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Sterol Regulatory Element-Binding Proteins Induce an Entire Pathway of Cholesterol Synthesis

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To evaluate the effects of sterol regulatory element-binding proteins (SREBPs) on the expression of the individual enzymes in the cholesterol synthetic pathway, we examined expression of these genes in the livers from wild-type and transgenic mice overexpressing nuclear SREBP-1a or -2. As estimated by a Northern blot analysis, overexpression of nuclear SREBP-1a or -2 caused marked increases in mRNA levels of the whole battery of cholesterogenic genes. This SREBP activation covers not only rate-limiting enzymes such as HMG CoA synthase and reductase that have been well established as SREBP targets, but also all the enzyme genes in the cholesterol synthetic pathway tested here. The activated genes include mevalonate kinase, mevalonate pyrophosphate decarboxylase, isopentenyl phosphate isomerase, geranylgeranyl pyrophosphate synthase, farnesyl pyrophosphate synthase, squalene synthase, squalene epoxidase, lanosterol synthase, lanosterol demethylase, and 7-dehydro-cholesterol reductase. These results demonstrate that SREBPs activate every step of cholesterol synthetic pathway, contributing to an efficient cholesterol synthesis.

Key Words: **cholesterol; sterol regulation; SRE; SREBP; transgenic mice; cholesterol genesis; gene regulation.**

Cholesterol is a crucial component of cellular membranes, but can be cytotoxic when it accumulates to excess. Therefore, cholesterol synthesis is tightly regulated by a feedback mechanism to maintain the appropriate cellular cholesterol level. Cholesterol synthesis is catalyzed by a group of microsomal enzymes including HMG CoA synthase and reductase known to commit rate-limiting steps. They are regulated at the transcriptional level and their transcriptional regulation is controlled by a family of transcription factors, sterol regulatory element-binding proteins (SREBPs) (reviewed in reference 1). SREBPs belong to a large class of transcription factors containing bHLH-Zip domains. Unlike other members of this class, SREBPs are synthesized as membrane-bound precursors that require a two-step proteolytic process of cleavage in order to release their amino-terminal bHLH-Zip-containing domains into the nucleus to bind to a specific DNA sequence; sterol regulatory element (SRE), and activate their target genes in a sterol-regulated manner (1–4).

Currently, there are three forms of SREBP that have been characterized, SREBP-1a, -1c, and -2. *In vivo* studies including transgenic and gene knockout mice have shown that SREBP-1c plays a more active role in regulating the transcription of genes involved in fatty acid synthesis, whereas SREBP-2 more specifically activates cholesterol synthesis, and SREBP-1a activates both (5, 6). Overexpression of SREBP-1a or -2 in the liver caused marked induction of cholesterol synthesis. Hepatic messenger RNA levels of HMGCoA synthase and reductase were highly activated as well as those of farnesyl pyrophosphate synthase and squalene synthase, which should explain a marked induction of cholesterol synthesis (7, 8). However, other enzymes in the cholesterol biosynthetic pathway are not fully investigated in these transgenic mice, or in terms of SREBP activation. In the current study, we measured mRNA levels of different enzymes involved in cholesterol synthesis. We found both SREBP1a and 2 activated gene expression of all cholesterogenic enzymes tested here. The data suggest that SREBP regulate cholesterol synthesis very efficiently by controlling gene transcription of most of the genes in the cholesterol synthetic pathway.

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TABLE 1

Sequences of PCR Primers for Cloning Mouse cDNA Probes

Gene	Primer (Sense:upper/Antisense:layer) 5′–3′	Predicted size (bp)	Species	GenBank Accession No.	References
Mevalonate kinase	GAATTCATGTTGTCAGAAGCCCTGC CGCTCGAGCGTCAGAGGCCCAGGGCTTGTC	1187	Mouse	AF137598	9
Phospho mevalonate kinase	AAATCCGGGAAGGACTTCGT TTGCTGTCGACTCTGCTCGA	365	Human, Mouse	NM_006556, AW701432	10, 11
Mevalonate pyrophosphate decarboxylase	TACCTCAATGACACCTCCAGGCGCA CAACCCCTTTCTCCAAATGGCACTG	420	Human, Rat	NM_002461, U53706	12
Isopentenyl pyrophosphate isomerase	ATCAGACATTCTGTCACAATGCCTG TTAGATCAACCTCTTCCAAGGGTA	391	Mouse, Rat	AA763802, AF003835	13
Geranylgeranyl pyrophosphate synthase	CACAGGCATTTAATCACTGGC AATGGCATGGATAGTGGGGA	569	Mouse	NM_010282	14
Squalene epoxidase	TCATTTCTGGAGGCCTCTCAGAATG CCTTTCAATGAACCAGATACTTCAT	909	Mouse	D42048	15
Lanosterol synthase	TGGCTGGCTGTCCTGAATGTTTA TTGGTGCCCTGCATTTTCAT	559	Mouse	AI746980	16, 17
Lanosterol demethylase	GGAAGGGAGTTGCATACGA GAAACAACACACCTGATGTCCTG	792	Human, Rat	NM_000786, U17697	18, 19
Lathosterol oxidase	TGCCGCCGATTACTACTTCT TGTGGTCCTTTCCCTTCAA	780	Mouse	AB016248	20, 21
Delta7-sterol reductase	TTGTGTACTACTTCATCATGGCATG GGGTTGAACTCAATTCCCATCAT	503	Mouse	AF057368	22
7α-hydroxylase	CCGAGTGATGTTTGAAGCT TAGGAACCGTCCTCAAG	607	Human, Rat	NM_000780.1, X17595	23
ACAT	ATAGCCAAGAAGAGGCTGCCG TCGTGGGATATTCTCTCTGA	689	Mouse	L42293	24

MATERIALS AND METHODS

Animals and dietary manipulation. Transgenic mice overexpressing either human SREBP-1a or -2 under the control of the rat phosphoenolpyruvate carboxykinase promoter were established as described (7, 8). Wild-type littermates of hemizygous Tg-SREBP-1a mice were used as controls. Three male animals from each group were fed high protein/low carbohydrate diet for 2 weeks to induce transgene expression. The animals were fasted 12 h prior to sacrifice.

Tissue preparation and total RNA extraction. Resected livers were immediately frozen in liquid nitrogen and stored at −70°C until use. Total RNA was extracted by TRIzol isolation method according to the protocol (Life Technologies, Gaithersburg, MD).

cDNA probes. cDNA probes for the mouse HMG CoA Synthase, HMG CoA Reductase, 7 αhydroxylase, farnesyl pyrophosphate synthase were prepared as described (7, 8). Probes for mevalonate kinase, phospho-mevalonate kinase, mevalonate pyrophosphate decarboxylase, isopentenyl dysphosphate isomerase, delta7 sterol reductase, squalene epoxidase, lanosterol synthase, lanosterol demethylase, lathosterol oxidase, geranylpyrophosphate synthase, and acyl CoA cholesterol acyltransferase (ACAT) were synthesized as follows. First-strand cDNA was prepared from mouse liver poly(A)+ RNA using RT-PCR kit (Takara) primed with oligo-dT. The cDNA was used as a template in PCRs with primer pairs listed in Table 1.

Northern blot analysis. Total RNA was prepared from mouse liver using TRIzol Reagent (Life Technologies, Inc.). For Northern gel analysis, equal aliquots of total RNA from three mice were pooled (total 10 μg), denatured with formaldehyde and formamide, subjected to electrophoresis in a 1% agarose gel, and transferred to Hybond N+ membranes (Amersham) for hybridization. The filters were hybridized with ^{32}P-labeled probes for 12 h at 42°C of 50% formamide, SDS, SSPE, Denhalt's hybridization buffer and was washed with 2 × SSC/1% SDS at 55°C for 20 min and exposed at BAS 2000 Fuji Phosphoimager.

RESULTS

To estimate the effects of overexpression of SREBPs on cholesterogenic genes, we used livers from transgenic mice overexpressing nuclear SREBP-1a and -2, both of which were reported to activate cholesterol synthesis. SREBP-1c, a transactivator for lipogenic enzymes, was shown to be inactive for induction of cholesterogenic enzymes (6). In these transgenic mice, the transgenes encode nuclear forms of SREBPs and thus, the proteins overexpressed from the transgenes directly enter the nucleus to activate their target genes. The transgenes were expressed under the control of the rat PEPCK promoter and animals were put on a low carbohydrate/high protein diet before sacrifice to induce the transgene expression. Previous estimation of transgene expression demonstrated that expression levels of nuclear SREBP-1a and -2 in these livers were roughly comparable at mRNA level. Therefore,

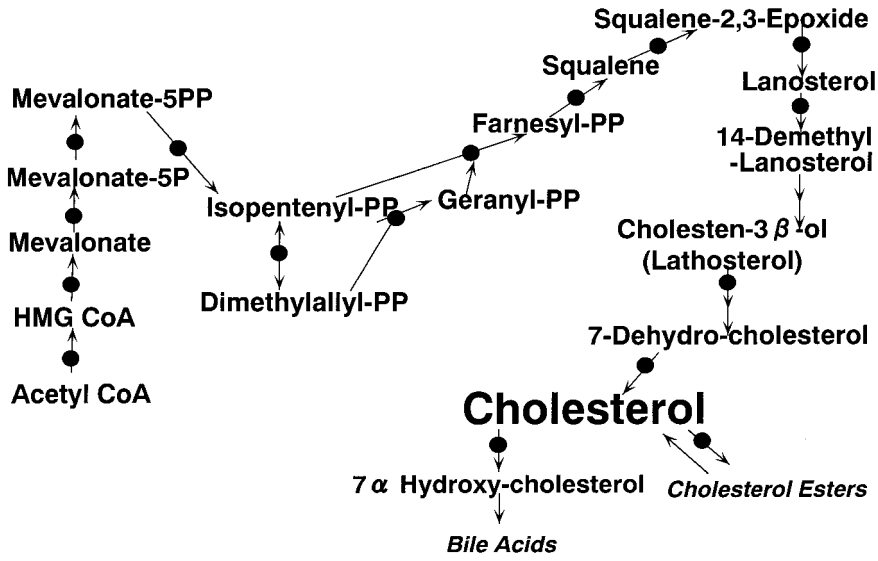

FIG. 1. Diagram of the cholesterol synthetic pathway.

Northern blot analysis of the SREBP target genes in these livers should provide an estimation of transcriptional activities of SREBP-1a and -2 for these genes.

Figure 1 depicts enzymes in the cholesterol synthetic pathway from acetyl CoA to cholesterol. The cDNA probes used for Northern blot analysis were prepared using RT-PCR with primers shown in Table 1.

Gene Expression of Cholesterol Synthetic Enzymes in the Pathway from Acetyl-CoA to Mevalonate-Pyrophosphate (Fig. 2)

HMG CoA synthase is an enzyme which produces HMG CoA from acetyl CoA. It is one of rate-limiting enzymes in this pathway, and promoter analysis on

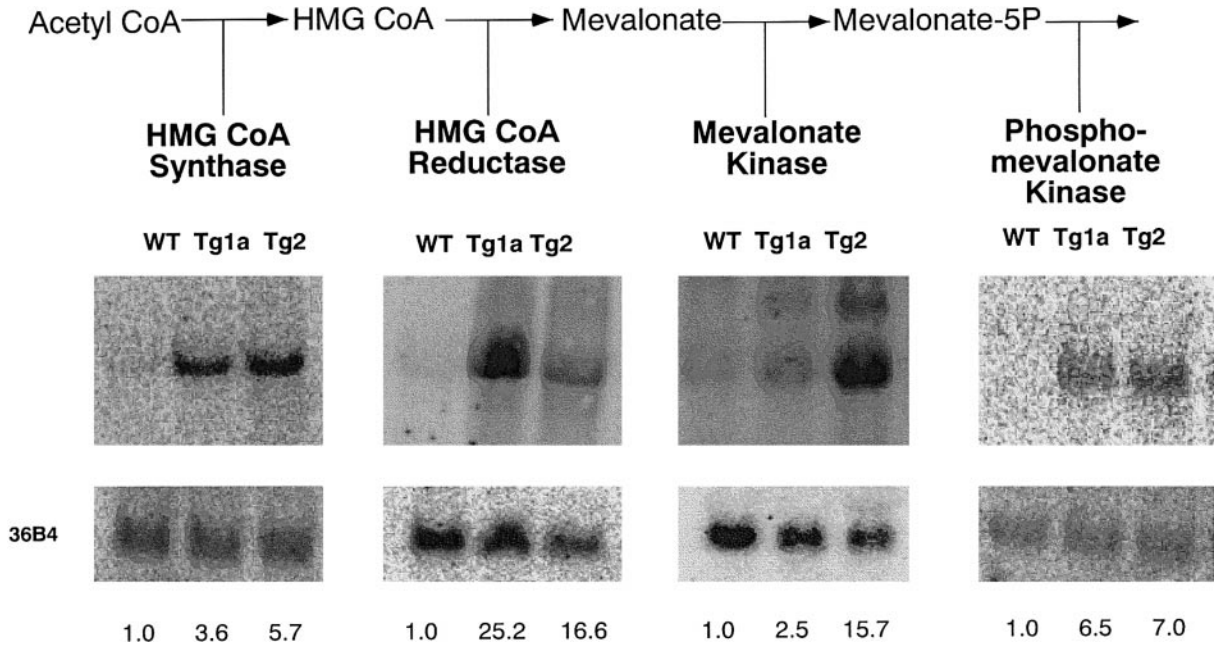

FIG. 2. Amounts of cholesterol biosynthetic gene mRNAs in livers of wild-type (WT), transgenic SREBP-1a (Tg1a), and –2 (Tg2) mice as measured by blot hybridization: Enzymes involved in conversion from acetyl CoA to mevalonate-pyrophosphate. Total liver RNA was pooled 10 μg were subjected to electrophoresis and blot hybridization with the indicated ^{32}P-labeled probe and with a control ^{32}P-labeled probe directed against 36B4. The amounts of radioactivity in each band was quantified as described under Materials and Methods. The fold increase in each mRNA of transgenic mice, relative to that of wild-type control mice, was calculated after correction for loading differences with 36B4. These values are shown below each blot.

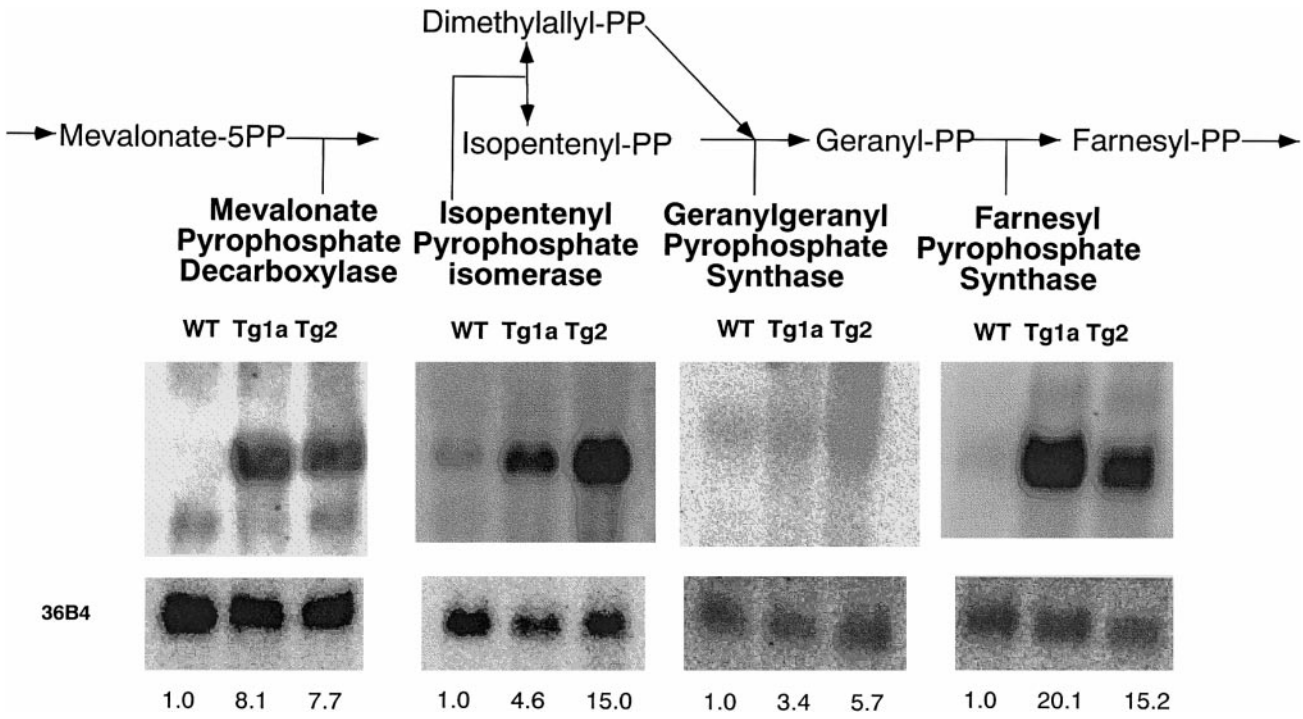

FIG. 3. Amounts of cholesterol biosynthetic gene mRNAs in livers of wild-type (WT), transgenic SREBP-1a (Tg1a), and -2 (Tg2) mice as measured by blot hybridization: Enzymes involved in conversion from mevalonate-pyrophosphate to farnesyl-pyrophosphate. Total liver RNA was pooled 10μg were subjected to electrophoresis and blot hybridization with the indicated ^{32}P-labeled probe and with a control ^{32}P-labeled probe directed against 36B4. The amounts of radioactivity in each band was quantified as described under Materials and Methods. The fold increase in each mRNA of transgenic mice, relative to that of wild-type control mice, was calculated after correction for loading differences with 36B4. These values are shown below each blot.

this gene has been extensively performed. Two SREs were known to be involved in sterol regulation mediated by SREBPs. We reconfirmed marked increases in its expression 25-fold in the livers of SREBP-1a transgenic mice (TgBP1a) and 13-fold in those of SREBP-2 transgenic mice (TgBP2) compared to wild-type livers (WT), which was consistent with previous reports. Similarly, mRNA level of HMG CoA reductase, another rate-limiting enzyme and established SREBP target gene, was also markedly increased by overexpressed SREBP-1a and -2 (13-fold and 4-fold, respectively). We measured mRNA level of mevalonate kinase that catalyzes the next step. It was 4-fold increased in TgBP1a and 3-fold in TgBP2 compared to WT. The expression of phosphomevalonate kinase was increased 6.5-fold in Tg-BP1a and 7-fold in Tg-BP-2.

Gene Expression of Cholesterol Synthetic Enzymes in the Pathway from Mevalonate-Pyrophosphate to Farnesyl-Pyrophosphate (Fig. 3)

As shown in Fig. 3, mevalonate pyrophosphate decarboxylase was induced by 8-fold both in SREBP-1a and -2 transgenic livers. Isopentenyl-phosphate isomerase was markedly (13-fold and 26–fold) activated by SREBP-1a and -2, respectively. Unlike other enzymes, activation of this enzyme by SREBP2 was more prominent than by SREBP-1a. Although induction of geranylgeranyl pyrophosphate synthase was relatively mild in both transgenic livers, farnesyl-pyrophosphate synthase was markedly upregulated, 20-fold in Tg-BP1a and 15-hold in Tg-BP-2 as consist with a previous observation (7, 8).

Gene Expression of Cholesterol Synthetic Enzymes in the Pathway from Farnesyl-Pyrophosphate to Lanosterol (Fig. 4)

Squalene synthase is an enzyme whose promoter was well established as a SREBP target (25). We observed 18-fold and 9-fold increase in squalene synthase mRNAs in TgSREBP-1a and -2 livers, respectively. The enzyme involved in the next step, squalene epoxidase was activated by SREBP-1a and -2, 10-fold and 9-fold, respectively. The following enzyme, lanosterol synthase was markedly increased 23-fold and 14-fold by SREBP-1a and -2, respectively.

Gene Expression of Cholesterol Synthetic Enzymes in the Pathway from Lanosterol to Cholesterol (Fig. 5)

Lanosterol demethylase, which was recently reported to be an SREBP target (26), was consistently

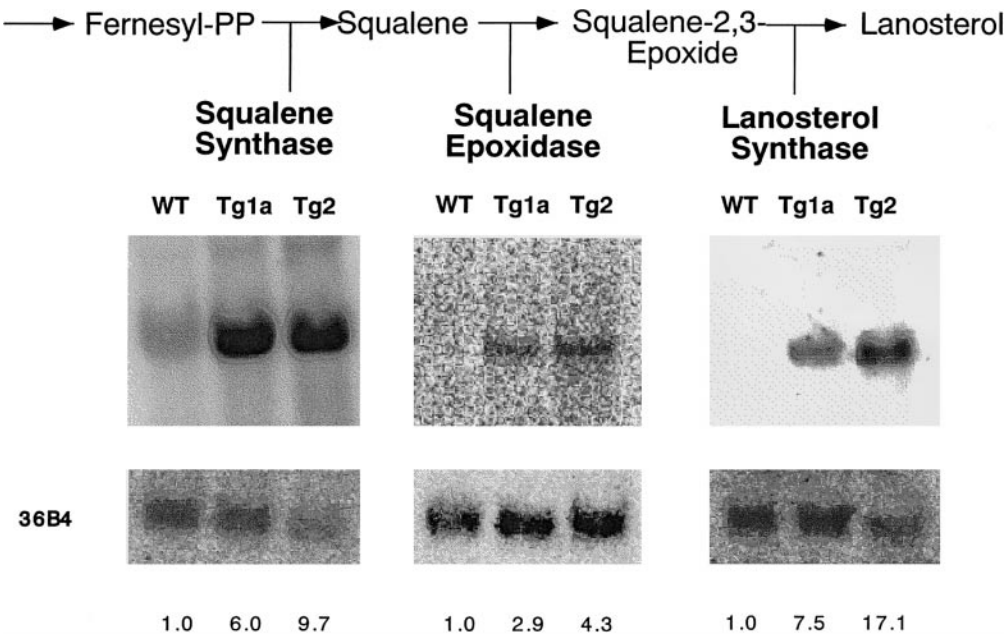

FIG. 4. Amounts of cholesterol biosynthetic gene mRNAs in livers of wild-type (WT), transgenic SREBP-1a (Tg1a), and -2 (Tg2) mice as measured by blot hybridization: Enzymes involved in conversion from farnesyl-pyrophosphate to lanosterol. Total liver RNA was pooled 10μg were subjected to electrophoresis and blot hybridization with the indicated ^{32}P-labeled probe and with a control ^{32}P-labeled probe directed against 36B4. The amounts of radioactivity in each band was quantified as described under Materials and Methods. The fold increase in each mRNA of transgenic mice, relative to that of wild-type control mice, was calculated after correction for loading differences with 36B4. These values are shown below each blot.

increased (8-fold by SREBP-1a and 11-fold by SREBP-2). Lathosterol oxidase was 5-fold and 7-fold upregulated by SREBP-1a and -2, respectively. Finally, delta7-sterol reductase that catalyzes the last step for cholesterol synthesis was also robustly increased by SREBPs (13-fold by SREBP-1a, and 5-fold by SREBP-2).

Gene Expression of Other Cholesterol-Related Genes (Fig. 6)

For comparison with cholesterogenic genes, we estimated expression of some enzymes which are not involved in cholesterol synthesis, but related to cholesterol metabolism. Expression of 7alpha-cholesterol hydroxylase rate-limiting enzyme for bile acid production was not significantly changed by overexpression of SREBP-1a or -2. There was a slight decrease observed in the mRNA levels of ACAT-1, catalyzing cholesterol ester formation, in the livers from both transgenic mice.

DISCUSSION

The current study demonstrates that essentially all the enzymes involved in cholesterol synthesis were markedly increased at the mRNA level in the livers overexpressing nuclear SREBP-1a and -2. It suggests that both SREBP-1a and -2 activate cholesterol synthesis by increasing gene expression of the individual enzyme at every step in the pathway. Cholesterol biosynthetic pathway is composed of many steps and is tightly under a negative feedback regulation by some intermediate substrates as well as the final product, cholesterol. If an intermediate product in the pathway accumulates because of relative slow rate of the next reaction, the accumulated molecule would further downregulate the previous steps, causing a cascade of repression and impairing the whole reaction. This can usually occur in the step of rate-limiting enzyme. However, the range of SREBP-regulation of HMG CoA synthase and reductase that are supposed to be rate-limiting enzymes in cholesterol biosynthetic pathway is tremendous. Therefore other steps could be rate-limiting. When demand for cellular cholesterol is increased, all the enzymes in the pathway need to be activated in a coordinated fashion by activated SREBPs to obtain the greatest rate of cholesterol synthesis. As for the enzymes we analyzed in the current study, relative activation of SREBP-1a vs SREBP-2 varies only in the range 0.5 to 2. There is no marked difference between SREBP-1a and -2 in the activation of cholesterogenic genes. In the differentiated tissues such as liver, SREBP-2 should be mainly involved in cholesterol synthesis whereas both SREBP-1a and –2

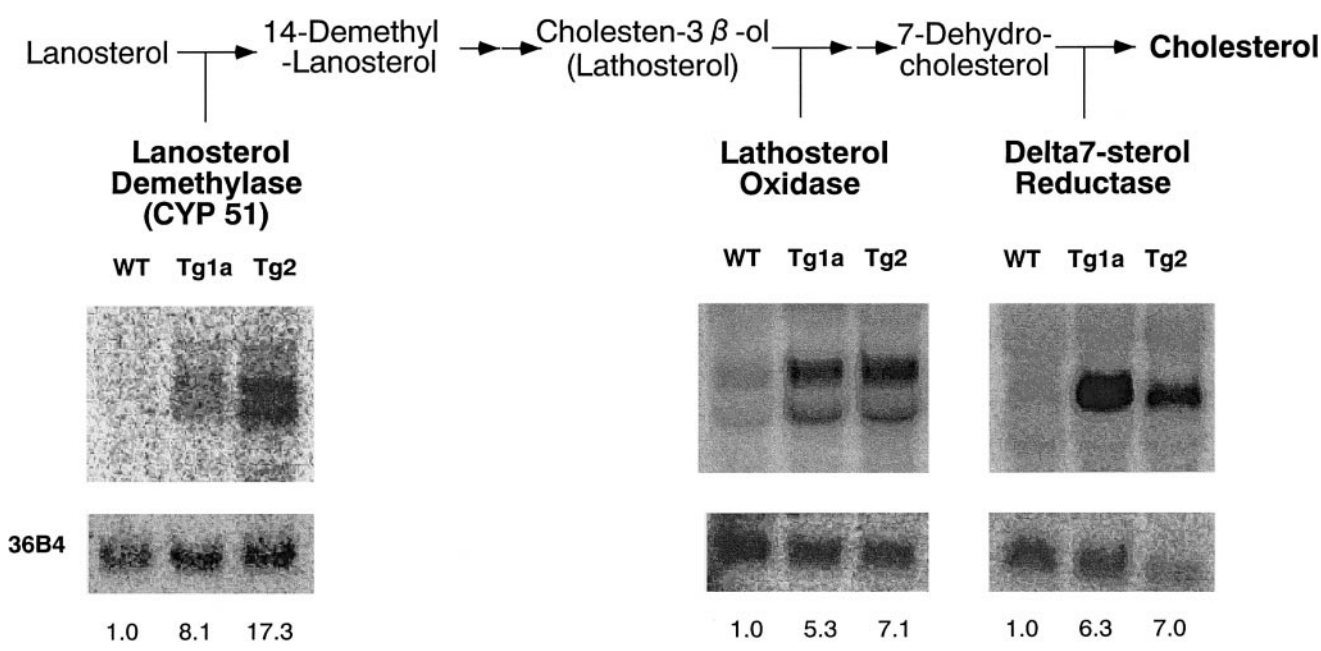

FIG. 5. Amounts of cholesterol biosynthetic gene mRNAs in livers of wild-type (WT), transgenic SREBP-1a (Tg1a), and -2 (Tg2) mice as measured by blot hybridization: Enzymes involved in conversion from lanosterol to cholesterol. Total liver RNA was pooled 10μg were subjected to electrophoresis and blot hybridization with the indicated ^{32}P-labeled probe and with a control ^{32}P-labeled probe directed against 36B4. The amounts of radioactivity in each band was quantified as described under Materials and Methods. The fold increase in each mRNA of transgenic mice, relative to that of wild-type control mice, was calculated after correction for loading differences with 36B4. These values are shown below each blot.

should participate in the sterol regulation in the cultured cells.

In the current study, we demonstrated SREBP activation of various enzymes in the cholesterol synthetic pathway. In some cholesterogenic genes such as HMG CoA synthase (27) and reductase (28), FPP synthase (29), squalene synthase (25), lanosterol 14α-demethylase (26), and very recently, 7deltasterol reductase (30),

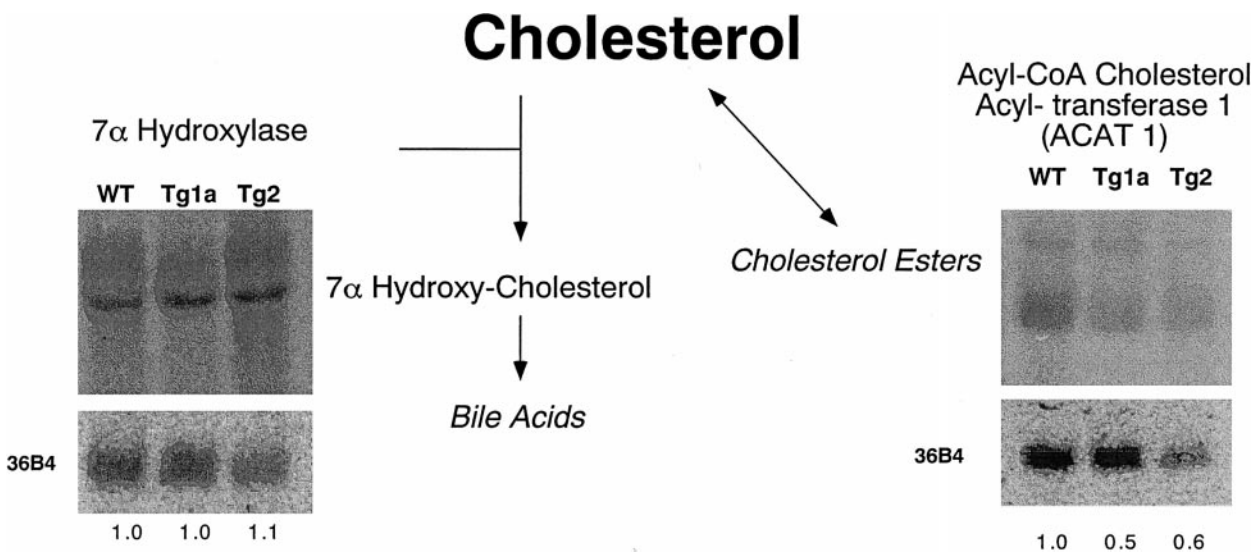

FIG. 6. Amounts of cholesterol-related gene mRNAs in livers of wild-type (WT), transgenic SREBP-1a, and -2 mice as measured by blot hybridization. Total liver RNA was pooled 10μg were subjected to electrophoresis and blot hybridization with the indicated ^{32}P-labeled probe and with a control ^{32}P-labeled probe directed against 36B4. The amounts of radioactivity in each band was quantified as described under Materials and Methods. The fold increase in each mRNA of transgenic mice, relative to that of wild-type control mice, was calculated after correction for loading differences with 36B4. These values are shown below each blot. ACAT-1, Acyl-CoA Cholesterol Acyl-transferase-1.

precise promoter analysis has been reported. They are well-established SREBP target genes. The sequence of 5′ flanking region of the mevalonate kinase gene was reported (31). The mevalonate kinase promoter contains a typical SRE flanked by GC-rich region, a potential Sp1 site and ATTGG, a binding site for NF-Y, both of which are crucial coactivators for SREBP. Therefore, this region is an SREBP binding and activation site of high probability. We surveyed public DNA data bank and searched upstream regions of other cholesterogenic genes. We found SRE-like sequences upstream of the genes for phosphomevalonate kinase (AF026069), isopentenyl pyrophosphate isomerase (AF291755), and lanosterol synthase (AP100469). However, it is necessary to perform detailed promoter analysis on these genes and the rest of cholesterogenic genes to confirm that they are SREBP targets. These studies will clarify the mechanism of the comprehensive regulation of cholesterol synthesis by SREBPs, which was proposed in the current study.

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