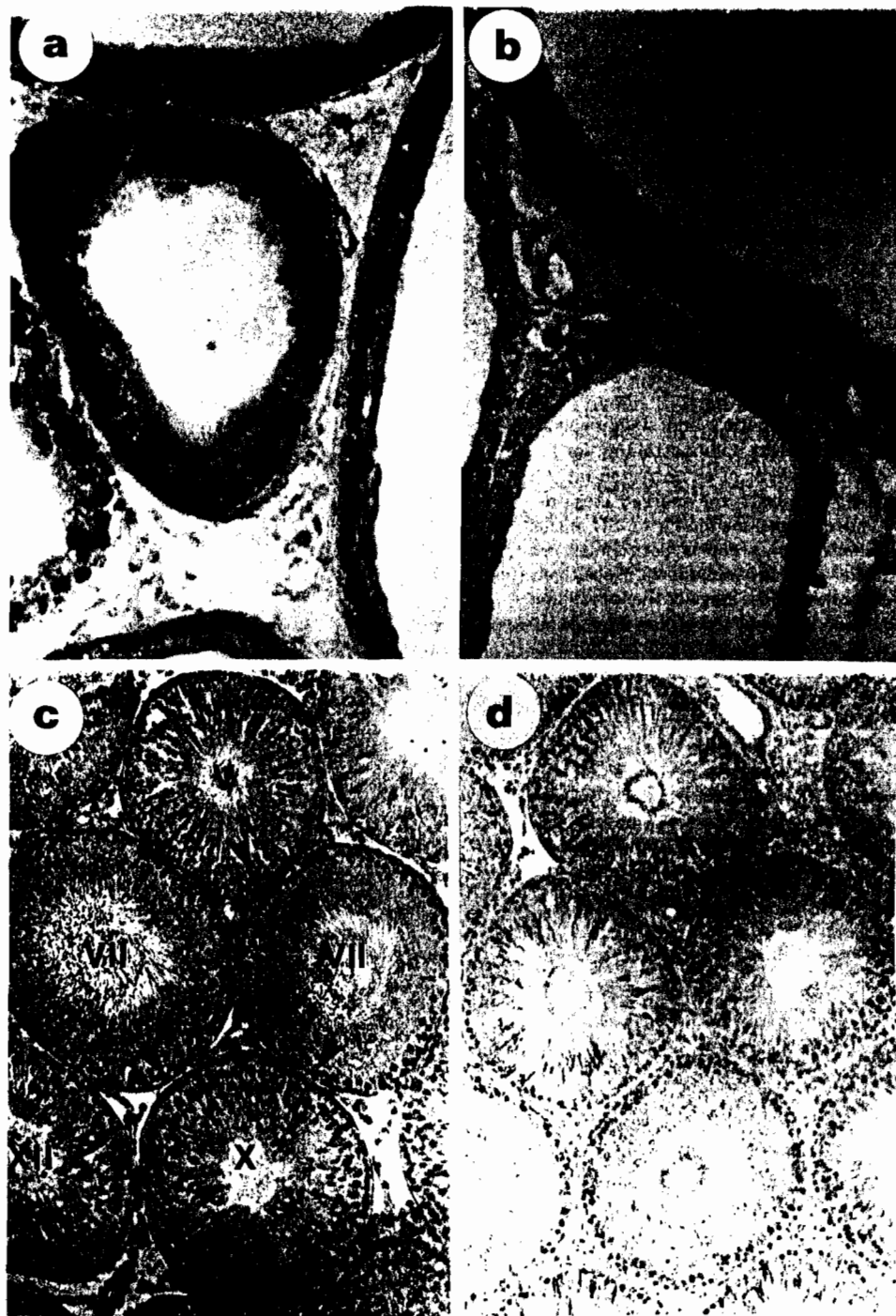


FIG. 1. Immunohistochemical localization of the AR in the prostate gland (*top*) and testis (*bottom*) of an untreated adult rat perfusion-fixed with Bouin's fixative. Note the pronounced nuclear staining of epithelial cells in the prostate (a) and of SCs (*arrowheads*) in the testis (c) when sections were incubated with an anti-serum to the AR and the absence of staining when sections were incubated with nonimmune serum (b) or preincubated with an excess of the peptide to which the antiserum was raised (d). Note also the marked stage dependence of immunostaining of SC nuclei in panel c (roman numerals indicate the stage). Magnification: a and b, $\times 225$; c and d, $\times 90$.

found in the nuclei of epithelial cells (Fig. 1a). This staining was absent in sections incubated with nonimmune serum (Fig. 1b).

EDS and EDS plus T replacement

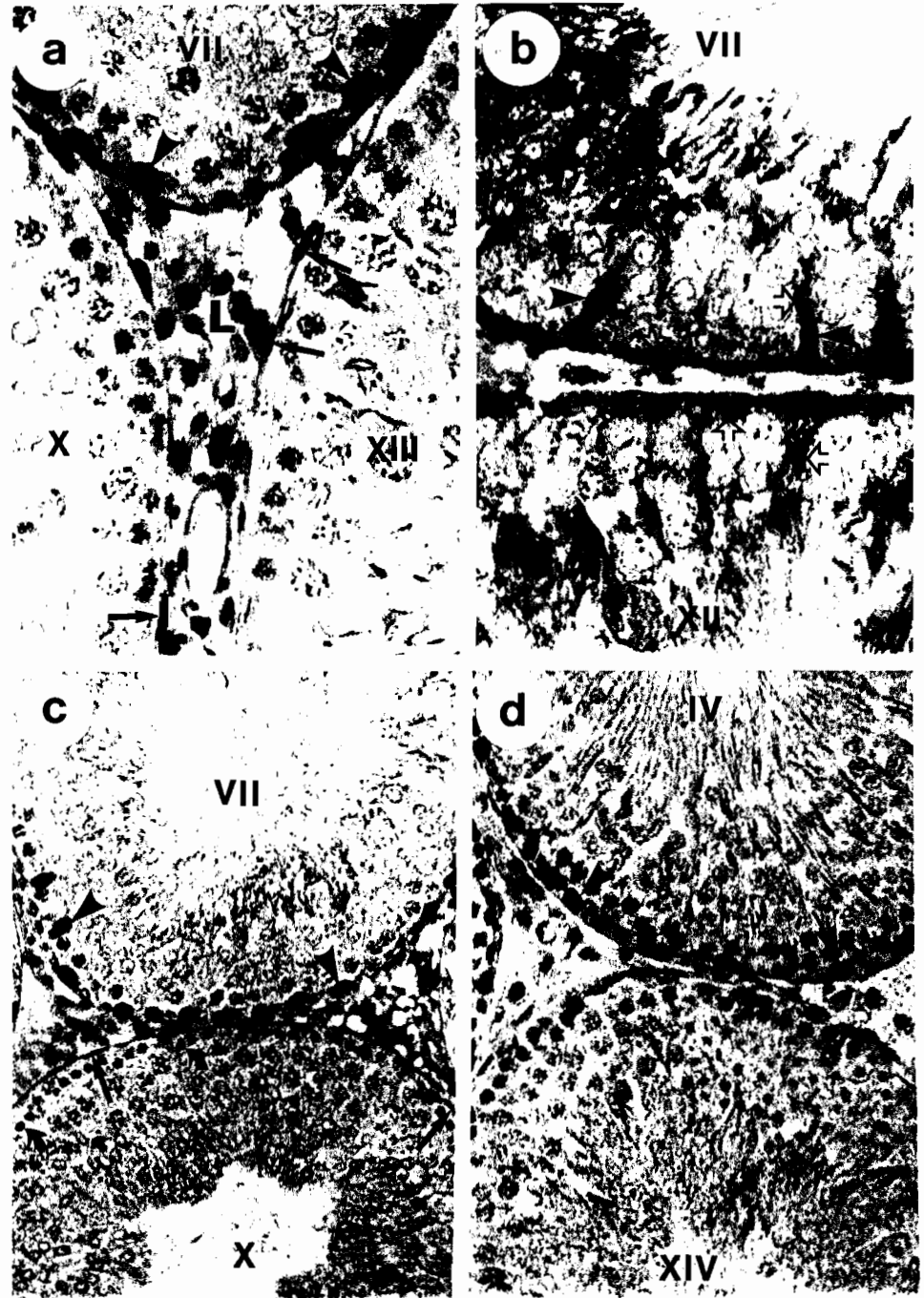
Removal of endogenous androgens by EDS treatment 6 days previously led to the virtual loss of nuclear immunostaining in the testes (Figs. 3 and 4). Replacement of T, beginning at the time of EDS treatment, led to the return of

AR, with a pattern of localization indistinguishable from that in normal animals (Figs. 3 and 4). However, quantitation of immunostaining in SC nuclei at the different stages of the spermatogenic cycle revealed that the level of immunostaining was reduced by approximately 30% in stages I-VII.

MAA administration

Administration of MAA 3, 7, 14, 21, or 28 days before death led to the specific loss of pachytene spermatocytes (day 3), early spermatids (days 7 and 14), or late spermatids

FIG. 2. Distribution and stage dependence of nuclear immunostaining for the AR in the testis of an untreated rat. All panels illustrate the marked stage-dependent immunostaining of SC nuclei (*arrowheads*), whereas peritubular cell nuclei (*arrows*) immunostain positively regardless of the stage of the tubule that they surround (a and c). Leydig cells (L) also demonstrate pronounced immunostaining. b, Double immunostaining for the AR (*arrowheads*) and the SC glycoprotein SGP-1 (*open arrowheads*) in tubules that are either expressing (stage VII) or not expressing (stage XII) the AR. c, *Curved arrows* indicate residual bodies in which there was no immunostaining. Magnification: a and b, $\times 360$; c and d, $\times 225$.



(days 21 and 28). The absence of these germ cell types did not affect AR immunolocalization (Fig. 4), and the pattern of immunostaining of SC nuclei exhibited the same stage specificity as that in control animals.

Immature animals

AR immunostaining on day 5 was most intense in peritubular myoid cells, although there was detectable staining in

SC and interstitial cells (Fig. 5a). The intensity of immunostaining in SC increased with the age of the animal from days 5–38. SC immunostaining showed no obvious difference between tubules at 5 or 14 days of age, but differences between tubules were apparent at 28 days of age and thereafter became progressively more pronounced, such that by 45 days of age, a stage-dependent pattern similar to that in the adult was evident (Fig. 5).

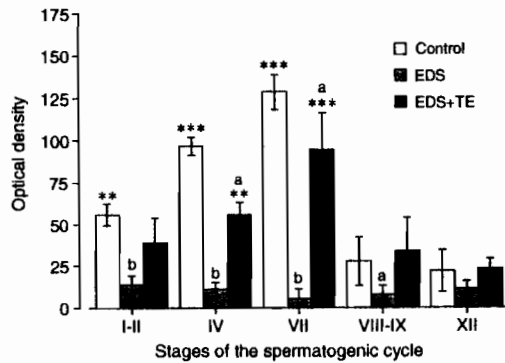


FIG. 3. Quantitative analysis of the intensity of SC nuclear immunostaining for the AR at different stages of the spermatogenic cycle in a control rat or a rat treated 6 days earlier with EDS alone or EDS supplemented with T esters (EDS + TE). Data are the mean \pm SD for 5 tubules/stage(s) and were based on the analysis of 10 nuclei/tubule. Tissue sections from each animal were processed in parallel for immunostaining to avoid interexperiment error. Comparable results were obtained in a separate experiment involving tissue from different animals. **, $P < 0.01$; ***, $P < 0.001$ (compared with values for stage XII in the same treatment group). a, $P < 0.05$; b, $P < 0.001$ (compared with respective control values for that stage).

Discussion

We have demonstrated that immunohistochemically detectable AR expression in SC is found only in specific stages of the spermatogenic cycle in the rat. AR were found in SC of tubules at stages II–VII, with greatest intensity in stage VII. Occasionally, weak staining was seen in stages I and VIII–XIV. Stage-dependent differences in immunostaining for the AR have been referred to previously (18), but were not detailed. Our findings are of considerable interest because the earliest effects of androgen withdrawal in the rat occur at stage VII (1–4). For example, histologically, the earliest changes observed after androgen withdrawal are noted in stages VII–VIII (2, 3); these effects can be prevented by T replacement (11). More recently, it has been shown that the secretion of specific proteins and overall protein secretion are controlled by T specifically at stages VI–VIII of the spermatogenic cycle (2). Our findings suggest that a mechanism underlying these stage-specific effects of androgens could be the stage-specific expression of the AR.

The reason for the stage-specific pattern of AR immunolocalization is unknown. FSH has been reported to increase AR [protein and messenger RNA (mRNA)] within 3–4 days in immature SC studied *in vitro* (19, 20). The stimulatory effect of FSH on AR binding was mimicked by (Bu)₂cAMP (19). It is of interest that FSH-stimulated cAMP accumulation has been reported to peak at stage IV (21), the cAMP response element-binding protein (CREB) mRNA has been shown to reach highest values in SC at stage V, and the CREB protein itself reaches peak levels in spermatids at stages VI–VIII (22). Other studies on expression of CREB mRNA report highest levels around the base of seminiferous tubules at stages VII–VIII, a pattern of expression that is androgen dependent (23). FSH binding (24) and FSH receptor mRNA (25) have been reported to peak at stage I. It is interesting to speculate, therefore, that FSH action in the stages preceding stage VII

leads to maximal AR levels by stage VII. In this way, FSH could stimulate increased AR in the specific stage at which androgens appear to exert their maximal effects. This hypothesis could also help to explain the interactive stimulatory effects of FSH and T on spermatogenesis and the findings that both FSH and T (LH) are required for quantitatively normal spermatogenesis in several species, including man (1, 26).

Our findings on the stage specificity of AR immunolocalization do not agree with those of an earlier study in rats of the ability of nuclei extracted from seminiferous tubules at various stages to bind [³H]methyltrienolone *in vitro* (27). In that study, higher levels of ligand binding were found in stages IX–XII and XIII–I than in stages II–VI or VII–VIII. The reasons for the difference between that study and ours are not clear. It is possible that variable numbers of nuclei were extractable at various stages of the cycle or that extraction of receptor protein from nuclei varied with the cycle in the earlier study. A recent study of human testes (28), using a polyclonal antibody raised against recombinant 55-kilodalton recombinant human AR, reported immunolocalization of AR to spermatogonia and spermatocytes in addition to the cell types in which we detected AR in the rat testis. However, the quality of preservation of testicular tissue in this study was suboptimal. Using our methodology, we have been unable to detect any convincing immunostaining for AR in germ cells in the rat (this study) or in the ram, human, or monkey (unpublished data). In the latter species, the pattern of AR immunostaining was comparable to that shown presently for the rat.

Withdrawal of T is known to lead to disruption of spermatogenesis (4, 10). We, therefore, tested the effects of T withdrawal, using EDS, and T replacement on AR immunolocalization. The ability to detect AR immunohistochemically was lost when endogenous androgens were removed by EDS treatment. Replacement of T, beginning at the time of EDS administration, led to a return of AR staining in a pattern indistinguishable from that in control animals. These results demonstrate that T can control the immunodetectability of its receptor in the testis. This could be due to T decreasing the rate of degradation of the AR (29), controlling the synthesis of its own receptor, for which there is previous evidence in prostate and other tissues (30, 31), or because the binding of T leads to a conformational change in the AR or displacement of a binding protein (32) such that an epitope is exposed to be bound by the antibody. Although the pattern of AR staining was very similar when comparing EDS- plus T-treated animals with normal controls, the quantitative levels of staining were somewhat lower in the former. This could be because FSH levels are suppressed by T replacement (1, 4); if FSH is important in maintaining normal AR expression, low FSH levels could lead to lower levels of AR immunostaining than those in controls.

There is accumulating evidence that where stage-dependent differences are found in the expression of particular genes and their protein products in SC, a major factor regulating these differences is the changing germ cell complement in the various stages (1). MAA has been used extensively to

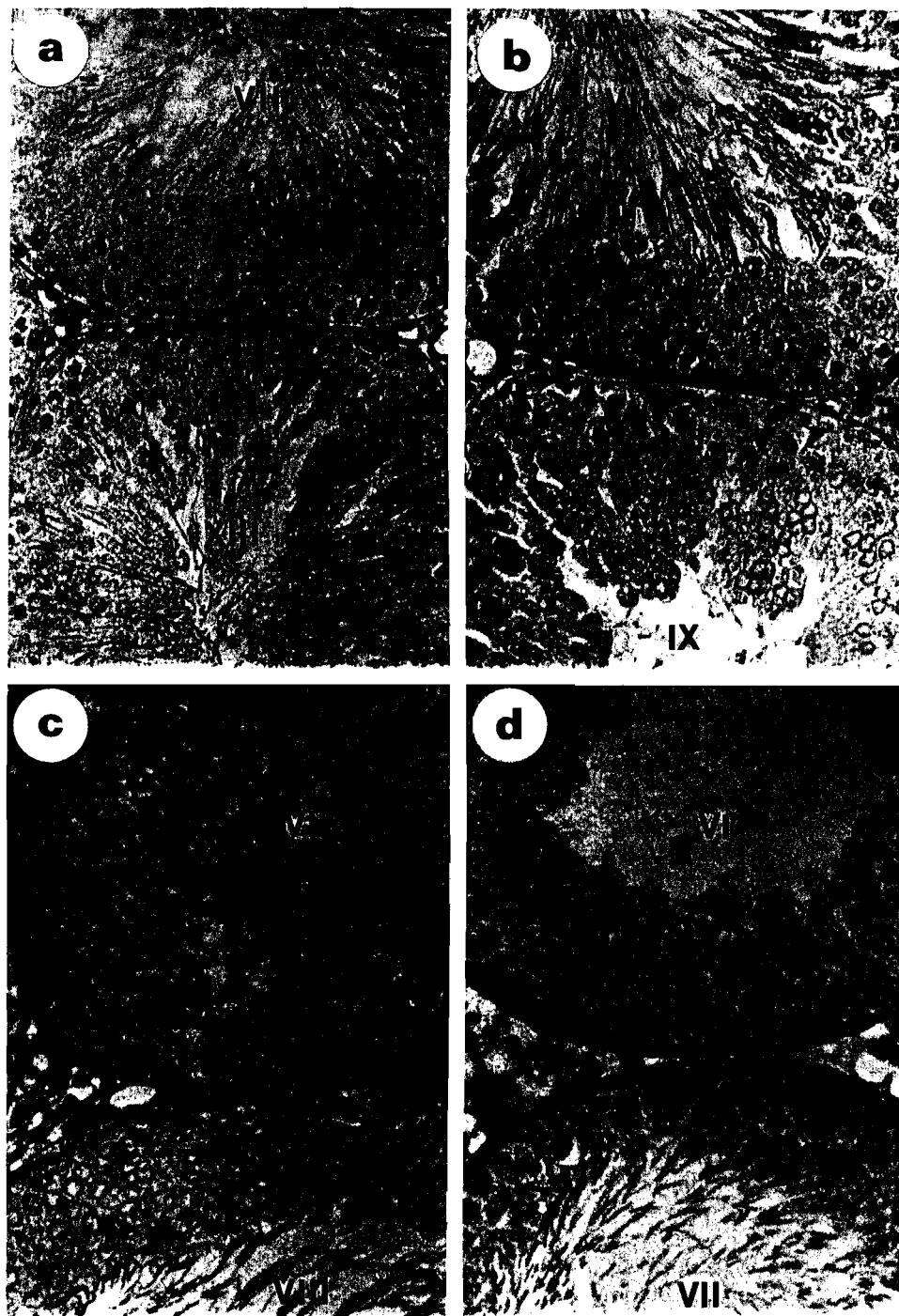


FIG. 4. Effect of EDS-induced T withdrawal (a), EDS treatment plus T administration (b), or MAA-induced depletion of specific germ cell types (c and d) on nuclear immunostaining for the AR in SCs (arrowheads) and peritubular myoid cells (arrows). Note the complete absence of immunostaining in a (open arrowhead shows a SC nucleus), but the restoration of a normal stage-dependent pattern of SC immunostaining when T was administered from day 0 to an EDS-treated rat shown in b (curved arrows = residual bodies). Selective depletion of pachytene spermatocytes from a stage VII tubule 3 days after MAA treatment (c) or depletion of round spermatids from a stage VII tubule and depletion of elongate spermatids from a stage VI tubule (both in d) 21 days after MAA treatment failed to alter the normal stage-dependent pattern of SC immunostaining for the AR. Magnification, $\times 225$.

cause selective maturation depletion of specific germ cell types at various times after administration (12, 33). This technique has successfully demonstrated that the production of several proteins by the SC is controlled by their adjacent germ cell population (12, 33, 34). However, in the present study we were unable to demonstrate that selective depletion of pachytene spermatocytes, round spermatids, or elongating spermatids led to any major change in AR immunolocalization. This, of course, does not eliminate the possibility that germ cells are involved in the local control of AR expression.

Immature animals demonstrated clear AR immunostaining

from day 5 of age. On day 5, staining was most intense in peritubular myoid cells, although there was detectable immunostaining in SC. Thereafter, SC immunostaining increased in intensity with age and became stage specific, with the maturation of spermatogenesis between days 21–35. The increase in AR staining with maturation is consistent with previous studies of AR using other techniques (35).

Our findings of marked stage-dependent differences in AR immunolocalization suggest that availability of the AR could be a critical factor in determining the stage specificity of androgen action in the rat testis. This seems particularly

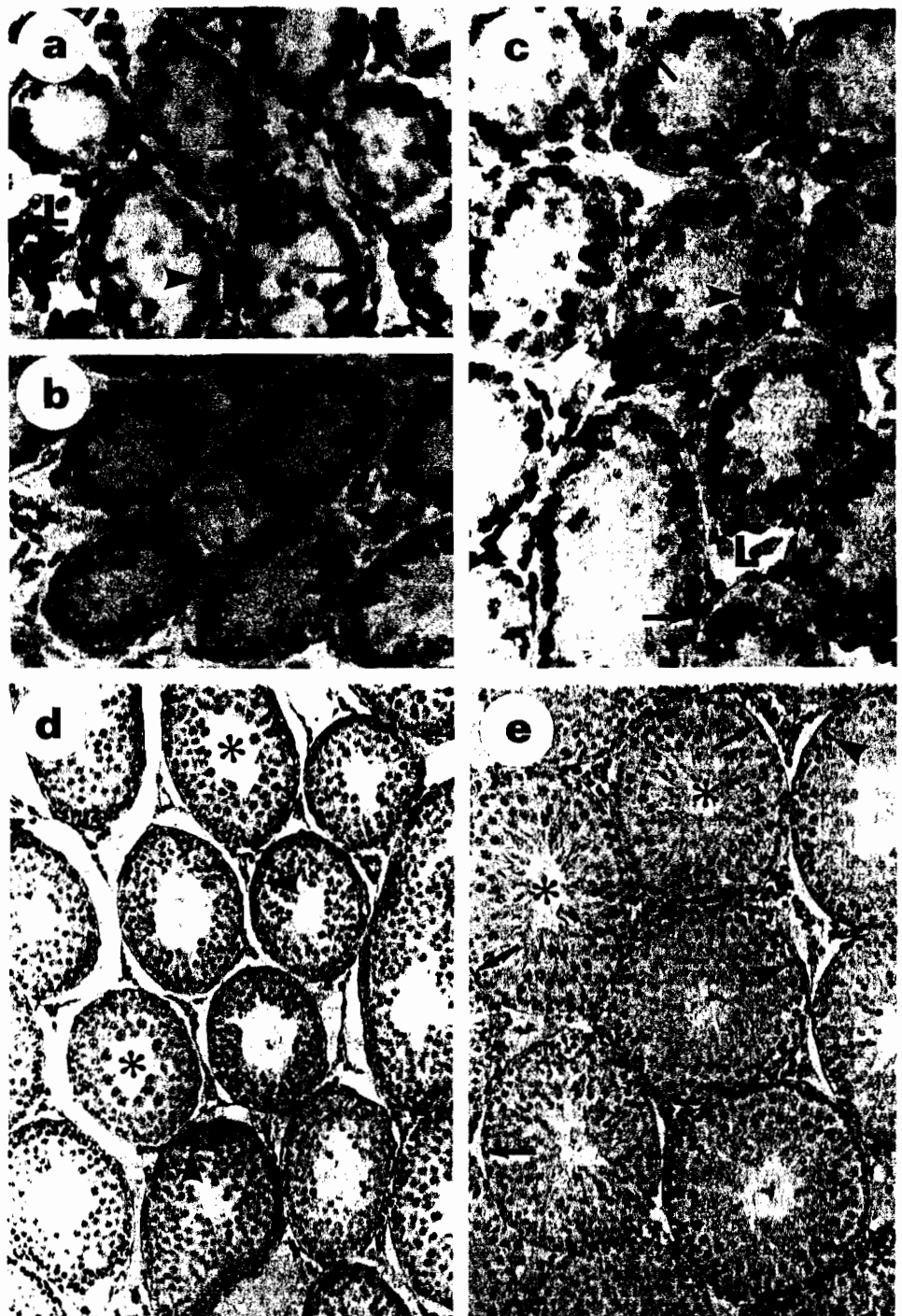


FIG. 5. Developmental changes in nuclear immunostaining for the AR in SCs (arrowheads), peritubular myoid cells (arrows), and Leydig cells (L). Testicular sections from rats aged 5 days (a and b), 14 days (c), 28 days (d), or 45 days (e) are illustrated. Note that at 5 and 14 days, immunostaining of peritubular myoid cell nuclei is very prominent, whereas immunostaining of SC nuclei is weak at 5 and 14 days, but thereafter increases progressively in intensity with age. Note also that immunostaining of SC nuclei is uniform at 5 and 14 days of age, shows evidence of stage-dependent differences at 28 days (d; asterisks identify two tubules with relatively weak SC immunostaining) that become as pronounced as in the adult at 45 days (e; asterisks identify two tubules in which no SC nuclear staining is detectable). b shows a control section (for a) at 5 days of age incubated with nonimmune serum. Magnification: a-c, $\times 225$; d and e, $\times 140$.

likely because T exerts major effects at stage VII (1-4), when AR staining is most pronounced. It will be of interest to assess the potential stage specificity of AR immunostaining using other antibodies to the AR. Such studies will help determine whether the patterns reported here are specific to a certain epitope on the AR or are characteristic of the protein as a whole. Similarly, it will be important to measure AR mRNA levels in SC at specific stages of the spermatogenic cycle. This may prove challenging because the protein is produced in peritubular myoid cells as well as in SC. If this problem can be overcome, the role of FSH in controlling

expression of the AR in SC needs to be assessed carefully, using FSH and LH depletion and repletion studies *in vivo* in both normal adult animals and animals around the time of puberty. Such work will be critical to furthering our understanding of the complex and interactive hormonal control mechanisms governing spermatogenesis.

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