Transcriptional activities of nuclear SREBP-1a, -1c, and -2 to different target promoters of lipogenic and cholesterogenic genes

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Abstract Recent studies on the in vivo roles of the sterol regulatory element binding protein (SREBP) family indicate that SREBP-2 is more specific to cholesterogenic gene expression whereas SREBP-1 targets lipogenic genes. To define the molecular mechanism involved in this differential regulation, luciferase-reporter gene assays were performed in HepG2 cells to compare the transactivities of nuclear SREBP-1a, -1c, and -2 on a battery of SREBP-target promoters containing sterol regulatory element (SRE), SRE-like, or E-box sequences. The results show first that cholesterogenic genes containing classic SREs in their promoters are strongly and efficiently activated by both SREBP-1a and SREBP-2, but not by SREBP-1c. Second, an E-box containing reporter gene is much less efficiently activated by SREBP-1a and -1c, and SREBP-2 was inactive in spite of its ability to bind to the E-box. Third, promoters of lipogenic enzymes containing variations of SRE (SRE-like sequences) are strongly activated by SREBP-1a, and only modestly and equally by both SREBP-1c and -2. Finally, substitution of the unique tyrosine residue within the basic helix-loop-helix (bHLH) portion of nuclear SREBPs with arginine, the conserved residue found in all other bHLH proteins, abolishes the transactivity of all SREBPs for SRE, and conversely results in markedly increased activity of SREBP-1 but not activity of SREBP-2 for E-boxes. These data demonstrate the different specificity and affinity of nuclear SREBP-1 and -2 for different target DNAs, explaining a part of the mechanism behind the differential in vivo regulation of cholesterogenic and lipogenic enzymes by SREBP-1 and -2, respectively.—Amemiya-Kudo, M., H. Shimano, A. H. Hasty, N. Yahagi, T. Yoshikawa, T. Matsuzaka, H. Okazaki, Y. Tamura, Y. Iizuka, K. Ohashi, J-i. Osuga, K. Harada, T. Gotoda, R. Sato, S. Kimura, S. Ishibashi, and N. Yamada. **Transcriptional activities of nuclear SREBP-1a, -1c, and -2 to different target promoters of lipogenic and cholesterogenic genes. *J. Lipid Res.* 2002.** 43: **1220–1235.**

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The liver synthesizes both cholesterol and fatty acids; however, their biosynthetic pathways are under distinct and separate regulation (1). In contrast to cholesterol synthesis, which is tightly regulated by a feedback system to maintain cellular cholesterol levels, fatty acid synthesis is driven primarily by the availability of carbohydrates. Despite these different patterns of regulation, recent evidence suggests that both biosynthetic pathways can be controlled by a common family of transcription factors designated sterol regulatory element binding proteins (SREBPs) (2, 3). SREBPs belong to a large class of transcription factors containing basic helix loop helix (bHLH)-Zip domains. Unlike other members of this class, SREBPs are synthesized as membrane-bound precursors that require cleavage by a two-step proteolytic process in order to release their amino-terminal bHLH-Zip-containing domain into the nucleus to bind to a specific DNA sequence, sterol regulatory element (SRE), and activate their target genes in a sterol-regulated manner (2). Another unique feature of this family is their DNA binding specificity. One family member, SREBP-1c, also named

Abbreviations: ACL, ATP citrate lyase; bHLH, basic helix loop helix; FAS, fatty acid synthase; FPP, farnesyl diphosphate; G6PD, glucose-6-phosphate dehydrogenase; GK, glucokinase; ME, malic enzyme; PK, pyruvate kinase; S14, spot 14; SRE, sterol regulatory element; SREBP, sterol regulatory element binding protein.

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ADD1, was cloned independently as a protein that binds to E-boxes, universal *cis*-elements for bHLH proteins, and presumably promotes adipocyte differentiation (4). Therefore, ADD1 has dual binding specificity to both classic palindromic E-boxes and nonpalindromic SREs (5). This unique binding specificity is attributed to the tyrosine residue in the basic region, which is unique to the SREBP family, whereas other common bHLH proteins have arginine at this position (5).

Although the list of SREBP target genes is expanding, they are functionally categorized into two groups. The first group includes cholesterol biosynthetic genes such as HMG-CoA synthase, HMG-CoA reductase, farnesyl diphosphate synthase, squalene synthase, and SREBP-2 itself, as well as the LDL receptor gene, each of which contain the classic SRE sequence (ATCACCCCAC) or its modified form SRE3 (CTCACACGAG), and adjacent cofactor (NF-Y or Sp1) sites in their promoters (6–9). The second group represents lipogenic enzyme genes that have been known to be nutritionally regulated at a transcriptional level (10, 11). They include acetyl-CoA carboxylase, fatty acid synthase (FAS), stearoyl CoA desaturase 1 and 2, glycerol-3-phosphate acyltransferase, diazepam-binding inhibitor/acyl-CoA-binding protein, and spot 14 (S14), for which promoter analyses revealed that SREBP binding and activation sites in their promoters seem to have more diverse consensus sequences than the classic SRE, and are tentatively designated SRE-like sequences (12–17). Some lipogenic enzyme genes such as S14 (16), FAS, liver-type pyruvate kinase (PK), and glucokinase (GK) contain E-boxes or E-box like sequences in their promoters that could confer carbohydrate, glucose, or insulin response sensitivity (18, 19), and could also be potential SREBP-targets due to the dual binding specificity of SREBPs to these sites (5).

To date, three SREBPs have been identified; SREBP-1a and SREBP-1c are produced from a single gene through the use of alternate promoters, and SREBP-2 is transcribed from a separate gene. All actively growing cultured cells studied to date produce predominantly SREBP-1a and SREBP-2, whereas most organs, including liver, from adult animals predominantly synthesize SREBP-1c and SREBP-2 (20). All three SREBPs are capable of activating each of the known target genes, although with differing efficiencies. SREBP-1c is weaker than SREBP-1a and SREBP-2 due to its shorter transactivation domain (21, 22).

To gain insight into the distinct roles of each SREBP isoform in vivo, transgenic mice that overexpress truncated, active nuclear forms of human SREBP-1a, -1c, or -2 in liver, were produced and characterized (21, 23, 24). The different SREBP-overexpressing transgenic animals showed different patterns of increase in hepatic synthesis and accumulation of cholesterol and/or fatty acids. These data suggest that the SREBP-1 isoforms are more selective in activating fatty acid biosynthetic genes, while SREBP-2 is more specific for controlling cholesterol biosynthesis.

From other lines of evidence, the physiological role of hepatic SREBP-1c as a nutritional regulator of lipogenic enzymes has been implicated by the distinct regulation of SREBP-1c and -2 in different nutritional conditions. The amounts of SREBP-1c protein and mRNA, but not those of SREBP-2, dramatically increase when mice are placed on a high carbohydrate diet or after refeeding fasted mice in a similar manner to changes in lipogenic enzyme mRNAs (25, 26). Furthermore, dietary polyunsaturated fatty acids, known to inhibit hepatic lipogenic enzyme expression, decreased the amount of nuclear SREBP-1c protein without affecting nuclear SREBP-2 protein (27–29); therefore, SREBP-1c seems to be a mediator of this polyunsaturated fatty acid regulation (16, 27). Finally, using survivors of SREBP-1 deficient mice, it was confirmed that SREBP-1 is responsible for nutritional induction of hepatic lipogenic enzymes whereas SREBP-2 controls cholesterol biosynthesis (30). All these in vivo data established distinct roles of SREBP-1c and -2 in hepatic lipogenesis and cholesterogenesis, respectively. Whereas SREBP-2 exerts sterol regulation through cleavage of the membrane-bound precursor protein to liberate the active nuclear form into the nucleus, SREBP-1 controls lipogenic enzymes by self-regulating its own transcription level. However, their precise molecular mechanisms are not fully understood. Present studies were performed to test the hypothesis that the molecular differences in transcriptional activities of nuclear SREBP isoforms can explain the distinct specificities in activation of cholesterol biosynthetic and lipogenic genes in vivo. Luciferase reporter gene assays in HepG2 cells were performed to compare transcriptional activities of SREBP wild-type isoforms and their YR mutant versions (substitution of tyrosine for arginine) for a whole battery of promoters of cholesterogenic and lipogenic genes. The results clearly demonstrated that SREBP-1a, -1c, and -2 showed differing DNA binding specificities to SRE, SRE-like, and E-box sequences, with SREBP-1a being the strongest activator for all of these targets. However, hepatic major isoforms SREBP-1c and -2 showed similar activities for lipogenic enzyme genes. Therefore, to account for distinct in vivo roles of SREBP-1 for lipogenesis, there must be a linked but distinct nutritional and metabolic regulation in the supply of nuclear forms of SREBP-1 and SREBP-2.

EXPERIMENTAL PROCEDURES

Materials

Standard molecular biology techniques were used. We obtained cholesterol and 25-hydroxycholesterol from Sigma, *N*-Acetyl-Leu-Leu-norleucinal-CHO (ALLN, calpain inhibitor I) from Calbiochem, Redivue [α-^{32}P]dCTP (6,000 Ci/mmol) from Amersham Pharmacia, and restriction enzymes from New England Biolabs. Plasmid DNAs for transfection were prepared with Endo-Free Plasmid Maxi kits (Qiagen).

Construction of expression, luciferase-reporter plasmids

Cytomegalovirus (CMV) promoter expression plasmids containing cDNA encoding human nuclear form of SREBP-1a and -1c (pCMV-SREBP-1a and -1c) were prepared as described (21).

Human truncated SREBP-2 expression plasmid (pCMV-SREBP-2) was prepared by inserting the same cDNA fragment used for the transgene construct of TgSREBP-2 (24) into the *Sma*I site of pCMV7. The tyrosine residue in the basic region of each SREBP expression plasmid (at amino acid 335 for SREBP-1a, 321 for SREBP-1c, and 342 for SREBP-2) was altered to an arginine residue by site-directed mutagenesis using a commercial kit (Transformer, Invitrogen). These YR mutated versions were designated pCMV-SREBP-1aM, pCMV-SREBP-1cM, and pCMV-SREBP-2M.

Luciferase gene reporter plasmids for artificial multimerized SRE promoter, human LDL receptor, HMG-CoA synthase, farnesyl diphosphate synthase, and fatty acid synthase gene promoters (SRE-Luc, pLDLR-Luc, pHMGCoASyn-Luc, pFPP-Luc, and pFAS-Luc, respectively) were prepared as previously described (21, 31). Luciferase reporter genes for promoters of mouse ATP citrate lyase (ACL), rat malic enzyme (ME), rat glucose-6-phosphate dehydrogenase (G6PD), rat liver-type pyruvate kinase (PK), rat spot 14 (S14), and rat GK genes (pACL-Luc, pME-Luc, pG6PD-Luc, pPK-Luc, pS14-Luc, and pGK-Luc) were constructed as follows. Each promoter was obtained by PCR using Pfu DNA polymerase (Stratagene), and rat or mouse genomic DNA as a template. The primers used were: rat ACL 5′ primer, AGCCCCTGAAGCGATCAGGCCACA (−300), 3′ primer, GGATCTCTCCAGCCGCCTGCAGCTG (+15) (32); rat ME 5′ primer, GAATTCGCATAGCCCAGAAGCTATAGCTGT (−783), 3′ primer, CGGGAGTGCGGCGGGCGGCGGCCGTGCGAC (+7), (33); rat G6PD 5′ primer, GTCGGCCAGAAGTGAAGAGGGCAGGAGCAG (−926), 3′ primer, TTTAGTTGCCGCTGCCAAACACGTTCACAG (+58) (34); rat PK 5′ primer, GACAGGCCAAAGGGGATCCAGCAGCATG (−197), 3′ primer, ACGTTGCTTACCTGCTGTGTCTGTGGGTCT (+12) (35); rat S14, 5′ primer CCTTCTAACTGGTTGAGCAGCTG (−250), 3′ primer, AACTCAGAGACCAGCAAACTGC (+12) (36); and rat GK 5′ primer CCCACGAGGATCCCCCACTG (−318), 3′ primer CTTCCGCACTAACGGGCCTGA (+11) (37).

A restriction site, *Kpn*I and *Hind*III (or *Xho*I), was added to each 5′ primer and 3′ primer, respectively. The PCR products were digested with *Kpn*I and *Hind*III (or *Xho*I), and were inserted into the respective sites of pGL2-Basic Vector (Promega). For pE-box-Luc, six copies of carbohydrate response element at position (−1448 to −1422) of the rat S14 gene (GCCAGTTCTCACGTGGTGGCCCTGTGC) (18) were inserted into the *Sma*I site of pGL2-Promoter Vector (Promega) in which the SV40 early promoter was added to the pGL2-Basic Vector. Other enhancer constructs were also produced by inserting the following oligonucleotides into *Sma*I/*Nhe*I sites of pGL2-Promoter Vector. SRE+Sp1: gctagcaaaatcaccccactgcaaactcctccccctgcgctagc; SRE-like+NFY (7 bp): gctagcaaaatcgcctgatgcaaactattggctgcgctagc; E-box: cccgggaaatcacgtgatgcgctagc; E-box+Sp1:cccgggaaatcacgtgatgcaaactcctcccctgcgctagc; E-box+NFY: cccgggaaatcacgtgatgcaaactattggctgcgctagc; SRE-like + NF-Y enhancer region from S14 promoter was produced by PCR using the following primers and S14-Luc as a template: 5′ primer: aaacccgggtttgtccctgggtag and 3′ primer: AAAGCTAGCTTGTTTTGAGCCAATCCC. PCR product was inserted into *Sma*I/*Nhe*I sites of pGL2-Promoter Vector.

Transfections and luciferase assays

Human hepatoma HepG2 cells were cultured in DMEM containing 25 mM glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate supplemented with 10% FCS. On day 0, cells were plated on a 12 well plate at 4.5×10^4 cells/well. On day 2 each expression plasmid (indicated amount or 0.5 μg), each luciferase reporter plasmid (0.5 μg), and an SV-β-galactosidase reference plasmid (pSV-β-gal, Promega, 0.5 μg) was transfected into cells using SuperFect Transfection Reagent (QIAGEN) according to the manufacturer's protocol. As a negative control, the same amount of basic plasmid CMV7 (38) was transfected in place of expression plasmids. Five hours after transfection, the cells were incubated in the same medium supplemented with cholesterol (10 μg/ml) and 25-hydroxycholesterol (1 μg/ml) to suppress endogenous SREBP activity for an additional 16 h prior to harvest. The amount of luciferase activity in transfectants was measured and normalized to the amount of β-galactosidase activity as measured by standard kits (Promega).

Gel mobility shift assay

Gel mobility shift assays were performed as previously described (19). DNA used as probes were as follows: SRE probe, the sequence from SRE and Sp1 sites of human LDL receptor gene promoter and used for construction of pSRE-Luc; E-box probe, the sequence from the S14 gene enhancer region used for construction of pE-box-Luc (refer to construction of expression, luciferase-reporter plasmids); DNA fragment A (GGCCCGCCCCTCACCCGTCGGTGCCCAGGTC) and B (TCGCCACCCCCTCTCGCCACCCACGCCCGCCCC) from the rat malic enzyme gene promoter (34) as indicated in Fig. 7. The DNA probes were labeled by fill-in reaction with Klenow enzyme and [α-^{32}P]dCTP after annealing the oligomers, and were purified on G50 Sephadex columns. The labeled DNA was incubated with a recombinant protein (100 ng) of human nuclear SREBP-1a, -2, or nuclear extracts (1 μg) from cells transfected with each CMV-SREBP expression plasmid as described in transfection and luciferase studies in a mixture containing 10 mM Tris-HCl, pH 7.6, 50 mM KCl, 0.05 mM EDTA, 2.5 mM $MgCl_2$, 8.5% glycerol, 1 mM dithiothreitol, 0.5 μg/ml poly(dI-dC), 0.1% Triton X-100, and 1 mg/ml nonfat milk for 30 min on ice. The DNA-protein complexes were resolved on a 4.6% polyacrylamide gel.

Immunoblot analysis

Some sets of HepG2 cells for transfection studies were incubated for the last 2 h in medium supplemented with ALLN (5 μg/ml). The nuclear extracts were prepared and subjected to immunoblot analysis using anti-human SREBP-1 or SREBP-2 antibody as the primary antibody as previously described (21).

Northern blot analysis of SREBPs in livers from transgenic mice

Transgenic mice overexpressing human SREBP-1a, -1c, and -2 under the control of the rat phosphoenolpyruvate carboxykinase promoter were established as described (21, 23, 24). Homozygous mice for Tg-SREBP-1c and -2 were obtained by mating hemizygotes. 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. Total liver RNAs were prepared using TRIZOL reagent (GIBCO BRL, Life Technologies). Northern blot analyses were performed with the indicated cDNA probes as previously described (23).

RESULTS

Distinct in vivo activation of cholesterogenic and lipogenic genes by SREBPs in livers of transgenic mice

Gene expression of different cholesterogenic and lipogenic enzymes in the livers of transgenic mice overexpressing nuclear forms of SREBP-1a, -1c, and -2 was estimated by Northern blot analysis in the same set of

experiments for direct comparison. Overexpressed transgene products were mature active forms that automatically enter the nucleus and activate the target genes irrespective of the cleavage processing of precursor proteins. The SREBP-1a, -1c, -2 transgenic lines were prepared so that the protein amounts of the transgene products in the liver nuclei were comparable (24). Therefore, transcriptional activities of the three isoforms for a target gene can be compared by mRNA levels of the gene in transgenic and wild type livers. As shown in **Fig. 1**, the patterns of induction of mRNAs by SREBP isoforms as compared with wild type were very similar for low density lipoprotein receptor (LDLR), HMG-CoA synthase, and farnesyl diphosphate (FPP) synthase genes. SREBP-1a and -2 were equally strong for activation of these cholesterogenic genes whereas SREBP-1c was essentially inactive. For lipogenic enzyme genes, SREBP-1a profoundly activated most lipogenic genes tested here (S14, FAS, ACL, ME, and G6PD). Although to a lesser extent than SREBP-1a, SREBP-1c and -2 overexpression induced mRNA levels of these genes to similar extents. Among glycolytic genes, liver-type pyruvate kinase (PK) was modestly activated while only a slight activation of glucokinase (GK) was detected by overexpression of SREBP-1a. Although it has been suggested that SREBP-1c could be a mediator of hepatic gene induction by insulin, overexpression of SREBP-1c in our transgenic mice did not induce PK or GK gene expression in the liver.

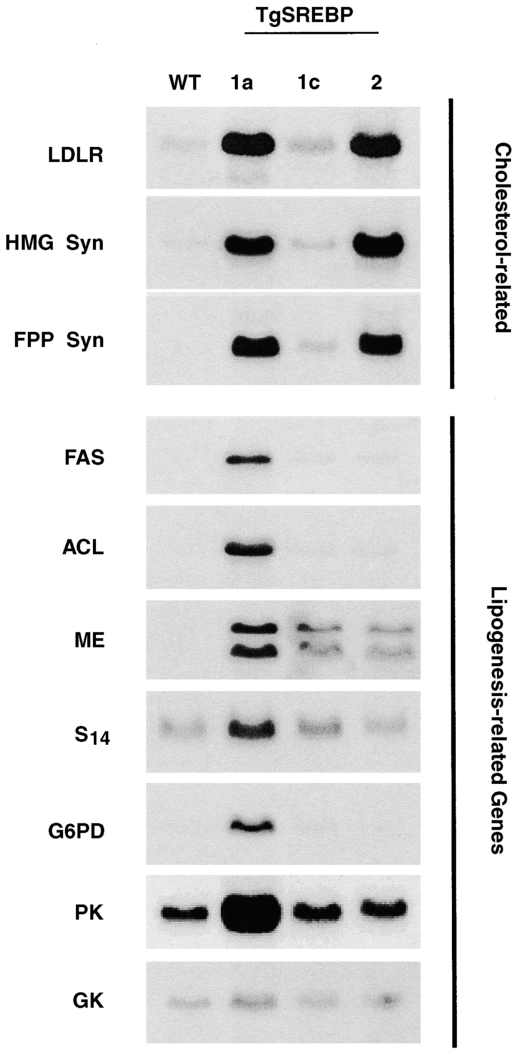

Fig. 1. Hepatic mRNA levels of cholesterogenic and lipogenic enzymes in nuclear SREBP-1a, -1c, and -2 transgenic mice as measured by Northern blot analysis. Transgenic mice overexpressing human nuclear SREBP-1a, -1c, and -2 under the phosphoenolpyruvate carboxykinase promoter were constructed as described (23, 24). Experimental animals were homozygous for SREBP-1c and -2 and hemizygous for SREBP-1a, to attain comparable levels of transgene products. Controls were wild-type littermates of SREBP-1a transgenic mice. Three male animals from each group were fed a high protein/low carbohydrate diet for 2 weeks and fasted 12 h prior to sacrifice. Aliquots of total RNA prepared from livers were pooled (15 μg) and subjected to Northern blot analysis with indicated cDNA probes as previously described (24).

Luciferase assays for cholesterogenic and lipogenic gene promoters

To further elucidate the molecular mechanism of different activities of SREBP isoforms for cholesterogenic and lipogenic enzymes, we set up luciferase reporter gene assays for different gene promoters and compared transcriptional activities of nuclear SREBP-1a, -1c, and -2 for them. **Figure 2** shows a schematic representation of the promoter constructs of the genes to be tested for the luciferase reporter gene assays. The promoters of cholesterogenic genes (LDLR, HMG-CoA synthase, and FPP synthase genes) have been extensively studied and shown to contain one or two functional classic SRE or SRE3 sites with adjacent cofactor binding sites (Sp1 or NF-Y), which are indispensable for SREBP activation (9, 39–41). In contrast, lipogenic enzyme gene promoters contain variable regulatory sequences for SREBP. They can be roughly separated into two groups: SRE-like containing promoters and E-box containing promoters. The former group includes FAS, ACL, ME, and S14. These genes contain regulatory sequences that resemble a classic SRE. In contrast, the latter groups are E-box containing promoters, which include S14, PK, GK, and FAS. Some of their E-box sequences have been reported to be responsible for nutritional regulation acting as carbohydrate, glucose, or insulin response elements (18, 19). Previous data from SREBP-1 knockout mice indicate that SREBP-1 could be somehow involved in nutritional regulation of these enzymes, possibly through these E-boxes. Because of the low stringency in defining putative SRE-like sequences, some genes, such as FAS and S14, can be included in both groups. The complexity of the FAS promoter will be discussed later. Although the exact binding sites of SREBPs in ME and G6PD promoters have yet to be identified by precise promoter analysis, their expressions have been shown to be controlled by SREBPs in studies with transgenic and knockout mice (30, 42). Putative elements responsible for the effect of SREBPs in these promoters are illustrated in Fig. 2. The ACL promoter contains an SRE-like sequence with a close NF-Y site that is responsible for SREBP activation (43).

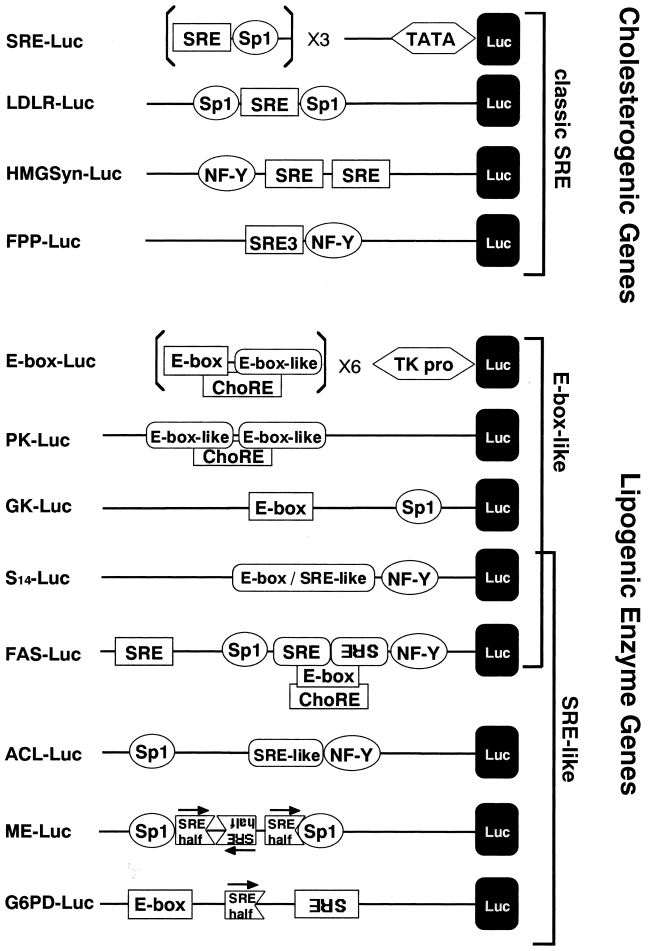

Fig. 2. Schematic representation of promoters of cholesterogenic and lipogenic enzyme genes used for luciferase reporter gene (Luc) assays in the current study. Cholesterogenic enzyme gene promoters contain one or two classic SREs accompanied by cofactor binding sites (NF-Y or Sp1). Lipogenic enzyme promoters are classified into two groups: SRE-like containing promoters and E-box containing promoters. SREBP binding sites for pyruvate kinase (PK), glucokinase (GK), malic enzyme (ME), and glucose-6-phosphate dehydrogenase (G6PD) gene promoters have not been identified, and thus binding of SREBPs to E-boxes or SRE-like sequences in those genes is putative. Figure is not to scale. FPP, farnesyl diphosphate synthase; S14, spot 14; PK, pyruvate kinase; GK, glucokinase; FAS, fatty acid synthase; ACL, ATP citrate lyase; ME, malic enzyme; G6PD, glucose-6-phosphate dehydrogenase.

Expression vectors encoding nuclear SREBP-1a, -1c, or -2 under the CMV promoter were constructed and the transcriptional activities compared for different promoter-luciferase constructs in human hepatoma HepG2 cells. We also set up a mutant version (M) for each SREBP expression vector in which an arginine residue was substituted for the tyrosine residue that is conserved in the basic region in the SREBP family. This tyrosine residue renders ADD1, the rat homolog for SREBP-1c, unique in terms of dual DNA binding specificity to SREs as well as to E-boxes (5). In contrast, other bHLH members have a conserved arginine at this position, and have binding affinity only to E-boxes, and not to SRE. The YR mutation of SREBPs was predicted to alter their binding specificity to their target genes and to give a clue as to functional and conformational distinction of SREBPs.

Prior to performing luciferase assays, the expression level of each SREBP nuclear form was estimated by an immunoblot analysis. Nuclear proteins were prepared from the same set of experiments as used for luciferase reporter assays. As shown in Fig. 3B, expression plasmids for SREBP-1a, -1c, -1aM, and -1cM produced similar amounts of the truncated proteins in the nuclear extracts of the transfected cells. The amounts of SREBP-2 and -2M were also similar. Although direct comparison of protein amounts expressed from SREBP-1 and -2 constructs is difficult, mRNA levels were similar and comparable (data not shown). Therefore, luciferase assays in the same set of experiments can afford quantitative results for relative transcriptional activities among different constructs.

Cholesterogenic enzyme gene promoters

The transcriptional activity of each SREBP expression plasmid for SRE was estimated using an optimum artificial SRE-reporter gene (44). In this SRE-Luc construct, three copies of a classic SRE (ATCACCCCAC)-Sp1 site, formerly designated repeats 2 and 3 in the analysis of the promoter of LDL receptor gene, were connected to a TATA box (44) (Fig. 2). After transfection, HepG2 cells were incubated in a medium containing FCS supplemented with cholesterol and 25OH cholesterol to suppress endogenous SREBP activities. As shown in **Fig. 3A**, we estimate the transcriptional activity of each SREBP isoform in a range between 0.1 ng and 500 ng of transfected expression plasmid DNA. The maximal effect was observed at 10 ng and, at higher concentrations, the activity was decreased, showing relatively high affinity and low saturation kinetics between a classic SRE and SREBPs. However, at all concentrations, relative activities among SREBP isoforms were similar. In our expression experiments, SREBP-1a is slightly stronger than SREBP-2, although the two isoforms were reported to have similar transcriptional activities with different protocols and cells (22). In contrast, SREBP-1c was 10-fold weaker and essentially inactive. Consistent with the previous report (5), the YR mutated SREBP-1c in our construct lost its luciferase activity completely (Fig. 3B). Predictably, no transcriptional activity was found in the mutated SREBP-1a because its bHLH domain is identical to that of mutated SREBP-1c. Furthermore, when we tried this mutation analysis for SREBP-2, arginine substitution almost completely abolished SREBP-2 activity for SRE as well. Therefore, the importance of this tyrosine residue for activation of the classic SRE is universal among all SREBP isoforms.

We chose LDL receptor, HMG-CoA synthase, and FPP synthase genes to estimate the effects of wild type and mutant SREBPs on the activities of native cholesterol-related gene promoters (**Fig. 4**). LDL receptor and HMG-CoA synthase promoters contain one copy and two copies of SRE (9, 39), respectively, while FPP synthase is regulated by a similar, but distinct sequence designated SRE3 (Fig. 2) (6). In HepG2 cells, SREBP-1a and -2 activated all three-gene promoters with similar efficiency while SREBP-1c

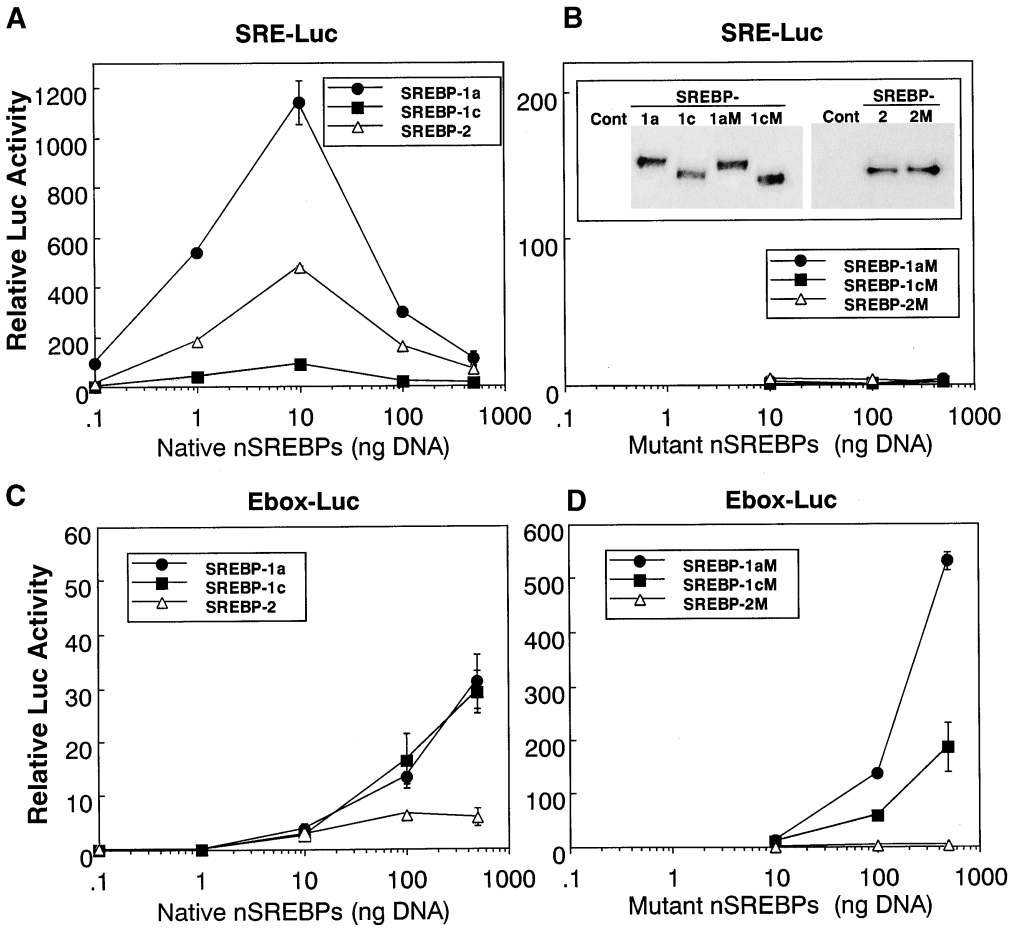

Fig. 3. Comparison of transcriptional activities of nuclear SREBP-1a (closed circle), -1c (closed square), -2 (open triangle) (A and C), and respective YR mutants (B and D) on SRE (A and B) and E-box (C and D) as measured by luciferase reporter gene assay in HepG2 cells. An artificial SRE-luciferease reporter plasmid, pSRE-Luc contains three sets of classic SRE and Sp1 site from LDL receptor promoter (refer to Fig. 2). An E-box enhancer luciferase reporter gene (pE-box-Luc) was constructed by integrating six copies of the carbohydrate response element of rat S14 gene (−1448 to –1422 bp) containing two E-boxes into the pGL2-Promoter vector (refer to Fig. 2). The HepG2 cells were transfected with the indicated DNA amount of CMV promoter-expression plasmid, pSRE-Luc or pE-box-Luc (0.5 μg), and pSV-β-gal (0.5 μg) as reference plasmid using SuperFect (Promega). Expression plasmids used were pCMV-SREBP-1a, pCMV-SREBP-1c, and pCMV-SREBP-2 encoding nuclear active forms of human SREBP-1a, -1c, and -2, respectively, under the CMV promoter, and YR mutated versions of the wild-type SREBP constructs (pCMV-SREBP-1aM, pCMV-SREBP-1cM, and pCMV-SREBP-2M) in which arginine was substituted for tyrosine residue in the basic region. A CMV7 empty vector (Cont) was used as a negative control. After transfection, the cells were incubated for 16 h in DMEM supplemented with 10% FCS, 1 μg/ml 25-hydroxycholesterol, and 10 μg/ml cholesterol to suppress endogenous SREBP activity. Luciferase activity was measured and normalized to β -galactosidase activity. The values are expressed as ratios of the value from control (Cont; open column). Each point represents the mean ± SE of three independent transfections. Inset in B: Immunoblot analysis of SREBP proteins expressed in transfected cells used for luciferase reporter assays. Another set of the cells for transfection was used to verify that the amounts of expressed SREBPs were comparable. Nuclear extracts were prepared and subjected to immunoblot analysis as previously described (15 μg) (21). Membranes were incubated with anti-human SREBP-1 or human SREBP-2 as primary antibody. ECL was used for visualization of the bands.

activation was very weak. These data are essentially consistent with the results from SRE-Luc as well as mRNA levels in transgenic studies (Fig. 1). The YR mutation in any of the SREBP constructs abolishes the activity of the LDL receptor promoter, as observed in SRE-Luc. In contrast, the YR mutation reduced the promoter activities of HMG-CoA and FPP synthase genes only 2- to 3-fold. The residual activities could be due to the abilities of the mutant SREBPs to weakly bind to modified SREs or other sequences in these promoters.

Lipogenic enzyme gene promoters

E-box-Luciferase reporter. It was previously reported that CACGTG-type E-box was preferentially picked up in the selection of the ADD1 binding motif from random oligonucleotides (5). This E-box is also present in the carbohy-

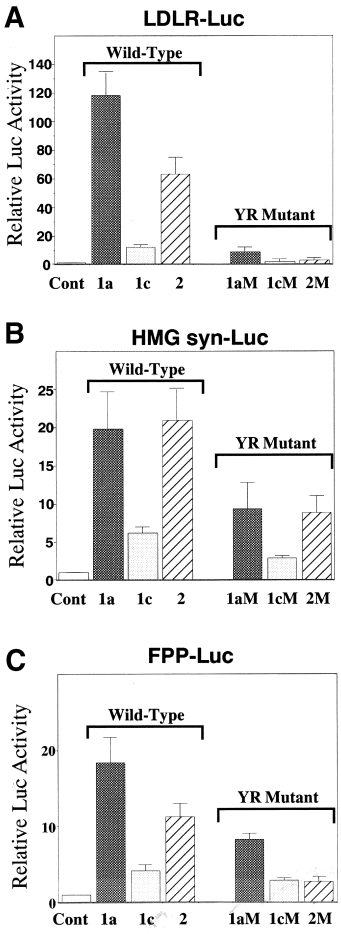

Fig. 4. Comparison of transcriptional activities of nuclear SREBP-1a, -1c, -2, and respective YR mutants on LDL receptor, HMG-CoA synthase, and FPP synthase gene promoters as measured by luciferase reporter gene assay in HepG2 cells. The HepG2 cells were transfected with the CMV promoter-expression plasmid as indicated below (1 μg), either pLDLR-Luc, pHMGSyn-Luc, or pFPPSyn-Luc as a reporter gene (0.5 μg, refer to Fig. 2), and pSV-β-gal (0.5μg) as reference plasmid using SuperFect (Promega). Expression plasmids used were pCMV-SREBP-1a, -1c, -2, -1aM, -1cM, -2M, and CMV7 (an empty plasmid as a control). After transfection, the cells were incubated for 16 h in DMEM supplemented with 10% FCS, 1 μg/ml 25-hydroxycholesterol, and 10 μg/ml cholesterol to suppress endogenous SREBP activity. Luciferase activity was measured and normalized to β -galactosidase activity. The values are expressed as ratios of the value from control (Cont; open column). Each bar represents the mean ± SE of four independent transfections.

drate response element of the S14 gene, whose expression in the liver has been known to be nutritionally regulated similarly to other lipogenic enzymes (18). The E-box is located 1.6 kb upstream of the transcription start site and does not have any promoter activity alone in preliminary experiments (data not shown), suggesting its potential enhancer activity. An enhancer reporter gene was constructed in which six copies of this carbohydrate response element were inserted into a luciferase gene reporter, which contains the SV40 promoter (pGL2-Promoter Vector) (Fig. 2). This reporter gene contains essentially only E-boxes fused to an intrinsic promoter and is designated E-box-Luciferase reporter (E-box-Luc) as a tester to estimate enhancer activities of each SREBP isoform on a representative E-box. As shown in Fig. 3, dose-activation curves of SREBPs for E-box-Luc were markedly distinct from those for SRE-Luc. SREBP-1a and -1c exhibited a similar activity for E-box in a dose-dependent manner (Fig. 3C). It is noteworthy that significant activation of E-box-Luc requires 10–100 ng of transfected SREBP expression plasmid DNA, which was higher than the DNA concentration for maximal activity for SRE. This suggests that SREBP-1 is more efficient for activation of SRE than E-boxes. In contrast, SREBP-2 had essentially no activity for E-box-Luc. Very interestingly, the YR mutation increased enhancer activities of SREBP-1a and -1c markedly, but did not restore any activity of SREBP-2 (Fig. 3D).

Based upon these results, we evaluated the dual binding specificity of each SREBP isoform for SRE and E-box (**Fig. 5**). This dual binding specificity was originally found for SREBP-1c/ADD1 (5). SREBP-1a shares the same bHLH protein with SREBP-1c and should have the same binding specificity. We performed the gel shift assays using the nuclear extracts derived from SREBP transfected cells (Fig. 5A). DNA containing an SRE and an Sp1 site, the same sequence as used for SRE-Luc and LDL-Luc, was labeled as an SRE probe. As shown in left panel of Fig. 5A, nuclear extracts from control cells in which endogenous SREBPs were suppressed by addition of 25-OH cholesterol and cholesterol in the medium, exhibited shifted bands unrelated to SREBPs, presumably due to an abundant presence of Sp1 family in the cells, since these bands disappeared after the addition of excess unlabeled DNA containing only Sp1 binding site. Nuclear extracts from the cells transfected with the SREBP-1a expression plasmid showed a new shifted band by SREBP-1a. Its specificity of SREBP-1 binding was confirmed by a supershift after addition of the specific antibody. In the middle panel, we compared SREBP-1a, YR mutant SREBP-1a (1aM), SREBP-2, and YR mutant SREBP-2 (2M) in binding to this SRE probe. As expected, SREBP-1aM did not cause the gel shift of the SRE. Similarly, SREBP-2 bound, but SREBP-2M did not bind, to the SRE. These data are consistent with their transcriptional activities for the SRE-containing promoter (SRE-Luc) as estimated by luciferase assays (Fig. 3). Therefore, the importance of the tyrosine residue in the basic region for binding to SRE (5) was verified for both SREBP-1a and -2. We examined binding of SREBPs to the E-box using a probe from E-box-Luc (right panel of Fig. 5A). We found that both SREBP-1a and -1aM bound to the E-box, which was consistent with the luciferase results. Unexpectedly, both SREBP-2 and -2M also bound to the E-box. This is a discrepancy with the results from luciferase assay that SREBP-2 was essentially inactive and SREBP-2M was completely inactive for the E-box containing promoter (Fig. 3C, D). Ability of SREBP-1a and -2 to bind to the E-box was reconfirmed using recombinant proteins (Fig. 5B). Competitive studies with excess SRE and E-box DNAs suggested that SREBP-2 appears to have a higher binding affinity for the SRE than for the E-box,

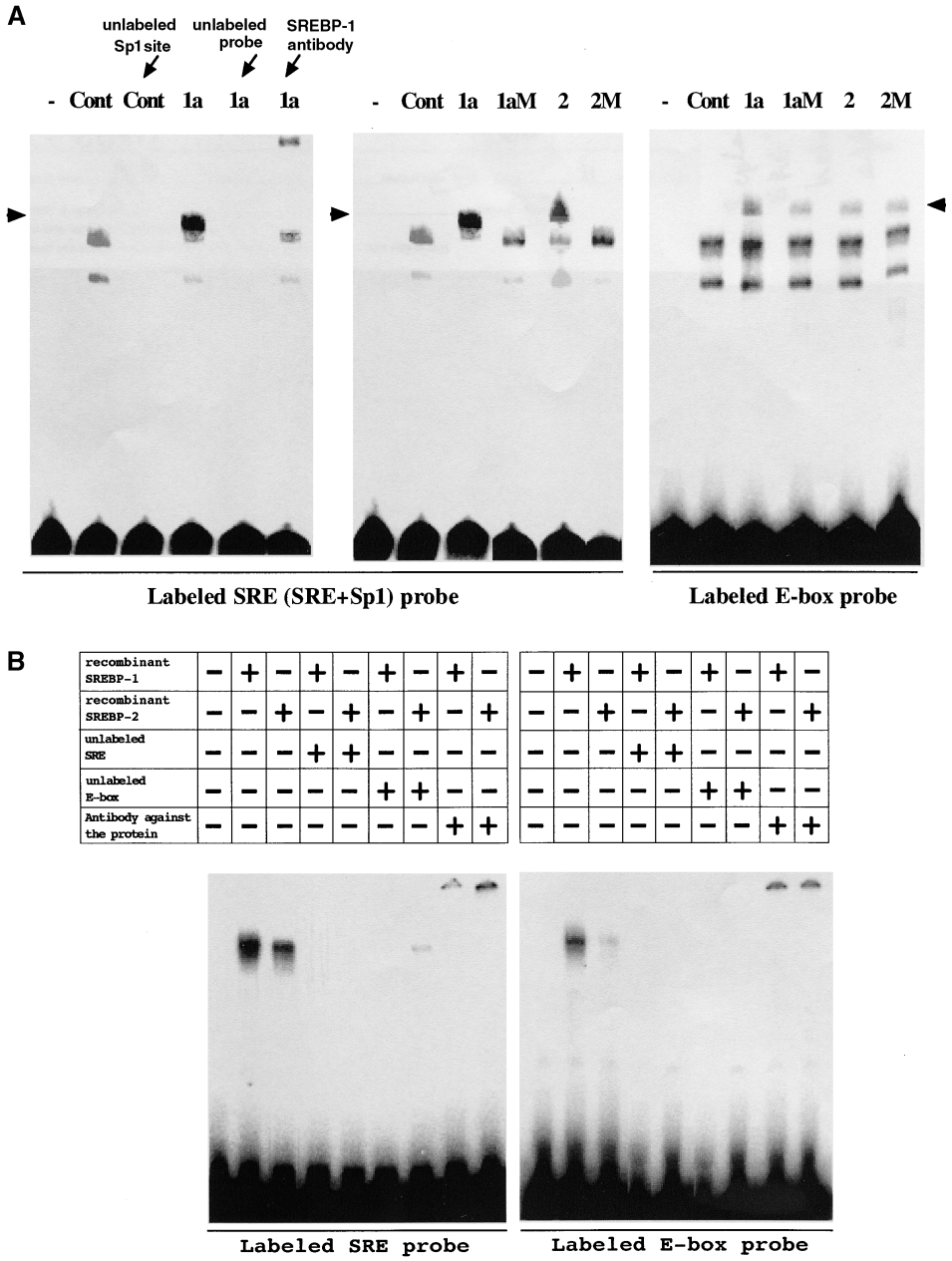

Fig. 5. Gel mobility shift assay for binding of native and YR mutant SREBPs expressed to SRE and E-box. A: Double stranded DNA fragments containing SRE-Sp1 site (derived from the human LDLR gene promoter, refer to Fig. 2) and E-box (derived from a carbohydrate responsive element of rat S14, refer to Fig. 2) were labeled with [α-^{32}P] dCTP. These labeled SRE and E-box probes were incubated with nuclear extracts from the cells transfected with pCMV-SREBP-1a, -2, -1aM, -2M, and CMV (an empty plasmid as control) and were run on polyacrylamide gels. Endogenous SREBPs were suppressed by pre-incubation of the cells with 25OH cholesterol and cholesterol. All the nuclear extracts including control exhibited shifted bands unrelated to SREBPs. These non-SREBP shifted bands were due to Sp1 binding to the SRE probe and to USF1 binding to the E-box probe (data not shown). In the left panel, specificity of SREBP-1a binding to the SRE probe (indicated by arrow) was confirmed by a supershift after addition of SREBP-1 antibody. The specificities of other bindings of SREBPs to the SRE and E-box (indicated by arrows) were confirmed by supershifts after addition of respective antibodies (data not shown). B**:** Gel mobility shift assay for binding of recombinant SREBP-1 and -2 to SRE and E-box. Labeled SRE and E-box probes were also incubated with recombinant nuclear SREBP-1 and -2 and subjected to gel mobility shift assay. Specificity of each protein binding to SRE and E-box was confirmed by supershift by addition of the respective specific antibody. In competitive assays, a 1,000-fold molar excess of indicated unlabeled probes was added prior to addition of the labeled probe.

relative to SREBP-1. This suggests that binding of SREBP-2 to the promoter might not be sufficient for activation of its downstream genes.

Pyruvate kinase and glucokinase gene promoters

The liver-type pyruvate kinase gene promoter contains a similar carbohydrate (glucose) response element to that of the S14 gene; two tandem E-box like sequences designated the L4 box (18). PK-Luc, containing this region, was tested as an SREBP target. Preliminary data show that the luciferase construct fused to 200 bp PK promoter containing this region was activated 2- to 3-fold by changing from low glucose (2.75 mM) to high glucose medium (25 mM) for 16 h for confirmation of this construct as a glucose response element. In addition, overexpression of USF1 (CMV-USF1), which is known to activate both PK and GK promoters, increased PK-Luc activity 3.1- ± 0.3-fold (three independent experiments). In contrast to the carbohydrate response element of S14 (E-box-Luc), the PK promoter did not significantly respond to SREBP overexpression (relative fold changes vs. control vector are: SREBP-1a, 1.1 ± 0.2; SREBP-1c, 1.6 ± 0.3; SREBP-2, 1.2 ± 0.3; SREBP-1aM, 1.6 ± 0.3; SREBP-1cM, 1.4 ± 0.1; SREBP-2M, 0.6 ± 0.1; mean ± SE from six independent transfections). As shown in Fig. 1, hepatic PK mRNA was modestly up regulated by SREBP-1a overexpression in the transgenic mice. It is not currently known whether this effect of SREBP-1a is direct or indirect; however, it can be concluded that the effect of SREBP on PK expression in vivo is not mediated through the carbohydrate response element of the PK gene. In our previous report, SREBP-1 disruption did not severely impair the high-sucrose induction of PK (30). Taken together, these results indicate that SREBP-1 is not significantly involved in the physiological regulation of PK. Another glycolytic and lipogenic enzyme, GK, was reported to have an insulin response element in its proximal promoter region. There is a CACGTG-type E-box close to this region. Consistent with the in vivo data (Fig. 1), GK-Luc was not significantly activated by any of the SREBP constructs (relative fold changes vs. control vector are: SREBP-1a, 1.0 ± 0.1; SREBP-1c, 1.8 ± 0.2; SREBP-2, 0.6 ± 0.05; SREBP-1aM, 1.4 ± 0.2; SREBP-1cM, 0.8 ± 0.1; SREBP-2M, 0.3 ± 0.1; mean ± SE from three independent transfections) whereas USF1 expression plasmid markedly induced the GK promoter (16 ± 2). These data suggest that SREBPs are not strong activators for GK, which is contrary to a previous report that adenoviral overexpression of SREBP-1c/ADD1 activates GK expression (45).

S14 proximal gene promoter

Recently the element responsible for SREBP-1c binding and activation of the S14 gene promoter was identified in the known polyunsaturated fatty acid suppressive region (16). This element (distinct from the carbohydrate response element used for E-box-Luc) is located at 140 bp upstream of the transcription start site. The sequence (TCGCCTGAT) exhibited a slight similarity to both E-box and SRE. The S14 promoter-luciferase containing this sequence with adjacent NF-Y site was estimated for SREBP activation (**Fig. 6A**). The S14 promoter was strongly activated by SREBP-1a, and by SREBP-1c and -2 with less efficiency at any concentration of the transfected DNA. The induction of hepatic S14 mRNA levels in transgenic mice (Fig. 1) was less marked than that in the luciferase assays. It is presumably because other transcription factors and hormones such as USFs and thyroid hormones regulate the long native promoter of S14 gene. However, this promoter along with the enhancer region (Fig. 3C) should play a role in nutritional induction of hepatic S14 gene expression (31). Interestingly, the YR mutation abolished the SREBP-2 activation of the S14 promoter Luc, but did not affect the SREBP-1 activation, suggesting that the molecular mechanism for SREBP activation of the S14 promoter could be different between SREBP-1 and -2. YR mutated SREBP-1a and -1c showed dose-dependent activation in all concentrations in contrast to native SREBPs whose peak activities were at 100 ng.

FAS gene promoter

The fatty acid synthase promoter is a unique case where two perfect SRE sites flank an E-box, also recognized as an insulin or carbohydrate response element (refer to Fig. 2) (19, 46). As depicted in Fig. 6B, wild-type SREBP1a had the strongest activity for the FAS promoter among SREBP isoforms. The highest activation was observed at 100 ng of DNA. SREBP-1c and -2 showed lower levels of activation than SREBP-1a, but similar dose-dependent patterns. The YR mutation significantly impaired the transcriptional activities for SREBP-1a and -1c at 100 ng and lower concentrations, but the activation by YR mutant SREBP-1a and -1c was dose-dependent and resulted in the same level of activation of FAS-Luc as respective native SREBP-1a and -1c at 500 ng. This is consistent with a previous report that YR mutated ADD1 still retained its binding to and transactivation of the FAS gene promoter in CAT assays (25). In contrast, the mutation caused SREBP-2 to lose its activity to FAS promoter, showing a discrepancy in the effect of YR mutation between SREBP-1 and -2.

ACL gene promoter

ATP citrate lyase catalyzes the reaction for the translocation of acetyl-CoA from mitochondria to cytosol and is critical for biosynthesis of both cholesterol and fatty acids in the cytosol. ACL is regulated in a lipogenic fashion and controlled by SREBP-1c in the liver (31). Recently, an SREBP binding and activation site in the rat and human ACL promoters was identified (43). It is an SRE-like sequence (TCAGGCTAG) accompanied by an NF-Y site. Mouse ACL promoter (300 bp), that contains the same SREBP binding site and a region responsible for insulin and polyunsaturated fatty acid effects, was fused to the luciferase reporter gene (ACL-Luc) (32, 47) (Fig. 2). As shown in Fig. 6C, SREBP-1a profoundly activated the

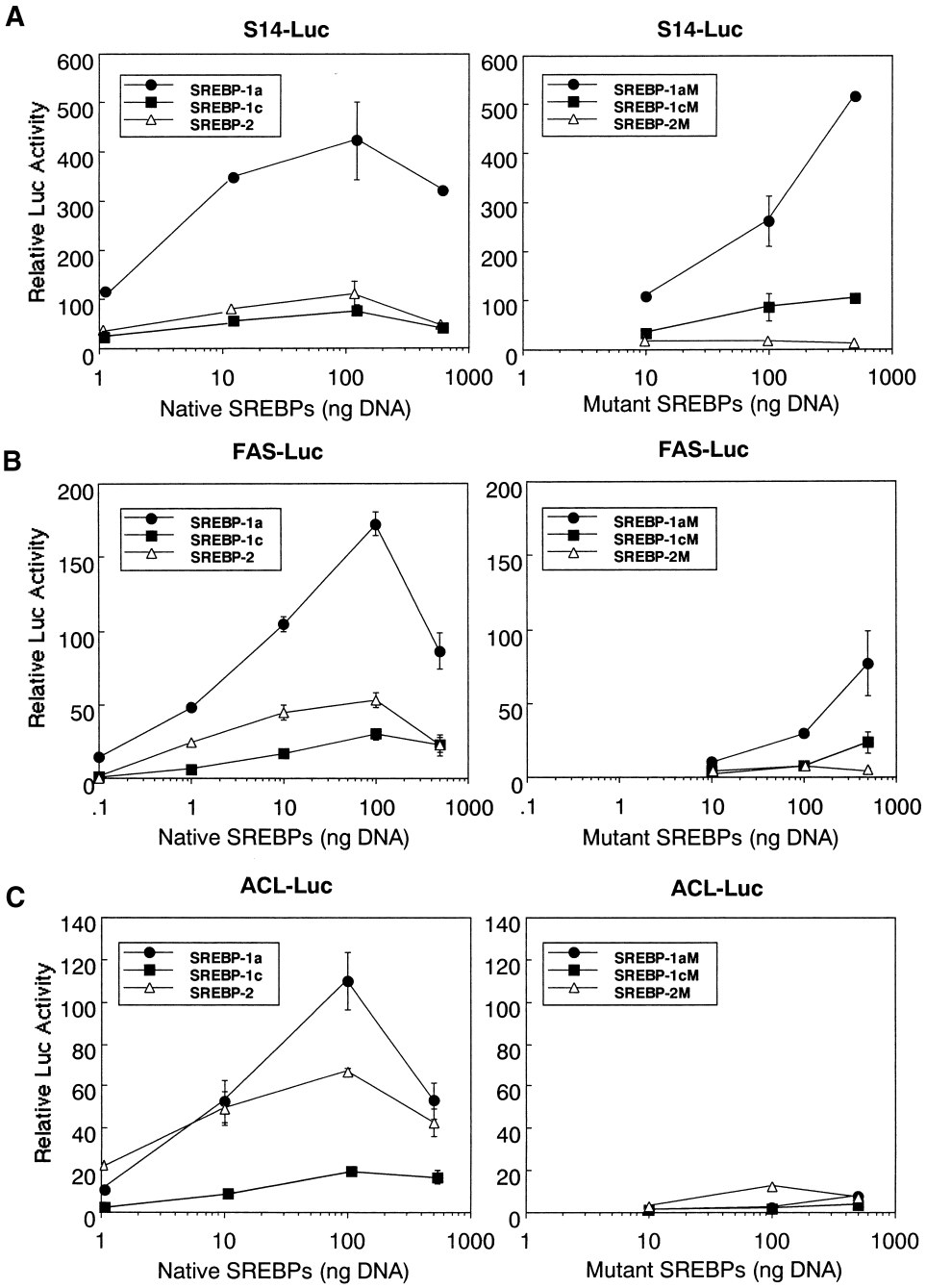

Fig. 6. Comparison of transcriptional activities of nuclear SREBP-1a (closed circle), -1c (closed square), -2 (open triangle), and respective YR mutants on the S14 (A), FAS (B), and ACL (C) gene promoters measured by luciferase reporter gene assay in HepG2 cells. Reporter plasmids p S14 Luc, pFAS Luc, and pACL Luc were constructed by fusing rat S14 proximal gene, rat FAS gene promoter, andmouse ACL gene promoter to pGL2-Basic Vector (refer to Fig. 2). The HepG2 cells were transfected with the indicated amount of CMV promoter-expression plasmid, p S14 Luc, pFAS Luc, or pACL Luc as a reporter gene (0.5 μg), and pSV-β-gal (0.5μg) as reference plasmid using SuperFect (Promega). Expression plasmids used were pCMV-SREBP-1a, -1c, -2, -1aM, -1cM, -2M, and CMV7 (an empty plasmid as control). The cells were treated and luciferase activity was corrected for by β -galactosidase activity measured as described in the legend of Fig. 4. The values were expressed as ratios of the value from control (Cont; open column). Each point represents the mean ± SE of three independent transfections.

ACL promoter with peak activity at 100 ng. Activation of ACL by SREBP-2 and SREBP-1c was lower, but significant. The YR mutation abolished the transactivity of each isoform.

Malic enzyme gene promoter

In the reported rat malic enzyme promoter sequence, no known authentic SRE site was found (33). However, there are several SRE half sites that have been shown to be

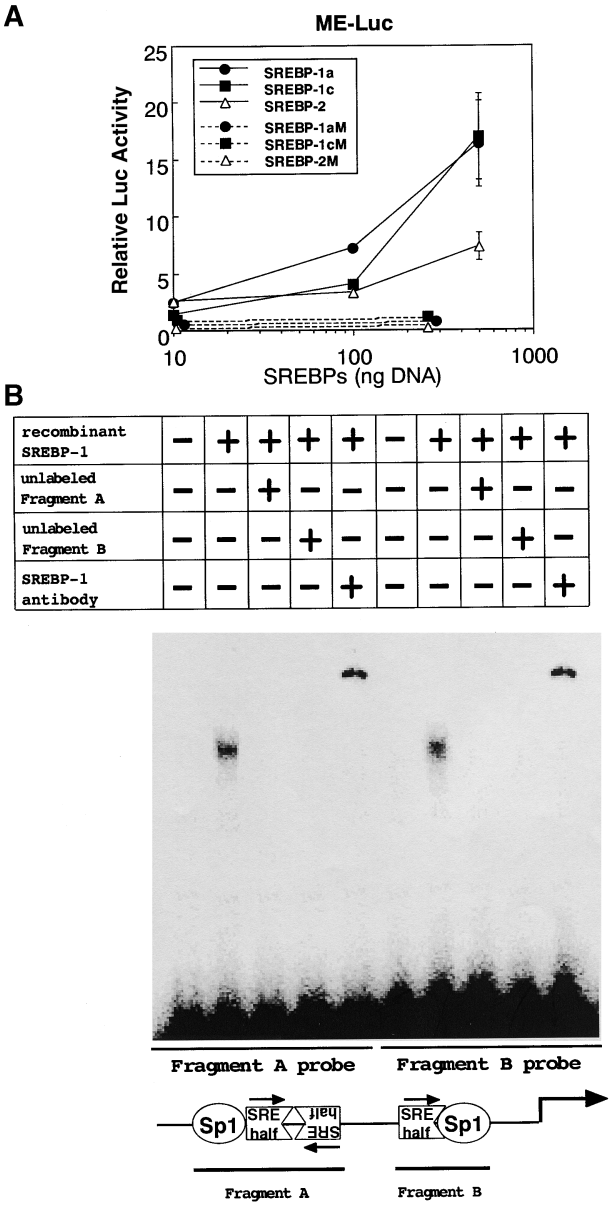

Fig. 7. Comparison of transcriptional activities of nuclear SREBP-1a (closed circle), -1c (closed square), -2 (open triangle), and respective YR mutants (dashed lines) on the malic enzyme gene promoter measured by luciferase reporter gene assay in HepG2 cells (A) and gel mobility shift assay for binding of SREBP-1 to the malic enzyme gene promoter (B). A: The HepG2 cells were transfected with the indicated amount of CMV promoter-expression plasmid, pME Luc (rat malic enzyme gene promoter fused to pGL2-Basic Vector, refer to Fig. 2) as a reporter gene (0.5 μg), and pSV-β-gal (0.5μg) as reference plasmid using SuperFect (Promega). Expression plasmids used were pCMV-SREBP-1a, -1c, -2, -1aM, -1cM, -2M, and CMV7 (an empty plasmid as control). The cells were treated and luciferase activity was corrected for by β -galactosidase activity measured as described in the legend of Fig. 4. The values were expressed as ratios of the value from control (Cont; open column). Each point represents the mean ± SE of three independent transfections. B: Double stranded DNA fragments containing two palindromic SRE half sites with an adjacent Sp1 site (Fragment A) and containing one SRE half site with an Sp1 site (fragment B) in the rat malic enzyme gene were labeled with [α-^{32}P]dCTP and were used as probes. The labeled fragment A and B were incubated with a recombinant nuclear SREBP-1 protein. Specificity of SREBP-1 binding to the fragment A was confirmed by supershifts after addition of SREBP-1 antibody. In competitive assays, a 1,000-fold molar excess of unlabeled fragment A or B was added prior to addition of the labeled probe.

involved in the binding of SREBP to the SRE-like region of the acetyl-CoA carboxylase promoter (Fig. 2)(46). Notably, at −228 bp, there is a cluster of SRE half sites (TCACCC) consisting of a region of two palindromic SRE half sites with an adjacent Sp1 site (fragment A in **Fig. 7**) and a region of a single SRE half site with an Sp1 site (fragment B in Fig. 7), which are potential SREBP binding sites. An 880 bp malic enzyme promoter-luciferase reporter gene was constructed and used in transfection studies with SREBP expression plasmids. As shown in Fig. 7A, SREBP-1c has activity equal to that of SREBP-1a and higher than that of SREBP-2. The fold activation by each isoform was relatively small, but dose-dependent. The YR mutation in each SREBP completely abolished this activity for the ME promoter. To show a direct binding of SREBP-1 to the malic enzyme gene promoter, we performed a gel shift assay (Fig. 7B). Both fragment A and B probes were shifted after the addition of an SREBP-1 recombinant protein, and specificity of its binding was validated by the disappearance of both bands in the presence of either excess unlabeled fragment A or B, and supershifts in the presence of SREBP-1 antibody. These indicate that SREBP-1 binds to both regions.

G6PD gene promoter

The reported sequence of the G6PD promoter (approximately 0.8 kb) contains E-box, SRE, or SRE-like sites without neighboring Sp1 or NF-Y sites (Fig. 2) (34). The G6PD promoter was fused to luciferase (G6PDLuc) and was used to assess activation by SREBPs. As shown in **Fig. 8**, G6PD-Luc was activated 20-fold by SREBP-1a at 100 ng and was only slightly (3–5 fold) activated by SREBP-1c and -2. These activations were slightly decreased by the YR mutation, the physiological significance of which is marginal.

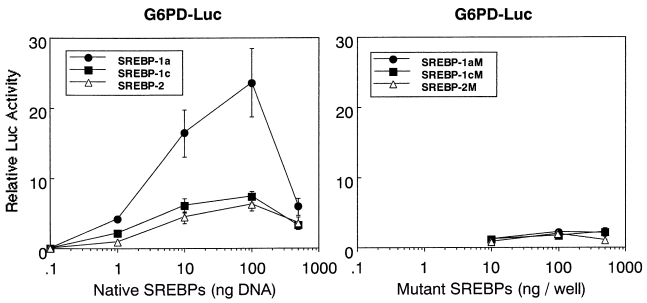

Fig. 8. Comparison of transcriptional activities of nuclear SREBP-1a (closed circle), -1c (closed square), -2 (open triangle), and respective YR mutants on the G6PD gene promoter measured by luciferase reporter gene assay in HepG2 cells. The HepG2 cells were transfected with the indicated amount of CMV promoter-expression plasmid, pG6PD Luc (rat G6PD gene promoter fused to pGL2-Basic Vector, refer to Fig. 2) as a reporter gene (0.5 μg), and pSV-β-gal (0.5μg) as reference plasmid using SuperFect (Promega). Expression plasmids used were pCMV-SREBP-1a, -1c, -2, -1aM, -1cM, -2M, and CMV7 (an empty plasmid as control). The cells were treated and luciferase activity was corrected for by β-galactosidase activity measured as described in the legend of Fig. 4. The values were expressed as ratios of the value from control (Cont; open column). Each point represents the mean ± SE of three independent transfections.

Representative SRE, SRE-like, and E-box enhancer constructs

To explore the general features on different specificities of SREBP isoforms for SRE, SRE-like and E-box containing promoters estimated in the current study, we made another set of enhancer constructs containing representative SRE, SRE-like, or E-box motif. As shown in **Fig. 9A**, we selectively chose SRE from LDLR promoter, SRE-like sequence from S14 promoter, and an E-box that was previously reported as the *cis*-element that had most preferential affinity for ADD1/SREBP-1c. Fig. 9B compares activation of these constructs by native and mutant SREBPs. The data were essentially similar to results from respective native promoters, demonstrating that SRE, SRE-like, and E-box in the SREBP target promoters could essentially represent all SREBP activations. Furthermore, it was found that the distance between SRE-like and NF-Y site was very important since SREBP activation was abolished by shortening the spacing length from the original 26 bp to 7 bp. The importance of spacing between SRE and NF-Y site has been reported for SRE and NF-Y site (48). A single copy of E-box is a very weak enhancer for SREBP activation, but strong for mutant SREBPs. Interestingly, mutant SREBP activation of E-box does not change in the presence or absence of neighboring NF-Y or Sp1 site. All these data suggest that SRE, SRE-like, and E-box have different features of SREBP activation in terms of SREBP isoform difference and requirement for cofactors.

DISCUSSION

The current studies clearly demonstrate different specificities of the three nuclear SREBP isoforms to different cholesterogenic and lipogenic enzyme gene promoters. Overall, the relative transcriptional activities of the SREBP isoforms for different genes in luciferase assays were essentially similar to the extents of fold increases in corresponding mRNA levels observed in livers of SREBP transgenic mice. This indicates consistency of overexpression tests for SREBPs between liver and HepG2 cells. It also supports the evidence that SREBPs are directly involved in activation of those gene promoters.

The most striking observation was found with the E-box-Luc construct. As demonstrated by gel shift assays in Fig. 5, SREBP-1 and -2 and their YR mutated versions could all bind to this E-box, demonstrating that the dual binding specificity for both SREs and E-boxes is true of not only SREBP-1c/ADD1, which was originally described (5), but also of SREBP-1a and SREBP-2. However, very interestingly, the luciferase assays demonstrated that SREBP-2 was essentially inactive and YR mutated SREBP-2 was completely null, whereas SREBP-1 could weakly activate E-box-Luc. SREBP-2 could bind to E-box, but could not activate its downstream gene unlike SREBP-1.

A recent paper by T. F. Osborne and colleagues elegantly showed that binding of SREBPs to SRE induces recruitment of cofactors Sp1 and NF-Y to adjacent sites to result in acetylation of histone H3 (49). It could be speculated that SREBPs require interaction with cofactors after binding to target DNAs to activate the downstream gene, but SREBP-2, unlike SREBP-1, might have an impaired interaction with these cofactors on the E-box containing promoter. This would be an intriguing example demonstrating that DNA binding of a transcription factor is a necessary, but not a sufficient condition for transactivation.

SREBP-1 can activate some other E-box or E-box-like sequences: CATGTG contained in the FAS promoter and CGCCTG in the S14 promoter, whereas it is essentially inactive for degenerated E-boxes in the PK promoter (CACGGG and CCCGTG). Therefore, there is some preference for SREBP-1 among E-box-like sequences. Although SREBP-1 seems to prefer a complete c-myc E-box, CACGTG, for binding (5), this sequence alone is not enough for promoter activation by SREBP-1 because this sequence was also found in the GK promoter, which SREBP-1 could not significantly activate (Fig. 2). It was reported that overexpression of SREBP-1c/ADD1 by adenoviral vector activated GK expression. We speculated that this GK induction could be indirect effect of SREBP-1. The YR mutation makes SREBP-1a and -1c superactive to the E-box-Luc while mutated SREBP-2 remained inactive. Although SREBP-1 and not SREBP-2 is functionally capable of activating E-boxes, still arginine appears to be preferable to tyrosine in the basic region, raising a question of its physiological action on E-box containing promoters. SRE-containing promoters absolutely require NF-Y or Sp1 as cofactors for SREBP activation (9, 40, 50, 51). In contrast, the current study suggested that these factors do not highly contribute to activation of E-box-Luc by native and mutant SREBP-1 (Fig. 9) and, thus, the mechanisms for SREBP activation of E-box and SRE should be distinct. Furthermore, the different dose-dependency for SRE-Luc

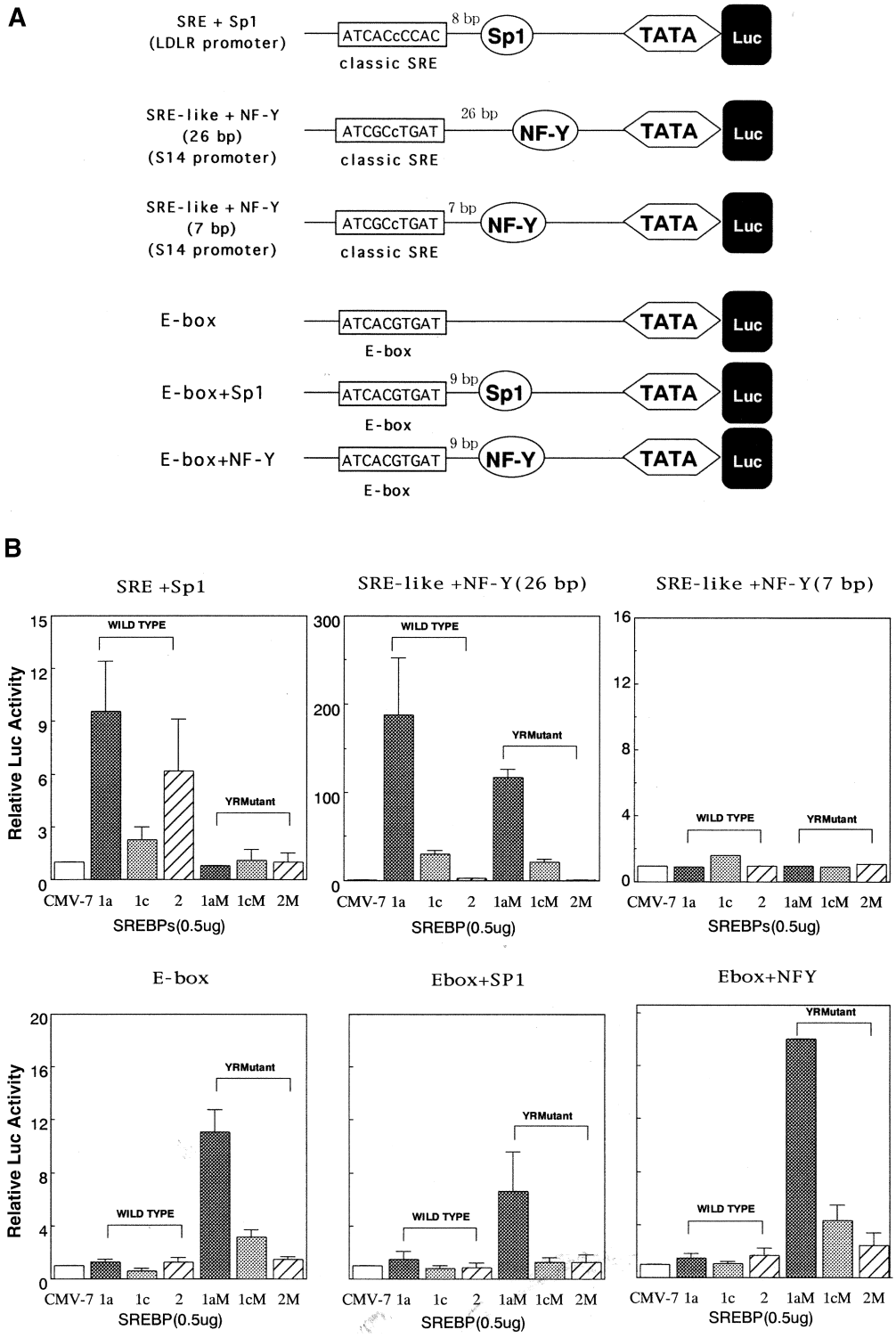

Fig. 9. Comparison of transcriptional activities of nuclear SREBP-1a, -1c, -2, and respective YR mutants on enhancer constructs containing representative SREBP binding sites (SRE, SRE-like, and E-box motifs) as measured by luciferase reporter gene assay in HepG2 cells. A: Construction of six enhancer luciferase reporters containing SRE (from LDLR promoter), SRE-like (from S14 promoter), and E-box (ATCACGTGAT) with or without Sp1 site or NF-Y site is shown. B: The HepG2 cells were transfected with the CMV promoter-expression plasmid, enhancer luciferase construct as indicated below (0.5 μg), and pSV-β-gal (0.5μg) as reference plasmid using SuperFect (Promega). Expression plasmids used were pCMV-SREBP-1a, -1c, -2, -1aM, -1cM, -2M, and CMV7 (an empty plasmid as a control). After transfection, the cells were incubated for 16 h in DMEM supplemented with 10% FCS, 1 μg/ml 25-hydroxycholesterol, and 10 μg/ml cholesterol to suppress endogenous SREBP activity. Luciferase activity was measured and normalized to β-galactosidase activity. The values are expressed as ratios of the value from control (Cont; open column). Each bar represents the mean ± SE of four independent transfections.

and E-box-Luc (Fig. 3) suggests that SREBP-1 has much higher affinity and more efficient activation for SRE than E-box. Taken together with the fact that SREBPs did not activate E-box containing GK and PK promoters, physiological relevance of SREBP to E-box containing promoters is currently unknown. A more precise base-by-base mutational analysis is needed to clarify the effects of SREBP-1 and -2 on different E-box sequences and to determine a physiological role of SREBP-1 in E-box containing gene promoters.

In contrast, for lipogenic enzyme genes containing SRE-like sequences, all the SREBP isoforms significantly activated their transcription. This indicates that any form of SREBP can stimulate expression of most lipogenic genes as long as the mature protein is targeted to the nucleus. In light of relative activities among the isoforms, SREBP-1a activation is extraordinarily strong. SREBP-1a contains a longer transactivation domain and thus has stronger transactivity than SREBP-1c. This trend is true of most lipogenic gene promoters with the exception of ME, upon which SREBP-1a and -1c activate similarly. In spite of a similar length amino-terminal transactivation domain, lipogenic enzyme gene promoters prefer nuclear SREBP-1a to nuclear SREBP-2 in terms of molecular specificity, although the difference is not so clear as that for E-boxes. The effect of the YR mutation on SRE-like containing promoters was partial, which places the features of SRE-like sequences between classic SREs and E-boxes. The relative transcriptional activities of nuclear SREBP-1a, -1c, and -2 for SRE-, SRE-like, and E-box containing promoters are summarized in **Fig. 10**.

The FAS promoter activity by each SREBP expression construct implicates something intriguing. The DNA binding site of FAS promoter for SREBPs is complicated to interpret. Two SREBP binding sites are connected in a palindromic fashion and both sites are important for sterol regulation (13). Meanwhile, the junction of these SREs composes an E-box, which has been shown to be responsible for the carbohydrate response of the FAS gene (Fig. 2). SREBP-1a was the strongest activator and the YR mutation did not affect the FAS promoter activity. In light of the presence of an E-box, it is reasonable that the YR mutation retains the binding of SREBP to this region, as observed in SREBP-1a and -1c, although relatively high amount of SREBPs was required. Meanwhile, SREBP-2 that is more specific to sterol regulation and is now known to have no activity for E-boxes (Fig. 3), was sensitive to the YR mutation for binding to this region (Fig. 5). This sensitive and fine-tuned activation property of the FAS promoter with SREBP-1 and -2 could explain why FAS is regulated in both sterol and lipogenic fashions. Another upstream SRE (Fig. 2) has been reported to be involved in in vivo nutritional regulation of FAS gene expression by SREBP (52).

In spite of an incredibly wide range of SREBP-1 control over all lipogenic genes, the extent of regulation of E-box containing lipogenic genes such as PK and GK by SREBPs seems considerably limited. In the current study, SREBP-1 is shown not to be involved in well-defined carbohydrate

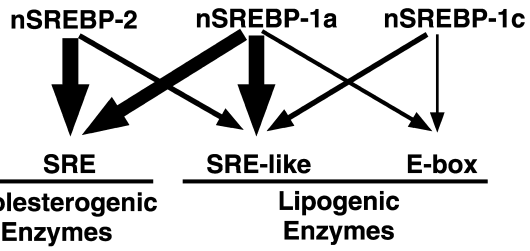

Fig. 10. Schematic representation of relative transcriptional activities of SREBP family to different DNA targets. Target sequences include classic SRE, which is usually found in the promoters of cholesterogenic enzyme and LDL receptor genes, various SRE-like sequences, and E-box (-like) sequences in lipogenic enzyme genes. Arrows and their boldness show specificities and transcriptional activities. nSREBP, nuclear SREBP.

or insulin responsive element in the PK and GK promoters. Consistently, in the recent studies with SREBP-1 knockout mice, although induction of most of lipogenic enzymes was severely impaired in the liver, some lipogenic enzymes such as PK retained lipogenic induction (30). The residual transcriptional activities for lipogenic enzymes in SREBP-1 knockout mice were more prominent in adipose tissues. All these data indicate that there must be other lipogenic regulators that control those lipogenic enzymes both in liver and adipose tissue. Furthermore, the marked superphysiological activity of YR mutated SREBP-1 for CACGTG-type E-box also prompts us to speculate the presence of an unknown bHLH protein that is structurally similar to SREBP-1 but has an arginine residue in the basic region, and thus controls E-box type lipogenic genes. This protein would not activate SRE, but activate E-box containing lipogenic enzyme genes and could participate in physiological regulation of these genes. Upstream stimulatory factors (USF), which have been shown to bind to PK, GK, S14, and FAS, could be involved; however, it does not appear that USF alone can account for this activity as the amount of nuclear USF did not change in these lipogenic conditions. Investigation for this residual activity could shed some light on the understanding of transcriptional factor(s) responsible for the carbohydrate response, which cannot be explained for by SREBP-1 and USF.

The current data may have different in vivo physiological relevance, as the major isoforms of SREBP in the liver are SREBP-1c and SREBP-2 (20). While cholesterogenic genes are activated by SREBP-2 and not by SREBP-1c, these two factors are almost equally active for lipogenic genes. However, our recent data from SREBP-1 knockout mice confirmed that SREBP-1c dominates hepatic nutritional regulation of lipogenic enzyme genes while SREBP-2 specifically regulates cholesterol biosynthesis (30). Taken together, it can be speculated that different methods of nuclear SREBP-1c and -2 supply are required for distinct nutritional and metabolic regulation of cholesterol and fatty acid biosynthesis. One possibility is that SREBP-1c controls hepatic lipogenesis by changing the bulk amount supply of nuclear SREBP-1c, overwhelming

nuclear SREBP-2, rather than by their molecular specificities to the promoters of lipogenic genes. It seems that in the regular fed state, SREBP-1c is much more abundant in the liver nuclei than is SREBP-2 and maintains basal lipogenesis. In lipogenic conditions such as in a refed state after fasting or placement on a high carbohydrate diet, mature SREBP-1c protein further accumulates in the nuclei and activates lipogenic genes; however, it is not strong enough for activation of cholesterogenic genes (27, 30). In contrast, SREBP-2 is not regulated by these nutritional changes (26, 30). This would make SREBP-1c a key transcriptional regulator for nutritional regulation of lipogenic enzymes in the liver. Another possibility is that nuclear SREBP-2 might have higher affinity to SRE than to SRE-like sequences, causing it to preferentially bind to and activate cholesterogenic gene promoters rather than lipogenic genes. In a cholesterol-depleted state, the reciprocal regulation of SREBP-1c and -2 is observed in livers from mice treated with lovastatin and Colestipol (53). Nuclear SREBP-2 is robustly increased whereas nuclear SREBP-1 is moderately suppressed. These changes result in marked induction of all cholesterogenic genes while some lipogenic genes such as FAS and ACL are concomitantly induced. Drs. Goldstein and Brown have extensively analyzed the mechanism for the release of nuclear SREBP-2 upon sterol regulation. It has been shown to be completely due to the activity of cleaving the SREBP-2-SCAP complex by site 1 protease with its translocation from rER to Golgi (2, 3, 54). In contrast, the mechanism of SREBP-1 activation in liver is currently unknown. Although SREBP-1c also requires SCAP for cleavage, its overall regulation seems to be at both cleavage and transcriptional levels. Recently, LXR has been identified as the dominant activator for SREBP-1c gene promoter, suggesting a link between lipogenesis and oxysterol regulation (55–57). Future studies are needed to elucidate the mechanism of transcriptional regulation of SREBP-1c (58).

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