Mitogen Induction of Nuclear Factors That Interact with a Delayed Responsive Region of the Transferrin Receptor Gene Promoter

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Abstract
Activation of the human transferrin receptor promoter by mitogenic stimulation of quiescent cells is a delayed event that reaches a maximum several hours after stimulation. Previous results have defined a region of the transferrin receptor gene promoter that is required for increased expression in mitogen-activated cells (W. K. Miskimins and D. B. Brown, Exp. Cell Res., 191: 328–331, 1990; Q. Ouyang et al., Mol. Cell. Biol., 13: 1796–1804, 1993). This region contains two elements (elements A and B) that appear to cooperate in the response to mitogenic stimulation. Serum stimulation of quiescent cells leads to the induction of nuclear factors that bind to both the A and B elements. Induction of these factors is also a delayed response to serum stimulation and reaches a maximum 6–9 h after stimulation. Element A, which is an unusual GC-rich sequence, forms several serum-inducible DNA-protein complexes, all of which depend on contacts within GC boxes. A major inducible complex of element A contains a factor that is supershifted by antibodies against the transcription factor Sp1. The B element appears to have overlapping binding sites for two types of factors. One of these sites binds factors that are competed off by an AP-1 consensus-binding site. The other B element site binds inducible factors that interact with GC boxes, identical to those observed for element A.

Introduction
Transferrin functions as the primary carrier of iron in mammalian systems, and its uptake into cells is mediated by the TR. Expression of TR is elevated significantly in proliferating cells in comparison to quiescent, noncycling cells (1–3). The increased expression of TR in proliferating cells appears to be critical for cell cycle progression since inactivation of the receptor causes the cells to arrest near the G1–S phase boundary (4–6).

Increased expression of TR in mitogen-activated cells involves increased transcription of the TR gene (7, 8). This is a delayed response, occurring several hours after mitogen stimulation and reaching a maximum prior to entry into S phase. By microinjection (9) and deletion analysis of TR gene promoter constructs stably expressed in Swiss/3T3 fibroblasts (10), we have mapped two adjacent cis-acting elements (Fig. 1, elements A and B) that are involved in the response of the TR gene to mitogen stimulation. These elements are of interest because their response to growth stimulation is delayed, and they are thus activated subsequent to the immediate/early phase of growth factor-induced cell proliferation. Little is known about the mechanisms that regulate elements that respond in a delayed manner to mitogen stimulation; thus, it is of interest to characterize the TR mitogen-responsive elements in further detail. We have found that factors that bind to both the A and B elements of the TR promoter are induced transiently by serum stimulation in 3T3 fibroblasts. The B element appears to be able to interact with two separate classes of factors that are induced with different kinetics. The major inducible protein-DNA complex of the A element appears to involve the transcription factor Sp1.

Results
Deletion mutagenesis has defined a mitogen-responsive region within the human transferrin receptor gene promoter (10). This region consists of two elements, A and B (see Fig. 1), both of which are necessary for full mitogen responsiveness of the TR promoter in serum-stimulated 3T3 fibroblasts. Activation of the TR promoter is a delayed response, with the major increase in expression from this promoter occurring after 6 h of mitogen stimulation of quiescent fibroblasts and prior to the onset of DNA synthesis (~12 h after serum addition; Ref. 10). Little is known about the mechanisms that regulate gene transcription during this critical phase of cell growth. Therefore, it is of interest to analyze the processes that lead to enhanced TR promoter activity in mitogen-stimulated cells. As a beginning, we have analyzed DNA-protein interactions within the mitogen-responsive region of the TR gene promoter using nuclear extracts from HeLa cells and from serum-stimulated 3T3 cells.

Fig. 2 shows a DNase I footprint analysis of the TR promoter mitogen-responsive region using extracts from logarithmically growing HeLa cells. In Fig. 2A the level of nuclear protein was titrated. Low levels of HeLa nuclear protein (1–2 µg) are sufficient to completely protect element A from DNase digestion, suggesting that an abundant protein(s) interacts with this sequence. However, no protection of element B was observed until very high levels of nuclear protein (~30 µg) were reached, suggesting either a low abundance or low affinity of the factor(s) that binds to this element. Fig. 2B shows a footprinting experiment in which a sufficient level of HeLa nuclear protein was added to the binding reaction to protect both the A and B elements, but competitor oligonucleotides specifying either element A or B also were included. When the A element oligonucleotide was included as a competitor it disrupted the footprint over the A element but had little effect on the B element footprint. When the B element oligonucleotide was included as a competitor it blocked the footprint over the B element.
However, it appears that the several DNase-hypersensitive sites that reside between element A and element B were diminished and that the A element footprint extends further upstream under these conditions. These results indicate that the A and B elements are able to interact independently with separate factors. Since both elements are involved in the activation of the TR gene promoter following mitogen stimulation of quiescent cells, it was expected that the activity of the factors that bind within this region would also be modulated in some manner. Therefore DNA-protein interactions were characterized further with the use of extracts from serum stimulated Swiss/3T3 cells.

For this analysis we have utilized oligonucleotide probes that encompass either element A (−57 to −37) or element B (−79 to −57; see Fig. 1). The binding of factors to each of these elements was characterized independently with the use of retardation gel analysis.

Fig. 3A shows the effects of serum stimulation on factors that bind within the A element. Several DNA-protein complexes are observed in extracts from quiescent cells (0 h), and the level of these complexes remains constant during the time course of serum stimulation. In contrast, there are several additional complexes that appear after serum stimulation (arrow and bracket, Fig. 3A). The major serum-
inducible complex of the A element (bracket) appears to be a doublet and is only transiently increased during the time course of serum stimulation. In Fig. 3A this doublet appears to increase up to 6 h after stimulation and then return to the level observed in quiescent cells by 12 h. In other experiments we have observed an increase in these complexes up to 9 h after stimulation, followed by a rapid return to basal levels (see Fig. 3C). In Fig. 3C, the levels of the major serum-inducible complexes (the doublet marked by a bracket in Fig. 3A) at various times after stimulation were quantitated by densitometric scanning and compared to the activation of the TR promoter as measured by CAT activity (10). These data show that there is a very close correlation between the appearance of these complexes and the increase in CAT activity in serum-stimulated cells. However the CAT enzyme is more stable than the inducible DNA-binding proteins, which return to basal levels by 12 h after stimulation.

The A element is a peculiar GC-rich sequence containing a single GC box (see Fig. 1). GC boxes are recognized by the transcription factor Sp1 (11), by other members of the Sp1 family of factors (12-14), and by several other DNA-binding proteins (15-18). Thus, an important question is whether the serum-inducible complexes of the A element involve the GC box and, if so, whether these complexes involve Sp1 or other GC box-binding proteins. Competition assays show that all of the inducible complexes are competed off by oligonucleotides carrying the A element (Fig.
to ser. This GC box is indicated in Fig. 1 and overlaps an AP-1-like motif. It is most likely that the ability of element B to compete for binding with the A element is due to this GC-rich motif. This is addressed further below.

A number of previous studies have identified nuclear factors that bind to GC-boxes (11–14, 16–18). The most well characterized of these is the transcription factor Sp1. Sp1 has not been implicated in the control of growth-dependent genes, but rather as a constitutive factor that is involved in the transcription of numerous genes. However, recent evidence has indicated that Sp1 expression undergoes substantial changes in various cell types during development (19) and that Sp1 plays a role in RB-mediated control of certain regulatory elements (20, 21). Thus, it was of interest to determine whether Sp1 is a component of the serum-inducible complexes of the TR promoter A element.

To address this, antibodies to Sp1 were tested for their ability to supershift the serum-inducible complexes of the A element. Fig. 4 shows the results with the use of extracts from 3T3 cells that had been stimulated by serum for 6 h. Anti-Sp1 supershifts the serum-inducible doublet (labeled by a bracket in Fig. 4) but has no effect on any of the other complexes. Note that one of the complexes of this doublet is not shifted completely by the Sp1 antibodies. Control experiments using antibodies to RB or fos had no effect on the pattern of complexes observed. We have also found that the complex that is supershifted by anti-Sp1 antibodies comigrates with the complex formed by purified recombinant human Sp1 (results not shown). These results indicate that one of the serum-inducible complexes of the A element TR mitogen responsive region involves Sp1.

Fig. 5 shows a UV cross-linking experiment using the labeled A element oligonucleotide and an extract from 3T3 cells that had been stimulated by serum for 6 h. In Fig. 5, Lane 1, the binding reaction was exposed directly to UV light and then fractionated by SDS-PAGE. Numerous proteins become labeled by this method as expected from the number of protein-DNA complexes observed by retardation gel analysis. However, two of the major bands observed have apparent Mr of ~110,000 and ~125,000. This is consistent with the possibility that Sp1 is involved in one of the observed complexes since Sp1 migrates as a doublet with apparent Mr of 95,000 and 105,000 (22). In the UV cross-linking analysis, covalent coupling of the labeled oligonucleotide would be expected to shift the molecular weight slightly. To address this possibility further, DNA-protein complexes were separated first by retardation gel electrophoresis and then subjected to UV cross-linking. The inducible complexes were excised from the gel and then separated by SDS-PAGE. In Fig. 5, Lane 2, the inducible doublet (see bracket in Fig. 3) of element A, which is supershifted by the Sp1 mAb, was analyzed. Only two labeled bands are visible with apparent Mr of approximately 125,000 and 110,000. In Fig. 5, Lane 3, the other serum-inducible complex of element A (see arrow in Fig. 3) was analyzed in a similar manner. For this complex a single major band of approximately Mr 88,000 was labeled. A similar UV cross-linking experiment was carried out with the use of the labeled Sp1 oligonucleotide probe (Fig. 5, Lanes 4 and 5), with results nearly identical to those obtained with the TR A element probe.

Fig. 6A shows the effects of serum stimulation on factors that bind within element B. One inducible complex is marked by an arrow. The induction of this complex shows kinetics similar to those observed for the A element. That is, it reaches a maximum around 6 h after stimulation and returns to basal levels by 12 h. The diffuse complex marked by a bracket (Fig. 6A) follows slightly different kinetics, reaching a maximum by 3 h and then declining to basal levels by 12 h after stimulation.

Competition experiments were carried out with the use of the 3-h postserum stimulation extract and the labeled B...
element probe. As expected, oligonucleotides carrying the B element (Fig. 6B) completely compete off the inducible complexes observed with this probe. It was surprising to find that the A element oligonucleotide, as well as the Sp1 oligonucleotide, compete off the slowest migrating complex (Fig. 6B, arrow) while enhancing the diffuse complex marked by a bracket.

Competition using the AP-1 oligonucleotide also produced somewhat unexpected results (Fig. 6B). This oligonucleotide completely eliminated the diffuse complex (Fig. 6B, bracket) but markedly enhanced the slower migrating complex (Fig. 6B, arrow). These results suggest that the B element contains two overlapping binding sites for serum-inducible proteins. These sites consist of a GC-rich sequence and an AP-1-like core sequence (see Fig. 1), suggesting that members of the Sp1 and AP-1 families could be involved in the observed complexes.

Supershift experiments were carried out using the B element probe and extracts from serum-stimulated 3T3 cells (Fig. 7A). For supershift experiments using anti-Sp1 antibodies, the unlabelled AP-1 oligonucleotide was added as a competitor to eliminate the diffuse complex (indicated by a bracket) and to enhance the complex involving the GC box (arrow). Anti-Sp1 is able to supershift this complex, suggesting that it is identical to the inducible complex of the A element that appears to involve Sp1 (Fig. 7A, Lane 2).

Supershift experiments were also carried out with the use of the labeled B element probe and an antibody that recognizes a conserved sequence within members of the jun family of transcription factors. For these assays, the TR A element oligonucleotide was added as a competitor to eliminate the complexes involving the GC box and enhance binding to the AP-1-like site. This antibody had little effect on the observed complex in 3T3 cell extracts under any conditions tested (Fig. 7A, Lanes 4 – 6).

We therefore carried out additional supershift experiments using purified recombinant c-jun homodimers and HeLa nuclear extracts (Fig. 7B). Three different antibodies were utilized for these experiments, one that recognizes multiple members of the jun family, one that is specific for c-jun, and one that recognizes multiple members of the fos family. For these experiments, the consensus AP-1 oligonucleotide was compared to the TRB’ oligonucleotide which retains the TR B element AP-1-like site but not the GC box. With the use of HeLa nuclear extracts, these two probes give similar patterns of protein-DNA complexes, except that the AP-1 oligonucleotide shows an additional slower migrating complex (Fig. 7B, Lanes 1, 7, 13, and 15). Both the anti-jun family and anti-fos family antibodies are able to produce a supershifted complex with the consensus AP-1 probe (Fig. 7B, Lanes 2 and 14). These same antibodies produce only weak supershifted complexes with the TRB’ probe (Fig. 7B, Lanes 8 and 16). The c-jun-specific antibody has little effect on the DNA-protein complexes observed with either of these probes (Fig. 7B, Lanes 3 and 8).

As expected, purified c-jun homodimers bind efficiently to the AP-1 consensus sequence (Fig. 7B, Lane 4) and the observed complex is supershifted by the anti-c-jun antibody (Fig. 7B, Lane 6). The antibody that recognizes a conserved sequence within several members of the jun family has a minimal effect on this complex (Fig. 7B, Lane 5). In contrast to the consensus AP-1 sequence, the AP-1-like site within the TR B element binds very poorly to c-jun homodimers.

**Discussion**

Activation of the TR promoter elements in response to mitogen stimulation of quiescent cells is a delayed event, occurring several hours after the addition of serum or growth factors (10). Here we show that factors that interact with these mitogen responsive promoter elements are also induced in a delayed manner, reaching maximal activity approximately 6–9 h after stimulation. Induction of these factors is transient, and they return to the levels observed in quiescent cells by 12 h after stimulation.

Serum stimulation of 3T3 cells leads to the induction of factors that bind to both the A and B elements of the TR mitogen-responsive region. Element B appears to have overlapping binding sites for 2 different types of serum-inducible factors. One of these sites is similar to the consensus-binding domain of the AP-1 family of transcription factors, and an oligonucleotide containing the core consensus AP-1-binding site is an effective competitor for one of the serum-inducible B element complexes. However, antibodies that recognize members of either the jun or fos family of factors have minimal effects on this inducible complex. In addition, c-jun homodimers bind very poorly to the B element. Thus, it will require further experimentation to identify the factor(s) present in this complex. Beard et al. (27) have shown previously that SV 40 infection of CV-1 cells leads to the induction of factors of approximately M, 55,000 and 47,000 that bind to the AP-1-like site of element B. Similar to our results, binding of these factors to
the B element was competed off by a consensus AP-1 oligonucleotide (27). In addition, Roberts et al. (28) isolated nuclear factors by affinity chromatography using an oligonucleotide spanning from −63 to −115 of the TR promoter. Although this sequence includes most of the TR B element, the isolated proteins (TREF1 and TREF2) do not bind with high specificity to the B element and are clearly not the same as the serum-inducible AP-1-like factors described in this report.

Element B also binds another serum-inducible factor that appears to involve a GC-rich region. That this binding site overlaps the AP-1-like site is indicated by the fact that competition with the AP-1 oligonucleotide dramatically enhances this complex. This GC-rich site in element B, although having a lower affinity, appears to bind the same serum-inducible factor that binds to element A, located immediately downstream in the promoter (discussed below). Since the B element has been shown to be critical for full mitogen responsiveness of the TR promoter (10), it is interesting that inducible factors that bind the AP-1-like site and inducible factors that recognize GC boxes can compete for binding in this region. This additional complexity of element B suggests that it could be targeted by multiple signalling mechanisms in response to mitogenic stimulation.

The A element of the TR promoter is an unusual, GC-rich domain, and previous experiments have shown that it can interact with multiple nuclear factors (23). Here we demonstrate that several serum-inducible DNA-protein complexes can form at this element and that the kinetics of induction are consistent with a role for these complexes in regulating the TR gene in response to mitogens. All of the serum-inducible complexes of element A are competed off by an oligonucleotide containing a 10-bp high affinity consensus sequence for the transcription factor Sp1. Furthermore, a major serum-inducible complex is supershifted by an antibody against Sp1. UV cross-linking experiments identify proteins within this complex with molecular weights similar to those of Sp1. Thus, it is most probable that Sp1 is a component of this inducible-complex. Sp1 generally is thought to be a constitutive factor that enhances the transcriptional initiation of numerous genes. However, several recent findings suggest that Sp1 may play a role in growth regulation. It has been shown to be inducible by infection of CV1 cells with simian virus 40 (24), which leads to a growth-like response. Saffer et al. (19) have found that there are large variations in Sp1 levels during development and between various tissues, suggesting a regulatory role for Sp1 in growth and development. Also, it has been shown recently that Sp1 is involved in mediating control of gene expression by the RB protein, a cell cycle-regulated protein (20, 21). Sif et al. (25) have found that several oncoproteins, including those encoded by v-rel, v-ras, and v-src, are able to stimulate gene expression through promoters containing Sp1 sites. Thus, Sp1 may play an important role in signalling mechanisms that regulate cell proliferation. Our data support the conclusion that Sp1 is involved in regulating a mitogen-dependent delayed response gene promoter.

Recently it has been shown that there is a family of Sp1-like factors that all bind to similar GC or GT-rich motifs (12–14). These factors are related highly within the DNA-binding domain but show greater divergence outside of this domain. The antibody used in these studies for supershift analysis was prepared by injection of a synthetic peptide that corresponds to the amino acid sequence immediately to the NH₂-terminal side of the zinc finger domains of human Sp1. This peptide sequence is conserved but not
identical in some of the other members of the Sp1-like transcription factor family. Therefore, we cannot conclude with absolute certainty that the supershifted complex contains Sp1 rather than another member of the Sp1 family of factors. However, the fact that cross-linking experiments identify a protein doublet consistent with the molecular weight of Sp1, and the fact that purified recombinant Sp1 precisely comigrates with the inducible complex, strongly suggest that Sp1 is indeed the DNA-binding protein present in the inducible complex.

In summary, we have found that serum stimulation of quiescent cells leads to the induction of factors that bind to the mitogen-responsive region of the TR promoter. Since activation of the TR promoter is a delayed event in growth-stimulated cells, it is very likely that these factors, which are also induced in a delayed manner, are involved in this process. A novel finding is that one of the major mitogen-inducible complexes that we have observed involves Sp1.

Materials and Methods
Preparation of Cell Extracts. Swiss/3T3 cells were grown to confluence in 150-mm culture dishes in DMEM supplemented with 10% calf serum. After reaching confluency, they were incubated for 24–48 h in serum-free medium consisting of a 1:1 mixture of DMEM and Waymouth’s 720 medium. The cells were stimulated by adding calf serum to 20%. At various times after stimulation, the cells were scraped from the dish and centrifuged for 5 min in a tabletop centrifuge at ~1000 rpm. The cell pellet was resuspended in 1 ml of buffer D2++ [20 mM HEPES (pH 7.9), 2.5 mM sucrose-50 mM KCl-0.5 mM DTT-0.5 mM phenylmethylsulfonyl fluoride-5 mM MgCl2, 0.1 mM ZnCl2] and centrifuged for 30 s at 3000 rpm in an Eppendorf microcentrifuge. The pellet was resuspended in 100 µl D2++ buffer containing 0.8 M KCl. The cell suspension was frozen in a dry ice ethanol bath for 5 min and then thawed on ice for 1 h. Cell debris was removed by centrifugation for 5 min in a microcentrifuge, and the supernatant was diluted 3-fold in D2++ buffer for retardation gel analysis.

DNase I Footprinting. The probe for footprinting analyses was prepared by digesting 20 µg of pTRSaf, a plasmid carrying TR promoter sequences from −114 to +261, with Sall. The Sall-digested plasmid was end labeled with the use of Klenow fragment in the presence of 250 µM dATP, dGTP, and thymidine triphosphate and 50 µCi [α-32P]dCTP. The reaction was incubated at room temperature for 1 h and chased with cold dCTP (250 µM final concentration) for 10 min. The labeled DNA was precipitated with ethanol and then digested with EcoRV. The appropriate 5’-end-labeled DNA fragment was isolated by electrophoresis on a 2% agarose gel in 45 mM Tris-45 mM borate-1 mM EDTA. This probe contains TR sequences from −114 to +11.

Footprinting reaction mixtures contained the end-labeled probe (0.5–1 ng), 1–30 µg of HeLa nuclear protein (26), and 100 µg/ml poly(dI-dC) in buffer consisting of 20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M KCl, 5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride. Reactions contained unlabeled oligonucleotide competitors as described in Fig. 2B. After incubation on ice for 20 min, DNase I was added at a final concentration of 0.1–4 µg/ml, and digestion was carried out at room temperature for 1 min. The reaction was immediately
stopped by addition of 1 reaction volume of stop buffer (0.6 M ammonium acetate-0.1% SDS-0.1 mM EDTA). The DNA was precipitated with ethanol, dissolved in 90% formamide-loading buffer, heated to 95°C for 10 min, and separated on an 8% polyacrylamide sequencing gel. After electrophoresis, the gel was fixed in 10% acetic acid-10% methanol for 20 min, transferred to 3MM Whatman filter paper, dried under vacuum, and exposed to Kodak XAR-5 film overnight at ~70°C.

Retardation Gel Analysis and UV Crosslinking. Oligonucleotide probes were prepared by end labeling with T4 polynucleotide kinase in the presence of [γ-32P]ATP. DNA-binding reaction mixtures (10–15 μl reaction volumes) contained 10–30 μg of protein extract diluted in buffer D2++, 1 μg poly(dI:dC), ~0.5 ng labeled oligonucleotide and, where indicated, unlabeled oligonucleotide competitors. Reactions were incubated 30 min on ice and then loaded onto a 5% polyacrylamide gel as described (23). After electrophoresis the gels were dried and exposed to Kodak XAR-5 film overnight at ~70°C.

For supershift assays using antibodies, the reactions were carried out as described previously with antibody present at the beginning of the binding reaction. Anti-SP1 and anti-c-fos were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-c-fos and c-Jun was purchased from Caltag Laboratories (South San Francisco, CA), and anti-RB was purchased from Oncogene Science (Uniondale, NY).

For UV cross-linking analysis of individual complexes, the retardation gel was exposed to a UV transilluminator (UVP Model TM-26) for 20 min. The wet gel was then exposed to X-ray film for several hours, and gel slices containing the complexes of interest were excised with a razor blade. The gel slices were saturated with SDS-sample buffer and then placed in the wells of a 1.5-mm-thick 8% SDS-polyacrylamide gel. After electrophoresis the gel was dried and exposed to Kodak XAR-5 film. For some experiments the binding reaction was exposed directly to UV light for 5 min, mixed with an equal volume of SDS sample buffer, and then separated by SDS-PAGE.

The sequences of the oligonucleotides used in this report are: TR −79 to 371, AGGAATGACCCACACGCCCTCTGGGGCCGGGGGCGCCTC; TRA −57 to 371, TGGGGCCGGGGGCGCCTC; TRB −79 to 63, AGGAATGACCCACCGGCCCTC; Sp1, TGGCGCGGGGCGCCGCAAG; and AP-1, AGCTTGATGATCCGAGGGATC. All oligonucleotides were used in double stranded form, and the other strand for each oligonucleotide was an exact complement.

Acknowledgments
We thank Dr. Robi Miskimins for numerous encouragements and critical evaluation of the manuscript.

References