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# Common double- and single-stranded DNA binding factor for a sterol regulatory element

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**ABSTRACT** **A cis-acting element necessary for sterol regulation, SRE-1, has previously been identified in the promoters of the low density lipoprotein receptor, hydroxymethylglutaryl (HMG)-CoA reductase, and HMG-CoA synthase genes. In this report we describe a nuclear factor, SRE-BF, isolated from Chinese hamster ovary nuclear extracts, that binds to the SRE-1 octanucleotide sequence. In addition to sequence-specific binding to SRE-1, as indicated by competition analysis with double-stranded DNA fragments, single-stranded oligomer DNA sequences also compete for binding in a sequence-specific fashion. Photochemical cross-linking experiments suggest that a common protein factor, with apparent molecular mass of 45–49 kDa, recognizes both single-stranded and double-stranded SRE-1. The binding specificity of SRE-BF to single-stranded SRE-1 closely correlates with the reported *in vivo* ability of SRE-1 to direct sterol responsiveness of transcription.**

Intracellular cholesterol content is regulated by integration of two pathways that govern the synthesis of endogenous cholesterol and the uptake of extracellular cholesterol. Cells depleted of cholesterol increase the number of low density lipoprotein (LDL) receptors and the activity of cholesterol biosynthetic enzymes. When exogenous cholesterol or one of its derivatives is supplied, either directly in soluble form or as lipoprotein particles, transcription of a number of gene products is decreased (1–3), resulting in the downregulation of pathways involved in uptake or synthesis of cholesterol. In this way, intracellular cholesterol homeostasis is regulated by a feedback loop.

A conserved DNA sequence has been identified in the 5′ flanking regions of several sterol-regulated genes: the genes encoding the LDL receptor (4, 5), hydroxymethylglutaryl (HMG)-CoA reductase (6), and HMG-CoA synthase (2). This octanucleotide region,

$$GTG\begin{matrix}G\\C\end{matrix}GGTG$$

sterol regulatory element 1 (SRE-1), has been shown to be an essential regulatory sequence in all of these promoters. Deletion or point mutations within this region significantly reduce levels of transcription under conditions of sterol depletion for the HMG-CoA reductase (6), HMG-CoA synthase (2, 7) and LDL receptor genes (3).

This region has been studied by a number of investigators. Gil *et al.* (8) reported cloning NF-1-like proteins that bound to TGG-containing sequences, which are also found in the SRE-1 region. Recently, a 19-kDa protein has been cloned (9) that binds to one of the two strands encoding the SRE-1 region of the HMG-CoA reductase promoter. This protein has no demonstrable affinity for the double-stranded sequence and is upregulated in the presence of sterols.

In this study we sought to characterize the nuclear proteins that bind to SRE-1 in order to understand the role of SRE-1 in sterol-mediated transcriptional regulation. The relationship between factor binding and the sterol regulatory activity of SRE-1 mutants was investigated. A 45- to 49-kDa SRE-1-specific DNA binding activity was identified that binds to the native, double-stranded DNA element and, surprisingly, binds preferentially to one of the two single strands of SRE-1.

## MATERIALS AND METHODS

**Plasmids.** Plasmids were constructed by standard techniques (10) and their structures were verified by DNA sequence analysis. pHMG-SRE resulted from ligation of a synthetic oligonucleotide containing the 20-base sequence (−141 through −160, see Table 1) from the hamster HMG-CoA reductase promoter (9) into the *Bam*HI/*Xba* I sites in pUC19. pLDL-SRE contains a 35-base sequence (−38 through −72) from the promoter region (repeat 2) of the human LDL receptor ligated into the *Bam*HI/*Xba* I sites of pUC19.

**Oligonucleotides.** Synthetic oligomers (Table 1) were prepared commercially by Oligos Etc. (Guilford, CT) on a modified Biotix (Danbury, CT) synthesizer using β-cyanoethyl phosphoramidite chemistry. Oligomer SRE-H contains a 20-base sequence (−141 through −160) that includes the SRE-1 sequence from the hamster HMG-CoA reductase promoter. Oligomer SRE-L is a 35-base sequence (−38 through −72, derived from the antisense strand) that includes the SRE-1 sequence from the promoter region of the human LDL receptor. Oligomers A–K represent cluster or point mutations introduced into oligomer SRE-L.

Double stranded competitor DNA was made by PCR amplification of the polylinker containing inserts of the pHMG-SRE and pLDL-SRE plasmids and of the wild-type pUC19 plasmid. The PCR amplifications were carried out as previously described (10) using M13 primer and M13 reverse primer from New England Biolabs. Each PCR cycle consisted of heat denaturation at 95°C for 30 sec, primer annealing at 37°C for 30 sec, and primer extension at 76°C for 30 sec. Amplified products were chloroform extracted, EtOH precipitated, suspended in 200 mM NaCl/10 mM Tris·HCl, pH 7.6/1 mM EDTA, and analyzed on 1.2% agarose gels.

**Gel Binding.** CHO cell extracts were prepared by modification of a previously described method (11). Double-stranded DNA mobility shift assays were carried out as described (12). Mobility-shift DNA-binding assays were carried out by mixing single-stranded DNA probe ($6–8 \times 10^3$ cpm), 2.25 μg of poly(dI-dC), 6 μg of CHO nuclear extract, and, for competition assays, 50–100 ng of unlabeled compet-

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Abbreviations: LDL, low density lipoprotein; HMG, hydroxymethylglutaryl; SRE, sterol regulatory element.

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Table 1. Oligonucleotides used

| Oligomer | Sequence |
|---|---|
| | −46 −54 −60 |
| SRE-H | gatccgagagatggtgcggtgccc |
| | −72 |
| SRE-L | gatctagcaggcggagtttgcagtggggtgattttcaaa |
| A | ---------- |
| B | ---------- |
| C | ---------- |
| D | ----------CGG---------- |
| E | ----------TAT---------- |
| F | ---------CC---------- |
| G | ---------AT---------- |
| H | ---------T---------- |
| I | ---------T---------- |
| J | ---C---------- |
| K | ----------G---------- |

SRE-H, sequences from the HMG-CoA reductase promoter; SRE-L, sequences from the LDL receptor promoter. Position numbers refer to those of the LDL receptor promoter. The boxed sequence represents SRE-1. Oligonucleotides A–F are truncated and/or mutated SRE-L sequences as indicated by dashed lines (unaltered) or by the mutation(s) introduced.

itor (final concentrations, 60 mM NaCl/10 mM Tris·HCl, pH 7.7/1 mM $MgCl_2$/0.02 mM EGTA/0.1 mM EDTA) and incubating for 10 min at 22°C. The reaction was then analyzed by pre-run 0.5× TBE/4.5% PAGE (1× TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3; acrylamide/bisacrylamide, 29:1) at 150 V for 1 hr. The gel was dried and exposed overnight.

**Single-Stranded $^{32}$P-End-Labeled Probes.** The M13 sequencing primer was phosphorylated with [$\gamma$-$^{32}$P]ATP. pHMG-SRE was cut with *Pst* I, denatured, annealed with the end-labeled M13 primer, and extended, in the presence of unlabeled dNTPs, with the large (Klenow) fragment of *Escherichia coli* DNA polymerase to synthesize the antisense single-stranded probe. The coding single-stranded probe was synthesized by annealing the end-labeled $^{32}$P-phosphorylated M13 reverse primer to *Asp*718-cut pHMG-SRE and extending as above. The single-stranded probes thus synthesized were then isolated by 8 M urea/5% PAGE (acrylamide/bisacrylamide, 19:1) and overnight elution at 37°C. The body-labeled single-stranded probes required for UV cross-linking experiments were synthesized by elongation with unlabeled M13 primer in the presence of [$\alpha$-$^{32}$P]dATP in the Klenow reaction.

**Double-Stranded $^{32}$P-Labeled Probes.** Double-stranded probes were prepared by either *Pst* I/*Eco*RI (antisense strand-labeled) or *Sma* I/*Hin*dIII (sense strand-labeled) digestion of pHMG-SRE, followed by filling in the 5′ overhang with [$\alpha$-$^{32}$P]dATP and excess unlabeled dCTP/dGTP/TTP. This leads to labeling of a unique strand of the double-stranded probe. The probe was isolated on a native 1× TBE/5% polyacrylamide gel and eluted by diffusion overnight into 10 mM Tris/1 mM EDTA, pH 7.6/100 mM NaCl at 37°C. Body-labeled double-stranded probes for UV cross-linking were prepared by cutting pHMG-SRE with *Pst* I, annealing with M13 primer, and then extending in the presence of [$\alpha$-$^{32}$P]dCTP in the standard Klenow reaction. The plasmid was digested a second time with *Asp*718 and the resulting double-stranded fragment was isolated as above.

**UV Cross-Linking.** UV cross-linking was done as previously described (13). In the standard binding reaction, 5–10 × $10^4$ cpm of the appropriate body-labeled probe was bound in 15 $\mu$g of CHO nuclear extract and then was UV cross-linked for 90 min. The reaction mixtures were then treated with DNase I and micrococcal nuclease for 30 min, and the products were separated on a Laemmli:10% SDS/polyacrylamide gel system.

## RESULTS

To study the nuclear proteins involved in sterol regulation, a 19-base-pair region containing the octanucleotide previously identified as SRE-1 of the HMG-CoA reductase promoter (6, 14) was cloned into the pUC19 polylinker (pHMG-SRE). A double-stranded end-labeled probe containing this SRE sequence was prepared, and gel shift analysis was carried out using nuclear extract prepared from CHO cells. As shown in Fig. 1 a specific complex was noted in the presence of the CHO nuclear extract. This binding was competitively inhibited only by SRE-1-containing DNA fragments (lanes b–e). Thus, the double-stranded SRE-1 binding activity, which will be referred to as SRE-BF, is sequence specific.

Because of several reports of the occurrence of single-strand-specific DNA binding to promoter sequences (9, 15), we examined the ability of single-stranded oligonucleotide sequences to compete binding of SRE-BF to the double-stranded SRE-1 probes (Fig. 1, lanes f–i). The binding of the double-stranded probe was competed by single-stranded oligomers in a sequence-specific fashion. A single-stranded oligomer containing one strand of the LDL receptor version of SRE-1, SRE-L, competitively inhibited the binding of the double-stranded probe at all concentrations examined. This binding was not competed by a single-stranded oligomer complementary to the SRE-L (lane j). Unrelated single-stranded oligomers had no effect on the double-stranded binding. Mixtures of double-stranded probe, labeled on either strand, with an unlabeled single-stranded oligomer (either SRE-L or its complement) in the absence of nuclear extract showed no anomalous migration on a native polyacrylamide gel, and thus no detectable strand-exchange reaction (data not shown).

To directly assess the relative affinities of single- and double-stranded binding at the SRE-1 site, competition analysis was performed. Graded amounts of competitor DNA preparations, consisting of either double-stranded or single-stranded SRE sequences, were mixed with the radiolabeled double-stranded SRE-1 probe, and the mixtures were then incubated in CHO nuclear extract. Bound probe was analyzed by the gel-retardation assay. The relative amount of probe bound was quantitated by densitometry of the autoradiogram of the resultant bound complex. The double-stranded SRE-1 complex was competed to half-maximal binding by 10-fold lower molar quantities of single-stranded SRE-1, compared with double-stranded SRE-1 molecules (Fig. 2).

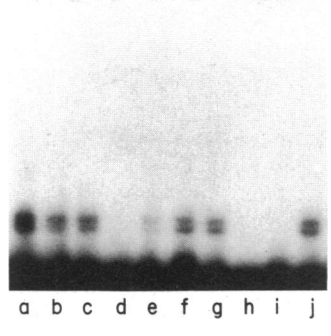


FIG. 1. Gel mobility and competition analysis. The *Sma* I/*Eco*RI-digested pHMG-SRE double-stranded probe (8 × $10^3$ cpm, ≈2.5 ng) was incubated with 6 $\mu$g of CHO nuclear extract and either 100 or 20 ng of competitor. Lanes: a, CHO nuclear extract with no added competitor; b and c, 100 and 20 ng of pUC19 polylinker; d and e, 100 and 20 ng of pHMG-SRE polylinker containing insert; f and g, 100 and 20 ng of M13 universal single-stranded oligomer primer; h and i, 100 and 20 ng of SRE-L oligomer; j, 20 ng of single strand complementary to SRE-L.

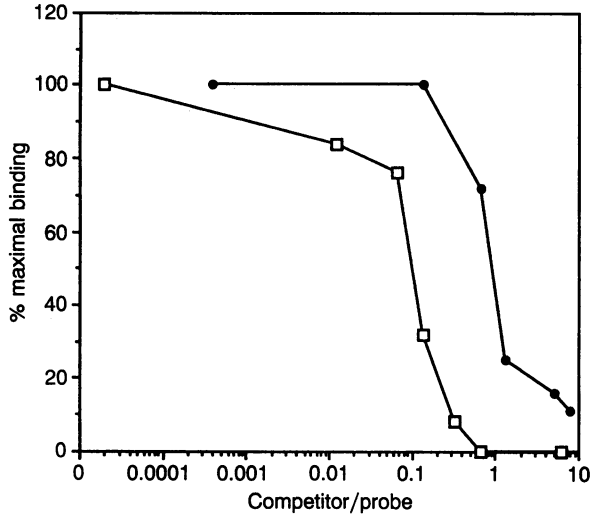


FIG. 2. Competition analysis using double- and single-stranded SRE-1 competitors. A double-stranded probe, generated by *Pst* I/*Eco*RI digestion of pHMG-SRE, was incubated with 6 μg of CHO nuclear extract at a final probe concentration of 2.5 nM. Graded amounts of unlabeled competitor double-stranded SRE (●) or single-stranded SRE (□) were added to the binding reaction. Relative amounts of bound material were quantitated by densitometry of the resulting autoradiogram and plotted as % maximal binding = (cpm bound in absence of competitor/cpm bound in the presence of competitor) × 100.

Because the single-stranded oligomers competitively inhibited binding to the double-stranded probe containing SRE-1, we investigated the nature of the binding to the single-stranded probes. The single-stranded probe prepared from the antisense strand formed a complex when incubated with CHO nuclear extract (Fig. 3*A*). This binding was competed by specific single-stranded oligonucleotides containing SRE-1 sequences but not by irrelevant or mutated single-stranded sequences (lanes B–I and Table 1). Anomalous

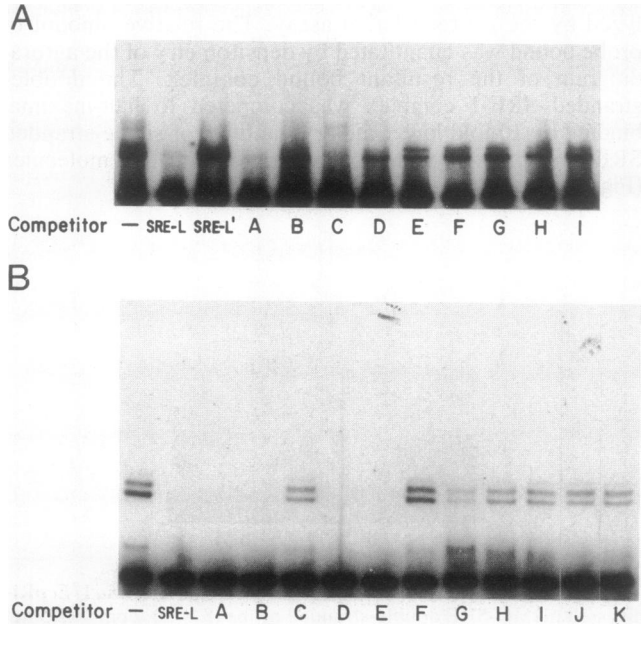


FIG. 3. Gel mobility and competition analysis of binding of the single-stranded (*A*) and double-stranded (*B*) SRE-1-containing probes. Probes ($8 \times 10^3$ cpm, ≈1.3 ng of single stranded or ≈2.5 ng of double stranded) were incubated with 6 μg of CHO nuclear extract and 100 or 50 ng, respectively, of SRE-L, SRE-L' (the sequence complementary to SRE-L), or competitor oligomer (see Table 1).

migration of the probe did not occur with identical mixtures of SRE-1 and the unlabeled competitors in the absence of nuclear extract. A sequence-specific complex was also detected when the homologous strand of the LDL receptor SRE was assayed (data not shown). No sequence-specific complex was detected when the single-stranded probes were prepared from the coding strand. It is thus likely that a common factor, SRE-BF, binds both single- and double-stranded SRE-1 probes, with higher affinity for the single-stranded form.

Because SRE-BF binds preferentially to single-stranded DNA, it is important to exclude the possibility that the observed double-stranded binding reflects binding to a small population of denatured DNA molecules. Double-stranded probes prepared individually by labeling one of the two strands of the double-stranded sequence formed identical complexes regardless of which strand was labeled. Since the single-stranded probe from the coding strand did not bind, the observed double-stranded binding cannot be due to denaturation of the double-stranded probe. Thus, binding of the double-stranded DNA requires a double-stranded DNA structure during the binding process and cannot be explained by the presence of an initially minor single-stranded DNA population.

There have been reports of sequence-specific binding of single-stranded nucleic acid sequences by heterogeneous nuclear ribonucleoprotein (16, 17). The complexes of SRE-BF and single-stranded cognate sequences formed in 6 μg of CHO nuclear extract were found to be resistant to digestion with 1 μg of pancreatic RNase but were completely digested by 10 ng of trypsin. Thus, SRE-BP is a protein-containing complex with no evident RNA component.

The sequence specificity of single-stranded binding was analyzed by using a series of mutant oligomers as competitors (Fig. 3*A*). The ability of the same oligomers to compete double-stranded DNA binding was also analyzed (Fig. 3*B*). The specificity for competition of the single-stranded probe was distinct from that for the double-stranded probe, as indicated by the ability of oligomer C to compete single-stranded binding (Fig. 3*A*) but not double-stranded binding (Fig. 3*B*) and the ability of oligomer B to compete double-stranded DNA probe (Fig. 3*B*) but not single-stranded DNA probe (Fig. 3*A*). The sequences necessary to compete single-stranded DNA binding were localized between −54 and −72 of the LDL receptor gene. This region overlaps but is distinct from the region of competition of double-stranded binding (positions −46 and −60). The sequence-specific nature of this binding is further supported by the inability of single point mutants to compete single-stranded binding (oligomers H–I). Table 1 summarizes the relationships of the oligomer competitors to each other and to the region previously determined to be important for sterol regulated transcription (3); Table 2 summarizes the requirements for SRE-BF binding to the double-stranded and single-stranded SRE region.

To characterize the relationship between the single-stranded and double-stranded SRE-1 binding activities, photochemical cross-linking of the complexes formed with single-stranded SRE-1 and double-stranded SRE-1 probes was performed. The complexes were digested with bovine DNase I and micrococcal nuclease, and the products were analyzed by SDS/PAGE under both reducing and nonreducing conditions. The proteins bound to single-stranded SRE-1 and to double-stranded SRE-1 comigrated at 45–49 kDa (Fig. 4). This series of bands may represent the presence of multiple proteins in the complex or partial proteolysis during the extract isolation. The addition of oligomer SRE-1 to the binding reaction completely abolished the formation of detectable cross-linked species (Fig. 4, lanes b and d). Control oligomers had no effect on the presence of the cross-linked complex. These results suggest that the same protein(s)

Table 2. Effects of mutations in SRE-1 as assayed by competition of single- and double-stranded SRE-1 binding assays and of these mutations on *in vivo* sterol regulation

| Oligomer | Competition: Single-stranded binding | Competition: Double-stranded binding | Sterol regulation *in vivo* |
|---|---|---|---|
| SRE-L | + | + | + |
| A | + | + | + |
| B | − | + | − |
| C | + | − | + |
| D | − | + | −* |
| E | − | + | −* |
| F | − | − | −* |
| G | − | − | −* |
| H | − | − | − |
| I | − | − | ± |
| J | + | − | + |
| K | + | − | + |

+, Ability of the oligonucleotide to compete in the indicated assay or of the indicated sequence to direct sterol-regulated transcription when assayed *in vivo* (see text); −, inability to compete or direct transcription.

*Sterol regulation is predicted to be negative based on the inactivation of transcription by one of the included point mutations.

complexes to the single-stranded and the double-stranded SRE-1 sequences.

## DISCUSSION

The experiments presented here identify a 45- to 49-kDa protein in CHO nuclear extracts, SRE-BF, that binds to the SRE-1 octanucleotide sequence. This DNA binding activity is competed specifically by double-stranded SRE-1-containing DNA fragments. Surprisingly, SRE-BF also binds to single-stranded DNA sequences derived from the wild type but not to the mutant SRE-1 sequence of the LDL receptor promoter. This binding activity is specific for the noncoding strand. The sequence specificity for single-stranded binding closely correlates with the reported (3) *in vivo* ability of SRE-1 to direct sterol responsiveness of transcription (Table 2). The sequences required for double-stranded binding partially overlap with those required for single-stranded binding.

The binding specificity of SRE-BF to double-stranded SRE-1 and to the antisense single strand of SRE-1 are clearly distinguishable, although there is partial overlap. The data

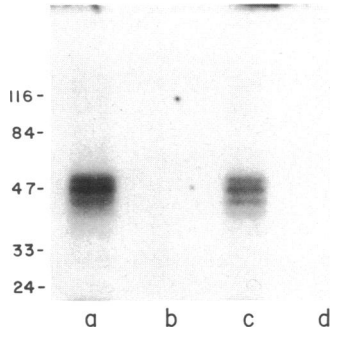


FIG. 4. UV cross-linking of SRE-1-binding proteins. Body-labeled single-stranded or double-stranded probe ($10^5$ cpm) was incubated with 15 $\mu$g of protein from CHO extract, irradiated with UV for 90 min, and then digested with DNase and micrococcal nuclease. Products were analyzed by denaturing gel electrophoresis and autoradiography. Lanes: a and b, single-stranded probe bound in the presence of the M13 sequencing primer or oligomer SRE-L; c and d, double-stranded probe bound in the presence of the M13 sequencing primer or oligomer SRE-L, respectively. Numbers on the left represent molecular mass × $10^{-3}$.

presented in Fig. 2 also argue cogently for a marked preference of SRE-BF for single-stranded cognate DNA sequences over double-stranded DNA sequences. Smith *et al.* (2) have identified a series of bases in the LDL receptor SRE-1 that are critical for transcriptional activation in the absence of sterols. Competition analysis of the single-stranded SRE-1 binding by various SRE-1 mutant oligonucleotides correlates well with the *in vivo* data. This suggests a possible role for SRE-BF single-stranded binding in sterol-regulated transcription.

Photochemical cross-linking experiments indicate that the two probes are bound by a factor(s) that comigrates in reducing denaturing polyacrylamide gels. It thus appears that a common factor recognizes and binds sequences in the SRE-1 region. The mechanisms involved in binding the two forms of DNA, however, are distinct. Since sequence-specific double-stranded binding occurs by contact with specific bases in the major groove of B-DNA, it has been possible in all cases examined to identify contact bases by methylation-interference studies. In contrast, the single-stranded DNA binding is not inhibited by any single purine base methylation (data not shown), despite the fact that sequence specificity is readily demonstrated by competition analysis. With exception of the period during chromosomal replication, DNA is not found as unwound or single-stranded forms. For SRE-BF binding to occur with high affinity would require a helicase activity to convert from B-DNA. Whether the SRE-BF has such helicase activity is not known.

Prokaryotic RNA polymerase is well known to bind to cognate single-stranded regions as a requirement for initiation of transcription. Although the majority of reported eukaryotic sequence-specific DNA binding proteins recognize native double-stranded sequences, there are reports of single-stranded protein–DNA complexes that are thought to be involved in the regulation of transcription. The estrogen receptor binds to the estrogen responsive element and has been shown to activate transcription of nearby genes (18–21). Analyses of the affinity of the estrogen receptor for both double-stranded and single-stranded versions of the estrogen-responsive element have shown a 60-fold preference for one of the two single strands (15). This situation is similar to the affinity preference of SRE-BF for SRE-1.

RPF-1, a factor that binds the sequence TGG found in numerous places in the HMG-CoA reductase promoter, including its SRE, identified by Gil *et al.* (8), appears to be distinguishable from SRE-BF. The TGG motif appears in multiple locations in the reductase promoter and is recognized by RPF-1. Although both factors exhibit sequence specific binding to the double-stranded SRE-1 region, proteins copurifying with RPF-1 migrate as a doublet with an apparent molecular mass of 31 and 33 kDa whereas the factor(s) identified here migrates with an apparent molecular mass of 45–49 kDa. When the cDNA for RPF-1 was isolated a related 48-kDa protein with similar binding specificity, NF-1/X, was isolated as well (22), but the DNA sequence specificity of RPF-1 and NF-1/X make it unlikely that either of these is related to SRE-BF. The affinity of RPF-1 and NF-1/X for single-stranded DNA templates has not been reported.

Point mutational analysis of SRE-1 *in vivo* (3) correlates well with SRE-BF binding of single-stranded SRE-1 *in vitro*. The binding of double-stranded SRE-1 by SRE-BP does not have the specificity of binding necessary for sterol regulation *in vivo*. It is possible that SRE-BF binding of single-stranded SRE-1 may be responsible for transcriptional regulation of the LDL receptor. A role of SRE-BF in DNA replication or other, nontranscriptional processes must also be examined. The fine specificity of double-stranded DNA binding by SRE-BF competed by double-stranded competitors remains to be established, and the correlation with previous point

mutational transcriptional analysis must be examined further. If SRE-BF binding of double-stranded SRE-1 is an obligate step in transcription, then either the required affinity for *in vitro* DNA binding is greater than the requirement for binding *in vivo* or binding to the double-stranded SRE-1 sequence occurs in concert with another factor.

There may be significant thermodynamic advantage to a protein–DNA complex in which the DNA is a single strand rather than a B-form double strand. A single-stranded DNA–protein complex would be expected to have greater flexibility, allowing protein–protein interaction at lower free energy input than the corresponding double-stranded DNA–protein complex. This suggests a possible thermodynamic advantage to an uncoiling mechanism in situations where interacting regulatory elements are closely apposed. The involvement of SRE-BF in functional events remains to be investigated. Whether sterols alter expression of or affinity of binding to single- or double-stranded SRE-1 probes is an important issue that remains to be resolved.

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