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Cell Cycle Progression and 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Are Regulated by Thyrotropin in FRTL-5 Rat Thyroid Cells*

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The incorporation of [^{14}C]acetate into cholesterol shows that FRTL-5 cells possess an active cholesterol biosynthetic pathway. When these cells were made quiescent, and synchronized by thyrotropin (TSH) starvation, in the presence of low serum (0.2%), addition of this hormone increased acetate conversion into cholesterol up to a maximum of 8-fold. Feedback inhibition of sterol synthesis by exogenous cholesterol occurs in FRTL-5 cells since, in the presence of higher serum concentration (5%), acetate conversion into cholesterol was significantly depressed. Even in high serum TSH increased sterol synthesis, albeit to a lesser extent. The time course of the TSH effect on cholesterol synthesis, strongly suggests that this process is necessary for quiescent FRTL-5 cells to enter the cell cycle. Thus, the rate of cholesterol synthesis was maximal 12–16 h after TSH challenge and declined thereafter, returning to levels slightly above the basal at 48 h. Thymidine incorporation into DNA, measured under identical conditions of TSH starvation/challenge, increased after 20 h, was maximal at 36 h, and returned to pre-TSH level at 70 h. The effect of TSH on cholesterol synthesis is not a general feature of lipid synthesis in FRTL-5 since [^{14}C]acetate incorporation into triglycerides after TSH treatment has a different magnitude and time course.

TSH increases cholesterol synthesis through the induction of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase. This is due to an increase in the level of 3-hydroxy-3-methylglutaryl-CoA reductase messenger RNA up to 8-fold caused by a proportional increase in the rate of gene transcription, as assessed by nuclear "run on" experiments.

The effect of TSH on cholesterol synthesis and reductase gene expression is likely to be mediated by cAMP since 8-bromo-cAMP mimicked the effect of the hormone.

The data presented suggest that an active cholesterol biosynthetic pathway is required for DNA synthesis to occur.

Substantial evidence has accumulated, which suggests that activation of sterol synthesis is associated with DNA synthesis and cell proliferation (1, and reviewed in Ref. 2). Challenging mouse lymphocytes with the nonspecific mitogen phytohemagglutinin causes the progression from a non-proliferating to a proliferating state, and an increased rate of DNA synthesis, which is preceded by an increase in the rate of sterol synthesis (1). However, it is still uncertain whether the metabolite active in promoting DNA synthesis is cholesterol itself or some other product of its complex biosynthetic pathway. Attention has been focused on the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)[1] reductase (mevalonate:NADP$^+$ oxidoreductase (CoA-acylating): EC 1.1.1.34) (3). In mammalian cells HMG-CoA reductase controls the rate of synthesis of many isoprenoids derived from mevalonic acid, the precursor common to cholesterol, dolichol, isopentenyl adenine, and ubiquinone. Inhibitors of HMG-CoA reductase such as 25-hydroxy-cholesterol or compactin, cause depletion of cellular sterols and arrest cell duplication. However, normal growth is restored by supplying mevalonate, not cholesterol (3–5).

The FRTL-5 thyroid cell line (6) is an excellent system for the study of the relationship between sterol synthesis and cell duplication since the progression from quiescence to proliferation can be induced by TSH, *i.e.* a specific, physiological mitogen (7, 9). In previous reports we have shown that, in the absence of TSH, FRTL-5 cells cease dividing and display major changes in membrane lipid composition of which the most notable is a more than 2-fold increase of membrane cholesterol (10); we have also shown that when FRTL-5 cells are deprived of TSH the binding of low density lipoproteins (LDL) increases more than 2-fold (11). TSH regulation of LDL receptor activity could explain, at least in part, why these cells accumulate cholesterol in the absence of the hormone. However, these findings leave unresolved the apparent discrepancy of a lower cholesterol content in an actively dividing cell population. One possible explanation is that proliferation of FRTL-5 requires an active cholesterol biosynthetic pathway rather than cholesterol itself. Indeed, cholesterol is a powerful regulator of its own biosynthesis (4). Thus, when the cell can take up cholesterol from the surrounding medium, it is protected from overaccumulation by suppression

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[1] The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; TSH, thyrotropin- or thyroid-stimulating hormone; LDL, low density lipoprotein; HBSS, Hanks' balanced salt solution; EGTA, ethylenebis[(oxyethylenenitrilo)]tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; CHAPS, 3-(3-cholamidopropyl) dimethyamine)-1-propanesulfonate; 8-Br-cAMP, 8-bromo-cAMP.

of the synthesis of cholesterol through the inhibition of the microsomal enzyme HMG-CoA reductase (4, 12–18).

In the present study we report on the effect of TSH on cholesterol biosynthesis and on the level of the rate-limiting enzyme HMG-CoA reductase.

EXPERIMENTAL PROCEDURES

Materials—TSH (26 ± units/mg) was purified from bovine pituitary extracts (19, 20). Insulin, MOPS, B-nicotinamide adenin dinucleotide phosphate (NADP), crystalline bovine serum albumin, salmon sperm DNA, 2-mercaptoethanol, polyvinylpirrolidone, Nonidet P-40, spermidine, and cycloheximide were obtained from Sigma. Actinomycin D was from Behring Diagnostics; agarose, phenol, proteinase K were purchased from Bethesda Research Laboratories. Ribonucleotide triphosphates, DNase I and pBR322 were obtained from Pharmacia LKB Biotechnology, Inc.; [α-^{32}P]dCTP (3000 Ci/mM), [α-^{32}P]UTP (800 Ci/mM), [^{3}H]thymidine (10 Ci/mM), and 2-[^{14}C]acetic acid (sodium salt, 53 mCi/mM), 26-[^{14}C]cholesterol (59 mCi/mmol), [^{14}C]3-hydroxy-3-methylglutaryl coenzyme A (55.5 mCi/mmol), and [^{3}H]mevalonic acid (2 Ci/mmol) were obtained from Du Pont-New England Nuclear. BA-85 nitrocellulose filters were purchased from Schleicher & Schuell.

Cells—FRTL-5 cells (ATCC No CRL-8305) are a strain of rat thyroid cells derived from a single cell subcultured from FRTL cells (7) which had been shifted from a medium containing 0.5% to one containing 5% bovine serum (7, 8). Unlike parental FRTL cells, this strain is viable for long periods of time in the absence of TSH (6, 9). In this condition the cells do not proliferate but can resume function and growth when re-exposed to TSH. Cells in the present study were a fresh subclone (F1) with all properties previously detailed (6, 9). They were routinely grown at 37 °C in a humidified atmosphere composed of 95% air and 5% CO_2, in Coon's modified F-12 medium supplemented with 5% calf serum and a mixture of six hormones and growth factors including: TSH (1×10^{-9} M), insulin (10 μg/ml), hydrocortisone (0.4 ng/ml), human transferrin (5 μg/ml), somatostatin (10 ng/ml) and glycil-L-histidyl-L-lysine acetate (2 ng/ml) (7, 8, 21, 22). The medium containing the complete mixture will be referred to as 6H. Depending on experimental needs, a similar mixture, but lacking TSH (referred to as 5H) was used. Also, as needed, the calf serum concentration was reduced to 0.2% during 48 h preceding the experiment. Unless otherwise stated cells were provided fresh medium every 3 days.

[^{3}H]Thymidine Incorporation into DNA—FRTL-5 cells were seeded at a density of 1×10^5/60-mm dish in 6H medium containing 5% calf serum. After 3 days, cells were washed with 3 ml of Hanks' balanced salt solution (HBSS) before fresh medium containing 5% calf serum but lacking TSH (5H medium) was added. After 5 more days, cells were again washed twice with HBSS before fresh 5H medium containing only 0.2% serum was added. Forty-eight h later (time 0), the experiment was started by replacing the 5H/0.2% serum medium (no TSH) with 3 ml of 6H, 0.2% medium (1×10^{-9} M TSH present) in a staggered fashion such that cells would be exposed to the latter medium for the noted time. During the last 2 h, 1×10^6 cpm [^{3}H]thymidine was added to each dish. The incubation was terminated by washing the cells twice with ice-cold HBSS before the addition of 3 ml of ice-cold 10% trichloroacetic acid. After 10 min over ice, the supernatant was carefully aspirated, 1 ml of diphenylamine solution was added, and DNA content as well as radioactivity were measured as previously reported (21, 22).

Total RNA Preparation and Slot-blot Analysis—Total RNA was isolated by the guanidinium thiocyanate/CsCl method (23). Total RNA slot-blots were performed using a Schleicher & Schuell Minifold II apparatus following the manufacturer's recommendations. RNA samples were mixed with three volumes of a denaturing solution (50% formamide, 2.2 M formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 0.5 mM EDTA), heated 5 min at 65 °C and diluted 2-fold in 19 × SSC (where SSC is 150 mM NaCl, 15 mM sodium citrate) (24). The samples were then applied to a BA-85 nitrocellulose membrane equilibrated in 10 × SSC. The blots were baked at 80 °C for 2 h under vacuum. Prehybridization (5 h at 42 °C), hybridization (18 h at 42 °C), and high stringency washes (15 min × 2 at 37 °C, 1 h at 60 °C, and 1 h at 55 °C) of the RNA blots were carried out as previously described (24).

Northern Blot Analysis—For Northern blot analyses, poly(A)$^+$ RNA was prepared by affinity chromatography on oligo(dT)-Sepharose columns from nonconfluent FRTL-5 cells maintained for 5 days in 5H, 5% serum medium (no TSH) and for 2 additional days in 5H, 0.2% serum medium, then either not exposed or treated with 1×10^{-9} M TSH for 12 h. Poly(A)$^+$-enriched RNA was fractionated on a 1% formaldehyde-agarose gel in 20 mM MOPS and transferred to Nytran membranes (Schleicher & Schuell) in 10 × SSC and 10 mM EDTA, pH 7.4, as described previously (25). After baking the filters at 80 °C for 1 h, they were prehybridized for 5 h in 50% formamide, 5 × SSPE (where SSPE is 150 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA), 5 × Denhardt solution (26), 200 μg/ml sonicated salmon sperm DNA, and 0.1% sodium dodecyl sulfate. Hybridization was carried out for 18 h at 42 °C in the prehybridization solution to which 2–5 × 10^6 cpm α^{32}P-labeled, nick-translated, purified probe (1–2 × 10^8 cpm/μg DNA) was added. The filters were washed as described above. A RNA ladder was included in each experiment as a size marker.

Filters were exposed to Kodak XAR-5 film (Eastman) with Du Pont Cronex intensifying screens (Du Pont) at −70 °C. Quantitative analysis of the autoradiograms was performed by laser densitometry on a LKB 2202 instrument.

Radiolabeled cDNA Probe Preparation—The full-length hamster HMG-CoA reductase cDNA was prepared by digesting the plasmid pRED 227 (27) (supplied by the American Type Culture Collection, Rockville, MD) with *Xba*I. Rat β-actin cDNA (28) (kindly provided by Dr. B. Paterson, National Cancer Institute, National Institutes of Health) and rat thyroglobulin cDNA (29) (kindly provided by Dr. R. Di Lauro, University of Udine, Udine, Italy) were used as negative and positive reference probes, respectively. Labeling of cDNAs was accomplished using a nick translation kit purchased from Bethesda Research Laboratories and [α-^{32}P]dCTP.

Transcriptional Activity in Isolated Nuclei—FRTL-5 cells were lysed at 40 °C in 140 mM NaCl, 1.5 mM MgCl, 10 mM Tris-HCl at pH 8.0, and 0.5% Nonidet P-40 (30). The *in vitro* nuclear run on transcription assay was performed as described by McKnight and Palmiter (31). Briefly, cell nuclei were isolated from either FRTL-5 cells maintained in 5H, 0.2% serum medium (without TSH), or cells exposed for 12 h to 6H, 0.2% medium (with TSH) and incubated with 25 μCi of [^{32}P]UTP for 30 min, at 24 °C. Nuclear RNA from cells challenged with TSH yielded nearly twice as many counts per minute as those yielded by nuclear RNA from TSH starved cells. RNA was purified, denatured, and hybridized to an excess of non-radioactive cDNA probes for HMG-CoA reductase, β-actin, and thyroglobulin. To rule out nonspecific hybridization, the plasmid PBR 322 was also added to the cDNA probes immobilized onto Nytran filters. Equal amounts of radioactivity (5×10^6 cpm in 3 ml of hybridization buffer) were added to each hybridization reactions. The blots were not treated with ribonuclease during the washes, which were performed in five changes, 45 min each, of a solution of 0.2 × SSC and 0.1% sodium dodecyl sulfate at 65 °C. Filter autoradiography and quantitative analysis were performed as described above.

Assay of HMG-CoA Reductase Activity—Non-confluent FRTL-5 cells in 5H, 0.2% serum medium were exposed to 1×10^{-9} M TSH in 100-mm dishes, for the noted times. Medium was aspirated, cells were washed with ice-cold HBSS scraped with a rubber policeman, collected in 3 ml of HBSS, and pelletted by centrifugation for 5 min at 1,000 rpm (Beckman J-6 centrifuge). Cell pellets in each time group were suspended in 100 μl of a cold lysis buffer containing 50 mM imidazole-HCl, pH 7.4, 100 mM sucrose, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM dithiothreitol, 50 μM leupeptin, and 15 mM CHAPS. The cell suspensions were sonicated with a microtip (Sonifier Cell Disruptor, model W 185) at the output setting of 2 for 10 s, at 4 °C, and stored frozen at −20 °C. Samples of each cell extract were thawed and centrifuged for 2 min at 13,000 rpm in a microcentrifuge (Fisher, model 235B). The supernatant was used for the determination of HMG-CoA reductase activity and protein content. Aliquots (60 μl) of each sample were preincubated for 30 min at 37 °C in a medium containing 150 mM potassium phosphate, pH 6.9, 20 mM KCl, 5 μM DTT, and 5 mg/ml bovine serum albumin. At the end of preincubation DL-HMG-3-[^{14}C]CoA (122 cpm/pmol) and [^{3}H]mevalonic acid (450,000 cpm) were added. Incubation was continued for 20 min at 37 °C and stopped by the addition of 50 μl of 10 N HCl. The tubes were centrifuged and an aliquot of each supernatant was applied to a column (1 ml) of Bio-Rex 5 (32); [^{14}C]mevalonate, containing [^{3}H]mevalonate as an internal standard, was eluted with 2 ml of water and radioassayed after addition of 10 ml of Ecolume (ICN). All assays were done in triplicate utilizing three individual dishes for each experiment. The variation of HMG-CoA reductase activity among triplicates was less than 7%. Protein was determined by the method of Bradford (33) using bovine serum albumin as the standard.

Measurement of the Rate of Incorporation of [^{14}C]acetate into Lipids—Non-confluent cells maintained in 5H medium supplemented with either 5% or 0.2% calf serum, as described above, and treated with either TSH (1×10^{-9} M) or 8-Br-cAMP (1×10^{-3} M) for the noted times, were incubated, during the last 2 h of the experimental treatment, with 1 μCi/ml of 2-[^{14}C]acetic acid (sodium salt). At the end of the incubation period, medium was aspirated, cells were washed with ice-cold HBSS, collected in 3 ml of HBSS, and pelleted as described for HMG-CoA reductase assay. Pellets were suspended in 1 ml of 2-propanol, sonicated at 4 °C with five bursts of 10 s each, and recentrifuged for 15 min at 2500 rpm (Beckman J6 centrifuge). The supernatant was aspirated and the pellet re-extracted as above. Both extracts were combined, dried under a stream of N_2, and dissolved in 0.5–1 ml of chloroform. Aliquots of 10 μl were applied to Silica Gel-60 thin layer chromatographic plates (E. Merck, Darmstad, West Germany), and developed in hexane/diethylether/acetic (70:30:1). This solvent system, in which phospholipids remain at the origin, provides excellent separation of free fatty acids, free cholesterol, triglycerides, and cholesterol esters (34). Each lipid fraction was located by comparison to high purity standards, scraped off the plate, and radioassayed in 10 ml of Ecolume (ICN). The incorporation of [^{14}C]acetate into cholesterol was essentially unaffected when the lipid extracts were saponified in alcoholic KOH before chromatography. This confirmed that only a negligible amount of label was incorporated in the cholesterol part of the cholesterol ester fraction, therefore, the analysis, in subsequent experiments, was limited to the non-esterified cholesterol fraction.

Measurements of Cholesterol Excretion—The efflux of newly synthesized cholesterol was measured from cells exposed to TSH for 14 h and pulsed, during the last 2 h, with 1 μCi/ml of 2-[^{14}C]acetic acid (sodium salt). The efflux of exogenously added 26-[^{14}C]cholesterol was measured from cells exposed to 0.4 μCi/ml of the tracer for 8 h, at 37 °C, while maintained in 5H, 0.2% calf serum medium (without TSH). Following the radiolabeling procedure the incubation media were removed, and the cells were washed three times with HBSS. Triplicate dishes, for each set, were used to measure total cell [^{14}C] cholesterol in 2-propanol extracts, as described above. Cells pulse labeled with 2-[^{14}C]acetic acid received 3 ml of 6H, 0.2% calf serum medium; cells radiolabeled with 26-[^{14}C]cholesterol were divided in two sets, one receiving 3 ml of 5H, 0.2% calf serum medium and the other 3 ml of 6H, 0.2% calf serum medium. Cells were maintained at 37 °C, in 5% CO_2, 95% air. After the indicated times efflux media were collected, and each dish was rinsed once with 3 ml of HBSS which was combined with the respective efflux medium. The cells were processed for lipid extraction in 2-propanol as described above. Efflux media were centrifuged for 10 min at $800 \times g$ to separate loose cells and transferred to stoppered conical tubes for lipid extraction according to established procedures (35). The lipid extracts were dried under a stream of air, dissolved in 1 ml of $CHCl_3$ and analyzed by thin layer chromatography as described above.

Measurement of Cholesterol—Total cholesterol was determined in 2-propanol extracts using a coupled enzyme assay with cholesterol oxidase, cholesterol esterase, peroxidase, 2-hydroxy-3-5-dichloro-benzenesulfonic acid, and 4-aminoantipyrine according to published procedures (35, 36). Values were normalized to cell protein measured (33) on the 2-propanol-insoluble pellet, dissolved in 0.1 N KOH.

RESULTS

TSH Effect on Cholesterol Synthesis and Cellular Cholesterol Level—In a typical experiment, nonconfluent FRTL-5 cells were maintained for 5 days in 5H, 5% calf serum medium (no TSH). During the subsequent 48 h cells were divided in two groups: one group was fed with the same medium, and the other with 5H medium containing only 0.2% serum. Following TSH addition (final concentration 1×10^{-9} M) to both sets of cells, the rate of [^{14}C]acetate incorporation into cholesterol began to increase after 4 h; it reached a maximum after 14 h and then returned to basal level after 72 h (Fig. 1*A*). The effect of TSH was markedly different, depending on the concentration of serum in the culture medium. In 0.2% serum, the basal level (*i.e.* in the absence of TSH) of [^{14}C]cholesterol/mg cell protein was nearly double the level in 5% serum. Although the time course of the TSH effect on the rate of cholesterol synthesis was similar at both serum concentrations, the magnitude of the effect was quite different. Thus, 14 h after TSH addition, when the effect of TSH was maximal, the rate of cholesterol synthesis was 8- and 4-fold over basal in 0.2 and 5% serum, respectively (Fig. 1*A*).

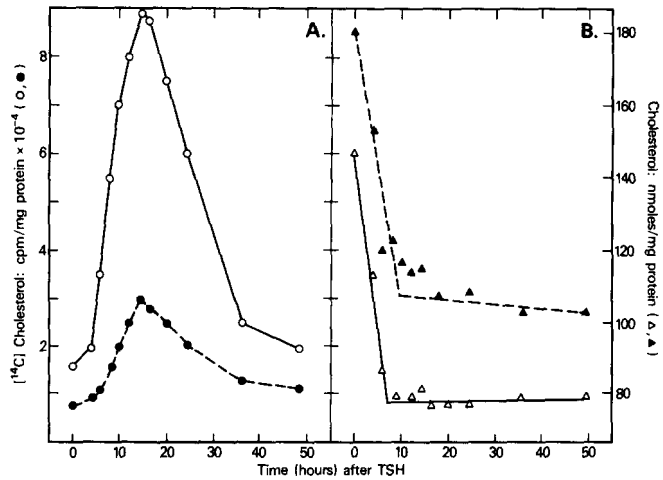

FIG. 1. **Time course of the effect of TSH on cholesterol synthesis (*A*) and total cell cholesterol (*B*) in FRTL-5 cells.** Non-confluent cells in 100-mm dishes were maintained in 5H (no TSH) medium with 5% calf serum for 5 days; 48 h before starting the experiment two separate sets of cells were fed either the same 5H, 5% serum medium (● in *panel A*, and ▲ in *panel B*) or 5H, 0.2% serum medium (○ in *panel A*, and △ in *panel B*). At the start of each experiment, groups of four dishes, for each serum concentration, had their medium replaced with the respective medium containing 1×10^{-9} M TSH. Further feeding with TSH containing media was continued in a staggered fashion, such that each set of cells would be exposed to the hormone for the noted length of time before harvest. Fresh medium, with or without TSH, was fed every 12 h. Two h before the end of the incubation 1 μCi/ml medium of [^{14}C]acetic acid (sodium salt) was added to each dish. At the end of the incubation time, medium was aspirated, the cells were rinsed with ice-cold HBSS, scraped off the dish with a rubber policeman, collected, and centrifuged in plastic tubes (Falcon 2059). Lipid extraction of the cell pellets, analysis by thin layer chromatography, and quantitative measurements, were performed as detailed under "Experimental Procedures." Chemical determination of cholesterol (*panel B*) was performed on the lipid extracts from cells in 5% (▲) or 0.2% (△) serum, as detailed under "Experimental Procedures," assuming that the extracted cholesterol accounted for 95% of the total cell content (35). Data are representative of at least five different determinations in which the variation was less than 10%.

While the rate of *de novo* cholesterol synthesis was transiently increased by TSH, the total cholesterol per cell protein was reduced by nearly 40% at 6 h after TSH, with a further decrease of about 10% through the remainder of the 72 h period investigated (Fig. 1*B*). The relative changes in total cholesterol were very similar at both serum concentrations. The changes could be attributed almost entirely to an absolute decrease of cholesterol since total cell protein did not change significantly, 6 h after TSH challenge. The decrease of total cellular cholesterol could be accounted for, at least in part, by the down-regulation of LDL receptor, which occurs in FRTL-5 upon exposure to TSH (11). However, the concomitant increase in the rate of cholesterol synthesis raised two questions: 1) what was the fate of the lost cholesterol? and, 2) was the contribution of newly synthesized cholesterol, to the free cholesterol pool, quantitatively important? These questions were addressed by evaluating the effect of TSH on the excretion of newly synthesized (endogenous) as well as exogenously added cholesterol. Newly synthesized cholesterol was pulse labeled by incubating cells, exposed to TSH for 14 h (the time required for the rate of cholesterol synthesis to reach its maximum, see Fig. 1), with [^{14}C]acetate during the last 2 h of

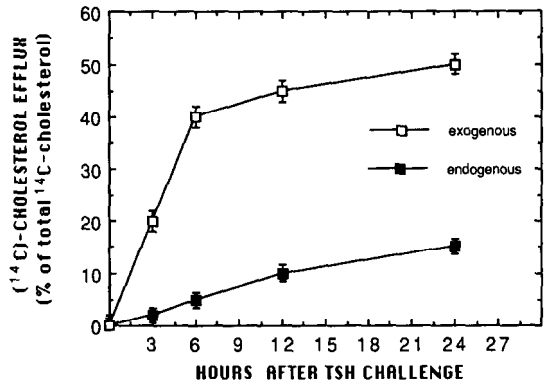

FIG. 2. **TSH stimulated efflux of newly synthesized (endogenous) and added (exogenous) [^{14}C]cholesterol from FRTL-5 cells.** Efflux of newly synthesized cholesterol (■) was measured in non-confluent cells and pulse labeled with 1 μCi/ml of 2-[^{14}C]acetic acid (sodium salt) during the last 2 h of a 14-h exposure to medium containing 1×10^9, after the TSH starvation procedure described in the legend to Fig. 1. Efflux of exogenously added cholesterol (□) was measured in non-confluent cells exposed to 0.4 μCi/ml of 26-[^{14}C] cholesterol for 8 h, at 37 °C, while in 5H, 0.2% calf serum medium (without TSH) as detailed in the methods. Data represent mean ± S.D. of values for medium [^{14}C]cholesterol from three different experiments, expressed as percent of the cellular free [^{14}C]cholesterol at each indicated time point. The values for the efflux of exogenous [^{14}C]cholesterol are corrected for the corresponding values measured from cells exposed to medium lacking TSH.

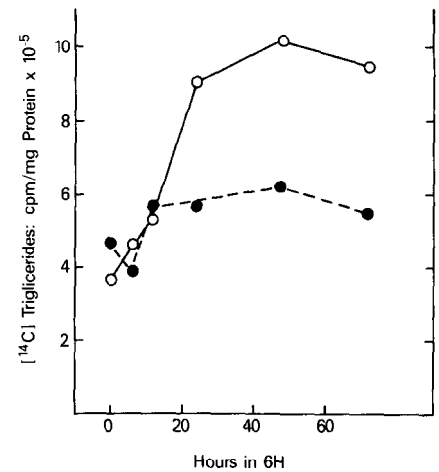

FIG. 3. **Time course of TSH promoted triglyceride synthesis in FRTL-5 cells.** Non-confluent cells were processed as described in the legend to Fig. 1. TSH (1×10^{-9} M) addition was carried out in a staggered fashion such that cells would be exposed to the hormone for the noted time (h in 6H). The lipid extracts used for cholesterol analysis, as detailed in Fig. 1, were used for measurements of [^{14}C] acetate incorporation into triglycerides, which were identified by thin layer chromatography, and quantitated as described under "Experimental Procedures." Lipid extracts were from cells in either 5% (●) or 0.2% (○) serum. *Data are representative of at least five determinations in which the variation was less than 10%.*

TSH treatment. Residual cell cholesterol, and cholesterol efflux, were measured during the subsequent 24 h. The rates of excretion of exogenous and newly synthesized cholesterol were very different during the first 6 h, but very similar thereafter. Thus, as shown in Fig. 2, only about 5% of newly synthesized cholesterol was lost in 6 h, whereas TSH caused a 40% loss of cell cholesterol radiolabeled from an exogenous source of [^{14}C]cholesterol. During the remainder of the 24 h examined, an additional 10% of total cell [^{14}C]cholesterol was excreted in both cases. The ability of FRTL-5 cells to dispose of cholesterol, even when exposed to low serum medium, is not surprising since these cells produce and secrete thyroglobulin (6–9) which is an efficient cholesterol carrier (38).[2] Since the loss of newly synthesized cholesterol from TSH-challenged FRTL-5 cells is modest, relative to the loss of total cell cholesterol, it appears that the contribution of TSH-induced cholesterol synthesis, to the free cholesterol pool in FRTL-5 cells, is negligible.

Synthesis of Triglycerides—The extent and the time course of the effect of TSH on cholesterol synthesis in FRTL-5 cells are not general features common to other lipids. In fact, analysis of triglycerides, in the same lipid extracts used for [^{14}C]cholesterol measurements, revealed that, in cells exposed to 0.2% serum, the rate of [^{14}C]acetate incorporation increased almost linearly for 24 h after TSH addition; it reached up to 2.5-fold the basal value and did not change significantly for the remaining 72 h examined (Fig. 3). In cells exposed to 5% serum the rate of triglyceride synthesis increased only about 25% 12 h after TSH addition and did not change significantly thereafter.

Effect of TSH on HMG-CoA Reductase Activity—The effect of 5% serum, on TSH-induced cholesterol synthesis, reflects feedback inhibition of exogenous cholesterol on HMG-CoA reductase, the rate-limiting enzyme of cholesterol biosynthesis (see below). Thus, it was possible that the effect of TSH was exerted through the induction of HMG-CoA reductase activity. This was tested in cells maintained under conditions identical to those described for [^{14}C]acetate incorporation experiments. Following TSH challenge of cells in 0.2% serum, HMG-CoA reductase activity increased measurably after 4 h; it reached a maximum of more than 6-fold the control level after 14 h, then decreased to a value nearly double of the control after 48 h (Fig. 4), in excellent agreement with the kinetics of TSH-promoted cholesterol synthesis (Fig. 1*A*). When a similar experiment was performed on FRTL-5 cells exposed to medium supplemented with 5% calf serum, no effect of TSH on HMG-CoA reductase activity could be measured 4 h after challenge, and only a 2-fold increase was detected after 14 h, which persisted after 48 h.

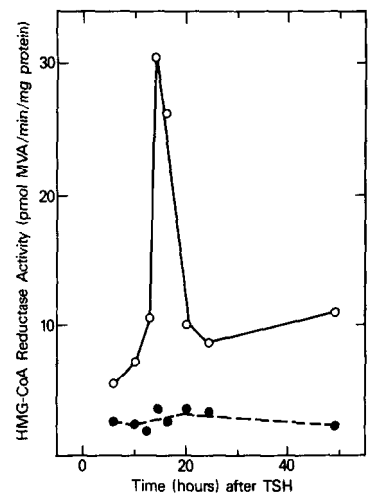

FIG. 4. **Time course of the effect of TSH on HMG-CoA reductase activity in FRTL-5 cells.** Non-confluent cells were maintained for 5 days in 5H medium (no TSH) with 5% serum, and for 2 additional days in 5H medium with 0.2% serum. At the start of the experiment cells were divided in two sets; one set was treated with 1×10^{-9} M TSH (○) in a staggered fashion, such that cells would be exposed to the hormone at the noted time, and the other set received medium without TSH (●). Fresh medium, with or without TSH, was fed every 12 h. The HMG-CoA reductase assay and calculation of activity were performed as detailed under "Experimental Procedures."

[2] M. Bifulco and S. M. Aloj, unpublished observations.

HMG-CoA Reductase mRNA Level Is Increased by TSH—Elevation of HMG-CoA reductase activity is the result of increased protein, caused either by decreasing its degradation or increasing its synthesis. We tested the ability of TSH to increase the level of HMG-CoA reductase messenger RNA, taking advantage of the availability of the full-length hamster reductase cDNA in the plasmid pRed-227 (27).

Cells were maintained under conditions identical to those described for [^{14}C]acetate incorporation experiments (see above). Following TSH challenge, total RNA was isolated at the noted time intervals (Fig. 5), and HMG-CoA reductase mRNA was measured by slot-blot hybridization to a ^{32}P-labeled *Xba*I restriction fragment of the plasmid pRed-227. Changes of HMG-CoA reductase mRNA level have a time course which is nearly superimposable on the time course of TSH-induced cholesterol synthesis and reductase activity. The highest level of HMG-CoA reductase mRNA was detected 12 h after TSH challenge (2 h before peak reductase activity), when it was more than 8-fold the basal; by 24 h mRNA level was decreased to control level. The level of β-actin mRNA, assayed on the same blots, did not change significantly throughout the 72 h examined (Fig. 5). It should be pointed out that the magnitude and the kinetics of the effect of TSH on HMG-CoA reductase mRNA and enzyme activity were identical if the cells were exposed to TSH only for 4 h rather than throughout the entire period (72 h) examined (data not shown). To prove that the effect of TSH on the level of HMG-CoA reductase mRNA was not the result of a cross-reaction of the labeled probe with another TSH induced mRNA, and to check if, and to what extent, the mRNA had been degraded, poly(A)$^+$-enriched RNA was prepared from control cells and from cells exposed to TSH for 12 h, and analyzed by Northern blot after fractionation by electrophoresis on formaldehyde-agarose gel. Fig. 6 shows that poly(A)$^+$-rich RNA from TSH-treated cells, when hybridized to the labeled HMG-CoA reductase cDNA, gives rise to a band whose intensity is 8–9-fold higher than that generated by poly(A)$^+$-rich RNA from TSH-starved cells. In both cases, only one mRNA species could be identified which migrates very close to the 28 S marker, suggesting that the size of HMG-CoA reductase mRNA in FRTL-5 cells is about 5 kilobases.

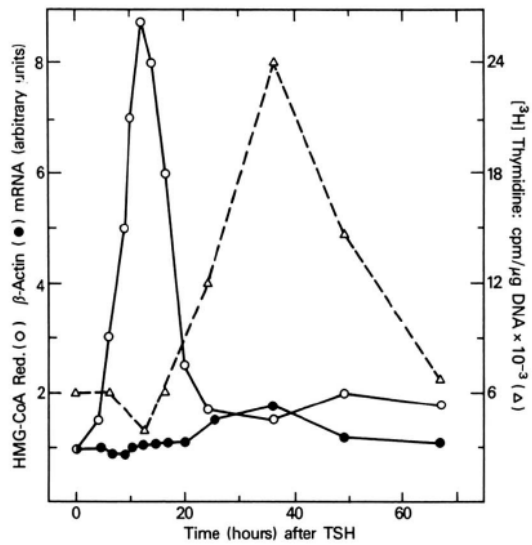

FIG. 5. **Time course of the TSH promoted changes of HMG-CoA reductase and β-actin messenger RNAs and [^{3}H]thymidine incorporation into DNA in FRTL-5 cells.** Non-confluent cells were maintained for 5 days in 5H medium (no TSH) with 5% serum, and for 2 additional days in 5H medium with 0.2% serum. At the start of each experiment, groups of five dishes had their medium changed with either 5H, 0.2% serum medium or this medium containing 1×10^{-9} M TSH. Further feeding with TSH was continued in a staggered fashion such that cells would be exposed the hormone for the noted time. Fresh medium, with or without TSH, was fed every 12 h. Total RNA was extracted, brought to identical concentration based on optical density at 260 nm, denatured, and blotted onto nitrocellulose filters as a series of 2-fold dilutions, starting at 5 μg of RNA, and hybridized to ^{32}P-labeled HMG-CoA reductase (○) or β-actin (●) probes, as described under "Experimental Procedures." Filters were exposed to x-ray film and mRNAs quantitated by densitometry in the linear range of the analysis. The densitometric values of RNA from cells not exposed to TSH were set arbitrarily at 1; all other values are relative to this. Replica plates were used to measure [^{3}H]thymidine incorporation into DNA, as described under "Experimental Procedures." Values for [^{3}H]thymidine incorporation into DNA (△) are normalized to DNA content which did not change significantly for 24 h after TSH challenge. Data are representative of at least three experiments in which differences were within 10%.

When FRTL-5 cells are exposed to TSH, DNA synthesis and cell duplication resume after a lag period which varies between 24–48 h, depending on the duration of TSH starvation, as well as the composition of the culture medium, *i.e.* the presence or absence of insulin and 5% serum (6–10). When the same FRTL-5 cell population used for the HMG-CoA reductase mRNA experiment was tested for the ability of TSH to activate DNA synthesis, after identical TSH starvation conditions (5 days in 5H, 5% serum medium and 2 additional days in 5H, 0.2% serum), the rate of [^{3}H]thymidine incorporation into DNA, measured with a 2-h pulse, increased 24 h after TSH challenge, was maximal after 36 h, and returned to basal level after 72 h (Fig. 5). Thus, a comparison of the time-dependent effects of TSH on HMG-CoA reductase mRNA and [^{3}H]thymidine incorporation into DNA indicates that induction of HMG-CoA reductase transcripts occurs before quiescent FRTL-5 cells enter the S phase.

TSH Elevates the Rate of Transcription in vitro of the HMG-CoA Reductase Gene—Factors which modify the level of mRNAs can change the rate of gene transcription, the rate of mRNA processing or degradation, or all of these. To assess whether the effect of TSH on HMG-CoA reductase mRNA in FRTL-5 cells was exerted at the transcriptional level, we performed a "run-on" assay on nuclei isolated 12 h after challenging the cells with TSH. After nuclei were incubated in the presence of [^{32}P]UTP for 30 min at 27 °C, ^{32}P-labeled nuclear RNA was isolated, denatured, and hybridized to an excess of several cDNA probes including HMG-CoA reductase, β-actin, thyroglobulin, and the plasmid pBR322, all immobilized on nylon filters. The β-actin and thyroglobulin probes were used as negative and positive control, respec-

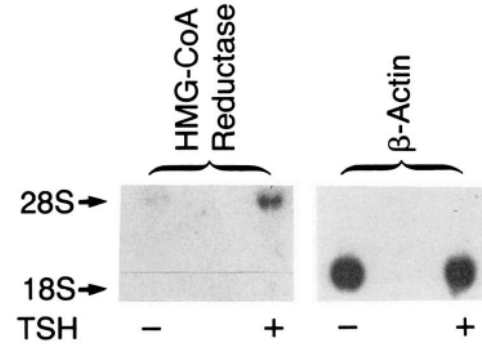

FIG. 6. **Northern blot analysis of FRTL-5 cells poly(A)$^+$-rich RNA.** Poly(A)$^+$-rich RNA was prepared from cells maintained for 5 days in 5H (no TSH) medium with 5% serum, and for 2 additional days in 5H medium with 0.2% serum, then exposed for 12 h to either the 5H, 0.2% serum medium (−TSH) or to this medium containing 1×10^{-9} M TSH (+TSH). Northern blot analysis was performed as described under "Experimental Procedures."

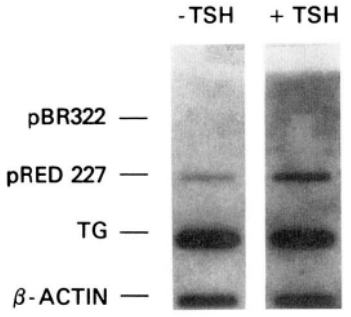

FIG. 7. **Effect of TSH on the rate of transcription of the HMG-CoA reductase gene in FRTL-5 cells.** Treatment of cells with 5H medium and with 5 and 0.2% serum was identical to that described in Figs. 3–5. Cell nuclei, isolated either from cells maintained in 5H, 0.2% calf serum (without TSH) or from cells exposed for 12 h to 1×10^{-9} M TSH, were incubated in the presence of 25 μCi of [^{32}P]UTP for 30 min at 24 °C (31). Nuclear RNAs were then purified, denatured, and hybridized (5×10^6 cpm in 3 ml of hybridization medium in both cases) with an excess of the cDNA probes indicated, and immobilized in Nytran filters. In order for the nuclear RNA from cells not exposed to TSH to show a clear band hybridizing with the HMG-CoA reductase probe (*pRED 227*), the filters were overexposed. This caused the thyroglobulin (*TG*) and β-actin hybridization band to become oversaturated. Densitometric comparison of +TSH and −TSH lanes showed that the intensity of the HMG-CoA reductase band was increased about 7-fold by TSH. The data are representative of at least three different experiments yielding essentially identical results.

tively, for the effect of TSH on transcriptional activity in FRTL-5 cells. The results of a typical run-on assay are reported in Fig. 7. From the densitometric intensities of the hybridization bands, within the linear range of analysis, it could be estimated that 12 h after TSH challenge the rate of HMG-CoA reductase transcription was increased 6–7-fold. The signals generated by β-actin and thyroglobulin cDNAs were much stronger than that of the HMG-CoA reductase cDNA; thus, in Fig. 6 they appear above saturation. However, at shorter exposure times, when the reductase signal was poorly detectable, it could be observed that the effect of TSH on β-actin transcription was modest, whereas it increased thyroglobulin transcription rate about 3-fold (37). As expected, there was no detectable hybridization with pBR322.

Effect of Cycloheximide and Actinomycin D on the TSH Promoted Induction of HMG-CoA Reductase Gene Expression—When FRTL-5 cells were treated with either the translation inhibitor cycloheximide (1 μg/ml) or the transcription inhibitor actinomycin D (0.5 μg/ml) 30 min before TSH challenge, the induction of HMG-CoA reductase mRNA was abolished (Table I). Both drugs had little effect on the level of mRNA from cells which were not exposed to TSH, whereas cycloheximide reduced significantly the level of thyroglobulin mRNA in both TSH-treated and -untreated FRTL-5 cells. The level of β-actin mRNA was not affected significantly by cycloheximide both in the absence or in the presence of TSH, whereas it was reduced by half by actinomycin D in TSH-treated cells. It is clear from the data of Table I that for TSH to induce HMG-CoA reductase mRNA protein synthesis should be preserved.

The Effect of TSH on HMG-CoA Reductase Gene Expression Is a cAMP-mediated Signal—TSH is a powerful inducer of cAMP production in FRTL-5 cells (7, 9, 21). It has been reported that cAMP also mediates TSH induction of malic enzyme in these cells (24, 53), therefore we tested whether the cyclic nucleotide was also involved in the effect of TSH on cholesterol synthesis and on HMG-CoA reductase gene expression. As shown in Fig. 8, when 8-Br-cAMP was used in

TABLE I

Effect of cycloheximide (Cyclo.) or actinomycin D (Act. D) on the level of HMG-CoA reductase, thyroglobulin, and β-actin messenger RNAs (arbitrary units) in FRTL-5 cells in the absence or in the presence of TSH

Non-confluent FRTL-5 cells were kept in 5H medium (no TSH), 5% serum for 5 days and for 2 additional days in 5H medium, 0.2% serum. At the start of the experiment medium was changed in separate sets of dishes with either the same 5H, 0.2% serum medium or with the same medium containing either 2 μg/ml cycloheximide or 0.5 μg/ml actinomycin D. Thirty min later TSH (1×10^{-9} M) was added, where noted, and the incubation was continued at 37 °C, in humidified 95% air, 5% CO_2, for 8 h after TSH addition. Total RNA extraction and analysis of mRNA were performed as described under "Experimental Procedures."

	Control	Cyclo.	Act. D	TSH	TSH + Cyclo.	TSH + Act. D
HMG-CoA reductase	1	0.8	1.1	5.0	1.1	1.4
Thyroglobulin	1	0.3	1.1	2.2	0.3	0.9
β-Actin	1	1.1	0.8	0.9	0.9	0.5

experiments similar to those described above, HMG-CoA reductase mRNA and enzyme activity (Fig. 8*A*) and [^{14}C] acetate incorporation into cholesterol (Fig. 8*B*) were similarly affected. The time course of the effect of 8-Br-cAMP was very similar to the time course of the effect of TSH, and also showed a transient effect; however, the maximum for all three parameters investigated was at 16 h rather than at 12–14 h (see Figs. 1, 4, and 5 for comparison). We cannot explain this difference other than speculating that it may reflect, at least in part, the time required for the cyclic nucleotide derivative to enter the cell and reach the active concentration. As was the case for TSH, 8-Br-cAMP did not affect significantly the level of β-actin mRNA (data not shown).

DISCUSSION

This study shows that TSH, a specific mitogen and physiological regulator of thyroid function, induces cholesterol synthesis and HMG-CoA reductase activity in FRTL-5 cells. These cells prove to be an excellent system to study cholesterol synthesis and its relationship to DNA synthesis and cell proliferation, since their growth depends on, and can be regulated by the presence of TSH in the culture medium (6–10). Previous attempts to investigate such relationships have used phytohemagglutinin-challenged mouse lymphocytes (1), hamster kidney cells released from double thymidine block (41), mouse fibroblasts, and smooth muscle cells stimulated by platelet-derived growth factor (PDGF) (42). These studies have established a temporal sequence of sterol synthesis and the transition from a quiescent to a proliferating cell population. TSH withdrawal and readdition is a means to control proliferation in FRTL-5 cells. However, when TSH is added to TSH starved, quiescent cells, HMG-CoA reductase messenger RNA, enzyme activity, and [^{14}C]acetate incorporation into cholesterol increase transiently and with identical kinetics. The increase is maximal when the rate of thymidine incorporation into DNA is negligible and disappears when thymidine incorporation into DNA is maximal. These findings show that induction of cholesterol biosynthesis occurs when FRTL-5 cells move from G_0 into G_1 and before the S phase. These data could be construed as to indicate that expression of HMG-CoA reductase is regulated by the cell cycle, they can also mean that the increase of sterol synthesis causes cell cycling. It may well be that these results are only seen when cells in G_0 are stimulated to resume cycling but is not sustained when cells maintain continuous division under optimal growth conditions. Indeed, recent elegant studies of

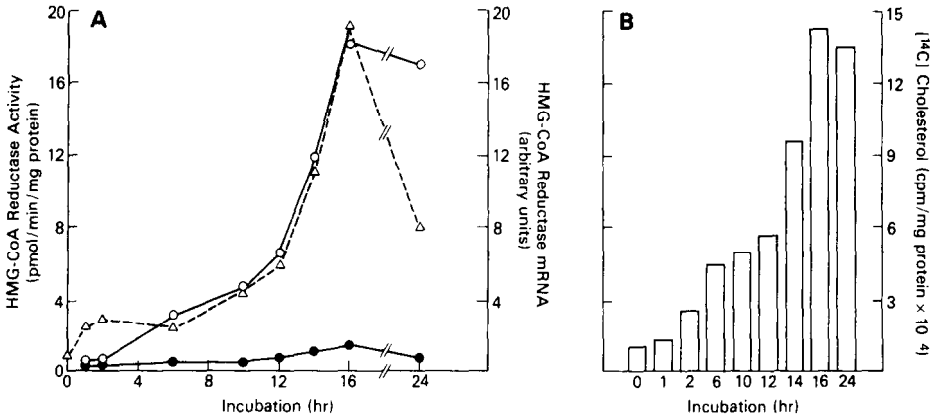

FIG. 8. **Time course of the effect of 8-Br-cAMP on HMG-CoA reductase messenger RNA level and enzyme activity (*A*) and on cholesterol synthesis (*B*) in FRTL-5 cells.** Treatment of cells with 5H medium with 5 and 0.2% serum was identical to that described in Figs. 3–6. At the start of the experiment separate sets of cells were fed with either the 5H, 0.2% serum medium or the same medium containing 1×10^{-3} M 8-Br-cAMP. Treatment with 8-Br-cAMP was continued in a staggered fashion such that cells would be exposed to the cyclic nucleotide derivative for the noted time. Fresh medium, with or without 8-Br-cAMP, was fed every 12 h. HMG-CoA reductase mRNA (△) and enzyme activity (○ + 8-Br-cAMP, ● control, *panel A*) were measured as detailed under "Experimental Procedures." Replicate plates were used for [^{14}C]acetate incorporation into cholesterol (*panel B*), as detailed in the legend to Fig. 1 and under "Experimental Procedures." Data are representative of two separate experiments yielding identical results.

HMG-CoA reductase activity, in very different cell systems undergoing exponential growth, have failed to show that increases of such activity are confined to a specific phase of the cell cycle (43). Thus, it could be speculated that isoprenoid synthesis has to overcome some kind of activation threshold which is much higher in cells traversing from quiescence to proliferation than in continuously dividing cells.

The behavior of FRTL-5 cells, with respect to cholesterol metabolism is unique. One would expect that cells preparing for mitosis would require an adequate supply of sterols to synthesize additional cell membrane. Hence, under mitogenic stimulation, both routes of sterol supply, *i.e.* biosynthesis and lipoprotein uptake, should be activated. Indeed, in Chinese hamster ovary cells, expression of HMG-CoA reductase and LDL binding activity appear to be coordinated (44). Contrariwise, challenging quiescent FRTL-5 cells with TSH, decreases LDL binding (11), while inducing reductase gene expression and enzyme activity, and causes a large decrease of cell cholesterol content. We have also observed that, adding cholesterol to TSH-treated, exponentially growing FRTL-5 cells, inhibits cell proliferation (10). However, it is important to note that stimuli which promote activation of HMG-CoA reductase result in greater production of mevalonate, which is the precursor of many non-sterol isoprenoids, in addition to cholesterol. Thus, it could be speculated that, when induced by TSH to undergo mitosis, FRTL-5 cells limit their cholesterol intake and overall content. As a result reductase activity is sustained at high level. This would provide sufficient sterols for new membrane biogenesis and, more importantly, maintain high levels of mevalonate and one or more of its non-sterol derivatives required for DNA synthesis. Blockade of mevalonate synthesis in FRTL-5 cells, with the reductase inhibitor mevinolin, causes arrest of DNA synthesis and cell duplication, which can be reversed by mevalonate addition in the absence or in the presence of lipoprotein cholesterol.[3]

The mechanism by which TSH induces accumulation of HMG-CoA reductase mRNA involves an increased rate of gene transcription. However, a cooperative message stabilizing effect cannot be ruled out. Since the increase of reductase mRNA is transient, and its magnitude and kinetics are independent of whether FRTL-5 cells are exposed to TSH for 4 h or during the entire 72 h examined, there is no approach to a true steady state from which one could estimate a meaningful half-life of the messenger RNA. Experiments performed *in vivo*, feeding hypophysectomized rats with thyroid hormone, have shown an increased rate of transcription of liver HMG-CoA reductase and also a stabilizing effect on the reductase mRNA (45). However, the two systems, and experimental conditions, are much too different for comparison. It is important to point out that involvement of thyroid hormone as a mediator of TSH action on reductase gene expression in FRTL-5 cells can be ruled out. Triiodothyronine has no measurable effect in 5% serum and only a very modest effect in 0.2% serum (data not shown); similarly triiodothyronine does not affect significantly the level of malic enzyme mRNA in FRTL-5 cells (24), whereas it is a powerful inducer of this enzyme in liver tissue (46) and in isolated hepatocytes (47).

The data presented support strongly the role of cAMP as a mediator of the stimulatory effect of TSH on HMG-CoA reductase gene expression and activity. We believe this is the first demonstration of a cAMP-mediated induction of this enzyme. However, the observation that TSH challenge to TSH starved FRTL-5 cells, through augmentation of cAMP production, decreases significantly their cholesterol content (10 and Figs. 1 and 2), raises the possibility that, in fact, cAMP may not act directly at gene transcription level, but rather through reduction of sterol-mediated suppression of reductase transcription (17, 48). Analysis of the 5′ end and untranslated regions of the hamster reductase gene (17, 48–50) has failed to show canonical sequences for cAMP responsive elements (51). However, there are indeed many proteins that bind to the promoter for HMG-CoA reductase which are thought to be responsible for transcriptional regulation (40, 52). It is conceivable that TSH, through its cAMP signal, could induce one or more such regulatory proteins active in promoting reductase gene transcription. If this will be proven to be true, a mechanism could emerge for positive regulation of an enzyme which, so far, is considered to be only negatively regulated (54). We believe that the FRTL-5 system lends itself to studies which will afford a better understanding of mechanisms involved in HMG-CoA reductase regulation.

[3] M. Bifulco and S. M. Aloj manuscript in preparation.

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