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Absence of correlation between in situ expression of cytochrome P450 17α hydroxylase/lyase and 3β-hydroxysteroid dehydrogenase/$^{\Delta 5-4}$ isomerase messenger ribonucleic acids and steroidogenesis during pubertal development in the rat testis

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Abstract

Changes in expression of Leydig cell 3β-hydroxysteroid dehydrogenase (3βHSD) and 17α-hydroxylase/C17–20 lyase ($P450_{17\alpha}$) messenger RNA (mRNA) during pubertal development have not been well characterized in the rat. In the present study, expression of 3βHSD and $P450_{17\alpha}$ were determined in frozen sections of testes of immature (days 21 and 28), pubertal (days 45 and 60) and adult (day 90) rats by in situ hybridization using digoxigenin-labeled riboprobes and quantified densitometrically. Measures of steroidogenesis in this study, 3βHSD and $P450_{17\alpha}$ enzyme activities per testis and plasma testosterone concentration, increased during pubertal development, peaking at 45–60 days of age. Expression of 3βHSD protein, a marker for Leydig cell function, was abundantly immunolocalized to the interstitial compartment of the testis. Quantified densitometrically, the amount of 3βHSD protein did not vary significantly during pubertal development. Transcripts of 3βHSD and $P450_{17\alpha}$ were expressed abundantly by clusters of immature Leydig cells in immature animals. However, in contrast to measures of steroidogenesis during pubertal development, mRNA of 3βHSD and $P450_{17\alpha}$ decreased to undetectable levels at the age of 45 and 60 days, respectively. The decline in mRNA of 3βHSD and $P450_{17\alpha}$ was confirmed by Northern analysis. Expression of 3βHSD and $P450_{17\alpha}$ transcripts rebounded in the adult at 90 days and were comparable to levels of expression observed in immature animals. These results show that during pubertal development the steady-state accumulation of mRNA of 3βHSD and $P450_{17\alpha}$ are not correlated with accumulation of 3βHSD protein, enzyme activities of 3βHSD and $P450_{17\alpha}$, or testosterone secretion. Possible explanations of the depletion of transcripts during pubertal development include: specific inhibition of transcription, increased mRNA instability, or high translational activity.

Keywords: Leydig cells; Rat; Messenger RNA; Cytochrome P450; Androgen biosynthesis; Puberty

1. Introduction

Development of the mature adult testis is initiated by the final generation of Leydig cells which starts just before pubertal development in the male rat [1]. Proliferation and differentiation of mesenchymal-like stem cells in the interstitium of the testis give rise to immature Leydig cells by day 28 [2,3] which continue proliferation until day 56 [3]. The number and size of Leydig cells increase in conjunction with increased Leydig cell steroidogenesis, expression of LH receptors, and testis secretion of testosterone [1]. Testoster-

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one, the principal circulating androgen in the adult, is essential for maintenance of spermatogenesis and expression of secondary sex characteristics.

Most studies of steroidogenesis during pubertal development have focused on the declining expression and activity of two steroid metabolizing enzymes, 5α-reductase and 3α-hydroxysteroid dehydrogenase, in Leydig cells which convert testosterone to biologically inactive compounds. Immature rat testes have high levels of 5α-reductase [4] and 3α-hydroxysteroid dehydrogenase activity which could minimize testosterone output [5]. In contrast, very little data exists on tissue localization and the temporal pattern of steroidogenic enzyme expression in the biosynthetic pathway to testosterone in rat testis through the period of pubertal development. A key enzyme in steroidogenesis is 3β-hydroxysteroid dehydrogenase/$^{\Delta 5-4}$ isomerase (3βHSD) which is often used as a histochemical marker for Leydig cells. It converts pregnenolone to progesterone, an obligatory intermediate in testosterone biosynthesis [6]. Testosterone biosynthesis is also dependent on 17α-hydroxylase/C17–20 lyase ($P450_{17\alpha}$), a single protein that catalyzes two sequential reactions in the rat testis. The first reaction is hydroxylation of progesterone which is followed by cleavage of the C-20,21 side chain from the steroid nucleus to yield androstenedione, the immediate precursor to testosterone [7].

Since 3βHSD and $P450_{17\alpha}$ are key enzymes responsible for androgen biosynthesis, the goal of this study was to examine the pattern of testicular 3βHSD and $P450_{17\alpha}$ gene expression during pubertal development by in situ hybridization, and to determine how changes in the abundance of 3βHSD and $P450_{17\alpha}$ mRNA were related to functional changes in Leydig cells as measured by plasma testosterone concentration and enzyme activities of 3βHSD and $P450_{17\alpha}$.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Animals were sacrificed by CO_2 asphyxiation at 21, 28, 45, 60 and 90 days of age. Rats were classified into the following stages of development: immature, pubertal, or adult at the age of days 21 and 28, 45 and 60, and 90, respectively. Animals used in this study were treated in accordance with the NIH Guide for the Care and Use of Experimental animals with the experimental protocol approved by the Institutional Animal Care and Use Committee of Howard University (protocol #9612). Testes from three animals per day were removed and weighed; testes were frozen in Tissue-Tek OCT compound (Miles, Elkhart, IN) in Peel-A-Way disposable embedding molds (Polysciences, Warrington, PA) for in situ hybridization and immunohistochemistry. An additional four rats for each of the specified age groups were sacrificed, blood was obtained from the heart, and blood plasma was isolated and stored at −20°C for steroid determination. Testes from these rats were frozen immediately with liquid nitrogen and stored at −80°C for RNA extraction or enzyme assays.

2.2. In situ hybridization

Rat testis cDNAs for 3βHSD and $P450_{17\alpha}$ were subcloned into pGEM-3Z (Promega, Madison, WI) and pSPT18/19 (Boehringer-Mannheim, Indianapolis, IN) plasmid vectors, respectively. The cDNAs for both enzymes were kindly provided by Dr. J. Ian Mason (University of Edinburgh, Scotland, UK). Digoxigenin-labeled RNA probes for rat testis 3βHSD and $P450_{17\alpha}$ probes were synthesized in an in vitro transcription reaction using the DIG/GENIUS System (Boehringer–Mannheim). Rat testis 3βHSD antisense and sense cRNA probes were complementary to approximately a 1.2 kb EcoR1–HindIII fragment of the rat testis cDNA sequence [6]. Rat testis $P450_{17\alpha}$ antisense and sense cRNA probes were complementary to approximately a 0.5 kb EcoR1–EcoR1 fragment of the cDNA sequence of the rat testis cDNA sequence [7]. The cRNA probes were treated with DNAase and purified with Nunc Trap® probe purification columns (Stratagene LaJolla, CA). Probe concentrations were determined spectrophometrically at 260/280 nm.

In situ hybridization with digoxigenin-labeled cRNA probes was carried out on frozen rat testis sections with modification [8,9]. Cryosections (8 μm) of rat testis tissue were thaw mounted onto Superfrost Plus, precoated slides (Fisher Scientific, Pittsburgh, PA). In situ hybridization was performed using one slide for the antisense cRNA probes for 3βHSD and $P450_{17\alpha}$ and a second slide for the sense strand negative control. Slides containing frozen testicular sections were fixed for one h at room temperature in freshly prepared 4% paraformaldehyde. The sections were prehybridized without coverslips with 40 μl of a 45% formamide hybridization cocktail (Amresco, Solon, OH) for 2 h at 55°C in a sealed box humidified with 2× SSC (1× SSC = 0.15 M sodium chloride and 0.015 M sodium citrate). The digoxigenin-labeled sense and antisense probes were heated denatured at 80°C for 5 min. Probe concentrations were diluted with hybridization cocktail to 250 ng/ml and 125 ng/ml for 3βHSD probes and $P450_{17\alpha}$ probes, respectively. The prehybridization cocktail was removed from slides and replaced with 40 μl of the probe cocktail. Testis sections were covered with siliconized cover slips and the slides were hybridized overnight at 55°C in a humidi-

fied chamber. The following day, unbound probe was removed from the slides by increasing SSC stringency washes performed at 55°C. Following the stringency washes, the slides were rinsed with TBS (tris-buffered saline) and blocked for 30 min with 5% normal goat serum. Alkaline phosphatase conjugated sheep anti-digoxigenin antibody was placed on sections and incubated overnight at 4°C for immunodetection of hybridized probes. The following day, slides were rinsed in TBS and treated for 10 min with alkaline phosphatase detection buffer (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5). Binary alkaline phosphatase chromogens, nitro blue tetrazolium chloride (NBT, 375 g/ml) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP,188 g/ml) in alkaline phosphatase detection buffer containing 1 mM levamisole were added to the sections. The color reaction was allowed to proceed for 1.5 h and then terminated by rinsing the slides in Tris–EDTA buffer. Slides were rinsed in deionized water, air dried, and the sections were covered with Crystal Mount (Biomeda, Foster City, CA).

2.3. Northern analysis

Northern analysis was conducted using polyA RNA extracted from testes at 28, 45, 60 and 90 days of age. Total RNA was isolated [10] and polyA RNA was extracted from total RNA using PolyATract®mRNA Isolation System (Promega, Madison, WI). Denatured polyA RNA was separated in a 1.25% agarose-formaldehyde-containing gel and transferred to nylon membrane and hybridized with ^{32}P-labeled cDNA probes as previously described with modification [11]. The sequences of the rat 3βHSD and $P450_{17\alpha}$ cDNA probes used for Northern blotting were the same as described above for the cRNA probes. Hybridization was visualized with STORM 660 phosphorescence imaging system (Molecular Dynamics, Sunnyvale, CA).

2.4. Immunohistochemistry

Frozen rat testes were sectioned (8 μm) and stained for 3βHSD using a specific 3βHSD antibody (kindly provided by Dr. J. Ian Mason, University of Edinburgh, Scotland UK) as described previously [12]. Briefly, frozen sections were fixed in Zamboni''s fixative (2% paraformaldehyde, 0.2% picric acid in 150 mM phosphate buffer, pH 7.5) and the primary antibody was detected by the peroxidase anti-peroxidase method. Negative control slides were prepared by substitution of rabbit IgG for the primary antibody and were devoid of any nonspecific background staining. We were unable to investigate localization and intensity of $P450_{17\alpha}$ expression in this experiment because the only antibody that we could obtain, an antibody to bovine $P450_{17\alpha}$ generated in a mouse, yielded only nonspecific staining in rat testis sections.

2.5. Evaluation of in situ hybridization and immunostaining

Stained slides were observed on a Zeiss Axioskop (Carl Zeiss, Thornwood, NY) microscope and the intensities of the in situ hybridization signals and of the 3βHSD immunostaining signals were determined by densitometry of acquired video images. The video camera from an Alpha Innotech IS-100 digital imaging system (Alpha Innotech, San Leandro, CA) was attached to the microscope and in situ and immunohistochemistry images were acquired at 100× and 40 × , respectively. Using the spot densitometry module of the IS-1000 software, four areas of each testis section were randomly chosen for analysis. The size of each area analyzed was standardized using the grid in the software package. Only optical density of stained Leydig cells within the standardized area was determined. This value, in arbitrary densitometric units (ADU), was corrected for background by subtracting optical density values obtained within adjacent seminiferous tubules.

2.6. Enzyme assays

Each frozen testis was homogenized prior to thawing in 4 vol of 0.25 M sucrose containing 1 mM EDTA, 0.1 M potassium buffered phosphate, pH 7.4. The homogenate was centrifuged at 10,000 × *g* for 15 min [13]. The protein content of the resulting supernatant was determined using BioRAD protein assay (Bio Rad, Hercules, CA). Aliquots (100 μl) of the supernatant were used for enzyme assays. 3βHSD was determined for each testicular homogenate in duplicate by measuring the conversion of pregnenolone to progesterone. Pregnenolone (50 nmol) was dissolved in 0.03 ml DMSO and added to 0.87 ml of 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 0.1 M NAD [13]. The reaction was initiated by the addition of 0.1 ml of the 10,000 × *g* supernatant. Duplicate incubations were carried out at 37°C for 20 min. The reaction was terminated by the addition of 0.1 ml of 1 N NaOH. An aliquot of the incubation mixture was assayed for progesterone content by radioimmunoassay. $P450_{17\alpha}$ hydroxylase/lyase activity was determined for each testicular homogenate in duplicate by measuring the conversion of progesterone to androstenedione. Progesterone (5 nmol) was dissolved in 0.05 ml DMSO and suspended in 2.0 ml of 0.05 M phosphate buffer, pH 7.4. The reaction mixture consisted of 0.1 ml of substrate, 0.8 ml of 50 mM phosphate buffer, pH 7.4 containing 0.5 mM NADPH [14]. The reaction was initiated by the addition of 0.1

ml aliquot of the supernatant. Incubations were performed for 30 min at 37°C. Reactions were terminated by the addition of 0.1 ml 1 N NaOH. An aliquot of the incubation mixture was assayed for androstenedione content by radioimmunoassay.

2.7. Hormone radioimmunoassays

An aliquot of each homogenate sample was diluted 100-fold with Dulbecco's phosphobuffered saline and 100 μl of diluted homogenate were quantified for progesterone and androstenedione in duplicate without solvent extraction using double antibody [^{125}I] ligand radioimmunoassay (RIA) kits (Pantex, Santa Monica, CA). The assays were performed according to the instructions from the manufacturer as previously described [15]. The minimum detectable amount of progesterone or androstenedione was 6 pg/tube. All samples were run in one assay and the mean coefficient of variation (CV) of samples duplicated within assays was 6 and 4% for progesterone and androstenedione, respectively. The antibodies showed negligible cross-reaction with a wide range of steroids. Plasma testosterone concentrations were determined in duplicate without solvent extraction using a double antibody [^{125}I] ligand RIA (ICN, Costa Mesa CA). The antiserum used showed minimal cross-reaction with dihydrotestosterone and with a wide range of other steroids. All samples were run in one assay and the mean CV of duplicated samples was 7%.

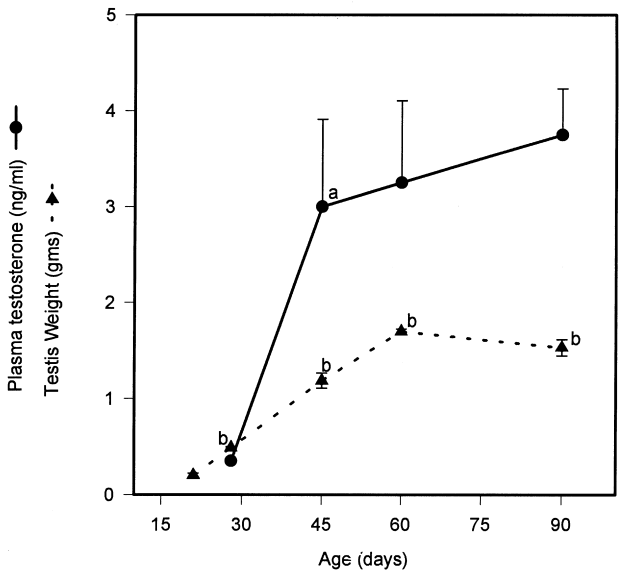

Fig. 1. Weight gain of rat testes and corresponding changes in plasma testosterone levels during postnatal development. The results are expressed as the arithmetic means ± SEM (n = 4 animals). (a) Significantly different ($p < 0.001$) from the preceding mean for plasma testosterone. (b) Significantly different ($p < 0.003$) from the preceding mean for testis weight.

2.8. Statistical analysis

Analysis of variance was performed using PROC MIXED in release 6.12 of the Statistical Analysis System software for personal computers [16]. Day of age was the independent variable in the statistical model and the PDIFF option was used for determining differences between means. Data points for plasma testosterone and 3βHSD and $P450_{17\alpha}$ enzyme activities were transformed to natural logarithms for analysis due to the correlation between means and variances.

3. Results

Changes in testis weight and plasma testosterone levels during pubertal development are shown in Fig. 1. Plasma testosterone increased nine-fold ($p < 0.001$) between 28 and 45 days of age. No significant change ($p > 0.05$) in plasma testosterone was observed between 45 and 90 days of age. Testis weight increased ($p < 0.003$) eight-fold between days 21 and 60 days of age. Testis weight and plasma testosterone were positively correlated ($r^2 = 0.750$, $p < 0.001$).

Immunohistochemical analysis showed that the 3βHSD protein was expressed in the interstitial compartment of the testis at all postnatal ages examined (Fig. 2). Numerous clusters of Leydig cells located in peri- and intertubular spaces exhibited strong immunostaining at 21 and 28 days of age. Both strongly and weakly stained Leydig cells were localized in the pubertal (days 45 and 60) and in the adult testis (day 90). Quantification of immunostaining by densitometry showed no significant difference ($p > 0.05$) among days with means ± SEM (n = 3) of 67.5 ± 14.3, 85.7 ± 14.9, 51.8 ± 13.4, 49.7 ± 6.6, and 53.1 ± 5.3 ADU for days 21, 28, 45, 60, and 90, respectively.

Digoxigenin-labeled 3βHSD and $P450_{17\alpha}$ cRNA probes hybridized in interstitial regions where the 3βHSD protein was observed by immunohistochemistry in serial sections (Figs. 3 and 4). Intense expression of 3βHSD and $P450_{17\alpha}$ was found in clusters of Leydig cells in immature animals on days 21 and 28. Thereafter, mRNA expression for the enzymes declined during the pubertal phase of development such that expression of 3βHSD mRNA was undetectable at 45 and 60 days of age; expression of $P450_{17\alpha}$ mRNA was undetectable at 60 days of age. The level of mRNA expression for both enzymes rebounded dramatically in the adult testis and was localized mainly in intertubular Leydig cells. Densitometric means for in situ hybridization intensity are shown in Fig. 5A. Analysis of variance indicated that the densitometric means with

arbitrary densitometric units > 0 were similar ($p > 0.05$). Note that days with undetectable mean values, days 45 and 60 for 3βHSD and 60 for $P450_{17\alpha}$, were excluded from the statistical analysis.

PolyA RNA isolated from whole testis was hybridized sequentially with ^{32}P- labeled rat testis 3βHSD and $P450_{17\alpha}$ cDNAs (Fig. 5B). A strong signal was present at day 28 for both enzymes. Thereafter, signal intensity declined such that faint signals were detected at 45 and 60 days of age.

The developmental pattern for 3βHSD and $P450_{17\alpha}$ enzyme activity per testis increased as a function of

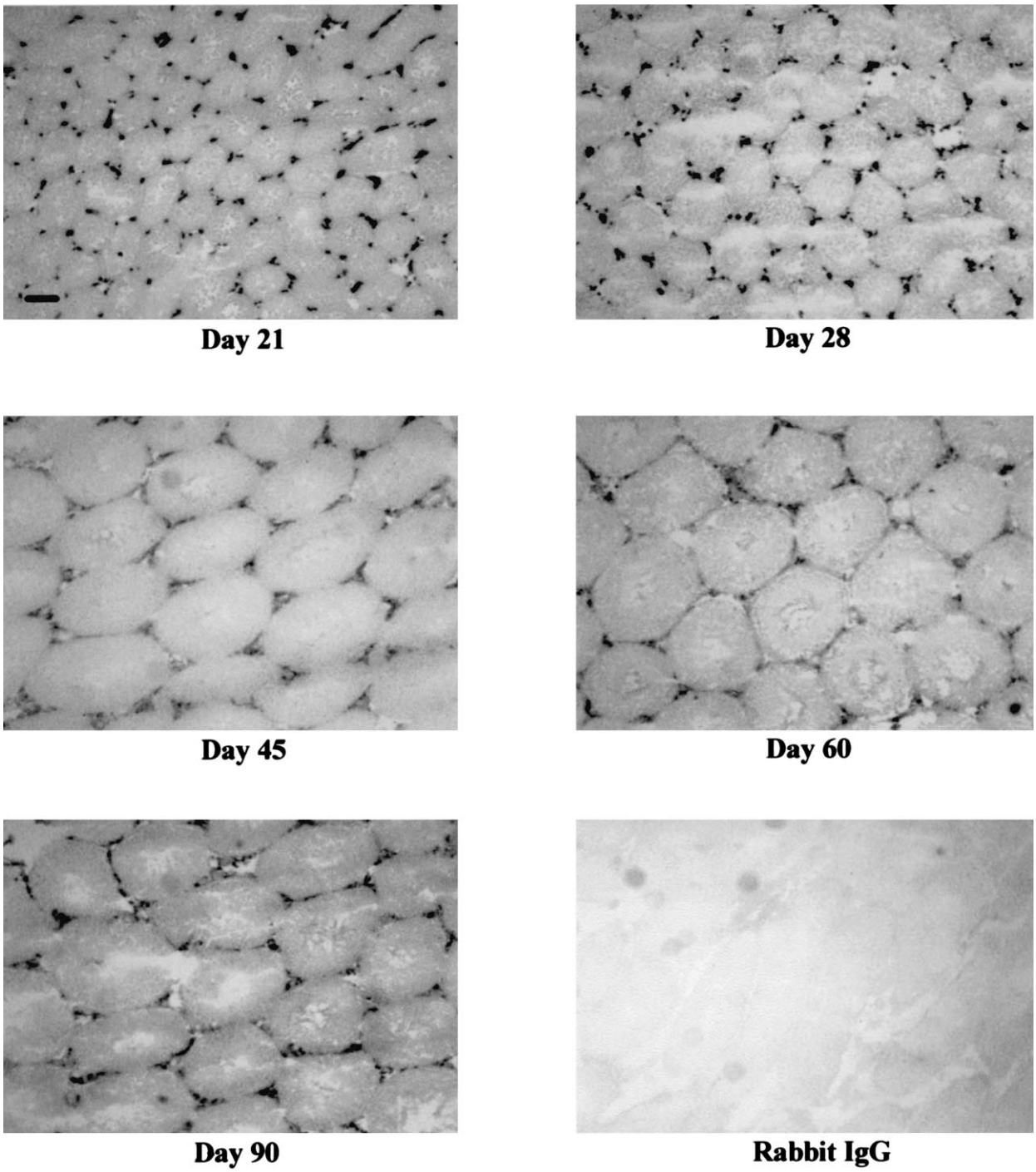

Fig. 2. The expression of 3βHSD protein in postnatal rat testes was investigated using immunohistochemistry. Seminiferous tubules and 3βHSD immunostaining of Leydig cells are shown for days 21, 28, 45, 60 and 90. The negative control was generated by substituting rabbit IgG for the primary antibody. The scale bar represents 100 μm. Original magnification: ×40.

age (Fig. 6). Enzyme activity of 3βHSD increased ($p < 0.03$) between days 21 and 60. Enzyme activity of $P450_{17\alpha}$ increased markedly between 21 and 45 days of age ($p < 0.02$) and plateaued thereafter. Postnatal growth of the testis was positively correlated with 3βHSD enzyme activity ($r^2 = 0.91$, $p < 0.05$) and $P450_{17\alpha}$ enzyme activity ($r^2 = 0.71$, $p < 0.05$).

4. Discussion

Our results show that steroidogenesis increased progressively during the pubertal period based on the results of enzyme assays for 3βHSD and $P450_{17\alpha}$ and plasma testosterone concentration. The major novel finding of this study was that mRNA expression for

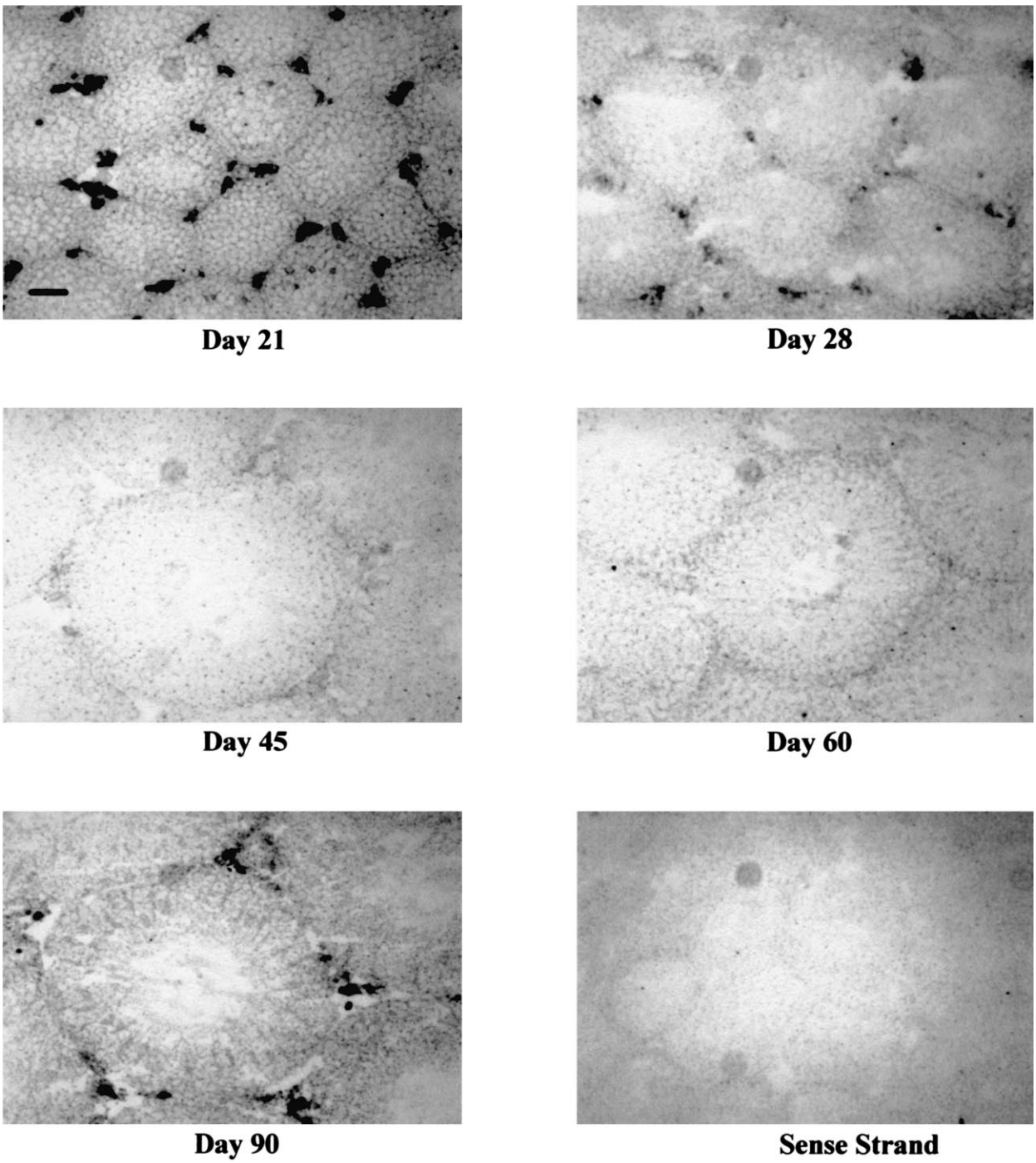

Fig. 3. The expression of 3βHSD RNA in Leydig cells during pubertal development was determined by in situ hybridization. Shown are representative sections of rat testes at days 21, 28, 45, 60 and 90 hybridized with 3βHSD cRNA antisense probe. For the negative control, the sense cRNA 3βHSD probe was substituted for the antisense probe. The scale bar represents 50 μm. Original magnification: ×100.

3βHSD and $P450_{17\alpha}$ decreased during the pubertal period to non-detectable levels as measured by in situ hybridization, in spite of increasing steroidogenesis.

In the rat, it is well established that growth of the testis, proliferation and differentiation of Leydig cells, and steroid biosynthesis increase during pubertal maturation [1]. However, the decrease in expression of mRNA for 3βHSD and $P450_{17\alpha}$ during the pubertal period and a rebound in expression in the adult testis has not been reported. Others [17] have concluded that the maturational change in steroidogenesis is due in part to a progressive increase in $P450_{17\alpha}$ mRNA expression. That study [17] was based on analysis of Leydig cells isolated from 21, 35 and 90 day old rats

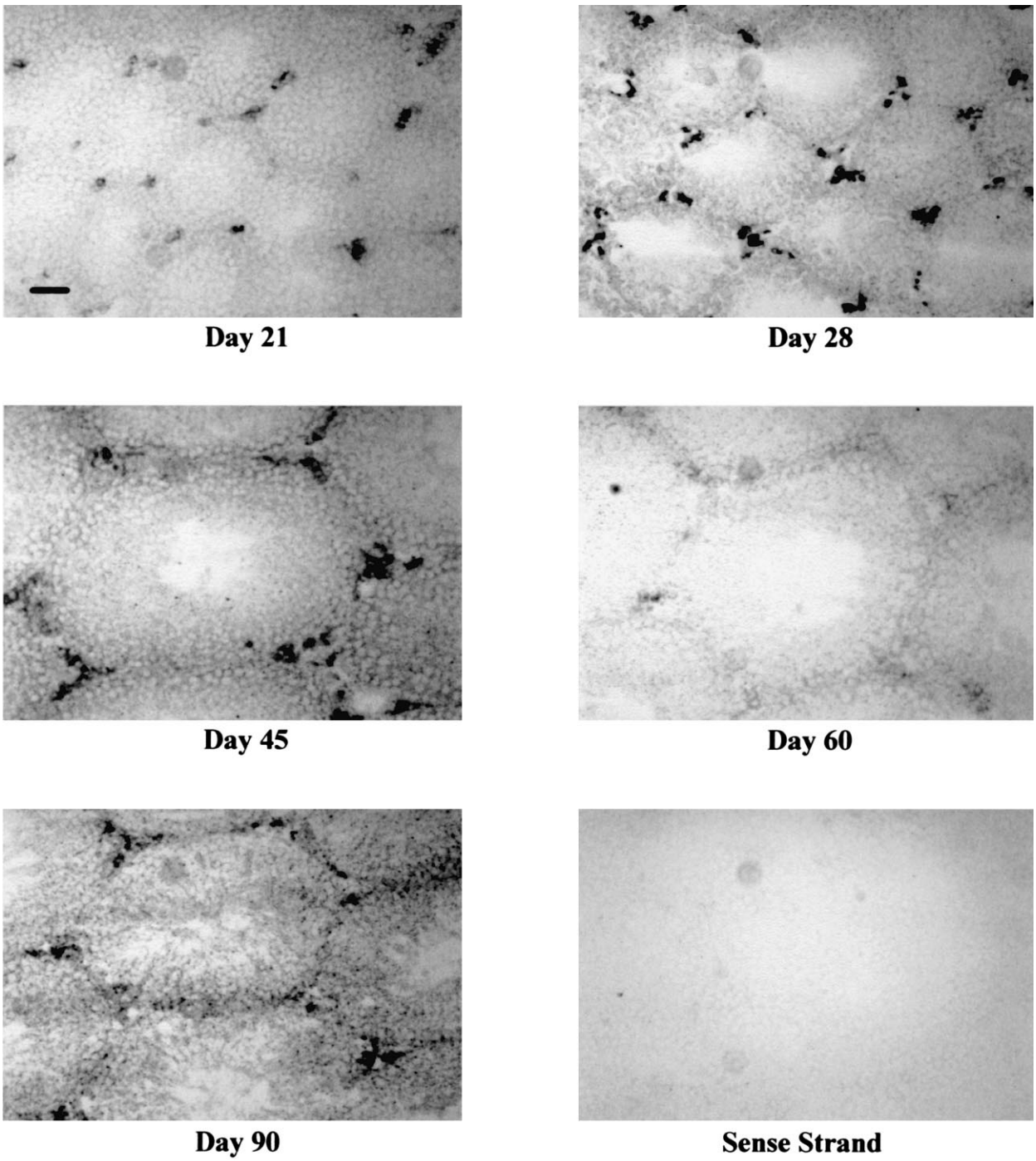

Fig. 4. The expression of $P450_{17\alpha}$ RNA in Leydig cells during pubertal development was determined by in situ hybridization. Shown are representative sections of rat testes at days 21, 28, 45, 60 and 90 hybridized with $P450_{17\alpha}$ cRNA antisense probe. For the negative control, the sense cRNA $P450_{17\alpha}$ probe was substituted for the antisense probe. Bar represents 50 μm. Original magnification: ×100.

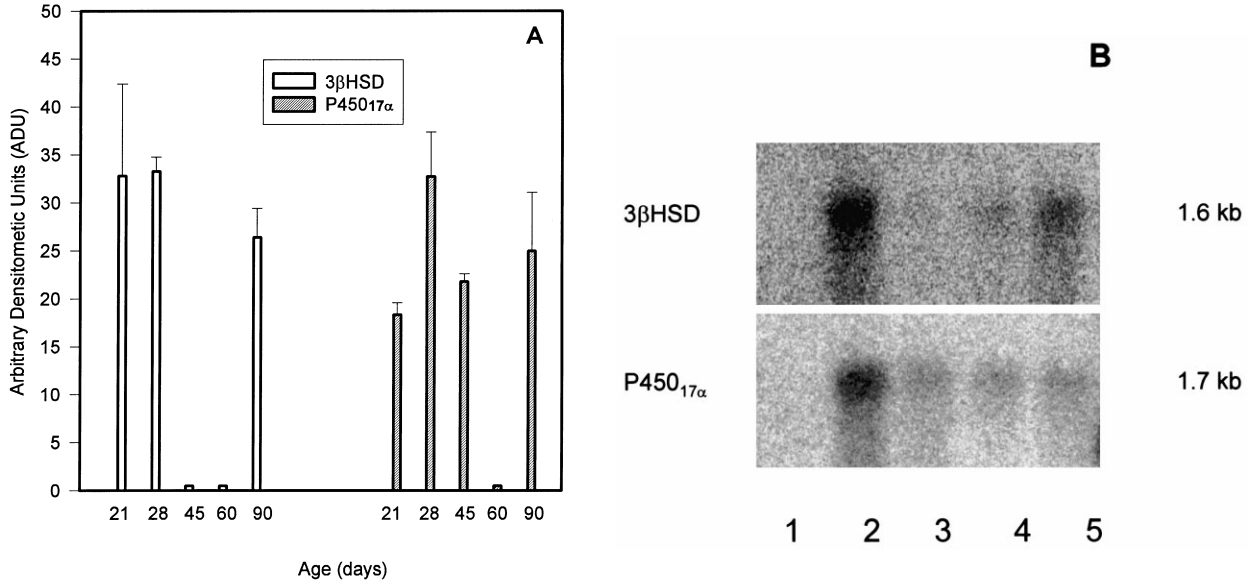

Fig. 5. Comparison of gene expression for 3βHSD and P450$_{17\alpha}$ during postnatal development of rat testis by in situ hybridization and Northern analysis. (A) In situ hybridization expression was quantified densitometrically for 3βHSD and P450$_{17\alpha}$ and expressed as the mean ± SEM (n = 3). Analysis of variance of densitometric means > 0 ADU indicated no significant differences ($p > 0.05$) among means. (B) Representative Northern blot of whole testes polyA RNA probed with 3βHSD and P450$_{17\alpha}$ cDNAs. Lane 1, negative control rat kidney (10 μg total RNA); lanes 2, 3, 4 and 5, represent days 28, 45, 60 and 90, respectively (5 μg polyA RNA).

which was not inclusive of the entire pubertal period, especially days 45 and 60 when P450$_{17\alpha}$ mRNA expression was low.

Enzyme activities for 3βHSD and P450$_{17\alpha}$ increased during maturation with maximum activity occurring during the pubertal phase of development when mRNA expression for 3βHSD and P450$_{17\alpha}$ was low or undetectable. Our findings of increased enzyme activity are in agreement with earlier studies of postnatal changes in 3βHSD [13] and 17α hydroxylase [18] enzyme activities.

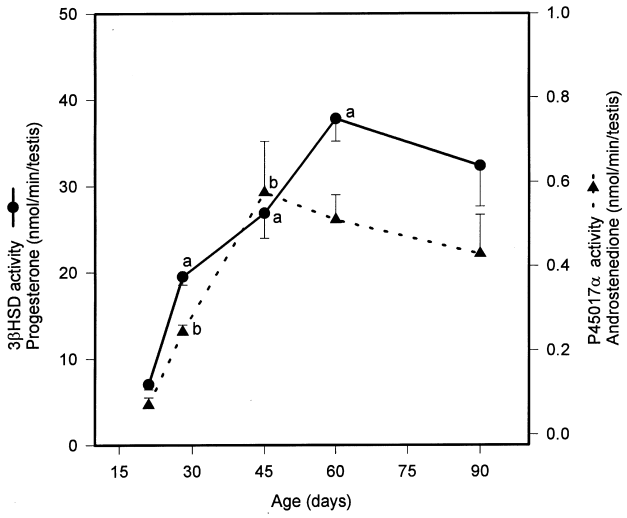

Fig. 6. Enzyme activities for rat testis homogenates were determined by the conversion of pregnenolone to progesterone for 3βHSD and progesterone to androstenedione for P450$_{17\alpha}$. Data points represent the arithmetic mean ± SEM (n = 4); each assay was performed in duplicate with two different aliquots of homogenate. (a) Significantly different ($p < 0.03$) from the preceding mean for progesterone concentration. (b) Significantly different ($p < 0.02$) from the preceding mean for androstenedione concentration.

The exact cause of the reduced abundance of the enzyme transcripts during pubertal development is not clear. Whereas the steroidogenic enzyme transcripts were non-detectable by in situ hybridization, the transcripts were not totally absent as shown by Northern analysis. The activity of the steroidogenic enzyme pathway indicates that there were sufficient enzymes present to produce the increased concentration of plasma testosterone during the pubertal period. The depletion of a transcript encoding a specific protein during a period of increased synthesis of the protein has been reported by other investigators. A substantial hormone-induced increase in ornithine decarboxylase (ODC) activity in immature rat granulosa cells was accompanied by a slight increase in the expression of ODC mRNA [19]. Moreover, an inverse relationship was found between testicular ODC activity and its gene expression during postnatal development in the rat [20]. The decreased abundance of mRNAs for P450$_{17\alpha}$ and 3βHSD in our study may reflect control of gene expression at the translational level. A high turnover of transcript during an active period of translation of transcripts into protein may prevent accumulation of transcripts until the rate of increase in

steroidogenesis is reduced at the conclusion of testicular maturation.

Another possibility for the decrease in 3βHSD and $P450_{17\alpha}$ gene expression during pubertal development is a change in mRNA stability. During puberty, it has been shown that chronic ACTH treatment in vivo increased adrenal steroidogenesis in guinea pigs, but coincidentally decreased the expression of $P450_{17\alpha}$ mRNA [21]. It was hypothesized from these findings that ACTH may have a post-transcriptional effect on steroidogenic gene expression by reducing the half-life of the mRNA.

A likely explanation for the pubertal pattern of $P450_{17\alpha}$ and 3βHSD gene expression in our study is the down regulation of transcription by testosterone. Androgen receptors are localized within Leydig cells [22] and their expression decreases during pubertal development [23]. In the immature animal, testosterone may be an important autocrine regulator of Leydig cell differentiation. Undifferentiated Leydig cells treated in vitro with androgen and LH increase their capacity to synthesize testosterone [24]. In contrast, in the adult testis, androgen end products may limit androgen biosynthesis by decreasing steroidogenic enzyme activity and gene expression. In vitro studies with adult Leydig cells show that testosterone treatment inhibited 3βHSD [25] and $P450_{17\alpha}$ enzyme activities [26]. Administration of testosterone to cultured Leydig cells after cAMP treatment resulted in a substantial decrease in $P450_{17\alpha}$ protein synthesis and enzyme activity [27]. Furthermore, androgens repressed the accumulation of RNA transcripts for 3βHSD [28] and $P450_{17\alpha}$ [29] in cultured mouse Leydig cells via an androgen receptor mechanism.

Down regulation of steroidogenic enzyme gene expression during pubertal development is akin to gonadotropin-induced Leydig cell desensitization. The inhibitory effect of a desensitizing dose of human chorionic gonadotropin on 3βHSD and $P450_{17\alpha}$ gene expression has been demonstrated in the rat [30,31]. Furthermore, it was shown that down regulation of 3βHSD gene expression in desensitized Leydig cells resulted from changes at the transcriptional level that were independent of the reduction in the number LH receptors [31]. It was suggested that the inhibitory effect could be related to negative feedback by androgen since testosterone production is stimulated markedly during the acute response to gonadotropin [32]. Androgen biosynthesis is dramatically reduced in desensitized Leydig cells following the acute response [31]. This could be a pharmacological response rather than a physiological regulatory mechanism. Our study with pubertal Leydig cells shows a progressive increase in testosterone biosynthesis in the presence of low 3βHSD and $P450_{17\alpha}$ gene expression. Under physiological conditions, LH secretion occurs in pulses of short duration [33] with a long-term tropic effect of increased steroidogenic enzyme activity [32].

Down regulation of steroidogenic enzyme gene expression in vivo coincides with the change in Leydig cell function as evidenced by increased testosterone output during sexual maturation in the rat. In contrast, the pubertal rise in testosterone secretion was associated with a significant increase in the activity of the enzyme proteins. Thus, 3βHSD and $P450_{17\alpha}$ enzyme activity does not closely parallel expression of their respective mRNAs. Possible explanations of the depletion of transcripts may include by high translational activity, increased messenger RNA instability, or a cell-specific inhibition of transcription during pubertal development. This phenomenon may be due, in part, to autocrine regulation of mRNA expression by testosterone.

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