Protein binding to the low density lipoprotein receptor promoter in vivo is differentially affected by gene activation in primary human cells

Jeff L. Ellsworth,[1,*] David B. Lloyd,[†] Andrew J. Carlstrom,[*] and John F. Thompson[†]

Department of Atherosclerosis and Lipid Metabolism,* Research Institute, Palo Alto Medical Foundation, 860 Bryant Street, Palo Alto, CA 94301, and Molecular Genetics and Protein Chemistry Department,† Pfizer Inc, Central Research Division, Eastern Point Road, Groton, CT 06340

Abstract Protein–DNA interactions within a region of the LDL receptor promoter involved in sterol-mediated feedback repression of transcription were examined using in vivo genomic footprinting with dimethylsulfate (DMS). A broad region of protein–DNA contacts spanning from repeat 1 to beyond the transcription start sites was observed in primary cultures of human skin fibroblasts and hepatocytes. Hypermethylation of guanine −59 within the sterol regulatory element-1 (SRE-1, repeat 2) occurred within a 4.0 h incubation of fibroblasts with media containing lipoprotein-deficient serum (LPDS) and cholesterol synthesis inhibitors. Methylation of this residue was reduced to control levels within 2.0 h after the addition of a mixture of 25-hydroxycholesterol and mevalonic acid. The time-dependent changes in DMS-reactivity of guanine −59 induced by the cholesterol synthesis inhibitors or oxysterols were paralleled by alterations in LDL receptor mRNA. In contrast to the results with fibroblasts, neither cholesterol synthesis inhibitors nor oxysterols produced consistent effects on the DMS-reactivity of guanine −59 in hepatocytes despite induction or repression of LDL receptor mRNA in these cells. Interestingly, no other changes in the protection pattern over repeats 1, 2, and 3 were apparent in either fibroblasts or hepatocytes. These results demonstrate that hypermethylation of guanine −59 within the SRE-1 is positively associated with activation of LDL receptor gene transcription in skin fibroblasts. Furthermore, the absence of demonstrable changes in DMS-reactivity of other purines within this region suggests that the LDL receptor promoter is poised to activate transcription with only minimal changes of protein binding to the proximal promoter in vivo.—**Ellsworth, J. L., D. B. Lloyd, A. J. Carlstrom, and J. F. Thompson.** Protein binding to the low density lipoprotein receptor promoter in vivo is differentially affected by gene activation in primary human cells. *J. Lipid Res.* 1995. **36:** 383–392.

Supplementary key words dimethylsulfate genomic footprinting • protein–DNA interaction • cholesterol synthesis inhibitors • feedback regulators

Mammalian cells in culture satisfy their sterol requirements by regulation of endogenous cholesterol synthesis and receptor-mediated endocytosis of low density lipoproteins (LDL) (1). When the demand for cell sterol is increased, the level of mRNA and activity of the LDL receptor and a number of enzymes in the cholesterol biosynthetic pathway such as 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) and HMG-CoA synthase are increased (1). When the demand for cell sterol is met, conversely, the amount of mRNA produced by these genes is reduced and cholesterol biosynthesis decreases. A DNA sequence, termed the sterol regulatory element-1 (SRE-1), appears to be required for sterol-mediated repression of these genes (2–4). The SRE-1 consensus, 5'-CACC(C/G)CAC-3', was hypothesized to represent the recognition sequence of a DNA binding protein whose level or activity is modulated by regulatory sterols. Various proteins that recognize single- and double-stranded SRE-1 sequences or sequences nearby have been reported in studies using in vitro protein/DNA binding assays (4–11). These studies have revealed binding sites for Sp1 (4), NF-1 (7, 8), Red 25 (7), SREBP-1 (10), and SREBP-2 (11). In vitro studies are limited, however, in that they may fail to detect DNA binding proteins that are present in low concentration or have overlapping specificities. Furthermore, binding sites that are unavailable in native chromatin may be falsely detected. For these reasons, we have used the technique of dimethylsulfate genomic footprinting (12) to examine the in vivo protein contacts on the LDL receptor SRE-1 and flanking sequences in human skin fibroblasts and primary

Abbreviations: LDL, low density lipoproteins; MEM, Eagle's minimal essential medium; EBSS, Earle's Balanced salt solution; SDS, sodium dodecylsulfate; PBS, phosphate-buffered saline; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase, EC 1.1.1.34; G3PD, glyceraldehyde-3-phosphate dehydrogenase, EC 1.2.1.12; SRE, sterol regulatory element; SSC, saline sodium citrate; PAGE, polyacrylamide gel electrophoresis; nt, nucleotides; DMS, dimethylsulfate; LPDS, lipoprotein-deficient serum.

[1]To whom correspondence should be addressed at present address: CV Therapeutics, 3172 Porter Drive, Palo Alto, CA 94304.

cultures of human hepatocytes. We provide the first evidence that the proximal promoter region of the LDL receptor gene is occupied by multiple proteins in vivo and these proteins are poised to activate transcription with minimal changes in the underlying DNA contacts.

EXPERIMENTAL PROCEDURES

Materials

Cytidine 5'-[α-^{32}P]triphosphate (~3,000 Ci/mmol), adenosine 5'-[γ-^{32}P]triphosphate (~6,000 Ci/mmol), and deoxyadenosine 5'-α-[^{35}S]thiotriphosphate (> 1,000 Ci/mmol) (all triethylammonium salts) were obtained from Amersham (Arlington Heights, IL). Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from Pharmacia-LKB (Piscataway, NJ). RNA transcription kits, RNase-Block II, Nuc Trap Push Columns, Epicurian Coli XL 1-Blue competent cells, and pBluescript II KS (+/−) were obtained from Stratagene (La Jolla, CA). RPA II kits were obtained from Ambion (Austin, TX). Proteinase K was obtained from BRL (Gaithersburg, MD). The plasmids pLDLR3 and pHcGAP were obtained from the American Type Culture Collection (Rockville, MD). RNase-free DNase I was purchased from Worthington (Freehold, NJ). Restriction enzymes were purchased from Stratagene or New England Biolabs (Beverly, MA). Sequenase 2.0 was obtained from United States Biochemical (Cleveland, OH). Ketoconazole and 5-cholesten-3β,25,diol were purchased from Sigma Chemical Co. (St. Louis, MO) and Steraloids (Wilton, NH), respectively. Mevinolin was a generous gift from Merck. All tissue culture supplies were purchased from GIBCO-BRL (Grand Island, NY) and all other chemicals were molecular biology grade and were purchased from Sigma or as described previously (13, 14).

Cell culture

Primary cultures of human hepatocytes were obtained from the Human Liver Research Facility at SRI International (Menlo Park, CA) and were isolated by the collagenase-biopsy perfusion method (15). Hepatocytes were plated on collagen-coated tissue culture flasks in CMH1 medium [Waymouth's 752/1 medium supplemented with 11.2 μg/ml alanine, 12.8 μg/ml serine, 24.0 μg/ml asparagine, 84.0 μg/ml gentamycin sulfate, 100.0 ng/ml amphotericin B, 0.168 μg/ml aminolevulinic acid, 5.0 μg/ml oleic acid, 5.0 μg/ml linoleic acid, 1.0 μg/ml D,L-tocopherol, 288 ng/ml testosterone, 272.0 ng/ml estradiol, 393.0 ng/ml dexamethazone, 7.9 μg/ml D-thyroxin, 30.0 ng/ml glucagon, 0.02 U/ml insulin, 70.4 μg/ml ascorbic acid, 0.1% ITS (final concentration 5.0 μg/ml transferrin, 5.0 μg/ml insulin, 5.0 ng/ml selenium), and 0.2% BSA] as described (15). After 2–4 h of culture to allow cell attachment, the media were replaced with fresh CMH1 and the cultures were returned to the incubator. After 6–18 h, incubations were initiated by the addition of drugs to the appropriate flasks of cells as indicated in the legends to the figures. Human skin fibroblasts were cultured in medium A (Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum, 292 μg glutamine/ml, 100 units of penicillin/ml, and 100 μg of streptomycin/ml) as described previously (13). The fibroblast cultures were refed with fresh medium A 24–48 h prior to each experiment. At the time of assay, each 100 mm^2 dish contained 2.8 × 10^6 cells for hepatocytes and 5.4 × 10^6 cells for fibroblasts.

Isolation and measurement of cellular RNA

Total RNA was isolated from hepatocytes (8.4 × 10^7 cells total) and fibroblasts (1.7 × 10^7 cells total) by the guanidinium isothiocyanate procedure (16) as described previously (13, 14). LDL receptor and G3PD riboprobes were prepared from PCR-generated templates using the plasmids pEB-LDLR KS(+) and pHX-G3PD KS(−) as described previously (14). The specific activities were ~8 × 10^7 and ~3.5 × 10^7 cpm/μg for the LDL receptor and G3PD probes, respectively. RNase protection analysis was performed using the RPA II kit as described previously (14).

Dimethylsulfate genomic footprinting

To methylate and isolate genomic DNA, the cell media were removed and replaced with Earle's Balanced Salt Solution containing 0.02 M HEPES, pH 7.4, prewarmed to 37°C. Dimethylsulfate was added to a final concentration of 0.1% and the dishes were incubated at room temperature for 1 min. The DMS-media were removed and the cells were washed four times with PBS prewarmed to 37°C, and 0.6 ml of genomic DNA prep buffer (1 mM Tris-HCl, pH 7.5, 0.4 M NaCl, 2 mM EDTA, 0.2% SDS) was added to each dish. After 10 min at room temperature, the lysate was collected, proteinase K was added to a final concentration of 0.2 mg/ml, and lysates were incubated for 18–20 h at 37°C. The DNA was then gently phenol-extracted twice and ethanol-precipitated. Genomic DNA was denatured at 90°C for 10 min in 0.2 ml of a solution containing 0.2 M KPO_4 and 1 mM EDTA. The samples were chilled on ice, 2 μl of 10 M NaOH was added, and the samples were heated for 5 min at 90°C. The cleaved DNA was extended with Sequenase and an oligonucleotide primer homologous to −255 to −240 in the LDL receptor promoter. A double-stranded oligonucleotide was ligated to the extended DNA as described (12) and amplified with one of the ligation oligonucleotides and a primer complementary to −187 to −167 in the LDL receptor promoter. Amplification was done for 25 cycles (1 min at 94°C, 2 min at 66°C, and 3 min at 76°C) with native Taq polymerase in a solution of 10 mM Tris-HCl (pH 8.35), 50 mM KCl, 1.2 mM $MgCl_2$,

and 0.2 mM dNTPs and the mixture was overlaid with mineral oil. The mixture was labeled by adding a 5' ^{32}P end-labeled oligonucleotide homologous to −185 to −155 of the LDL receptor promoter, fresh Taq polymerase, dNTPs and buffers. The samples were incubated 2 min at 94°C, 2 min at 70°C, and 10 min at 76°C. After ethanol precipitation, the samples were resuspended in loading buffer and electrophoresed on a 6% polyacrylamide gel containing 7 M urea. The gel was dried and exposed to X-ray film.

Other methods

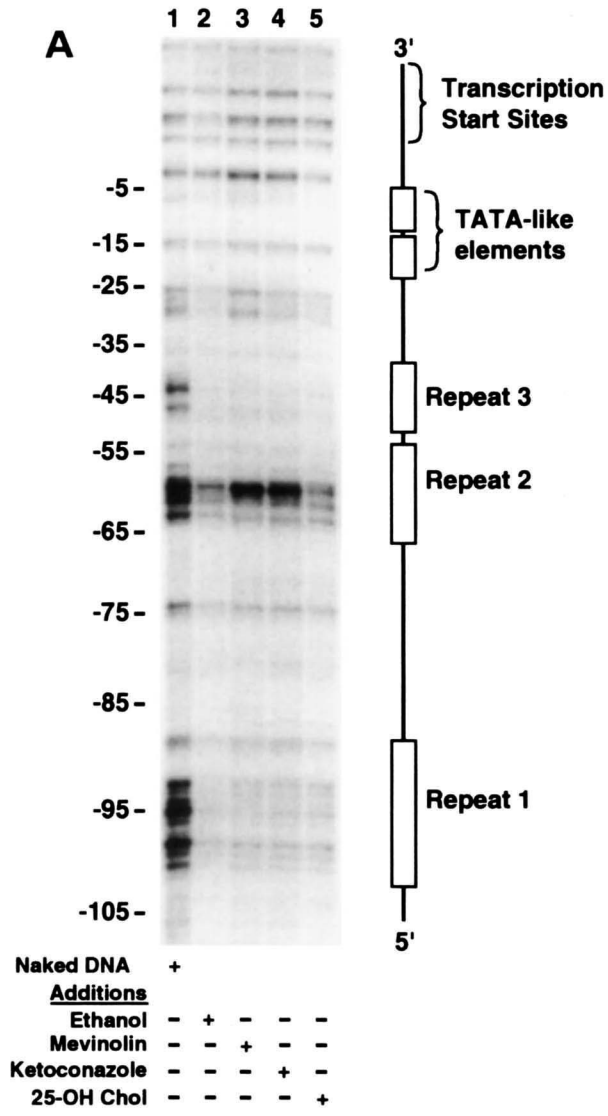
A 100-ng sample of Hpa II-digested pBR322 was end-labeled to a specific activity of 4×10^7 cpm/μg with Klenow polymerase for use as size standards. Statistical analyses were performed by the Rank Sum Test.

RESULTS

Some of the close contacts between a protein and DNA can be identified using the alkylating agent dimethylsulfate (DMS) which enters the nucleus rapidly and alkylates the N7 of guanine in the major groove and, at a slower rate, the N3 of adenine in the minor groove of DNA (17, 18). Proteins bound at, or adjacent to, these purines may either reduce or enhance the frequency of methylation in vivo in comparison to protein-free DNA. To assess whether the DMS-reactivity patterns of the LDL receptor promoter were sensitive to changes in cellular cholesterol metabolism, cells were incubated with DMS before and after treatment with cholesterol synthesis inhibitors (mevinolin or ketoconazole) to increase, or, with feedback regulators (25-hydroxycholesterol and mevalonic acid), to decrease, transcription of the LDL receptor gene (14). Primers to visualize the lower strand of the LDL receptor promoter were used. The base composition of the downstream region prevented us from obtaining consistent results with the upper strand. This was not a serious limitation, however, as only three guanine residues are found within the upper strand region encompassing repeats 1, 2, and 3 of the LDL receptor promoter. Compared to protein-free DNA (**Fig. 1A,** lane 1), a broad region of protection was observed in the DMS-reactivity patterns over repeats 1, 2, and 3, from 5' of repeat 1 at −103 to just 5' of the start sites of transcription in fibroblasts (Fig. 1A, lane 2). The protected residues were: guanines −100, −98, −95, −94, −92, −88 and adenine −96 within repeat 1; guanines −63, −61, and −59 within repeat 2; and guanines −47 and −43 within repeat 3. The guanines in the lower strand of repeat 3 are difficult to distinguish due to low basal DMS-reactivity. Methylation protection of guanines −58 and −60 was also observed in this experiment; however, the degree of protection of these residues varied among different trials. The DMS-reactivity patterns of mevinolin- or ketoconazole-treated fibroblasts (Fig. 1A, lanes 3 and 4) were similar to that of cells incubated with vehicle alone; interestingly, however, these agents produced hypermethylation of guanine −59 within repeat 2. No consistent changes in the DMS-reactivity patterns of other guanines within repeat 2 were noted nor were differences in DMS-reactivity noted between fibroblasts incubated with vehicle or a mixture of 25-hydroxycholesterol and mevalonic acid (Fig. 1A, lane 5).

To evaluate whether the changes observed in DMS-reactivity of guanine −59 were associated with alterations in LDL receptor gene transcription, parallel cultures were incubated as described above and the level of LDL receptor mRNA was measured by ribonuclease protection. Treatment with mevinolin (Fig. 1B, lane 2) or ketoconazole (Fig. 1B, lane 3) increased LDL receptor mRNA by 3.8 ± 2.1-fold (mean ± SD, n = 4, $P < 0.05$) and 2.9-fold (range = 1.6- to 4.2-fold), respectively. Incubation with 25-hydroxycholesterol and mevalonic acid (Fig. 1B, lane 4), conversely, decreased LDL receptor mRNA by 83 ± 11% (mean ± SD, n = 4, $P < 0.05$). In another study, qualitatively similar results were obtained when DMS-footprinting and LDL receptor mRNA measurements were carried out with either human hepatoma HepG2 cells or the human T-cell line Jurkat (D. B. Lloyd and J. F. Thompson, unpublished results). Sterol-dependent alterations in LDL receptor gene transcription are thus directly correlated with changes in DMS-reactivity of guanine −59. With the exception of this single base, no other consistent changes in DMS-reactivity were noted. This demonstrates that activation or repression of the LDL receptor gene in human skin fibroblasts is not associated with major changes in protein–DNA contacts within the proximal promoter region of the LDL receptor gene in vivo.

To examine the correlation between DMS-reactivity and transcription in greater detail, fibroblasts were incubated for various times at 37°C in the presence and absence of either oxygenated sterols or mevinolin. Parallel cultures of fibroblasts were used for genomic footprinting and for measurement of LDL receptor mRNA. Relative to protein-free DNA (**Fig. 2A,** lane 1), a broad region of protection was observed over repeats 1, 2, and 3 with DNA from cells treated with vehicle alone (Fig. 2A, lane 2). Incubation with media containing 10% lipoprotein-deficient serum for 24 h produced hypermethylation of guanine −59 within repeat 2 (Fig. 2A, lane 3). No changes in DMS-reactivity of other bases within this region were observed. After 25-hydroxycholesterol and mevalonic acid were added to these cells, hypermethylation of this residue was reduced at 2.0 h and remained low for the 10.0 h tested (Fig. 2A, lanes 4–8). The level of LDLR mRNA fell more than 3-fold between 2.0 and 6.0 h after addition of oxysterols and remained low for up to 10.0 h (Fig. 2B). Incubation with lipoprotein-deficient se-



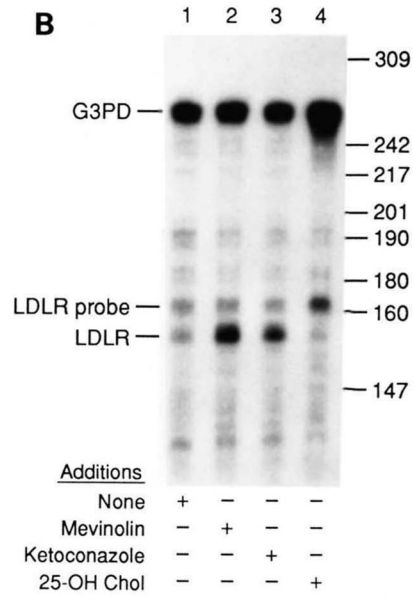
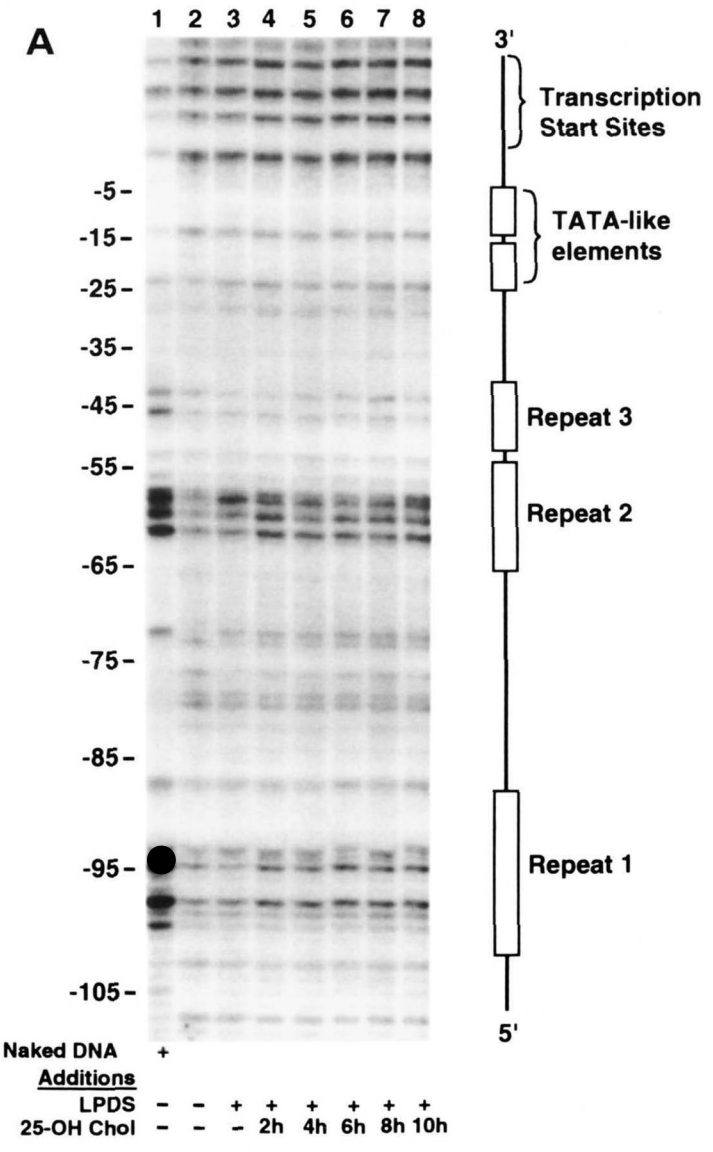
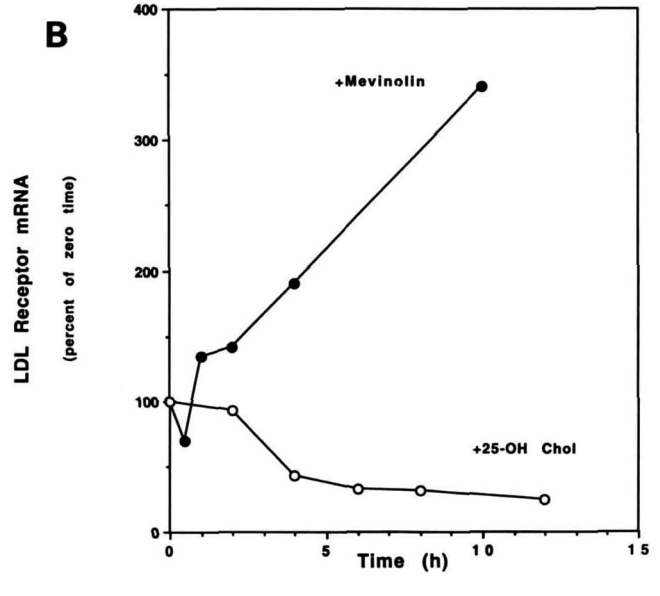


Fig. 1. Dimethylsulfate reactivities of the LDL receptor promoter in fibroblasts. A: Lower strand guanine ladders of genomic DNA isolated from DMS-treated human skin fibroblasts. DNA was isolated 12 h after addition of vehicle alone (ethanol, 0.2%), 2.0 μM mevinolin, 10.0 μM ketoconazole, or 2.0 μg 25-hydroxycholesterol/ml + 0.2 mg mevalonic acid/ml (lanes 2, 3, 4, 5, respectively). Lane 1 contains protein-free genomic DNA treated with dimethylsulfate in vitro. The positions of repeats 1–3 and the TATA-like elements relative to the transcription start sites are shown for reference. Enhanced DMS reactivity is denoted by the arrowhead. B: Ribonuclease protection analysis of 10.0 μg of total RNA isolated from parallel cultures of fibroblasts after treatment with the agents as described in A. The sizes of ^{32}P-labeled Hpa II fragments of pBR322 in nucleotides are shown on the right.

rum and mevinolin, in contrast, produced a time-dependent hypermethylation of guanine −59 (Fig. 2C) and induction of LDL receptor mRNA (Fig. 2B) that were observed at 4.0 h and maintained for the 12.0 h tested. The apparent 4.0 h lag in induction of guanine −59 hypermethylation and elevation of LDL receptor mRNA by sterol synthesis inhibitors is probably due to the time required in cell culture systems for depletion of cellular regulatory sterols. These data thus demonstrate that oxysterols and mevinolin produce reciprocal changes in DMS-reactivity of guanine −59 within repeat 2 of the LDL receptor promoter that are paralleled by changes in LDL receptor mRNA. Moreover, these changes are established early and remain largely unchanged for greater than 10 h of incubation with these agents.

To examine tissue-specific changes in DMS-reactivity in the LDL receptor promoter, primary cultures of human hepatocytes were used. The pattern observed with vehicle alone was qualitatively similar to that observed with fibroblasts, Jurkat (D. B. Lloyd and J. F. Thompson, unpublished results), and HepG2 (D. B. Lloyd and J. F. Thompson, unpublished results) cells with protections at guanines −100, −98, −95, −94, −92 and adenine −93 within repeat 1, guanines −63, −61, −60, −59, and −58 within repeat 2, and guanines −47 and −43 within repeat 1 (**Fig. 3A,** lane 2). No consistent differences in the DMS-reactivity were noted, however, between hepatocytes incubated with ketoconazole (Fig. 3A, lane 3), or oxysterols (Fig. 3A, lane 4) despite induction (76%) and repression (72%), respectively, of LDL receptor mRNA (Fig. 3B). Hypermethylation of guanine −59 was not observed in genomic DNA of hepatocytes from different donors treated with ketoconazole, suggesting that regulation of LDL receptor gene transcription in fibroblasts and





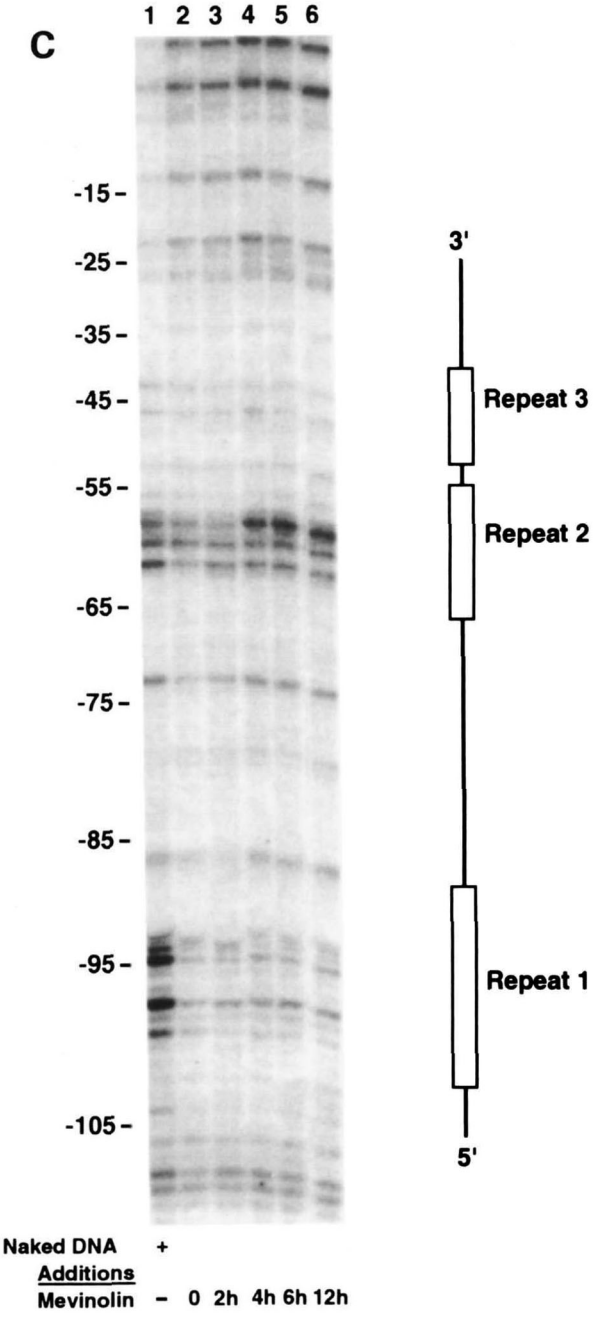
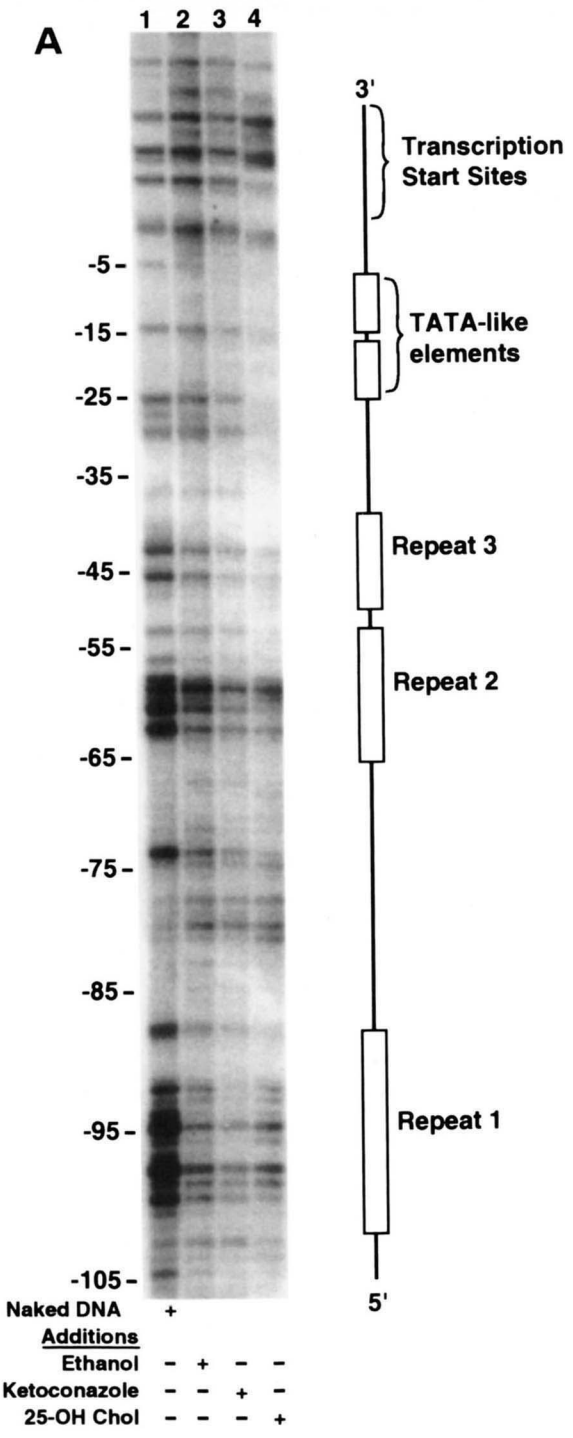


Fig. 2. Time course of oxysterol- and mevinolin-mediated changes in DMS reactivities of the LDL receptor promoter in fibroblasts. A: Lower strand guanine ladders of genomic DNA isolated from DMS-treated human skin fibroblasts as in Fig. 1A except that cells were incubated for 24 h with media containing 10% lipoprotein-deficient serum (13) and genomic DNA was isolated at 0, 2, 4, 6, 8, and 10 h after the addition of 25-hydroxycholesterol and mevalonic acid. Lane 1 contains protein-free DNA that was treated with DMS in vitro. B: Ribonuclease protection analysis of 10 μg of total RNA isolated from cultures similar to A and C, by methods described in Fig. 1B. C: As is A, except that genomic DNA was isolated at 0, 2, 4, 6, and 12 h after the addition of media containing 10% lipoprotein-deficient serum and mevinolin in the amounts described in the legend to Fig. 1A. Lane 1 contains protein-free DNA that was treated with DMS in vitro.



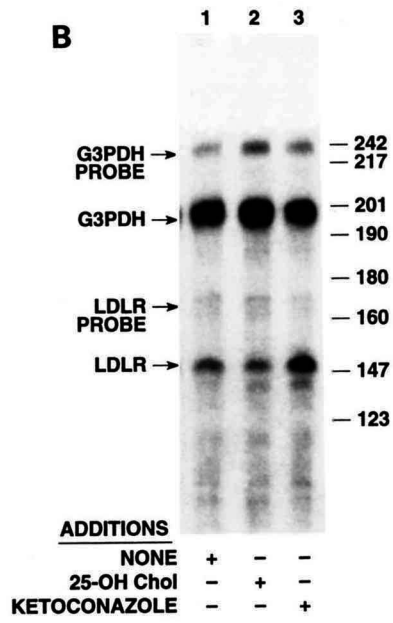


Fig. 3. Dimethylsulfate reactivities of the LDL receptor promoter in primary cultures of hepatocytes. A: Lower strand guanine ladders of genomic DNA isolated from DMS-treated human hepatocytes. DNA was isolated as in Fig. 1A, 12 h after addition of vehicle alone (ethanol, 0.2%), 20 μM ketoconazole, or 10 μg 25-hydroxycholesterol/ml + 0.2 mg mevalonic acid/ml (lanes 2, 3, and 4 respectively). Lane 1 contains protein-free genomic DNA treated with DMS in vitro. B: Ribonuclease protection analysis of 20 μg of total RNA isolated from parallel cultures of hepatocytes incubated as in A.

hepatocytes may differ in this region. A summary of these data is presented in **Fig. 4.** These data demonstrate that, with the exception of differences in DMS-reactivity of guanine −59 within repeat 2, the protein:DNA contacts over repeats 1, 2, and 3 of the LDL receptor promoter are largely similar in fibroblasts and primary cultures of human hepatocytes. These interactions are present before incubation with mevinolin, ketoconazole, or oxygenated sterols and are largely unchanged during subsequent gene activation or repression.

DISCUSSION

Studies of CHO cells transfected with fragments of the 5'-flanking region of the LDL receptor gene have defined

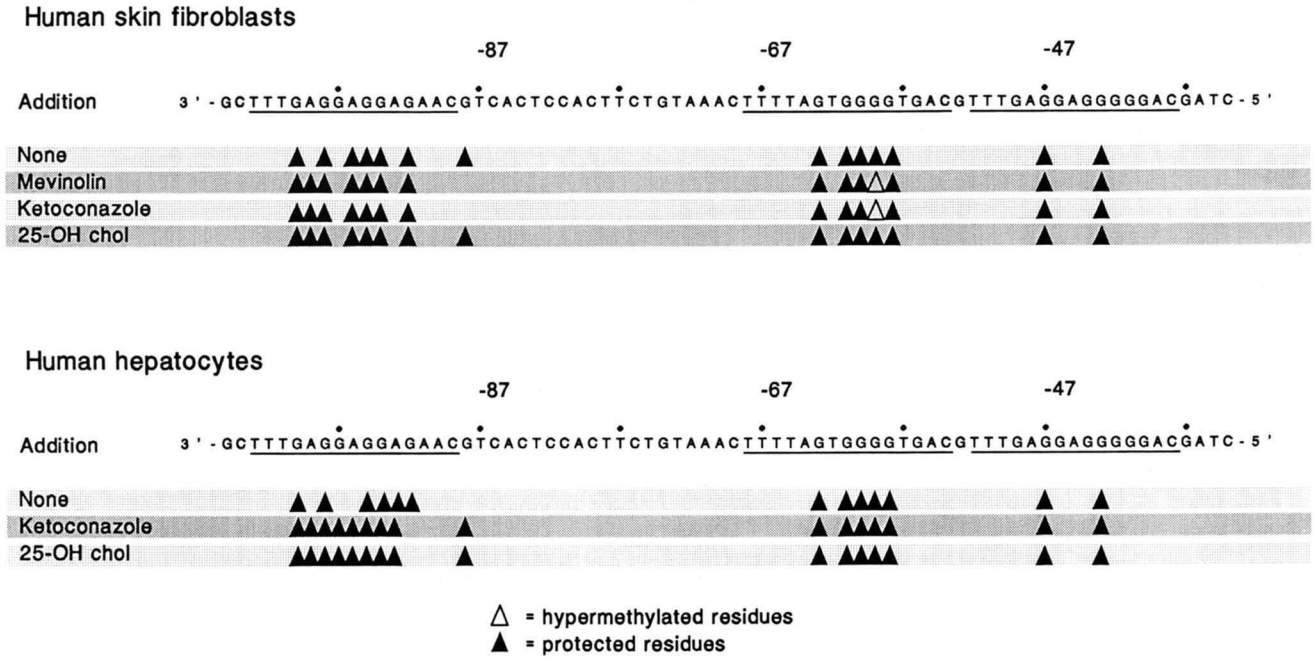


Fig. 4. Summary of in vivo protection and enhancement of DMS-reactivity in protein-bound, as compared with protein-free, samples from fibroblasts and hepatocytes. Average of three experiments with fibroblasts and two experiments with hepatocytes from two different donors. The designation "None" under Addition represents control dishes of cells that received ethanol alone.

the minimal sequence elements required for basal transcription and repression of transcription by sterols (4, 5, 19). These studies have shown that the proximal promoter region of the LDL receptor gene contains three imperfect direct repeats, designated 1–3, that are necessary for regulated transcription. The second repeat in this sequence was found to mediate repression of transcription in the presence of sterols and thus was named the sterol regulatory element or SRE-1 (4). These elements are thought to play a role in transcription by binding sequence-specific, *trans*-acting proteins. Indeed, in vitro binding studies have revealed that repeats 1 and 3 of the LDL receptor promoter can bind the transcription factor Sp1 (4). The LDL receptor SRE-1 does not bind Sp1 in vitro, but was shown to bind a number of proteins of 59–68 kDa (5, 6, 10, 11). Mutations in the SRE-1 that blocked in vitro binding of these proteins abolished sterol-mediated repression of a heterologous gene, suggesting that they are mediators of sterol responsiveness.

While the previous studies of protein binding to the proximal promoter region of the LDL receptor gene have been very useful, they have relied on in vitro methods for assessing protein–DNA interactions; and, thus, may not accurately reflect binding site interactions in native chromatin with physiological concentrations of proteins. The low concentration of the SREBP, for example, prevents its detection by in vitro DNase I-footprinting (4). Binding of the protein to the SRE-1 can be readily detected as the protein is purified (5, 6). To circumvent this difficulty, we have used the technique of genomic footprinting by ligation-mediated PCR (LM-PCR) to examine the protein contacts on the LDL receptor promoter in situ. Compared with DNA from parallel cultures treated with DMS in vitro, genomic DNA from fibroblasts and hepatocytes showed a broad region of protection that encompassed repeats 1, 2, and 3 of the LDL receptor promoter under all conditions tested. These in vivo protection patterns are generally similar to the in vitro DNase I footprinting results of Dawson et al. (4) in which HeLa cell nuclear extracts and a fragment of the LDL receptor promoter extending from −141 to +25 were used. The major difference noted, as discussed above, was the methylation protection of the SRE-1 seen in the current studies. Each of the residues protected in repeats 1, 2, and 3 has been shown to be required for transcription in studies using single or multiple nucleotide mutagenesis (4, 5, 19), further supporting the hypothesis that these residues are likely to be involved in expression of the LDL receptor gene in vivo. The similarities observed in the DMS-reactivity patterns within this region across several different cell lines and in primary hepatocyte cultures further support this view. Genomic footprinting by LM-PCR is most powerful at allowing comparison of protein binding in cells grown under different conditions. While information about basal protein binding can be inferred by comparison of modification patterns obtained with naked DNA relative to cellular DNA, these interpretations must be tempered by the realization that subtle artifacts can

arise. The general agreement between the in vitro and in vivo data are reassuring but not proof that the postulated interactions have occurred.

The changes in DMS reactivity of guanine −59 observed in the present study were correlated with activation or repression of LDL receptor gene transcription in skin fibroblast cultures. The marked hypersensitivity of guanine −59 to DMS may signify a distortion in chromatin which could be created by the formation of a higher-order, multi-protein complex. Both the proteins implicated in binding the SRE-1 (10, 11) as well as a putative regulatory oxysterol-binding protein (20, 21) contain leucine zipper motifs that have been implicated in protein–protein interactions. When proteins binding at the Sp1 sites, distal elements, and RNA polymerase are factored in, a large, interactive complex would result. Previous examples of large, multi-protein complexes formed over similar lengths of DNA have also resulted in sites of hypermethylation when the linear array of proteins is assembled into a higher-order complex (22). In fibroblasts, the removal of a regulatory oxysterol from the preexisting protein-DNA structure could allow the generation of such a complex and yield the observed hypermethylation. Other types of DNA distortion such as bending could also be involved (23–26). Taken together, our genomic footprinting results suggest a primary role for the SRE-1 in sterol-dependent regulation of the LDL receptor gene in skin fibroblasts in vivo.

The importance of the SRE-1 in sterol-dependent regulation of LDL receptor gene expression in primary cultures of human hepatocytes is less clear. Although this region was protected from methylation under control conditions, inhibition of sterol synthesis with ketoconazole did not produce hypermethylation of any guanines within this region, despite an induction in the level of LDL receptor mRNA. These data suggest that differences between fibroblasts and hepatocytes in the mode of regulation of transcription may occur within the SRE-1 of the LDL receptor gene. In another study (D. B. Lloyd and J. F. Thompson, unpublished results), the DMS-reactivity patterns of guanine −59 in HepG2 cells were found to more closely resemble that seen in fibroblasts than primary hepatocytes. This suggested that HepG2 cells may have lost the ability to express a tissue-specific regulator of LDL receptor gene transcription and therefore reflected regulation seen in fibroblasts. A number of liver-specific transcription factors, such as C/EBP (27), are deficient in HepG2 cells. Thus, it is not surprising that regulation observed in this cell line differs from that seen with primary hepatocytes. Whatever the mechanism, previous studies from our laboratory and others (13, 28–33) have documented that regulation of LDL receptor gene expression in liver-derived cells is more complex than in less specialized cells such as fibroblasts. Interactions with more distal elements of the promoter that do not require high degrees of local folding may be involved in liver-specific expression. The lack of hypermethylation of guanine −59 in primary hepatocytes is consistent with this notion and suggests that some of the tissue-specific differences in regulation may occur at the level of transcription. Alternate possibilities cannot be ruled out at this time, which emphasizes the need for additional research on regulation of LDL receptor gene transcription in hepatic versus nonhepatic tissues.

Our data suggest that the protein architecture over repeats 1–3 of the LDL receptor promoter in vivo is largely maintained regardless of the activation state of the gene. Although we cannot rule out the possibility that one or more protein(s) bound to this region in the inactivated state is rapidly replaced by another protein with identical DNA contacts in the activated state, a simpler hypothesis would suggest that the principal DNA-protein contacts remain the same with novel protein–protein interactions causing the local DNA distortions observed in fibroblasts. Confirmation of this, or any, model will require a better understanding of all the protein components involved in this process. The data presented in the current study clearly do not support the hypothesis that the SRE-1 is unbound by protein in the absence of transcription and simply binds a factor when it becomes available during gene activation. Suggestions that increased Sp1 binding is important in activation (33) also appear to be less likely for the cell types studied here. It seems likely that the protein/DNA configuration over this region is established before induction or repression. There is precedence for this mechanism in other systems: protein/DNA contacts are maintained before and after induction of the *c-fos* gene by growth factors (34), of GAL4 binding to sequence elements within upstream activating sequences in yeast (35), of the interferon α-1 gene promoter by virus (36), and in protein binding to the human α globin locus control region during differentiation of mouse erythroleukemia cells (37). The rate-limiting step in activation of the LDL receptor gene in this scenario would involve modification of DNA-bound proteins (38), binding of other factors to the protein/DNA complexes (39), or both. In this manner, the transcription apparatus over the LDL receptor promoter would be poised to respond rapidly to changes in cellular level of sterol.

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REFERENCES

1. Goldstein, J. L., and M. S. Brown. 1990. Regulation of the mevalonate pathway. *Nature.* **343:** 425–430.
2. Osborne, T. F., G. Gil, J. L. Goldstein, and M. S. Brown. 1988. Operator constitutive mutation of 3-hydroxy-3-methylglutaryl coenzyme A reductase promoter abolishes protein binding to sterol regulatory element. *J. Biol. Chem.* **263:** 3380–3387.
3. Smith, J. R., T. F. Osborne, M. S. Brown, J. L. Goldstein, and G. Gil. 1988. Multiple sterol regulatory elements in promoter for hamster 3-hydroxy-3-methylglutaryl-coenzyme A synthase. *J. Biol. Chem.* **263:** 18480–18487.
4. Dawson, P. A., S. L. Hofmann, D. R. van der Westhuyzen, T. C. Sudhof, M. S. Brown, and J. L. Goldstein. 1988. Sterol-dependent repression of the low density lipoprotein receptor promoter mediated by a 16-base pair sequence adjacent to binding site for transcription factor Sp1. *J. Biol. Chem.* **263:** 3372–3379.
5. Briggs, M. R., C. Yokoyama, X. Wang, M. S. Brown, and J. L. Goldstein. 1993. Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. I. Identification of the protein and delineation of its target nucleotide sequence. *J. Biol. Chem.* **268:** 14490–14496.
6. Wang, X., M. R. Briggs, X. Hua, C. Yokoyama, J. L. Goldstein, and M. S. Brown. 1993. Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. II. Purification and characterization. *J. Biol. Chem.* **268:** 14497–14504.
7. Osborne, T. F., M. Bennett, and K. Rhee. 1992. Red 25, a protein that binds specifically to the sterol regulatory region in the promoter for 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J. Biol. Chem.* **267:** 18973–18982.
8. Gil, G., J. L. Osborne, J. L. Goldstein, and M. S. Brown. 1988. Purification of a protein doublet that binds to six TGG-containing sequences in the promoter for hamster 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Biol. Chem.* **263:** 19009–19019.
9. Stark, H. C., O. Weinberger, and J. Weinberger. 1992. Common double- and single-stranded DNA binding factor for a sterol regulatory element. *Proc. Natl. Acad. Sci. USA.* **89:** 2180–2184.
10. Yokoyama, C., X. Wang, M. R. Briggs, A. Admon, J. Wu, X. Hua, J. L. Goldstein, and M. S. Brown. 1993. SREBP-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. *Cell.* **75:** 187–197.
11. Hua, X., C. Yokoyama, J. Wu, M. R. Briggs, M. S. Brown, J. L. Goldstein, and X. Wang. 1993. SREBP-2, a second basic-helix-loop-helix-leucine zipper protein that stimulates transcription by binding to a sterol regulatory element. *Proc. Natl. Acad. Sci. USA.* **90:** 11603–11607.
12. Mueller, P. R., and B. Wold. 1989. In vivo footprinting of a muscle specific enhancer by ligation mediated PCR. *Science.* **246:** 780–786.
13. Ellsworth, J. L., C. Chandrasekaran, and A. D. Cooper. 1991. Evidence for sterol-independent regulation of low density lipoprotein receptor activity in Hep-G2 cells. *Biochem. J.* **279:** 175–187.
14. Ellsworth, J. L., A. J. Carlstrom, and J. Deikman. 1994. Ketoconazole and 25-hydroxycholesterol produce reciprocal changes in the rate of transcription of the human LDL receptor gene. *Biochim. Biophys. Acta.* **1210:** 321–328.
15. Allen, K. L., and C. E. Green. 1993. Isolation of human hepatocytes by biopsy perfusion methods. *In* In Vitro Methods of Toxicology. C. A. Tyson and J. Frazier, editors. Vol. 1. Academic Press, New York. 262–270.
16. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162:** 156–159.
17. Gilbert, W., A. Maxam, and A. Mirzabekov. 1976. Contacts between the LAC repressor and DNA revealed by methylation. *In* Control of Ribosome Synthesis. N. C. Kjeldgaard and O. Maaloe, editors. Munksgaard, Copenhagen. 139–148.
18. Lawley, P. D., and P. Brooks. 1963. Further studies on the alkylation of nucleic acids and their constituent nucleotides. *Biochem. J.* **89:** 127–138.
19. Sudhof, T. C., D. R. van der Westhuyzen, J. L. Goldstein, M. S. Brown, and D. W. Russell. 1987. Three direct repeats and a TATA-like sequence are required for regulated expression of the human low density lipoprotein receptor gene. *J. Biol. Chem.* **262:** 10773–10779.
20. Dawson, P. A., N. D. Ridgway, C. A. Slaughter, M. S. Brown, and J. L. Goldstein. 1989. cDNA cloning and expression of oxysterol-binding protein, an oligomer with a potential leucine zipper. *J. Biol. Chem.* **264:** 16798–16803.
21. Ridgway, N. D., P. A. Dawson, Y. K. Ho, M. S. Brown, and J. L. Goldstein. 1992. Translocation of oxysterol binding protein to Golgi apparatus triggered by ligand binding. *J. Cell Biol.* **116:** 307–319.
22. Thompson, J. F., L. Moitoso de Vargas, C. Koch, R. Kahmann, and A. Landy. 1987. Cellular factors provide a mechanism for coupling recombination with growth phase: characterization of a new component in the lambda site-specific recombination pathway. *Cell.* **50:** 901–908.
23. Kerppola, T. K., and T. Curran. 1993. Selective DNA bending by a variety of bZIP proteins. *Mol. Cell. Biol.* **13:** 5479–5489.
24. Shuey, D. J., and C. S. Parker. 1986. Bending of promoter DNA on binding of heat shock transcription factor. *Nature.* **323:** 459–461.
25. Shuey, D. J., and C. S. Parker. 1986. Binding of *Drosophila* heat-shock gene transcription factor to the hsp70 promoter. *J. Biol. Chem.* **261:** 7934–7940.
26. Thompson, J. F., and A. Landy. 1988. Empirical estimation of protein-induced DNA bending angles: applications to lambda site-specific recombination complexes. *Nucleic Acids Res.* **16:** 9687–9705.
27. Agellon, L. B., P. Zhang, X. C. Jiang, L. Mendelsohn, and A. R. Tall. 1992. The CCAAT/enhancer-binding protein trans-activates the human cholesterol ester transfer protein gene promoter. *J. Biol. Chem.* **267:** 22336–22339.
28. Ellsworth, J. L., C. Brown, and A. D. Cooper. 1988. Stimulation of LDL receptor activity in Hep-G2 cells by a serum factor(s). *J. Cell Physiol.* **135:** 213–223.
29. Reagan, J. W., L. R. Miller, and R. W. St. Clair. 1990. In vivo clearance of low density lipoprotein in pigeons occurs by a receptor-like mechanism that is not down-regulated by cholesterol feeding. *J. Biol. Chem.* **265:** 9381–9391.
30. Grove, R. I., C. E. Mazzucco, S. F. Radka, M. Shoyab, and P. A. Kiener. 1991. Oncostatin M up-regulates low density lipoprotein receptors in Hep-G2 cells by a novel mechanism. *J. Biol. Chem.* **266:** 18194–18199.
31. Roach, P. D., S. Balasubramaniam, F. Hirata, M. Abbey, A. Szanto, L. A. Simons, and P. J. Nestel. 1993. The low-density lipoprotein receptor and cholesterol synthesis are affected differently by dietary cholesterol in the rat. *Biochim. Biophys. Acta.* **1170:** 165–172.
32. Rudling, M., and B. Angelin. 1993. Loss of resistance to dietary cholesterol in the rat after hypophysectomy: importance of the presence of growth hormone for hepatic low

density lipoprotein-receptor expression. *Proc. Natl. Acad. Sci. USA.* **90:** 8851–8855.
33. Hamanaka, R., K. Kohno, T. Seguchi, K. Okamura, A. Morimoto, M. Ono, J. Ogata, and M. Kuwano. 1992. Induction of low density lipoprotein receptor and a transcription factor Sp1 by tumor necrosis factor in human microvascular endothelial cells. *J. Biol. Chem.* **267:** 13160–13165.
34. Herrera, R. E., P. E. Shaw, and A. Nordheim. 1989. Occupation of the c-fos serum response element in vivo by a multiprotein complex is unaltered by growth factor induction. *Nature.* **340:** 68–70.
35. Selleck, S. B., and J. E. Majors. 1987. In vivo DNA-binding properties of a yeast transcription activator protein. *Mol. Cell. Biol.* **7:** 3260–3267.
36. Palmieri, M., and M. G. Tovey. 1990. Genomic footprinting: detection of putative regulatory proteins in the promoter region of the interferon α-1 gene in normal human tissues. *Mol. Cell. Biol.* **10:** 2554–2561.
37. Strauss, E. C., N. C. Andrews, D. R. Higgs, and S. H. Orkin. 1992. In vivo footprinting of the human α-globin locus upstream regulatory element by guanine and adenine ligation-mediated polymerase chain reaction. *Mol. Cell. Biol.* **12:** 2135–2142.
38. Hunter, T., and M. Karin. 1992. The regulation of transcription by phosphorylation. *Cell.* **70:** 375–387.
39. Pugh, B. F., and R. Tjian. 1990. Mechanism of transcriptional activation by Sp1: evidence for coactivators. *Cell.* **61:** 1187–1197.