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Assessment of Mechanisms of Thyroid Hormone Action in Mouse Leydig Cells: Regulation of the Steroidogenic Acute Regulatory Protein, Steroidogenesis, and Luteinizing Hormone Receptor Function*

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ABSTRACT

Recently, we demonstrated that triiodothyronine (T_3) stimulated steroid hormone biosynthesis and steroidogenic acute regulatory (StAR) protein expression in mLTC-1 mouse Leydig tumor cells through the mediation of steroidogenic factor 1 (SF-1). We now report a dual response mechanism of T_3 on steroidogenesis and StAR expression, and on LH receptor (LHR) expression and binding in mLTC-1 cells. T_3 acutely (8 h), induced a 260% increase in StAR messenger RNA (mRNA) expression over the basal level which was coincident with an increase in progesterone (P) production. In contrast, chronic stimulation with T_3 (beyond 8 h), resulted in an attenuation of StAR expression and P production. This attenuation was most likely caused by a decrease in cholesterol delivery to the inner mitochondrial membrane as demonstrated by incubations with the hydrophilic steroid precursors, 22R hydroxycholesterol and pregnenolone, which restored P synthesis. In similar studies, chronic treatment with T_3 increased the levels of cytochrome P450scc mRNA by 83%, whereas those of cytochrome P450 17α-hydroxylase and 3β-hydroxysteroid dehydrogenase decreased. The diminished response in steroidogenesis following chronic T_3 exposure was not a result of alterations in StAR mRNA stability, but rather was due to inhibition of transcription of the StAR gene. Similar acute stimulatory and chronic inhibitory responses to T_3 were found when LHR mRNA expression and LHR ligand binding were examined. Transfections with an LHR or StAR promoter/luciferase reporter construct demonstrated that a 173-bp fragment of the LHR promoter containing an SF-1 binding motif was involved in T_3 response, as was the SF-1 recognition site at −135 bp in the StAR promoter. Furthermore, the importance of SF-1 in T_3 function was also verified employing mutation in the bases of SF-1 sequences using electrophoretic mobility shift assays. The potential physiological relevance of these findings was demonstrated when similar responses were obtained in mice rendered hypo and hyperthyroid. Collectively, these observations further characterize the thyroid-gonadal connection and provide insights into the mechanisms for a dual regulatory role of thyroid hormone in Leydig cell functions. (*Endocrinology* **142:** 319–331, 2001)

THE ACUTE response of steroid biosynthesis to hormonal stimulation is initiated by the delivery of cholesterol from the outer to the inner mitochondrial membrane, by a process dependent on *de novo* protein synthesis (1–3). Among the candidate proteins involved in this delivery, a 30-kDa mitochondrial phosphoprotein, now called the steroidogenic acute regulatory protein (StAR), has been demonstrated to possess the necessary characteristics to mediate this process (2). The purification of StAR from MA-10 mouse Leydig tumor cells and its subsequent cloning and expression revealed that it was a novel mitochondrial protein that could mediate intramitochondrial cholesterol transport (4). Pulse-chase experiments and tryptic peptide mapping indicated that in response to hormone stimulation a 37-kDa, short-lived, precursor protein was rapidly synthesized and that the mature 30-kDa form of StAR was derived from this precursor through import and processing by the mitochondria (2, 5, 6). Synthesis of StAR protein is sensitive to protein synthesis inhibitors, whereas the activity of P450scc enzyme or cholesterol delivery from cellular stores to the outer mitochondrial membrane are not acutely affected by these inhibitors (1, 2, 7).

Thyroid hormone, through its nuclear receptor, plays a crucial role in regulating differentiation, growth, and metabolism in higher organisms (8, 9). Thyroid hormone receptors (TRs) and steroid hormone receptors share many properties, including ligand-dependent activation, nuclear site of action, sequence-specific DNA recognition sites and the ability to regulate gene transcription. A novel C-erb-A gene, encoding the TR, has been detected in a human testicular complementary DNA (cDNA) library (10). To date, multiple TRs, binding T_3 with high affinity and specificity, have been detected in different tissue (11). The level of TR expression in rat testis is highest during fetal and perinatal life, and decreases thereafter, whereas the morphological and functional development of the testis has been shown to be dependent upon thyroid hormone action (12–14). Recent studies show that mesenchymal and immature Leydig cells

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contain higher levels of TR messenger RNA (mRNA) than adult Leydig cells (15). It has also been reported that Leydig cells increase in number in adult rat after neonatal hypothyroidism (16).

A recent report from this laboratory (17) demonstrated the presence of a single class of high-affinity thyroid hormone receptors and demonstrated T_3-induced StAR expression and progesterone (P) production in both primary and tumorous (mLTC-1, Ref. 18) murine Leydig cells. Furthermore, mLTC-1 cells, which express the nuclear receptor SF-1, coordinately increased T_3-mediated StAR expression and steroidogenesis. Conversely, overexpression of DAX-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X-chromosome) markedly diminished SF-1 expression and concomitantly abolished the T_3 responses.

The importance of thyroid hormone has long been implicated in mammalian reproduction because disturbed thyroid function is frequently associated with abnormalities in sexual function including impaired fertility (12, 19). Several lines of evidence suggest that thyroid malfunction affects hormones involved in reproduction, especially LH, FSH, and testosterone (T). Thyroid hormone deficiency also results in alterations in testicular morphology, and these responses can vary greatly from species to species (12, 20, 21). A preliminary report demonstrates that hypothyroidism in rats accounts for augmentation of LH/hCG binding to ovarian membranes, whereas chronic administration of T_3 markedly diminished its level (22). Despite the apparent importance of thyroid hormone in mammalian reproduction, little information is available on its role in the regulation of gonadal steroidogenesis and LHR function.

Specific T_3 binding has also been demonstrated in mechanically dispersed Leydig cells of goat testes, where the effects of T_3 on androgen production are mediated through a thyroid hormone-induced protein (TIP) that has not yet been characterized (23). Thyroid hormone has been shown to potentiate cellular differentiation and LH/hCG receptor formation in pig granulosa cells *in vitro* in response to FSH stimulation (24). In MA-10 mouse Leydig tumor cells, it has been reported that longer stimulation with cAMP analog resulted in suppression of StAR mRNA, StAR protein, and P production (25). However, the mechanisms responsible for the long term attenuating responses of Leydig cells to T_3 remain to be established.

The present findings document, for the first time, that chronic exposure to T_3 significantly reversed its observed increased acute response (8 h) on StAR expression and steroidogenesis, and that this reversal is in large part due to decreased cholesterol supply to the mitochondrial inner membrane. In addition, T_3 acutely increased LHR gene expression and ligand binding, whereas its longer effects progressively suppressed these levels. The findings in cultured cells correlated with *in vivo* data obtained in mice with experimentally induced hypo and hyperthyroidism. Furthermore, mLTC-1 cells transfected with LHR promoter-driven luciferase constructs demonstrated a positive thyroid hormone response element within 173-bp upstream of the transcription start site of the LHR gene. These findings provide novel evidence that thyroid hormone plays a dual role in regulating LHR function, and control of steroidogenesis.

Materials and Methods

Animals and treatments

Three-week-old mice (C57 Black/6 Strain), weighing 20–28 g, were housed under a controlled lighting schedule (14-h light/10-h dark), and fed commercial diet and water *ad libitum*. The animals were maintained in accordance with the guidelines of the Turku University Ethical Committee for the Use and Care of Experimental Animals. Mice were injected with 2-thiouracil (THU) and thyroxine (T_4) (both at 600 μg/kg BW) in alkaline saline on alternate days to produce recurrent periods of hypo and hyperthyroidism respectively, for 3 weeks. Simultaneously, a control group was injected with physiological saline. Following 3 weeks of injection, and approximately 8 h after the last injection, mice were killed by cervical dislocation, and serum T_3, T_4, LH (IFMA, AGG Wallac, Inc. Turku, Finland), PRL (IRMA, Orion-Farmos Diagnostica, Turku, Finland) levels were determined. The testes were excised under sterile conditions and placed in ice-cold HEPES-buffered Waymouth's medium (Life Technologies, Inc., Paisley, Scotland, UK) containing 0.365 g/liter L-glutamine (Life Technologies, Inc., Glasgow, Scotland, UK), 0.25% BSA (Sigma, St. Louis, MO), 10000 U/liter penicillin and 50 mg/liter streptomycin, and Leydig cells were isolated (see below).

Isolation, purification, and culture of primary mouse Leydig cells

Mouse testicular Leydig cells were prepared as described previously (17). Briefly, after removing the testicular capsule, interstitial cells were dissociated from the testicular tissue by treating with 0.2% collagenase (20 min, 34 C, 95% O_2/5% CO_2), separated by filtration, washed, and purified by continuous Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation (1.01–1.126 kg/liter). Cell types were separated and collected based on their buoyant densities with Leydig cells being found in the zone equivalent to 1.07 kg/liter of Percoll. The purity of Leydig cells in this fraction, as assessed by 3β-hydroxysteroid dehydrogenase (3β-HSD) staining, was found to be 75–80% (17, 26). Cells were subcultured in Waymouth's medium supplemented with 9% heat-inactivated horse serum (Life Technologies, Inc.) and 4.5% FCS (Bioclear UK Ltd., Mile Elm Calne, Wiltshire, UK) containing penicillin and streptomycin.

RNA extraction and RT-PCR

Total RNA was extracted from isolated Leydig cells of different experimental groups employing the single step acid guanidinium thiocyanate-phenol-chloroform extraction method (27). The isolation and amplification of mouse (mLTC-1) StAR (4), LHR (28), and L19 (29) cDNAs were carried out as previously described (17). Briefly, the following primer pairs were used:

StAR (forward) 5′-GACCTTGAAAGGCTCAGGAAGAAC-3′ (bases −51 to −27)

StAR (reverse) 5′-TAGCTGAAGATGGACAGACTTGC-3′ (bases 931 to 908)

LHR (forward) 5′-CTCTCACCTATCTCCCTGTC-3′ (bases 179 to 195)

LHR (reverse) 5′-TCTTTCTTCGGCAAATTCCTG-3′ (bases 878 to 858)

L19 (forward) 5′-GAAATCGCCAATGCCAACTC-3′ (bases 154 to 173)

L19 (reverse) 5′-TCTTAGACCTGCGAGCCTCA-3′ (bases 559 to 540)

To control for variation in RT-PCR efficiency, a 405-bp fragment of the L19 ribosomal protein gene was coamplified in each sample. RT and PCR of the target genes were sequentially run in the same assay tube, as described previously (17, 30). Two micrograms of total RNA were reverse transcribed and the cDNAs generated were further amplified by PCR in a 50 μl reaction mixture containing 1 nmol/liter of oligoprimers, 200 mmol/liter of a deoxy-NTP mixture, including [α^{32}P]-CTP, 20 U RNasin, 12.5 U avian myeloblastosis virus reverse transcriptase (AMV-RT) and 2.5 U Dynazyme-DNA polymerase in 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.1% Triton X-100, pH 8.8) (Finnzymes, Espoo, Finland). The reaction was initiated (see Ref. 17 for StAR) for mouse LHR at 50 C for 10 min (RT), followed by denaturation at 97 C for 3 min. The PCR was then run for 30 cycles, defining denaturation at 96 C for 1.5 min, annealing at 53 C for 1.5 min and extension

at 72 C for 2 min (PTC-200, Peltier Thermal Cycler, MJ Research, Inc.). A final cycle of extension at 72 C for 10 min was included. The molecular sizes of the PCR products (StAR, LHR, and L19) were determined in 1.2% agarose gel. The gels were then vacuum dried, and exposed to Fuji Photo Film Co., Ltd. x-ray film (Tokyo, Japan) at 4 C for 1–3 h. The relative mRNA levels of StAR, LHR, and L19 were quantified by phosphorimaging and densitometry (Tina 2.0 Program, Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany).

Preparation of mitochondria, and immunodetection of the 30-kDa StAR protein

Isolation of mitochondria was carried out from control and treated mLTC-1 cells as described previously (25, 31). In brief, cells were washed with 0.01 mol/liter PBS, collected and homogenized at 4 C (30 strokes at 1200 rpm) with a Potter-Elvehjem homogenizer, fitted with a serrated pestle. The homogenate was centrifuged at 600 × *g* for 20 min to remove broken cell debris and nuclei, and the resulting supernatant was further centrifuged at 10,000 × *g* for 25 min. The pellet containing mitochondria was washed twice in the same buffer and pelleted each time at 9,000 × *g* for 15 min.

Mitochondrial protein (20 μg) from each group was solubilized in sample buffer (25 mmol/liter Tris-Cl, pH 6.8, 1% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue) and loaded onto a 12% SDS-polyacrylamide gel (Mini Protean II System, Bio-Rad Laboratories, Inc., Hercules, CA), as described by Laemmli (32). Electrophoresis was performed at 200 V for 1 h, and proteins were electrophoretically transferred onto a nitrocellulose membrane (Hybond, Amersham Pharmacia Biotech, Buckinghamshire, UK), and the membranes were processed as described previously (31). The immunodetection of StAR protein was performed using an ECL Western blotting detection kit (Amersham Pharmacia Biotech), and the membranes were exposed for 1–5 min to Fuji Photo Film Co., Ltd. x-ray film and quantitated as above.

Northern blot analysis

Total RNA (20 μg) from different treatment groups was assessed using Northern hybridization analysis. Briefly, antisense complementary RNA probes, a *Not*I fragment (960 bp) of the mouse StAR cDNA, a *Bgl*II fragment (410 bp) of the extracellular domain of the rat LHR, and an *Eco*RI fragment (298 bp) of P450scc were produced by *in vitro* transcription (Promega Corp., Madison, WI) with T_7 RNA polymerase, dNTPs and [α^{32}P]-UTP (Amersham Pharmacia Biotech). For preparing cDNA probes, an *Eco*RI-*Pst*I fragment (780 bp) of SF-1, a *Sac*I-*Bgl* II fragment (906 bp) of 3β-HSD, and a *Not*I fragment (1713 bp) of P450c17 cDNA were labeled with [α^{32}P]-CTP using the Prime-a-Gene labeling method (Promega Corp.). The labeled probes were purified using Sephadex G-50 nick columns (Amersham Pharmacia Biotech). Prehybridization (12 h) and hybridization (16 h) of the ribo- and cDNA probes were carried out at 66 C and 42 C, respectively, under stringent conditions as previously described (17, 31, 33). Following hybridization, the membranes were washed twice at room temperature for 20 min with 2× SSC containing 0.1% SDS, followed by 1–3 h at 66 C or 42 C with 0.1× SSC and 0.1% SDS until removal of the background counts. To assess the variation of mRNA levels, the membranes were subjected to rehybridization with a cDNA probe of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and exposed to Fuji Photo Film Co., Ltd. x-ray film for 36–48 h at −80 C.

[^{125}I]iodo-hCG binding

Highly purified hCG (CR-127, NIDDK, NIH, Bethesda, MD) was radioiodinated with Na[^{125}I]-iodide (IMS 300, Amersham Pharmacia Biotech), using a solid phase lactoperoxidase method (34). The specific activity of the labeled hormone was found to be between 28–32 μCi/μg. The hCG binding studies were carried out under optimized conditions, as described previously (35). In brief, after washing, 3×10^5 cells were incubated with [^{125}I]iodo-hCG (~10^5 cpm/incubation) either in the absence (total) or presence (nonspecific) of 50 IU of unlabeled hCG (Pregnyl, Organon Oss, The Netherlands). The binding affinity was determined by Scatchard analysis, by incubating similar aliquots of cell suspension with increasing amounts of labeled hCG (1.5 to 60 × 10^4 cpm/tube) in the presence or absence of a fixed concentration (50 IU/tube) of Pregnyl. The reaction was terminated after overnight incubation by addition of 3 ml of ice-cold Dulbecco's-PBS containing 0.1% BSA. After centrifugation, the supernatant was discarded by aspiration and the pellet was counted in a γ-spectrometer (1260 Multigamma II, AGG Wallac, Inc.).

Plasmids, transfections, and determination of luciferase activity

The murine LHR promoter/luciferase fusion genes were constructed as described previously (36). Briefly, the 2040-bp fragment of the 5′-flanking region (−1/−2040 bp), 173-bp deleted fragment from 2040-bp (−174/−2040 bp) and 173-bp (−1/−173 bp) fragment (in relation to translation initiation codon) of the mouse LHR gene, were linked to the coding sequence of the luciferase reporter construct (36, 37). The role of SF-1 mediated T_3 function was also assessed in mLTC-1 cells using 2 μg of pBKCMV-hDAX-1 (obtained from Dr. R. Yu, Northwestern University Medical School, Chicago, IL) expression plasmid. The mLTC-1 cells were transfected at 60–70% confluency by using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals GmbH, Mannheim, Germany). Two micrograms each of an LHR promoter construct, and a pSV-β-galactosidase expression vector (Promega Corp.), were used for transfection under optimized conditions (17), following instructions of the manufacturer. Thirty-six hours after transfection, cells were stimulated without or with T_3 for 8 h and assessed for either SF-1 binding (EMSA) or reporter assay (luciferase measurement). Luciferase activity was determined from cell lysates by chemiluminescence following addition of luciferin using the 1251 luminometer (BioOrbit, Turku, Finland) (32). The corresponding β-galactosidase activity was measured from the same sample to determine the variation in transfection efficiency.

The 5′-flanking regions of the mouse StAR gene were generated or obtained from Dr. B. J. Clark (Department of Biochemistry, University of Louisville School of Medicine, Louisville, KY). Full-length and deleted fragments of the 5′-StAR gene were placed upstream of the luciferase reporter gene into the pGL2 basic vector (Promega Corp.). The plasmids carrying mutations in the SF-1 sites (−135 and −42 bp) were generated by site-directed mutagenesis. Briefly, SF-1 sites at positions −135 and −42 bp were mutated from (CCAAGGTGG to TACGTAGTT) and (AGGCTG to TACGTA), respectively. For promoter analysis, mLTC-1 cells were cotransfected with 1 μg of plasmids and 1 μg of pRL-SV40 vector (a plasmid that constitutively expresses Renilla luciferase) to normalize the transfection efficiency. Following 36 h, cells were stimulated and luciferase activity was determined, as above.

Preparation of nuclear extracts and electrophoretic mobility shift assays (EMSA)

The nuclear extracts (NE) from different experimental groups, were prepared as described previously (36), with slight modifications to improve their purity. Briefly, after washing the cells twice with PBS, they were collected (10^7 cells/300 μl), and kept for 5 min in ice-cold lysis buffer A containing protease inhibitors (20 mmol/liter HEPES, 20 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 mg/liter leupeptin, 2 mg/liter aprotinin, pH 8.0). Following centrifugation, the crude nuclear pellet was resuspended and allowed to swell for 15 min at 4 C in 75 μl buffer C (buffer A containing 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, pH 7.9), followed by intermittent mixing of the suspension for another 15 min. After removing the debris by centrifugation at 12,000 × *g* for 5 min, the NE was assayed directly or stored at −80 C.

The doubled-stranded DNA probes were engineered and synthesized from the mouse LHR promoter (37) sequences [consensus (con) and mutated (mut) bases in the SF-1 binding sites are *underlined*], by heating sense (s) and antisense (as) primers to 72 C for 5 min in annealing buffer (10 mM Tris-Cl, 100 mM NaCl, 1 mM EDTA, pH 7.5), followed by cooling at room temperature.

SF-1-cons 5′- GGGTGGCCACAGTTCAAGGTCAAGGAGAA-3′
SF-1-conas 5′- GGGTTCTCCTTGACCTTGAACTGTGGCCA-3′
SF-1-muts 5′- GGGTGGCCACAGTGTAATATCAAGGAGAA-3′
SF-1-mutas 5′- GGGTTCTCCTTGATATTACACTGTGGCCA-3′

The 5′-GGG overhangs present in the double-stranded oligonucleo-

tides (100 ng) were labeled with [α^{32}P]-dCTP (3000 Ci/mmol, Amersham Pharmacia Biotech) using Klenow (Promega Corp.) fill-in reaction at 37 C for 1 h, and probes were purified by Nick column (Amersham Pharmacia Biotech). Ten micrograms of NE were incubated in 20 μl reaction buffer (12 mM HEPES, 60 mM KCl, 12% glycerol, 4 mM Tris-Cl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.9) containing 2 μg of poly (dI-dC):poly (dI-dC). The NE protein was incubated at 4 C for 30–40 min in the presence of molar excess (0- to 10-fold) cold competitors (unlabeled consensus SF-1 and mutated SF-1 nucleotides), followed by additional incubation for 45–50 min with approximately 0.5 ng of labeled probe. The reaction mixture (protein-DNA complexes) was then subjected to electrophoresis in low ionic strength 0.25× TBE buffers (90 mM Tris, 90 mM boric acid and 2 mM EDTA) on 5% polyacrylamide gels. The gels were then vacuum dried, exposed to Fuji Photo Film Co., Ltd. x-ray film and quantified.

Determination of pregnenolone, progesterone (P), and testosterone (T)

The concentrations of pregnenolone, P and T in the media were determined following extraction with diethyl ether using specific RIAs as previously described (38–40).

Data analysis

The data presented are the mean ± SEM. Statistical analysis was performed by one-way ANOVA, using the Statview program (Abacus Concepts Inc., Berkeley, CA), followed by Fisher's protected least significant differences test (Fisher's PLSD). $P < 0.05$ was considered statistically significant.

Results

Acute and chronic effects of T_3 on StAR mRNA, StAR protein and steroidogenesis in mLTC-1 cells

The data presented in Fig. 1 show that acute (8 h) stimulation with T_3 (37.5 pM) resulted in a 260 ± 15% increase in StAR mRNA expression (determined by quantitative RT-PCR) over basal level, whereas the elevated levels clearly declined following longer exposure to T_3 (Fig. 1A). The concentration of T_3 used in these experiments was based on our previous observations (17). Similar results were obtained when mitochondrial protein samples were measured for content of the 30-kDa StAR protein. In these samples, a 170 ± 10% increase in immunoreactive StAR protein was seen fol-

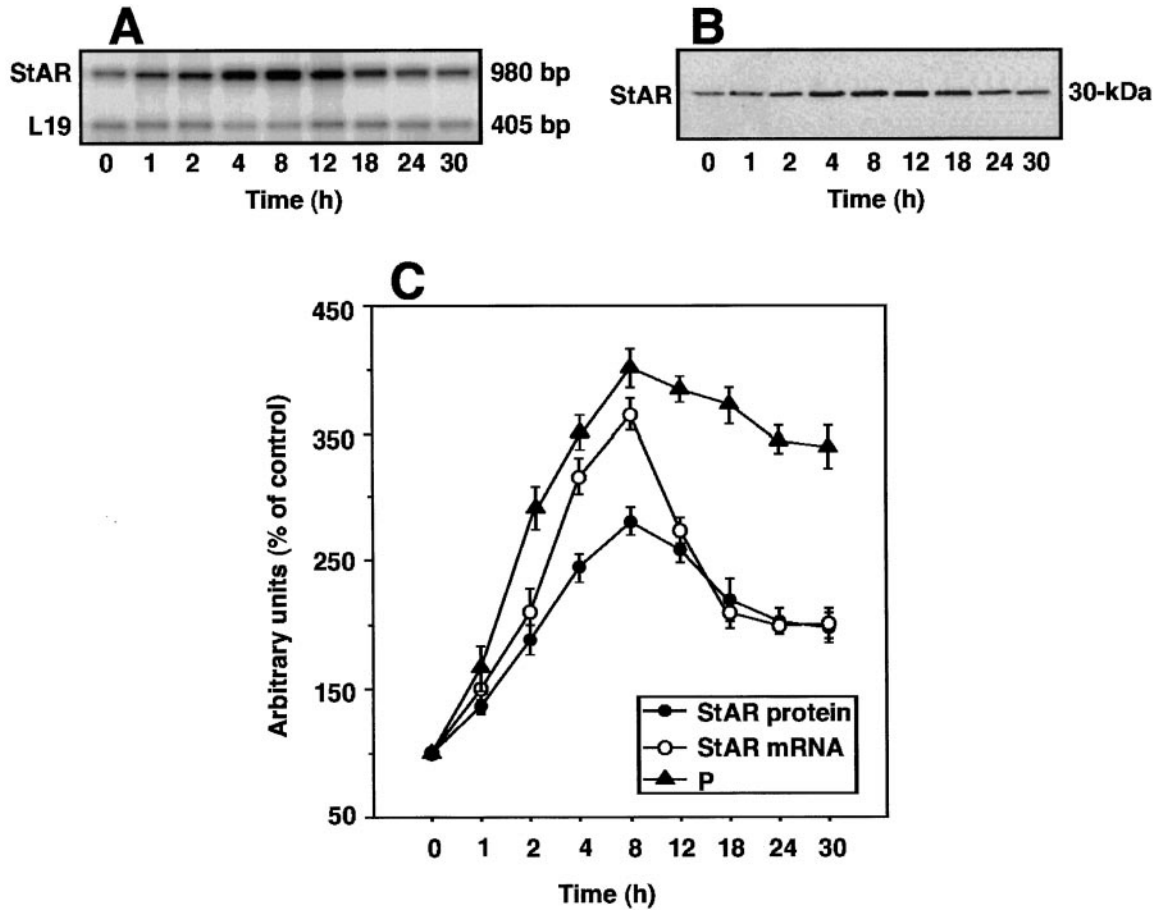

FIG. 1. Effects of T_3 on StAR mRNA, StAR protein, and P levels in mLTC-1 cells. Cells were stimulated with T_3 (37.5 pM) at indicated times (0–30 h), total RNA was extracted from the different treatment groups and subjected to RT-PCR analysis as described in *Materials and Methods*. The variation in RT-PCR efficiency was evaluated by coamplifying a 405-bp fragment of ribosomal L19 protein gene with each sample. The RT-PCR products were resolved in 1.2% agarose gels, dried, and exposed to x-ray films. A, Representative autoradiogram showing T_3-induced StAR mRNA expression at different time points. For immunodetection of the 30-kDa StAR protein content, 20 μg of mitochondrial protein were analyzed from different groups. B, Representative autoradiogram showing immunoblotting of StAR protein content. Likewise, P production was monitored at each time point. The arbitrary densitometric units (ADU) of the StAR mRNA, StAR protein and the corresponding P responses at the indicated times (C) are expressed as percent of control (0 = 100%). The values are the mean ± SEM of four independent experiments.

lowing 8 h of T_3 treatment, and a subsequent decline occurred thereafter up to 30 h (Fig. 1B). P concentration in the media observed at 8 h was increased to 300 ± 18% by T_3, and thereafter declined, following StAR expression. Compilation of the StAR mRNA, StAR protein and P data demonstrated an intimate correlation on T_3-induced StAR expression and steroidogenesis (Fig. 1C).

The effect of T_3 on StAR mRNA and P production requires transcription and ongoing protein synthesis

The involvement of transcription and *de novo* protein synthesis in T_3-stimulated StAR mRNA expression and steroidogenesis was assessed next. mLTC-1 cells stimulated for 8 h with T_3 (37.5 pM) in the presence of actinomycin D (ACT.D) or cycloheximide (10 mg/liter each) showed significant inhibition ($P < 0.0001$) of StAR mRNA expression in response to each inhibitor. T_3 alone had a profound stimulatory effect on StAR mRNA (Fig. 2). A full-length mouse StAR probe hybridized with two major transcripts at 3.4 and 1.6 kb, and two minor ones at 2.7 and 1.4 kb. All transcripts were of sufficient size to encode functional StAR protein, and showed coordinate regulation (200–500%) by T_3. P production in those cells exhibited similar response, suggesting an intimate association between StAR expression and steroid production (Fig. 2C).

Long-term T_3-mediated inhibition of P synthesis decreases cholesterol mobilization to the inner mitochondrial membrane

The mechanism of the T_3-mediated suppression of levels of StAR expression and P production beyond 8 h was studied next. mLTC-1 cells treated for 8 h with T_3 (37.5 pM) documented an approximately 300% increase in P production (Fig. 3A). Subsequently, cells stimulated for 30 h with T_3 displayed a >50% reduction in P synthesis, when compared with 8 h stimulation. Incubation with either 22R hydroxycholesterol (30 μM, a membrane-permeant analog of cholesterol), or pregnenolone (20 μM, Sigma), in combination with T_3 for 8 h, additively ($P < 0.01$) increased P production. When these incubations were extended to 30 h, a partial restoration of the T_3-mediated inhibition of P synthesis was found, reaching 61 ± 4.6% and 72 ± 3.7% of the respective 8 h levels (Fig. 3A). These findings suggest that the inhibitory effect of T_3 occurs, at least in part, before pregnenolone production. Another explanation is increased metabolism of the P accumulated during the first 8 h of culture between 8–30 h. The

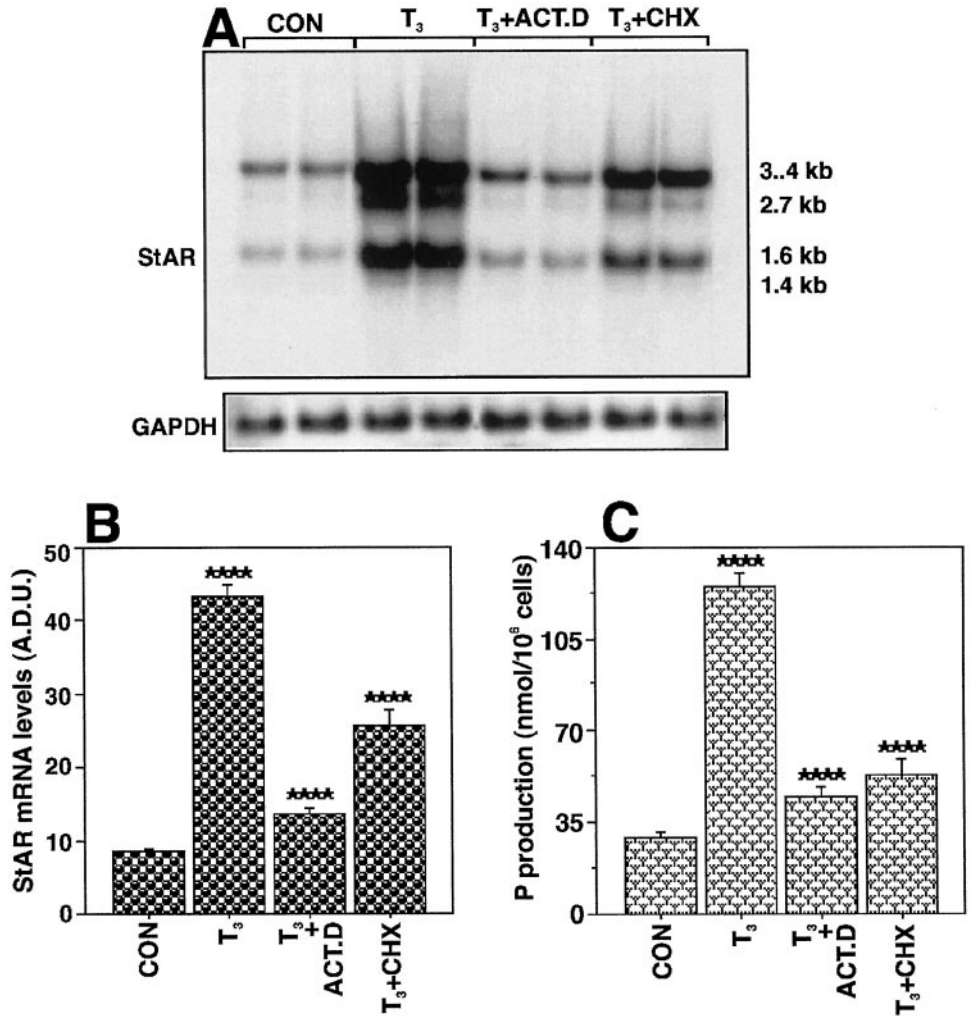

FIG. 2. The dependence of T_3 effects on StAR mRNA expression and P production on transcription and ongoing protein synthesis. The mLTC-1 cells were stimulated for 8 h in the absence (CON) or presence of T_3 (37.5 pM), T_3 plus ACT. D (10 mg/liter) and T_3 plus CHX (10 mg/liter). Twenty micrograms of total RNA from each group were probed with full-length mouse StAR cDNA. A, Representative autoradiogram among three experiments with similar results. The apparent molecular sizes of the different StAR transcripts are indicated on the right. The ADU values of major StAR transcripts were quantified, corrected with corresponding levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and presented in B. C, P concentrations in media of the same samples (± SEM, n = 4). The *asterisks* denote significant differences in the following comparisons: control *vs.* T_3, T_3 *vs.* T_3+ACT. D and T_3 *vs.* T_3+CHX, ****; $P < 0.0001$; ACT. D, Actinomycin D; CHX, cycloheximide.

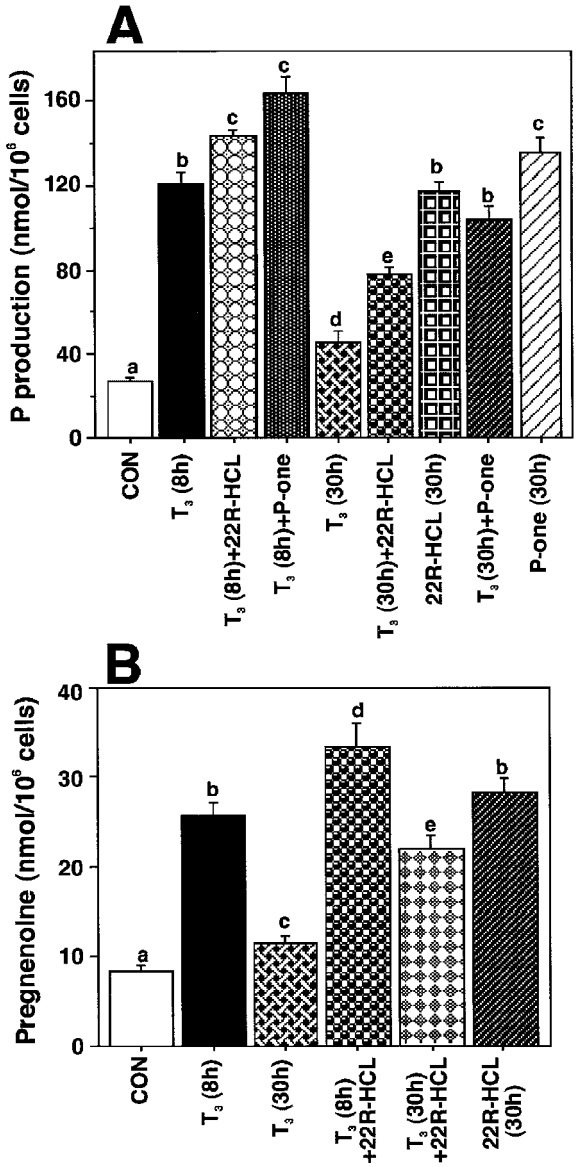

FIG. 3. Long-term T_3-mediated attenuation of P and pregnenolone synthesis. The mLTC-1 cells were stimulated without (CON) or with T_3 (37.5 pM) for 8 and 30 h. The media were then replaced by serum-free Waymouth's medium alone or containing steroid precursors, 22R hydroxycholesterol (22R-HCL; 30 μM) and pregnenolone (P-one; 20 μM), and followed by stimulation for an additional 8 h. Cell were also stimulated with steroid precursors alone for comparison at indicated times. P levels in the media were monitored by RIA (A). B, Chronic involvement of T_3 in pregnenolone production. Cells were stimulated in the absence (CON) or presence of T_3 (37.5 pM) for 8 and 30 h. The media were then replaced by serum-free Waymouth's medium alone or supplemented with the steroidogenic enzyme inhibitors, 3β-HSD (Trilostane, 10 μM) and P450c17 (SU 10603, 25 μM), in the absence or presence of 22R hydroxycholesterol (22R-HCL; 30 μM). Pregnenolone accumulation in the media was monitored following 8 h incubation by RIA. The values are the mean ± SEM of four independent experiments. *Different letters above the bars* indicate that these groups differ significantly at least at $P < 0.05$.

acute and chronic effects of T_3 on P synthesis were further assessed by determining testosterone (T) production. In a similar experimental paradigm, acute and chronic effects of T_3 on T production of mLTC-1 cells, though quantitatively much lower, were qualitatively similar to those of P (data not shown).

To corroborate the above observations, the role of StAR protein in cholesterol translocation was studied using inhibitors of the Δ^4 and Δ^5 steroidogenic pathways, *i.e.* the 3β-HSD inhibitor, trilostane (10 μM, Sanofi Pharmaceuticals, Inc., Malvern, PA) and the P450c17 inhibitor, SU 10603 (25 μM). As demonstrated in Fig. 3B, mLTC-1 cells stimulated with T_3 for 8 h significantly ($P < 0.001$) increased pregnenolone production, whereas a marked decrease occurred in these levels at 30 h. Incubation of cells pretreated for 30 h with T_3 in the presence of 22R hydroxycholesterol for 8 h significantly restored (80 ± 5.3% of 8 h T_3 stimulated) pregnenolone synthesis. These data indicate that longer exposure of T_3 decreases cholesterol delivery to the mitochondrial inner membrane and probably does so through repression of the StAR protein.

Long-term effect of T_3 on StAR mRNA stability and on steroidogenic enzyme mRNA levels

Considering the effects of long-term T_3 incubation, StAR mRNA stability was evaluated. Cells prestimulated in the absence or presence of T_3 (37.5 pM) for 10 h were further incubated with actinomycin D (10 mg/liter) for up to 24 h. As shown in Fig. 4, in both transcriptionally arrested control and T_3-treated cells, the half-lives of StAR mRNA were found to be approximately 11 h, indicating that long-term exposure to T_3 does not affect StAR mRNA stability, and thus, the decreased StAR mRNA levels are a result of decreased transcription.

We then assessed the effects of T_3 treatment on the expression levels of several steroidogenic enzyme genes, *i.e.* those of P450scc, P450c17 and 3β-HSD, which catalyze the metabolic steps at various phases of steroid hormone biosynthesis. Northern analysis (Fig. 5) revealed that stimulation with T_3 (37.5 pM) for longer time periods resulted in an 83 ± 5.4% increase in P450scc mRNA. Conversely, chronic T_3 incubation (30 h) caused significant ($P < 0.01$) decreases in P450c17 and 3β-HSD mRNA levels by 56 ± 3.5% and 34 ± 3.1%, respectively.

Influence of T_3 on [^{125}I]iodo-hCG binding and on LHR mRNA expression

The effects of T_3 (37.5 pM) on [^{125}I]iodo-hCG binding and LHR mRNA expression are presented in Fig. 6. [^{125}I]iodo-hCG binding was maximally increased at 16 h (228 ± 10.4% of control) following incubation with T_3. Longer exposure to T_3 markedly reduced the hCG binding (46 ± 5.5% at 72 h) in a time-dependent manner when compared with controls (Fig. 6A). Both the acute and chronic effects of T_3 on LHR mRNA expression were analyzed by Northern hybridization. An LHR-specific probe (nucleotides 441–849) revealed multiple transcripts of 6.9, 4.2, 2.6, and 1.8 kb in these cells, and all of them were coordinately upregulated by acute T_3 stimulation, whereas down-regulation of the elevated levels occurred following longer treatment (Fig. 6B). Under these incubation conditions, no significant changes were observed in the hCG binding affinity (K_d) (data not shown). The effects of T_3 on LHR

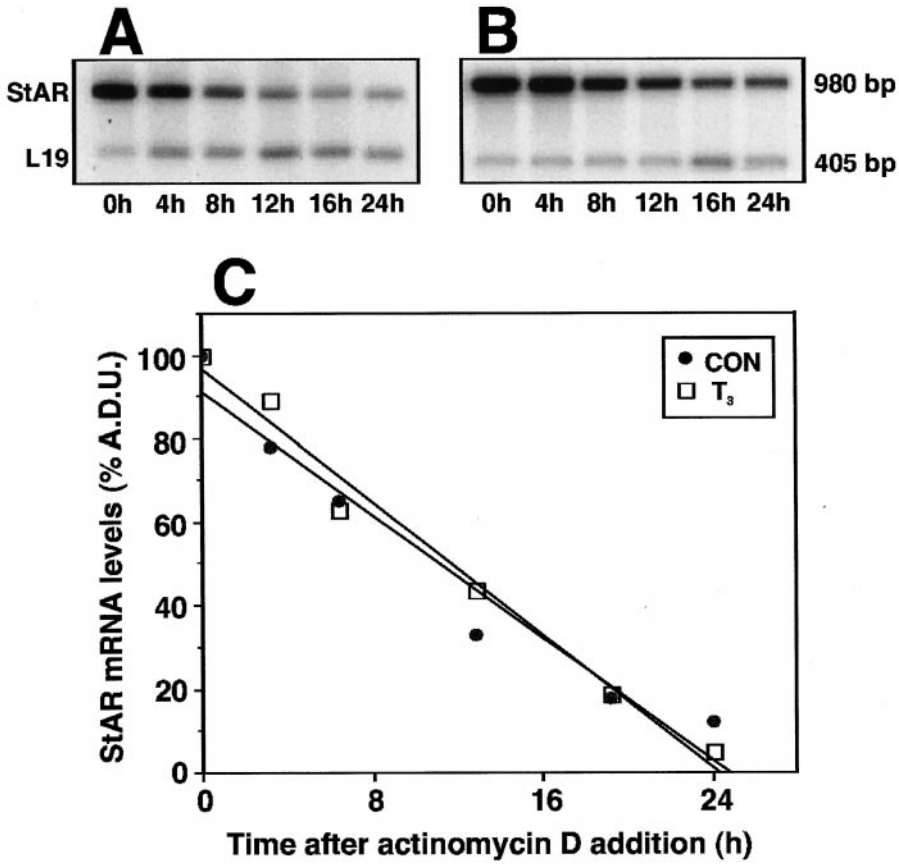

FIG. 4. Effect of T_3 on StAR mRNA stability in mLTC-1 cells. Cells were stimulated in serum-free Waymouth's medium without (CON) or with T_3 (37.5 pM) for 10 h. At time zero, actinomycin D (10 mg/liter) was added to culture media and the incubation was continued up to an additional 24 h. Total RNA was extracted at indicated times, and 2 μg of the RNA from each group were subjected to RT-PCR analysis, as described in the legend of Fig. 1. The expression levels of the StAR mRNA in control (A) and in T_3-treated groups (B) are illustrated. C, ADU values of StAR mRNA in control (CON) and T_3-treated (T_3) cells after correction for intensities of the corresponding L19 bands. A representative autoradiogram from three similar experiments is presented.

levels closely correlated with the binding data, and strongly implicate the involvement of thyroid hormone in the regulation of LHR gene expression.

Serum T_3, T_4, LH, T, and PRL levels, and testicular weights of mice rendered hypo and hyperthyroid

The physiological relevance of thyroid hormone action on the regulation of LHR levels was assessed in mice rendered hypo and hyperthyroid by treatments with THU and T_4, respectively. The hormone levels in the serum of these animals are summarized in Table 1. Serum T_3 and T_4 levels were decreased with THU and increased with T_4 treatments, respectively, providing evidence for the altered thyroid status of the treated mice. Serum LH increased moderately but consistently in hyperthyroid animals and decreased in the hypothyroid group. There were no noticeable alterations in serum T levels; however, serum PRL levels increased significantly following THU treatment when compared with controls. No changes were observed in testis weights (Table 1).

[^{125}I]iodo-hCG binding and LHR mRNA levels with altered thyroid hormone status

The results presented in Fig. 7 illustrate [^{125}I]iodo-hCG binding to Leydig cells isolated from testes of mice of the different treatment groups. Interestingly, hypothyroidism caused a 175% elevation in binding (Fig. 7A). In contrast, T_4 treatment significantly decreased hCG binding to 56% of that seen in controls. Scatchard analysis revealed that the affinity of hCG binding showed no major changes with the mean K_d values being 4.47 ± 0.11, 4.29 ± 0.26, and $4.52 \pm 0.13 \times 10^{-10}$ M in hypothyroid, hyperthyroid and control groups, respectively (Fig. 7B).

Employing RT-PCR analysis, LHR mRNA levels were significantly increased ($80 \pm 7.2\%$) by THU and decreased ($P < 0.01$) by T_4 treatments in comparison to control values (Fig. 8). Northern blot analysis employing an LHR specific probe confirmed the RT-PCR results, and revealed that all of the LHR mRNA splice variants were coordinately regulated (data not shown).

Effect of T_3 on LHR promoter activity

To corroborate these findings, and to further understand the mechanisms of thyroid hormone action in regulating LHR levels, fragments of the LHR promoter were linked upstream of the firefly luciferase reporter gene. mLTC-1 cells transfected with the longest LHR promoter construct (−1/−2040 bp) displayed significantly elevated (183% of control) luciferase activity following T_3 induction (Fig. 9). Conversely, cells transfected with 173-bp deleted segment (−174/−2040 bp), which includes a nuclear SF-1 binding site, displayed reduced luciferase activity ($66 \pm 5.6\%$ of full-length promoter) and no response to T_3 was observed. In contrast, the T_3-mediated luciferase activity increased to 136% of the basal level when mLTC-1 cells were transfected with the promoter fragment consisting of the first 173-bp

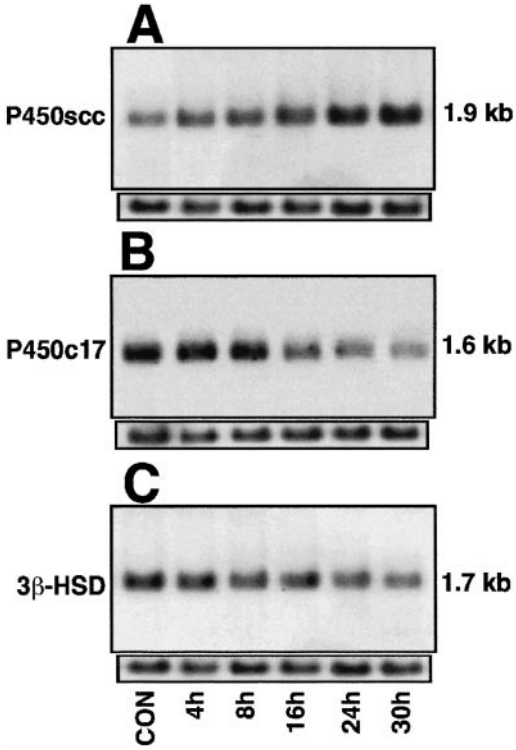

FIG. 5. Acute and chronic effects of T_3 on P450scc, P450c17 and 3β-HSD mRNA levels. The mLTC-1 cells were incubated without (CON) or with T_3 (37.5 pM) for 4–30 h. Total RNA was extracted from different groups, and subjected to Northern blotting with specific probes using 20 μg RNA, as described in *Materials and Methods*. A representative autoradiogram of each group with similar results (n = 3–5) is presented. The apparent molecular sizes of the different steroidogenic enzyme mRNAs (A, P450scc; B, P450c17; C, 3β-HSD) are indicated on the right. The GAPDH mRNA expression of each group in the corresponding lane demonstrates equal loading of RNA (*lower panels*).

(−1/−173 bp) upstream of the translation start site, indicating the presence of putative thyroid hormone binding motif(s) in this basal promoter region.

Mutation in the bases of the SF-1 recognition sequences affect the T_3 function

Our previous results in mLTC-1 cells demonstrated that SF-1 is a key component in T_3 function (17). To gain more insight into these mechanisms, we carried out EMSA studies with oligonucleotides corresponding to the consensus SF-1 binding site. Radioactively labeled oligonucleotide containing an SF-1 consensus site revealed a single specific protein-DNA complex. As illustrated in Fig. 10, T_3 treatment significantly augmented an apparent dose-dependent increase in protein-DNA complexes, using nuclear extracts prepared from mLTC-1 cells. Competition binding studies demonstrated that the binding to protein-DNA complexes was effectively inhibited by increasing doses of the homologous sequence (cold competitor). Importantly, mutation of bases in the SF-1 consensus sequences abolished the binding competition obtained with consensus SF-1 (Fig. 10). Interestingly, cells expressing DAX-1 markedly suppressed the basal and T_3-stimulated formation of protein-SF-1 DNA complexes, suggesting a relationship between T_3 action and SF-1.

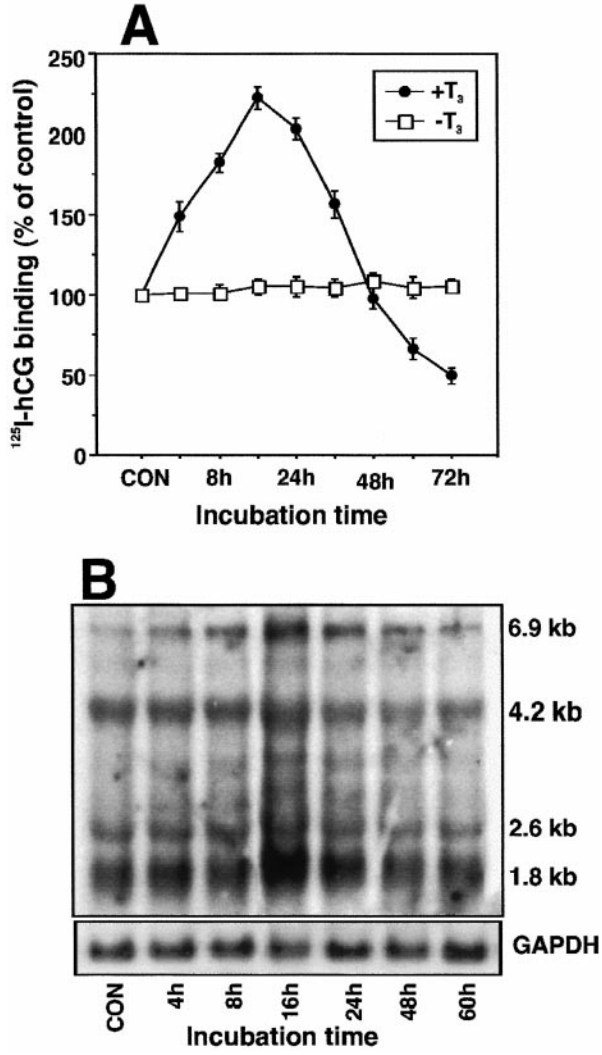

FIG. 6. Influence of T_3 on [^{125}I]iodo-hCG binding and LHR mRNA expression in mLTC-1 cells. Cells were stimulated in the absence (−T_3) or presence of T_3 (+T_3; 37.5 pM) at indicated times (0–72 h), and [^{125}I]iodo-hCG binding was assessed as described in *Materials and Methods*. A, [^{125}I]iodo-hCG specific binding to control and T_3-stimulated mLTC-1 cells, with fixed concentration of labeled hCG (~10^5 cpm/tube). The results are compiled from four independent experiments and represent the mean ± SEM of quadruplicate determinations. B, Time-dependent effect of T_3 on LHR mRNA expression by Northern hybridization analysis. The mLTC-1cells were stimulated in the absence (CON) and presence of constant concentration of T_3 (37.5 pM) for times indicated. A specific complementary RNA probe corresponding to nucleotides 441–849 of the extracellular part of rat LHR gene was used for hybridization with 20 μg of total RNA per group. The apparent molecular sizes of the different LHR splice variants at 6.9, 4.2, 2.6, and 1.8 kb are indicated on the *right*. The GAPDH mRNA level of each fraction demonstrates equal loading of RNA (*lower panel*). Similar results were obtained from three independent experiments.

To further understand these mechanisms, function of the 5′-flanking region of the mouse StAR gene was studied in response to T_3. The data demonstrated that the proximal −966-bp (p-966StAR/Luc) fragment was highly responsive to T_3 and was similar to full-length (3.6 kb) promoter activity, among the different constructs (−966, −426, −254 and −110 bp) examined (data not shown). Using this promoter frag-

TABLE 1. Serum hormone levels, and testicular weights of mice treated with thiouracil or thyroxine

Groups	T_3 (ng/ml)	T_4 (ng/ml)	LH (μg/l)	Testosterone (ng/ml)	Prolactin (μg/l)	Testis wt (mg)
Control (9)	1.14 ± 0.13	43.4 ± 3.2	0.18 ± 0.05	2.39 ± 0.08	11.2 ± 3.5	162 ± 7.1
Thiouracil (11)	0.59 ± 0.08^b	21.6 ± 6.8^b	0.10 ± 0.03^b	2.51 ± 0.11	18.9 ± 3.3^a	158 ± 8.4
Thyroxine (10)	2.26 ± 0.16^b	62.7 ± 5.8^b	0.25 ± 0.06^a	2.44 ± 0.13	7.3 ± 4.2^a	166 ± 6.2

The mice were injected with saline (control), thiouracil or thyroxine for 3 weeks, and killed thereafter by cervical dislocation. Serum samples were collected and measured hormonal levels as mentioned in *Materials and Methods.* Testicular weights of mice of the different groups were also determined. The number of animals per group is indicated in *parentheses*. The results are the mean ± SEM. *Letters* represent significant differences from respective control groups, $^a P < 0.05$, $^b P < 0.01$.

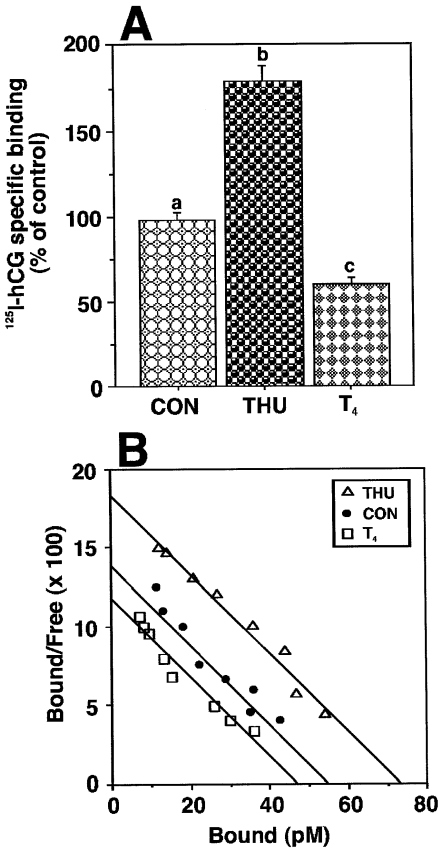

FIG. 7. The capacity and affinity of [^{125}I]iodo-hCG binding to the isolated primary mouse Leydig cells from different treatment groups. The Leydig cells were isolated and purified separately from control (CON), hypo (THU), and hyperthyroid (T_4) mice as described in *Materials and Methods*. [^{125}I]iodo-hCG binding (A) was carried out in isolated Leydig cells (3×10^5 cells/incubation) from different groups, as demonstrated in the legend of Fig. 6. B, Affinity of hCG binding as determined by Scatchard analysis, by incubating equal aliquots of cell suspensions with increasing concentrations of [^{125}I]iodo-hCG (1.5–60×10^4 cpm/assay). The values are the mean ± SEM of 9–11 mice belonging to the same treatment group. *Different letters above the bars* indicate that these groups differ significantly at $P < 0.01$.

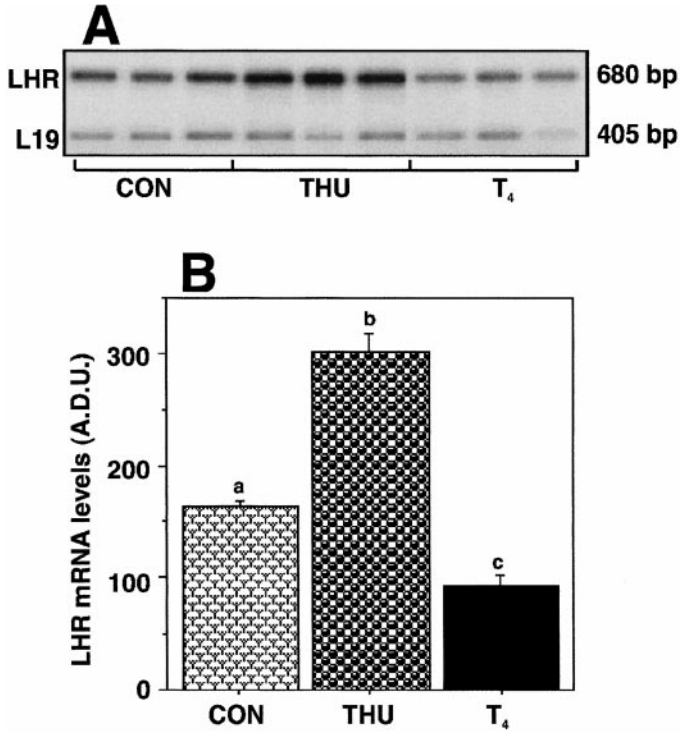

FIG. 8. LHR mRNA in isolated Leydig cells of mice following experimentally induced hypo and hyperthyroidism. Total RNA was extracted from control (CON) and treated groups (THU and T_4), and subjected to RT-PCR analysis. A, Representative autoradiogram showing levels of LHR mRNA in the different groups (three of each). The ADU value of each band was quantified and corrected for intensity of the corresponding L19 bands (B). The results are the mean ± SEM of triplicate determinations. *Different letters above the bars* indicate that these groups differ significantly from each other at $P < 0.01$.

ment, the functional involvement of SF-1 recognition sites (at positions −135 and −42 bp) in T_3 function were evaluated (Fig. 11). Transient transfection of mLTC-1 cells with a p-966StAR/Luc plasmid carrying a mutation of the SF-1 site at position −135 bp, accounted for an approximately 40% decrease of basal and T_3-stimulated luciferase activity compared with p-966StAR/Luc alone. Mutation at both SF-1 sites (positions −135 and −42 bp) did not show further impairment of basal and T_3-induced luciferase activity. Although PGL2 control showed low stimulation of luciferase activity by T_3, it does not confound the conclusion concerning functional significance of the −135 bp SF-1 site in StAR promoter function. These results indicated the importance of the SF-1 site at position −135 bp, and further documented the specific involvement of SF-1 in T_3 function.

Discussion

The critical function of the StAR protein in hormone-regulated acute steroid biosynthesis is now well established. We recently demonstrated that thyroid hormone action could regulate steroidogenesis and StAR expression in mLTC-1 mouse Leydig tumor cells (17). Acute treatment with T_3 coordinately induced StAR gene expression and ste-

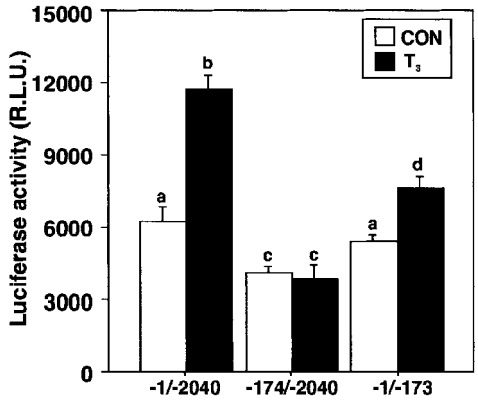

FIG. 9. Effect of T_3 on LHR promoter-driven luciferase activity by transient transfections in mLTC-1 cells. Cells were transfected with full-length (−1/−2040 bp), 173-bp deleted fragment from full-length (−174/−2040 bp), and 173-bp fragment alone (−1/−173 bp) of the murine LHR promoter-driven luciferase reporter constructs, as described in *Materials and Methods*. After 36 h, cells were stimulated in the absence (CON) or presence of T_3 (37.5 pM) for 8 h. Luciferase activity was determined and normalized with the corresponding β-galactosidase value, and expressed as relative luciferase response (RLU). The data are the mean ± SEM of three experiments in quadruplicates. *Letters above the bars* indicate that these groups differ significantly at least at $P < 0.05$.

roid production, whereas its chronic action remarkably diminished these responses. In the present study we evaluated the mechanisms involved in more prolonged exposure to T_3 on StAR expression, steroidogenesis and LHR expression in murine Leydig cells. To our knowledge, these findings provide the first evidence that long-term treatment with T_3 attenuated the acute increase observed in steroidogenesis and that it is most likely due to reduction of cholesterol supply to the mitochondrial inner membrane caused by inhibition of StAR synthesis. In addition, we present herein data that thyroid hormone is involved in regulation of LHR function in mouse Leydig cells *in vivo*.

The key second messenger in trophic hormone stimulated steroidogenesis is cAMP which, in turn, triggers a regulatory cascade resulting in mobilization and delivery of cholesterol to the inner mitochondrial membrane where P450scc catalyzes its conversion to pregnenolone. The transport of cholesterol is the first, truly regulated and rate-limiting step in steroidogenesis, and it is mediated by the StAR protein (1, 2, 4, 5). Recently, mechanisms of StAR action have been evaluated by N- and C-terminal truncated forms, which demonstrate the importance of the C-terminus, and provide evidence that StAR acts on the outside of the mitochondria to transfer cholesterol (41, 42).

Our results show that acute induction of P production and StAR expression in mLTC-1 cells by T_3 requires on-going RNA and protein synthesis, which are in general agreement with previous observations (2, 17, 43). Chronic T_3 treatment, which can clearly be seen at 30 h, diminished the stimulatory responses on P synthesis and StAR expression obtained at 8 h. Inhibition of P production did not appear to be as pronounced as inhibition of StAR expression suggesting the possible involvement of StAR independent processes in P production. Consistent with our observations, transcriptional inhibition in MA-10 cells markedly affected synthesis of the StAR protein, whereas 10–20% of the steroidogenic potency of the cells occurred through a StAR-independent mechanism (44). In mLTC-1 cells, four StAR transcripts were observed, two major (3.4 and 1.6 kb) and two minor ones at 2.7 and 1.4 kb, all of which showed coordinate regulation following T_3 stimulation (17). Our data also document that the decrease of StAR expression following T_3 treatment was due to StAR gene transcription, rather than degradation of the message. The half-life of the StAR mRNA in transcriptionally arrested control and T_3-treated cells was found to be approximately 11 h in both cases. Thus, the present findings clearly show that T_3 regulates steroidogenesis through induction of the StAR protein that, in turn, is preceded by SF-1 expression. In an earlier report, we demonstrated that mLTC-1 cells expressing SF-1 coordinately augmented StAR expression and steroid production, whereas DAX-1 overexpression drastically decreased these responses (17). Consideration of our previous data led to the hypothesis that longer T_3 stimulation may induce DAX-1 expression. DAX-1 has been reported to repress SF-1 function, but no clear-cut regulation of DAX-1 protein was observed in relation to acute or chronic T_3 treatments (data not shown).

The present results confirm our earlier observations and extend them by illustrating the mechanisms of chronic T_3-mediated attenuation of the acute stimulated levels of StAR expression and steroidogenesis. This was clearly shown in experiments in which addition of 22R hydroxycholesterol or pregnenolone to the T_3-inhibited cells significantly reversed the inhibition of P synthesis. These results were also confirmed by measuring pregnenolone production from 22R hydroxycholesterol in the presence of the 3β-HSD inhibitor trilostane and the P450c17 inhibitor SU 10603. These inhibitors have recently been demonstrated to be potent in inhibiting pregnenolone metabolism in bovine adrenocortical cells (45). In addition, P production was greatly reduced (~90%) by these inhibitors in luteinized primate granulosa cells (46). It is also possible that longer exposure to T_3 decreases the low-density lipoprotein (LDL) receptors, which have been demonstrated to be instrumental in cholesterol delivery to adrenocortical cells (47).

Contrary to the inhibitory effect that chronic treatment with T_3 had on steroidogenesis and StAR, P450scc mRNA levels increased significantly during T_3 stimulation. These results clearly reinforce and support the data obtained in cultured rat or MA-10 cells, which indicated that treatment with either hCG, $(Bu)_2$cAMP or forskolin increased the expression of P450scc mRNA and protein (48, 49). However, P450c17 and 3β-HSD mRNAs displayed moderate but consistent decreases following longer exposure to T_3. Previous studies also demonstrated that chronic stimulation with cAMP represses the levels of T-mediated stimulation of P450c17 and 3β-HSD mRNA levels in cultured mouse Leydig cells (50). This possibility cannot be excluded in mLTC-1 cells, as they possess basal StAR expression and T production in contrast to MA-10 mouse Leydig tumor cells. On the other hand, increased levels of 3β-HSD activity, protein synthesis, and mRNA levels were also reported in cultured rat Leydig cells in relation to LH, cAMP or forskolin stimulation (51).

The role of thyroid hormone in gonadal function has been well documented and is known to affect the development,

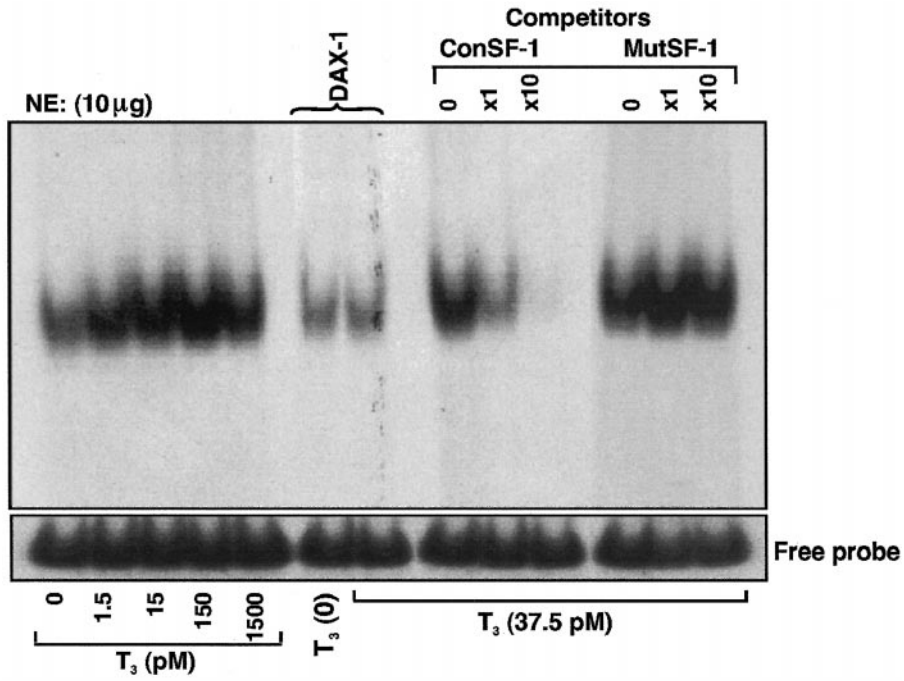

FIG. 10. Specificity of SF-1 binding in relation to T_3 action on mLTC-1 cells. Cells were stimulated for 8 h in the absence or presence of increasing (0–1500 pM) or fixed (37.5 pM) concentration of T_3, and subjected to preparation of nuclear extracts (NE). Ten micrograms of NE obtained from different treatment groups were incubated with different molar ratios of cold competitors [corresponding to SF-1 consensus (ConSF-1) or mutational (MutSF-1) sequences], followed by an additional incubation with ^{32}P-labeled consensus SF-1 oligonucleotide (0.5 ng) as described in *Materials and Methods*. Protein-DNA complexes were subjected to electrophoresis through a 5% nondenaturing polyacrylamide gel for approximately 2 h at 210 V; gels were then dried and visualized by autoradiography. Specificity of SF-1 binding in relation to T_3 (37.5 pM) was also assessed with cells expressing DAX-1. Similar results were obtained from four independent experiments. Free probes of the corresponding lanes are shown in the *lower panel*.

growth, and function of essentially all organs and tissues and has been implicated in mammalian reproduction leading to gonadal abnormalities with impaired fertility (8–11). The present findings provide interesting evidence that acute T_3 treatment markedly elevates LHR mRNA expression and receptor binding, whereas chronic treatment dramatically decreased these levels. The biphasic response of thyroid hormone on LHR regulation demonstrated the antagonistic interrelationship between the thyroid and testis in gonadotropin action.

To address the physiological relevance of thyroid hormone action on LHR expression, we carried out experiments in hypo and hyperthyroid mice, where serum T_3 and T_4 levels decreased in the former and increased in the latter. Our present results with mouse serum hormone levels corroborate previous findings, which demonstrate that prepubertal hypothyroidism is related to decreased serum LH and FSH, normal or low T levels, and hypersecretion of PRL in most cases, together with increasing number of Leydig cells (15, 16, 21, 24, 52). The *in vivo* results provided evidence indicating the effect of hypothyroidism in the augmentation of LHR expression, whereas conversely, hyperthyroidism diminished these responses. These results may be interpreted as indicating that increased testicular LHR levels in hypothyroid mice sensitize this organ to LH. In contrast, decreased LHR levels in hyperthyroidism have an opposite effect, and the maintenance of normal T secretion requires elevated LH secretion. In agreement with this observation, previous studies have demonstrated an enlargement of the interstitium with increased numbers of Leydig cells in hypothyroid mice, whereas fibrous thickening of the interstitium with reduced Leydig cell numbers were observed with T_4 treatment (not illustrated) (12, 14, 15). A discrepancy seems to exist with regards to *in vitro* and *in vivo* receptor function, possibly due to the repressor-like activity of thyroid hormone in receptor

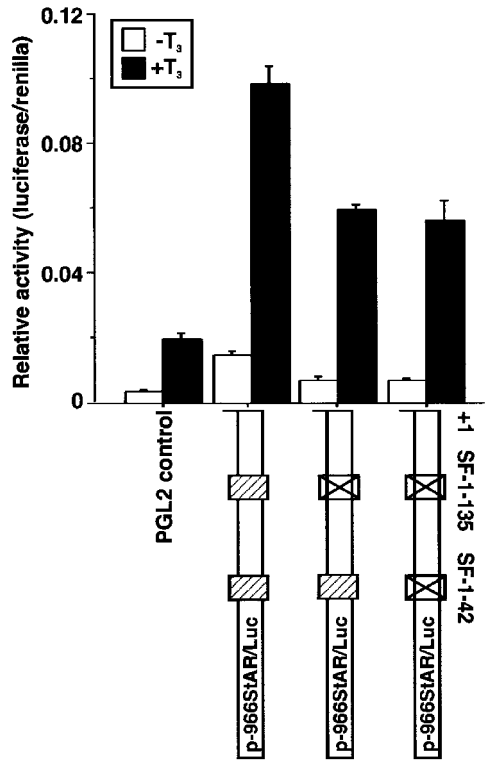

FIG. 11. Effects of alterations of bases in the SF-1 recognition site on T_3-mediated StAR promoter activity. The mLTC-1 cells were cotransfected with p-966StAR/Luc, and p-966StAR/Luc carrying mutations at −135 bp SF-1 site alone or in combination with −42-bp plasmids, together with pRL-SV40 (renilla) as described in *Materials and Methods*. Following 36 h, cells were stimulated in the absence ($-T_3$) or presence of T_3 ($+T_3$; 37.5 pM), and luciferase activity in the cell lysates was determined after 8 h of stimulation and normalized with pRL-SV40 vector, and expressed as relative activity (luciferase/renilla). PGL2 basic plasmid was used as control. The data are the mean ± SEM of four independent experiments.

protein interaction. However, it should be taken into account that pituitary TSH levels may interact with LHR and inhibit *in vivo* receptor function, although other factors might also be involved.

The effects of thyroid hormone on adult testis have not been thoroughly investigated, possibly because of the findings that T_4 action *in vivo* did not affect their oxygen consumption (53). Although thyroid hormone has nonnuclear actions, the major actions of T_3 are thought to be mediated by nuclear T_3 receptors (TRs) (54). TR is a member of the steroid/thyroid hormone receptor superfamily that binds to the hexameric motif AGGTCA, termed the nuclear receptor half-site, to activate gene transcription (55). The cloning and characterization of the 5′-flanking region of the mouse and rat LHR gene demonstrated that the basal promoter region (173 bp) is highly GC rich containing AP1 and several SP1 sites, which are involved in transcriptional regulation (36, 37, 56). The importance of this region was further confirmed, by expressing either the 173-bp fragment alone or its deletion from the full-length fragment (2040 bp) in mLTC-1 cells. Comparison with the 2040-bp fragment, in relation to T_3-mediated luciferase activity indicated the presence of putative thyroid hormone response element(s) in this region. These results support our previous findings that regulation of T_3-induced StAR expression and P production is dependent on SF-1, as inhibition of the latter by DAX-1 abolished the T_3-mediated responses (17).

To evaluate the mechanisms involved in thyroid hormone action, and to further understand the role of SF-1, EMSA studies were carried out with T_3-stimulated nuclear extracts using oligonucleotides corresponding to an SF-1 binding site. Our data suggest potential involvement of SF-1 in T_3 function, as mutations in the SF-1 consensus sequences rendered it incapable of competing with protein-DNA complexes. The importance of SF-1 was also assessed in cells expressing DAX-1 in relation to T_3. Further insight into these mechanisms was observed in studying the 5′-flanking analyses of the mouse StAR gene, which demonstrate that the proximal 966-bp fragment is sufficient for full promoter activity in transiently transfected mLTC-1 cells. Concerning the p-966-bp fragment, it is noteworthy that mutations in the bases of the SF-1 site at position −135 bp significantly decreased both basal and T_3 stimulated StAR promoter activity. These data suggest that the −135-bp SF-1 element is important for basal StAR expression and not for the T_3 response, which further supported previous findings with regard to the cAMP-dependent StAR promoter activity in MA-10 cells (43, 57). These results further strengthen the crucial involvement of SF-1 in thyroid hormone-regulated gonadal function.

Importantly, the 5′-flanking region of the SF-1 promoter contains an E box, a CAAT box, and SP1 elements that are required for steroidogenic-specific expression of the SF-1 gene in the adrenal gland and gonads (58). There is a conspicuous lack of thyroid hormone response elements (TREs) in the promoter sequences of SF-1 (58), in the human and mouse StAR (43, 59), and in murine and rat LHR (37, 56), suggesting the involvement of additional factor(s). Indeed, recent studies demonstrate interactive cooperation of SF-1 and C/EBPβ (CCAAT/enhancer binding protein-β) in the regulation of StAR gene transcription and steroidogenesis (57). In addition, the binding motif of the GATA-4 transcription factor, located at position −61/−66 in the mouse and human StAR promoters, is also involved in mediating the acute response to hormones (60).

Taken together, our data clearly demonstrate that thyroid hormone acutely increased StAR expression, steroidogenesis, and LHR levels, whereas these responses were suppressed with chronic treatment. The latter effects of T_3 occurred partly due to diminished cholesterol delivery to the mitochondrial inner membrane. The precise nature of the TR isoform(s) in Leydig cells, and other factors involved in the thyroid hormone action on LHR regulation, will need to be addressed in further detail. Importantly, the present clear-cut *in vitro* and *in vivo* findings provide evidence for the role of thyroid hormones in LHR regulation in mouse Leydig cells, explaining further the pathophysiology of gonadal function in association with thyroid malfunction.

Acknowledgments

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