Effects of tri-iodothyronine on alternative splicing events in the coding region of cytochrome P450 aromatase in immature rat Sertoli cells

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Abstract

Transient postnatal hypothyroidism in male rats induces a prolonged proliferation of immature Sertoli cells. This change in Sertoli cell replication at young ages is coincident with enhanced and prolonged aromatase activity that leads to a marked increase in the conversion of androgens into estrogens. Both events are drastically inhibited by tri-iodothyronine (T_3) replacement either *in vivo* or *in vitro*. This study, after the immunolocalization of aromatase in cultured rat Sertoli cells, examined the effects elicited by T_3 on this enzyme, by simultaneously investigating three functional levels of aromatase: mRNA expression, protein content, and enzymatic activity. The immunolocalization of cytochrome P450 aromatase (P450 arom) was shown in the cytoplasm of cultured Sertoli cells from 15- and 21-day-old rats. Western blot analysis revealed an enhancement of aromatase protein content upon stimulation with N^6,2′-*O*-dibutyryladenosine-3′:5′-cyclic monophosphate ($(Bu)_2$cAMP) that was clearly down-regulated by T_3. The presence of a functional P450 arom protein in purified Sertoli cells was confirmed by the measurement of [^{3}H]H_2O released after incubation with [1β-^{3}H]androst-4-ene-3,17-dione. With 100 nM T3, a decrease in both P450 arom mRNA levels and aromatase activity was observed. The aromatase enzymatic activity was strongly stimulated by $(Bu)_2$cAMP and markedly down-regulated by T_3. In contrast, the strong increase in aromatase mRNA upon $(Bu)_2$cAMP stimulation was apparently unaffected by T_3 administration. This paper shows how the identification of an altered transcript induced by T_3 coding for putative truncated and inactive aromatase protein might explain such a decrease in aromatase activity in T_3-treated cells. On the basis of these results, it is concluded that at least two mechanisms could be involved in the down-regulatory effect of T_3 on aromatase activity in prepuberal Sertoli cells. The first mechanism is linked to a possible direct modulatory role for T_3 in the regulation of the aromatase promoter, whilst the second one is represented by the induction of altered transcripts coding for truncated and inactive aromatase proteins.

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Introduction

Aromatase is an enzyme complex located in the endoplasmic reticulum within the cells in which it is expressed. In the testes, it has been immunolocalized in the Leydig cells of humans (Inkster *et al.* 1995), rats (Kurosumi *et al.* 1985), rams (Bilinska *et al.* 1997) and stallions (Almadhidi *et al.* 1995). In mice (Nitta *et al.* 1993), brown bears (Tsubota *et al.* 1993) and roosters (Kwon *et al.* 1995), aromatase is present not only in Leydig cells, but also in the seminiferous tubules.

In cultured human Sertoli cells (Foucault *et al.* 1992) and in immature rat Sertoli cells (Papadopoulos *et al.* 1986), aromatase activity has been detected by measuring the amount of estradiol synthesized from androgen precursors (i.e. testosterone and androstenedione), and by direct evaluation of enzymatic activity assessed from the release of [^{3}H]H_2O (Rosselli & Skinner 1992). However, there is an absence of data concerning the immunolocalization of aromatase in Sertoli cell cultures.

The inhibitory effect of tri-iodothyronine (T_3) on immature Sertoli cell proliferation was concomitant with the inhibitory effects of T_3 on aromatase activity (Jannini *et al.* 1995). Thyroid hormone receptors encoded by the c-*erb*A_α gene are conspicuous in the developing testis, and are maximally expressed in Sertoli cell nuclei during fetal and early postnatal life; their numbers decrease significantly throughout the prepubertal period and are virtually absent in the adult (Jannini *et al.* 1995). The close correlation between Sertoli cell mitogenesis and c-*erb*A_α

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mRNA expression in the normal postnatal testis and the concomitant prolongation of both c-*erb*A_α mRNA expression and Sertoli cell mitogenesis in transient neonatal hypothyrodism (induced by propylthiouracil or methimazole), suggests that high c-*erb*A_α expression and the consequent high content of thyroid receptors (TRs) are markers of proliferating Sertoli cells (van Haaster *et al.* 1992, Bunick *et al.* 1994).

The prolonged proliferation phase observed in immature Sertoli cells prepared from hypothyroid rats was associated with an increase in thyroid receptors (Palmero *et al.* 1993), a dramatic enhancement of aromatase activity (Panno *et al.* 1994) and increased estrogen-receptor content (Panno *et al.* 1996*b*). Hormone replacement (with T_3) markedly shortened Sertoli cell replication (van Haaster *et al.* 1993) and dramatically reduced both aromatase activity (Panno *et al.* 1996*b*) and estrogen-receptor content (Panno *et al.* 1996*a*). On the basis of the latter data, it was reasonable to postulate that T_3 modulates a short autocrine loop in which the formation of estradiol sustains the mitogenic activity of prepubertal Sertoli cells (Sisci *et al.* 1997).

Thus, it became attractive to suggest a link between the effect of T_3 on aromatase activity and the mitogenic activity of prepubertal Sertoli cells. This leads to the consideration of T_3 as a hormone, which, together with follicle-stimulating hormone (FSH), may serve to regulate both events in prepubertal Sertoli cells.

Even though FSH has an important role in controlling aromatase activity via the stimulation of cAMP (Dorrington & Armstrong 1975), the mechanism by which the expression of aromatase is controlled in Sertoli cells has not been defined. Both basal and FSH-induced estradiol production in prepubertal rat Sertoli cells are inhibited either *in vitro* or *in vivo* by T_3 exposure (Panno *et al.* 1994, Ulisse *et al.* 1994). An inhibitory effect on aromatase activity through negative interference with cAMP production has been ruled out, demonstrating that T_3 *per se* does not influence cAMP levels substantially in basal conditions, or in conditions of FSH stimulation (Ulisse *et al.* 1994). Thus, these data suggest that the inhibitory effect of T_3 on aromatase activity is downstream from cAMP formation.

Estradiol production, as an index of aromatase activity, has been reported in rat Sertoli cells to be maximal up to the second postnatal week and to decline dramatically at the onset of spermatogenesis in the third postnatal week (Dorrington & Armstrong 1975). This addresses a possible intrinsic cell mechanism able to down-regulate aromatase activity during the above-mentioned period encompassing the final stages of Sertoli cell maturation.

Therefore, in this study, after immunolocalizing cytochrome P450 aromatase (P450 arom), we used 2- and 3-week-old animals to study the regulatory effects of T_3 on immature rat Sertoli cells. The experiments investigated aromatase expression – under basal conditions and upon stimulation by N^6,2′-*O*-dibutyryladenosine-3′:5′-cyclic monophosphate ($(Bu)_2$cAMP; Sigma, St Louis, MO, USA) – focusing simultaneously on three levels contributing to the expression of aromatase, namely mRNA expression, protein content, and enzymatic activity.

Materials and Methods

Materials

Male Wistar rats (aged 15 and 21 days) were purchased from Morini (Reggio Emilia, Italy). Ham's F-12/Dulbecco's modified Eagle's medium (DMEM-F12), bovine calf serum, 3,3′,5-tri-iodo-L-thyronine, $(Bu)_2$cAMP, hyaluronidase, trypsin inhibitor, DNase I, collagenase/dispase, the BSA protein standard and activated charcoal were purchased from Sigma Chemical Co.; [1β-^{3}H]androst-4-ene-3,17-dione was obtained from New England Nuclear (Boston, MA, USA); Dextran T70 was from Amersham Pharmacia Biotech UK Ltd (Little Chalfont, Buckinghamshire, UK); the Bradford protein assay kit was from Bio-Rad (Richmond, CA, USA); and the enzymes, buffers and nucleotides used for reverse transcription/PCR (RT-PCR) were purchased from Promega (Madison, WI, USA).

Sertoli cell cultures

The testes of immature (15- and 21-day-old) Wistar rats were minced and then subjected to an enzymatic treatment with collagenase–dispase (0·05%), soybean trypsin inhibitor (0·005%), deoxyribonuclease (0·001%) in F-12:DMEM (1:1, v/v) for 15 min at 37 °C. After an additional enzymatic treatment with the cocktail mentioned above, the pellet was subjected to a third enzymatic digestion in the presence of hyaluronidase (0·5%), soybean trypsin inhibitor (0·005%) and deoxyribonuclease (0·001%). The cells were filtered, washed and plated for 48 h (under an atmosphere of 5% CO_2 in air) at 37 °C in DMEM supplemented with 5% bovine serum, in order to attach the Sertoli cells. On Day 2, the culture medium containing germ cells was replaced by fresh culture medium without serum, to prevent the growth of residual peritubular or germ cells still attached to the Sertoli cells (Steinberger & Jakubowiak 1993). On Day 4, the cells were subjected for 3 min at room temperature to a hypotonic treatment with 20 mM Tris–HCl buffer, pH 7·0 (Galdieri *et al.* 1981). This treatment is a rapid and efficient method for eliminating germ cells; they are ruptured without producing any adverse effects on Sertoli cell function (provided that there is a 24 h recovery period at 32 °C) (Wangle *et al.* 1986). The culture dishes were then washed with fresh medium and incubated for 24 h at 32 °C before any treatments were begun.

After these procedures, peritubular cell contamination, evaluated by cytochemical detection of alkaline

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phosphatase activity (Palombi & Di Carlo 1988), was not evident. Furthermore, Leydig cell contamination, evaluated by cytochemical detection of 3β-hydroxysteroid dehydrogenase activity (Rommerts *et al.* 1985), was found to be absent.

Immunocytochemical staining

Rat Sertoli cells were cultured for 48 h on chamber slides in DMEM supplemented with bovine serum (5%) (under an atmosphere of 5% CO_2 in air) at 37 °C. The Sertoli cells were fixed for 30 min in freshly prepared paraformaldehyde (2%). To inhibit endogenous peroxidase activity, hydrogen peroxide (3% in absolute methanol) was used for 30 min. The cells were then incubated for a further 30 min with 10% normal goat serum to block the non-specific binding sites. After that, cells were processed for immunocytochemistry. They were first incubated (overnight at 4 °C) with primary antibody, a rabbit polyclonal antiserum raised against the human placental P450 arom (1:50), provided by Dr Yoshio Osawa (Hauptman–Woodward Medical Research Institute, Buffalo, NY, USA). A second incubation was performed with secondary antibody, a biotinylated goat-anti-rabbit (1:600) immunoglobulin G (Vector Laboratories, Burlingame, CA, USA), for 1 h at room temperature; this was followed by incubation with avidin–biotin–horseradish peroxidase complex (Strept ABC complex/HRP; Vector Laboratories). The peroxidase reaction was developed by incubation with 3–3′-diaminobenzidine tetrachloride dihydrate for 3 min (Stable DAB; Sigma Chemical Co.). After each step of this procedure, cultured cells were rinsed with Tris buffer saline (0·05 M Tris–HCl plus 0·15 M NaCl; pH 7·6) containing 0·05% Triton X-100 (TBS-T); in the last wash, only TBS was used. As control, the primary antibody was replaced by normal rabbit serum at the same concentration. For morphological analysis, May–Grumwald–Giemsa staining of cultured cells was performed.

Western blot analysis

Cells were collected in a lysis buffer (HEPES, 50 mM; NaCl, 150 mM; $MgCl_2$, 1·5 mM; EGTA, 1 mM; glycerol, 10%; Triton X-100, 1%) and the protein concentration was measured using the Bradford method (Bradford 1976). A 50 μg sample of protein was analyzed for each specimen, except for the placenta samples (5 μg). The proteins were separated on SDS-polyacrylamide (11%) gel and then electroblotted onto a nitrocellulose membrane. The blots were incubated overnight at 4 °C with rabbit polyclonal antiserum directed against the human placental P450 arom (1:50). The antigen–antibody complexes were detected by incubating the membranes at room temperature with peroxidase-coupled goat anti-rabbit immunoglobulin G; they were developed using the ECL Plus Western blotting detection system (Amersham Pharmacia Biotech). Blots were then exposed to film, and bands of interest were quantified by densitometry (using a Model 620 densitometer; Bio-Rad). The results obtained as optical density arbitrary values were converted into percentages of the control ('% control'); the values from samples from untreated cells were taken as 100%. Human placental extract was used as a positive control.

Aromatase activity assay

The aromatase activity in culture medium containing subconfluent Sertoli cells was measured by using the tritiated water release assay, with 0·5 μM [1β-^{3}H]androst-4-ene-3,17-dione (New England Nuclear) as the substrate (Lephart & Simpson 1991). Incubations were performed at 37 °C for 5 h under an atmosphere of 5% CO_2 in air. The results obtained were expressed as picomoles per hour and were normalized to mg protein (pmol/h/mg protein), and, where indicated, converted into percentages of the control ('% control'); the values for samples from cells not treated with T_3 (basal or $(Bu)_2$cAMP-stimulated) were taken as 100%.

RNA isolation

Total cellular RNA was extracted from cultured Sertoli cells by using the 'Total RNA Isolation System' kit (Promega). The purity and integrity of the RNA were checked spectroscopically and by gel electrophoresis before the analytical procedures were performed.

RT-PCR assay

Aromatase mRNA was analysed by the RT-PCR method. Total RNA (250 ng or 1 μg) was reverse-transcribed, in a final volume of 20 μl, using 200 U cloned Moloney murine leukemia virus (M-MLV) reverse transcriptase in the presence of 0·4 μg oligo-dT (12–18 mer), 0·5 mM deoxy-NTP and 24 IU RNasin for 30 min at 37 °C, then heat-denatured for 5 min at 95 °C. The cDNAs obtained were further amplified by a PCR using 1 μM primers (Gibco BRL–Life Technologies Italia srl, San Giuliano Milanese, Milan, Italy) designed to amplify the P450 arom highly conserved sequence (289 bp long), including the helical and aromatic regions (Fig. 1A: upstream Aro-Ex8, 5′-GCTTCTCATCGCAGAGTAT CCGG-3′, located in exon 8; downstream Aro-Ex9, 5′-CAAGGGTAAATTCATTGGGCTTGG-3′, located in exon 9; amplified product 289 bp (Ex9)) (Levallet *et al.* 1998*a*). However, for the selective detection of altered transcripts with different 3′-end regions (coding for putative inactive protein previously reported for germ cells and rat ovary (Lephart *et al.* 1990, Levallet *et al.* 1998*b*)), we used the following antisense primers: (1) Aro-Ex10, 5′-GGA ATCGTTTCAAAAGTGTAACCAG-3′, encompassing

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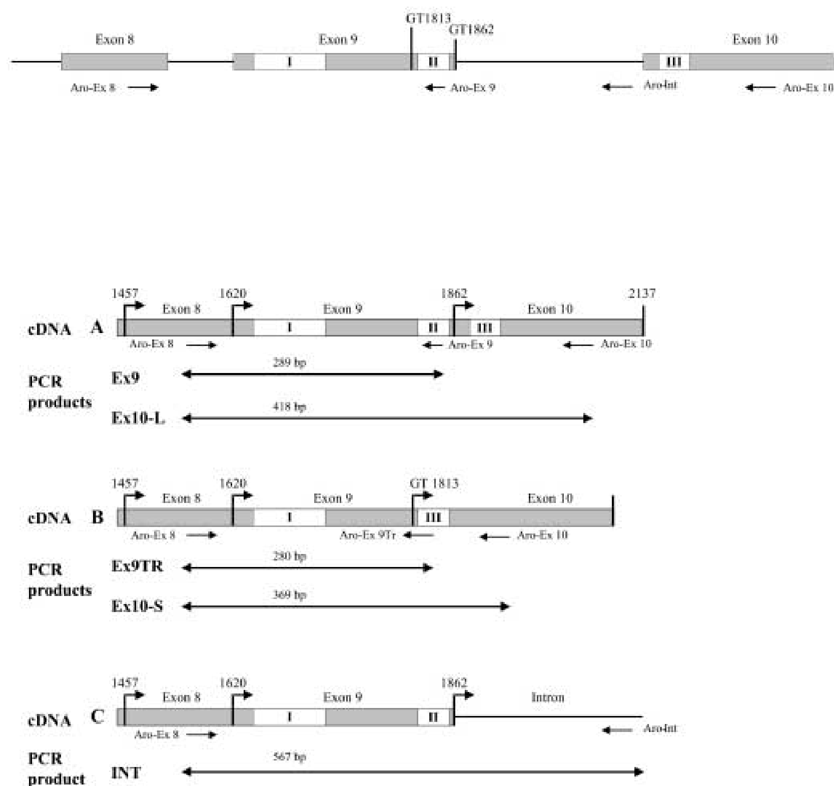

Figure 1 Schematic structure of the 3′-end region of the rat aromatase gene and the aromatase mRNAs detected by RT-PCR. Top: schematic structure of exons 8–10 of the rat aromatase gene. The positions of the introns and exons of the gene, as well as important structural determinants of the gene itself (the helical region, the aromatic region, and the heme-binding region, labeled I, II and III respectively) are shown. Segments of the coding region are shown as thick bars, and intronic segments are shown as thin bars. Bottom: schematic structure of three different mRNAs derived from this segment of the rat aromatase gene. 'cDNA A' represents a cDNA in which normal splicing has taken place. 'cDNA B' represents a transcript in which the GT dinucleotide at 1813 has been used as a splice donor site in place of the normal splice donor (which is at residue 1862). 'cDNA C' is that which results when the intron following exon 9 is not processed normally. The sizes of the PCR products detected for each of these cDNAs, using different combinations of primers, are indicated.

23 nucleotides of exon 10 (1949–1973) (Fig. 1A); (2) Aro-Ex9Tr, 5′-GGCTGAAAATACCTGTAGGGA ACTCG-3′, which matches the three nucleotides before the 49 nucleotides lacking region of exon 9 and the first 23 nucleotides at the beginning of exon 10 (Fig. 1B); (3) Aro-INT, 5′-AAGTCTTTGCCAAATTAAGGA CGC-3′, located in the intronic sequence between exon 9 and exon 10 previously described by Lephart (Lephart *et al.* 1990) (Fig. 1C). The PCR was performed during 35 cycles (1 min at 94 °C, 1 min at 60 °C, 2 min at 72 °C) in the presence of *Taq* DNA polymerase (2 U/tube) and 2·2 mM magnesium chloride in a final volume of 50 µl. To check for the presence of DNA contamination, an RT-PCR was performed on 1 µg total RNA without M-MLV reverse transcriptase (the negative control). An internal control (using water instead of RNA) for each RT-PCR was performed to investigate RNA contamination of the reaction mixture. For each sample, 5 µl PCR amplification products were analysed on 4% agarose gels and stained with ethidium bromide. Standard DNA (100 bp DNA ladder; Promega) was run to provide the appropriate size marker. The specificity of amplification was validated for each reaction by the strong positive signal detected when using total RNA extracted by rat ovary.

Quantitative competitive RT-PCR

Quantitative RT-PCR experiments were performed using a known amount of total RNA added to increasing dilutions of an RNA standard (see below). After RT and PCR with the P450 arom-specific primers, the amplified products of 289 bp (samples) and 260 bp (standard)

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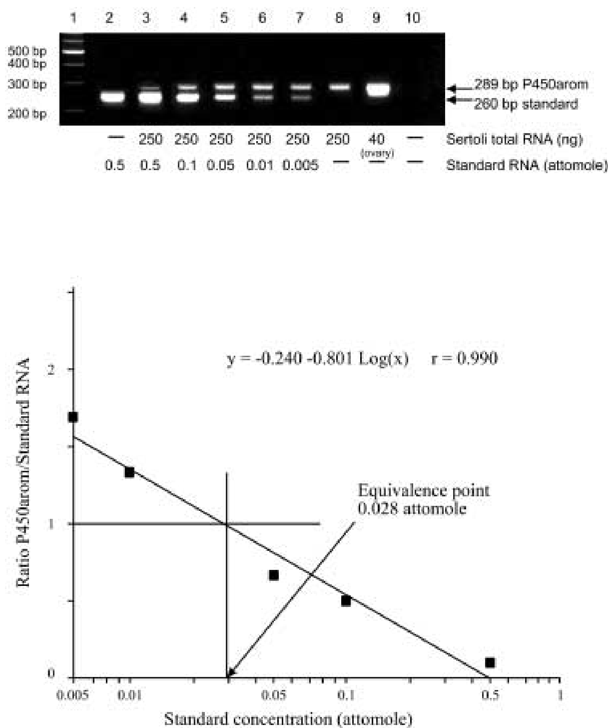

Figure 2 Quantitative competitive RT-PCR of total RNA from immature rat Sertoli cells. P450 arom mRNA was co-amplified with serial dilutions of RNA standard. Lane 1: 100 bp DNA ladder (Promega). Lane 9: RT-PCR performed using rat ovary mRNA as the positive control. Lane 10: negative control in which RT-PCR was performed on 1 µg total RNA without M-MLV reverse transcriptase (primer Aro-Ex8/Aro-Ex10). An internal control (water instead of RNA) for each RT-PCR was performed to investigate RNA contamination of the reaction mixture (not shown). The amplified products were subjected to electrophoresis on 4% agarose gel; the gel was photographed and analyzed by densitometry. The P450 arom mRNA content was estimated by the determination of the equivalence point, according to the equation established by Menzo *et al.* (1992).

obtained were separated on a 4% agarose gel stained with ethidium bromide, then photographed and analysed by densitometry (using a Model 620 densitomere; Bio-Rad). The arbitrary values obtained were utilized to build a curve by plotting the ratio between the 289 bp and 260 bp values versus the logarithm of the standard concentration used. The 260 bp values were corrected by the coefficient 289 bp/260 bp. P450 arom mRNA content was estimated by the determination of the equivalence point (ratio = 1) according to the equation established by Menzo *et al.* (1992) (Fig. 2).

RNA standard preparation

The RNA standard was prepared by internal nucleotide deletion of the 289 bp amplified product. Total RNA from rat ovary was subjected to RT and PCR with the appropriate primers. The 5′STD primer contained the following three specific sequences: (1) the complementary sequence for the DNA-binding region of T7 RNA polymerase; (2) the primer Aro-Ex8 (underlined); (3) a 27-base sequence (position 1607–1634) located 29 bp downstream from the Aro-Ex8 primer (bold): 5′STD, 5′-TAATACGACTCACTATAGGGAGAGCTTCTCATCGCAGAGTATCCGG-**CACACTGTTGTTGTTGACAGAGACATA**-3′. The 3′STD primer resulted from the association of the oligo(dT) and the specific Aro-Ex9 sequences (underlined) 3′STD: 5′-TTTTTTTTTTTTTTTTTCAAGGGTAAATTCATTGGGCTTGG-3′. The cDNA of interest now contained sequences for the T7 polymerase; it had a 29 bp deletion relative to the native mRNA strand. Using 20 IU RNasin, 2·5 mM of each ribonucleotide and 20 IU T7 RNA polymerase, this cDNA strand was then transcribed to an internal competitive RNA standard of 260 bp. The RNA standard was treated with 1 IU RQ1 RNase-free DNase (Promega) to digest the cDNA templates. The RNA was extracted and resuspended in diethyl pyrocarbonate-treated water.

Extraction from agarose gel and sequencing of P450 arom RT-PCR transcript

The RT-PCR products were extracted and purified from agarose gel by using the QIAquick gel extraction kit (Promega) and sequenced using radioactive dideoxy chain terminating method (Sequenase kit; Amersham Pharmacia Biotech).

Statistical analysis

Each data point represents the mean ± S.E.M. of three experiments. Data were analysed by ANOVA, using the STATPAC computer program (Glantz 1988).

Results

Immunocytochemistry

The purified Sertoli cells from 15- and 21-day-old rats were recognizable by their typical morphological features evidenced by May–Grumwald–Giemsa staining (Fig. 3a and d). Strong P450 arom immunoreactivity was observed in the cytoplasm of cultured Sertoli cells from 15-day-old rats, whereas only moderate cytoplasmic immunoreactivity was found for 21-day-old rats; furthermore, no reaction was detected in the nuclei (Fig. 3b and e). No immunostaining was observed in the cells of either age group when processed without primary antibody (Fig. 3c and f).

Effects of T_3 on P450 arom protein expression in Sertoli cells

A 55 kDa specific protein in Sertoli cells co-migrated with P450 arom human placental protein (Fig. 4). $(Bu)_2cAMP$

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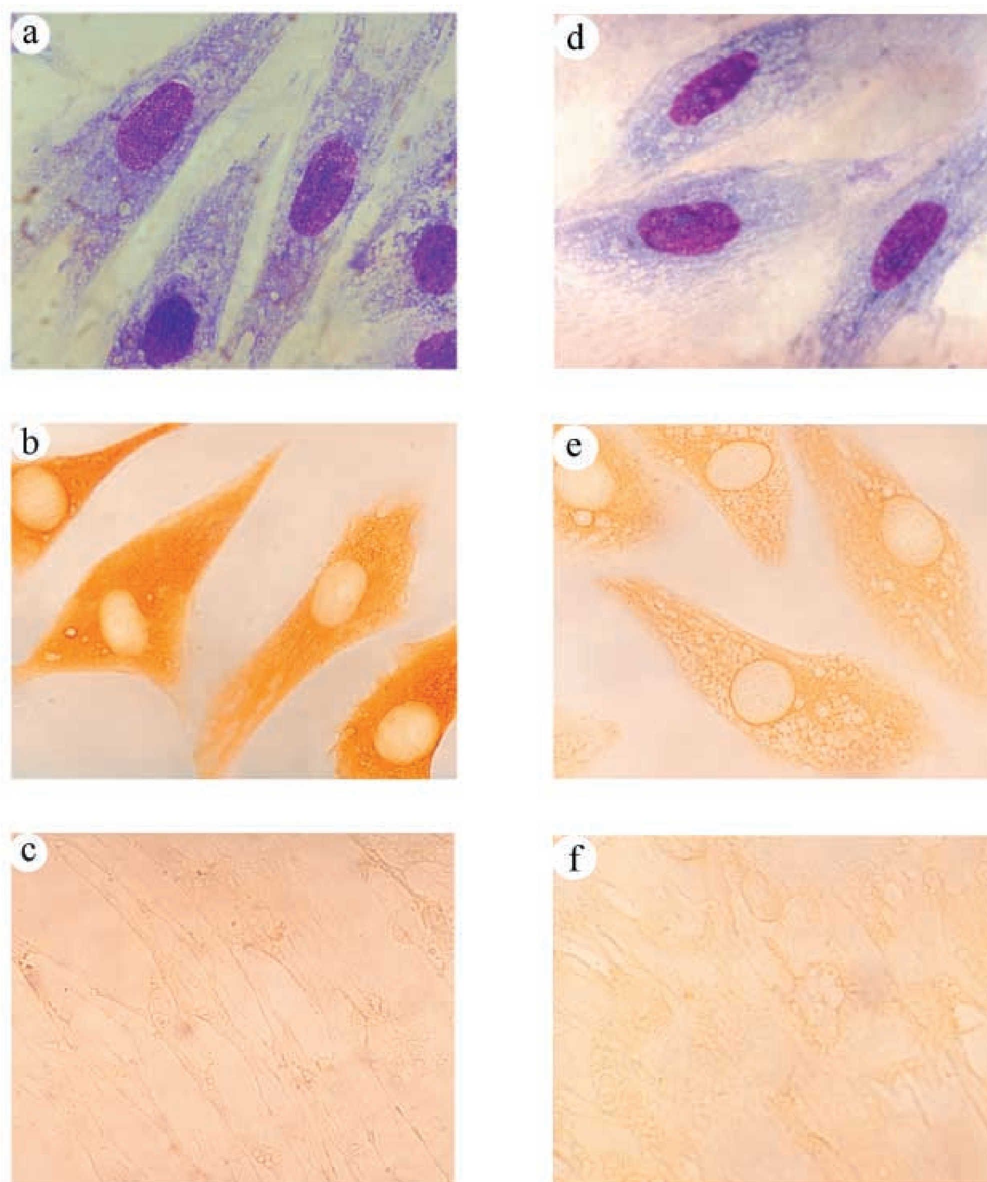

Figure 3 Morphology and P450 arom immunoreactivity in cultured Sertoli cells from immature rats. (a–c) Cultured Sertoli cells from 15-day-old rats. (a) May–Grunwald–Giemsa staining. (b) Strong P450 arom immunoreactivity in the cell cytoplasm; unstained nuclei. (c) Negative immunoreaction in control culture. (d–f) Cultured Sertoli cells from 21-day-old rats. (d) May–Grunwald–Giemsa staining. (e) Moderate P450 arom immunoreactivity in cell cytoplasm; immunonegative nuclei. (f) No immunoreaction in control culture. Magnification: × 850.

administration slightly enhanced aromatase protein content (15 ± 4·0% (P<0·05 with respect to the control) in 15-day-old rats and 18 ± 5·5% (P<0·05 with respect to the control) in 21-day-old rats). This increase was reduced by 40 ± 5·5% (P<0·01 with respect to the $(Bu)_2$cAMP-stimulated samples in 15-day-old rats) and by 20 ± 4·5% (P<0·05 with respect to the $(Bu)_2$cAMP-stimulated samples in 21-day-old rats) in samples treated with T_3 and $(Bu)_2$cAMP (Fig. 4); similar results were obtained using 100 nM T_3 (data not shown). Basal levels were not influenced by T_3 treatment (Fig. 4).

Effect of T_3 on aromatase activity in Sertoli cells

Aromatase activity measurement in Sertoli cells of 15- and 21-day-old rats was 0·055 ± 0·004 and

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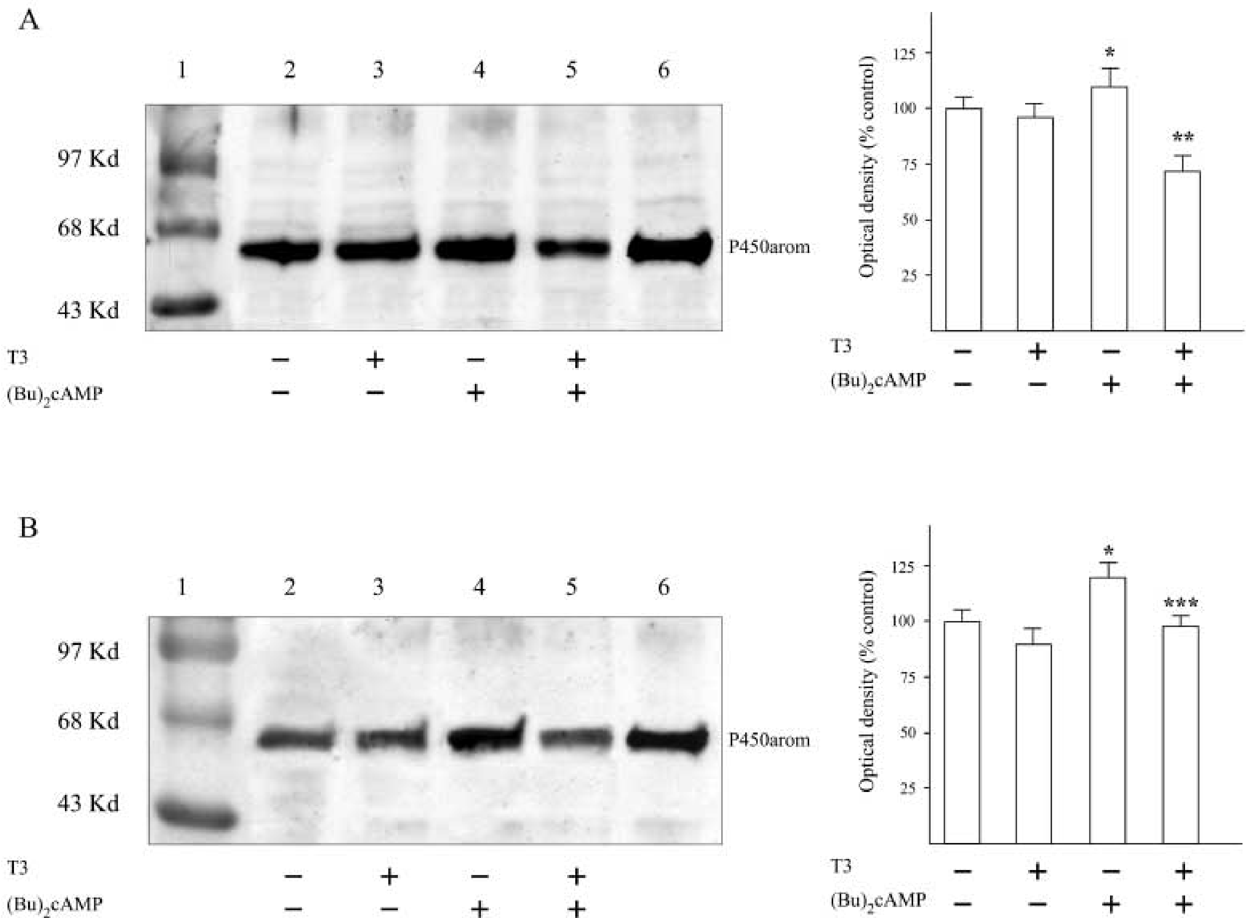

Figure 4 Western immunoblotting of P450 arom in cultured Sertoli cells from 15-day-old rats (A) and 21-day-old rats (B). After isolation (see Materials and Methods), cells were cultured for 72 h in DMEM-F12 in the absence (lane 2) or presence of T_3 (1 nM) (lane 3), or were treated with $(Bu)_2$cAMP (0·5 mM) in the final 24 h (lane 4) or with T_3 (1 nM) for 72 h combined with $(Bu)_2$cAMP (0·5 mM) in the final 24 h (lane 5). Human placenta extract (lane 6) was used as the positive control; 50 µg protein was analyzed for each specimen (except placenta; 5 µg). Lanes 1 show prestained high-range-molecular-mass markers (Inalco, Milan, Italy). The autoradiographs on the left-hand side of the Figure show the results of one representative experiment, whereas the histograms on the right-hand side show the mean values ± S.E.M. from densitometric analyses of three separate experiments. The results obtained (optical density arbitrary values) were converted into percentages of the control ('Optical density (% control)'), samples from untreated cells being taken as 100%. *P<0·05 with respect to the controls; **P<0·01 and ***P<0·05 with respect to the $(Bu)_2$cAMP-stimulated samples.

0·065 ± 0·006 pmol/h/mg protein respectively. In the rat Leydig tumor cell line designated as R2C (American Type Culture Collection no. CCL-97) used as positive controls, the level was 130 ± 4·5 pmol/h/mg protein, which is similar to the basal values reported in the literature (Young *et al.* 1997).

Basal aromatase activity in Sertoli cells from 15-day-old rats was partially inhibited after incubation of T_3 for 72 h (T_3 (1 nM) = 54·5 ± 3·0% inhibition, P<0·01; T_3 (100 nM) = 51·7% ± 7·0% inhibition, P<0·01 with respect to the control) (Fig. 5). The degree of inhibition was less when these experiments were performed using Sertoli cells from 21-day-old rats (T_3 (1 nM) = 25·7 ± 4·8% inhibition, P<0·01; T_3 (100 nM) = 28·7 ± 6·0% inhibition, P<0·01 with respect to the control). Shorter periods of treatment did not affect the basal aromatase activity of Sertoli cells at all ages examined (data not shown).

Treatment with $(Bu)_2$cAMP (0·5 mM) for 24 h strongly stimulated (a 30–50-fold increase; data not shown) the enzymatic activity in both ages examined; this stimulation was partially reversed by T_3 treatment for 72 h at both ages investigated (15-day-old rats: $(Bu)_2$cAMP+T_3 (1 nM) = 39·4 ± 2·1% inhibition, P<0·01; $(Bu)_2$cAMP+T_3 (100 nM) = 49·1 ± 8·2% inhibition, P<0·01; 21-day-old rats: $(Bu)_2$cAMP+T_3 [1 nM] = 22·9 ± 3·1% inhibition P<0·05; $(Bu)_2$cAMP+T_3 (100 nM) = 24·4 ± 3·6% inhibition, P<0·05 with respect to the $(Bu)_2$cAMP-stimulated samples) (Fig. 5). A significant difference was observed between the percentage inhibition by T_3 (used at physiological concentration (1 nM)) on $(Bu)_2$cAMP-induced

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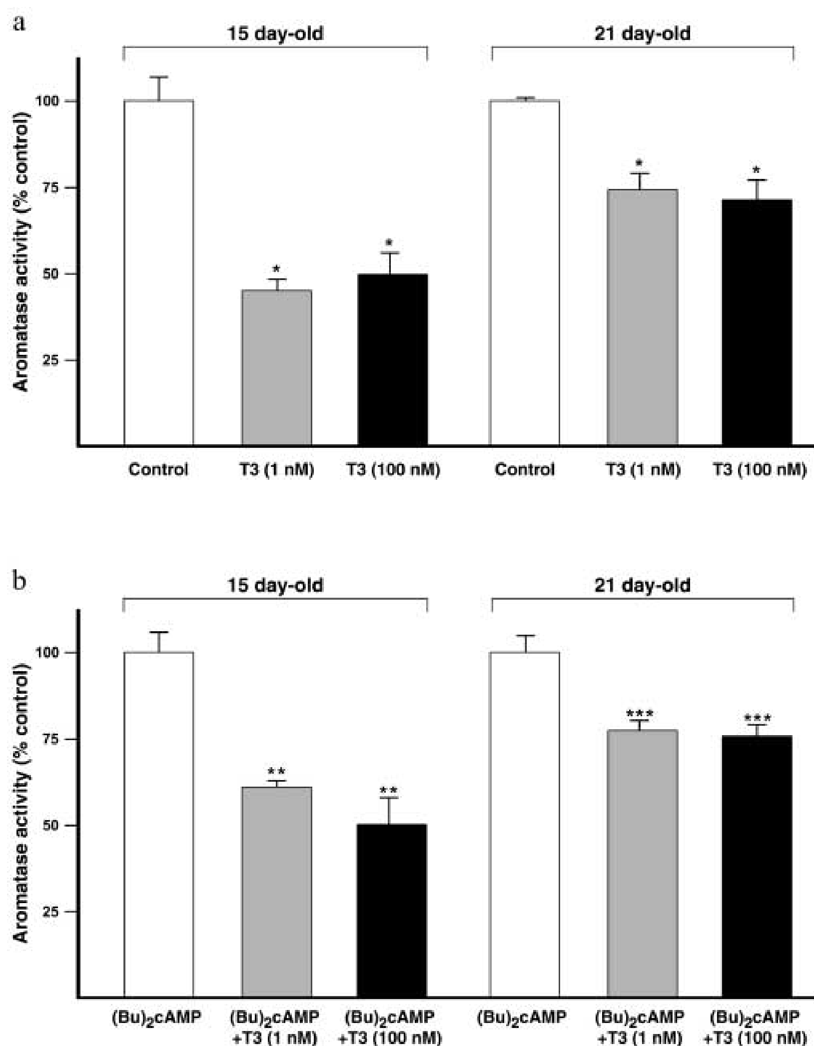

Figure 5 Effects of T_3 on aromatase activity in basal (a) or $(Bu)_2$cAMP-treated (b) cultured Sertoli cells from 15- and 21-day-old rats. After isolation (see Materials and Methods), cells were cultured for 72 h in DMEM-F12 in the absence (control) or presence (T_3 (1 nM or 100 nM)) of T_3, or treated with $(Bu)_2$cAMP (0·5 mM) in the final 24 h ('$(Bu)_2$cAMP)') or with T_3 (1 nM or 100 nM) for 72 h combined with $(Bu)_2$cAMP (0·5 mM) in the final 24 h ('$(Bu)_2$cAMP+T_3'). Aromatase activity was evaluated by measuring the tritiated water released by Sertoli cell cultures after incubation with 0·5 µM [1β-^{3}H]androst-4-ene-3,17-dione at 37 °C for 5 h. The results obtained were expressed as picomoles [^{3}H]H_2O released per hour and were normalized for mg protein (pmoles/h/mg protein) and converted into percentages of the control ('% control'); samples from cells not treated with T_3 (control) or $(Bu)_2$cAMP were taken as 100%. Values represent the means ± S.E.M. of three different experiments. *$P<0·01$ with respect to the controls; **$P<0·01$ and ***$P<0·05$ with respect to the $(Bu)_2$cAMP-stimulated samples.

aromatase activity in 15-day-old rats (54·5 ± 3·0%) and that measured in 21-day-old rats (25·7 ± 4·8%, $P<0·05$).

Effects of T_3 on P450 arom mRNA expression: evidence of normal and altered transcripts

In the group of two-week-old rats exhibiting higher percentages of inhibition of aromatase activity, we investigated the effects of T_3 on basal and $(Bu)_2$cAMP-stimulated P450 arom mRNA levels by using quantitative competitive RT-PCR (with primers Aro-Ex8 and Aro-Ex9). T_3 was able to inhibit mRNA basal expression only at the 100 nM dose (control=0·11 ± 0·005; T_3 (100 nM)=0·064 ± 0·004 attomoles/µg total RNA, $P<0·01$) (Fig. 6). The $(Bu)_2$cAMP stimulation does not appear to be substantially influenced by T_3 exposure

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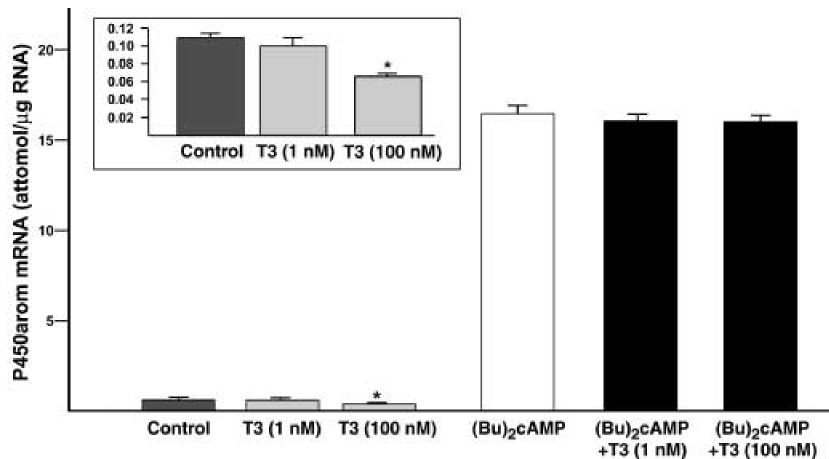

Figure 6 Effects of T_3 on aromatase mRNA levels in cultured Sertoli cells from 15-day-old rats, as determined by quantitative competitive RT-PCR. Cells were cultured for 72 h in DMEM-F12 in the absence (control) or presence of T_3 ('T_3') at the concentrations indicated or treated with $(Bu)_2cAMP$ (0·5 mM) in the final 24 h ('$(Bu)_2cAMP$') or with T_3 (1 nM and 100 nM) for 72 h combined with $(Bu)_2cAMP$ (0·5 mM) in the final 24 h ('$(Bu)_2cAMP+T_3$'). Total RNA was extracted, reverse-transcribed and amplified by the Aro-Ex8 and Aro-Ex9 primers for 35 cycles with serial dilutions of the RNA standard. The results obtained were expressed as attomoles/µg total RNA. Values represent the means ± S.E.M. of three different experiments; $^*P<0{\cdot}01$ with respect to the control.

(despite the down-regulatory effects on both protein aromatase content and aromatase activity).

In order to explain this apparent discrepancy, we must also take into account the possibility that the aromatase mRNA assays may be measuring different mRNA species coding for inactive protein that is unable to convert androgens into estrogens. To investigate the possible presence of these different mRNA species in immature Sertoli cells, we used appropriate primers to expand the region of the aromatase mRNA amplified to include all three functional regions (i.e. the helical, aromatic, and heme-binding regions). By utilizing the primers Aro-Ex8 and Aro-Ex10 (Fig. 1A and B), in the presence of T_3 treatment in basal conditions and upon stimulation with $(Bu)_2cAMP$, we obtained, together with the normal expected fragment of 418 nucleotides (Ex10-L), a shorter fragment of 369 nucleotides (Ex10-S) (Fig. 7A). Nucleotide sequence analysis of these products revealed that the shorter segment is the same as that reported by Levallet (Levallet *et al.* 1998*b*) and results from an alternative splicing site on a GT dinucleotide at position 1813–1814 (Fig. 1C). This change leads to an alteration of the reading frame and to the appearance of a stop codon, yielding a putative protein lacking both the aromatic and heme-binding regions. When we amplified the cDNA of P450 arom by using primers Aro-Ex8 and antisense primers Aro-INT (located in the intronic sequence described by Lephart *et al.* (1990)) (Fig. 1C), we obtained the expected fragment of 567 nucleotides (INT) only upon $(Bu)_2cAMP$ stimulation (Fig. 7A). To prove the identity of these two altered transcripts, we re-amplified them with internal antisense primers. When we used Aro-Ex9Tr, we obtained the expected fragment of 280 bp (Fig. 7B). Moreover, to confirm that the aromatic region is internal to the INT fragment, we used the antisense primer Aro-Ex9 and reproduced the band of the expected size (289 nucleotides; Fig. 7B). The identities of the transcripts obtained were further verified by nucleotide sequence analysis.

Discussion

In this work, complementary approaches were used to investigate the role of T_3 in aromatase expression in prepuberal Sertoli cell cultures. Morphological analysis and cytochemical evaluation of phosphatase alkaline and 3-βHSD activity excluded the presence of germ, peritubular and Leydig cell contamination in Sertoli cell cultures. In fact, the typical features of Sertoli cells (represented by the characteristic nucleus with one large, centrally located nucleolus and two chromocenters containing all of the centromeric heterochromatin; Jean *et al.* 1983, Russell 1993) were observed in culture cells.

Aromatase immunolocalization was revealed in the cytoplasm of cultured Sertoli cells from immature rats at both ages investigated. Using the same polyclonal antiserum against human placental P450 arom, Western analysis revealed a 55 kDa specific protein simultaneously in rat Sertoli cells and in human placenta used as a positive

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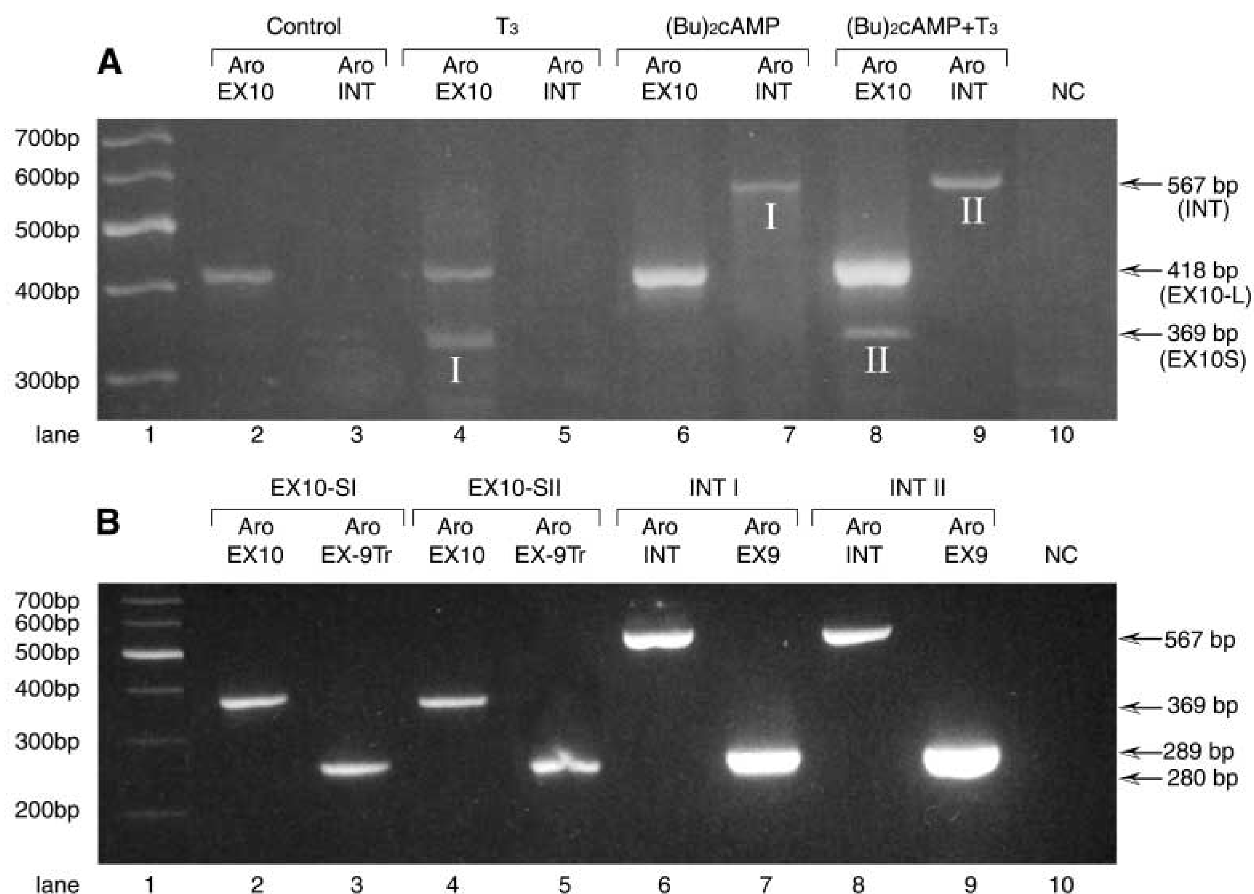

Figure 7 Amplification of various P450 arom mRNAs extracted from cultured rat Sertoli cells from 15-day-old rats. (A) Sertoli cells were cultured for 72 h in DMEM-F12 in the absence (control) or presence of T_3 ('T_3'; 1 nM) or treated with $(Bu)_2$cAMP (0·5 mM) in the final 24 h ('$(Bu)_2$cAMP') or with T_3 (1 nM) for 72 h combined with $(Bu)_2$cAMP (0·5 mM) in the final 24 h ('$(Bu)_2$cAMP+T_3'). For each treatment, two aliquots (1 µg each) of total RNA were amplified by RT-PCR, using different antisense primers (Aro-Ex10 (first aliquot; lanes 2, 4, 6 and 8) and Aro-INT (second aliquot; lanes 3, 5, 7 and 9)) and the same sense primers (Aro-Ex8). The expected 418 bp transcript (Ex10-L) was obtained in all samples amplified with Aro-Ex10, while an additional shorter transcript of 369 bp (Ex 10-S I and II) was obtained in only T_3-treated samples (lanes 4 and 8). A 567 bp transcript (INT I and II) was obtained in samples treated with $(Bu)_2$cAMP (lanes 7 and 9). Lane 1: 100 bp DNA ladder (Promega). Lane 10 (negative control): RT-PCR was performed on 1 µg total RNA without M-MLV reverse transcriptase (primer Aro-Ex8/Aro-Ex10). (B) Electrophoresis of cDNA after purification and re-amplification. Aliquots of each of four different transcripts (Ex 10-S I and II, INT I and II) were purified and reamplified using the same sense primer Aro-Ex8 and different antisense primers, as indicated in the Figure. Lane 1: 100 bp DNA ladder (Promega). Lane 10 (negative control): RT-PCR was performed on 1 µg total RNA without M-MLV reverse transcriptase (primer Aro-Ex8/Aro-Ex10). An internal control (water instead of RNA) for each RT-PCR was performed to investigate RNA contamination of the reaction mixture (not shown). All of the amplified products were separated on 4% agarose gel stained with ethidium bromide.

control. Western blot analysis revealed that an enhancement of aromatase protein content upon $(Bu)_2$cAMP stimulation was clearly down-regulated by T_3. The presence of a functional P450 arom protein in purified Sertoli cells was confirmed by the measurement of $[^3H]H_2O$ released after incubation with $[1\beta\text{-}^3H]$androst-4-ene-3,17-dione.

Despite the consistent detection of aromatase activity and immunoreactive aromatase protein, our studies identified a few discrepancies between the aromatase content evaluated by Western blot analysis and the aromatase enzymatic activity. T_3 does not seem to have a substantial influence on basal aromatase content; the enzymatic activity, however, appears to be markedly down-regulated.

Crucial effects of T_3 on Sertoli cell differentiation have also been documented (Jannini *et al.* 1995), and, even though FSH has early effects on Sertoli cell proliferation and aromatase activity that contrast with the effects of T_3, additive effects of two hormones on several parameters of Sertoli differentiation (Müllerian inhibiting substance, androgen receptors, inhibin α) have been reported (Cooke *et al.* 1994, Arambepola *et al.* 1998*a*,*b*). Therefore, the

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induced decrease in aromatase activity may be included in the large spectrum of differentiative effects induced by T_3 in immature Sertoli cells.

The inhibitory effects of thyroid hormone on the estradiol response to FSH occur downstream of cAMP formation (Ulisse *et al.* 1994). In 2-week-old animals, the more pronounced inhibitory effect elicited by T_3 on $(Bu)_2$cAMP-induced aromatase stimulation with respect to the older group may be mediated by a higher TR content. Therefore, to improve our evaluation of the effects of T_3 on aromatase mRNA expression, we preferred to focus only on the 2-week-old rats, since thyroid receptor levels are more elevated at this age (Jannini *et al.* 1995).

With T_3 at 100 nM, we observed a decrease in both aromatase mRNA levels and aromatase activity. The first event somehow addresses a possible regulatory effect of T_3 on aromatase promoter. Our recent studies (data not shown) indicate that the same promoter (promoter II), proximal to the translation start site, regulating P450 arom in fetal gonads, in ovaries of humans, rats, chicken and in two rat Leydig tumor cells, controls aromatase mRNA in Sertoli cells. This has been proved in cultured Sertoli cells prepared from immature rat testis during the crucial time of interest, even though we cannot exclude the contribution of a small amount of aromatase mRNA derived from other promoters (Young *et al.* 1999). In this promoter II at position −90, a steroidogenic factor-1 sequence containing a thyroid responsive element half-site is recognizable (Young & McPhaul 1998); thus, it is possible that a direct effect of thyroid hormone on this regulatory region of aromatase could explain the inhibitory effects on aromatase mRNA when we treated Sertoli cells with high doses of thyroid hormones. We could hypothesize that the latter effect would be somehow be overcome upon $(Bu)_2$cAMP stimulation, since, in such circumstances, no inhibitory effects induced by T_3 were noticed.

In the course of our studies, we identified unspliced and alternatively spliced forms of aromatase mRNA. The RNAs were of two types; the two unusual transcripts (Ex10-S and INT) identified here were previously reported in rat germ cells but were absent in mature Sertoli cells (Levallet *et al.* 1998*b*). We have demonstrated, for the first time, alternative splicing events during aromatase gene expression in immature Sertoli cells stimulated by hormonal treatment. Alternative splicing events in the coding sequences of aromatase mRNA are present in various species (rodents, porcine animals, bovine animals, fish) and tissues (testis, ovary, placenta) (Vanselow & Furbass 1995, Fukada *et al.* 1996, Choi *et al.* 1997, Furbass *et al.* 1997), but the mechanisms involved are not clear (Levallet *et al.* 1998*a*). For instance, the apparent discrepancy between the unchanged aromatase mRNA levels and the decrease in aromatase activity which occurs upon combined treatment with $(Bu)_2$cAMP and T_3 might be explained by the concomitant presence of a T_3-induced altered transcript not revealed by our quantitative RT-PCR and coding for putative inactive aromatase proteins.

The effects on the predicted protein products differ for the two mRNAs. The altered transcript (determined by a comparison between the predicted nucleotide sequence and the rat ovary sequence of 508 amino acids (Hichey *et al.* 1990)), induced by T_3 leads to a truncated protein of 428 amino acids, with an alteration of protein sequence following amino acid 405 and lacking both the heme-binding and aromatic domains. However, the altered transcript observed upon $(Bu)_2$cAMP treatment (simulating FSH stimulation) for 24 h would, according to its nucleotide sequence, lead to the synthesis of a truncated protein of 426 amino acids lacking the heme-binding domain because of the appearance of a stop codon 5 amino acids after the end of exon 9. Inspection of the Western blot (Fig. 4) failed to detect such a truncated aromatase protein species, suggesting that they do not accumulate to any significant extent. Although such shortened proteins are not evident in our immunoblots, it is not clear as to whether these two proteins could be distinguished electrophoretically. We must take into account the possibility that the polyclonal antiserum against human placental aromatase used properly here for immunostaining was inadequate for precise discrimination of changes in aromatase protein content occurring after hormonal treatments. Nevertheless, we cannot rule out the possibility that hormonal treatment(s) *per se* might also influence the functional status of translated aromatase protein.

The induced altered transcript revealed upon $(Bu)_2$cAMP treatment, concomitant with the strong enhancement of aromatase mRNA, may somehow represent an incipient mechanism regulating aromatase along the induced differentiation. This may contribute to an explanation of the progressive inhibitory effect on aromatase induced by chronic FSH treatment, as observed in prepubertal Sertoli cell cultures (Schteingart *et al.* 1995).

In conclusion, on the basis of our results, it emerges that at least two mechanisms could be involved in the down-regulatory effect of T_3 on aromatase activity in prepuberal Sertoli cells. The first mechanism is linked to a possible direct modulatory role for T_3 in the regulation of aromatase promoter activity (though the molecular mechanism remains to be clarified); the second mechanism is represented by the induction of altered transcripts coding for truncated and inactive aromatase proteins.

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