Histochem Cell Biol (2008) 129:631–642
DOI 10.1007/s00418-008-0397-8

ORIGINAL PAPER

Thyroid hormone receptor α_1–β_1 expression in epididymal epithelium from euthyroid and hypothyroid rats

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Accepted: 30 January 2008 / Published online: 26 February 2008

Abstract The objectives of the present work were to assess whether epithelial cells from the different segments of epididymis express TRα_1–β_1 isoforms, to depict its subcellular immunolocalization and to evaluate changes in their expression in rats experimentally submitted to a hypothyroid state by injection of 131I. In euthyroid and hypothyroid groups, TR protein was expressed in epididymal epithelial cells, mainly in the cytoplasmic compartment while only a few one showed a staining in the nucleus as well. A similar TR immunostaining pattern was detected in the different segments of the epididymis. In hypothyroid rats, the number of TR-immunoreactive epithelial cells as well as the intensity of the cytoplasmic staining significantly increased in all sections analyzed. In consonance to the immunocytochemical analysis, the expression of TRα_1–β_1 isoforms, assessed by Western blot revealed significantly higher levels of TR in cytosol compared to the nuclear fractions. Furthermore, TR expression of both α_1 and β_1 isoforms and their mRNA levels were increased by the hypothyroid state. The immuno-electron-microscopy showed specific reaction for TR in principal cells associated with eucromatin, cytosolic matrix and mitochondria. The differences in expression levels assessed in control and thyroidectomized rats ascertain a specific function of TH on this organ.

Keywords Hypothyroidism · Thyroid hormone receptor α_1–β_1 isoforms · Epididymis · Immunohistochemistry

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Introduction

Thyroid hormone (TH): the pro-hormone thyroxine (T_4) and the active hormone 3,5,5′-triiodothyronine (T_3) are essential for normal development, growth and metabolism (Harvey and Williams 2002; Yen 2001). The classical genomic actions of TH are mediated by the thyroid hormone receptors (TR). The TR are members of the nuclear receptor superfamily and act as hormone inducible transcription factors.

There are two TR genes (TRA and TRB), from which several isoforms are generated. The TRA gene encodes for TRα_1, the non-hormone binding splicing variant TRα_2 and the truncated products TR$\Delta\alpha_1$ and TR$\Delta\alpha_2$. Through alternative promoter usage, the TRB gene yields TRβ_1, TRβ_2, TRβ_3 and the truncated variant TR$\Delta\beta_3$, which is unable to bind T_3 (Bassett et al. 2003). Genetic studies have revealed that TR isoforms differ in their physiological roles and in their tissue distribution (Forrest and Vennström 2000; Zinke et al. 2003).

In male reproductive tract, TH has been implicated in differentiation and maturation of fetal tissues including the

testis (Cooke 1991). Moreover, TH plays an integral role in the development and proliferation of Sertoli cells (Buzzard et al. 2003; Holsberger et al. 2003; Holsberger and Cooke 2005). In thyroidectomized rats, degenerative changes have been shown to occur in the seminiferous epithelium resulting in the inability of Sertoli cells to support spermatogenic cells (Oncu et al. 2004). Recently, Holsberger et al. (2005) have shown that TRα_1, rather than TRβ_1, is critical for T_3 actions on Sertoli cells. Furthermore, other regulatory effects of TH have also been reported in other cell types from male reproductive tract. For instance, the differentiation from mesenchymal cells into Leydig progenitor cells as well as into mature Leydig cells requires TH (Ariyaratne et al. 2000a; Mendis-Handagama et al. 1998; Mendis-Handagama and Ariyaratne 2001). Moreover, hypothyroidism accelerates the differentiation of Leydig cells (Teerds et al. 1998; Ariyaratne et al. 2000b).

Direct effects of TH have also been reported in the rat epididymis, a component of the testicular excurrent duct system, the epididymis is a highly specialized organ involved in the transport, maturation, and storage of spermatozoa (Del Rio et al. 2000; Kala et al. 2002; St-Pierre et al. 2003). The epididymal epithelium contains several cell types: principal, basal, clear, narrow, halo and apical cells. Principal cells are the most abundant cell type and play a major role in secretion and absorption (Robaire and Hermo 1988). Also, the epididymis can be divided morphologically and functionally into four segments: the initial segment, caput, corpus, and cauda epididymidis (Serre and Robaire 1998; Jones 1998); they exhibit regional differences in gene expression (Jervis and Robaire 2001) and differential response to androgen withdrawal, aging, and stress indicating that each region represents discrete regulatory units (Kirchhoff 1999). Even though most functions of the epididymis depend upon sex steroids (Kala et al. 2002), other regulatory molecules are also implicated in the control of epididymal functions. It has been shown that hyperthyroidism differentially influences epididymal glycosidases that were expected to interfere with sperm maturation and fertilization events (Maran et al. 2001). On the other hand, it has been shown that neonatal hypothyroidism causes an increase in adult epididymal weight (Cooke and Meisami 1991). However, Del Rio et al. (2003) have reported that hypothyroidism in adult rat provokes morphological changes in the caput and corpus epididymidis with a decrease in the number of epithelial cells, which could adversely affect the maturation, and motility of the sperm.

Even though direct effects of TH have been reported in the rat epididymis (Del Rio et al. 2000; Kala et al. 2002; St-Pierre et al. 2003) as well as in other cells of the male reproductive system, TR localization was only reported for Leydig and Sertoli cells (Hardy et al. 1996; Holsberger et al. 2005). The goals of the present study were: (1) to assess whether epididymal epithelial cells from the different areas of epididymis express TR$\alpha_{(}$–$\beta_{(}$ isoforms, (2) to depict their subcellular immunolocalization, and (3) to evaluate if the TR expression is modified when rats are submitted to hypothyroid state. The knowledge of the localization of TR isoforms in the epididymis could help to elucidate T_3 effects on this portion of the male reproductive system.

Materials and methods

Animals

Adult male rats of the Wistar strain, aged 2 months old, were used in this investigation. They were housed in air-conditioned quarters with a light–dark cycle (14–10 h) and provided with free access to tap water and commercial rodent chow.

In order to induce a hypothyroid state, a thyroidectomy through an ip injection of 270 μCi of ^{131}I per rat was performed (hypothyroid group) (Del Rio and Quirós 1983) while other animals remained euthyroid (control group). After 30 days of thyroidectomy, the rats were decapitated within 10 s of removal from their cage, avoiding any stress or external stimuli. Arterial and venous blood drained from the head and trunk was collected, allowed to clot, and the serum stored frozen at −20°C for subsequent radioimmunoassay to determine total T_3 (TT_3) and total T_4 (TT_4) levels.

The epididymides were excised and cut into their different topographical regions: initial segment, caput, corpus and cauda. In addition, livers from control rats were removed and served as positive controls for different assays.

Animal conditions complied with the Guidelines on the Handling and Training of Laboratory Animals published by the Universities Federation for Animal Welfare, and the local Institutional Animal Care Committee.

Circulating levels of T_3 and T_4

Iodothyronine serum levels were measured by electrochemiluminescence immunoassay (EQLIA) from Roche Diagnostics GmBH, Mannheim, Germany, using commercial Elecsys System 2010 (Elecsys Corporation, Lenexa, KS, USA). For statistical purposes, the serum from nine control and thyroidectomized male rats was analyzed.

Cell culture, transfection and luciferase assay

Cos-7 cells, a simian virus-40-transformed monkey kidney cell line, which do not express TR (Martinez-Arrieta et al. 1999), were seeded at 3×10^5 cells on coverslips

(22 × 22 mm) placed at the bottom of sterile 60 mm dishes 24 h prior to transfection. They were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA), supplemented with 10% fetal bovine serum, antibiotics and glutamine in a humidified incubator (5% CO_2) and incubated at 37°C. The cells were transfected with 5 µg of CDM 8 vector expressing TRβ_1 as well as with a RSV-β-galactosidase control plasmid (1 µg) by calcium phosphate co-precipitation according to Ausubel et al. (1996). After 2 h incubation, cells were shocked with 15% glycerol in phosphate-buffered saline, and then submitted overnight to a steady mixing in DMEM medium supplemented with 10% fetal bovine serum stripped of T_3 with 5% (w/v) Dowex 1 × 8–400 resin (Sigma, St. Louis, MO, USA) previous to ultrafiltration.

Light microscopy immuno-labelling and morphometry

In order to detect TR protein in epididymis from control and hypothyroid rats, we selected the TRα_1 antibody (FL-408, Santa Cruz Biotechnology Inc., USA) that was successfully applied by other authors (Nicoll et al. 2003; St-Pierre et al. 2003). Besides, this antibody was able to recognize both TRα_1 and TRβ_1 thyroid hormone receptor subunits.

For the immunolocalization of TR at light microscope level, the initial segment, caput, corpus and cauda epididymidis of control ($n = 9$) and hypothyroid rats ($n = 9$) were fixed by immersion in 4% buffered formalin, dehydrated in graded ethanols, embedded in paraffin and cut into 4 µm thick sections. As previously described (Mukdsi et al. 2004), the sections were washed in PBS and treated with 3% H_2O_2 for 10 min at room temperature to block endogenous peroxidase. The slides were treated with 1% bovine serum albumin (BSA) and anti-TRα_1 antibody (1:300) overnight in a wet chamber at 4°C, followed by washing in PBS and incubation with a biotin-labelled second antibody against rabbit IgG (1/150) at room temperature for 30 min. Then the slides were tested with a preformed ABC complex (Vector Labs, Burlingame, CA, USA). The immuno-labelling was detected with 3–3 diaminobenzidine (Sigma, St. Louis, MO, USA) in 0.1 M Tris buffer, pH 7.2, containing 0.03% H_2O_2. In all experimental models the exposure time to 3–3 diaminobenzidine was 7 min. Sections were counterstained with hematoxylin, dehydrated and mounted with Entellan (Merck, Darmstadt, Germany).

To validate the consistency of TR expression in epididymal epithelial cells, the silver enhancement technique (Danscher and Ryter-Norgard 1983) was employed as a complementary method to detect the TR protein. Briefly, epididymis and liver paraffin sections, and transfected Cos-7 cells were incubated overnight at 4°C with a 1:300 dilution of the same antibody against TR previously applied. Then, the samples were incubated with an anti-rabbit IgG gold complex (Electron Microscopy Sciences, Hatfield, PA, USA) diluted 1:20 for 1 h at 37°C. In order to visualize gold complexes, the slides were rinsed thoroughly with deionized water and incubated with a silver enhancement complex (Sigma, USA) for 7 min and coverslipped with glycerol (Merck, USA).

Liver fragments of control male rats and Cos-7 cells transfected with pCDM 8 vector expressing TRβ_1 were used as positive controls of TR protein. Non-transfected Cos-7 cells served as negative control.

To assess primary antibody specificity, additional slides were incubated in parallel replacing primary antibody with normal rabbit serum. To control non-specificity binding of the secondary antibody, the primary antibody was replaced with PBS–BSA.

For morphometric assessment of the TR positive epithelial cells in control and hypothyroid rats, one large section from each epididymis region was mounted onto glass slides and immunostained for TR. In each experimental condition, a total of 2,000 epididymal epithelial cells per epididymis were counted determining the TR-immunoreactive cells number. The morphometry was made at 400× magnification using a Zeiss Axioskop 20 microscope. All TR-immuno-labelled epithelial cells, either with strong or weak staining evaluated as specific labelling, were considered. The TR-immunoreactive cells from hypothyroid rats were compared to the control and expressed as percentage of increase. Three epididymides per experimental condition, from a total of three independent experiments, were analyzed. Statistical analysis was carried out by using an analysis of variance (ANOVA) followed by the Tukey test.

Electron microscopy immuno-labelling

For ultrastructural studies by immuno-electron-microscopy, epididymides from control and hypothyroid rats were fixed in 1.5% (v/v) glutaraldehyde and 4% (w/v) formaldehyde in 0.1 M cacodylate buffer pH 7.3 at room temperature for 5–6 h and osmium fixation was omitted. The epididymides were dehydrated in a series of increasing concentrations of ethanol, and embedded in LR White (London Resin Corporation, UK). Thin sections were cut with a diamond knife on a Porter-Blum MT2 and JEOL JUM-7 ultramicrotome, mounted on 250-mesh nickel grids and incubated overnight on a drop of the same anti-rat TRα_1 antiserum (1/200), applied in light microscope immunocytochemistry. To detect TR positive structures, an anti-rabbit gold complex (Electron Microscopy Sciences, Hatfield, PA, USA) was employed diluted 1:20.

To validate the specificity of the immunostaining, negative controls were performed as described for immunohistochemistry at light level: (1) replacement of primary

antiserum with a normal rabbit serum followed by the IgG/colloidal gold complex (Sigma Chemical Co.) in order to detect bindings of the immunoglobulin molecules, and (2) replacement of primary antiserum with 1% BSA in PBS to evaluate inespecific interactions of the gold complex. The grids were examined using a Zeiss Leo 906-E electron microscope.

Preparation of nuclear and cytosolic extracts

Nuclear extracts were prepared according to Sugawara et al. (1993) with modifications (Mukdsi et al. 2004). Briefly, whole epididymides of control ($n = 9$) and hypothyroid rats ($n = 9$) were minced and homogenized in a Wheaton glass tissue grinder in 2 ml 0.32 M sucrose, 3 mM $MgCl_2$, 40 mM KCl, 10 mM HEPES (pH 7.5), 1 mM DTT, and the protease inhibitor cocktail: 0.5 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml leupeptin, and 2 μg/ml aprotinin at 4°C. The homogenate was centrifuged at $1{,}000\times g$ for 10 min. To purify the nuclei, the pellet was re-suspended in 5 ml 0.32 M sucrose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Tris–HCl (pH 7.5), 1 mM DTT and the protease inhibitor cocktail (SMCT buffer). Then, 250 μl of 10% Nonidet P-40 was added and the solution incubated on ice for 10 min before being centrifuged at $250\times g$ for 5 min. The pellet was washed once with SMCT and then re-suspended in 2 vol 20 mM HEPES (pH 7.8), 0.6 M KCl, 0.02 mM $ZnCl_2$, 0.2 mM EGTA, 0.5 mM DTT, 0.1 mM PMSF and 1 μg/μl pepstatin, and incubated on ice for 30 min. The suspension was centrifuged at $20{,}000\times g$ for 30 min at 4°C. The supernatant, containing the high salt nuclei extract, was dialyzed against 20 mM HEPES (pH 7.8), 5 mM 2-mercaptoethanol, 50 mM NaCl, 2 mM EGTA, 10% (vol/vol) glycerol, and 0.1 mM PMSF, and then centrifuged at $10{,}000\times g$ for 15 min. For cytosolic preparation, the initial $1{,}000\times g$ supernatant was centrifuged at $105{,}000\times g$ for 50 min in a fixed angle rotor and the supernatant saved. Aliquots of nuclear extracts and cytosolic fractions were stored frozen at −86°C after estimation of total protein concentration by using a Bio-Rad kit (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis

The levels of TRα_1 and β_1 isoforms in nuclear and cytosolic extracts from control and hypothyroid rat epididymis were determined by Western blot analysis using the same antibody applied in light microscope immunocytochemistry, as it is recommended for the detection of TRα_1 and β_1 isoforms by Western blotting based on their different molecular weight (47 and 55 kDa for TRα_1 and β_1 signals, respectively). Besides, this antibody detects the full length of TRα_1 corresponding to amino acids 1–408 (FL-408, Santa Cruz Biotechnology Inc., USA).

Nuclear extracts from livers of control male rats and TRβ_1 transfected Cos-7 cells were used as positive controls of TRα_1–TRβ_1 and TRβ_1 expression, respectively. Non-transfected Cos-7 cells served as negative controls of the expression of both TR isoforms (Martinez-Arrieta et al. 1999). Epididymis, liver, transfected and non-transfected Cos-7 cells extracts (30 μg protein) were run on 12% acrylamide gel. To assess the corresponding molecular weight, Full Range Rainbow molecular weight marker was used (Amersham-Life Science, Bucks, England). Proteins were transferred to a nitrocellulose membrane, and non-specific binding was blocked with PBS containing 5% non-fat dried milk, and 0.1% Tween 20 (blocking buffer) at room temperature. The membranes were rinsed and then incubated for 1 h with a 1:300 dilution of anti-rat TRα_1. Subsequently, the blots were incubated with a peroxidase-conjugated anti-rabbit secondary antibody (Jackson Immunoresearch Labs Inc, West Grove, PA, USA), diluted in blocking buffer (1/5,000). The membranes were then thoroughly rinsed in PBS/0.1% Tween-20. The HRP-coupled secondary antibody was revealed using ECL Western blot detection reagents (Amershan Biosciences, Bucks, UK) following the manufacturer's instructions. Emitted light was captured on Hyperfilm (Amersham-Pharmacia-Biotech, Bucks, UK). The expression of β-actin (1/5,000; monoclonal anti β-actin; Sigma) was used as an internal control to confirm equivalent loading of total protein. Semiquantitative signals were determined by densitometric analysis using the Scion Image software (version beta 4.0.2; Scion Corporation, NIH, Baltimore, USA). The levels of TRα_1 and β_1 isoforms of control and hypothyroid rat epididymis were statistically analyzed using the Student t test and the results were expressed as means ± SD (significance was reported at $P < 0.05$). Three epididymides per experimental condition, from a total of three independent experiments were analyzed. Statistical testing and calculation of the Western blot data were performed using the InStat V2.05 program from GraphPad Inc.

Total RNA extraction

Total RNA was prepared by the one-step acid-guanidinium method of Chomczynski and Sacchi (1987). In brief, 1 g of epididymis was homogenized in 10 ml denaturing solution (4 M guanidinium isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% *N*-lauroyl sarcosine and 0.1% *h*-mercaptoethanol). After phenol–chloroform–isoamyl alcohol (50:49:1) extraction, RNA was precipitated in isopropanol, recovered by centrifugation and washed; RNA was dissolved in diethyl pyrocarbonate-treated water, quantified and checked for purity by spectrophotometry at 260 and 280 nm.

Reverse transcription (RT)-polymerase chain reaction (PCR) for the estimation of TRα_1 and β_1 mRNA levels

Messenger RNA was reversed transcribed and amplified by PCR (RT-PCR) according to standard methods. Briefly, 1 μg of total RNA was incubated with 0.1 μM of degenerated oligo dT12VG primer at 65°C for 3 min. After 3 min on ice, the following reagents were added: 20 U RNase inhibitor (RNaseOUT, Promega, Madison, WI, USA), 4 μl of 5× RT buffer (250 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT), 0.5 mM of each dNTP and 200 U M-MLV RT (Moloney murine leukemia virus reverse transcriptase, Promega, Madison, WI, USA). After 1 h at 37°C, remnant M-MLV RT was inactivated at 95°C for 5 min. The expression of TRα_1 and β_1 mRNAs was normalized with the housekeeping β-actin mRNA that was measured in parallel tubes. For PCR, primer design and optimizations were carried out with software downloaded from the National Center for Biotechnology Information and Jellyfish, 3.0 version. Primers were designed to distinguish cDNA and genomic DNA/pseudogenes (Kreuzer et al. 1999). Primers were from Sigma (Sigma–Aldrich from Buenos Aires, Argentina) and designed to amplify bands of 591, 650 and 273 bp for TRα_1, β_1 mRNA and β-actin mRNAs, respectively; according to the following sequence: TRα_1 forward: 5′-TTCAGCGAGTTTACCAAG ATCATCAC-3′, TRα_1 reverse: 5′-TTAGACTTCCTGAT CCTCAAAGACCTC-3′; TRβ_1 forward: 5′-GTGACCGT GTAGAGTAGATG-3′, TRβ_1 reverse: 5′-CTCCACACCA AGTCTACAGC-3′and β-actin forward: 5′-CGGAACCG CTCATTGCC-3′, β-actin reverse: 5′-ACCCACACTGTG CCCATCTA-3′ (Montesinos et al. 2006; Susperreguy et al. 2007). PCR was carried out in a 20 μl final vol: 1.5 mM $MgCl_2$, 4 μl 5× PCR buffer, 1 U Taq-polymerase (Promega, Madison, WI, USA), 0.25 mM each dNTP (Promega, Madison, WI, USA) and 2 μl RT product. TR cDNAs were amplified with 0.5 μM forward and reverse TR primers, and β-actin cDNA with 0.25 μM primers. A negative control (sterile water instead of RT product) was included in each PCR run. The PCR amplification was performed on a Cycler PCR System (Bio-Rad, Hercules, CA, USA). The thermal profile was: 94°C 5 min; (34× for TR and 26× for β-actin): 94°C 1 min, 56°C 1 min; 72°C 2 min; and 72°C 10 min. The mass of total RNA for RT, the number of cycles for PCR and $MgCl_2$, primer and dNTP concentrations were selected experimentally (data not shown). RT-PCR products were resolved by electrophoresis in 2% agarose gel followed by ethidium bromide staining. The PCR-amplification products were checked by sequencing (MACROGEN, USA). The intensities of the bands from RT-PCR assays were determined by scanning densitometry (Scion Corporation, NIH, Baltimore, USA). The levels of mTRα_1 and β_1 isoforms of control and hypothyroid rat epididymis were statistically analyzed using ANOVA–Student–Neuman–Keuls ($P < 0.01$).

Results

Circulating levels of TH under different thyroidal states

Thyroidectomy generated by ip injection of 270 μCi of ^{131}I produced significant decreases in circulating TT_3 and TT_4 ($P < 0.05$) confirming the hypothyroid state of the rats: control rats: TT_3: 1.9 ± 0.6 (nmol/l)/TT_4 total: 42.7 ± 19.9 (nmol/l); hypothyroid rats: TT_3: 0.6 ± 0.2 (nmol/l)/TT_4: 7.1 ± 7.7 (nmol/l).

Immunocytochemistry of TR expression

To establish whether TR isoforms are expressed in rat epididymis and to evaluate the effect of hypofunction of the thyroid gland on their expression, sections taken from the different segments of the epididymidis, and incubated with anti-rat TR antibody, were analyzed by light and electron microscopy immunocytochemistry.

As shown in Fig. 1, both control and hypothyroid groups expressed the TR protein in epididymal epithelial cells. All positive cells showed label in the cytoplasmic compartment (Fig. 1a, b), while only a few one showed a strong staining in the nucleus as well (Fig. 1c). Several negative epithelial cells for TR protein were visualized as well (Fig. 1a, b). In both experimental models, a similar TR immunostaining pattern was detected in the different segments of the epididymis showing a scattered cytoplasmic distribution (Fig. 2a, b, d). The caput epididymidis from control was the exception where the labelling appeared to be clearly polarized to the apical cytoplasm (Fig. 2c). In hypothyroid rats (Fig. 2b, d), the intensity of TR immunostaining detected in the different segments of the epididymis was more noticeable than that achieved in the control group (Fig. 2a, c). The analysis of TR-immunoreactive cells percentages showed that the hypothyroidism provoked a significant augmentation in the number of immunostained cells ranging the 21% compared to those detected in epididymides derived from control animals.

In order to confirm the TR-immunoreactivity detected in epididymis using ABC complex, we applied a silver enhancement immunohistochemistry technique. In both experimental models, cytoplasmic staining was evident as a granular pattern of TR protein with little or no nuclear staining in epididymal epithelial cells (Fig. 3a). In addition, no differences in TR expression in terms of intracellular localization of TR were detected when compared to those observed with the ABC technique. Hepatocytes from control rats (Fig. 3b) and transfected Cos-7 cells (Fig. 3c) used

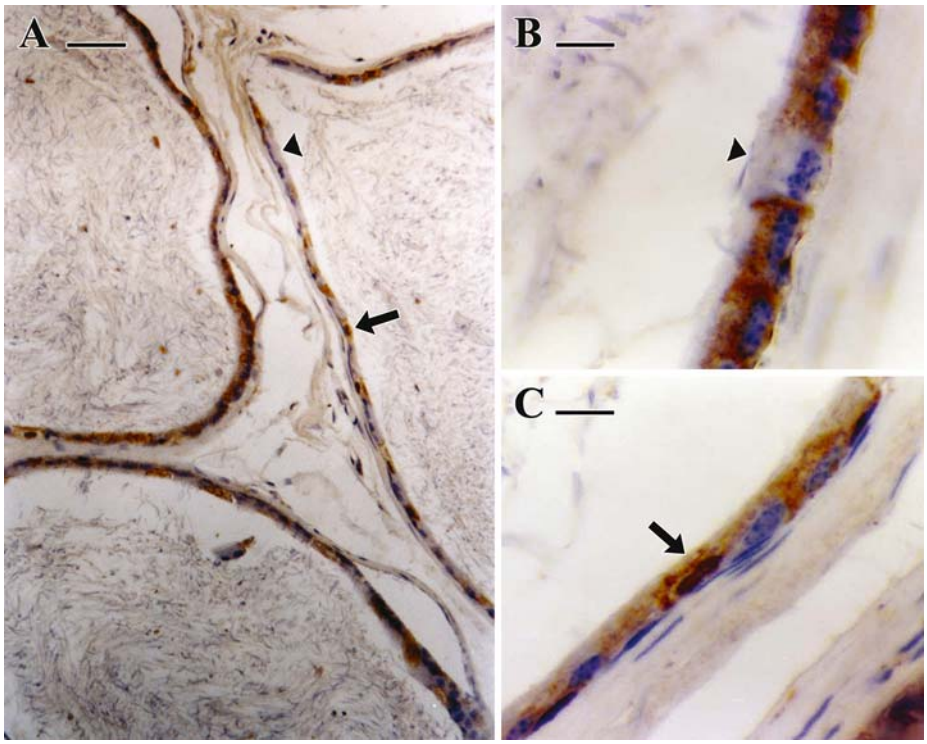


Fig. 1 Immunocytochemical detection of TR on sections of epididymis from control and hypothyroid rats. **a** Photomicrograph of cauda epididymidis from control rat displaying cytoplasmic and nuclear (*arrow*) TR immunostaining in several epithelial cells. Some TR-negative epithelial cells were also observed in intact animals (*arrowhead*). *Bar* 150 μm. **b** In hypothyroid rat, numerous epididymal epithelial cells from caput epididymidis exhibited a disseminated expression of TR at cytoplasmic level whereas few negative cells were seen (*arrowhead*). *Bar* 30 μm. **c** Micrograph of cauda epididymidis from hypothyroid rat showing few cells exhibiting positive staining in the nuclear compartment (*arrow*). *Bar* 30 μm

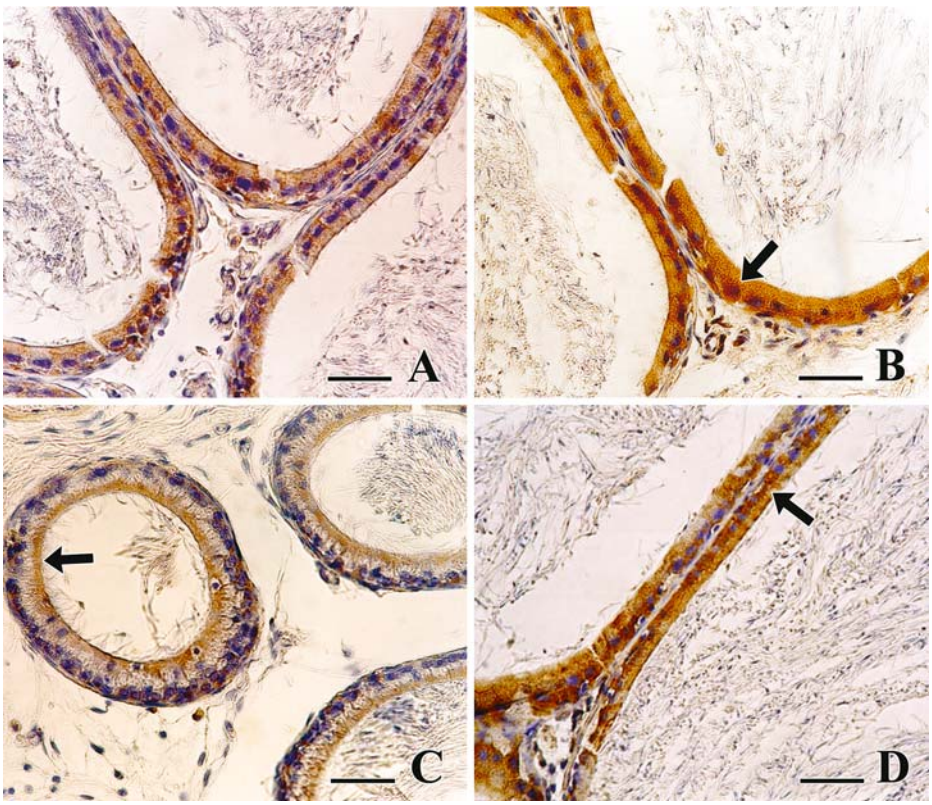
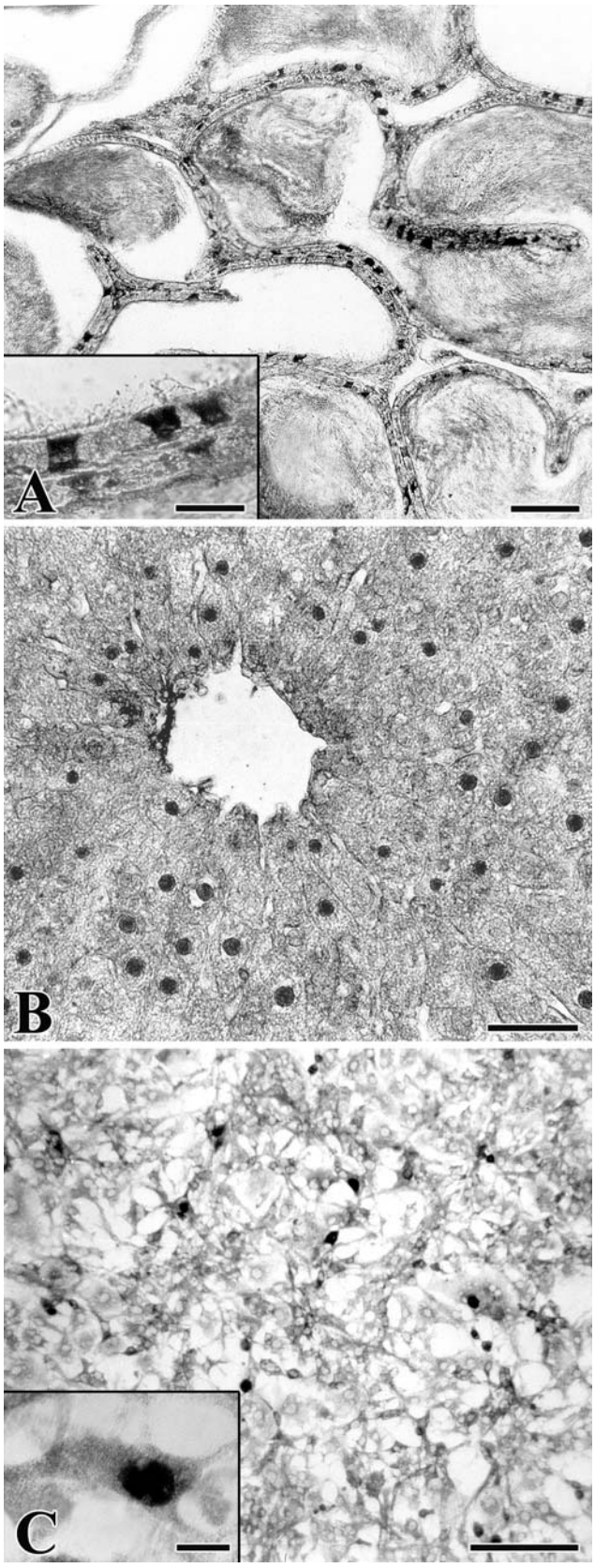


Fig. 2 **a** In the corpus epididymidis from control rats, TR immunostaining was evident throughout the cytoplasm of epithelial cells. *Bar* 100 μm. **b** In epithelial cells from the corpus epididymidis of hypothyroid rats, a heavily concentrated, *dark-brown* cytoplasmic staining indicative of higher TR protein levels than in the control group was detected. Some cells exhibited nuclear immuno-labelling (*arrow*). *Bar* 100 μm. **c** In caput epididymidis from control rats, TR proteins were mainly localized in the apical cytoplasm of epithelial cells (*arrow*) accompanied by a weak reaction at the base. *Bar* 100 μm. **d** A strong and widespread cytoplasmic TR immunoreaction was observed in the majority of epithelial cells from the cauda epididymidis of hypothyroid rats. Occasional positive nuclei were seen in this segment (*arrow*). *Bar* 100 μm

as positive controls displayed a remarkable nuclear immunoreactivity confirming the role of TR in the nuclear compartment as a transcription factor. As expected, non-transfected Cos-7 cells were negative when they were incubated with the primary antibody. In all cases, no staining was observed in sections incubated with PBS–BSA 1% or rabbit normal serum, which were used as negative controls of the immunocytochemical reaction.



◀ **Fig. 3** Immuno-labelling of TR in epididymis, liver and Cos-7 cells revealed by a silver enhanced technique. **a** TR expression in the epididymis of a control rat. *Bar* 200 μm. The *inset* is a magnified view illustrating the cytoplasmic staining of these isoforms in epithelial cells. *Bar* 75 μm. **b** The nuclear staining exhibited by hepatocytes serves as a positive control for TR immunoreaction. *Bar* 100 μm. **c** Light microscope micrograph of immunoassayed transfected Cos-7 cells. *Bar* 100 μm. *Inset* representative cell showing a discernible expression of TR protein in the nucleus. *Bar* 10 μm

Since the immunostaining at light microscope led us to prove the presence of TR in both compartments of epididymal epithelial cells, we applied the ultrastructural immunogold technique with the aim to identify the TR-positive cell structures throughout the different portions of the epididymis. In both experimental models, the epididymal cells disclosed a specific TR labelling, mainly in the principal cells of the epididymal epithelia. The TR was noted in the nucleus associated with the eucromatin (Fig. 4a). At the cytoplasmic compartment, TR was related to the cytosolic matrix and mitochondria (Fig. 4b). In addition, some basal cells from control rats showed a conspicuous nuclear immuno-reactivity for this receptor (Fig. 5). The positive TR immunostaining was abolished when sections were incubated with pre-immune serum or PBS–BSA, followed by IgG-gold probe (data not shown).

TR expression at protein and mRNA levels

Besides subcellular localization of TRα_1–β_1 isoforms at light and electron microscopy we examined the changes in their expression profile by Western blotting of cytosolic and nuclear protein extracts as well as in their mRNA levels by RT-PCR.

As shown in Fig. 6a, two strong bands of approximately 47 and 55 kDa consistent with TRα_1 and β_1 signals were noted in the lanes of cytosolic fraction of control and hypothyroid rats, whereas only a weak TRβ_1 band was detected in nuclear extracts. It is important to mention that TRβ_1 signals from cytosolic fractions were visualized as a doublet of 52 and 55 kDa, a finding in consonance with previous studies conducted in rats and human beings (Tagami et al. 1993; Chamba et al. 1996; Montesinos et al. 2006). Significantly higher levels of TR were revealed in cytosolic versus nuclear fractions ($P < 0.01$). This finding was in consonance to the immunocytochemical analysis of TR proteins using light microscopy. The relationship of TRα_1/TRβ_1 densitometric signals achieved in cytosolic extracts (mean ± SD, 3.9 ± 0.4) was similar to the reported for brain TRα_1/TRβ_1 ratio (Ercan-Fang et al. 1996). On the contrary, the prevalence of TRα_1 isoform in rat epididymis was in contrast to liver TR isoform distribution (Fig. 6b). It is surprising that

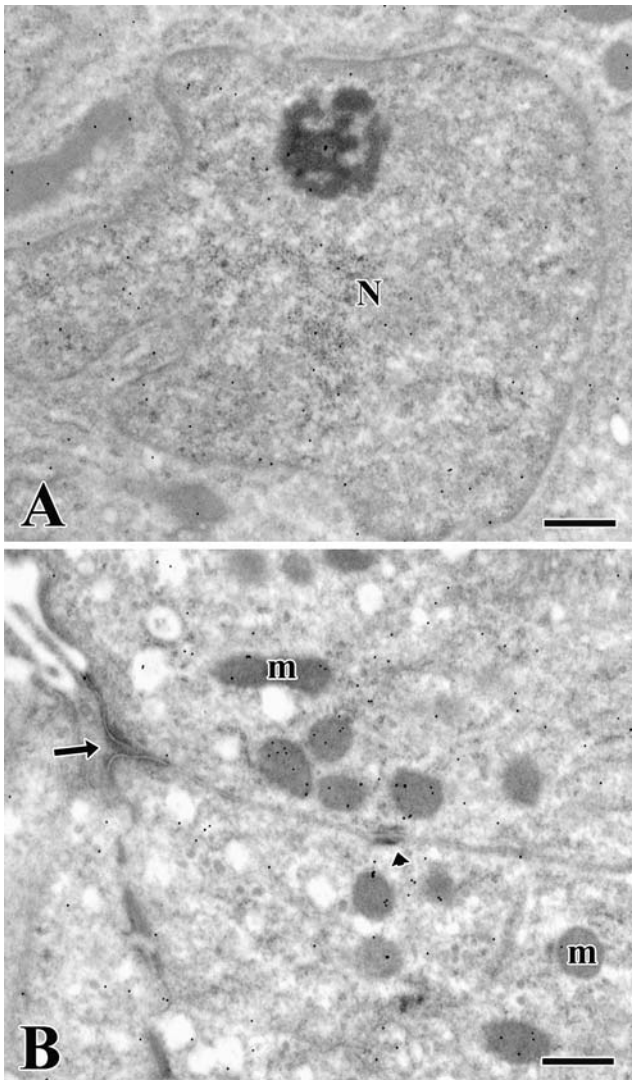


Fig. 4 Immuno-electron micrographs of principal epithelial cells from the corpus epididymidis. **a** Gold particles are situated in the nucleus (N) mainly associated with the euchromatin, but TR protein expression is also detected at cytoplasmic level of control rat. *Bar* 1 μm. **b** The TR immunostaining is localized in the cell cytoplasm particularly in mitochondria (m) and in the cellular matrix from the epididymal principal epithelial cell of hypothyroid rat. Tight junction (*arrow*) and desmosome (*arrowhead*) as part of the junctional complex can be observed between adjacent epithelial cells. *Bar* 1 μm

although higher TRα_1 levels were detected in cytosolic fraction of rat epididymis in both experimental models, this TR isoform could not be observed in nuclear extracts (Fig. 6a).

Densitometric analysis of both TR isoforms from cytosolic extracts revealed levels significantly increased under hypothyroid conditions compared to the control (arbitrary units, mean ± SD: TRα_1 = control: 5.2 ± 0.4 versus hypothyroid: 6.8 ± 0.5, $P < 0.01$; TRβ_1 = control: 1.2 ± 0.2 versus hypothyroid: 2.3 ± 0.3, $P < 0.01$). In the nucleus compartment from both experimental models, no densitometric analyses were conducted with TRβ_1 due to the weak signal registered.

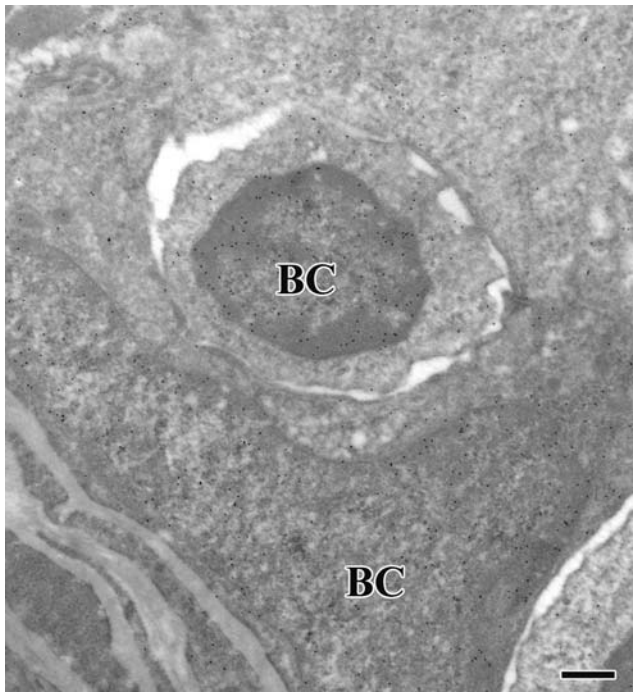


Fig. 5 Electron microscopic immunocytochemistry on a basal cell (*BC*) from the epididymis of a control rat, exhibiting a stronger immunoreactivity for TR protein in the nucleus compared to the cytoplasmic compartment. *Bar* 1 μm

As expected, liver from control male rat and transfected Cos-7 cells showed TRα_1–β_1 and TRβ_1 expression, respectively, at nuclear level. No transfected Cos-7 cells were negative for TR (Fig. 6b).

Having demonstrated that hypothyroidism promoted an increase in the TRα_1 and β_1 expression assessed by immunocytochemistry and Western blotting, it was of interest to evaluate if this altered thyroidal state would also modify the mRNA expression of both TR isoforms. With this aim, RT-PCR was performed to estimate TRα_1 and β_1 mRNA levels in epididymis from hypothyroid and control rats. As shown in Fig. 7, the TRα_1 and β_1 mRNA expressions were significantly augmented under thyroidectomy when compared to control values ($P < 0.01$). Also, TRα_1 mRNA level was significantly higher than β_1 ($P < 0.01$), which correlated with the higher TRα_1 protein level achieved by Western blotting from cytosolic extracts.

Discussion

The comprehensive analysis of the present data demonstrates the presence of specific TRα_1 and β_1 isoforms in epithelial cells derived from adult rat epididymis emphasising the potential importance of thyroid hormone in this portion of the male reproductive tract. Although TRs are classically described as ligand-dependent transcription factors (Yen

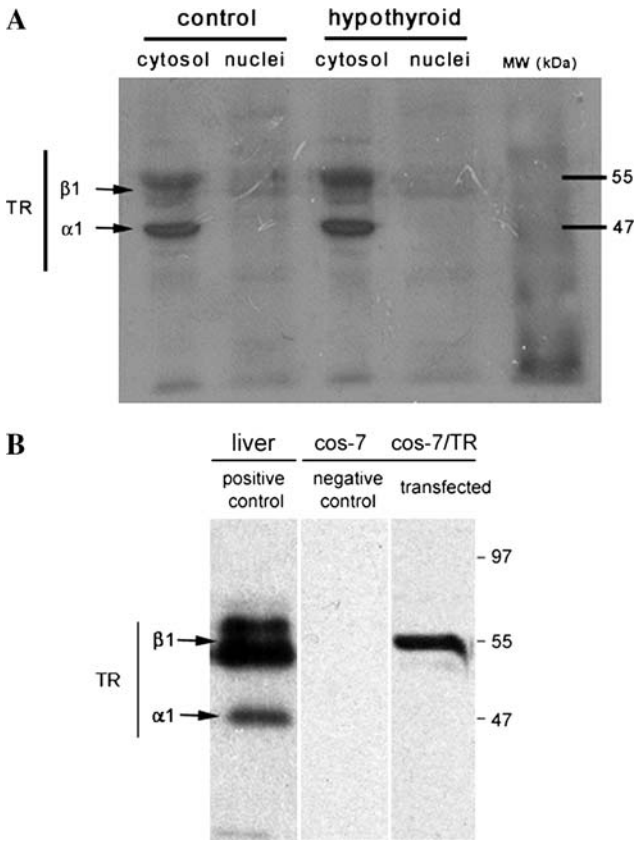


Fig. 6 Western blotting expression of TRα_1 and β_1 isoforms proteins of nuclear and cytosolic fraction of epididymis from control and hypothyroid rat (**a**); liver nuclear extracts from control rat and total extracts of transfected and non-transfected Cos-7 cells (**b**). The bands detected in epididymis extracts correspond to the molecular weight of the isoforms (47 and 55 kDa, respectively). Data are from a representative experiment from a total of three with similar results. Three different samples were analyzed from individual animals in each group. Data were statistically analyzed by Student *t* test and the results are expressed as mean ± SD (significance was reported at $P < 0.05$)

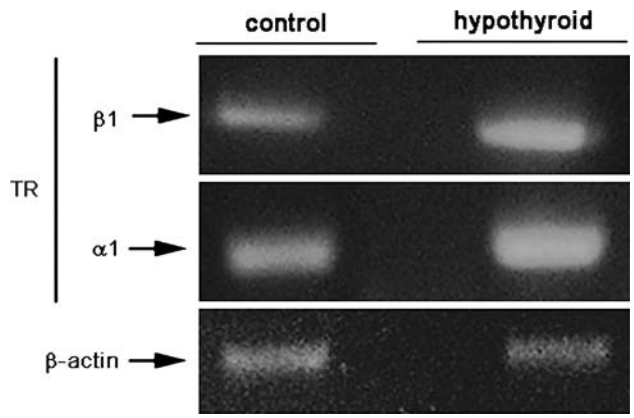


Fig. 7 Representative RT-PCR analysis for the estimation of TRα_1 and TRβ_1 mRNA levels from the epididymis of a control (*lane 1*) and a hypothyroid (*lane 2*) rat. One micrograms of total RNA was reverse transcribed and then TRα_1 and TRβ_1 (*upper panels*) and β-actin (*lower panel*) were amplified by PCR. Data are from one representative experiment with three samples in each group of a total of three with similar results

2001; Yin et al. 2002; Beaudet et al. 2005), the immunohistochemistry applied throughout the different portions of epididymis revealed a predominant cytoplasmic localization, with a remarkable immuno-labelling occurring principally under a hypothyroid state where the majority of epithelial cells were immuno-positive for TR. This finding was in concurrence with those achieved by Western blotting where both TRα_1 and β_1 isoforms were significantly detected in cytosolic extracts. Even though St-Pierre et al. (2003) demonstrated the presence of TRα_1 in rat epididymis as a single band from total protein, in the present study, we were able to detect both TRα_1 and β_1 isoforms applying the same primary TR antibody, mainly localized in the cytosolic compartment where the relationship of TRα_1/TRβ_1 densitometric signals was similar to that described for the brain (Ercan-Fang et al. 1996). Besides, the analysis of TRα_1 and β_1 mRNA levels by RT-PCR confirmed the effects of the deprivation of thyroid hormone on TR expression in epididymis where an increase in both isoforms was observed on animals submitted to a hypothyroid state.

At ultrastructural level, we confirmed the presence of TR in the cytoplasm and occasionally in nucleus. At the cytoplasm the TR was detected mainly in the cytosolic matrix and associated to mitochondria from principal epithelial cells, even though a few positive basal cells were also seen. The confirmation of cytoplasmic TR staining using different immunohistochemical techniques as well as Western blot methodology confirms that epididymis epithelial cells mainly express cytoplasmic TR protein.

Changes in the immunostaining of TR observed in the present study could probably be the direct consequences of the lack of T_3 binding to its nuclear receptors due to thyroid insufficiency. Our results could indicate that in vivo depletion of TH might up-regulate TR expression in adult rat epididymis as was informed for other tissues as brain and liver (Zandieh-Doulabi et al. 2004; Constantinou et al. 2005). The differences in the levels of TR expression under thyroidectomy compared with that observed in the control model ascertain a specific function for TH on this organ. In correlation with our results, Del Rio et al. (2003) by light and electron microscopy demonstrated remarkable epididymis morphological alterations after thyroidectomy indicative of lack of activity; been probable that the depression of cell activity, due to the lack of T_3, could trigger an increased expression of TR in a desperate effort to recover normal activity level.

Despite their main function as hormone-dependent transcription factors, the cytoplasmic localization of TR in epididymis in not surprising as they were also detected in cytosol of rat bone marrow mast cells (Siebler et al. 2002), human hypothalamic areas (Alkemade et al. 2005), human umbilical vein endothelial cells (Diekman et al. 2001), rat hepatocytes (Zandieh Doulabi et al. 2002) and mouse heart

(Stoykov et al. 2006). Also, it has been shown that in living cells, TR may shuttle rapidly between the nuclear and cytoplasmic compartment (Hager et al. 2000; Bauman et al. 2001). Translocation of the TR is suggested to be ligand dependent (Zhu et al. 1998; Maruvada et al. 2003) and to require multiple protein interactions with different cofactors (Bauman et al. 2001). The cytoplasmic localization, in addition to nuclear localization, appears to be a general phenomenon in the human brain for steroid hormone receptors (Hager et al. 2000; Maruvada et al. 2003; Scheller and Sekeris 2003; Stoykov et al. 2006).

Even though T_3 is known to exert many of its actions through the classical genomic regulation of gene transcription, a number of T_3 effects occur rapidly and are unaffected by inhibitors of transcription and protein synthesis (Wrutniak-Cabello et al. 2001). These effects were named as "non-classical" or "non-genomic" actions of T_3 (Davis and Davis 2003) and represent a transcription-independent effect on protein trafficking. Moreover, non-genomic actions of TH have been described at the plasma membrane, in the cytoplasm and in cellular organelles. They have been involved in the modulation of Ca^{2+} and glucose transport, activation of ERK/MAPK and regulation of the phospholipid metabolism (D'Arezzo et al. 2004; Bergh et al. 2005; Cao et al. 2005; Moeller et al. 2006; Menegaz et al. 2006).

An important finding in this work was the immuno-electron localization of TR on mitochondria in epididymal epithelial cells from control and hypothyroid rats, in agreement with Sato et al. (2006), who showed the presence of TRα_1 in mitochondria and nuclei of rat tongue muscle by immuno-electron-microscopy. In this respect, several authors have described that TH exerts different effects on mitochondrial biogenesis affecting respiration and increasing the expression of the mitochondrial-DNA (Wrutniak-Cabello et al. 2002; Weitzel et al. 2003). These direct effects of T_3 on mitochondria appear to occur via binding to TR localized in the mitochondria (Weitzel et al. 2003; Psarra et al. 2006). Besides, it has been shown that this organelle contains truncated TRα_1 isoforms p28 and p46 as well as TR-related proteins (Casas et al. 2000, 2003; Morrish et al. 2006; Sato et al. 2006). The 46 kDa truncated TRα_1 isoform (p46) has a similar T_3 binding affinity to the full length TRα_1 (Wrutniak et al. 1995; Bassett et al. 2003). The immuno-electron staining of TR registered in mitochondria from epididymal epithelial cells may account for the effects of thyroid hormone on this organelle, considering the homology that exists between TRα_1 epitope (1–410) and the p46 epitope (36–410) (Bassett et al. 2003).

The presence of TR in the nucleus of basal cells from control rats may be involved in the proliferation of cellular precursors and their differentiation into epididymal principal epithelial cells, as was demonstrated for the proliferation and differentiation of mesenchymal cells into Leydig progenitor cells (Mendis-Handagama and Ariyaratne 2001, 2004).

The expression of TR in both the nuclear and cytoplasmic compartment suggests an active role for TH in epithelial cells of epididymis acting through classical and non-classical pathways. The importance of non-genomic signalling as a complementary route for cell regulation by nuclear receptors has recently become evident. This rapid mechanism is utilized not just by peptide hormones, but also by steroids and other lipid-related substances, resulting in amplification and fine-tuning of the signals. In summary, these studies raise novel questions regarding the role of thyroid hormones in epididymis epithelial cells. The role of the unusual TR subcellular localization in epididymal epithelial cells also needs to be unravelled. However, the presence of TR in these cells as well as the different TR profile under an altered thyroidal state strongly suggests the involvement of T_3-specific functions. Further studies will disclose thyroid hormone role in the epididymis.

Acknowledgments The authors wish to thank Mrs. Mercedes Guevara for her excellent technical assistance. We are particularly indebted to Biochem. Félix D. Roth for his precious help in computer imaging. We would also like to thank native speaker Dr. Paul Hobson for revising the English of the manuscript. This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Fondos para la Investigación Científica y Tecnológica (FONCyT) and the Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (SECyT).

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