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Interaction of Multiple Factors With a GC-Rich Element Within the Mitogen Responsive Region of the Human Transferrin Receptor Gene

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Abstract Nuclear factors from HeLa cells were isolated by elution of DNA-cellulose bound proteins with a double stranded synthetic oligonucleotide corresponding to the region from −34 to −79 of the human transferrin receptor (TR) gene promoter. The eluted proteins were further purified and separated from the oligonucleotide by ion exchange chromatography. Proteins within the resulting fraction bound with specificity to the TR promoter. Retardation gel analysis and competition with specific double-stranded oligonucleotides show that multiple factors present in this fraction compete for binding within the same region of the TR promoter. Footprinting experiments demonstrate that these factors contact a GC-rich element that is within the region that is required for enhanced expression of the gene in proliferating cells. One of the factors protects an extended DNA sequence but still contacts the GC-rich element.

Key words: transcription factors, Sp1, Ap1, CREB, DNase I footprinting, oligonucleotide competition

The transferrin receptor (TR) is expressed at low levels in quiescent, non-dividing cells and at much higher levels in cells that are actively proliferating. Previous experiments have demonstrated that a region of the TR gene promoter between −78 and −34 is involved in activation of the gene in response to mitogens (Miskimins and Brown, 1990; Ouyang and Miskimins, unpublished results). By footprinting experiments it has been shown that this region contains two elements that are protected from DNase I cleavage by HeLa cell nuclear proteins (Miskimins et al., 1986). In vivo experiments carried out by microinjection of competitor oligonucleotides have shown that the entire region containing both protein binding sites is necessary and that competition with either site alone is insufficient to block the mitogenic response (Miskimins and Brown, 1990). These results suggest that there is cooperativity between these two elements or that an accessory protein that is coupled to both DNA-binding sites may be involved. Alternatively, it is possible that an additional, previously undetected factor that binds to a sequence overlapping both sites is responsible for these observations.

To further investigate the DNA-protein interactions within this region of the TR promoter, we have partially purified factors that bind to the mitogen responsive region by affinity elution of proteins from DNA-cellulose. Analysis of the purified fraction by retardation gels and DNase I footprinting shows that several specific complexes can assemble over the GC-rich element of the TR promoter and that multiple DNA-binding proteins compete for binding to this element.

METHODS

Materials

HeLa cell nuclear extract was prepared by the method of Dignam et al. (1983), except that after extraction with buffer C the sample was frozen immediately without dialysis and stored at −70°C until used. Double-stranded DNA-cellulose was either purchased from Sigma Chemical Co. or prepared by the method of Alberts and Herrick (1971) and contained 3–8 mg of DNA per gram of powder.

The sequences of the oligonucleotides used in this report are as follows: TR(−79/−34), AG-

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GAAGTGACGCACAGCCCCCCTGGGGGC-CGGGGGCGGGGCCA; Sp1, TG-CACGGGGCGGGGCCTGCA; CRE, CCCGGGTGACGTCACGGGGA; Ap1, AGCTT-GATGAGTCAGCCGGATC. All oligonucleotides were used in double stranded form and the other strand for each oligonucleotide was an exact complement.

Chromatography

A column (1 × 10 cm) of DNA cellulose was packed and equilibrated with Buffer D2 (20 mM HEPES, pH 7.9, 0.25 M sucrose, 50 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM $MgCl_2$, 100 μM $ZnCl_2$). HeLa nuclear extract (~200 mg protein) in buffer C was diluted with 3.5 volumes of buffer D2 and passed over the DNA-cellulose column. The column was washed until the absorbance at 280 nm reached background levels. Proteins were then eluted with 1 column volume of buffer D2 containing 25 μg/ml (up to 50 μg/ml in some experiments) of specific double stranded oligonucleotide. In initial experiments, 1×10^6 cpm of labelled oligonucleotide was added as a tracer. The peak fractions, as determined by absorbance at 280 nm, were pooled and immediately applied to a 6.6 × 60 mm column of DEAE-Sephacel equilibrated with buffer D2. After extensive washing with the same buffer the bound proteins were eluted with a linear 0.05–0.5 M KCl gradient.

Gel Retardation Assays

A labelled probe was prepared by cleaving the plasmid pTRSaf, which contains 365 base pairs (bp) of TR sequence including 114 bp of 5′ flanking sequence and the first 251 bp downstream of the transcriptional start site, with Sal I. The linearized plasmid was then labelled by end-filling with Klenow polymerase in the presence of α-^{32}P-dCTP followed by digestion with Ava I. The resulting fragment was purified by electrophoresis on a 1.5% agarose gel in 45 mM Tris, 45 mM borate, and 1 mM EDTA. The band was excised and isolated by centrifugation through a mini-column containing a fritted disc (Vogelstein, 1987) and used without further manipulation.

The binding reaction contained 3 μl of each column fraction or 3 μl of nuclear extract (diluted 1:3.5 with buffer D2), ~0.5 ng labelled probe, and 1 μg of poly(dI-dC) · (dI-dC) in a total volume of 5 μl. After incubation on ice for 10 min, the reaction was loaded onto a 0.75 mm thick polyacrylamide gel containing 22.5 mM Tris, 22.5 mM borate, and 0.5 mM EDTA. After electrophoresis the gel was dried and exposed to Kodak XAR-5 film at −70°C.

DNase I Footprinting

For footprinting, the binding reaction described above was scaled up 2–10-fold. Prior to loading the samples onto the gel, a one-tenth volume of 0.1 μg/ml DNase I in 10 mM $CaCl_2$ was added. The reactions were incubated at room temperature for 1 min and then loaded onto a polyacrylamide gel and electrophoresed as described above. The wet gel was exposed to film for 3 h and the protein-DNA complexes were excised. The gel slices were incubated in 0.6 ml of 0.6 M ammonium acetate, 0.1% sodium dodecylsulfate (SDS), 0.1 mM EDTA at 37°C overnight. The gel slice was removed and the eluted DNA fragments were precipitated with 3 volumes of ethanol. The samples were then subjected to electrophoresis on a 7% polyacrylamide sequencing gel and the bands detected by autoradiography.

RESULTS

Figure 1 shows a diagram of the region of the TR promoter that is involved in the proliferation-dependent expression of the gene. The bars labelled A and B show the regions that are protected in DNase I footprinting experiments using HeLa cell nuclear extract (Miskimins et al., 1986). As mentioned above, both regions A and B are required for full mitogen responsiveness. Region A is very GC-rich and contains a high affinity consensus site for the transcription factor Sp1 (Kadonaga et al., 1986). Region B contains an element that is somewhat similar to the consensus sequences for both CREB and Ap1 transcription factors. We have attempted to isolate factors that are involved in the interactions throughout this entire proliferation-responsive region of the gene. To do this we synthesized an oligonucleotide that spans the sequence from −37 to −79 (indicated by lower bar in Fig. 1) and utilized it in the affinity elution procedure described below.

HeLa nuclear extract was passed over a DNA-cellulose column as described in Methods. After extensive washing, the column was eluted with 1 column volume of buffer containing 25 μg/ml of the double stranded oligonucleotide spanning the region from −37 to −79 of the TR gene. This

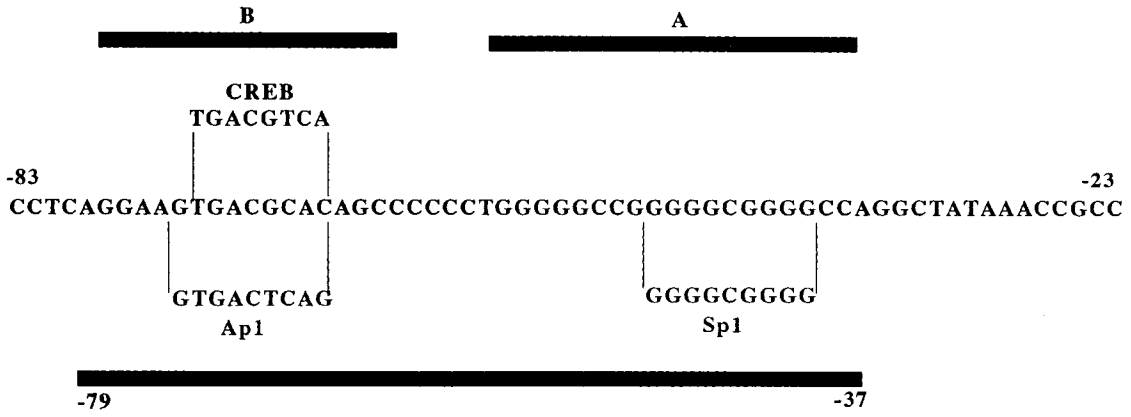


Fig. 1. A diagram of the regulatory region of the TR promoter. The bars at the top labelled A and B represent sequences that are protected from DNase I in footprinting experiments using HeLa nuclear extracts. The lower bar indicates the region included in the oligonucleotide used for the affinity elution purification procedure. Also indicated are regions of similarity to the consensus binding sites for the transcription factors Sp1, CREB, and Ap1.

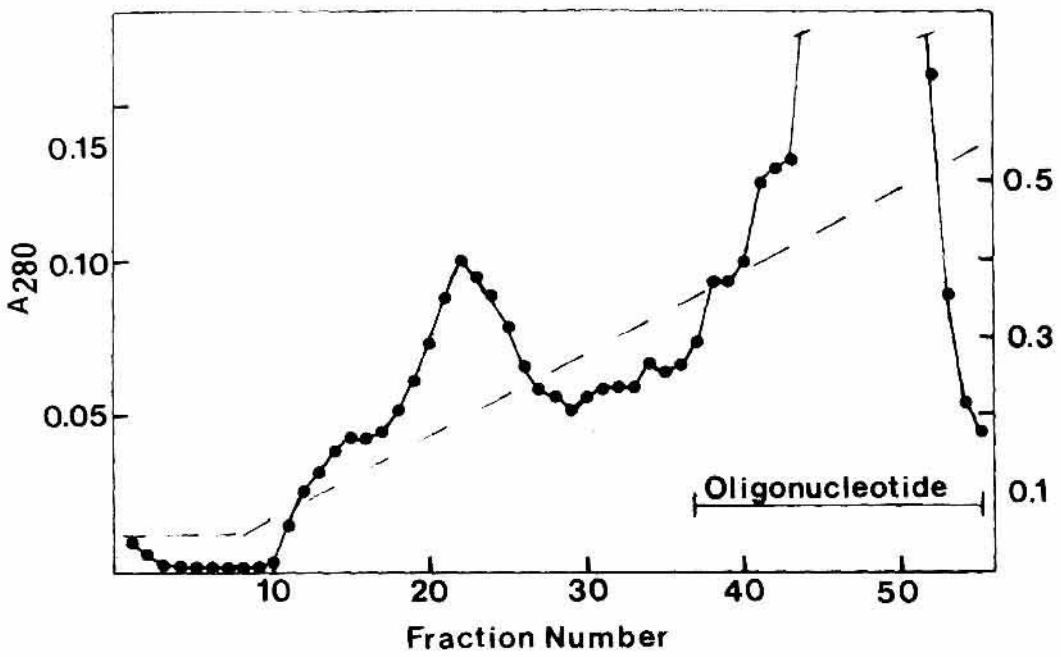
concentration of oligonucleotide is far less than the 3–8 mg/ml of DNA immobilized in the column and should allow specific release of proteins that bind with high affinity to the oligonucleotide. The protein-DNA complexes that eluted from the column were immediately applied to a DEAE-Sephacel column to separate the oligonucleotide from the bound proteins (Fig. 2, top panel). The oligonucleotide binds very tightly to the positively charged matrix and elutes at high salt as indicated by the bar in the top panel. A small proportion of the protein flowed through the column, but the remainder eluted at a salt concentration between 0.1 and 0.3 M.

The DEAE-Sephacel fractions were assayed by gel retardation assays using a restriction fragment from the TR gene promoter region that includes sequences between −114 and +25 (Fig. 2, middle panel). All of the TR promoter binding activity was found in fractions 16 to 34. Multiple protein-DNA complexes were observed and a similar pattern of complexes is observed in the crude HeLa nuclear extract. At least four slower migrating complexes are observed with fractions 16–22 (brackets in Fig. 2 middle panel). Two faster migrating complexes are observed with fractions 22–34 (arrow in Fig. 2 middle panel). All of these complexes are competed off by the unlabelled oligonucleotide used in the affinity elution (not shown).

The bottom panel of Figure 2 shows a silver stained SDS-polyacrylamide gel of fractions from the DEAE column. The lanes labelled A are equivalent to fraction 19 in the top panel and the lanes labelled B are equivalent to fraction 25 in the top panel. Fractions from two completely separate and independent preparations are shown demonstrating that the procedure is highly reproducible. Lanes A contain 10–12 visible bands that range in apparent molecular weight from 26 K to 120 K. Lanes B contain a major band of 116 K apparent molecular weight and a minor band that migrates as a ~50 K molecular weight protein.

To further analyze the specificity of binding to the TR promoter these fractions were analyzed by DNase I footprinting. Figure 3 shows that proteins in the fractions equivalent to fraction 19 (see Fig. 2) give a strong footprint over the region from −38 to −54 and that there is weaker protection from DNase digestion extending upstream to at least −81. Using the sample equivalent to fraction 25 (see Fig. 2), we were unable to detect any protected region within the TR promoter sequences.

The purified fraction (equivalent to fraction 19 in Fig. 2) produces several specific DNA-protein complexes with the TR promoter fragment, yet gives a relatively simple DNase I footprint. It was therefore analyzed in greater detail by retardation gel, footprinting, and competition analyses, as described below. Using higher resolution retardation gel analysis with an end-labelled probe that includes TR promoter sequences from −114 to +25, 4 distinct protein-DNA complexes are clearly resolved (Fig. 4. lane 1). These were labelled complexes A, B, C, and D. Lane 2 shows an identical binding reaction, except that 1.5 pmol of the TR(−79/−37) oligonucleotide was included as a cold competitor. This represents approximately a 160-fold excess of competitor relative to the labelled probe. Un-


0.15
0.10
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A280
0.5
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Oligonucleotide
10
20
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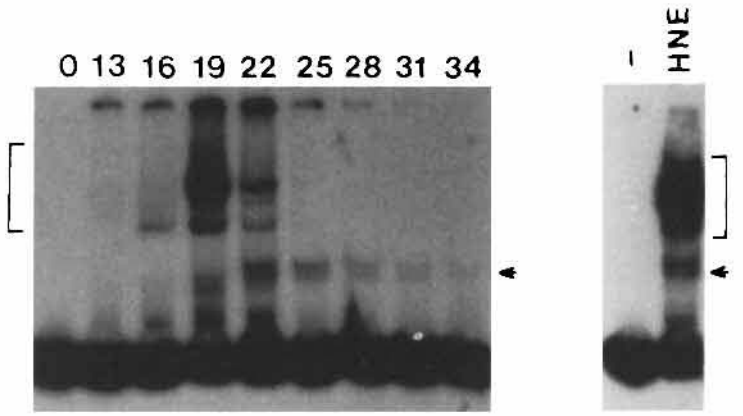
40
50
Fraction Number


0 13 16 19 22 25 28 31 34
-
HNE

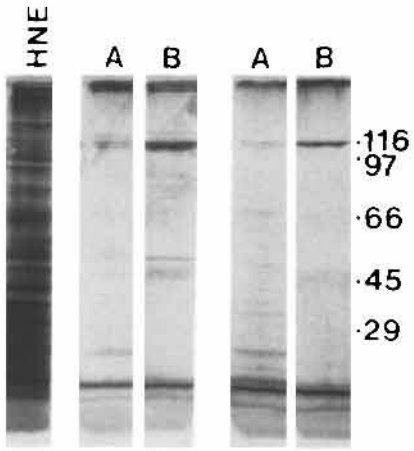

HNE
A
B
A
B
116
97
66
45
29

Figure 2.

der these conditions complexes A and B decrease in intensity, while complexes C and D are enhanced. If the level of this oligonucleotide competitor is titrated to even higher levels, all four of the complexes are competed off (data not shown). These results suggest that all of the complexes (A through D) involve specific interactions with the TR sequences between −79 and −37, but that complexes A and B are higher affinity complexes possibly involving multiple protein factors and cooperative binding.

Within this region of the TR promoter there is a high affinity recognition site for the transcription factor Sp1 (Kadonaga et al., 1986). This element is located between −48 and −39 and thus is entirely within the TR(−79/−37) oligonucleotide. Therefore an oligonucleotide containing the core Sp1 recognition sequence, but with flanking sequences different from those of the TR sequence, was synthesized and used as a competitor in the retardation gel assay (Fig. 4, lane 3). All four complexes (A, B, C, and D) were competed off by the Sp1 oligonucleotide, suggesting that they all require protein contacts within the Sp1 binding site. Surprisingly, 2 new complexes were observed (labelled E and F) under these conditions. Neither of these complexes was observed in the absence of the Sp1 oligonucleotide. When an oligonucleotide containing either a CRE or an Ap1 binding site was added, in addition to the Sp1 oligonucleotide, complex E was competed off (lanes 4 and 6). However, neither the CRE (lane 8) nor the Ap1 oligonucleotide (not shown) had any effect on the pattern of complexes when added alone. Complex F was competed off when the TR(−79/−37) oligonucleotide was added in addition to the Sp1 oligonucleotide (lane 5). Thus the nuclear factor involved in formation of this complex recognizes sequences specific to the TR gene regulatory region.

The complexes observed in Figure 4 were further analyzed by DNase I footprinting (Fig. 5). Attempts to footprint complexes D and E were unsuccessful. However, complexes A, B, and C

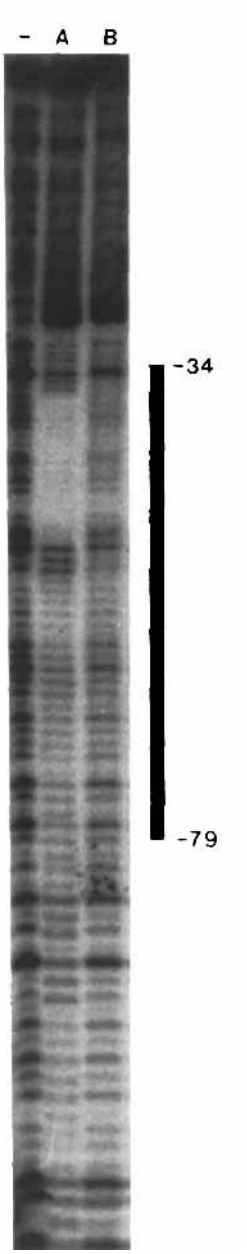


Fig. 3. DNase I footprinting of the TR promoter region using the affinity eluted fractions after DEAE-Sephacel chromatography. The lanes show the pattern of DNase generated bands in the absence of any protein (−), in the presence of a sample equivalent to fraction 19 in Figure 2 (A), or in the presence of a sample equivalent to fraction 25 in Figure 2 (B).

Fig. 2. Isolation of TR promoter binding proteins by the affinity elution procedure. The **top panel** shows the profile from a DEAE-Sephacel column eluted with a linear KCl gradient. The column was loaded with oligonucleotide-bound nuclear factors that were eluted from a DNA-cellulose column, as described in Methods. The solid line shows the profile of absorbance at 280 nm and the dashed line indicates the KCl concentration. The bar shows the region where the oligonucleotide elutes, as determined by including a radioactive tracer. The **middle panel** shows a retardation gel analysis of the column fractions. The labelled probe was a fragment that contains the TR promoter region from −114 to +25. The right side of the middle panel shows the complexes observed using unfractionated HeLa cell nuclear extract (HNE). The **bottom panel** shows silver stained SDS-polyacrylamide gels of the column fractions. The fractions from two completely separate preparations are shown. The lanes labelled A are equivalent to fraction 19 in the top and middle panels and the lanes labelled B are equivalent to fraction 25. The numbers on the right indicate the positions of molecular weight markers in Kd.

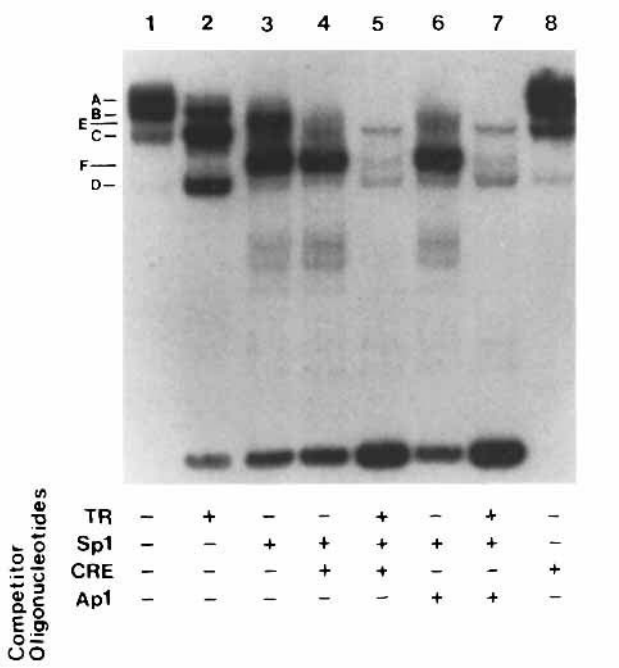


Fig. 4. Retardation gel analysis of the partially purified fraction. All lanes are the same, except that unlabelled competitor oligonucleotides were added as indicated at the bottom of the figure. Sequences of the oligonucleotides are given in the Methods section. The labelled probe is a restriction fragment containing TR promoter sequences from −114 to +25.

all give nearly identical footprints with strong protection over the GC-rich element between −34 and −54. This is consistent with the fact that all of these complexes are disrupted by competition with the Sp1 oligonucleotide. Interestingly, complex F, which is only observed when the Sp1 oligonucleotide is included in the reaction mixture as an unlabelled competitor (see Fig. 4), protects the same GC-rich element but the footprint extends upstream an additional 8–10 base pairs. This suggests that the formation of this complex involves the GC-rich element, but requires contacts with base pairs outside of the Sp1 consensus sequence.

Figure 6 shows a gel retardation analysis of the same protein fraction using 3 separate labelled probes. In lane 1 the probe is the same TR promoter restriction fragment used above and, as expected, 4 complexes are observed (labelled A, B, C, and D as in Fig. 4). In this experiment the level of protein was decreased 2-fold, as compared with the results shown in Figure 4 and under these conditions complex D is enhanced. Under these conditions, as in Figure 4, all four complexes are disrupted by adding the Sp1 oligonucleotide as an unlabelled competitor

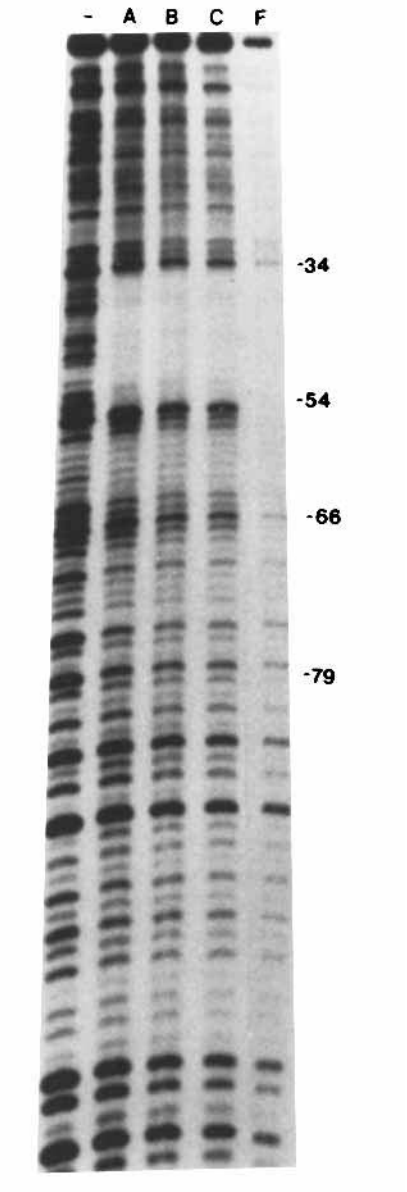


Fig. 5. DNase I footprinting of specific DNA-protein complexes. The letters above each lane correspond to the complexes in Figure 4. The lane labelled (−) is the no protein control. After DNase treatment the complexes were resolved by retardation gel electrophoresis. After exposure to film for several hours, the individual complexes were excised from the gel and the DNA fragments eluted. The fragments were then analyzed on a sequencing gel.

(not shown). These results further suggest that the slower migrating complexes involve multiple proteins and specific protein-protein interactions. In lane 2 the Sp1 oligonucleotide was end-labelled and used as the probe. Only 2 complexes are observed and these comigrate with complexes C and D in lane 1. This is intriguing, since all four complexes (A, B, C, and D) are

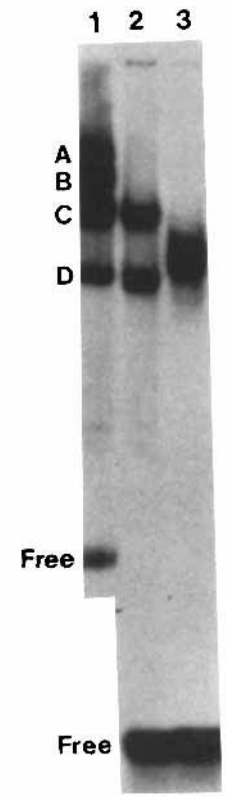


Fig. 6. Retardation gel analysis of the purified fraction using three different labelled probes. In **lane 1** the probe is an end-labelled restriction fragment containing TR promoter sequences from −114 to +25. In **lane 2** the probe is an end-labelled oligonucleotide containing an Sp1 consensus binding site, and in **lane 3** the probe is an end-labelled oligonucleotide containing a consensus binding site for CREB.

competed off by the same Sp1 oligonucleotide when it is used as an unlabelled competitor and all footprint identical regions of the TR promoter. Thus complexes A and B appear to require the Sp1 consensus binding sequence, but do not assemble without additional sequence information from the TR promoter region. Finally, lane 3 shows the results when the CRE oligonucleotide was end-labelled and used in the binding reaction. With this probe a complex that migrates between complexes C and D is observed. On shorter exposures this complex appears as a doublet. Similar complexes are observed with the Ap1 oligonucleotide (not shown). Thus the affinity purified fraction does contain proteins that interact with Ap1/CRE-like sequences, but these apparently are prevented from binding to the TR regulatory region by factors interacting with the GC-rich element (see Fig. 4).

DISCUSSION

These results demonstrate that proteins that bind to the TR promoter can be eluted from DNA-cellulose by a double-stranded oligonucleotide. In the purified fractions the protein-DNA complexes observed by gel retardation assays are very similar to the complexes observed using crude nuclear extracts. The specificity of the factors for the TR promoter sequence is shown by DNase I footprinting. The advantages of the procedure described here are that it is extremely simple and fast and does not require immobilization or oligomerization of the oligonucleotide. Large oligonucleotides with complex protein-binding domains can be utilized. This may facilitate isolation of accessory factors that function to couple multiple transacting factors to the basic transcriptional machinery. However, it is more difficult to do multiple passes over the columns, which have been demonstrated to be extremely useful for complete purification of proteins by the standard oligonucleotide affinity column procedure (Kadonaga and Tjian, 1986). It may, however, be a very convenient first step in the purification of a sequence-specific DNA binding protein. In addition, it represents a very simple and useful way to rapidly prepare a fraction containing substantially elevated specific DNA binding activity, but little of the interfering activities present in a crude nuclear extract.

The protein fraction that we have isolated by this method has allowed us to make several interesting observations about the interaction of nuclear factors with the proliferation-responsive region of the TR promoter. First, at least 4 DNA-protein complexes can be observed that require contacts with the Sp1 consensus site within the TR promoter region. However, only 2 of these complexes are observed when a labelled probe containing an Sp1 binding site, but different flanking sequences, is used. Thus the assembly of the other two complexes (A and B) is dependent on sequences that are unique to the TR promoter. It is possible that these complexes involve specific protein-protein interactions, one protein being directly in contact with the GC-box and the binding of additional proteins being stabilized by contacts both with the flanking TR sequence and the GC-box binding protein.

Formation of these complexes (A, B, C, and D) at the GC-rich element blocks the binding of additional nuclear factors within this region of the TR promoter. This is demonstrated by the

addition of an oligonucleotide containing an Sp1 consensus binding site as a competitor. This disrupts all four of the initial complexes (A, B, C, and D), but results in the appearance of two new, previously unseen complexes (E and F). One of these factors is competed off by oligonucleotides containing either the CREB or Ap1 binding sites and therefore presumably is binding to a similar site found immediately adjacent to the GC-rich element (element B in Fig. 1). The other factor binds within the GC-rich region, but shows an extended protected region in DNase I footprinting experiments.

This complex pattern of DNA-protein interactions described here is relevant to the mechanism by which the TR promoter responds to mitogen stimulation. In quiescent cells there is a low or basal level of expression of the endogenous TR gene or of a reporter gene linked to the TR promoter (Miskimins et al., 1986; Ouyang and Miskimins, unpublished results). Serum or growth factor activation of cells leads to a 5–7-fold increase in expression. This increase occurs late after mitogen addition with the maximum level reached just before the onset of DNA synthesis (Miskimins et al., 1986). We have shown that deletion of the sequences between −55 and −78 partially impairs the ability of the promoter to respond to serum (Ouyang and Miskimins, unpublished results). This region encompasses protein binding region B, which contains the motif that is similar to the consensus sequences for Ap1 and CREB (see Fig. 1). Deletions that remove this site as well as the GC-rich element (region A in Fig. 1) completely abolish the responsiveness to mitogens. We have demonstrated here that complexes assembled at the Sp1 consensus element within the GC-rich region can obstruct binding of a factor that interacts with region B (CRE/Ap1-like), as well as binding of a separate factor that gives an extended footprint over the GC-rich element. One possibility that is consistent with these findings is that mitogen activation of cells leads to inactivation or loss of factors that bind the Sp1-like site in the TR promoter. This allows binding of other factors to both region A and B and these factors cooperatively stimulate transcriptional activity from the promoter.

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