Induction of Epidermal Growth Factor Receptor Gene Transcription by Transforming Growth Factor β1: Association with Loss of Protein Binding to a Negative Regulatory Element

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Abstract

Transforming growth factor β (TGF-β) is a potent modulator of cell growth in many systems. In normal rat kidney fibroblasts, TGF-β1 increases epidermal growth factor (EGF) receptor gene transcription and synergizes with EGF to stimulate growth in soft agar, a characteristic of the transformed phenotype. In order to identify the target of TGF-β1 action, we have used a series of 5′ deletion mutants of the EGF receptor promoter linked to a chloramphenicol acetyltransferase reporter gene (ERCAT). The TGF-β response element(s) was localized to a cis-regulatory region which resides between positions −919 and −860 relative to the ATG translation initiation codon of the EGF receptor promoter. This 60-base pair region contains a repressor of the EGF receptor promoter and a TGF-β inhibitory element that mediates TGF-β1 suppression of trans/in/stromelysin gene transcription through binding of a CAGATG sequence complex. Cotransfection of c-fos, c-jun, or both expression vectors with the intact or 5′−deleted ERCA constructs identified several EGF-responsive inhibitory regions within the EGF receptor promoter, but these did not localize to the −919 to −860 promoter region. Mobility shift assays showed binding of the 60-base pair DNA fragment to proteins in extracts from untreated normal rat kidney cells; the binding was specifically competed by oligonucleotides containing a CAGATG sequence but not by oligonucleotides containing the EGF receptor repressor or the TGF-β inhibitory element. TGF-β1 treatment but not anti-Fos antibody caused a decrease in specific 60-base pair DNA-protein complex formation. These results provide evidence for a model in which TGF-β1 stimulates EGF receptor gene transcription through a Fos-independent mechanism involving loss of binding of proteins to the CAGATG element in the EGF receptor gene promoter.

Introduction

TGF-β, a major constituent of platelets, is a modulator of growth and differentiation in a number of diverse systems (1–3). First studied as a component of sarcoma growth factor, an activity isolated from the conditioned media of sarcoma virus-transformed cells (4), TGF-β was shown to act synergistically with TGF-α as an autocrine stimulator of the growth of these transformed cells. Both factors also enable NRK fibroblasts to grow in soft agar, a characteristic of the transformed phenotype (4, 5). TGF-α is now known to be a functional analogue of EGF, mediating its effects through the EGF receptor (reviewed in Ref. 6). The predominant form of TGF-β1 consists of two identical M, 12,000 subunits linked by disulfide bonds. Other closely related forms of TGF-β with similar biological activity have also been isolated (1).

TGF-β exerts effects on cells through interactions with specific cell surface receptors. Three types of receptors that specifically bind TGF-β with high affinity have been identified (7). They have apparent molecular weights of 55,000, 80,000, and 280,000, respectively, and are found on many cell types including NRK fibroblasts. The TGF-β type II receptor has recently been cloned and found to be a serine/threonine kinase, which implicates phosphorylation as an important mediator of TGF-β signaling (8).

The molecular mechanism of action of TGF-β subsequent to receptor binding remains largely uncharacterized. One effect of TGF-β common to a number of phenotypically diverse systems is its ability to stimulate synthesis of extracellular matrix components, notably collagen and fibronectin (9, 10). Another consequence of TGF-β action that is more cell type specific is its stimulation of EGF receptor gene transcription (11). It is likely that both of these effects contribute to the ability of TGF-β to synergize with EGF in promoting mitogenesis and soft agar growth of NRK cells (9).

In this study, we demonstrate that a cis-acting regulatory element (between −919 and −860 relative to the ATG translation initiation codon) in the EGF receptor promoter is responsible for TGF-β1 induction of the EGF receptor in NRK cells. This region contains a negative cis-acting regulator of EGF receptor gene transcription and binds trans-acting factors. We have recently localized a negative regulator of the EGF receptor promoter to an 8-bp domain in this region from −877 to −870 in human A431 and HeLa cells (12). This 60-bp region also contains consensus sequences for a TGF-β inhibitory element. TGF-β1 treatment

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3 The abbreviations used are: TGF-β, transforming growth factor β; NRK, normal rat kidney; EGF, epidermal growth factor; bp, base pairs; CAT, chloramphenicol acetyltransferase; kb, kilobase(s); TIE, TGF-β inhibitory element; RB, retinoblastoma; ITS, insulin-transferrin-selenium acid; DMEM, Dulbecco's modified Eagle's medium; TLC, thin-layer chromatography.
as well as competing oligonucleotides which contain a sequence of CAGATC inhibit DNA binding of the specific transacting factor complex. These results suggest a model whereby the loss of transacting factor(s) binding to a represor region of the EGF receptor promoter leads to activation of the EGF receptor gene by TGF-β1.

Results

Regulation of the Human EGF Receptor Gene Promoter in Rat NRK Cells. It has previously been established by nuclear runoff assays that TGF-β1 increases the rate of transcription of the EGF receptor gene in NRK cells (11). To examine the nature of this transcriptional regulation further, we transfected a plasmid containing the EGF receptor promoter linked to the CAT reporter gene into NRK cells. This plasmid, pERCAT1, contains a 5′ proximal 1.1-kb fragment of the human EGF receptor promoter (−1109 to −16 relative to the ATG translation start site) subcloned into the expression vector pSVOCAT (13). As shown in Fig. 1, the human EGF receptor gene promoter is functional in the rat cell line. No activity was observed when the control expression vector pSVOCAT was transfected into NRK cells (data not shown).

Fig. 2. Deletion analysis of the EGF receptor promoter to identify the TGF-β1 responsive element. EGF/CAT constructs (schematically represented in Fig. 1) were transfected into NRK cells and treated with 100 pmol TGF-β1; CAT activities were measured as described in "Materials and Methods." A, the results of one representative experiment are shown here. B, the results represent the means of four independent experiments ± SD.

To investigate the regulation of the human EGF receptor gene promoter in rat NRK cells, we used deletion constructs containing various portions of the 5′ flanking region of the human EGF receptor promoter that were subcloned into the CAT expression vector pSVOCAT. Transfection of these constructs into NRK cells revealed that this promoter contains two negative regulatory elements located at −919 to −860 and −484 to −386 and three positive regulatory elements located at −859 to −779, −385 to −178, and −177 to −105, respectively (Fig. 1). A similar pattern of basal transcriptional activity has been observed following transfection of the same human EGF receptor promoter constructs into human A431, human HeLa, and monkey CV-1 cells (12). These results suggest that the basal regulatory elements in the EGF receptor promoter are similar in human, monkey, and rat cells.

Effect of TGF-β1 on Mutants of the EGF Receptor Promo-"
TGF-β1 Alters the Interaction of Proteins with a Negative Regulatory Element in the EGF Receptor Promoter.

Since a negative regulatory element was identified within the promoter region responsive to TGF-β1 (−919 to −860), we determined whether TGF-β1 treatment alters the interaction of protein(s) with this region by gel-shift analysis. When enriched nuclear extracts from NRK cells were incubated with the end-labeled 60-bp DNA fragment, several retarded species were resolved (Fig. 3). Binding of the labeled DNA probe to the major retarded complexes (denoted B) was significantly inhibited by competition with a 100-fold excess of the unlabeled 60-bp DNA fragment but not by competition with a 100-fold excess of pBR322 DNA fragments. Extensive analysis of binding of the labeled DNA probe to the higher mobility bands suggested that this binding was nonspecific, since in other experiments binding to these bands was either blocked by both unlabeled 60-bp DNA and pBR322 DNA fragments, not completely blocked, or variably inhibited by unlabeled 60-bp DNA. These results suggest that the complex denoted B results from specific binding of the 60-bp fragment.

Binding of the 60-bp probe to the specifically bound protein complexes was altered when protein extracts from TGF-β1-treated cells were used. As shown in Fig. 4, DNA binding to the B complexes was significantly reduced after TGF-β1 treatment of NRK cells. Taken together, these results are consistent with a model in which the transcriptional activation of the EGF receptor gene promoter by TGF-β1 results from the inhibition of a protein that binds a negative regulatory sequence.

A TIE/Fos Protein Complex Does Not Appear to Mediate the Effect of TGF-β1 on the EGF Receptor Promoter. This 60-bp region within the EGF receptor promoter contains the sequence 5′-GAGTTGGTGC-3′, which corresponds to the consensus sequence for the TIE that mediates TGF-β suppression of trans/in/stromelysin gene transcription (14). The TIE (GNTTGGTG) has been reported to act through binding of a Fos-containing protein complex (14). To examine the possibility that a c-Fos protein complex mediates the effect of TGF-β1 on the EGF receptor gene promoter, cotransfected c-fos and c-jun expression vectors separately or together with the pERCAT1 reporter plasmid. As shown in Fig. 5A, cotransfection of pERCAT1 with c-fos, c-jun, or both expression vectors resulted in inhibition of EGF receptor promoter activity. Transfection of c-fos or c-jun alone gave a 2-fold inhibition of CAT activity, whereas transfe-
Fig. 5.  A, inhibition of EGF receptor promoter activity by c-Fos or c-Jun. NRK cells were cotransfected with 4 µg of pERCAT1, 5 µg of pRSVLacZ, and either 16 µg of pCMV vector (control), 16 µg of pCMVFos (Fos), 16 µg of pCMVJun (Jun), or 8 µg of pCMVFos plus 8 µg of pCMVJun (Fos+Jun). CAT activity was measured as described in “Materials and Methods.” Each value represents the mean of four independent experiments; bars, SD. Transfection efficiency was normalized by measuring β-galactosidase activity. B, deletion analysis of EGF receptor promoter to identify Fos/Jun response elements. NRK cells were cotransfected with 4 µg of the indicated EGFR/CAT constructs, 5 µg of pRSVLacZ, and either 16 µg of the pCMV vector or 8 µg of pCMVFos plus 8 µg of pCMVJun. CAT activity was determined as described in “Materials and Methods.” Each value represents the mean of three independent experiments; bars, SD. Transfection efficiency was normalized by measuring β-galactosidase activity.
Fig. 6. Competitive binding analysis of specific nuclear protein complex formation with the 60-bp TGF-β1 responsive element. Protein-DNA complexes were resolved following incubation of the 3'-labeled 60-bp probe DNA (−919 to −450) with NRK cell nuclear extracts by the gel mobility shift assay as described in "Materials and Methods." DNA-protein complexes (B1-B7) and free probe (F) are indicated. Lanes 1, no extract; Lane 2, 5 μg of enriched extract from NRK cells. Lanes 3–10: to the incubation mixture was added a 100-fold excess of oligomer 1 (unlabeled probe; Lane 3); oligomer 2 (Lane 4); oligomer 3 (Lane 5); oligomer 4 (Lane 6); oligomer 5 (Lane 7); or oligomer 6 (Lane 8). Lanes 9–10: to the incubation mixture was added a 1000-fold excess of a c-Myc binding site oligomer 5'-CGAAGCACACCACGTGTCGGCTGTCGGCGACAAAG (Lane 9) or a mutated c-Myc binding site oligomer 5'-CGAAGCACACCACGTGTCGGCTGTCGGCGACAAAG (Lane 10). Lanes 11–13: nuclear extracts were preincubated with anti-SP1 antibody (SP1-Ab, Lane 11); anti-c-Fos antibody (Fos-Ab, Lane 12); or anti-Fos antibody (Fos-Ab, Lane 13) prior to incubation with the DNA. The sequences of oligomers 1–8 are indicated at the bottom of the figure. The underlined sequences refer to the TIE, bHLH, and EGF receptor repressor sequences, respectively (oligomer 1), or to the c-Myc binding consensus sequence (oligomer 7). The boxed sequences indicate mutated bases.

1. GCCGTCTCTGTGACCGGATTGTGCTCTATTCTGACATTTTCCATTTATGCTGTGACAA
2. GCCGTCTCTGTGACCGGATTGTGCTCTATTCTGACATTTTCCATTTATGCTGTGACAA
3. GCCGTCTCTGTGACCGGATTGTGCTCTATTCTGACATTTTCCATTTATGCTGTGACAA
4. GCCGTCTCTGTGACCGGATTGTGCTCTATTCTGACATTTTCCATTTATGCTGTGACAA
5. AGAGTCTATTGCTGGGGCTGGCT
6. CCTATTCTGGCCGAGATGCT
7. Myc site
8. Myc site (Mutant)

The CAGATG Domain Competes with the 60-bp Fragment for Specific Protein Binding. In addition to the TIE, the 60-bp fragment also contains a recently described re-
pressor element (12). To directly assess the role of these elements in the action of TGF-β on EGF receptor transcription, we investigated the interaction of protein(s) with this region by gel-shift analysis.

To identify elements within the 60-bp region that participate in protein-DNA complex formation, we synthesized 60-bp oligonucleotides with 6–8 random bp substituting for nucleotides within the consensus sequences specifying the TIE (oligomer 2), the EGF receptor repressor element (oligomer 3), or the CAGATG sequence (oligomer 4). The latter sequence contains the CA–TG motif shared by bHLH binding protein domains (15). We then determined whether a 100-fold excess of the unlabeled mutant oligonucleotides could effectively compete with the labeled unmutated oligonucleotide for protein binding. As shown in Fig. 6, both of the mutated 60-bp oligomers 2 and 3 competed for binding with a potency comparable to that of the wild-type (Fig. 6, Lanes 2–5). These results suggest that neither the TIE nor the EGF receptor repressor element are required for protein complex formation with the 60-bp fragment. In contrast, mutation of the CAGATG sequence to a random sequence within the 60-bp fragment (oligomer 4) almost entirely eliminated its ability to compete with the wild-type 60-bp fragment for protein binding (Fig. 6, Lane 6). Addition of a 100-fold excess of a 19-mer oligonucleotide that contained the EGF receptor repressor element (oligomer 5) did not inhibit binding of the labeled 60-bp fragment to protein (Fig. 6, Lane 7). Consistent with these results, a 100-fold excess of oligomer 6, which is a 20-mer that contains the CAGATG sequence but lacks the TIE and EGF receptor repressor elements, completely blocked binding of the 60-bp fragment and subsequent complex formation (Fig. 6, Lane 8). Furthermore, a 1000-fold excess of an oligomer that contains a classic binding site for c-Myc, a bHLH protein, efficiently competed for binding of proteins to the 60-bp wild-type oligonucleotides probe (oligomer 7; Fig. 6, Lane 9), but a 1000-fold excess of a mutated c-Myc binding site oligomer had no effect (oligomer 8; Fig. 6, Lane 10). Taken together, these results indicate that neither the TIE nor the EGF receptor repressor element are required for protein interaction with the 60-bp fragment and implicate CAGATG, which contains the consensus sequence for bHLH binding proteins, in the formation of the specific protein-DNA complex.

**Discussion**

Previous studies have shown that TGF-β1 increases transcription of the gene encoding the EGF receptor in NRK fibroblasts (11). In this study, we have used a CAT reporter gene linked to the 1.1-kb region immediately 5′ to the translation start site of the human EGF receptor gene to localize the target of TGF-β1 regulation. Using a series of deletion CAT constructs, we identified a 60-bp region on the promoter from −919 to −860 that is responsive to TGF-β1. This region acts as a transcriptional repressor for the basal promoter. Treatment of cells with TGF-β1 results in loss of specific protein binding to this 60-bp fragment, consistent with a model in which TGF-β1 prevents binding of a repressor(s) to the EGF receptor promoter.

These results also illustrate the evolutionary conservation of the EGF receptor promoter. The proximal 1.1 kb of the human EGF receptor promoter was functional when transcribed into NRK cells. Examination of the activity of a series of deletion CAT constructs indicated that the 1.1-kb EGF receptor promoter contains two negative elements and three positive elements. This same pattern of basal activity has been observed in three other cell types transfected with the same deletion CAT constructs: human epidermal carcinoma (A431) cells, human carcinoma (HeLa) cells, and monkey CV-1 cells (12). These results are generally in agreement with previous studies using CV-1 cells (13, 16, 17), with the exception of the CAT deletion constructs pE7CAT4 and pE7CAT5 that were used to test the effect of deleting the 60-bp fragment. Presumably, these discrepancies reflect differences in experimental technique or cellular conditions rather than species since the 60-bp fragment identified as the target of TGF-β1 action appears to function as a repressor in rat, monkey, and human cells (12).

There are three potential elements within the 60-bp fragment that may respond to TGF-β1 treatment. At the 5′-end is a TIE-like element similar to that described previously for the transin/stromelysin gene (14). In the latter case, evidence suggests that TGF-β suppresses gene transcription through binding of a Fos-containing protein complex. However, our results indicate that Fos expression has no effect on the transcriptional activity of the 60-bp fragment within the EGF receptor promoter. We have also recently identified a repressor element within the EGF receptor 60-bp fragment that is located near the 3′-end (12). Finally, there is another sequence (CAGATG), which is a potential binding site for bHLH proteins (15), in the middle of the 60-bp fragment. To determine which of these sites is responsible for the specific binding of proteins to the 60-bp fragment, we used gel mobility shift assays. Oligonucleotides containing the CAGATG sequence were able to mimic the action of TGF-β by inhibiting complex formation. These results suggest that protein complexes binding to the CAGATG sequence are the target of TGF-β action in NRK cells.

Although several elements within the EGF receptor promoter have been characterized to date, the CAGATG element has not been identified previously. The EGF receptor gene promoter does not have either a "TATA" box or a "CAAT" box, but it has multiple "GC" boxes and multiple transcription initiation sites (18). One EGF receptor-specific transcription factor has been partially purified and characterized. EGF receptor-specific transcription factor binds to an element located at position −248 to −233 (bp) relative to the AUG translation initiation codon (17), stimulates in vitro transcription of the EGF receptor promoter 5- to 10-fold, and acts on promoters that lack TATA elements (19, 20). Another factor termed GCB binds to G-C-rich DNA sequences in the EGF receptor promoter and represses transcription (21). This M, 91,000 protein binds strongly to two upstream regions of the EGF receptor promoter between −270 and −225 bp and weakly to regions between −150 to −90 bp. Stimulation of the EGF receptor promoter by EGF, phorbol 12-myristate 13-acetate, and cyclic AMP is mediated by a 36-bp proximal element (−112 to −77) that acts in an orientation-independent manner (22). In contrast to transcriptional elements in most promoters, most of the elements described above are located downstream of the major in vivo transcription initiation site within the EGF receptor promoter (18). The results presented here identify a new domain upstream of the transcription initiation site as a target for TGF-β action and presumably EGF receptor promoter regulation in NRK cells. Since this sequence (CAGATG) shares the CA–TG binding motif of the bHLH family of proteins, the protein that binds this sequence may be a member of the bHLH family.
The activation of the EGF receptor gene in NRK cells by TGF-β1 has physiological importance in this cell system. TGF-β in association with EGF can stimulate NRK cell anchorage-independent growth, a phenotype of cell transformation (5). This effect is cell type specific and presumably reflects the nature of the protein complexes formed with the 60-bp fragment within the EGF receptor promoter. One possible explanation for the cell type specificity is differences in promoter elements and binding proteins. In NRK cells, we have shown that specific complex formation results from protein binding to the CAGATG element; in other cells such as CV-1 cells and HeLa cells, complex formation in this region results from protein binding to another repressor element (12).

There is considerable precedent for cell type specificity in TGF-β signaling. Opposing physiological responses to TGF-β, such as growth inhibition versus stimulation or induction of one gene but repression of another, have been observed between different cell types and even within the same cell. Thus, it is likely that there are multiple mechanisms for TGF-β regulation of gene expression. Piettenpol et al. (23) have presented results indicating that the inhibition of c-myc gene expression is an important step in the inhibition of skin keratinocyte proliferation by TGF-β. In this case, the block in c-myc expression by TGF-β1 occurred at the level of transcriptional initiation. A cis-acting element (TCE) in the 5′ regulatory region of c-myc has been identified which mediates TGF-β1 and RB suppression of c-myc (24, 25). Kerr et al. (14) have shown that TGF-β1 suppression of transin/stromelysin gene transcription is mediated through binding a Fos-containing protein complex to the TIE. Robbins et al. (26) have shown that RB negatively regulates c-fos expression in fibroblastic cells and have identified a cis-acting element (termed the retinoblas-toma control element) in the promoter region of c-fos that mediates this response. The c-myc promoter region known to be necessary for transcriptional inhibition by TGF-β1 also contains a response element similar to the RB control element. These results implicate RB in TGF-β-mediated inhibition of gene transcription of proteins such as Myc and Fos, which are required for cell growth.

For TGF-β stimulation of growth, other mechanisms for gene regulation have been described. TGF-β1 increases the steady-state RNA levels of several extracellular matrix components such as types I, III, and V collagen and fibronectin in fibroblastic cells (9, 10). A nuclear factor 1 binding site has been shown to mediate the transcriptional activation of a type I collagen promoter by TGF-β1 (27). TGF-β1 also induces expression of its own promoter, which is mediated by an AP-1 complex (28). In the EGF receptor promoter, no nuclear factor type 1 or AP-1 binding motifs are present, and there is no evidence that TGF-β stimulates binding of an activating protein complex in NRK cells.

Our results suggest a model for the action of TGF-β1 on the EGF receptor promoter in NRK cells. TGF-β1 may stimulate modification of a bHLH protein-containing transcriptional repressor, possibly by phosphorylation, thereby down-regulating its binding and resulting in activation of EGF receptor gene transcription. It is likely that a similar mechanism could apply to other targets of TGF-β1 activation as well.

Materials and Methods

Enzymes and Reagents. Restriction enzymes and the Kle- no fragment were purchased from either Life Technologies, Inc. or New England Biolabs. O-Nitro-phenyl-β-D-galactopyranoside, acetyl coenzyme A, and other general chemicals were obtained from Sigma Chemical Co. Poly(dI-dC) was obtained from Pharmacia. [14C]Chlor-amphenicol was purchased from Amersham. ITS-premix and EGF were obtained from Collaborative Research. Porcine TGF-β1 was provided by R & D systems, Inc. Media and reagents for tissue culture were obtained from Life Technologies, Inc.. The c-Fos antibody, SP1 antibody, EGR1 antibody, and Myc binding site oligomer were from Santa Cruz Biotechnology, Inc.

Plasmids. EGF receptor/CAT expression vectors pERCAT1, pERCAT4 through pERCAT10, and pERCAT15 were constructed as described (13). c-fos and c-jun expression vectors pCMVFos and pCMVJun were constructed as described (29). pRSVLacZ was constructed as described (30).

Cell Culture and Transfection. NRK cells were grown in DMEM supplemented with 10% calf serum and penicillin-streptomycin. Cells were incubated in a humidified 37°C-5% CO2 incubator. The CaPO4 precipitation method was used for transfection into NRK cells (31). Briefly, 5 × 10⁶ cells were plated in a 100-mm tissue culture dish overnight. CAT construct (20 μg) and 5 μg pRSVLacZ were coprecipitated and added to the cells. After 20 min at room temperature, the cells were incubated for 4 h at 37°C. Cells were then washed twice with phosphate-buffered saline before fresh medium was added, and cells were incubated at 37°C for 36 to 48 h.

Transfection and Treatment. In experiments involving multiple treatment conditions, a modified DEAE-dextran procedure was used for transfection (32). Briefly, 5 × 10⁵ cells were plated in a 100-mm tissue culture dish overnight. Just before transfection, the cells were washed twice with phosphate-buffered saline. CAT construct (16 μg) was mixed with the DEAE-dextran solution and added to the cells. After 15 min at room temperature, the cells were supplemented with DMEM medium containing 10% calf serum plus 100 μm chloroquine and incubated for 4 h at 37°C, followed by a glycerol shock treatment for 1 min. The transfected cells were removed by trypsinization, pooled, and subcultured in DMEM containing 10% calf serum for 24 h. Cell culture medium was changed to DMEM containing ITS and 1 mg/ml bovine serum albumin for another 24 h. Following treatment, cells were collected after an additional 24-h incubation. CAT assays were done as described below using the same protein concentrations.

Cotransfection Experiments. CAT construct (4 μg) was cotransfected with 16 μg of the pCMV vector, 16 μg of pCMVFos, 16 μg of pCMVJun, 8 μg of pCMVFos and 8 μg of pCMVJun by the CaPO4 precipitation procedure. pRSV-LacZ (5 μg) was also cotransfected in this set of experiments. β-galactosidase and CAT assays were done 36 to 48 h after transfection as described below.

CAT Assay and β-Galactosidase Assay. Cells were harvested with a rubber policeman at 48 h posttransfection, washed with phosphate-buffered saline, and then resuspended in 100 ml of 0.25 M Tris (pH 7.8). Four cycles of freeze-thawing alternating between −80°C and 37°C were used to lyse the cells. Cell debris was spun down in a microcentrifuge, and 100 ml of cell extract was collected. The extract (10 to 20 ml) was tested for β-galactosidase
activity (31) to monitor transfection efficiency. Cell extracts were added to a reaction buffer containing 0.65 ml of 0.1 m phosphate buffer (pH 7.4), 0.05 ml of 30 mm MgCl₂, and 0.05 ml of 3.36 m β-mercaptoethanol. O-nitrophenyl-β-D-galactopyranoside (0.75 ml; 0.13 g in 100 ml of phosphate buffer) was then added, and the reaction mixture was incubated at 37°C until a yellow color was observed. The reaction was stopped with 0.5 ml of 1 M Na₂CO₃, and the absorbance at a wavelength of 410 nm was measured. Normalized quantities of cell extracts were then used for the CAT assay (33). The cell extracts were heated to 65°C for 15 min (34) before they were added to the CAT reaction mixture. Reaction time was adjusted among experiments according to the transfection efficiency but was never more than 4 h. The reaction mixture was extracted with ethyl acetate, dried, and redissolved in ethyl acetate, and the products were separated by silica gel TLC. The TLC plate was then exposed to Kodak XAR5 film. The spots corresponding to the positions of the [14C]chloramphenicol and the acetylated products were cut from the TLC plate and counted in an LKB scintillation counter.

**Protein-DNA Binding Assay.** Gel retardation assays were used to demonstrate the actual physical binding of protein factors to DNA fragments. Nuclear extracts from NRK cells were isolated and enriched on Heparin Sepharose CL-6B as described (35). For experiments involving TGF-β1, confluent cultures of NRK cells were made quiescent by incubation for 1 day in DMEM supplemented with ITS-premix and 1 mg/ml bovine serum albumin and then treated with 100 ng TGF-β1 for 12 h. Nuclear extracts were prepared as described above. The DNA fragment was 3′-end labeled with [α-32P]dATP and Klenow DNA polymerase. An enriched nuclear extract (5 μg) was incubated in the presence of 30,000 cpm (0.2ng) [32P] end-labeled probe and 1 μg poly(dl-dC) in buffer D (25 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.9, 1 mm EDTA, 5 mm dithiothreitol, 150 mm NaCl, and 10% glycerol (v/v)). The specificity of the protein-DNA complex was determined by competition assay in which a 100-fold excess of unlabeled DNA was incubated with the nuclear extract for 10 min before the addition of labeled fragment. Experiments involving antibody binding to DNA-protein complexes were performed by adding antisera (50 μg/ml final concentration) to nuclear extracts and incubating at 4°C for 2 h before addition of the probe. After 20 min of incubation at 22°C, the reaction was stopped with gel buffer containing 0.25% bromophenol, and the sample was loaded onto a native 5% polyacrylamide gel as described previously (36). After electrophoresis, the gel was dried and then exposed to Kodak XAR film.

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**References**


