Purification of a Protein Doublet That Binds to Six TGG-containing Sequences in the Promoter for Hamster 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase*

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The gene for 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the rate-controlling enzyme of cholesterol biosynthesis, is transcribed at a relatively high level when cellular sterols are depleted and is repressed when sterols accumulate. We have previously reported that the regulatory region of the hamster reductase gene contains eight different sequences that bind nuclear proteins as determined by DNase I footprinting assays. We here report the purification of a single activity that accounts for six of these footprints. This activity was found in a doublet of proteins (designated reductase promoter factor 1, RPF-1) that have apparent molecular weights of 33,000 and 35,000. They were isolated by DNA affinity chromatography using oligonucleotides corresponding to either of two footprinted sequences. The 33- and 35-kDa species were present as monomers, as indicated by gel filtration and gradient ultracentrifugation. Oligonucleotides corresponding to any one of the six footprinted sequences prevented the binding of RPF-1 to all of the other sequences, indicating that all six bind to a single site in RPF-1. The only sequence shared by all six footprinted sequences is the trinucleotide, TGG, both of whose guanosines were found to contact with RPF-1, as determined by methylation interference assays. The footprinted sequence that binds RPF-1 with highest affinity contains the palindrome, TGG(N2)CCA, which conforms to the consensus sequence for binding NF-1, a nuclear protein that stimulates replication of adenovirus-2. Purified RPF-1 was shown to bind to the adenovirus NF-1 binding site with high affinity. Although the apparent molecular weight of the RPF-1 doublet was lower than the molecular weight range for NF-1 proteins (52,000–66,000), it is likely that the 33–35-kDa doublet is derived from a larger NF-1-like protein as a result of proteolysis. We conclude that RPF-1 belongs to a group of TGG-binding proteins that includes NF-1 and other proteins previously described as CCAAT binding proteins. This protein binds to six sites in the promoter region for hamster 3-hydroxy-3-methylglutaryl CoA reductase, where its function remains to be determined.

The pathway of cholesterol biosynthesis is regulated by multiple mechanisms, including end product repression of gene transcription (1). In particular, the gene encoding 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), an early enzyme in the cholesterol biosynthetic pathway, is transcribed at relatively high rates when cells are deprived of sterols and is repressed when sterols accumulate within the cell (2–4).

The mechanism for sterol-mediated repression of HMG-CoA reductase is beginning to be elucidated. A fragment of DNA comprising of 277 base pairs (bp) upstream of the major transcription initiation site contains the information necessary for expression and sterol-mediated repression. When placed upstream of a reporter gene and introduced into cultured cells by transfection, this fragment allows regulated expression (2). This fragment also supports transcription in vitro in the presence of a HeLa whole cell extract (3). A segment of this region comprising nucleotides −141 to −277 confers sensitivity to sterol-dependent regulation when inserted into the promoter for herpes simplex virus thymidine kinase (4).

The 277-bp promoter region of HMG-CoA reductase contains eight sequences that are protected from DNase I digestion by proteins in an extract from hamster liver nuclei (3) (Fig. 1). When the nuclear extract was passed over a heparin agarose column, the proteins giving rise to six of the eight footprints emerged in a single peak (3). These six footprints, designated FP1, -2B, -2C, -2D, -5, and -6, are denoted by asterisks in Fig. 1.

The region corresponding to footprints 2C and 2D is believed to play a role in sterol-mediated repression. When nucleotides in either of these two regions were substituted with random sequences, sterol-mediated repression was abolished (4). Footprint 2D contains a sequence (boxed in Fig. 1) that shows a 7/8-bp match with a sequence in the regulatory region of the gene for the low density lipoprotein (LDL) receptor (4). Transcription of the LDL receptor gene is also repressed by sterols, and alteration of the sequence corresponding to the boxed element lowers gene expression and prevents sterol-mediated repression (5, 6). Thus, footprint 2D is a potential target for the binding of a sterol-modulated transcriptional regulatory protein.

In the current studies we have used the technique of oligonucleotide affinity chromatography to purify a pair of proteins that produces six of the footprints in the HMG-CoA reductase promoter, including footprint 2D.

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†The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; bp, base pair(s); LDL, low density lipoprotein; NF-1, nuclear factor 1; RPF-1, reductase promoter factor 1; SDS, sodium dodecyl sulfate; CTF, CCAAT-box-binding transcription factor.
EXPERIMENTAL PROCEDURES

Materials

Male golden Syrian hamsters were maintained as previously described (3). Other materials were obtained from previously reported sources (2-4).

Buffers

All buffers contained 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 50 μM leupeptin added just prior to use. Buffer A contained 50 mM Tris-chloride (pH 7.9 at 25 °C), 1 mM disodium EDTA, 50% (v/v) glycerol; Buffer B, same as buffer A plus 12.5 mM MgCl₂; Buffer C, same as buffer A plus 7.5 mM MgCl₂.

Purification of Reductase Promoter Factor-1 (RPF-1)

All operations were carried out at 0–5 °C. A nuclear extract was prepared from livers of 300 hamsters (1500 g of liver) as described (3). The extract was dialyzed against two changes of buffer B containing 0.1 M KCl, centrifuged for 15 min at 100,000 × g, and the supernatant fraction (175 ml, 4.56 g of protein) was retained.

Step 1. Heparin-agarose Chromatography—The dialyzed nuclear extract was applied to a column of heparin-agarose (2.5 × 15.5 cm) equilibrated in buffer B containing 0.1 M KCl at a flow rate of 48 ml/h. The column was washed with ~325 ml of the same buffer and was eluted with 300 ml of buffer B containing 0.45 M KCl. The eluted protein was concentrated to about 4 ml by ultrafiltration through a Diaflo PM-30 membrane in a stirred Amicon cell and dialyzed against buffer C containing 0.1 M KCl.

Step 2. Oligonucleotide Affinity Chromatography—Double-stranded oligonucleotides corresponding to footprint regions 2B and 2D of the hamster HMG-CoA reductase promoter (Fig. 1) were prepared as described below. The oligonucleotides were polymerized to form oligomers and coupled to Sepharose as described by Kadonaga and Tjian (7). Mixed oligomers ranging from 7-mers to 500-mers were used. Five ml (71 mg) of protein of the heparin-agarose fraction were loaded onto 20 ml of FP2B-Sepharose equilibrated in buffer C containing 0.1 M KCl. After washing the column with 200 ml of the same buffer, RPF-1 was eluted with 80 ml of buffer C containing 0.5 M KCl. The eluted protein was concentrated to about 63 ml by ultrafiltration through a Diaflo PM-30 membrane in a stirred Amicon cell and dialyzed against buffer C containing 0.1 M KCl.

Preparation of Double-stranded Oligonucleotides

Synthetic oligonucleotides corresponding to each strand of the individual footprint regions of the hamster reductase promoter (Fig. 1) were synthesized on an Applied Biosystems model 380 DNA synthesizer and purified using a Sephadex G-50 column. The synthesis was scaled up by a factor of 5-10-fold from the standard assay procedure. The oligonucleotide pair was designed such that after hybridization there would be a four-nucleotide single-stranded tail of GATC on one strand (approximately 250,000 cpm), which was uniquely labeled on one strand (approximately 250,000 cpm), was treated with 2 ml of dimethyl sulfate for 5 min at room temperature as described by Maxam and Gilbert (10). The methylated probe was used before piperidine cleavage in a gel shift assay that was carried out with an aliquot of the mixture described above. After electrophoresis, the wet gel was exposed to XAR-5 film for 10-36 h at -70 °C. The autoradiograms were used to align the gel, and the bands corresponding to the free and protein-bound probe were located and separately excised. The amount of radioactivity in each band was determined by liquid scintillation counting. A background value of 50–75 cpm was subtracted from each value before determining the percentage of input DNA bound.

Methylation Interference Assay

Oligonucleotide pairs were annealed as described above except that each member was 32P-labeled before annealing to the complementary nonlabeled oligonucleotide. Each resulting double-stranded probe, which was uniquely labeled on one strand (approximately 250,000 cpm), was treated with 2 ml of dimethyl sulfate for 5 min at room temperature as described by Maxam and Gilbert (10). After electrophoresis, the wet gel was exposed to XAR-5 film for 10–60 min at room temperature, and the free and protein-bound DNA probes were located, separately excised, and electrophoresed from the gel. The samples were precipitated with ethanol and subjected to piperidine cleavage followed by electrophoresis in 10% denaturing polyacrylamide gels (9). The gels were exposed to XAR-5 film for 10–36 h at -70 °C.

Other Assays

The protein content of the nuclear extract and the heparin-agarose fraction of hamster liver was measured by the method of Bradford (11). The protein content of purified RPF-1 was estimated by comparison of intensity of silver stain bands of the marker protein carbonic anhydrase (Mr 31,000) with those of phosphorylase b, Mr 97,000; bovine serum albumin, Mr 66,000; ovalbumin, Mr 45,000; carbonic anhydrase, Mr 31,000; and soybean trypsin inhibitor, 21,000.

RESULTS

Fig. 1 shows the sequence of the promoter region of the hamster HMG-CoA reductase gene. The eight previously described footprints are indicated. The sequence within FP2D that is similar to the regulatory sequence in the promoter of the LDL receptor gene is boxed. In addition, we have indicated the most 5′ site for transcription initiation, which is the predominant site used in vitro (9).

Fig. 2, lane 2, shows these footprints as they are observed when a 32P-labeled promoter fragment is digested with DNase I after incubation with a crude hamster liver nuclear extract. When the nuclear extract was subjected to heparin-agarose chromatography, the proteins giving rise to all of the eight footprints eluted between 0.1 and 0.45 M KCl (lane 3). This fraction from the heparin-agarose column was then subjected to affinity chromatography on a Sepharose column containing oligomers of one of the footprinted sequences (footprint 2B),
The high salt eluate from this column (lane 6) contained proteins that gave rise to footprints 1, 2B, 2C, 2D, and 6. The protein that gave rise to footprint 5 was also found in this fraction, but this footprint is not apparent at the concentrations used in the assays of Fig. 2. The high salt eluate did not contain proteins that gave rise to footprints 3 and 4. The six footprints (1, 2B, 2C, 2D, 5, and 6) are hereafter designated as the RPF-1 (reductase promoter factor-1) footprints. The active protein fractions from the first footprint 2B column were subjected to another cycle of chromatography on footprint 2B-Sepharose, and again the protein(s) that produced all of the RPF-1 footprints adhered to the column and was eluted with salt (lane 9). The protein that had gone through two cycles of such affinity chromatography was designated reductase promoter factor-1 (RPF-1). RPF-1 was capable of producing footprints 1, 2B, 2C, 2D, and 6 (lanes 10–12). It also could produce footprint 5 when assayed at appropriate concentrations (see below). This fraction did not contain material that gave rise to footprints 3 or 4.

The findings of Fig. 2 showed that a single protein, or complex of proteins (RPF-1), was retained on a footprint 2B column, yet it produced six footprints in the HMG-CoA reductase promoter. To determine whether RPF-1 would be retained on a column prepared with oligonucleotides from one of the other binding sites, we passed the heparin-agarose fraction over a column containing oligonucleotides corresponding to footprint 2D. After two cycles of chromatography on this column, the eluted fraction contained proteins that again bound to all six of the RPF-1 footprints (Fig. 2, lanes 13–15). Thus, affinity chromatography on either footprint 2B or footprint 2D produced a fraction that gave rise to the same series of footprints in the HMG-CoA reductase promoter. RPF-1 did not adhere to a column containing an oligonucleotide corresponding to footprint 4D (data not shown), indicating that its retention on the FP2B and -2D oligonucleotides was specific.

Fig. 3 shows the appearance of RPF-1 on SDS-polyacrylamide gel electrophoresis, as visualized with a silver stain. Lanes 2 and 3 show the profile of proteins in the crude nuclear extract and the heparin-agarose fraction, respectively. When this latter fraction was subjected to two cycles of chromatography on oligonucleotides corresponding to FP2B, a doublet of proteins in the molecular weight range of 33,000–35,000 was obtained (lane 4). When the heparin-agarose fraction was chromatographed on the oligonucleotide column corresponding to footprint 2D, an identical appearing protein doublet was obtained (lane 5). Although this protein doublet adhered to both oligonucleotides, the optimal conditions for adherence were different. The proteins adhered to the footprint 2B column in the presence of 7.5 mM MgCl₂. Adherence to the footprint 2D column did not occur unless MgCl₂ was omitted from the buffer.

To demonstrate directly that the 33–35-kDa doublet of proteins was capable of binding to the footprint 2B sequence, we performed a preparative gel shift assay (Fig. 4A). Purified RPF-1 was subjected to nondenaturing polyacrylamide gel electrophoresis either alone (Fig. 4A, lane 1) or in the presence of 32P-labeled DNA fragment corresponding to footprint 2B (lane 3). An additional control lane contained the oligonucleotide without protein (lane 2). RPF-1 retarded the mobility of the 32P-labeled oligonucleotide (compare lane 3 with lane 2). The portion of the preparative gel corresponding to the position of the retarded band was cut out from all three lanes (dashed boxes in Fig. 4A). This region was subjected to analytical SDS-polyacrylamide gel electrophoresis and silver staining (Fig. 4B). The starting RPF-1 fraction contained the 33–35-kDa doublet (lane 4). When the RPF-1 fraction was subjected to electrophoresis in the absence of DNA (lane 1), this doublet was not recovered in the position corresponding to the retained band (lane 5). However, when RPF-1 was subjected to electrophoresis together with the DNA fragment (lane 3), the portion of the gel containing the labeled DNA (dashed box) also contained the 33–35-kDa doublet (lane 7). This experiment demonstrates that both proteins in the 33–35-kDa doublet are part of a complex with the footprint 2B oligonucleotide and that the formation of this complex changes the mobility of the proteins as well as the oligonucleotide.

To determine whether the 33- and 35-kDa proteins were present as monomers in the absence of DNA, we subjected the RPF-1 proteins to analytical gel filtration on Sephacryl S-300. Fractions from the column were assayed for the ability to retard the footprint 2B binding site (Fig. 5). The retardation activity appeared in fraction 83, which was between the elution positions of ovalbumin (molecular weight 43,000) and myoglobin (molecular weight 17,000) and corresponded to a calculated molecular weight of 30,000. A similar molecular weight was obtained when the RPF-1 fraction was subjected to ultracentrifugation on a glycerol gradient and assayed by the same procedure as shown in Fig. 5 (data not shown). Together, these results indicate that the 33–35-kDa protein exist as monomers in the absence of DNA.

To determine whether all of the footprinted regions were binding to a common site on the 33–35-kDa proteins, we performed a series of competition experiments using the footprinting assay and the pooled heparin-agarose fraction that contained RPF-1 (Fig. 6). When this fraction was incubated with the reductase promoter fragment, all eight footprints were seen (lane 2). An excess of a control DNA, corresponding to the sequence recognized by nuclear factor Sp1 (7), did not compete for any of the footprints, even when present at a
FP2B
jected to two cycles of chromatography on a column containing gel electrophoresis as described. From the second FP2B affinity column, 25 µl of the flow-through fraction. From the first FP2B affinity column, 6, 12, or 30 ng of the heparin-agarose fraction. From the first FP2B affinity column, 0.3 µg of RPF-1 purified on the FP2B affinity column (lane 4), and 0.3 µg of RPF-1 purified on the FP2D affinity column (lane 5) were subjected to SDS-polyacrylamide gel electrophoresis, and the protein bands were detected with a silver stain. Protein standards of known molecular weight were subjected to electrophoresis in lane 1.

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<th>No. Protein</th>
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Fig. 2. Purification of RPF-1 as monitored by DNase I footprinting. RPF-1 was purified from hamster liver as described under "Experimental Procedures." A ³²P end-labeled DNA probe prepared from pRed CAT-1 (2) was incubated in the absence of protein (lanes 1 and 16) or in the presence of RPF-1 at different stages of purification. Lane 2, 52 µg of hamster liver nuclear extract; lane 3, 14 µg of the heparin-agarose fraction. From the first FP2B affinity column, 10 µl of the flow-through (lane 4), 25 µl of the 0.125 M KCl wash (lane 5), and 10 µl of the 0.5 M KCl eluate (lane 6) were assayed. From the second FP2B affinity column, 25 µl of the flow-through (lane 7), 25 µl of the 0.125 M KCl wash (lane 8), and 10 µl of the 0.5 M KCl eluate (lane 9) were assayed. After concentration of the eluate from the second FP2B column, 6, 12, or 30 ng of protein were assayed (lanes 10–12). Another aliquot of the heparin-agarose pool was subjected to two cycles of chromatography on a column containing oligonucleotides corresponding to FP2D. Aliquots (6, 12, or 30 ng) of this protein were also assayed (lanes 13–15). The fractions were subjected to partial DNase I digestion and denaturing polyacrylamide gel electrophoresis as described (3). The gel was exposed to x-ray film for 18 h at −20 °C with an intensifying screen. F, flow-through fraction; W, wash fraction; E, eluted fraction.

Fig. 3. SDS gel electrophoresis of RPF-1 purified by affinity chromatography on affinity columns containing FP2B or FP2D. 26 µg of hamster liver nuclear extract (lane 2), 29 µg of the heparin-agarose fraction (lane 3), 0.3 µg of RPF-1 purified on the FP2B affinity column (lane 4), and 0.3 µg of RPF-1 purified on the FP2D affinity column (lane 5) were subjected to SDS-polyacrylamide gel electrophoresis, and the protein bands were detected with a silver stain. Protein standards of known molecular weight were subjected to electrophoresis in lane 1.

Fig. 4. Preparative gel shift electrophoresis of purified RPF-1 followed by analytical SDS gel electrophoresis of the eluted protein. RPF-1 was purified as described under "Experimental Procedures." Panel A, RPF-1 (1.2 µg) was incubated with 2.4 pmol of ³²P-labeled FP2B probe and loaded onto a 5% polyacrylamide gel as described under "Experimental Procedures." Lane 1, RPF-1 alone; lane 2, ³²P-labeled FP2B probe alone; lane 3, RPF-1 plus ³²P-labeled FP2B probe. After exposure to x-ray film for 5 min, gel slices containing the [³²P]FP2B/RPF-1 complex and the corresponding regions of the control lanes (shown by the dashed box) were cut out and electroeluted overnight in 25 mM Tris, 100 mM glycerine containing 1% SDS. The eluted samples were concentrated by precipitation with acetone, resuspended in SDS sample buffer, and loaded onto an analytical 8% polyacrylamide SDS gel. Panel B, silver stain of the analytical SDS gel. Lane 4, 0.2 µg of RPF-1 loaded directly on SDS gel; lanes 5–8, eluted samples from lanes 1–3, respectively; lane 8, molecular weight markers.

500-fold molar excess (lanes 3 and 4). DNA fragments corresponding to footprint 1 competed for all of the footprints that are generated by RPF-1 (lanes 5 and 6). This DNA did not compete for footprints 3 or 4, which are produced by different proteins than RPF-1. Each of the other RPF-1 DNA fragments also competed for all of the RPF-1 footprints, but not footprints 3 or 4 (lanes 7–18). The highest affinity competition was provided by footprint 2β, which competed for all of the footprints when it was present in only a 50-fold excess. Even at this high concentration, however, this competition was specific, since the same excess did not compete for footprint 4. As expected, the footprint 4 DNA fragment competed for
footprint 4 but not for any of the other footprints (lanes 13 and 14).

A more quantitative approach to test for a common binding site with different affinities involves the use of analytical gel shift assays. In the experiment shown in Fig. 7 (left), a DNA fragment corresponding to each of the footprinted sequences was radiolabeled and incubated with purified RPF-1. The RPF-1 proteins bound each of these DNA fragments, and all of the complexes had similar mobilities. The sequence corresponding to footprint 1 was the labeled probe, footprint 2B was the most efficient competitor DNA fragment, and that it binds footprint 2B with the highest affinity. The preceding data suggest that a single site in RPF-1 binds to footprint 4. A similar order of affinities was obtained through competition gel shift assays (Fig. 8). A 32P-labeled fragment corresponding to footprint 2B was incubated with RPF-1 in the presence of increasing concentrations of unlabeled competitor DNA corresponding to each of the other footprint sequences. Unlabeled footprint 2B gave 50% competition when it was present in less than a 10-fold molar excess with respect to 32P-labeled footprint 2B. Footprints 1, 2C, 2D, and 6 required approximately a 100-fold excess to give 50% competition. The DNA fragment corresponding to footprint 5 required a 500-fold excess to give similar competition. Footprint 4 did not give significant competition at any of the concentrations tested.

We next sought to determine whether the same order of affinity would be observed if the labeled binding site corresponded to a different footprint, namely footprint 2D (Fig. 9).
FIG. 7. Binding of a purified RPF-1 to individual footprint regions of HMG-CoA reductase promoter measured by gel shift assay. Left, $^32$P end-labeled double-stranded DNA probes corresponding to each footprint region (see Fig. 1) were prepared and used in standard gel shift assays as described under "Experimental Procedures." The gel was dried and exposed to x-ray film for 12 h at $-20^\circ$C. The specific probe and the amount of purified RPF-1 protein is indicated for each lane. The positions of migration for both the free $^32$P-probe and the protein-bound $^32$P-probe are indicated on the right. Right, the spots corresponding to the free and bound probes were excised from each lane of the gel, and the amount of $^32$P was quantified by liquid scintillation counting. The percentage of the input DNA bound by RPF-1 is plotted as a function of the amount of protein added.

FIG. 8. Oligonucleotide competition for binding of purified RPF-1 to region FP2B of HMG-CoA reductase promoter measured by gel shift assay. Left, RPF-1 (2.5 ng) was incubated with $^32$P-labeled FP2B probe (12 fmol) and processed by the standard gel shift assay with the following modifications. Where indicated, a 10-, 50-, or 500-fold molar excess of unlabeled competitor DNA fragment was included in the binding reaction. The identity of each competitor DNA is indicated in the figure and corresponds to the specific promoter region noted in Fig. 1. Right, the data from the gel was quantified as described under "Experimental Procedures." The percentage of binding to FP2B in the absence of competitor was set at 100%, and all the values are plotted relative to this level.

FIG. 9. Oligonucleotide competition for binding of purified RPF-1 to region FP2D of HMG-CoA reductase promoter measured by gel shift assay. Left, RPF-1 (5 ng) was incubated with $^32$P-labeled FP2D probe (12 fmol) and processed for the standard gel shift assay as described in the legend to Fig. 8. Right, the data from the gel was quantified as described in the legend to Fig. 8.

reason we tested the ability of an oligonucleotide corresponding to the NF-1 binding site from the adenovirus-2 origin to compete for the binding of RPF-1 to the HMG-CoA reductase footprints. Fig. 10 shows that footprint 2B DNA competed all of the footprints when present at a 30-fold molar excess (lanes 3–6). In the same experiment, an unlabeled fragment corresponding to the sequence of the adenovirus-2 origin also competed for all of the footprints when present at a 30-fold molar excess (lanes 7–10).
RPF-1 also bound the adenovirus-2 origin fragment when studied by a gel shift assay (Fig. 11). The retained band with the adenovirus origin DNA was in an identical location to the retained band obtained with the FP2B, and a protein titration revealed that RPF-1 binds to the adenovirus-2 origin fragment with higher affinity than it binds to footprint 2B.

When it binds to the adenovirus-2 origin sequence, NF-1 is known to make contacts with the two Gs in the TGG and with the two Gs on the lower strand that are complementary to the CCA sequence (16). All of the sequences from the HMG-CoA reductase promoter that bind RPF-1 contain the trinucleotide TGG (Fig. 12). However, footprint 2B is the only one that contains the CCA in the correct position to generate a palindromic site.

To determine whether RPF-1 makes contact with the Gs in the TGG sequences in the HMG-CoA reductase footprints, we performed a methylation interference assay (Fig. 13). Double-stranded fragments corresponding to the various footprints were end-labeled either on the coding strand or the noncoding strand. The Gs were subjected to incomplete methylation, the RPF-1 binding sites are aligned according to the common TGG motif, and the position of each sequence element relative to the start of transcription is shown at the extreme right. Sequences that are identical in at least four of the six RPF-1 binding sites are boxed. The guanosine contact points for binding of RPF-1 to the upper and lower strands of the reductase promoter DNA (Fig. 13) are denoted by filled and open circles, respectively. The lower strands of FP2C and 5 were not assayed.
at methylated guanosines. The cleaved fragments from the bound and free fractions were then subjected to polyacrylamide gel electrophoresis. The results showed that in each case methylation of either of the two Gs in the TGG sequence blocked the ability of the oligonucleotide to bind to RPF-1. For example, in the sequence corresponding to footprint 1, the two Gs occur at positions -240 and -241. When either of these two Gs were methylated, the corresponding DNA was found in the free fraction. The bound fraction did not contain any bands corresponding to cleavage at positions -240 or -241. This indicated that when either of these residues was methylated the DNA did not bind to RPF-1.

Similar results were obtained with all of the other RPF-1 binding footprints (Figs. 12 and 13). In each case binding to RPF-1 was blocked when either of the two guanosines following the thymidine were methylated. Footprint 2B was also prevented from binding to RPF-1 when the guanosines complementary to the CCA sequence were methylated (positions -187 and -188 on the lower strand) (Fig. 13). In the case of footprints 1 and 6, two guanosines on the lower strand were also crucial. In footprint 2D two guanosines on the upper strand and two guanosines on the lower strand bound to RPF-1 in addition to the adjacent guanosines in the TGG sequence. The two guanosines on the lower strand occur in the same position as the lower strand guanosines in footprint 2B and the adenovirus origin (Fig. 12). However, these guanosines are preceded by a guanosine in footprint 2D rather than by a thymidine. For technical reasons it was not possible to obtain clear results with the methylation interference assay for the lower strands of footprints 2C and 5. The upper strand of footprint 2C contains two other TGG trinucleotides in addition to the TGG that is highlighted in Fig. 12 (see Fig. 1). Neither of these other TGGs contacted the RPF-1 binding site, since methylation of these Gs did not interfere with binding (Fig. 13). Thus, a TGG is necessary but not sufficient for binding to RPF-1.

**DISCUSSION**

In this paper we report the purification of a doublet of proteins, designated RPF-1, that binds to six different sequences in the regulatory region of the hamster HMG-CoA reductase gene promoter. DNA fragments corresponding to each of the six sequences compete for the binding of all of the others, strongly suggesting that a single binding site is involved. This binding site accounts for six of the eight previously reported footprints of the hamster HMG-CoA reductase promoter (3).

**Characterization of RPF-1**—Purified RPF-1 contained two major proteins with molecular masses of 33 and 35 kDa as determined by SDS gel electrophoresis. In the absence of DNA, these proteins are present as monomers as revealed by gel filtration (Fig. 5) and density gradient ultracentrifugation.
(data not shown). Both of these proteins bound to DNA as determined by preparative gel shift electrophoresis (Fig. 4). Both proteins were isolated by affinity chromatography using polymerized binding sites corresponding to either footprint 2B or footprint 2D (Fig. 2), suggesting that both proteins bind to all of the footprinted sequences. We cannot exclude the possibility that only one of the two proteins binds to DNA and the other protein binds to the first protein when the latter is bound to DNA. RPF-1 seems to bind to each of the footprinted sequences independently, and there is no evidence for cooperative interaction between binding sites. In experiments not shown, we have produced substitution mutations in footprint regions 1, 2B, 2C, and 2D. A mutation in each of these footprints abolished the binding of RPF-1 to that footprint but did not affect the binding to the other footprints.

We cannot exclude the possibility that one or both of the RPF-1 proteins are derived from a larger protein through proteolysis even though all buffers contained high concentrations of two protease inhibitors, phenylmethylsulfonyl fluoride and leupeptin. Indeed, variable amounts of molecular weight proteins were present in different RPF-1 preparations, raising the possibility that proteolysis has occurred. The same 33- and 35-kDa species were detected even when we used a mixture of protease inhibitors that included pepstatin A, chymostatin, antipain, and sodium metabisulfite (data not shown).

All of the sequences recognized by RPF-1 have only one feature in common: they all contain the trinucleotide TGG. The sequence that bound with highest affinity (footprint 2B) also contained the inverse complementary sequence (CCA) seven nucleotides downstream. The other five footprinted sequences did not contain this CCA. Methylation interference assays revealed that in all six footprinted regions the pair of adjacent guanosines in the TGG sequences made contacts with RPF-1. When these sequences were methylated, all of the oligonucleotides lost the ability to bind to the protein. In the footprint 2B sequence, the pair of guanosines complementary to the downstream CCA sequence also bound to RPF-1, thus suggesting that RPF-1 contacts guanosine residues in adjacent major grooves on the same side of the DNA helix in footprint 2B. In each footprint region (except 2C), at least one other guanosine made contact with RPF-1 as revealed by the methylation interference assay. Footprint 2C contains two TGG sequences in addition to the one that contacted RPF-1 (Fig. 13). Methylation of the guanosines in the other two TGG sequences did not prevent RPF-1 binding, a finding that supports the notion that sequences in addition to TGG are required for RPF-1 binding.

The function of RPF-1 is unknown, but we do know something about the functions of each of the footprint regions that bind RPF-1. In a previous study, Chinese hamster ovary cells were transfected with a plasmid containing the 277-bp fragment of the HMG-CoA reductase promoter fused to the coding region of a reporter gene, and the amounts of mRNA were measured (4). The relative transcription rates are summarized in Table I. The wild-type promoter was transcribed at a relatively high rate in the absence of sterols and was repressed by 4-fold when sterols were present. Scrambling of the sequences corresponding to the highest affinity RPF-1 binding sites (footprints 1 and 2B) had no significant effect on transcription either in the presence or absence of sterols. Mutation of footprints 2C and 2D disrupted regulation of transcription, but in opposite ways. Mutation of footprint 2C led to a constitutively low level of transcription that was not repressed when sterols were added. Disruption of footprint 5 had little effect on transcription. Disruption of footprint 6 led to a 2.5-fold increase in transcription both in the absence and presence of sterols. Because of the different effects of each mutation, it is not yet possible to assign either a positive or negative transcriptional role to RPF-1. If this protein does play a role in transcription, it must do so in concert with other proteins that modulate its effect when it binds to different footprint regions.

Although the sequences of the HMG-CoA reductase promoters from hamster and human show a high degree of identity, the TGG trinucleotides in footprints 2B and 1, the two highest affinity RPF-1 binding sites in the hamster sequence, are not conserved in the human (3, 17). In contrast, the TGG trinucleotides in the other four footprint sequences are conserved. On the basis of these findings and the lack of effect of mutations in footprints 2B and 1 in the hamster promoter (Table I), it seems unlikely that these two footprint sequences play a crucial role in transcription. However, we cannot exclude the possibility that these two sequences perform redundant functions in the hamster promoter and that mutations of both would be necessary to produce a functional defect. It is also possible that in the human promoter these high affinity RPF-1 binding sites have been translocated to a more distant 5' site. These possibilities seem unlikely, however, and we favor the notion that footprints 1 and 2B are not crucial for transcription.

When RPF-1 binds to footprint 2D, the guanosine contact pattern is different from that seen with the other binding sites (Fig. 13). This specificity suggests that RPF-1 has a unique function when bound to footprint 2D, possibly in sterol-mediated regulation. Footprint 2D contains a sequence that shows a 7/8-bp match with a sequence (designated repeat 2) in the LDL receptor promoter that is crucial for regulation (boxed sequence in Fig. 1) (5, 6). This sequence begins with the second G in the TGG trinucleotide. Repeat 2 does not contain this TGG, and it does not bind RPF-1, as determined by gel retardation and footprint assays. It seems likely that the boxed sequences in footprint 2D and the similar sequence in repeat 2 of the LDL receptor promoter bind a sterol-dependent negative transcription factor. RPF-1 might compete for the binding of this factor to footprint 2D, and thus it may act to modulate the sensitivity of the HMG-CoA reductase promoter to sterol-dependent repression.

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**Table I**

| Mutated footprint region | Relative expression
<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>-Sterols</td>
</tr>
<tr>
<td>None</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>2B</td>
<td>3</td>
</tr>
<tr>
<td>2C</td>
<td>1</td>
</tr>
<tr>
<td>2D</td>
<td>5</td>
</tr>
<tr>
<td>3 (5' end)</td>
<td>4</td>
</tr>
<tr>
<td>3 (middle)</td>
<td>1</td>
</tr>
<tr>
<td>3 (3' end)</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
</tr>
</tbody>
</table>

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2. G. Gil, T. F. Osborne, J. L. Goldstein, and M. S. Brown, unpublished observations.
TGG-binding Proteins and HMG-CoA Reductase Promoter

Disruption of the two footprint regions in the HMG-CoA reductase promoter that are not attributable to RPF-1, namely, footprints 3 and 4, consistently reduced transcription (Ref. 4 and Table I). These sequences are almost totally conserved in the human promoter (3, 17). It seems likely that these two sequences bind proteins that activate transcription of the HMG-CoA reductase gene.

Relation of RPF-1 to Previously Described TGG-binding Proteins—The only feature that all of the RPF-1 binding sites share is the trinucleotide TGG. In recent years several research groups have described a confusing list of proteins that bind to DNA sequences containing this trinucleotide (8, 13–15). All of these TGG-binding proteins make contacts with the pair of guanosines following the thymidine, as determined by methylation interference analysis (16, 18, 19). The most well characterized of these proteins in animal cells is nuclear factor-1 (NF-1). This factor was originally detected because of its ability to bind to a TGG-containing sequence in the replication origin of adenovirus-2, where it acts in concert with another cellular factor to stimulate DNA replication (8, 13). Subsequently, NF-1 was determined to be identical to a transcription factor, CTF-1, that binds to a CCA-containing sequence that is required for high level transcription of the herpes simplex virus thymidine kinase gene and the α-globin gene (14). Optimal binding of CTF/NF-1 occurs when the sequence TGG is followed by its inverse complement (CCA) with a spacing of 7 nucleotides (18). However, NF-1 can also bind, albeit with lower affinity, to DNA sequences in which only a single TGG or CCA is present (14). Purified CTF/NF-1 contains a relatively large amount of the 33-35-kDa members of this trinucleotide (8, 13–15). The two subunits are separated and must be recombined in order to bind to DNA. These workers also reported that NF-1 could be dissociated into heterodimeric subunits by a similar procedure. RPF-1 does not seem to share these properties. The protein adheres to negatively charged resins and is eluted with high salt as a single active peak. Moreover, unlike the proteins described by Chodosh et al. (19, 20), the RPF-1 proteins appear to be present as monomers in the absence of DNA as determined by gel filtration and gradient ultracentrifugation.

Based upon the above considerations, we conclude that RPF-1 belongs to an incompletely defined family of TGG-binding proteins. It is clear that RPF-1 is not specific for the HMG-CoA reductase promoter. It must recognize a large number of sequences in DNA. Whether this protein functions in transcriptional regulation and/or DNA replication or whether it is some determinant of chromatin structure remains to be determined.

Note Added in Proof—Partial amino acid sequence analysis obtained from each of the two purified RPF-1 polypeptides shows that they are distinct but related proteins, now designated RPF-A and RPF-B. In addition, the sequence of the full length cDNA encoding the smallest RPF-1 polypeptide (RPF-B) demonstrates that the 33-kDa form as purified in the current study is generated by proteolysis of a 62,729-kDa protein (Gil, G., Smith, J. R., Goldstein, J. L., Slaughter, C. A., Orth, K., Brown, M. S., and Osborne, T. F. (1988) Proc. Natl. Acad. Sci. U. S. A., in press).

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REFERENCES