Cholesterol biosynthesis from lanosterol: molecular cloning, chromosomal localization, functional expression and liver-specific gene regulation of rat sterol Δ^8-isomerase, a cholesterogenic enzyme with multiple functions

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Sterol Δ^8-isomerase (SI) (EC 5.3.3.5), also known as emopamil binding protein or sigma receptor, catalyses the conversion of the 8-ene isomer into the 7-ene isomer in the cholesterol biosynthetic pathway in mammals. Recently, mutations of SI have been found to be associated with Conradi–Hünermann syndrome in humans. To investigate the *in vitro* and *in vivo* modes of molecular regulation of SI and its role in cholesterol biosynthesis in mammals, we isolated a full-length cDNA encoding rat SI. The deduced amino-acid sequence of rat SI predicts a 230-residue protein (26737 Da) with 87% and 80% amino-acid identity to mouse and human counterparts. The rat SI gene was mapped to chromosome 12q1.2 using fluorescence *in situ* hybridization (FISH). The biological function of the cloned rat SI cDNA was verified by overexpressing recombinant Myc–SI in *Saccharomyces cerevisiae*. It showed a characteristic pattern of inhibition on exposure to *trans*-2-[4-(1,2-diphenylbuten-1-yl)phenoxy]-*N*,*N*-dimethylethylamine (tamoxifen; $IC_{50} = 11.2\ \mu M$) and 3β-[2-(diethylamino)ethoxy]androst-5-en-17-one (U18666A; $IC_{50} = 4.2\ \mu M$), two well known potent inhibitors of SI. Northern-blot analysis of 3-week-old rats compared with 2-year-old rats showed that SI mRNA expression in both age groups was restricted to liver, where a 70% reduction in mRNA levels was observed in 2-year-old rats. The FISH studies revealed ubiquitous expression of SI mRNA in rat hepatocytes. The *in vitro* studies showed that the SI mRNA was highly suppressed by 25-hydroxycholesterol in H4IIE cells. Treatment of H4IIE cells grown in medium supplemented with fetal bovine serum with tamoxifen for 24 h resulted in a dose-dependent induction of SI mRNA, with a concomitant suppression of sterol regulatory element binding protein-1 mRNA. Interestingly, this effect was not seen in emopamil-treated cells. The *in vivo* experiments also indicate that both mRNA expression and enzymic activity of SI in liver were induced approx. 3-fold in rats fed 5% (w/w) cholestyramine plus 0.1% (w/w) lovastatin in normal chow for 2 weeks. With this newly cloned rat SI cDNA, it becomes possible to gain molecular understanding of previously unknown and tamoxifen-mediated gene regulation of SI that is involved in cholesterol metabolism, ischaemia and genetic diseases.

Key words: tamoxifen, emopamil, transcription, H4IIE, FISH.

INTRODUCTION

Cholesterol biosynthesis in mammals requires more than 32 enzymes and their co-operative regulation in the cell (for review see [1,2]). In the later stage of cholesterol biosynthesis from 4,4′,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol (lanosterol) [3–5], which has recently been established based on the determination of the previously unknown site of sterol Δ^{24}-reductase reaction [6], sterol Δ^8-isomerase (SI) (EC 5.3.3.5) catalyses the conversion of the 8-ene isomer into the 7-ene isomer, which is the only reversible reaction in the 19 steps of cholesterol biosynthesis from lanosterol in mammals [7–9]. This enzyme renders the sterol more suitable for the subsequent Δ^{24}-reduction [6] and confers an important physiological role (see below). SI is known to be modulated by several well known sterol-biosynthesis inhibitors, such as *trans*-2-[4-(1,2-diphenylbuten-1-yl)phenoxy]-*N*,*N*-dimethylethylamine (tamoxifen; a chemotherapeutic agent used for the treatment of breast cancer), 3β-[2-(diethylamino)-ethoxy]androst-5-en-17-one (U18666A) and *trans*-1,4-bis-(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride (AY-9944) (Scheme 1) [8–10]. In addition to its role in sterol isomerization in cholesterol biosynthesis, SI also functions as an emopamil-binding protein (EBP) and as a sigma receptor [10–12], to which a number of structurally diverse molecules, including the immunosuppressant SR31747A [12,13] and emopamil [11], are known to bind.

Studies on SI have recently attracted our attention, because mutations in the gene encoding SI cause X-linked dominant Conradi–Hünermann syndrome in humans [16] and 'tattered' in mice [17], which is one of the disorders with aberrant punctate calcification in cartilage, or chondrodysplasia punctata (CDP). By quantitative sterol analysis of a variety of tissues, five patients

Abbreviations used: AY-9944, *trans*-1,4-bis-(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride; CDP, chondrodysplasia punctata; CHILD, **c**ongenital **h**emidysplasia with **i**chthyosiform erythroderma and **l**imb **d**efects; CL-diet, 5% cholestyramine plus 0.1% lovastatin in normal chow; DHC, dehydrocholesterol; DHCR, DHC reductase; DIG, digoxigenin; EBP, emopamil-binding protein; FBS, fetal bovine serum; FISH, fluorescence *in situ* hybridization; HDL, high-density lipoprotein; lanosterol, 4,4′,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol; LDL, low-density lipoprotein; LPDS, lipoprotein-deficient serum; OHC, hydroxycholesterol; ORF, open reading frame; SD, Sprague–Dawley; SI, sterol Δ^8-isomerase; SREBP-1, sterol regulatory element binding protein-1; tamoxifen, *trans*-2-[4-(1,2-diphenylbuten-1-yl)phenoxy]-*N*,*N*-dimethylethylamine; U18666A, 3β-[2-(diethylamino)ethoxy]androst-5-en-17-one.

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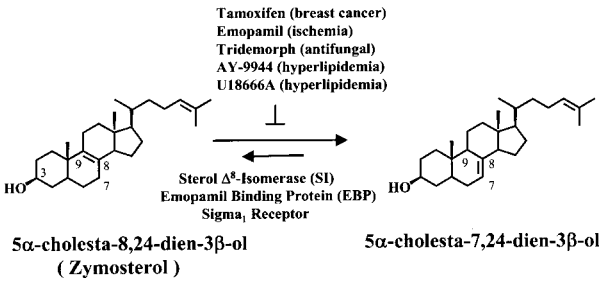
The nucleotide sequence data reported will appear in the GenBank® Nucleotide Sequence Database under the accession number AF071501.



Scheme 1 Reversible enzymic reaction of SI, and inhibitors of SI used in this study

The mark ⊥ denotes blocking of the reaction, catalysed by SI, by various inhibitors. Potential targets for each drug are indicated in parentheses.

suffering from CDP as well as CHILD (**c**ongenital **h**emidysplasia with **i**chthyosiform erythroderma and **l**imb **d**efects) syndrome, were identified with abnormally increased levels of 8-dehydrocholesterol (DHC) and cholest-8(9)-en-3β-ol, suggesting a deficiency in SI enzymic activity in these patients [16–19].

The gene encoding rat SI has not been cloned previously, and its transcriptional regulation *in vitro* or *in vivo* has not been reported either. In order to investigate the molecular regulation of SI gene expression by various modulators and the role of SI in cholesterol biosynthesis, we isolated a full-length cDNA encoding rat SI and examined transcriptional regulation of the SI gene. We demonstrate in the present study that the expression of the SI gene is regulated transcriptionally *in vitro* (in cultured cells) and *in vivo* (in diet-fed-rats) by widely known regulatory agents ranging from tamoxifen, an oestrogen receptor antagonist and SI-specific inhibitor, to cholesterol-lowering drugs.

MATERIALS AND METHODS

Materials

A rat liver cDNA library (oligo[dT] primed and randomly primed) and a rat multiple tissue Northern blot were purchased from ClonTech Laboratories (Palo Alto, CA, U.S.A.). A Multiple Choice™ Rat Brain Northern Blot was purchased from OriGene Technologies Inc. (Rockville, MD, U.S.A.), [α-^{32}P]dCTP (3000 Ci/mmol) and Hyper X-ray film, rainbow prestained protein markers and the ECL® Western-blot detection system were obtained from Amersham Pharmacia Biotech. A random-primed DNA labelling kit was purchased from Roche Molecular Biochemicals, a total RNA isolation kit was obtained from Invitrogen and a DNA preparation kit was from Qiagen. Cholesterol, 7-DHC and 25-hydroxycholesterol (OHC) were purchased from Steraloids (Wilton, NH, U.S.A.). The sources of the following drugs or agents are indicated in parentheses: AY-9944 (Wyeth–Ayerst, Princeton, NJ, U.S.A.); cholestyramine (LG Chem, Pharmaceutical Division, Seoul, Korea); Lovastatin® (Dr K.-W. Cho, Choongwae Pharmaceutical Co., Suwon, Korea); tamoxifen (Sigma); U18666A (Dr R. J. Cenedella, Kirksville College of Osteopathic Medicine, Kirksville, MO, U.S.A.); (+) emopamil (Dr H. Glossmann, Institut für Biochemische Pharmakologie, Universität Innsbruck, Innsbruck, Austria). Oligonucleotide primers were synthesized using a DNA synthesizer (Applied Biosystems, Foster City, CA, U.S.A.). RPMI 1640 medium, fetal bovine serum (FBS), penicillin/streptomycin, Dulbecco's modified Eagle's medium, and L-glutamine were purchased from Life Technologies. Anti-Myc monoclonal antibody (9E10) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). All other reagents were of the best grade available.

Screening and isolation of SI cDNA from a rat liver cDNA library

A 0.7 kb fragment of human SI cDNA [12], covering the entire open reading frame (ORF) and obtained by reverse transcriptase-PCR, was labelled with [α-^{32}P]dCTP using a random-primed labelling kit according to the suggested procedures for a specific activity $> 1.6 \times 10^9$ d.p.m./μg. The labelled fragment was denatured at 100 °C for 10 min, and used as a probe to screen $> 3 \times 10^6$ plaques from a rat liver cDNA library according to standard protocols provided by ClonTech. After tertiary screening, nine positive clones were used for further analyses using slot-blot hybridization [20]. After digestion of nine clones with *Eco*RI, two different clones were identified, SI-1 (1.1 kb) and SI-2 (0.8 kb) (results not shown).

DNA sequencing and analysis of rat cDNA

A DNA fragment (1.1 kb) that was isolated by *Eco*RI digestion of cloned DNA from SI-1 was sequenced on both strands by the dideoxy sequencing method using a *Taq* Dye Deoxy Terminator Cycle sequencing kit (Applied Biosystems). Sequence and structure analyses of the cloned cDNA and its predicted protein product were carried out using BLAST programs in GenBank®. A rat cDNA clone similar to human EBP (GenBank® accession number Z37986), *Mus musculus* SI (GenBank® accession number X97755), *Arabidopsis thaliana* SI (GenBank® accession number AF030357) and ERG2 (GenBank® accession number M74037) were analysed with the BLASTX algorithm. Alignments and hydrophobicity analysis were calculated with the Wisconsin Sequence Analysis Package according to the algorithms of Kyte and Doolittle [21]. Analysis of transmembrane segments was performed using the following prediction programs: PHD_htm at EMBL, TMpred at ISREC (the Swiss Institute for Experimental Cancer Research; http://ulrec3.unil.ch/software/TMPRED_form.html), SOSUI (a transmembrane-region prediction program) at Tokyo University of Agriculture and Technology (http://www.tuat.ac.jp/~mitaku/adv_sosui), and HMMTOP prediction as described previously [22]. Topology of rat SI structure was predicted by at least three of the four programs. The alignment of these sequences with rat SI was performed using the CLUSTALW program.

Heterologous expression of Myc-tagged rat SI in *Saccharomyces cerevisiae*

Rat SI cDNA was subcloned into c-*myc*-YEp351ADC1, and transformation of *S. cerevisiae JB811* (*ade2-1 leu2-3,112 pep4-3 trp1-289 ura3-52*) was performed as described previously [12]. Cells were harvested when D_{600} reached 1.2, and were lysed with glass beads in 50 mM Tris/HCl (pH 7.4), 1 mM EDTA and 0.1 mM PMSF. The microsomes were prepared as described previously [12]. Immunoblotting with a 9E10 c-Myc monoclonal antibody following SDS/PAGE analysis was performed as previously described [23]. Protein concentrations were determined by the method of Bradford [24], using BSA as a standard.

Cell culture

Cells were grown in RPMI-1640 medium (Gibco BRL) containing 10 % (v/v) FBS (FBS medium) and incubated in air/CO_2 (19:1) at 37 °C. Cells (5×10^6) were seeded in 100 mm dishes containing 10 ml of FBS medium. Rat hepatoma cells (H4IIE), mouse fibroblast cells (L929) and CHO cells formed confluent

monolayers in 24 h, while human hepatoblastoma cells (HepG2) formed confluent monolayers in 5 or 6 days. The cells were washed and replaced with 10 ml of FBS medium or RPMI-1640 medium containing 10 % (v/v) lipoprotein-deficient-serum (LPDS) medium.

Animals, diet feeding, drug treatment, plasma lipid analysis and preparation of microsomes

Male Sprague–Dawley (SD) rats (30–900 g body weight) ranging from 3-weeks-old to 2-years-old were obtained from Yonsei Medical Center, Seoul. The rats were maintained on Purina rodent chow (Purina Mills, Richmond, IN, U.S.A.) containing 20 % protein, 4.5 % fat, 6 % fibre, 7 % ash, 0.5 % calcium and 0.5 % phosphate, under a reverse light cycle (light 18:00–6:00 h) as previously described [25]. In the diet feeding experiments, each group (3–4 rats/group) was fasted for 12 h and then fed diets, such as 5 % cholestyramine plus 0.1 % lovastatin in normal chow (CL-diet) or 0.01 % tamoxifen in chow for 2 or 4 weeks [26,27]. At the end of the treatment, the rats were fasted for 24 h and killed by decapitation at the midpoint of the dark period (22:00 h). Their livers were excised and processed for microsome preparation as previously described [25]. For the analysis of plasma lipids, at the end of the treatment, rats were fasted for 24 h and killed by CO_2 inhalation. Blood was drawn by heart puncture as described in [25] and analysed for serum cholesterol. Plasma cholesterol was determined using an automatic analyser (Hitachi 7150). High-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol were calculated as described previously [28]. Enzyme assay was carried out using 100 μM 5α-cholesta-8,24-dien-3β-ol (also known as zymosterol) that had been suspended in the detergent Tyloxapol (WR-1339) (detergent/sterol, 75:1, v/v) and 1–2 mg of microsomal protein as described previously [8]. For the *in vitro* inhibition experiments, for both rat liver microsomes and yeast overexpressed protein, drugs or agents were dissolved in dimethyl sulphoxide (Me_2SO) such that the final concentration of Me_2SO was less than 0.3 % (w/v) of the incubation mixture [3]. The sterol concentration in enzyme assay samples was measured using a Hewlett Packard GC 5890 II flame-ionization detector (SAC™-5 capillary column from Supelco™, catalogue no. 24156; 5 % diphenyl/95 % dimethylsiloxane; 30 m × 0.25 mm; 0.25 μm inner diameter; flow rate = 2.44 ml/min] using 5α-cholestane as a standard [6,25].

RNA preparation and Northern-blot analysis

For the measurement of the effects of drugs, diet and aging on SI gene expression, total RNA from drug-treated rat hepatoma cells or livers of either CL-diet-fed, tamoxifen-fed or normal-diet-fed rats (3-weeks-old and 2-years-old) was prepared using a total RNA isolation kit (Invitrogen) according to the manufacturer's protocol. RNA (15 μg) from each tissue was separated on a 1.2 % (w/v) agarose gel in 30 % (v/v) formaldehyde and blotted. RNAs were probed with ^{32}P-labelled rat SI cDNA (GenBank® accession number AF071501). A rat β-actin cDNA probe was used as a control. After hybridization [42 °C; 50 % (v/v) formamide] blots were washed with 2 × SSC containing 0.1 % (w/v) SDS (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate) followed by 0.1 × SSC containing 0.5 % (w/v) SDS at 65 °C. A rat multiple tissue Northern blot (ClonTech) and a Multiple Choice™ Rat Brain Northern Blot (Origen) were probed with ^{32}P-labelled rat SI cDNA. The blots were exposed to Hyper X-ray film with intensifying screens at −70 °C for longer than 48 h. The bands that appeared in the blots were quantified with a BAS 2500 system (Fuji Photo Film Co.). Rat SI mRNA signals obtained from different tissues by the Northern-blot hybridization were normalized to those of β-actin mRNA expression.

In situ hybridization of SI mRNA

For the detection of rat SI expression in liver, *in situ* hybridization was performed with digoxigenin (DIG)-labelled rat SI cRNA probes as previously described [29,30]. To prepare RNA probes for *in situ* hybridization, PCR was performed using a rat SI cDNA insert (1.1 kb *Eco*RI-digested clone SI-1) with the primers 5′-GTTTGCTGTGTGCACATTCATT-3′ and 5′-ACAGCAC-ATCCCCATATATCTG-3′. PCR products were cloned into a pGEM vector containing the T7 and Sp6 promoters. Antisense and sense RNA probes were prepared by *in vitro* transcription (from nt 306–595 of the rat SI cDNA sequence; GenBank® accession number AF071501) using T7 and Sp6 RNA polymerase, respectively, in the presence of 0.4 mM DIG-linked UTP in a 20 μl reaction mixture, according to the manufacturer's instructions. Male SD rats (8 weeks old) were perfused with 0.01 M PBS (pH 7.5) and 4 % (w/v) paraformaldehyde in PBS under anesthesia by the intravenous injection of sodium pentobarbital. Immediately after perfusion, liver was fixed in fresh 4 % (w/v) paraformaldehyde in PBS for at least 24 h at 4 °C, and then cryoprotected by incubation for 6 h in 12 % sucrose in PBS and overnight in 18 % sucrose in PBS at room temperature. Serial frozen sections (10 μm thick) were cut under sterile conditions and mounted on the slide (Fisher Co., Pittsburgh, PA, U.S.A.). Slices of rat liver (10 μm) were fixed in 4 % (w/v) paraformaldehyde for 30 min and incubated at 56 °C overnight with the hybridization buffer containing 1 μg/ml DIG-labelled cRNA probe. The composition of the buffer was 50 % (v/v) formamide, 5 × SSC and 40 μg/ml single-stranded DNA. After hybridization, the sections were washed in 2 × SSC and then in 0.1 × SSC for 1 h. The sections were then incubated in anti-DIG Fab antibody for 2 h before washing, and the hybridization signals of the rat SI gene were detected by labelling with Nitro Blue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate. The slides were observed under a light microscope (Olympus, Tokyo, Japan).

Chromosomal localization of the rat SI gene

A 2.4 kb genomic fragment of rat SI was used for chromosomal localization by replicative R-banded fluorescence *in situ* hybridization (FISH). Chromosome slides for FISH were prepared from the lymphocyte cultures of SD rats as described previously [31]. Either thymidine (150 μg/ml) or bromodeoxyuridine (150 μg/ml) was added to the cultures for replicative R-banding. Colcemide (0.05 μg/ml) was added 20–30 min before cell harvesting. Cell suspensions (3 %, v/v) were collected, treated with 0.075 M KCl, fixed in methanol/glacial acetic acid (3:1, v/v) and then dried in air. The chromosome slides were stained with 1 μg/ml Hochest 33258 in Sorenson's phosphate buffer (66.6 mM Na_2HPO_4/66.6 mM KH_2PO_4, pH 6.8) for 5 min, then rinsed and mounted with the same buffer under coverslips. The slides were heated at 75 °C and exposed to HBO-20 W Black light (Toshiba). Rat SI cDNA, a 2.4 kb of *Eco*RI-digested genomic fragment, was labelled using a nick translation kit with biotin-16-dUTP (Roche Molecular Biochemicals) according to the manufacturer's protocol. The labelled probe was ethanol-precipitated with 625 μg/ml sonicated salmon sperm DNA or tRNA and then denatured. The denatured probe was mixed with an equal volume of hybridization solution [final concentrations in mixture; 50 % (v/v) formamide, 2 × SSC, 10 % (w/v) dextran

sulphate and 1 mg/ml BSA]. Chromosomal slides were incubated with 20 μl of mixture containing 100 ng of DNA at 37 °C overnight. Amplification methods were used for detection of hybridization signals. After serial rinsing with 1 × SSC, 2 × SSC and 4 × SSC the chromosomal slides were incubated with anti-biotin antibody (Vector Laboratories, Burlingame, CA, U.S.A.). After rinsing, the chromosomal slides were incubated with anti-goat IgG coupled to fluorescein (Nordic Immunology, Capistrano Beach, CA, U.S.A.), and were stained with 1 μg/ml propidium iodide. Chromosome slides were observed using Zeiss filter sets at wavelengths of 450–490 nm (B-2A and B-2E), 510–560 nm (G-2A) and near 365 nm (UV-2A). The slides were photographed on Kodak Ektachrome (ASA 100) film.

Table 1 Comparison of the nucleotide and amino acid sequence of rat SI with sequences of different species

Species	Isomerase-related protein	Sequence identity or similarity (%) Nucleotide Identity	Amino acid Identity	Amino acid Similarity	Reference
Human	EBP	81	80	86	[12]
Mouse	SI	91	87	93	[14]
Rat	SI	100	100	100	This paper
Plant	SI	26	34	57	[32]
Fungus	SI	2	13	47	[33]

RESULTS

Cloning, sequence analysis and structural features of rat SI cDNA

Screening of a rat liver cDNA library (ClonTech) with the human SI cDNA probe yielded two positive clones (i.e. SI-1 and SI-2) from approximately $> 3 \times 10^6$ plaques screened. The rat SI cDNA (clone SI-1) is 1071 nt long, and consists of a 102-nt 5′-untranslated region that is followed by a 693-nt coding region and a 276-nt 3′-untranslated region (Figure 1, left-hand panel). As anticipated, the nucleotide sequence of rat SI showed the highest sequence similarity to mouse SI (Table 1). That is, the coding region/ORF of the rat SI cDNA sequence (693 nt) showed 87, 80, 34 and 13 % identity with mouse (GenBank® accession number X97755), human (GenBank® accession number Z37986), *Arabidopsis thaliana* (cress; GenBank® accession number AF030357) [32], and yeast [33] sequences respectively. Like human SI cDNA, rat SI cDNA contains only a single

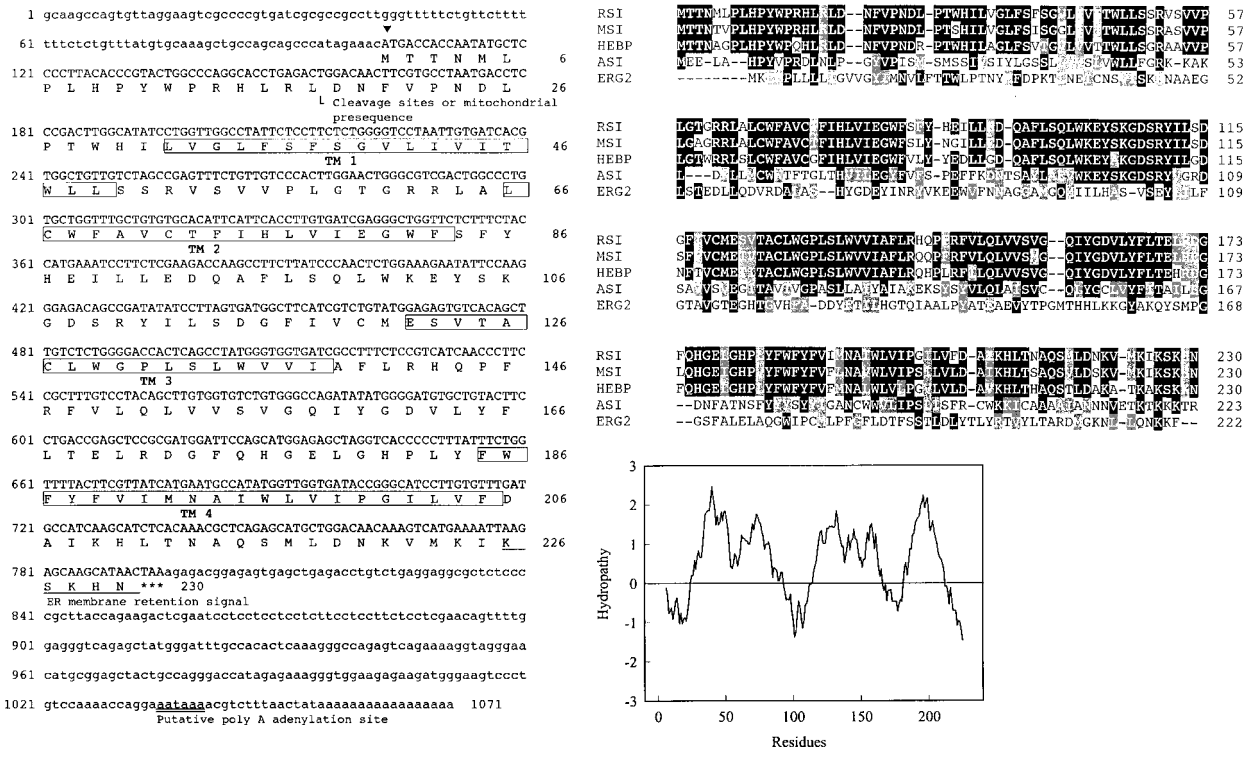


Figure 1 Nucleotide sequences and structural features of rat SI cDNA

Left-hand panel: the nucleotide sequence and the deduced amino-acid sequence. The numbers on the left refer to the nucleotide sequence, and the numbers on the right refer to the amino-acid sequence. Lower case nucleotides indicate the 102 additional 5′ nucleotides and the 276 additional 3′ nucleotides in clone SI-1. Location of the codon for the translation initiator methionine, ATG (▼), and the translation stop codon TGA (***) are indicated. The position of the AATAAA polyadenylation signal in the 3′-untranslated region is double-underlined. TM, transmembrane. Top right-hand panel: sequence alignment between SIs from different species. Residues of the rat SI (RSI) sequence (GenBank® accession number AF071501) are either identical (dark shading) or similar (light shading) to amino acids in the corresponding human EBP (HEBP) sequence (GenBank® accession number Z37986), and the mouse (MSI; GenBank® accession number X97755) and *A. thaliana* (ASI; GenBank® accession number AF030357) SI sequences. Bottom right-hand panel: Kyte–Doolittle hydrophobicity plot of the deduced SI protein sequence. Hydropathy analysis with an average window size of 19 amino acid residues plotted at one-residue intervals. On the ordinate, hydrophobicity and hydrophilicity are indicated by positive and negative numbers respectively. Domains that appear above the central line are predicted to be hydrophobic.

polyadenylation site [12]. The deduced amino-acid sequence of SI (Figure 1, left-hand panel) predicts a 230-residue protein with a calculated molecular mass of 26737 Da, identical to human (230 amino acids) [12] and mouse SI enzymes (230 amino acids) [14] (Figure 1, top right-hand panel). The relatively high degree (i.e. 57% for plant/rat SI to 93% mouse/rat SI, except yeast) of sequence similarity between the nucleotide and amino acid sequences of SIs of different species suggests that they may be involved in fundamental physiological processes that are evolutionally conserved between animals and plants (Table 1 and Figure 1, top right-hand panel).

Several structural motifs are conserved between the mouse, human and rat proteins. They are: hydrophobic regions, the transmembrane domains (boxed), and the endoplasmic-reticulum membrane retention-signal sequence (underlined) (Figure 1, left-hand panel). In transmembrane domains 2–4 of rat there are also six amino acid residues (i.e. His^{77}, Glu^{81}, Glu^{123}, Thr^{126}, Asn^{194} and Trp^{197}) that are thought to line the catalytic cleft in the human EBP [34]. As in the case of human EBP cDNA, there is also a conserved cytoplasmic domain (WKEYXKGDSRY; where single-letter amino-acid notation is used) between transmembrane domains 2 and 3 in rat SI that is believed to be important for enzyme function [34]. The rat SI polypeptide contains a highly conserved motif, GQIYGD (residues 157–162), which is thought to represent a potential N-myristoylation site [35,36]. The deduced amino-acid sequence of SI also contains a total of 15 serine residues (6.5%) and nine threonine residues (3.9%) located throughout the backbone, which may serve as putative phosphorylation sites for serine/threonine kinases as previously suggested [37]. There are also two putative protein kinase C phosphorylation sites (i.e. Ser^{50}-Ser^{51}-Arg^{52} and Thr^{60}-Gly^{61}-Arg^{62}) [38,39], and two casein kinase II phosphorylation sites (i.e. Ser^{105}-Lys^{106}-Gly^{107}-Asp^{108} and Ser^{216}-Met^{217}-Leu^{218}-Asp^{219}) [37]. The deduced amino-acid sequence of rat SI contains one potential amidation site at Thr^{60}-Gly^{61}-Arg^{62}-Arg^{63} [40,41]. A Kyte–Doolittle hydropathy plot [21] revealed that there is a large proportion of hydrophobic amino acids throughout the polypeptide backbone of the deduced amino-acid sequence (Figure 1, bottom right-hand panel). It also indicates that the conserved region of hydrophobicity may contain as many as four transmembrane domains, a feature shared by other cholesterogenic microsomal enzymes [e.g. 7-DHC reductase (DHCR)] [42].

Chromosomal localization of the SI gene by replicative R-banded FISH

The chromosomal assignment of rat SI was determined by replicative R-banded FISH with 2.4 kb genomic DNA probes. We examined 30 typical metaphase chromosomal spreads of two SD rats [31]. A single hybridization signal of rat SI genes was localized to rat chromosome 12q1.2 (Figure 2). The human EBP gene was previously reported to be localized to chromosome Xp11.22-p11.23 [16].

Functional analysis of the cloned SI cDNA

To verify the function of the cloned rat SI cDNA, a control expression vector for the cDNA (i.e. c-*myc*-YEp351ADC1) encoding a Myc-tagged SI protein (Myc–SI) was transfected into *JB811 S. cerevisiae* cells [42]. As expected, transfected yeast cells expressed an approximately 28 kDa rat SI protein, which was recognized by the anti-Myc monoclonal antibody 9E10 (Figure 3, upper panel). No protein was detected in the immunoblot of yeast cells transfected with control vector. Immunoblotting of Myc–SI, resolved by SDS/PAGE (12% polyacrylamide), showed excellent agreement with the calculated molecular mass of SI (i.e. 28054 Da). Rat SI activity in yeast crude membranes is illustrated in Figure 3 (middle panel). The overexpressed cloned Myc–SI fusion protein showed a similar pattern of inhibition to that previously seen in the rat liver microsomal protein on exposure to tamoxifen ($IC_{50} = 11.2\ \mu M$) or U18666A ($IC_{50} = 4.2\ \mu M$) when enzyme activities were determined under conditions where the activities were linear with respect to time and substrate concentration (Figure 3, lower panel). The overexpressed protein was not inhibited significantly by other sterol biosynthesis inhibitors, such as lovastatin (a specific inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase) [43] or miconazole (a specific inhibitor of lanosterol 14α-demethylase) [28,44] (Figure 3, lower panel).

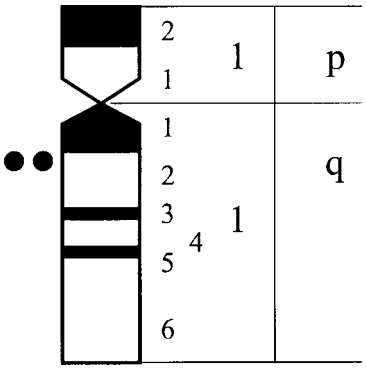


Figure 2 Assignment of the rat SI gene to chromosome 12q1.2

An idiogram of chromosome 12 summarizes the FISH analysis using the 2.4 kb genomic DNA clone covering exon 1 of the rat SI gene. Each dot represents the localization of double fluorescent signals on banded chromosome 12.

Distribution of SI mRNA

The distribution of mRNA encoding SI in different rat tissues was examined by Northern-blot analysis using a rat SI cDNA ORF probe. The signals of SI mRNA on a blot were normalized to those of β-actin mRNA [42]. As shown in Figure 4 (top panel), the approx. 1.3 kb SI transcript was highly expressed in liver, which corresponds well to the known distribution of the enzyme activity (S.-H. Bae and Y.-K. Paik, unpublished work). A detectable signal was only seen in the kidney and brain. We examined whether the predominant distribution pattern or quantities of SI mRNA in liver changed during aging. Total RNA was prepared from 3-week-old rats and 2-year-old rats (2–3 rats/group) and was subjected to Northern-blot analysis. As shown in Figure 4 (bottom panel), SI mRNA was only expressed in the liver of both age groups, suggesting that the distribution pattern of SI mRNA in liver remained the same throughout life. However, there was an approx. 70% reduction in mRNA level in the older rats, indicating that quantitative declines of gene expression occurred in liver, as seen for other genes (e.g. the cytochrome P450 gene) [45] during aging. In addition, it was noted that there was a marked difference with regard to the LDL cholesterol level between the 3-week-old rats and the 2-year-old rats. That is, the serum LDL cholesterol in 2-year-old rats (58 mg/dl, $n = 2$) was 70% higher than that in 3-week-old rats (34.5 mg/dl), while differences in other lipids were less than 20%. For example in older rats ($n = 2$), total cholesterol was 10% higher (88 versus 98 mg/dl), triacylglycerol was 13% lower (100 versus 88.5 mg/dl) and HDL cholesterol was 18% higher (53 versus 66 mg/dl). It is not yet clear whether

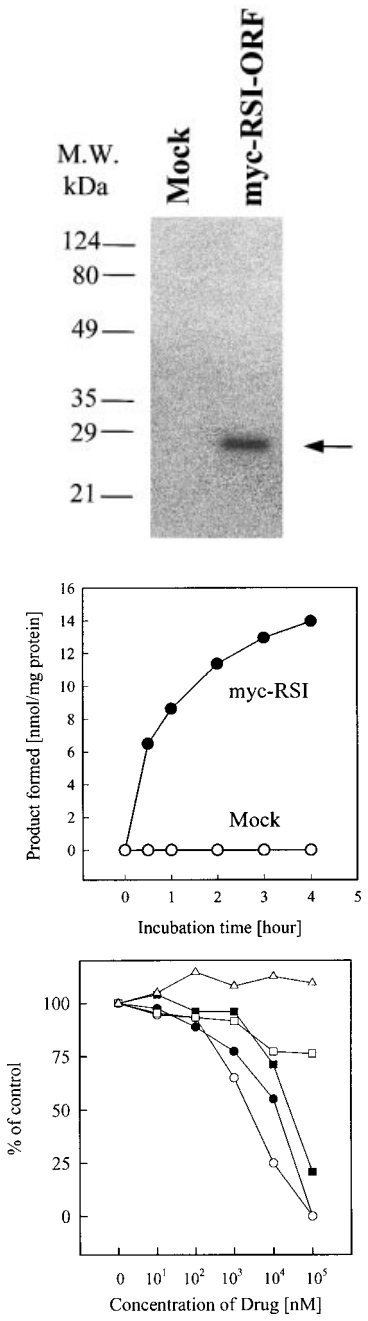


Figure 3 Heterologous expression of a Myc-tagged rat SI cDNA (Myc–RSI-ORF) in *S. cerevisiae* strain *JB811* (*ade2-1 leu2-3,112 pep4-3 trp1-289 ura3-52*)

Upper panel: Western-blot analysis was carried out as previously described [39] using 10 μg of microsomal protein that was resolved by SDS/PAGE (12% polyacrylamide). The blot was immunostained with 80 ng/ml 9E10 c-Myc antibody and was analysed with the ECL® Western-blotting detection system using a horseradish peroxidase-conjugated secondary antibody. The arrow indicates the migration of a c-Myc immunoreactive band with a molecular mass corresponding to 28 kDa. Middle panel: time course of overexpressed rat SI activity. Microsomes (0.5 mg) prepared from strains transformed with the vector (mock, ○; Myc–RSI-ORF, ●) were incubated anaerobically for the indicated times. Each value represents the average of duplicate assays from two separate experiments. Lower panel: inhibition of Myc–RSI by tamoxifen (●) (IC_{50} = 11.2 μM), U18666A (○) (IC_{50} = 4.2 μM), AY-9944 (■), miconazole (□) and lovastatin (Δ). Each drug was dissolved in Me_2SO or deionized water, the concentration of which was kept at less than 0.3% (w/v). Data represent the means of two separate experiments. Enzyme assays were carried out for 60 min in the presence of each inhibitor. The initial measurement for the inhibition of SI activity by each drug started at a concentration of 10 nM.

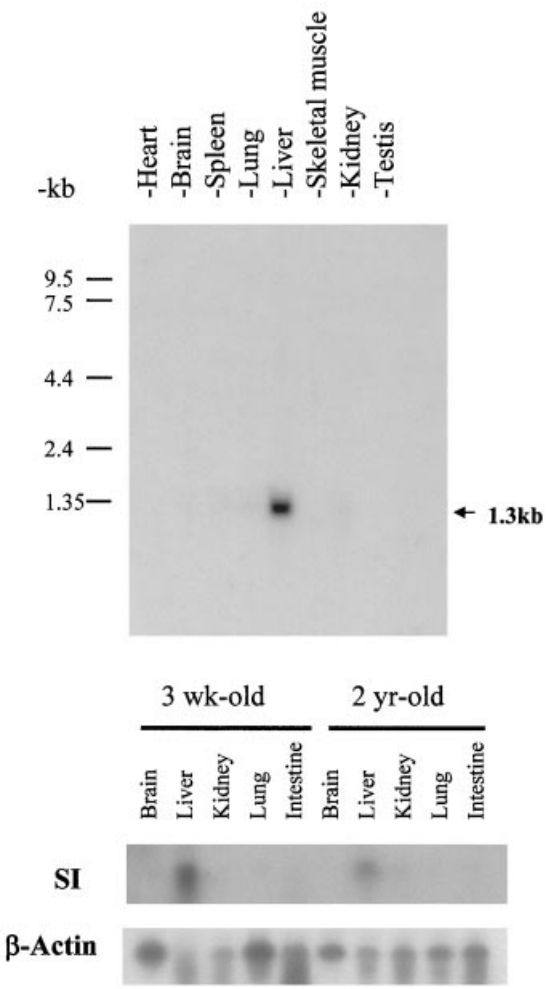


Figure 4 Tissue distribution of rat SI mRNA

Top panel: Northern-blots with 2 μg of polyadenylated RNA (rat multiple tissue Northern Blot; ClonTech) were probed with the ^{32}P-labelled rat SI cDNA ($> 10^8$ c.p.m./ng) and exposed for 16 h. The arrow indicates the migration of the 1.3 kb SI mRNA. Bottom panel: Northern blots with 20 μg of total RNA prepared from tissues of 3-week-old rats and 2-year-old rats. Shown below is the same blot for β-actin.

there is a direct correlation between the lowered level of SI gene expression and the increased serum LDL cholesterol. To determine the distribution of SI within the liver, we used *in situ* hybridization with a SI ^{32}P-labelled riboprobe. Rat liver was used because it has an abundant distribution of SI mRNA (Figure 4, top panel). The hybridization signal of the rat SI gene was ubiquitously distributed throughout the liver section (results not shown). There was no distinctive hybridization signal in Kupffer cells and ductal cells in liver, or in blood vessels (results not shown). The hybridization signal of the rat SI gene was mainly detected in the cytoplasm of hepatocytes. Our results may provide evidence that the rat SI gene may be involved in liver function, since rat SI mRNA was only detected in hepatocytes, not in connective tissue or blood vessels.

In vitro inhibition of endogenous SI gene expression by intracellular sterols

Having established that SI mRNA is highly expressed in hepatic tissues (Figure 4, top and bottom panels), we examined whether

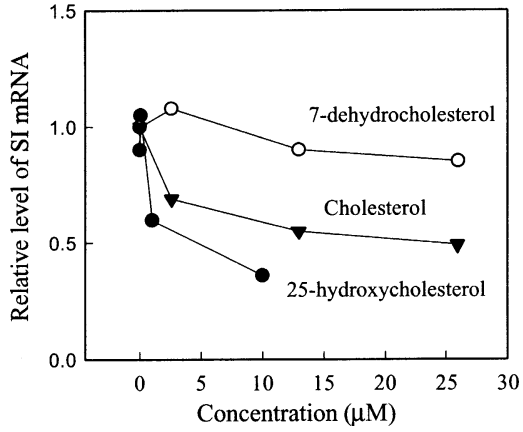


Figure 5 Effects of sterols on the expression of the SI gene

H4IIE cells were cultured in LPDS medium with the following additions: 0, 0.01, 0.1, 1 and 10 μM 25-OHC (●), or 0, 2.6, 13 and 26 μM 7-DHC (○), or cholesterol (▼). Following incubation for 24 h, total RNA was prepared from the cells, and 20 μg of RNA was subjected to Northern-blot analysis. The filter was exposed to Hyper film MP (Amersham Pharmacia Biotech) with an intensifying screen at −70 °C for 5 days. Shown here are the values normalized to that of the β-actin signal.

the endogenous SI gene can also be regulated by sterols. To determine the effects of sterols on mRNA expression in H4IIE cells, we analysed the level of SI mRNA in H4IIE cells that had been treated by representative sterols, such as 25-OHC, 7-DHC and cholesterol, at non-toxic concentrations [46,47]. Differential suppression of SI mRNA level was observed when cells were treated with sterols in LPDS medium (Figure 5). Although the concentration applied to the cells was relatively high (μM range), the sterol-mediated suppression of SI gene transcription was most prominent in the cells that were treated with 25-OHC, which is consistent with the sterol-mediated regulatory mode of other cholesterogenic genes. Although it was difficult to determine their IC_{50} values, because the inhibitory effect of two sterols (7-DHC and cholesterol) were significantly lower than that of 25-OHC, the rank order of inhibition for the sterols tested was 25-OHC > cholesterol > 7-DHC (Figure 5).

Induction of SI gene expression by tamoxifen, but not by emopamil, in the liver-derived cell lines

To examine a mechanistic insight into the regulation of SI gene expression by two representative SI ligands in the H4IIE cells, Northern-blot analyses were performed using a SI cDNA as a probe after treating the cells with drugs. Tamoxifen and emopamil were chosen because (1) they are typical ligands and/or inhibitors for SI [9–11], and (2) despite their specific inhibitory activities on SI [10], their molecular nature of inhibition of SI gene expression is not well understood. As shown in Figure 6 (upper and middle panels), a dose-dependent increase in the SI mRNA level by addition of tamoxifen, but not by emopamil, was observed when cells were treated with these different inhibitors for 24 h in FBS medium. However, the magnitude of tamoxifen-mediated SI gene induction in H4IIE cells was only seen when cells were grown in FBS medium, suggesting that the cellular sterols or other factors present in FBS medium may play a critical role in the expression of the SI gene. It seems important to note that rat SI mRNA was not induced by emopamil treatment in either medium, indicating that emopamil may exert its binding activity on SI by a different mechanism. For instance, besides their

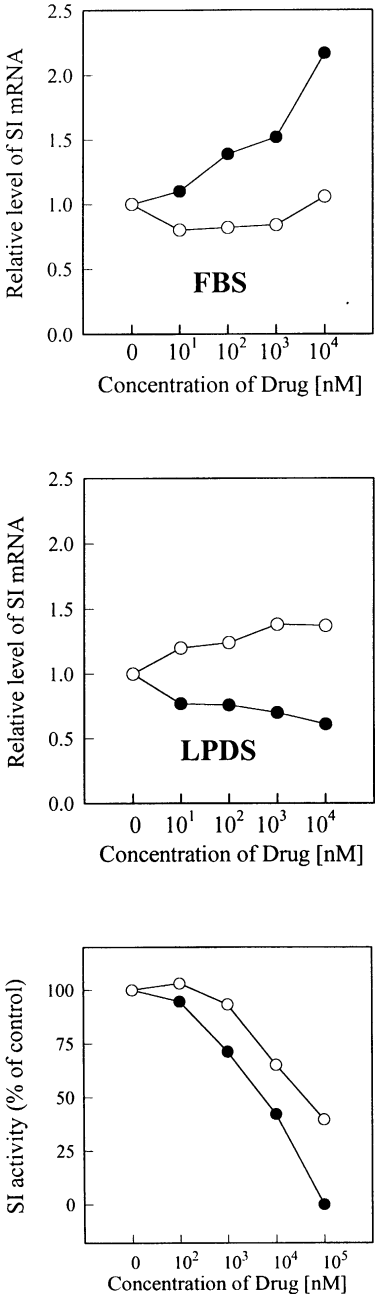


Figure 6 Differential effects of tamoxifen and emopamil on mRNA expression and enzyme activity of SI in H4IIE cells

H4IIE cells were cultured in FBS (upper panel) or LPDS (middle panel) medium with the following additions: 0, 10, 100, 1000, or 10000 nM tamoxifen (●) or emopamil (○) in FBS or LPDS respectively. Following incubation for 24 h, total RNA was prepared from the cells, and 20 μg of RNA was subjected to Northern-blot analysis. The filter was exposed to Hyper film MP (Amersham Pharmacia Biotech) with an intensifying screen at −70 °C for 5 days. Data were quantified and the average intensity ($n = 2$) of the SI mRNA band was plotted relative to the value for the SI mRNA band at 0 nM tamoxifen or emopamil. All mRNA levels in this Figure were determined using the BAS 2500 system and normalized to β-actin mRNAs determined after rehybridization. Lower panel: the *in vitro* inhibition of microsomal SI enzymic activity of H4IIE cells by tamoxifen (●) or emopamil (○).

different IC_{50} values for SI (7.2 μM for tamoxifen and 62 μM for emopamil) at the enzymic protein level (Figure 6, lower panel), they also appeared to act quite differently on SI gene expression when the cells were grown in FBS medium. Therefore SI mRNA

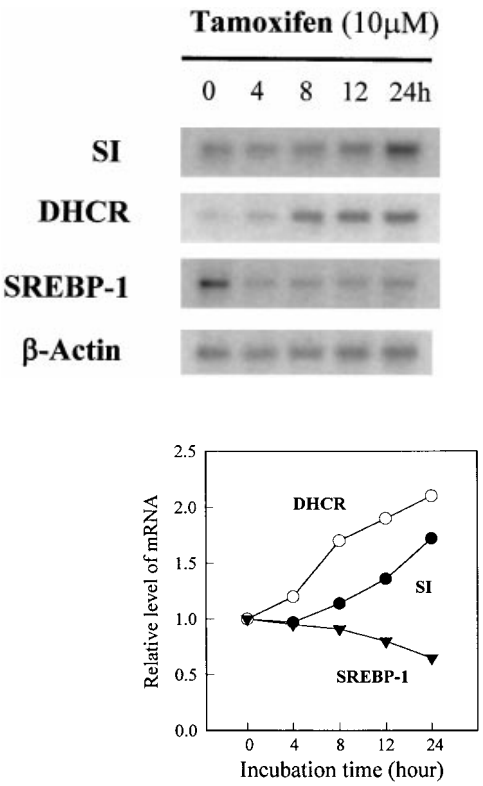


Figure 7 Time course of induction of genes for SI, DHCR and SREBP-1 by tamoxifen treatment

Top panel: the changes in mRNA levels of SI, DHCR, and SREBP-1 genes. Bottom panel: the quantification of mRNA obtained form Northern blots shown in the top panel. All mRNA levels in this Figure were determined using a BAS 2500 system and were normalized to β-actin mRNA levels, which were determined after rehybridization.

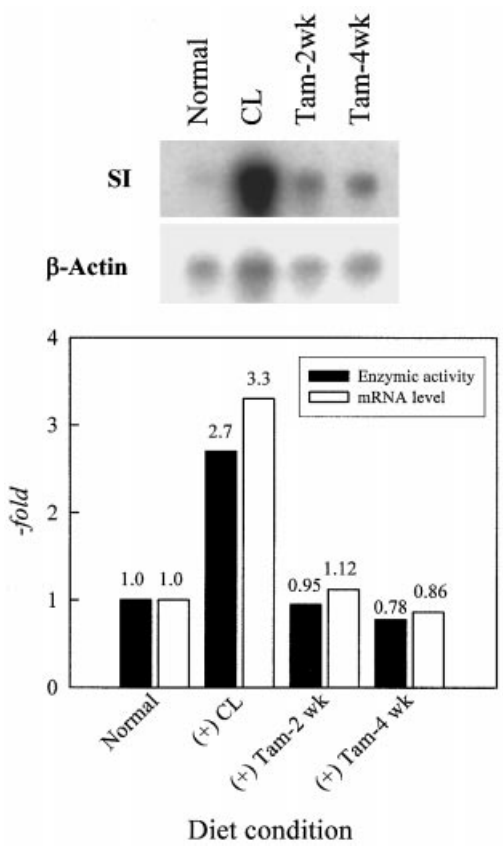


Figure 8 *In vivo* effects of cholesterol-lowering drugs on the expression of SI mRNA in rat liver

Total RNA was isolated from each liver of 3–4 rats in each feeding group. Northern-blot analysis was carried out using 15 μg of total RNA from normally fed, CL-diet- or tamoxifen-fed rats (top panel). Quantification of blots was performed using a BAS 2500 system (Fuji Photo Film Co.; bottom panel). Data are shown as means, obtained from two different blots following normalization of the SI-specific hybridization signals to those of β-actin. (+)CL, CL-diet; (+)Tam-2 wk: 0.01% (w/w) of tamoxifen in chow for 2 weeks; (+)Tam-4 wk: 0.01% (w/w) of tamoxifen in chow for 4 weeks.

expression is regulated by cellular sterol levels, which might be depleted by tamoxifen treatment, similar to that observed for many important genes of cholesterol biosynthesis. Consequently, to examine whether tamoxifen-mediated SI gene induction was related to a mode of sterol regulatory element binding protein-1 (SREBP-1) gene expression, Northern-blot analysis was performed on the RNAs that were isolated from the cells that had been harvested at every 4 h after tamoxifen treatment in FBS medium. As shown in Figure 7, SI mRNA increased in a time-dependent manner while that of SREBP-1 was gradually suppressed during the same period. These results suggest that tamoxifen-mediated induction of the SI gene in FBS medium seems to be negatively related to SREBP-1 gene expression.

In vivo effects of cholesterol-lowering drugs on the expression of rat SI mRNA in liver

In our previous report [9], the hepatic microsomal SI activity was found to be induced *in vivo* more than 3-fold by feeding rats a diet containing lipid-lowering drugs (i.e. the CL-diet). To determine whether this type of hepatic microsomal enzymic induction of SI by CL-diet feeding was in fact due to the transcriptional activation of SI gene expression in liver, male rats were fed the CL-diet for 2 weeks. As shown in Figure 8 (top and bottom panels), in the CL-diet-fed rats both hepatic SI mRNA (expressed as the density of the hybridized bands in the blot as measured by densitometric scanning) and the specific enzymic activity (expressed as nmol/min per mg of protein) were increased by approx. 3.3-fold [226 (control) compared with 747.6 (CL-diet); values were normalized to the β-actin signal, $n = 2$] and 2.7-fold [0.4 ± 0.09 (control) compared with 1.08 ± 0.12 (CL-diet], $n = 3$], respectively, relative to the control group in three independent experiments. None of the other tissues (e.g. heart, brain and kidney) showed any increases in SI mRNA levels (results not shown). Thus the *in vivo* SI induction in liver by the CL-diet shown in the present study and previously seen [9] was mainly due to an elevation of hepatic SI mRNA level in rats.

To understand a molecular mechanism of action of tamoxifen against SI activity as previously reported [10], Northern-blot analysis was performed on total RNAs extracted from livers of rats fed tamoxifen for 2 or 4 weeks. Surprisingly, the level of SI mRNA essentially remained unchanged in liver at this dose of tamoxifen [0.01% (w/w) in chow]. The discrepancy between the *in vitro* and the *in vivo* SI gene regulation by tamoxifen remains to be determined. This result, however, suggests that different mechanisms of action by tamoxifen may exist between the *in vitro* (in cultured cells) and the *in vivo* system (in animals). Failure of the same dose of tamoxifen to alter the level of expression of SI mRNA (2 week-fed group, from 226 to 254;

4 week-fed group, from 226 to 194.8; values normalized to β-actin signal $n = 2$) suggests that the mechanism of action of this inhibitor may occur more rapidly at the protein level, as seen previously [10], than at the transcriptional level. We do not rule out the possibility that the long-term ingestion of higher doses of tamoxifen (e.g. > 16 months) [48] may modulate the level of transcription of the SI gene *in vivo*.

DISCUSSION

It is worth noting that SI has three unique features with regard to both enzymic properties and its related diseases (e.g. hypercholesterolaemia, ischaemia and genetic diseases, such as CDP and CHILD syndrome) [13–19]. First, unlike other cholesterol biosynthesis enzymes, SI does not require any reducing cofactor (i.e. NADPH) or metal ions for catalysing the fastest enzyme reaction among the distal enzymes in cholesterol biosynthesis from lanosterol [7,8,10]. In fact our sequence information for cloned SI cDNA revealed the absence of a putative NADPH binding site that normally exists in other members of the sterol biosynthesis enzyme family [23,42]. Secondly, although it has multiple functions as the binding target for breast cancer drugs (e.g. tamoxifen), anti-ischaemic drugs (e.g. emopamil) and antifungals (e.g. tridemorph) [11], there may be different mechanisms of action with respect to regulation of expression and tissue-specificity of the SI gene. For instance, when H4IIE cells were treated with either tamoxifen or emopamil, only tamoxifen-treated cells showed a dose-dependent induction of SI mRNA (Figure 6). Thirdly, mutations in the SI gene are related to multiple genetic diseases, such as CDP and CHILD syndrome [16–19]. The common transmembrane domain 2–4 of the SI protein is evolutionarily well-conserved, so mutations in the SI gene, especially, may be very important for understanding the role for SI in genetic diseases of human and the tattered (*Td*) mouse. For example, heterozygous mutations in human CDPX2 patients including three mis-sense (E80K, S133R and R147H), one nonsense (R63X), one frameshift (216-217insT) and two splice site (IVS2-2delA, IVS3 + 1GT) mutations have been found in transmembrane domains 2–4 [16]. Glu^{80} was found to be required for both catalytic activity and inhibitor binding of SI [34]. A single amino acid substitution (G107R) in SI was also found to be the cause of the defect in *Td* mice, as well as seven unrelated CDPX2 patients [17]. In addition, SI has relatively high hydrophobicity (Figure 1, bottom right-hand panel), and a combination of detergents are required to solubilize the SI enzyme from the microsomes for purification [8,9].

SI also shares some common features with other cholesterogenic enzyme(s). Our results demonstrated that SI mRNA expression was increased when H4IIE cells were cultured in the absence of regulatory sterols (e.g. 25-OHC) (Figure 5) or endogenous sterols compared with when the same cells were fed sterols (i.e. cultured in FBS) (results not shown). It is well known that the addition of cholesterol to cultured cells only weakly suppresses the SI gene, whereas the addition of 25-OHC brings about a sharp decrease in transcription of the SI gene. Although it did not show a typical dose-dependent inhibition (because of its higher inhibitory concentration), the inhibitory activity of 25-OHC might result from its dual roles: suppression of *de novo* cholesterol biosynthesis and stimulation of esterification of pre-existing sterols as previously described [49]. This pattern of reduced gene expression by exogenous sterols is similar to that of other distal cholesterogenic enzymes, such as squalene synthase [50].

There are at least two differences between EBP and SI with respect to the pattern of gene expression. First, despite their same function as SI or EBP, there is a clear difference in their tissue-specific mRNA expression patterns in rodents. That is, EBP (in guinea pig) is expressed in a wide variety of tissues, including liver, ileum, colon, kidney, adrenal gland, testis, ovary, and uterus [14] while SI mRNA (in rat) is expressed in a liver-specific manner (Figure 4, top panel). Secondly, although tamoxifen and emopamil function as common ligands for both SI and EBP, only the former was able to induce the SI mRNA in the liver-derived cell lines (Figure 6). This suggests that emopamil and tamoxifen may not share the same active site and therefore they may exhibit different action mechanisms for modulating their target protein(s) in cultured cells.

The inhibition of cholesterol biosynthesis by tamoxifen has been well documented in cultured cells, such as oestradiol receptor-dependent MCF-7 cells, oestradiol receptor-independent BT20 cells [51], and HepG2 cells [49]. Our previous results suggested that tamoxifen directly inhibits SI as a prime target, thereby significantly lowering the cholesterol level in the mammalian cells [10]. When tamoxifen was added to H4IIE cells, the SI gene was induced in both a time- and dose-dependent manner (Figure 7). This induction of the cholesterogenic gene is generally well documented, such as compactin-mediated induction of the HMG-CoA reductase gene [53], in which the SREBP pathway might be commonly involved [52]. It is not yet clear why SREBP-1 mRNA was suppressed paradoxically under the conditions in which the cellular sterol level might be reduced by tamoxifen treatment (Figure 7). It was also noted that the magnitude of induction for DHCR mRNA was much higher than that for SI mRNA, suggesting that the sterol-sensing domain present in DHCR (not in SI) [42] may be activated under tamoxifen-mediated sterol depletion. In our preliminary data, the 5′-flanking region of both SI and DHCR genomic sequences contain SRE sequences and several Sp-1 binding sites, which seem to be involved in sterol-mediated suppression of the SI gene (H. J. Shin and Y.-K. Paik, unpublished work). This requires further studies into the transcription factors that may be involved in tamoxifen-mediated regulation of these genes.

Although the molecular processes leading to the progressive decline of enzyme activities during aging are not yet clear, there is compelling evidence that changes in gene expression, and concomitant changes in mRNA level, may underlie age-related gene regulation. In this regard, the relative expression level of SI mRNA in liver significantly declined during aging (up to 70 % in 2-year-old rats) (Figure 4, bottom panel). Our main intention for this experiment, however, was to examine any quantitative or qualitative changes in SI gene expression in rats of two different age groups. More studies are required to determine whether the age-related changes in SI mRNA levels in liver may be a consequence of an alteration in gene transcription, altered processing of the transcripts or others.

In conclusion, our results on the molecular characterization of SI cDNA and the primary structure of the enzyme presented in this study will be very useful in establishing the mechanism of tissue-specific regulation of the SI gene, as well as the physiological function of this enzyme, especially in brain disease (sigma receptor) [13] and the Conradi-Hünermann syndrome [16–18].

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