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Research

Expression of prostaglandin D synthetase during development in the mouse testis

P. J. Baker and P. J. O'Shaughnessy*

Division of Veterinary Physiology and Pharmacology, Department of Veterinary Preclinical Studies, University of Glasgow Veterinary School, Bearsden Rd, Glasgow G61 1QH, UK

Prostaglandin D synthetase is expressed relatively highly in the testis and reproductive tract of a number of species, including the mouse. In adult mouse testis, expression is confined largely to the Leydig cells and in this study changes in the expression and localization of prostaglandin D synthetase mRNA during testis development were examined. Initial studies using RT–PCR and isolated testicular compartments indicated that prostaglandin D synthetase expression in the neonatal testis was predominantly within the seminiferous tubules. *In situ* hybridization studies confirmed that prostaglandin D synthetase mRNA appears to be expressed only in the tubules of neonatal mouse testes and only in the interstitial tissue of the adult testis. TaqMan real-time PCR was used to quantify prostaglandin D synthetase mRNA content during development using an exogenous mRNA as a control standard. Expression per testis decreased after birth to < 10% at day 15 before recovering again by days 25–30. After day 30, expression per testis increased 40-fold during final development to adulthood. Studies using RT–PCR showed that early expression before day 15 was restricted to the tubular compartment, whereas the subsequent increase in expression after day 30 was restricted to the interstitial compartment. Database analysis showed that the 3′ end of the prostaglandin D synthetase transcript was subject to alternate splicing. Both splice isoforms were shown by RT–PCR to be present throughout development and without a major change in expression pattern. These results indicate that expression of prostaglandin D synthetase mRNA shifts during development from the tubular compartment of the fetal or neonatal testis to the developing adult Leydig cells, with expression in the Leydig cells increasing markedly after puberty. These changes are similar to those observed for 17β-hydroxysteroid dehydrogenase type III and may indicate that this developmental process is not uncommon in the testis.

Introduction

Prostaglandin D synthetase is a 30 kDa peptide that acts to catalyse isomerization of prostaglandin H_2 to prostaglandin D_2 (Urade *et al.*, 1995). In addition, the primary structure of prostaglandin D synthetase identifies it as a member of the lipocalin superfamily, the members of which are involved in binding and transport of small lipophilic ligands such as retinoids and steroids (Pervaiz and Brew, 1987). Relatively high expression of prostaglandin D synthetase is observed in the testis and reproductive tract of several species including mice (Hoffman *et al.*, 1996; Gerena *et al.*, 2000a), rats (Sorrentino *et al.*, 1998), humans (Blodorn *et al.*, 1996; Tokugawa *et al.*, 1998) and cattle (Rodriguez *et al.*, 2000; Gerena *et al.*, 2000b). Within the testis there is apparent species variation in the site of prostaglandin D synthetase expression, as the protein or mRNA is localized to the Sertoli cells in bulls and rats, and to Leydig cells in mice (Hoffman *et al.*, 1996; Sorrentino *et al.*, 1998; Gerena *et al.*, 2000a,b; Rodriguez *et al.*, 2000). In humans, both the seminiferous tubules and Leydig cells have been reported as the principal site of prostaglandin D synthetase expression (Bludorn *et al.*, 1996; Tokugawa *et al.*, 1998). The likely function of prostaglandin D synthetase within the testis is not clear. Spermatogenesis is dependent on adequate concentrations of vitamin A and the retinoid binding–transport capacity of prostaglandin D synthetase may be important in maintaining vitamin A in the tubules. Alternatively, prostaglandin D_2 is involved in a number of physiological processes including vasodilation (Dumitrascu, 1996) and hormone release (Terao *et al.*, 1995), and may, therefore, play a regulatory role in normal testis function. The developmental profile of prostaglandin D synthetase mRNA expression in the testis is not known and in this study changes in the level and site of prostaglandin D synthetase expression during development in the mouse testis were examined.

*Correspondence
Email: P.J.O'Shaughnessy@vet.gla.ac.uk

Materials and Methods

Animals and tissues

Normal mice were bred at the University of Glasgow Veterinary School and maintained as required under United Kingdom Home Office regulations. The mice used were derived from F1 hybrids of C3H/HeH and 101/H strains.

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Table 1. Primers and probes used for real-time PCR

Target		Primers and probes (5′–3′)
Luciferase	Forward primer	TCGAAGTATTCCGCGTACGTG
	Reverse primer	GCCCTGGTTCCTGGAACAA
	Probe	TGTTCACCTCGATATGTGCATCTGTAAAAGCA
Prostaglandin D synthetase	Forward primer	GGGAATCCCAAGAGACCCAG
	Reverse primer	GCTCTGAGCAAATGGCTGC
	Probe	AGGACCAAACCCATCCACAGCATGC

The day of birth was designated as day 1 and the mice were killed on days 1, 5, 10, 15, 20, 25, 30 and 40, and when adult animals were aged 90–180 days. Seminiferous tubules and interstitial tissue were separated mechanically as described by O'Shaughnessy *et al.* (2000) and interstitial tissue webs and tubules were stored frozen in liquid nitrogen until used for RNA extraction.

Reverse transcription and polymerase chain reaction

RNA was extracted using Trizol (Life Technologies, Paisley) and was reverse-transcribed using random hexamers and Moloney murine leukaemia virus reverse transcriptase (Superscript II; Life Technologies) as described previously (O'Shaughnessy and Murphy, 1993; O'Shaughnesssy and Mannan, 1994). The PCR reactions were performed in Tris–HCl buffer (75 mmol l^{-1}, pH 9.0 at 25°C) containing 20 mmol ammonium sulphate l^{-1}, Tween-20 (0.01% v/v), 2 mmol $MgCl_2$ l^{-1}, 0.2 mmol l^{-1} each dNTP, Taq polymerase (2 U per 100 µl), 200 nmol l^{-1} each primer and template (0.1–2.0 ml) in a total reaction volume of 30 µl.

The primers used were based on GenBank sequences (AB006361 and X89222) for mouse prostaglandin D synthetase: forward: 5′-TCAACAAGACAAGTTCCTGG; and reverse: 5′-TGAATTTCTCCTTCAGCTCG. The expected product size was 390 bp. The PCR products were separated on a 1% (w/v) agarose gel and visualized using ethidium bromide.

The following primers were used to examine expression of alternate transcripts of prostaglandin D synthetase: forward: 5′-GGCCTCACAGAGGACAT; and reverse: 5′-CTTGAGAGTGACAGAGCAAG. The expected size of the full-length product was 140 bp, whereas the shortened transcript is 107 bp. Products of the PCR reactions were separated on a 4% NuSieve:agarose (3:1) gel (Flowgen, Ashby de la Zouch).

Products from PCR reactions were separated on 1% (w/v) agarose gels and visualized with ethidium bromide.

Real-time PCR

For quantification of the prostaglandin D synthetase mRNA content of testes during development a real-time PCR approach was used, which used the TaqMan PCR method after reverse transcription of the isolated RNA (for review of real-time PCR methodology see Bustin, 2000). In brief, the TaqMan assay uses the 5′-nuclease activity of DNA polymerase to cleave a specific probe that hybridizes to the target amplicon during the annealing and extension phase of the PCR. Each probe contains a fluorescent dye reporter at the 5′ end and a quencher dye at the 3′ end that will normally inhibit the reporter emission. Therefore, cleavage of the probe separates the reporter and quencher dyes, resulting in increased fluorescent emission of the reporter, which is monitored by a suitable detector. The probe also provides an added degree of specificity to the assay. To measure cDNA concentrations the threshold cycle (C*t*) at which fluorescence is first detected above baseline is used and a standard curve is drawn between starting cDNA concentrations and C*t*. In the present study, arbitrary standards were generated by serial dilutions of cDNA prepared from adult testis interstitial tissue. An external standard was used to allow prostaglandin D synthetase mRNA content to be expressed per testis and to control for the efficiency of RNA extraction, RNA degradation and the reverse transcription step. The external standard was luciferase mRNA (Promega UK, Southampton) and 5 ng was added to each testis at the start of the RNA extraction procedure. Prostaglandin D synthetase cDNA content was then expressed relative to luciferase cDNA content in the same sample, to allow direct comparison of expression per testis between different samples.

Primers and probes for use in the TaqMan method were designed using Primer Express (Applied Biosystems, Warrington) (Table 1). RNA was extracted and reverse-transcribed as above with the exception that residual genomic DNA was removed by DNAse treatment (DNA-free; Ambion Inc., supplied by AMS Biotechnology), because the TaqMan primers were not specifically designed to produce an amplicon spanning an intron–exon boundary. PCRs were carried out in a 25 µl volume using a 96-well plate format. Components for real-time PCR were purchased from Oswel Ltd (Southampton) other than the primers and probes, which came from MWG Biotech (Milton Keynes). Each PCR well contained 1 × reaction buffer (with passive reference), 5 mmol $MgCl_2$ l^{-1}, 200 mmol dNTPs l^{-1} (including dUTP), 300 nmol each primer l^{-1}, 200 nmol probe l^{-1} and 0.02 U enzyme μl^{-1} (Hot GoldStar; Oswel). Reactions were performed and fluorescence detected on a GeneAmp 5700 system (Applied Biosystems). For each sample, a replicate was run omitting

the reverse transcription step and a template negative control was run for each primer–probe combination.

In situ *hybridization*

Testes from fetuses at day 18 of gestation or from adult mice were placed in Bouin's fixative and prepared for *in situ* hybridization as described by Baker *et al.* (1999). The 390 bp prostaglandin D synthetase RT–PCR product from adult testis (described above) was cloned into pCRscript (Stratagene Ltd, Cambridge) and sequenced using big dye terminators (Applied Biosystems) to prepare probes for *in situ* hybridization. Sense and antisense cRNA probes were prepared using T3 and T7 polymerase and were labelled with [^{35}S]dUTP. Tissue sections (7 mm) were hybridized overnight at 60°C with probe (100 000 c.p.m. ml^{-1}) in hybridization buffer (50% (v/v) deionized formamide, 10% (w/v) dextran sulphate, 50 mmol dithiothreitol l^{-1}, 500 mg calf thymus DNA ml^{-1}, 1 × Denhardt's solution, 20 mmol Tris–HCl l^{-1}, pH 8.0, 5 mmol EDTA l^{-1} and 10 mmol sodium phosphate l^{-1}, pH 6.8). After hybridization, the slides were washed, dehydrated and allowed to air dry. Autoradiography was carried out using Ilford K5 emulsion (Ilford) and the slides were stained with Meyer's haematoxylin and eosin (Merck Ltd, Lutterworth).

Results

Localization of prostaglandin D synthetase mRNA expression in the testis

RT–PCR. In initial studies to examine localization of prostaglandin D synthetase in mouse testis the interstitial and tubular compartments from adult and neonatal testes were isolated and cDNA was prepared. Amplification of this cDNA using primers designed to amplify prostaglandin D synthetase indicated that prostaglandin D synthetase was expressed predominantly in the interstitial compartment of the adult testis and in the tubular compartment of the neonatal testis (Fig. 1).

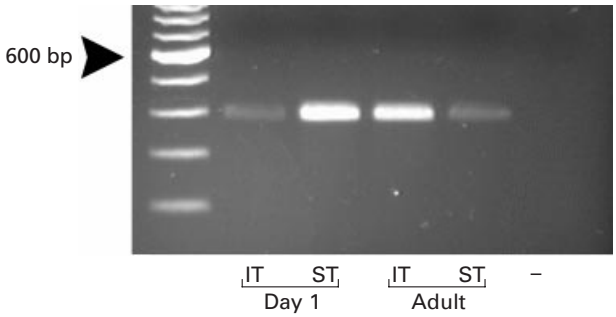

Fig. 1. Expression of prostaglandin D synthetase in different compartments of neonatal (day 1) and adult mouse testis. cDNA was prepared from interstitial tissue (IT) or seminiferous tubules (ST) of neonatal or adult testes and amplified by PCR over 30 cycles using primers specific to prostaglandin D synthetase. A 100 bp ladder was included in the first lane and a tissue blank (–) in the last lane.

In situ *hybridization.* A cRNA probe was hybridized to sections from adult and late fetal testes to confirm localization of prostaglandin D synthetase expression. In the adult testes, grains were clearly localized to the interstitial tissue with little or no apparent hybridization within the tubules (Fig. 2d,f). In the fetus, hybridization was limited to the tubular areas (Fig. 2a,b) and Leydig cells appeared to be largely devoid of grains (arrowhead, Fig. 2b).

Developmental changes in prostaglandin D synthetase expression

Real-time PCR. Curves were prepared using serial dilutions of an arbitrary sample of cDNA and the threshold cycle (C*t*) at which amplification was first detectable above background was plotted against the relative cDNA concentration (Fig. 3a) to characterize amplification of prostaglandin D synthetase and luciferase cDNA using TaqMan probes. The slope of the curve is inversely proportional to the efficiency of the reaction with a slope of –1.4427 equivalent to 100% efficiency. It is necessary to show that the curves are not significantly non-parallel (that the amplification efficiencies are not significantly different) to be able to compare prostaglandin D synthetase cDNA content directly with that of luciferase and this was tested using two-factor ANOVA. The efficiencies of the PCR reactions were similar for probes used in this study (96.6% versus 95.6%, Fig 3a) and the interaction factor from ANOVA was not significant. Therefore, the ratio of prostaglandin D synthetase cDNA:luciferase cDNA was calculated from the difference between the C*t* values (Δ C*t*).

Developmental changes in mRNA content per testis. Accumulated data from a study of changes in prostaglandin D synthetase mRNA expression during postnatal testis development are shown (Fig. 3b). These results show that expression decreases after birth by > 90% to a nadir at day 15. Thereafter, there is an increase in expression until days 25–30, at which time expression per testis is similar to that on the day of birth. After day 30, expression of prostaglandin D synthetase mRNA increases more than ten-fold up to day 40 with a further four- to fivefold increase up to adulthood (Fig. 3b).

Changes in localization of expression. RT–PCR was performed using RNA extracted from tubules and interstitium of mice aged 1, 5, 10, 20, 30, 40 and 90 days to determine the age at which expression of prostaglandin D synthetase mRNA changes from the tubular compartment of the testis to the interstitial compartment (Fig. 4). On days 1 and 5, expression was confined largely to the tubular compartment with only low expression apparent in the interstitial tissue. At days 10 and 20 there was no detectable prostaglandin D synthetase expression (up to 30 cycles of

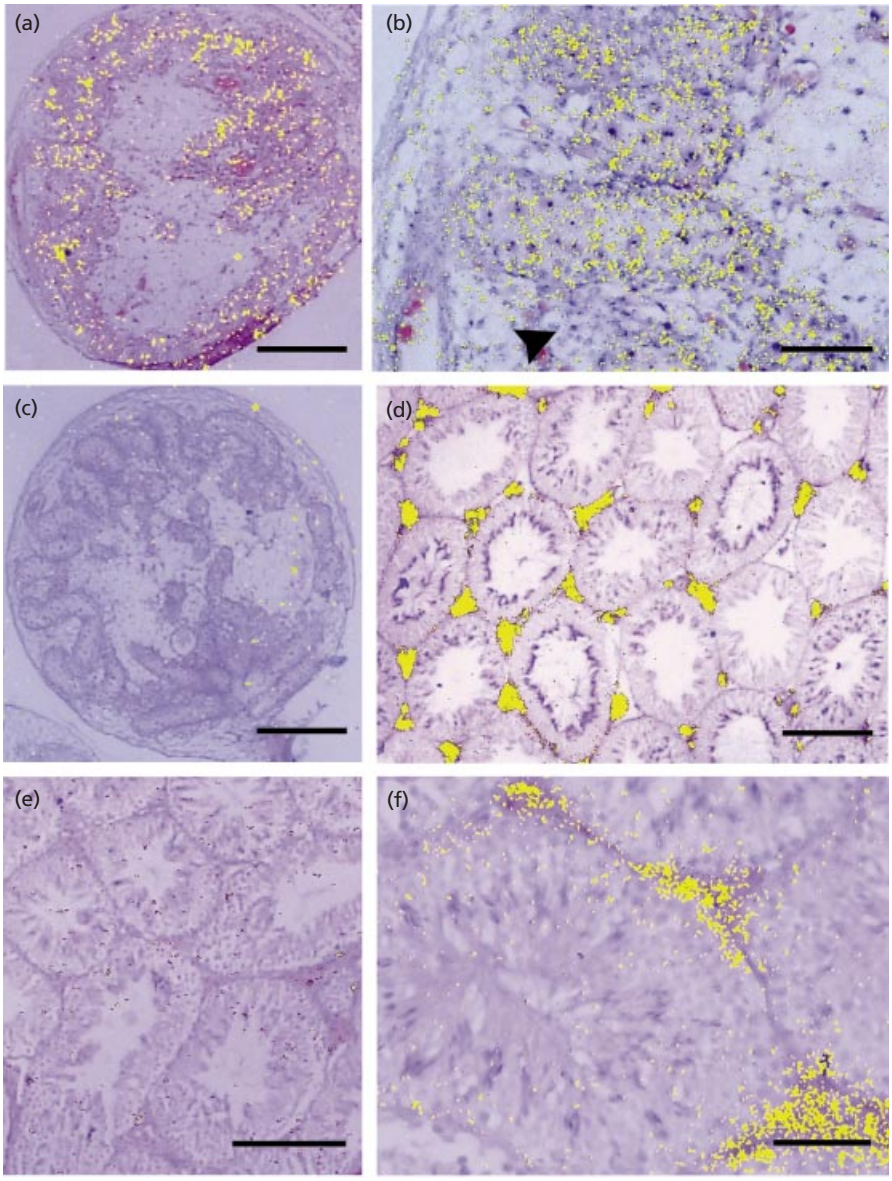

Fig. 2. *In situ* hybridization showing prostaglandin D synthetase mRNA expression in fetal (day 18 of gestation) and adult mouse testes. (a) and (d) are low-power photomicrographs showing anti-sense probe hybridization to fetal and adult testes, respectively. (b) and (f) are higher power photomicrographs showing anti-sense probe hybridization to fetal and adult testes, respectively. The black arrowhead in (b) indicates a group of fetal Leydig cells that show no hybridization. (c) and (e) show hybridization by a sense control probe in fetal and adult testis. Scale bar represents 200 µm (a,c,d,e) or 50 µm (b,f).

PCR) in either compartment. By day 30, expression was detectable again but was confined mainly to the interstitial compartment and this localization was maintained in day 40 and adult mice.

Alternate splicing. There are two sequences of prostaglandin D synthetase in GenBank and comparison with the mouse EST database identified two different 3′ endings that differed by 33 bases. The longer ending is exemplified by AB006361, whereas the shorter ending lacks bases 641–673 of this sequence (Fig. 5). Primers were designed that spanned the alternate region and RT–PCR was performed using RNA from day 1, day 30 and adult testes to show which isoform is predominant *in vivo* and to determine whether there is a change in expression during development. The results indicate that both isoforms are expressed at all three ages and that expression of the two isoforms appears similar, at least after day 1 (Fig. 6).

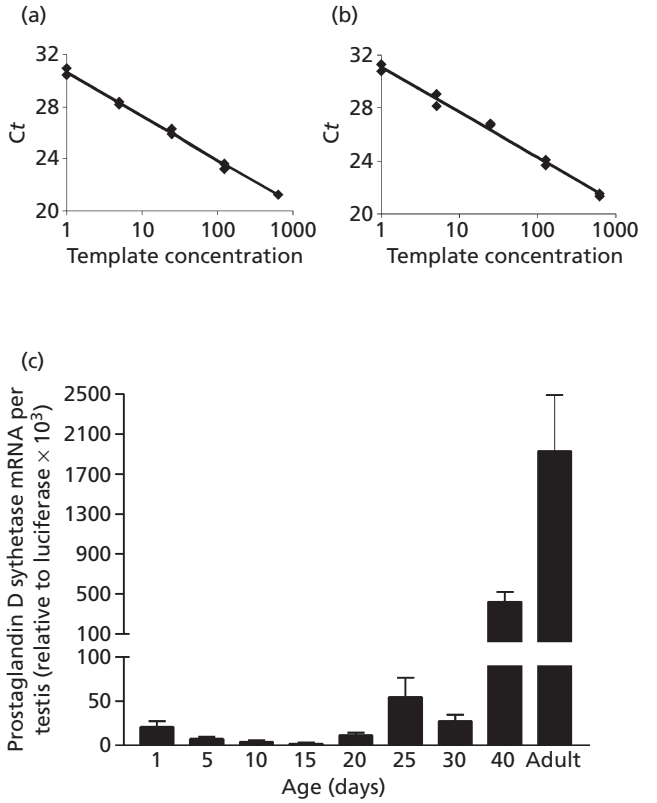

Fig. 3. Real-time PCR amplification of (a) prostaglandin D synthetase and (b) luciferase cDNA. Standard curves prepared by serial dilutions of adult testis cDNA. The log of the relative template concentration was plotted against the threshold cycle (C*t*) and regression analysis was used to generate the best-fit line. The formula given for each curve (prostaglandin D synthestase: $y = -1.4797\ln(x) + 30.702$; and luciferase: $y = -1.4903\ln(x) + 31.127$) describes that line and the slope of the line can be used to determine the efficiency of the PCR reaction. In this case amplification of prostaglandin D synthetase was 96.6% efficient and luciferase was 95.6% efficient. (c) Cumulative data showing prostaglandin D synthetase mRNA content, relative to added luciferase control, during development. Results are mean ± SEM (n = 3–6 animals in each group).

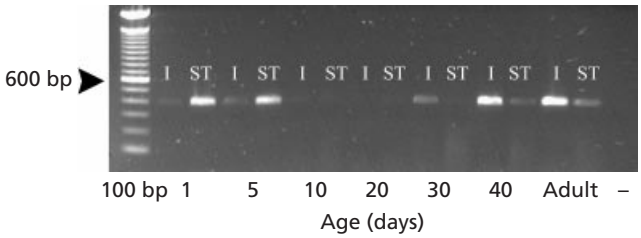

Fig. 4. Expression of prostaglandin D synthetase in interstitial tissue (I) and seminiferous tubules (ST) from mice aged day 1 to adulthood. Interstitial tissue and seminiferous tubules were isolated from animals of different ages and cDNA was prepared. This cDNA was amplified for 30 cycles with primers specific to prostaglandin D synthetase. A 100 bp ladder was included in the first lane and a tissue blank (–) was included in the last lane.

Discussion

The results of the present study indicate that testicular expression of prostaglandin D synthetase is confined largely to the tubules during fetal and postnatal development and that this expression then shifts to the Leydig cells at about the time of puberty. The shift to the Leydig cells is followed by a marked increase in expression, which starts between day 30 and day 40 and continues into adulthood. These changes in expression pattern are very similar to the changes in 17β-hydroxysteroid dehydrogenase type III expression observed during development of the mouse testis (O'Shaughnessy *et al.*, 2000), thereby indicating that shifts in specific mRNA expression from the tubules to the developing adult Leydig cells may not be uncommon.

In the present study, real-time PCR was used to quantify prostaglandin D synthetase mRNA content during development. A housekeeping gene such as β-actin or GAPDH is commonly used in this kind of study as an internal standard to correct for differences in total mRNA content between samples. In developmental studies of the testis, problems arise because not only is there an overall change in the total mRNA content as the tissue grows but there is also a marked change in the proportion of different cell types in the testis as spermatogenesis progresses. An internal standard can be useful under these conditions only if it is expressed equally in all cells at all stages of development, which is probably not the case for the commonly used standards and, in particular, β-actin (Velculescu *et al.*, 1999; Medhurst *et al.*, 2000). An external mRNA was used as a control in the present study to avoid this problem. Provided that an equal amount of this mRNA is added to all tissues and provided that it behaves in the same way as endogenous RNA, then expressing endogenous mRNA relative to the external standard allows direct comparison of expression between tissues of different mass and composition to be made.

The results of the quantitative study of prostaglandin D synthetase expression during development indicate that there is a decrease in expression after birth followed by a marked increase in expression around puberty. Localization studies using RT–PCR and different testicular compartments indicate that the decrease after birth is due to loss of expression in the tubules, whereas the increase in expression after day 20 is due to increasing expression within the Leydig cells. The adult population of Leydig cells arises at about days 7–10 in mice (Baker *et al.*, 1999; Nef *et al.*, 2000) and the timing of prostaglandin D synthetase expression indicates that it arises relatively late in adult Leydig cell development. The rat testis shows a similar increase in expression after puberty, although there are no clear changes in expression between birth and day 10 in rats (Samy *et al.*, 2000). This may be due to the less sensitive methods used by Samy *et al.* (2000), although it is not clear which cell type is the major source of prostaglandin D synthetase in rats. Sertoli cells have been shown to express prostaglandin D synthetase in rats (Sorrentino *et al.*, 1998)

.....GAT AAG TGC ATT CAA GAG TAA acgcaggtgagagaagtcagtcagagggctggtcacatggtga
... D K C I Q E stop
cctggcctcaggactcccttgctctgtcactctcaagatcccagccctggctccccaaagtacctctacaccctccagctttgccttgac

aaagaaataaaagtccaaagcaagtc

Fig. 5. Sequence of the prostaglandin D synthetase 3′ region showing the 3′ end of the open-reading frame and the 3′ untranslated region. The alternately spliced sequence within the 3′ untranslated region is outlined in a box. The polyadenylation site is underlined.

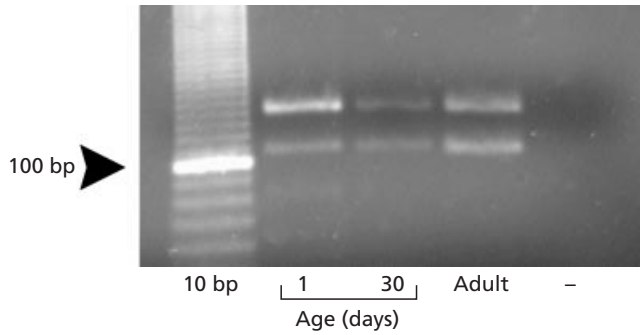

Fig. 6. Expression of alternate transcripts of prostaglandin D synthetase by testes from mice at different ages. cDNA was prepared from whole testes of animals aged 1 day, 30 days and adult and amplified using primers designed specifically to span both alternate transcripts. Lane 1 contained a 10 bp ladder and the last lane was a control without tissue (–). The expected sizes of the transcripts were 140 bp and 107 bp.

but there have been no localization studies in the whole testis.

As with 17β-hydroxysteroid dehydrogenase type III, it is not clear why prostaglandin D synthetase expression in the tubules decreases after birth. The Sertoli cells are still undergoing active proliferation at this time (Vergouwan *et al.*, 1993) and occluding junctions are starting to develop between cells (Nagano and Suzuki, 1976; Kluin *et al.*, 1984). Androgen receptor expression is first apparent in the Sertoli cells at about this time (You and Sar, 1998), indicating that the cells are becoming androgen-sensitive. It is possible that this may act as a trigger to alter the pattern of gene expression within the Sertoli cells. It has been suggested on the basis of immunocytochemical localization that Sertoli cells at stages VI–VIII continue to express prostaglandin D synthetase (Gerena *et al.*, 2000) in adult mice. However, two studies using *in situ* hybridization (present study and Hoffman *et al.*, 1996) have failed to detect significant expression in the Sertoli cells, suggesting either that mRNA content is very low or that the protein has a different site of origin.

The shift in prostaglandin D synthetase expression from Sertoli cells to the Leydig cells may implicate this protein in androgen biosynthesis, particularly as the pattern of expression is similar to that of 17β-hydroxysteroid dehydrogenase type III, which is involved intimately in androgen synthesis by the Leydig cells. Prostaglandin D synthetase binds to retinoic acid and retinal and it may act as a retinoid-binding protein (Tanaka *et al.*, 1997). Retinoids have been implicated in the growth, development and function of many different cell types, including Leydig cells (Chaudhary *et al.*, 1991; Lefevre *et al.*, 1994; Lee *et al.*, 1999), and it is possible that prostaglandin D synthetase may regulate retinoid concentrations in the environment of the Leydig cells. Alternatively, retinoids are required for maintenance of spermatogenesis (Mitranond *et al.*, 1979; Van Pelt and de Rooij, 1990) and Leydig cell-derived prostaglandin D synthetase may act as a carrier in the movement of retinoids to the tubule lumen. It is interesting that the source of prostaglandin D synthetase varies among species, with Leydig cells in mice and humans the major or only source, whereas in bulls Sertoli cells are the only source (this study; Hoffman *et al.*, 1996; Gerena *et al.*, 2000b; Rodriguez *et al.*, 2000). This finding implies either that the role of prostaglandin D synthetase varies among species or that within the testis the source is not relevant, with prostaglandin D synthetase able to function at a distance from the cell of origin as required. It should be noted that prostaglandin D synthetase-null mice are fertile, indicating that the role of prostaglandin D synthetase in the testis can be assumed by other factors such as the cellular retinoic acid-binding proteins (Eguchi *et al.*, 1999).

The significance of alternate splicing in the 3′ untranslated region of the prostaglandin D synthetase transcripts is not known. Regions within the 3′ untranslated region can regulate the stability of mRNA and it is possible that these alternate transcripts have different stabilities in the cell. It is noticeable that the sequence around the splice sites corresponds poorly to the consensus sequences for eukaryote splicing (Smith *et al.*, 1989). In addition, the five bases immediately upstream of the alternately spliced sequence (GGTGA) are identical to the five bases at the 3′ end of the spliced sequence. It is possible that if the spliced region is a short exon, or is at the downstream end of an exon, then either sequence may be used as the 5′ splice donor site leading to constitutive splicing. Despite the expression of prostaglandin D synthetase in two different cell types during development, the results of the present study showed that there were no marked changes in the abundance of the splice forms, which is consistent with this hypothesis of constitutive splicing.

The results of the present study show that expression of prostaglandin D synthetase mRNA shifts during development from the seminiferous tubules of the fetal or neonatal

testis to the adult Leydig cell population in the post-pubertal testis. There are striking similarities with the developmental pattern of 17β-hydroxysteroid dehydrogenase type III, indicating that the adult Leydig cell population assumes a number of the functions that the Sertoli cells express during fetal development.

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