Cell Proliferation and Expression of the Transferrin Receptor Gene: Promoter Sequence Homologies and Protein Interactions

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Abstract. A 365-bp fragment from the 5′ region of the human transferrin receptor gene has been subcloned and sequenced. This fragment contains 115 bp of flanking sequence, the first exon, and a portion of the first intron. It contains a TATA box, several GC-rich regions, and is able to efficiently promote expression of the bacterial CAT gene in mouse 3T3 cells. Sequence comparisons demonstrate that this DNA segment has homology to the promoter regions of the human dihydrofolate reductase gene and the mouse interleukin 3 gene, as well as to a monkey DNA sequence that has homology to the SV40 origin and promotes expression of an unidentified gene product. Several high molecular mass proteins that interact with the transferrin receptor gene promoter have been identified. The activity of these proteins is transiently increased in 3T3 cells that have been stimulated by serum addition. This increase precedes a rise in transferrin receptor mRNA levels in the cytoplasm, which in turn precedes entry of the cells into S phase. DNase I footprinting of the transferrin receptor promoter reveals several protein binding sites. Two of the sites are within the conserved GC-rich region of the promoter. One of these binding sites probably interacts with Spl, while the second interacts with an uncharacterized protein.

CELLS are provided with the essential nutrient iron through the action of transferrin receptors (TR).[1] Surface receptors and iron-loaded transferrin are internalized to acidic intracellular vesicles where the iron is released. The receptor and apotransferrin are then recycled to the cell surface and reutilized. Through this process, intracellular iron is available for heme-containing proteins, oxidative enzymes, and other iron-requiring enzymes. The requirement for iron in many physiological processes necessitates the presence of a low level of TR on nearly all cell types. However, under certain conditions, the number of cell surface receptors is greatly increased. In maturing reticulocytes a large supply of intracellular iron is needed for hemoglobin synthesis. This need is met by expression of a large number of cell surface TR. Likewise, when erythroleukemia cells are induced to differentiate in vitro, there is a severalfold increase in TR number that precedes the production of hemoglobin (15). The number of cell surface TR is also greatly increased in rapidly proliferating cells (21, 40, 13). Many transformed cells have an elevated level of TR. Mitogen-stimulated lymphocytes and fibroblasts increase their cell surface TR manyfold. In contrast, when induced to differentiate, most cell types stop cell division and display a sharp reduction in TR number (37).

The marked increase in TR levels in proliferating cells appears to be a necessity for DNA synthesis. Functional receptors are required for the cell to complete S phase (30, 39). When treated with mAbs that bind to the TR, cells are prevented from growing and accumulate in S phase (39). This effect is not overcome by supplying other forms of iron to the cell, and appears to be related to receptor aggregation at the cell surface (22).

As a protein that is expressed at elevated levels in proliferating cells and provides an important S phase function, the TR belongs to a group of similarly expressed proteins and enzymes. Included in this group of proteins are the enzymes of deoxynucleotide biosynthesis and DNA replication. The levels of mRNA coding for many of these enzymes, as well as for TR, have been shown to increase upon mitogen stimulation. This suggests that common regulatory mechanisms may control expression of the genes coding for this class of proteins. Such mechanisms may be both transcriptional (14, 9) and posttranscriptional (23, 12). Our aim is to define the molecular nature of these controls. Here we present the sequence of the human TR gene promoter, a striking homology of this sequence with the dihydrofolate reductase (DHFR) gene promoter and the interleukin 3 gene promoter, and the mitogen-dependent expression of a group of proteins that interact with defined regions of this sequence.

Materials and Methods

Plasmid Construction and DNA Sequencing

A 365-bp Sal I/Xho I fragment from the 5′ end of the human genomic se-

1. *Abbreviations used in this paper*: DHFR, dihydrofolate reductase; TR, transferrin receptor.

quence in phage TR76 (20) was gel-purified and inserted into the Sal I site of M13 mp9 using standard procedures. Clones containing the insert in both orientations were isolated and sequenced by the dideoxy chain termination procedure. The sequence was independently confirmed by the method of Maxam and Gilbert (25).

To construct CAT expression plasmids using the TR365 fragment, the purified Sal I/Xho I fragment was converted to a blunt-ended fragment by end-filling with the Klenow fragment. HindIII linkers were ligated to the fragment, which was then digested with HindIII and gel-purified to remove excess linkers. The fragment was ligated to HindIII-digested pSVO CAT (11), which had been treated with alkaline phosphatase. The mixture was used to transform *Escherichia coli* HB 101. Plasmids containing the 365-bp fragment in the two different orientations with respect to the CAT gene were identified by restriction analysis and Maxam/Gilbert sequencing.

Transient Expression Assays

20 μg of plasmid DNA was added as a calcium phosphate precipitate to 150-mm^2 tissue culture flasks containing 1 × 10^6 Swiss/3T3 cells that had been plated 24 h earlier. The medium was replaced after 24 h and the cells harvested after 48 h. The cells were resuspended in 100 μl of 0.25 M Tris-Cl, pH 7.8. The cells were disrupted by three freeze-thaw cycles and debris was pelleted by centrifuging for 2 min in an Eppendorf microcentrifuge. Extracts were either assayed immediately for CAT activity or stored frozen at −20°C.

CAT assays were carried out by the method of Gorman et al. (11). Reactions contained 0.5 M Tris-Cl, pH 7.8, 0.5 mM acetyl coenzyme A, 1 μCi [^{14}C]chloramphenicol, and 30 μl of extract in a volume of 150 μl. They were incubated at 37°C for 60 min. Chloramphenicol was extracted in ethyl acetate and dried under vacuum. The sample was redissolved in 20 μl of ethyl acetate, spotted on a silica gel thin layer plate, and chromatographed in chloroform/methanol (95:5). The plate was then exposed to Kodak XAR-5 film.

Low Ionic Strength Polyacrylamide Electrophoresis and DNase I Footprinting

A labeled fragment for footprint analysis was prepared by digesting pTR-CAT-16 with Taq I and end-filling with Klenow fragment in the presence of [^{32}P]dCTP. The DNA was recovered by ethanol precipitation and then digested with Ava I. The appropriate fragment was gel-purified by agarose electrophoresis and electroelution. The electroeluted fragment was bound to a 1-cm column of DEAE-Sephacel in a Pasteur pipette and eluted with 1 M NaCl, 6.5 M urea, 10 mM Tris-Cl, pH 7.6. The fragment was then ethanol-precipitated and redissolved in H_2O.

Protein-DNA complexes were analyzed by a procedure similar to that of Fried and Crothers (10). Binding reactions (20 μl) contained 10 mM Hepes, pH 8.0, 60 mM KCl, 5 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 5% polyethylene glycol (PEG), 1 mM dithiothreitol (DTT), 1 μg poly(dI-dC)·poly(dI-dC), 0.2 μg plasmid DNA, 0.5 ng end-labeled probe, and 0–10 μg HeLa nuclear extract prepared by the procedure of Dignam et al. (5). Incubation was carried out on ice for 30 min. The samples were then applied to gels composed of 3.5% acrylamide, 10 mM Hepes, pH 8.0, and 0.5 mM EDTA. The electrode buffer consisted of 10 mM Hepes, pH 8.0, 0.5 mM EDTA. Electrophoresis was done at 200 V for ~2 h.

Footprinting reactions (50 μl) contained 10 mM Hepes, pH 8.0, 60 mM KCl, 5 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 5% PEG, 1 mM DTT, 2.5 μg poly(dI-dC)·poly(dI-dC), 0.5 μg plasmid DNA, 50 ng of end-labeled probe, and ~10 μg of HeLa cell nuclear extract. Incubation was carried out on ice for 30 min. The samples were then placed at room temperature and 5 μl of 5–50 μg/ml DNase I in 10 mM $CaCl_2$ was added. After 1 min the reactions were stopped by adding EDTA to 50 mM and placing the samples on ice. Protein-DNA complexes were resolved on polyacrylamide gels as above. The wet gels were exposed to XAR-5 film for 1–2 h. The radioactive bands were excised from the gel and incubated overnight in 0.1 mM EDTA, 0.1% SDS, and 0.5 M ammonium acetate. The acrylamide was removed and the DNA fragments recovered by ethanol precipitation. The DNA was resuspended in 90% formamide and 10 mM NaOH, heated to 90°C for 10 min and loaded on a 7% sequencing gel.

Protein Blotting

Protein blotting experiments were carried out as described (28) except that nuclei were lysed directly in SDS-containing sample buffer. DNA was sheared by pipetting and the sample directly loaded on the polyacrylamide gel.

Results

The TR Gene Promoter

The TR gene is 33-kbp long and consists of at least 19 exons (20, 26). Less than 200 bp 5′ of the first exon are required for the human gene to be expressed in mouse Ltk$^-$ cells (20). This suggested that promoter elements were contained within this short DNA sequence. We have subcloned and sequenced a 365-bp sequence that contains 115 nucleotides up-

A

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  1  GATCTTGTCA  AGCACCTCGC  GAGCGTACGT  GCCTCAGGAA  GTGACGCACA  GCCCCCCTGG
 61  GGGCCGGGGG  CGGGGCCAGG  CTATAAACCG  CCGGTTAGGG  GCCGCCATCC  CCTCAGAGCG
121  TCGGGATATC  GGGTGGCGGC  TCGGGACGGA  GGACGCGCTA  GTGTGAGTGC  GGGCTTCTAG
181  AACTACACCG  ACCCTCGTGT  CCTCCCTTCA  TCCTGCGGGG  CTGGCTGGAG  CGGCCGCTCC
241  GGTGCTGTCC  AGCAGCCATA  GGGAGCCGCA  CGGGGAGCGG  GAAAGCGGTC  GCGGCCCCAG
301  GCGGGGCGGC  CGGGATGGAG  CGGGGCCGCG  AGCCTGTGGG  GAAGGGGCTG  TGGCGGCGCC
361  TCGAG
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B

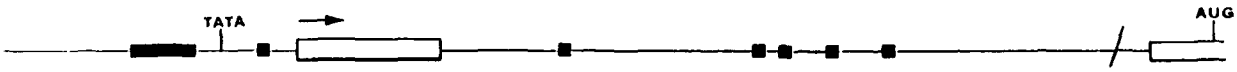


Figure 1. Nucleotide sequence of the 5′ region of human TR gene. (*A*) The sequence of a 365-bp fragment that contains 115 bp of 5′ flanking sequence, an untranslated exon, and part of the first intron. (*B*) A diagram of the relevant features is shown. *Open boxes* indicate exon sequences. *Closed boxes* indicate the GGGGC repeats. The slash indicates the 3′ end of the sequence above.

stream of the mRNA start site, the first exon, and part of the first intron (Fig. 1 *A*). A map illustrating the relevant features of this fragment (TR365) is shown in Fig. 1 *B*. A TATA box is present 27 + 2 bp upstream of the transcriptional start site, deduced from the primer extension experiments of Schneider et al. (36). Upstream of the TATA box is a highly GC-rich region that consists largely of four repeats of the sequence GGGGC. These are arranged as one inverted copy followed by three direct repeats of this sequence. An additional six copies of the sequence GGGGC occur within the TR365 fragment for a total of 10 repeats of this pentamer within the 300 bp surrounding the mRNA start site. In comparison, the sequence GGGGC occurs only once in 5 kb of the TR cDNA, emphasizing the unusually high frequency of this sequence at the 5′ end of the TR gene.

As a first step in defining regulatory elements within the TR365 fragment, we have used the bacterial CAT (chloramphenicol acetyltransferase) gene system to assay for promoter function. The TR365 sequence was cloned in both orientations into the HindIII site of pSVO-CAT (11) to generate the plasmids pTR-CAT-16 and pTR-CAT-18. The plasmids were introduced into Swiss/3T3 cells by calcium phosphate precipitation. Cell extracts were made 48 h later and assayed for CAT activity (Fig. 2). The plasmid pTR-CAT-16, which contains the TR365 fragment in the 5′-3′ orientation with respect to the CAT gene, showed a significant level of expression when compared with the negative control, pSVO-CAT, which lacks a promoter sequence. In the opposite orientation (pTR-CAT-18) TR365 displayed no promoter activity. While the promoter activity of the TR365 sequence is somewhat lower than that of pSV2-CAT, we have observed that placing the SV40 72-bp repeats downstream of the CAT gene in pTR-CAT-16 elevates the expression to a level comparable to pSV2-CAT. These experiments demonstrate that elements within the TR365 sequence display a strong mammalian promoter activity.

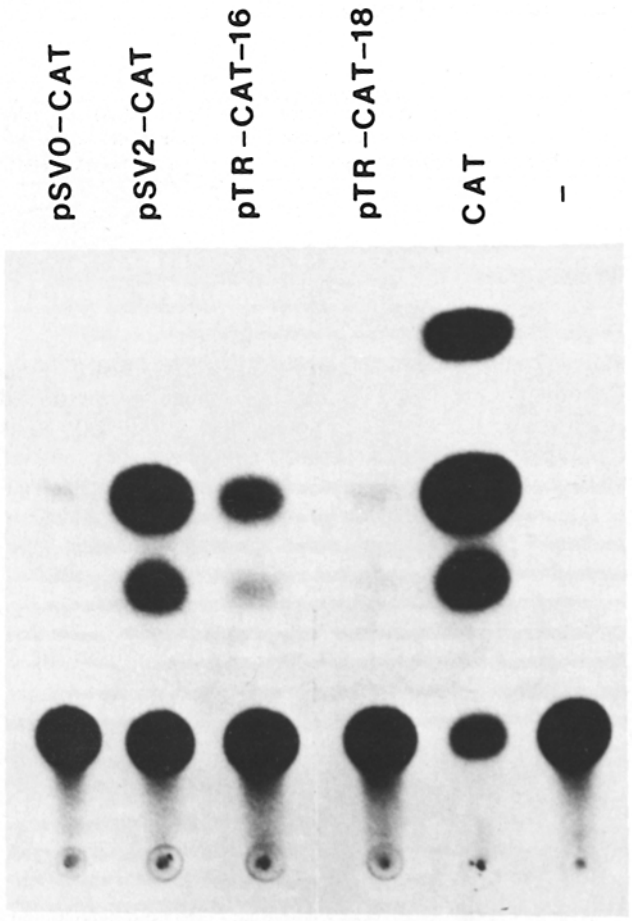


Figure 2. Promoter activity of the 365-bp fragment from the 5′ end of the TR gene. The plasmids indicated above each lane were introduced into Swiss/3T3 cells by calcium phosphate precipitation. The promoter efficiency of each construct was measured by assaying CAT activity 48 h after transfection. pSVO-CAT is a negative control that lacks a promoter sequence 5′ to the CAT gene. pSV2-CAT contains the SV40 early promoter and enhancer sequences (Gorman et al., 1982). pTR-CAT-16 contains the TR 365-bp fragment in the 5′-3′ orientation with respect to the CAT gene, while pTR-CAT-18 contains the same fragment in the opposite orientation. In the lane labeled CAT, the assay contained purified CAT enzyme and (−) indicates [^{14}C]chloramphenicol alone. The origin is at the bottom of the figure.

Sequence Homologies to the TR Promoter

As noted above, the stretch of GC bp just upstream from the TATA box in the TR gene is comprised of four repeats of the sequence GGGGC, three direct and one inverted (Fig. 3). A similar pattern of repeats has been reported in several other genes (32, 41, 29). The human DHFR gene contains a sequence that is homologous with the three direct GGGGC repeats of the TR gene (Fig. 3). Notably, the DHFR sequence is in an identical location to the TR repeats just upstream from the TATA-like sequence. Similarly, the murine interleukin 3 gene contains a homologous GC-rich region that is also just 5′ to the TATA box. In this gene the first three GGGGC repeats going upstream from the TATA box are inverted from those found in the TR gene. A DNA segment (pCaOri7.01) that was isolated from monkey cells by its homology to the SV40 origin region and is known to promote bidirectional transcription of the adjacent genes (34) also has homology to the GGGGC repeats of the TR gene. This DNA fragment does not contain a TATA box but does have three direct GGGGC repeats. In addition, the conserved GC-rich region in each of these genes contains at least one consensus Spl binding site (GGGCGG) as defined by Dynan and Tjian (8).

The biological significance of the conserved sequence described above is unknown. It is significant, however, that in three genes the repeats are located in nearly identical positions in relation to the TATA boxes and the transcriptional initiation sites. In addition, all three genes code for products that are induced by mitogenic stimulation. One hypothesis suggested by these observations is that the conserved GC-rich sequences represent cis-acting regulatory sequences involved in activation of a class of genes that respond to common signals during the progression of cells to S phase.

Protein Binding Sites within the GC-rich Flanking Sequence

To test the hypothesis suggested by the observations described above, we have begun to analyze the DNA-protein interactions within the TR gene promoter (28). The sites of protein binding within this region have been defined by DNase I footprinting. A double-stranded probe was prepared

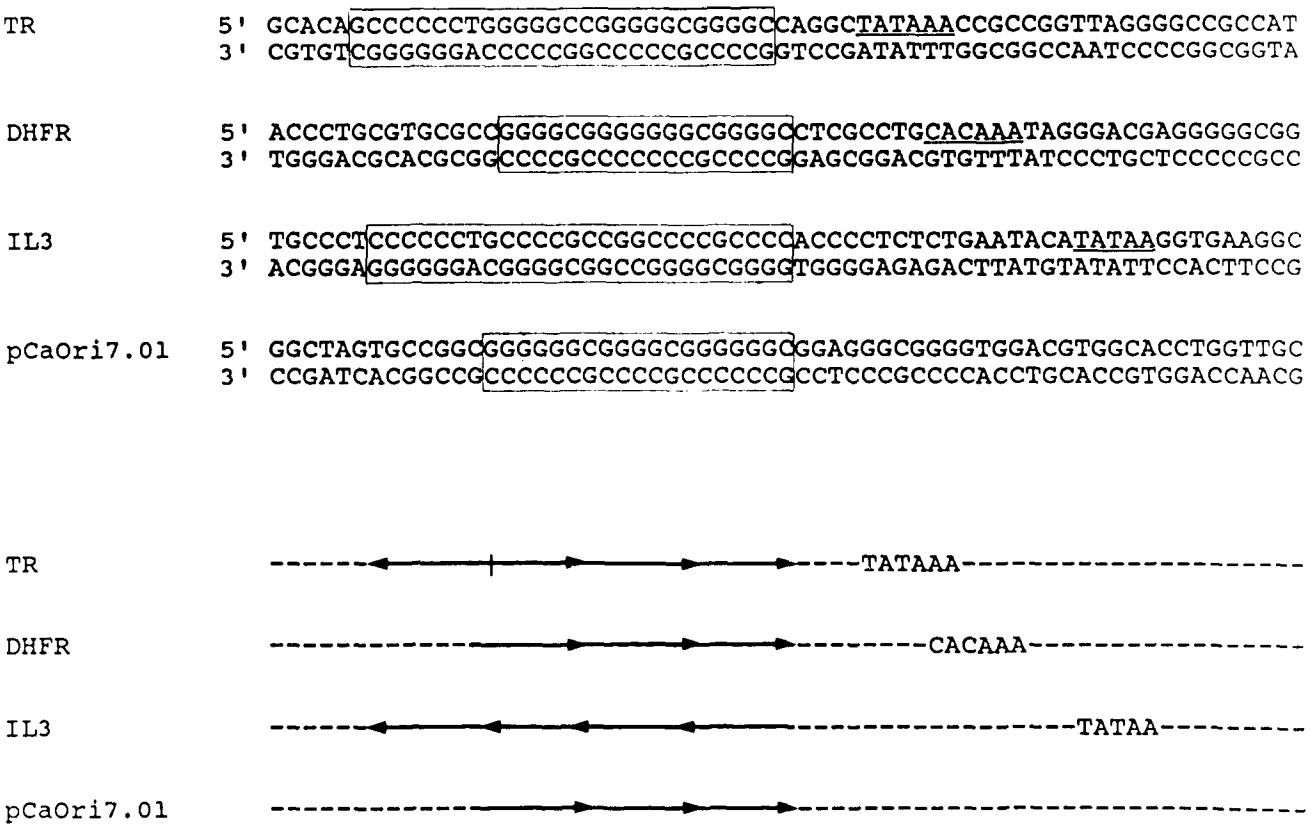


Figure 3. TR promoter has homology to other promoters. The promoter regions of human DHFR, mouse interleukin 3 (IL3), and a monkey promoter from a functionally undefined gene (pCaOri7.01) were found to have GC-rich regions homologous to the TR promoter. The regions of homology are boxed. The TATA boxes are underlined. The diagram at the bottom of the figure shows the direction of the GGGGC repeats relative to the TR sequence.

by cleaving the pTR-CAT-16 plasmid with Taq I, labeling the ends with ^{32}P using the Klenow fragment of polymerase I, cleaving with Ava I, and purifying the labeled fragment by preparative agarose electrophoresis. This procedure resulted in a 140-bp fragment labeled on the 3′ end of the template strand containing the GC-rich region of the promoter and extending 53 bp downstream of the TATA box. Complexes formed between this probe and nuclear proteins from HeLa cells were analyzed by low ionic strength polyacrylamide electrophoresis (10). Complexes of two different mobilities could be detected by this procedure (Fig. 4). At the highest protein concentrations (Fig. 4, lanes *4* and *5*) a protein-DNA complex (complex II) that migrated only a short distance into the acrylamide gel was formed. At lower protein concentrations (Fig. 4, lanes *2* and *3*), however, a complex with intermediate mobility (complex I) was observed. The two complexes observed may represent protein binding to multiple sites within the sequence and is consistent with previous findings (38). Complex I was subjected to further analysis by DNase I footprinting experiments that were carried out using a procedure similar to that described by Carthew et al. (2). The labeled probe was incubated with a crude HeLa cell nuclear extract (5) and then partially digested with DNase I. DNA-protein complexes were separated by electrophoresis on low ionic strength polyacrylamide gels (see Materials and Methods) and detected by autoradiography. DNA fragments were extracted from the gel slices and analyzed on 7% acrylamide sequencing gels. The results are shown in Fig. 5. Two regions of protection are clearly observed. The first region covers 22 bp just upstream of the TATA box. The protein binding to this binding site protects much of the conserved GC-rich region described above. Precisely in the center of this region is a consensus Spl recognition sequence (8). It is therefore likely that protection in this region is due to this protein. Also noteworthy in this experiment is that enhanced cleavage sites on both sides of the protected region are induced by protein binding. The second protected region covers a 22-bp sequence just upstream from the first. This

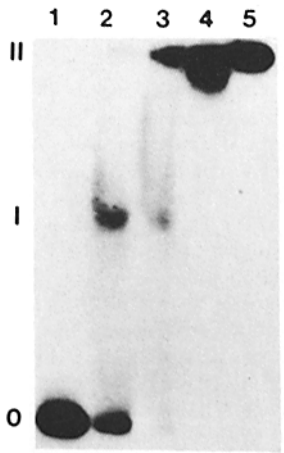
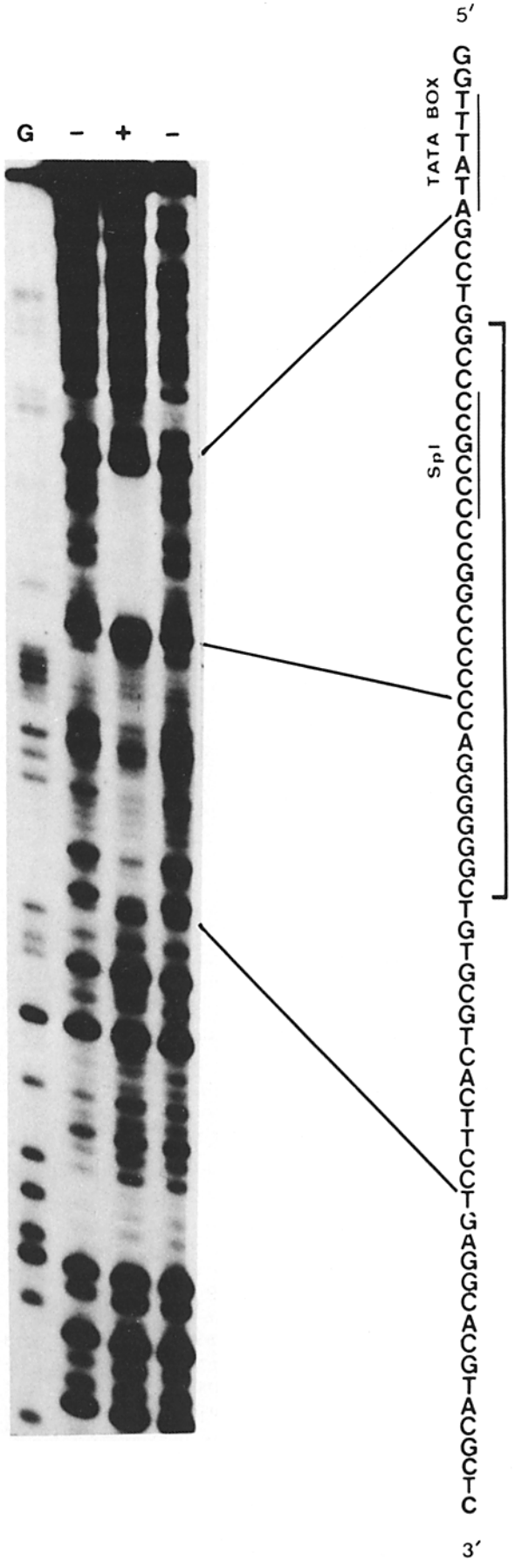


Figure 4. TR promoter DNA-protein complex formation. A 140-bp DNA fragment containing the 5′ flanking region of the TR gene promoter, including the conserved GC-rich sequence and the TATA box, and 25 bp of the first exon was labeled with ^{32}P at the 3′ end of the template strand. Complexes between this fragment and HeLa cell nuclear proteins were analyzed on 3.5% acrylamide gels. Lanes *1–5* contain 0, 1, 3, 5, and 10 μg of nuclear extract proteins, respectively. The position of unbound DNA probe is marked by (0).



sequence contains the remainder of the conserved GC-rich region and extends some distance upstream as well. Again, enhanced cleavage sites flank the protein binding site. This protected site has the sequence GCTGTGCGTC at its center. A homologous sequence is found in a similar location in the human DHFR promoter (see Fig. 8 and Discussion).

Mitogen-dependent Stimulation of Proteins that Interact with the TR Promoter

It has been shown that the level of cell surface TR is greatly increased in rapidly proliferating cells (21, 40). This increase in surface receptors coincides with an increase in mRNA, which codes for the receptor (31, 4), suggesting that receptor levels are under transcriptional or posttranscriptional control. There is increasing evidence linking transcriptional control in eukaryotes with sequence-specific trans-acting DNA binding proteins. We have identified several high molecular weight proteins that interact with the 365-bp fragment that contains the TR gene promoter (28). These proteins bind with high affinity and specificity to this DNA fragment. The in vivo significance of these protein-DNA interactions is at present unknown. However, if they are involved in transcriptional control of the receptor gene, their activity may be modulated by mitogen stimulation of cell growth. We have tested this idea using Swiss/3T3 mouse fibroblasts. These cells were allowed to become quiescent by growing to confluence in 5% FCS followed by a 5-d incubation without a change of medium. The quiescent cells were then stimulated to grow by changing to medium containing 15% FCS. At the times indicated, nuclei were isolated and DNA-protein binding to the TR gene promoter was studied as previously described (28). This method involves separating the proteins by SDS-polyacrylamide electrophoresis, transferring the proteins to nitrocellulose, and incubating the immobilized proteins with specific labeled DNA probes. Proteins of apparent molecular masses of 120, 105, and 95 kD are detected at significant levels in the quiescent cells (Fig. 6). Each of these proteins is induced to a higher level by serum stimulation, all reaching a maximum DNA binding activity at ~3 h after increasing the serum concentration. Between 3 and 12 h poststimulation, there is a very rapid drop in the level of these three proteins, with almost no detectable activity beyond 18 h.

The binding activity of an 88-kD protein is quite different. Almost no binding to the TR365 probe is observed in the nuclei of quiescent cells. Upon serum stimulation, the activity is rapidly induced and reaches a maximum after ~6 h. Again, between 6 and 18 h after increasing the serum concentration there is a rapid decline in the level of this protein.

Fig. 7 compares the effect of serum stimulation on the 88-

Figure 5. Footprint analysis. DNA-protein complexes were treated with DNase I and separated on 3.5% acrylamide gels as in Fig. 4. Complex I was excised from the gel and the DNA fragments eluted and analyzed on 7% acrylamide–8.3 M urea sequencing gels. The lanes are (*G*) Maxam/Gilbert DNA sequence marker; (−) no protein added; (+) 7.5 μg HeLa nuclear extract protein added. The protected sequences are shown at the right. Brackets mark the GC-rich conserved sequence (Fig. 3). The TATA box and consensus Spl binding sequences are indicated. The arrows indicate DNase-sensitive sites induced by protein binding. Note that the sequence shown is the template strand and therefore complimentary to the sequence shown in Fig. 1.

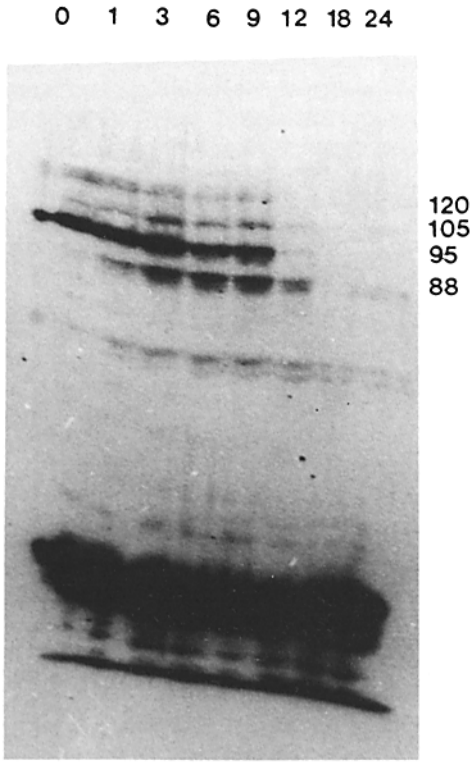


Figure 6. Serum stimulation of nuclear proteins that interact with the TR gene promoter. Quiescent Swiss/3T3 cells were stimulated with 15% FCS. At the times (h) indicated above, each lane nuclear proteins were extracted and fractionated by SDS PAGE. The proteins were electrophoretically transferred to nitrocellulose and incubated with the ^{32}P-labeled TR promoter as described (Miskimins et al. [28]).

kD nuclear protein to induction of cytoplasmic mRNA, which codes for the TR and the induction of DNA synthesis. DNA synthesis begins ~12 h after increasing the serum concentration to 15% and reaches a maximum at 24 h. TR mRNA is present at a low level in the cytoplasm of quiescent cells and increases severalfold upon stimulation, reaching a maximum near the onset of DNA synthesis. This result is consistent with previous reports (31, 4). The activity of the 88-kD nuclear protein that interacts with the TR gene promoter reaches a maximum just before the increase in cytoplasmic mRNA.

Discussion

The human TR gene is one of many genes that have highly GC-rich promoter regions. This group includes the genes for DHFR, arginosuccinate synthetase, adenosine deaminase, HPRT, HMG-coenzyme A reductase, metallothionein, interleukin 3, type II procollagen, epidermal growth factor receptor, APRT, superoxide dismutase, and others. This disparate group of genes has no obvious relationship in terms of expression patterns or function of their products. Many of these genes are expressed in multiple cell types and often lack TATA and CAAT boxes in the GC-rich 5′ flanking regions. It has been suggested that promoters with this structure allow constitutive, low level expression of the gene products (27, 33). A subset of genes with GC-rich promoters, including the TR gene, code for products that are expressed at increased levels during the transition from quiescence to active cell growth. We have shown here that several of the promoters for these mitogen-responsive genes contain common sequence motifs, suggesting a common mode of regulation. Although we have not yet analyzed the transcriptional rates of the TR gene, we and others (31, 4) have observed an increase in TR message after mitogen stimulation. A similar increase in message for several other gene products that are required for entry into S phase of the cell cycle has been observed (35, 9, 3, 24, 16). Increased levels of DHFR mRNA in serum-stimulated cells is due in part to enhanced transcriptional levels (35, 9), and the DHFR promoter has homology to the TR promoter. We have therefore begun an analysis of the factors that control expression of the TR gene and other coordinately regulated genes.

A common feature of these GC-rich promoters is the presence of the hexanucleotide GGGCGG, often in multiple copies. This sequence is the recognition site for the HeLa cell transcription factor Spl (8). This protein has been shown to enhance transcription in vitro from several promoters, including the SV40 early promoter (8) and the HSV-thymidine kinase promoter (18). A single copy of this hexanucleotide is present upstream of the TATA box in the TR gene promoter. It is found within the GC-rich region that is conserved in the TR, DHFR, and interleukin 3 genes (see Fig. 3). We have observed a strong protein binding site within this region using DNase I protection assays. The Spl consensus sequence is precisely in the center of this binding site and it is most likely that protection is due to Spl binding. It has been shown that Spl does not bind tightly to every GGGCGG sequence and that the affinity is enhanced by flanking sequences on both sides of this hexanucleotide. The best binding sites for Spl are the sequences G_4CG_4C or TG_3CG_4C (19). The high affinity site G_4CG_4C is found in the protected region of the TR promoter, strengthening the conclusion that the binding we observe is due to Spl. Furthermore, Spl binding to an analagous sequence in the mouse DHFR gene and to the monkey promoter in pCaOri7.01 has also been demonstrated (7, 6).

We have observed a second protein binding site, just upstream of the consensus Spl binding site, that overlaps the conserved GC-rich region. In the core of this protected region the sequence GCTGTGCGTC is found. A related sequence is found in a similar position in the human DHFR gene, although in the opposite orientation (Fig. 8). Eight of nine nucleotides in the DHFR match the sequence of this TR promoter protein binding site:

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5′ GCTGTGCGPyC  TR
   | |  | | | |  |
5′ GC-GTGCGPyC  DHFR
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We can speculate that the protein that binds to this site may interact with Spl to modulate the transcriptional activity of the TR and other genes (such as DHFR) that are coordinately regulated. The promoters of these genes may function in a manner analagous to the HSV-thymidine kinase promoter, which requires, in addition to Spl, a second factor (CTF) for optimal activity (18). Alternatively, it is possible that Spl allows constitutive, low level expression of the TR gene in most cells, while the additional promoter binding proteins stimulate increased production of TR in proliferating cells.

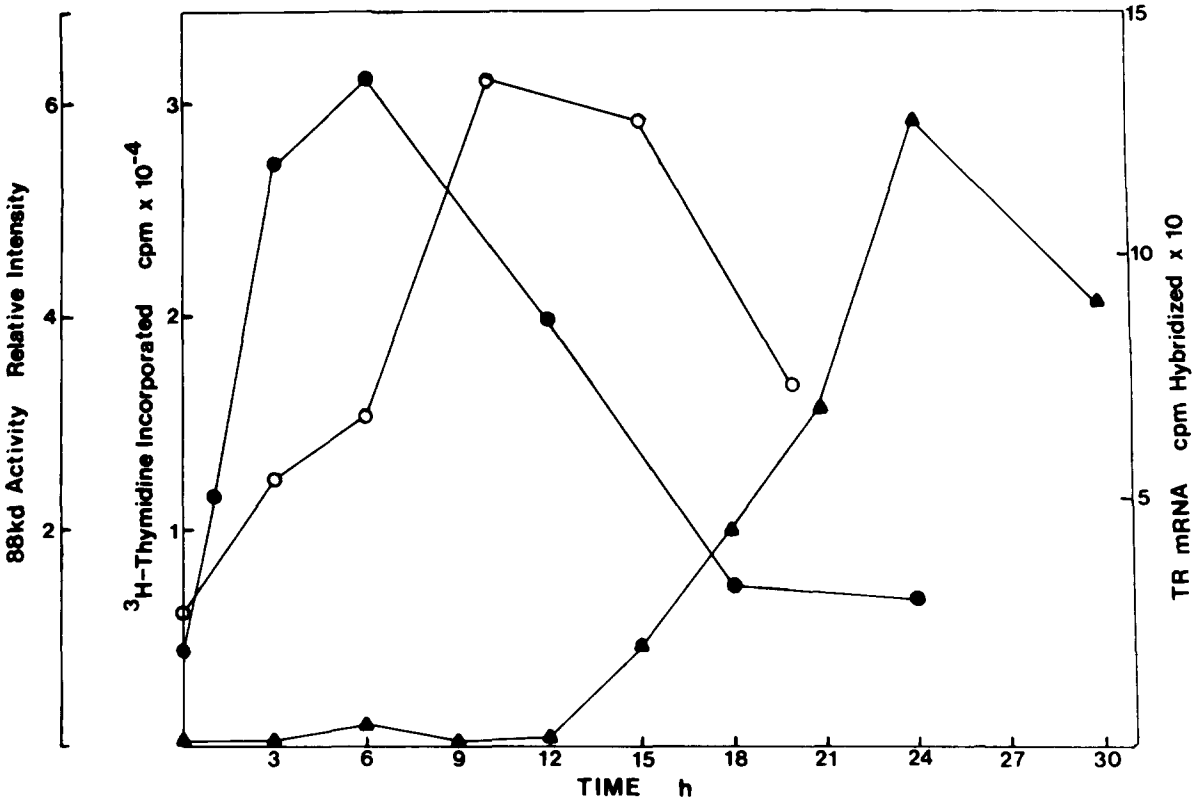

Figure 7. Stimulation of DNA synthesis, TR mRNA, and 88-kD protein by serum. Quiescent Swiss/3T3 cells were induced to undergo mitosis by raising the serum concentration in the medium to 15%. DNA synthesis (*solid triangle*) was monitored by assaying the incorporation of [^{3}H]thymidine into TCA-insoluble material. The level of TR mRNA (*open circle*) was determined using dot blot hybridization as described by Bresser et al. (1). The binding activity of the 88-kD protein (*solid circle*; see Fig. 6) was quantitated by scanning the autoradiograph with a densitometer.

In preliminary experiments we have identified two additional protein binding sites in the TR promoter immediately upstream of the two sites described above (data not shown). The multiple sites of protein interaction suggest a complex mechanism for regulation of TR expression. Purification of the proteins involved and demonstration of their effects on transcription in vitro and in vivo will be required to fully understand their involvement in TR gene expression and cell proliferation.

Using a protein blotting procedure, we have identified several proteins that appear to bind strongly to the TR promoter (28). It is unknown at present whether any of these proteins recognize the specific sequences described above or if they have any physiological relevance to TR gene expression. Jones et al. (17) have recently demonstrated that Spl has an apparent M_r of 95 kD on SDS-polyacrylamide gels. It is possible therefore, that the 95-kD protein that we detect by protein blotting is Spl. It is also intriguing that the binding activity of one of the proteins (88 kD) detected by this procedure is induced by mitogen action, as is the TR itself. The level of binding activity of this protein parallels the increase in TR mRNA after serum stimulation of 3T3 cells (Fig. 7). It will be of interest to further characterize this protein in order to establish whether or not it plays a role in TR expression or has any significance in cell growth control.

The observation that there is conserved sequence homology in the promoters of the TR gene and several other mitogen-responsive genes, along with the demonstration that these conserved sequences serve as sites for protein interactions in the TR promoter, suggests a mechanism for coor-

Figure 8. Comparison of protein binding sites in the TR gene promoter with the DHFR promoter sequence.

dinate gene expression during cell growth. As more genes within this class are cloned and sequenced, and as the proteins involved are purified and characterized, it will be possible to systematically test this hypothesis and its implications for control of cell growth.

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