THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 52, Issue of December 26, pp. 33181–33190, 1997
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Printed in U.S.A.

Transforming Growth Factor-α Enhances Cyclin D1 Transcription through the Binding of Early Growth Response Protein to a *cis*-Regulatory Element in the Cyclin D1 Promoter*

(Received for publication, August 6, 1997)

Yu-Xin Yan‡, Hiroshi Nakagawa‡, Myung-Ho Lee§, and Anil K. Rustgi‡¶‖

From the ‡Gastrointestinal Unit and the ¶Hematology-Oncology Unit, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114 and the §Pulmonary Division, Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02114

Cyclin D1 is a critical oncogene involved in the regulation of progression through the G_1 phase of the cell cycle, thereby contributing to cell proliferation. This is mediated through interaction of cyclin D1 with its catalytic partners, the cyclin-dependent kinases, and the subsequent phosphorylation of the retinoblastoma protein. Cyclin D1, in turn, is regulated by mitogenic stimuli. We demonstrate that transforming growth factor-α (TGFα) induces cyclin D1 mRNA in esophageal squamous epithelial cells, and this appears to correlate with increased cyclin D1 protein expression and cyclin-dependent kinase 6 activity. The induction of cyclin D1 transcription by TGFα is mediated in part through the induction of the early growth response protein (Egr-1) and its subsequent binding of Egr-1 to a *cis*-regulatory region spanning nucleotides −144 to −104 of the cyclin D1 promoter. The Egr-1 binding activity to the cyclin D1 promoter appears to require *de novo* protein synthesis and is not influenced by Sp1 binding to overlapping Sp1 motifs. Taken together, these data provide evidence that TGFα enhances cyclin D1 transcription through the induction of Egr-1 binding to a *cis*-regulatory region in the cyclin D1 promoter. This has important mechanistic implications into the transcriptional regulation of cyclin D1 by an essential proproliferative growth factor and cell cycle progression.

Cyclin D1 is critical in the progression of the cell through G_1 phase. The overexpression of cyclin D1 in cultured cells shortens G_1 phase and causes a more rapid entry into S phase (1, 2). Conversely, microinjection of antisense cyclin D1 oligonucleotide or cyclin D1 antibody in SaoS2 osteosarcoma cells will arrest cells in G_1 phase (3). Antisense cyclin D1 has been shown to inhibit the growth of human colon cancer cells (4). Cell cycle progression through G_1 phase is regulated by the association of cyclin D1 with its catalytic protein partners, the cyclin-dependent kinases (cdks).[1] In particular, cyclin D1 preferentially associates with either cdk4 or cdk6, an association that is prevented by the interaction of cdks with their inhibitors (5, 6). The cdk inhibitors can disrupt cyclin D1-cdk kinase activity. G_1 arrest is associated with the overexpression of the cdk inhibitors. A key cellular target for the cyclin D1·cdk4 or cyclin D1·ckd6 complex is the retinoblastoma tumor suppressor gene product (pRB) (7, 8), and in this context, pRB is progressively phosphorylated, resulting in the release of important transcriptional factors, such as E2F (9, 10).

Aside from the role of cyclin D1 in the normal eukaryotic cell cycle, there is compelling evidence for the contribution of cyclin D1 to cancer development and progression. For example, it is frequently overexpressed in cancers through gene amplification in squamous epithelial cancers (11–15) and chromosomal translocation in parathyroid adenomas (16) and centrocytic lymphomas (17). The oncogenecity of cyclin D1 has been appreciated through its ability to complement a defective adenovirus *E1a* oncogene in the transformation of cultured cells (18) and also to cooperate with *ras* in transforming such cells (19). Furthermore, it induces mammary hyperplasia and carcinoma in lactating transgenic mice (19) and oral-esophageal dysplasia in transgenic mice (20).

An appreciation of the role of cyclin D1 in the cell cycle is possible through an understanding of the regulation of cyclin D1 by growth factors. This regulation is complex, with the involvement not only of predominantly transcriptional mechanisms but also posttranscriptional mechanisms. An initial study examined the effects of serum and growth factors on cyclin D1 (and cyclin D3) expression in normal human diploid fibroblasts (21). Serum supplementation increased the cyclin D1 mRNA level, with a peak at about 12 h prior to onset of S phase. In addition, a number of different growth factors, such as epidermal growth factor, fibroblast growth factor, insulin growth factor, and platelet-derived growth factor increased cyclin D1 mRNA and, correspondingly, DNA synthesis (22). However, cyclin D1 mRNA decreased upon serum depletion, high cell density, and senescence (22).

To study the transcriptional regulation of the cyclin D1 promoter, different groups have cloned the promoter and 5′ and 3′ untranslated regulatory regions of cyclin D1, corresponding to approximately 3 kilobases by one group (23) and approximately 1.8 kilobases by another group (24). A serum inducible region between −944 and −848 has been noted with DNase footprinting showing the critical element between −928 and −921 (23).

* This work was supported by the American Digestive Health Foundation/American Gastroenterology Association Fiterman and Funderburg Awards (to A. K. R.), American Cancer Society Faculty Research Award JFRA-649 (to A. K. R.), National Institutes of Health Grants DK40561 and DK53377 (to A. K. R.), and Department of Energy Award DE-FG-2-91-ER61228 (A. K. R.). Oligonucleotides were synthesized through Center for the Study of Inflammatory Bowel Disease Molecular Biology Core National Institutes of Health Grant DK43351. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed: Gastrointestinal Unit, Jackson 904, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114. Tel.: 617-724-3740; Fax: 617-726-3673; E-mail: rustgi@helix.harvard.mgh.edu.

[1] The abbreviations used are: cdk, cyclin-dependent kinase; pRB, retinoblastoma tumor suppressor gene product; TGFα, transforming growth factor-α; EGR, early growth response; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline.

Furthermore, cotransfection of the cyclin D1 promoter and c-*jun* activates a potential cAMP response element site at −52 (23). It has also been shown that c-*myc* decreases cyclin D1 mRNA in Balb/3T3 fibroblasts, perhaps through the down-regulation of the initiator element in the cyclin D1 promoter similar to the one found in the adenovirus major late promoter, which is bound by USF *in vitro* (25). This is in contrast to the finding that c-*myc* induces cyclin D1 expression in Rat-1 cells, although potential *cis*-regulatory elements within the cyclin D1 promoter were not investigated (26).

Given that cyclin D1 and *ras* cooperate to transform cultured cells and that *ras* results in elevated cyclin D1 levels in NIH 3T3 cells (27), it has been also demonstrated that transforming *ras* mutants induce the cyclin D1 promoter in human trophoblasts (JEG-3), in the mink lung epithelial cell line (Mvl.Lu), and in the Chinese hamster ovary fibroblast cell line (28). Site-directed mutagenesis of AP1 like sequences at −954 abolished the p21-*ras*-dependent activation of cyclin D1 expression. AP1 sequences were also required for activation of the cyclin D1 promoter by c-*jun* (28).

Cyclin D1 is critical in the proliferation and transformation of squamous epithelial cells, especially in the head/neck and esophagus; however, the transcriptional regulation of cyclin D1 by growth factors in this cell type remains to be elucidated. It is also clear that transforming growth factor-α (TGFα), a peptide that binds the epidermal growth factor receptor, is important in these processes, but the ultimate targets for the pro-proliferative effects of TGFα remain to be elucidated in squamous epithelial tissues (29, 30). Linking the effects of TGFα to cyclin D1 transcription would enhance our understanding of mechanisms underlying proliferation in squamous epithelial cells. Therefore, we have examined the effects of TGFα on cyclin D1 mRNA and have linked the increase of cyclin D1 transcription by TGFα in part through the induction of the early growth response (EGR) transcriptional factor binding activity with a *cis*-regulatory element in the cyclin D1 promoter. The physiological effect of this induction appears to be increased cdk6 kinase activity and a shorter G_1 phase.

EXPERIMENTAL PROCEDURES

DNA Plasmid Constructions—A 1884-base pair *Pvu*II fragment (−1749 to +135, designated as Dlpro-1749) contains the promoter and the 5′ and 3′ untranslated regulatory regions of the *PRAD1*/cyclin D1 gene (24). This was subjected to restriction enzyme digestions to make a series of deletion constructs in the promoterless vector, pA3LUC (31), which also contains the firefly luciferase gene. These deletions were generated from the Dlpro-1749 fragment by removing different fragments from the 5′ end using specific restriction enzyme sites followed by blunt end ligation: Dlpro-1095, −1095 to +135 (*Eco*RI/*Pvu*II); Dlpro770, −770 to +135 (*Msc*I/*Pvu*II); Dlpro-558, −558 to +135 (*Pml*I/*Pvu*II); Dlpro-327, −327 to +135 (*Sma*I/*Pvu*II); Dlpro-144, −144 to +135 (*Nar*I/*Pvu*II); and Dlpro-78, −78 to +135 (*Sau*3A1/*Pvu*II). The deletion Dlpro-104 was generated by polymerase chain reaction using custom-designed primers with additional restriction sites for insertion into the pA3LUC vector: sense primer, 5′-CTAGCCCGGGTCCCGCTC-CCATTCT-3′; antisense primer, 5′-CTAGAAGCTTCTGTGGGTCCTG-GCT-3′.

All restriction enzymes were obtained from New England Biolabs (Beverly, MA). Each construct was confirmed by DNA sequencing (Sequenase, version 2.0, United States Biochemicals, Cleveland, OH).

In addition, four pairs of oligonucleotides were designed and synthesized for sense and antisense strands of the cyclin D1 promoter spanning nucleotides −144 to −104: wild type, 5′-CGCCCGCGCCCCCTC-CCCCTGCGCCCGCCCCCGCCCCCC-3′; mutant 1, 5′-CGCCCGCGC-CC**TA**TCCCCCTGCGCCCGCCC**TA**GCCCCCC-3′; mutant 2, 5′-CGCC-CGCGCCC**TA**TCCCCCTGCGCCCGCCCCCGCCCCCC-3′; and mutant 3, 5′-CGCCCGCGCCCCCTCCCCCTGCGCCCGCCC**TA**GCCCCCC-3′.

After annealing, each double-stranded fragment was subcloned into the *Hin*dIII and *Xho*I sites of the pT81 plasmid, which contains a minimal herpes simplex virus thymidine kinase promoter fused to the luciferase reporter gene (32).

Cell Culture and Transient Transfection—The esophageal squamous carcinoma cell lines TE-1 (a gift from T. Nishihira) and T.T (obtained from the Japanese Cancer Resource Bank) were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum (Sigma) and 100 units/ml each of penicillin and streptomycin (Sigma). For preparation of total RNA and nuclear proteins, the cells were serum starved (0.1% serum) for 24 h and treated with or without 50 ng/ml TGFα (R&D Systems, Minneapolis, MN) for different time periods prior to harvesting of cells. As a protein translation inhibitor, 10 μg/ml cycloheximide (Sigma) was used.

Transient transfections by the calcium phosphate method (5 Prime → 3 Prime, Inc., Boulder, CO) were performed in triplicate in 6-well tissue culture plates (Becton Dickinson Co., Lincoln Park, NJ). Equal numbers of cells (3×10^5 cells/ml) were seeded in each well 24 h prior to transfection. After the medium was replaced with fresh medium, the cells were transfected with 2 μg of reporter plasmid containing the different cyclin D1 promoter deletions in 250 μl of transfection buffer. After 12–18 h incubation with the transfection solution, cells were shocked with 15% glycerol buffer (3 volumes of glycerol, 7 volumes of sterile distilled H_2O, and 10 volumes of 2 × DNA precipitation buffer per manufacturer's instructions) for 90 s. The cells were then grown in Dulbecco's modified Eagle's medium containing 0.1% fetal calf serum for 48 h at 37 °C. For TGFα stimulation experiments, cells were washed twice with PBS after glycerol shock, incubated in 0.1% fetal calf serum-Dulbecco's modified Eagle's medium for 24 h, and stimulated with different doses of TGFα, ranging from 50 to 150 ng/ml final concentration. Cells were harvested 48 h after transfection.

Luciferase Assay and Human Growth Hormone Assay—Cells were washed twice with PBS buffer, harvested in 200 μl of cell culture lysis reagent (Promega, Madison, WI), and incubated at room temperature for 15–20 min. The cell lysate was vortexed briefly and centrifuged for 1 min. Then, 40 μl of lysate was mixed with 100 μl of luciferase assay reagent (20 mM Tricine, 1.07 mM $MgCO_3$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μM coenzyme A, 530 μM ATP, and 470 μM luciferin). Assays were carried out in a luminometer (Analytical Luminescence Laboratory). Luciferase activity was expressed in rate of light units. Corrections for transfection efficiency were done by cotransfecting pXGH5 (the mouse metallothionein I promoter fused to the human growth hormone structural gene sequence). This plasmid is included in the human growth hormone transient gene expression assay system (Nichols Institute, San Juan Capistrano, CA), and human growth hormone activity was quantitated according to the manufacturer's instructions. After standardization with human growth hormone activity, a mean luciferase activity (generated from transfections done in triplicate) was obtained, and S.D. was calculated. Transfections were repeated and reproduced in at least three independent experiments. Variation between experiments was not greater than 10%.

RNA Extraction and Northern Blot Analysis—Northern blot analysis was done as described previously (14). Briefly, total RNA was purified by the acid guanidinium thiocyanate-phenol chloroform extraction method. RNA samples (10 μg) were fractionated in a 1% formaldehyde agarose gel and transferred onto a Hybond-N nylon membrane (Amersham Corp.). The UV-cross-linked membrane was hybridized in a rapid hybridization buffer (Amersham Corp.) with a 1.4-kilobase cyclin D1 cDNA random prime labeled with [α-^{32}P]dCTP (NEN Life Science Products) and washed under high stringency conditions: once with 2 × SSC, 0.1% SDS at room temperature for 20 min, once with 2 × SSC, 0.1% SDS at 65 °C for 15 min, and once with 0.1 × SSC, 0.1% SDS at 65 °C for 15 min. The membrane was exposed to X-OMAT-AR film (Eastman Kodak Co.) at −70 °C. Autoradiographic signals were quantitated by scanning (Hewlett-Packard ScanJet IIP) and analyzed with the NIH Image software.

RNA stability was assessed with a transcription inhibitor, dichloro-β-diribofuranosylbenzimidazole (DRB), and Northern blot analysis was performed under identical conditions.

Flow Cytometry—Cells were grown in serum-free medium as described above in the presence or absence of TGFα (50 ng/ml). Cells were collected at various time points (0–24 h) with or without TGFα treatment. Then, cells were centrifuged at 1000 rpm for 4 min. The cell pellet was resuspended in 0.5 ml of PBS, fixed in 5 ml of 70% ethanol, and stored at −20 °C overnight. The cells were then washed twice with PBS and resuspended in 1 ml of a solution containing 3.8 mM sodium citrate and 10 mg/ml propidium iodide. After 10 mg/ml RNase treatment at 37 °C for 20 min, the samples were analyzed by a fluorescence-activated cell sorter (FACScan, Beckton Dickinson).

Western Blot Analysis—Lysates from exponentially growing TE-1 cells with and without TGFα (50 ng/ml) treatment were harvested at various time points (0–24 h) and prepared in a buffer (50 mM HEPES,

pH 7.4, 0.1% Nonidet P-40, and 250 mM NaCl) with 1 mM protease inhibitors (aprotinin, phenylmethanesulfonyl fluoride, leupeptin, and chymostatin) (all from Boehringer Mannheim) and 10 mM phosphatase inhibitors (sodium vanadate and sodium fluoride) (14). 150 mg of total protein of each sample was separated on a 10% SDS-polyacrylamide gel. Following electrophoresis, proteins were transferred to Immobilon membranes (Millipore) at 10 V for 12 h at 4 °C. Blocking was performed in 5% milk, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Tween-20 for 1 h. The primary antibody (cyclin D1 monoclonal antibody HD11, a gift of Ed Harlow) was used at a 1:4 dilution. The secondary antibody was peroxidase conjugated goat anti-mouse immunoglobulin (Cappel; 1:5000 dilution). The detection system was by chemiluminescence (ECL; Amersham Corp.). Autoradiographic signals were quantitated by scanning (Hewlett-Packard ScanJet IIP) and analyzed with the NIH Image software.

Kinase Assay—Conditions were identical to harvesting TE-1 cells with and without TGFα (50 ng/ml), as for Northern and Western blot analyses. For kinase assays, cells were washed with PBS, scraped in 1 ml of kinase buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween-20, 10% glycerol supplemented with 1 mM dithiothreitol, 1 mM protease inhibitors (aprotinin, leupeptin, and chymostatin), and kept on ice for 20 min with occasional vortexing. The cell lysates in the Eppendorf tubes were sonicated on full power two times for 1 min each time and then spun at 14,000 rpm for 15 min at 4 °C. Protein concentration was determined by the Bradford assay. In a total volume of 500 μl of buffer, 150 mg of cell lysate was incubated with 200 ng of either cdk4 or cdk6 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4 °C for at least 1 h. Protein A-Sepharose CL-4B beads (Pharmacia Biotech Inc.) were washed with the buffer (without inhibitors) three times and then resuspended in an equal volume of buffer. Then, 30 μl of mixed slurry beads were added to each tube and rocked at 4 °C for at least 1 h. The beads were rinsed three times with buffer and then twice with 1 × kinase reaction buffer (5 × kinase reaction buffer: 250 mM HEPES, pH 7.2, 50 mM $MgCl_2$, 25 mM $MnCl_2$, 5 mM dithiothreitol). The kinase reaction (total volume 30 μl) contained beads, 1 × kinase reaction buffer, 1 mM ATP, 1 mM dithiothreitol, 10 μCi of [γ-^{32}P]ATP, and double distilled H_2O, along with a carboxyl-terminal fragment of the retinoblastoma protein (pRB) fused to glutathione *S*-transferase (a gift of E. Harlow and P. Hinds) as a substrate. Kinase assays were performed for 45 min at 37 °C with mild vortexing at 10 and 20 min. The reaction was stopped with 30 μl of protein sample buffer and placed on ice. The samples were electrophoresed on a 12.5% SDS-polyacrylamide gel to determine phosphorylation efficiency. The gel was Coomassie-stained, dried, and exposed to X-OMAT-AR film at −70 °C. Autoradiographic signals were quantitated by scanning (Hewlett-Packard ScanJet IIP) and analyzed with the NIH Image software.

Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSAs)—Nuclear extracts from cultured cells were prepared essentially as described by Schreiber *et al.* (33) except that the buffers were supplemented with a mixture of 1 mM protease inhibitors (aprotinin, chymostatin, and pepstatin) (all from Boehringer Mannheim). Protein concentration was determined by the Bradford assay (34). The extracts were frozen in liquid nitrogen and stored at −70 °C until use.

Oligonucleotides were synthesized by the phosphoramidite procedure (Applied Biosystems, Inc.) and purified by gel electrophoresis. To make the DNA oligonucleotide probes, 5 pmol of a double-stranded oligonucleotide was labeled by the Klenow fill-in reaction in a buffer consisting of 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 7.5 mM dithiothreitol, 33 μM dATP, 33 μM dGTP, 33 μM dTTP, 0.33 μM [α-^{32}P] dCTP, 1 unit of DNA polymerase I Klenow fragment (Amersham Corp.) and then purified with a NAP5 column (Pharmacia). Gel EMSAs were carried out by incubating 5 μg of crude nuclear extract with 5 fmol of ^{32}P radioactively labeled oligonucleotide probe (20,000 cpm) in 20 μl of binding reaction containing 10 mM Tris-HCl, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol, and 1.0 μg of poly(dA-dT)/poly(dA-dT) (Pharmacia). After incubation at room temperature for 15 min, samples were loaded onto 4% polyacrylamide, 0.25 × Tris borate gels and electrophoresed at 10 V/cm for 2 h. The gels were dried under vacuum and exposed to X-OMAT-AR film at −70 °C. For competition experiments, the nuclear extract was preincubated with 200-fold molar excess of unlabeled competitor oligonucleotides prior to the addition of the ^{32}P radioactively labeled oligonucleotide probe. The sequences of the oligonucleotides are as follows: cyclin D1 promoter, −140, 5′-CGGCGT-TGGCGCCCGCGCCCCC-3′; cyclin D1 promoter, −130, 5′-CGCCCCC-TCCCCCTGCGCC-3′; cyclin D1 promoter, −110, 5′-GCGCCCGCCCC-CGCCCCCCTCC-3′; Egr wild type, 5′-CGCCCTCGCCCCCGCGCCGG-3′; Egr mutant, 5′-GGATCCAGCTAGGGCGAGCTAGGGCGA-3′; Sp1, 5′-GATCGATCGGGCGGGGCGATC-3′; glucocorticoid response element, 5′-GATCAGAACACAGTGTTCTCTA-3′.

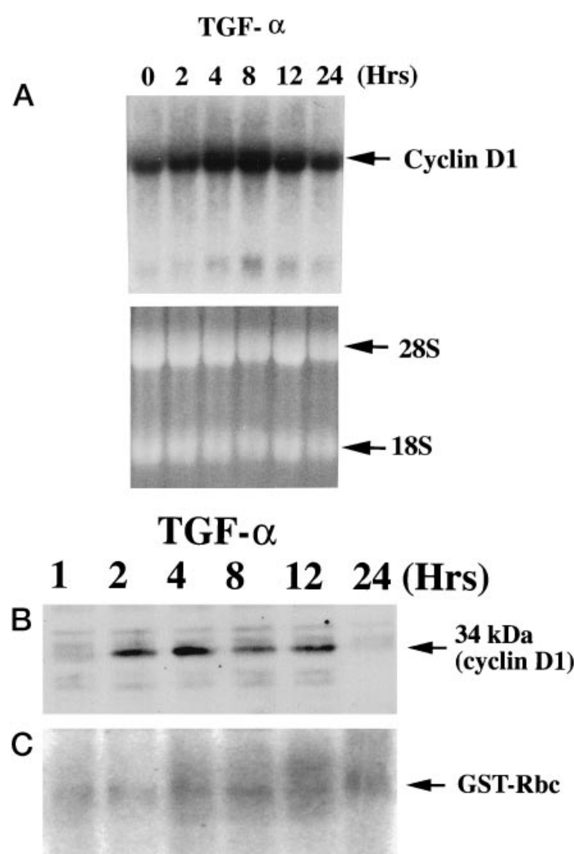


FIG. 1. *A*, Northern blot analysis. RNA was extracted from exponentially growing TE-1 cells stimulated with TGFα and electorphoresed in a 1% formaldehyde agarose gel. After transfer to a Hybond-N membrane and UV cross-linking, the membrane was hybridized with the random-primed ^{32}P radioactively labeled human cyclin D1 cDNA probe, washed under high stringency conditions, and then exposed to x-ray film. Equal loading of RNA was shown by ethidium bromide staining. *B*, Western blot analysis. Total protein was extracted from exponentially growing cells stimulated with TGFα and electrophoresed in a 10% SDS-polyacrylamide gel. After transfer to an Immobilon-P membrane, the membrane was stained with Ponceau S to confirm equal loading of proteins. The membrane was probed with cyclin D1 HD11 antibody, and detection was by chemiluminescence. *C*, kinase assay. Total protein was extracted from exponentially growing TE-1 cells stimulated with TGFα and immunoprecipitated with cdk6 antibody. After a kinase reaction with GST-retinoblastoma carboxyl terminus substrate and [γ-^{32}P]ATP, products were electrophoresed in a 12.5% SDS-polyacrylamide gel.

Immune-supershift assays were performed using affinity-purified polyclonal antibodies, anti-Sp1 (Santa Cruz Biotechnology, Inc.), anti-Egr-1 (a gift of Dr. V. Sukhatme) (35), or anti-AP2 (a gift of Dr. T. Williams) and preincubated with the crude nuclear extract at room temperature for 10 min prior to the addition of the ^{32}P radioactively labeled oligonucleotide probe.

RESULTS

TGFα Induces Cyclin D1 mRNA and Protein with an Increase in cdk6 Kinase Activity—Exponentially growing TE-1 cells were serum starved for 48 h and treated with TGFα (50 ng/ml), and RNA was harvested at different time points after treatment. Northern blot analysis showed that cyclin D1 mRNA (4.7-kilobase transcript) was induced 3-fold above basal level (quantitated by laser densitometry), and this induction was evident by 4 h after treatment (Fig. 1*A*). The response was sustained until about 8 h after treatment, and then the cyclin D1 mRNA level returned to its basal level. A similar effect on cyclin D1 mRNA was seen after treatment of TE-1 cells with epidermal growth factor (data not shown). However, no effect

TABLE I
FACScan analysis of TE-1 cells with and without TGFα stimulation

Time	% G_1		% S		% G_2/M	
	TGFα −	TGFα +	TGFα −	TGFα +	TGFα −	TGFα +
h						
0	64.9	64.8	15.8	14.5	19.3	20.7
2	67.2	66.8	15.5	13.1	17.2	20.0
4	70.4	66.2	11.3	13.4	18.4	20.4
8	64.8	59.8	15.7	15.9	19.5	24.3
12	66.3	55.8	21.1	25.6	12.6	18.6
24	63.0	47.6	19.6	22.5	17.4	29.8

on cyclin D1 was evident when cells were maintained in serum-free medium (data not shown). It should be emphasized that the effect of TGFα on cyclin D1 mRNA is consistent with the level of cyclin D1 mRNA induction observed with other growth factors, such as basic fibroblast growth factor, platelet-derived growth factor, and insulin growth factor 1 (22, 36–38).

To determine the relationship of cyclin D1 protein expression to cyclin D1 mRNA expression after TGFα stimulation, Western blot analysis was performed (Fig. 1*B*). This demonstrated that cyclin D1 protein was induced 3-fold (based upon laser densitometry) with TGFα stimulation, peaking at 4 h after treatment and consistent with the time period of mRNA induction. The cyclin D1 protein is not induced under serum-free conditions (data not shown). It should be noted, however, that the protein was not detectable at time point 24 h, a time point well beyond the induction of the mRNA. It is possible, given the known short $t_{1/2}$ of cyclin D1 protein, that it is degraded through proteolysis and TGFα depletion (Ref. 39 and see "Discussion").

We next tested whether the increase in cyclin D1 RNA and protein correlated with an effect on cdk4 and/or cdk6 kinase activity and ultimately on progression through the G_1 phase. Fig. 1*C* reveals that there was a 2-fold induction (based upon laser densitometry) in cdk6 activity by 8 h after treatment as measured by phosphorylation of the pRB substrate (GST-retinoblastoma carboxyl terminus substrate), although the overall magnitude of the cdk6 kinase activity was low. There was no increase in cdk4 kinase activity (based upon laser densitometry; data not shown). We would emphasize that these results are consistent with a report of preferential cdk6 kinase activation in oral squamous carcinoma cell lines but not cdk4 kinase activity (Ref. 40 and see "Discussion"). Moreover, the magnitude of cdk6 kinase activity is similar, perhaps underscoring the common biological properties of oral and esophageal squamous cancer cell lines.

Recognizing that a significant effect of increased cyclin D1 overexpression is a shortened G_1 phase, we performed flow cytometric analysis on cells with and without TGFα stimulation. As Table I shows, there is indeed a shortened G_1 interval and more cells entering S phase by 12 h after treatment. This is consistent with other reports of cyclin D1 overexpression on cell cycle kinetics (1, 41). Of note, there are also more cells in G_2/M by 24 h as a function of TGFα stimulation, which may also help to explain undetectable cyclin D1 protein on the Western blot because of proteolysis in the setting of mitogen depletion (40).

To help determine whether the effects of TGFα on cyclin D1 mRNA are related to mRNA stability *versus* transcriptional mechanisms, we treated exponentially growing TE-1 cells with dichloro-β-diribofuranosylbenzimidazole (25 μg/ml), an inhibitor of transcription, for 30 min (42, 43). TGFα (50 ng/ml) was then added, and RNA was extracted over the next 24 h for Northern blot analysis. Fig. 2 shows that in the presence of dichloro-β-diribofuranosylbenzimidazole, cyclin D1 mRNA was stable up to 4 h after treatment, and then it decreased by 50% at 4 h (based upon laser densitometry). These data suggest that

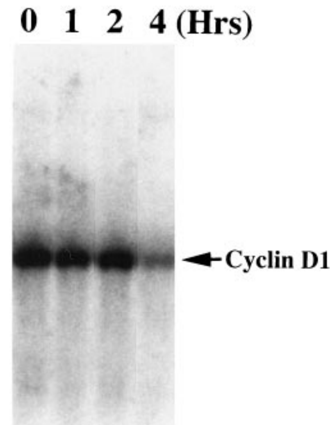


FIG. 2. **Northern blot analysis.** After dichloro-β-diribofuranosylbenzimidazole (an inhibitor of transcription) treatment of TE-1 cells for 30 min, TE-1 cells were stimulated with TGFα, and RNA was extracted and electorphoresed in a 1% formaldehyde agarose gel. After transfer to a Hybond-N membrane and UV cross-linking, the membrane was hybridized with the random-primed ^{32}P radioactively labeled human cyclin D1 cDNA probe, washed under high stringency conditions, and then exposed to x-ray film. Equal loading of RNA was confirmed by ethidium bromide staining (not shown).

TGFα does not induce a significant change in cyclin D1 mRNA stability. Thus, the increase in the cyclin D1 mRNA level observed following TGFα stimulation was most likely due to increased transcription. Nuclear run-on assays did not reveal a measurable increase in the cyclin D1 transcription rate after 30 min (data not shown). However, this assay may not be sufficiently sensitive to detect a transcriptional change that requires 4 h to effect an increase in the cyclin D1 mRNA level following TGFα treatment. For this reason, we turned to transient transfection assays to assess the effect of TGFα on cyclin D1 transcription.

To evaluate transcriptional mechanisms underlying TGFα induction of cyclin D1 mRNA in TE-1 cells, we next tested cyclin D1 promoter deletion-luciferase reporter gene constructs in transient transfection assays. A panel of cyclin D1 promoter deletion constructs (Fig. 3*A*) was transfected in exponentially growing TE-1 (80–90% subconfluent) cells by the calcium phosphate method followed by glycerol shock after 12–18 h of incubation with the transfection solution. The cells were then grown in serum-free medium for approximately 48 h prior to harvesting the cells for luciferase assays. The luciferase activity was standardized to human growth hormone activity. All transfections were compared with the promoterless pA3LUC vector. Fig. 3*B* depicts cyclin D1 promoter basal activity with a range of relative luciferase activity evident from −1749 to −144. There was a decrease in basal activity from −1095 to −770 and an increase from −770 to −558. However, basal activity was reduced substantially from −104 to +135. These transfections yielded an identical pattern of cyclin D1 basal activity in a different esophageal squamous cancer cell line, T.T (data not shown).

To determine whether TGFα induces *cis*-regulatory elements within the cyclin D1 promoter, the transfections were repeated with TGFα stimulation. After transfection and glycerol shock, cells were serum starved for 24 h and then treated with various doses of TGFα for another 24 h prior to harvesting for luciferase assays. It should be emphasized that serum starvation was done to deplete TGFα in the tissue culture media and any other growth factors that could potentially induce the cyclin D1 promoter. Thus, the effect of TGFα alone could be evaluated. In this fashion, the different concentrations of TGFα tested ranged from 50 to 150 ng/ml, reflecting reported physiological

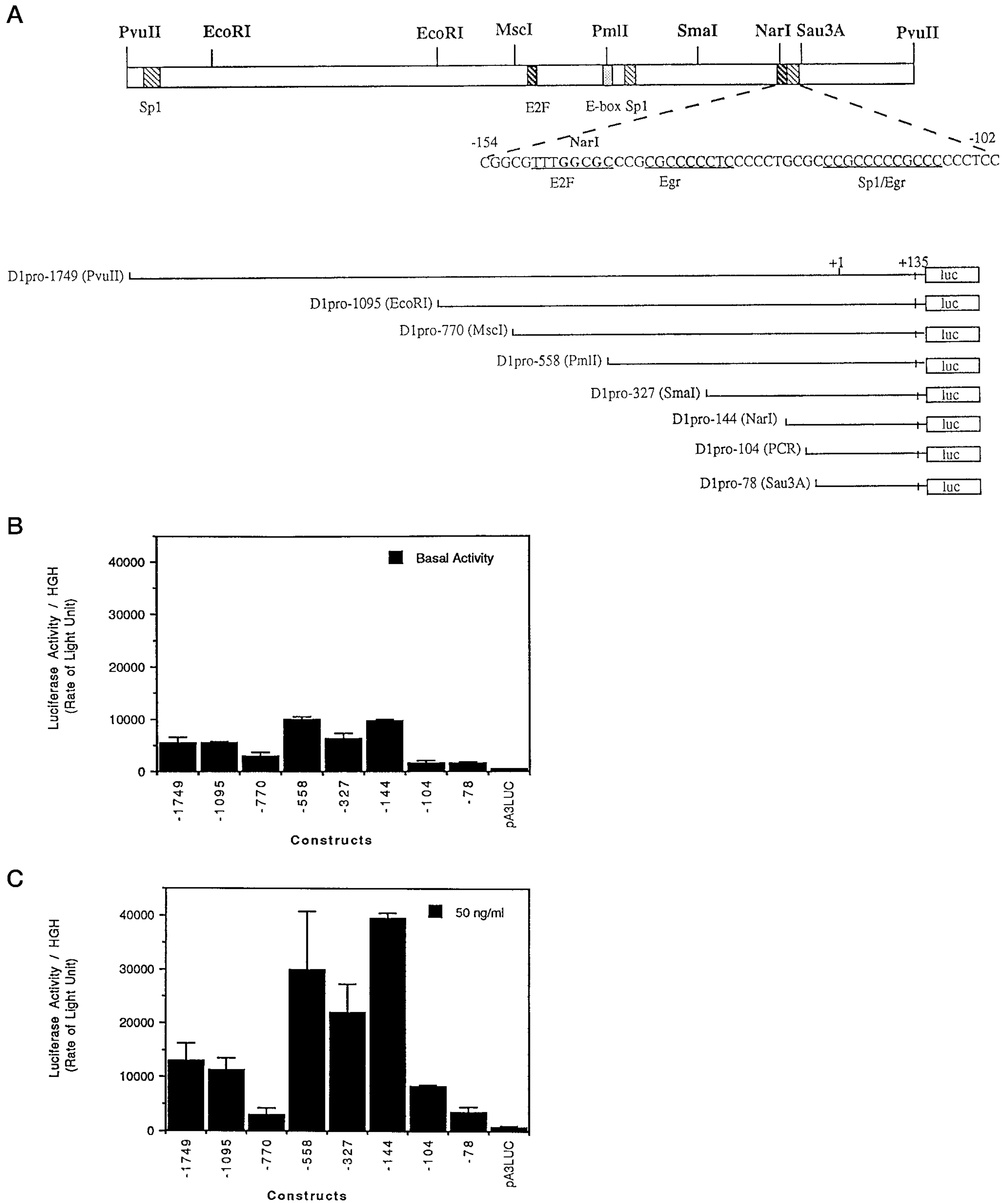


FIG. 3. *A*, restriction enyzme map of the cyclin D1 promoter and 5′ flanking sequences is shown, along with the cyclin D1 promoter deletion constructs fused to the luciferase reporter gene. *B*, cyclin D1 promoter basal activity. The serial cyclin D1 promoter deletions were transfected into TE-1 cells by the calcium phosphate method, and luciferase activity was measured and standardized to human growth hormone activity. Experiments were performed in triplicate and repeated independently three times. *C*, effect of TGFα on cyclin D1 promoter activity. The serial cyclin D1 promoter deletion constructs were transfected into TE-1 cells by the calcium phosphate method, and the cells were stimulated with TGFα (50 ng/ml). Luciferase activity was measured and standardized to human growth hormone activity. Experiments were performed in triplicate and repeated independently three times. For panels *B* and *C*, means and standard deviations are depicted. The details of the transfection conditions are outlined under "Experimental Procedures." An identical pattern was observed with TGFα at concentrations of 100 and 150 ng/ml (data not shown).

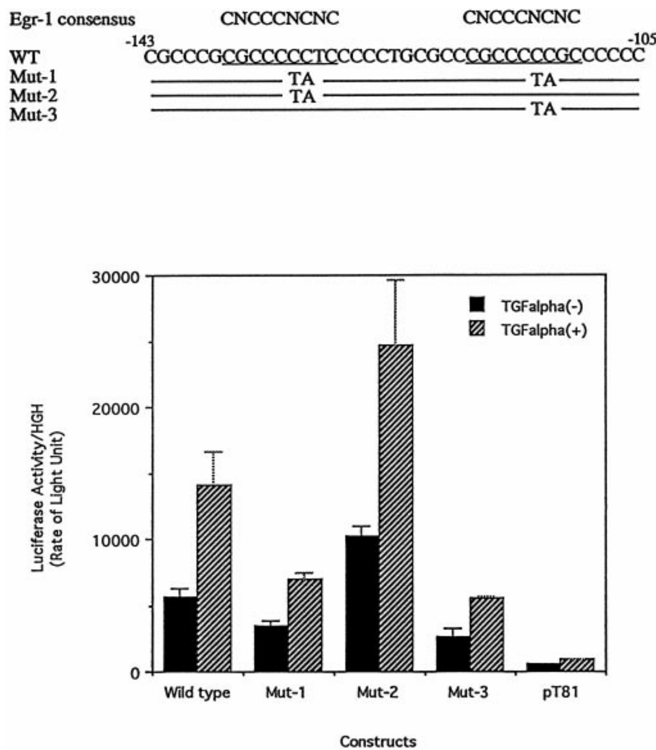


FIG. 4. **Transfection of −144 to −104 region (wild type and mutants) in pT81 plasmid (heterologous minimal promoter herpes simplex thymidine kinase luciferase reporter gene).** Transient transfections were performed by the calcium phosphate method. TE-1 cells were either stimulated with TGFα (50 ng/ml) or not treated at all. Luciferase activity was standardized to human growth hormone activity. Transfections were performed in triplicate and repeated independently three times.

levels of TGFα *in vivo* (30, 44). Induction of cyclin D1 promoter by TGFα in TE-1 cells yielded a relatively consistent pattern of the different doses tested. Fig. 3*C* shows the representative pattern by 50 ng/ml TGFα-treated TE-1 cells. These experiments demonstrated that TGFα affects the cyclin D1 promoter basal activity in a complex manner through a combination of transactivation and transrepression. Areas of significant changes in luciferase activity were observed between −770 to −558 and −144 to −104.

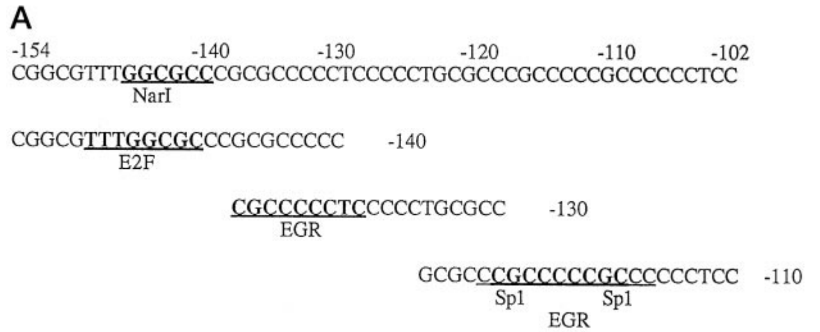
To study potential TGFα-responsive positive *cis*-regulatory elements in the cyclin D1 promoter, we focused on the region between −144 and −104 because induction was maximal in this region. However, we recognize there are likely other positive, as well as negative, *cis*-regulatory elements in other regions of the cyclin D1 5′ untranslated regulatory region; these elements are likely to be important. The −144 to −104 region contains a potential E2F binding site overlapped with a *Nar*I restriction enzyme site, two putative Egr protein motifs, and two putative Sp1 motifs. These two Sp1 motifs overlap with the 3′ Egr motif (Fig. 3*A*). The −144 to −104 region was then subcloned in the heterologous herpes simplex virus thymidine kinase minimal promoter fused to the luciferase reporter gene in the pT81 plasmid. In addition, three mutant oligonucleotides corresponding to this region were subcloned as well, with mutation of the putative Egr motifs, either individually or in combination. These constructs were transfected into TE-1 cells. As shown in Fig. 4, the 40-base pair fragment could still respond to TGFα stimulation. Among the mutant constructs, the response to TGFα stimulation was maintained as long as the 3′ putative Egr/Sp1 motifs were preserved, as was observed with mutant 2. In fact, the response to TGFα stimulation was greater with mutant 2 than with the wild-type construct, suggesting that the 5′ and 3′ motifs may have different affinities for Egr and may also bind repressor proteins to flanking nucleotides, especially at the 5′ Egr motif.

Multiple Nuclear Transcriptional Factors Interact with cis-regulatory Elements between −144 and −104 of the Cyclin D1 Promoter—To explore the interaction of nuclear transcriptional factors with potential *cis*-regulatory elements between −144 and −104, EMSAs were performed with nuclear extracts from TE-1 and T.T cells grown under the same conditions as those employed in the transfection studies. Three ^{32}P radioactively labeled double-stranded oligonucleotides were used as DNA probes, designated as −140, −130, and −110 (Fig. 5*A*), respectively. Fig. 5, *B* and *C*, shows that probes −110 and −130 bound multiple protein complexes in both TE-1 and T.T nuclear extracts (Fig. 5, *A* and *B, lanes 1–3* and *6–8*). The majority of the DNA-protein complexes appeared to be sequence-specific because a 200-fold molar excess of unlabeled oligonucleotide probe abolished the binding activity to the ^{32}P radioactively labeled probes (Fig. 5, *A* and *B*, *lanes 4* and *9*), and an unrelated oligonucleotide, glucocorticoid response element, did not compete away binding of nuclear proteins with the specific probes (Fig. 5, *A* and *B, lanes 5* and *10*). One binding activity appeared to be induced in nuclear extracts prepared from cells treated with TGFα (Fig. 4), although it was present in other nuclear extracts (Fig. 5, *A* and *B*, *lanes 1, 3, 6,* and *8*). This complex was competed away with a 100-fold molar excess of unlabeled oligonucleotide probe containing the Egr-1 consensus motif (Fig. 5, *A* and *B*, *lane 11*) but not with an unlabeled oligonucleotide probe containing a mutated Egr-1 motif (Fig. 5, *A* and *B*, *lane 12*). This result suggests that the Egr-1 protein or an Egr-1-like protein can bind *specifically* to the −130 and −110 probes.

The −144 to −104 region of the cyclin D1 promoter also contains a putative E2F site centered at −140. However, we found that radioactively labeled oligonucleotide −140 did not bind Egr (data not shown), nor did unlabeled −140 compete away Egr binding to −130 or −110. Furthermore, no transcriptional factors that bound oligonucleotide −140 were induced by TGFα based on EMSA (data not shown).

The TGFα-induced trans-acting Factor Is the Early Growth Response Protein Egr-1—To identify nuclear *trans*-acting factors that bind to the Egr motifs within cyclin D1 promoter nucleotides −144 and −104, immune-supershift assays were done with an anti-Egr-1 antibody. This antibody specifically recognizes the 80-kDa Egr-1 protein, and it also does not cross-react with the homologous 55–60-kDa Egr-2 protein. Fig. 6*A* shows that the Egr-DNA specific complex (*lane 1*) was abolished by preincubating with the anti-Egr-1 antibody (Fig. 6*A, lane 4*) but not with a control antibody (anti-AP2) (Fig. 7*A, lane 3*). The −130 probe also demonstrates supershift of the DNA-Egr complex specifically by the Egr-1 antibody (Fig. 6*B*). Although they are not shown, similar results were obtained with TE-1 nuclear extracts and the −130 and −110 probes. Therefore, the Egr-1 protein is a transcriptional factor induced by TGFα in binding to the Egr motifs within nucleotides −144 and −104.

Because the putative Egr sites in the probes −130 and −110 do not overlap (Fig. 5*A*), Egr binding activities may represent independent events. To further investigate any cross-reactivity of DNA binding proteins to the adjacent G+C-rich sequences, competition experiments were done for the probe −110 with a 100-fold molar excess of unlabeled oligonucleotides. As shown in Fig. 7*A*, the unlabeled oligonucleotide −110 competed away the Egr complex with higher efficiency (Fig. 7*A, lane 2*) than the unlabeled oligonucleotide −130 (Fig. 7*A, lane 6*). Another unlabeled oligonucleotide −140 did not interfere with the Egr complex (Fig. 7*A, lane 7*). Similar results were obtained with



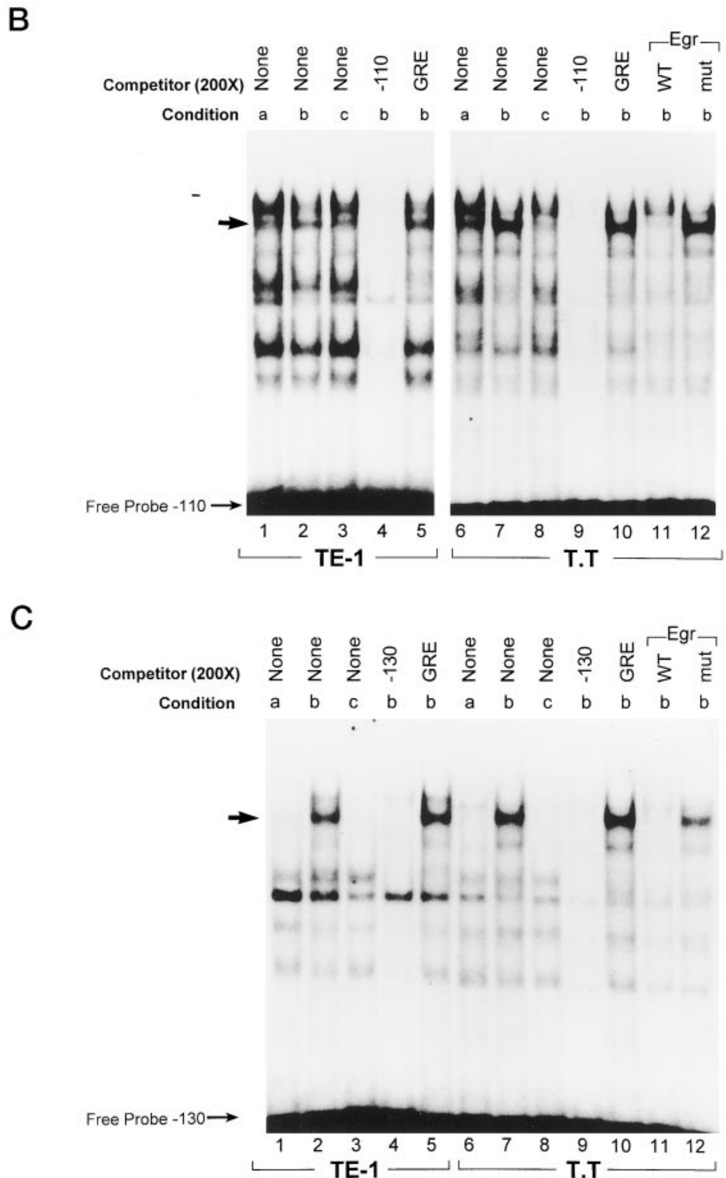


FIG. 5. **EMSA showing interaction of the G+C-rich elements (−144 to −104) in the cyclin D1 promoter with nuclear proteins from esophageal squamous carcinoma cells treated with or without TGFα (50 ng/ml).** *A*, three double-stranded oligonucleotides, designated as −140, −130, and −110, were designed to generate EMSA probes and competitors covering a G+C-rich nucleotide sequence between −144 and −104 in the cyclin D1 promoter that contains a putative E2F site and two putative Egr sites, one of which overlaps Sp1 consensus motifs. *B*, EMSA. The ^{32}P radioactively labeled oligonucleotide −110 probe was mixed with nuclear extracts prepared from TE-1 and T.T cells grown under distinct conditions, namely in medium containing 0.1% fetal calf serum (designated as condition *a*), medium containing 0.1% fetal calf serum stimulated with TGFα 50 ng/ml for 4 h (designated as condition *b*), or medium containing 10% fetal calf serum without TGFα stimulation (designated as condition *c*). Competition experiments were carried out with a 200-fold molar excess of unlabeled identical −110 oligonucleotide, glucocorticoid response element, wild-type Egr, and mutant Egr. The sequences of the oligonucleotides are shown under "Experimental Procedures." *C*, the EMSA conditions and reagents are identical to those in *B*, except that the ^{32}P radioactively labeled oligonucleotide −130 was used.

TE-1 nuclear extracts with the same −130 and −110 probes (data not shown). Moreover, the −110 oligonucleotide entirely competed away Egr binding to the ^{32}P radioactively labeled −130 oligonucleotide (data not shown). These data are consistent with the notion that the Egr-1 protein has a stronger binding affinity to the Egr motif in the −110 oligonucleotide than in the −130 oligonucleotide. This could potentially be explained by differences in flanking nucleotides and/or the presence of binding of other nuclear transcriptional factors that bind the same DNA regions in a sequence-specific fashion and may do so in a coordinated fashion. The differential binding affinities of Egr for the motifs present between −144 and −104 may also help to explain, in part, the results obtained with the pT81 transfections.

Sp1 May Not Play a Critical Role in TGFα-mediated Transcriptional Regulation of Cyclin D1 in the Cells of Esophageal Squamous Epithelial Origin—Competition experiments were performed to determine the possible involvement of the ubiquitous Sp1 nuclear transcriptional factor in DNA binding activity through the putative Sp1 consensus motif present within the −110 oligonucleotide probe. A 200-fold molar excess of Sp1 oligonucleotide did not interfere with any of the DNA binding

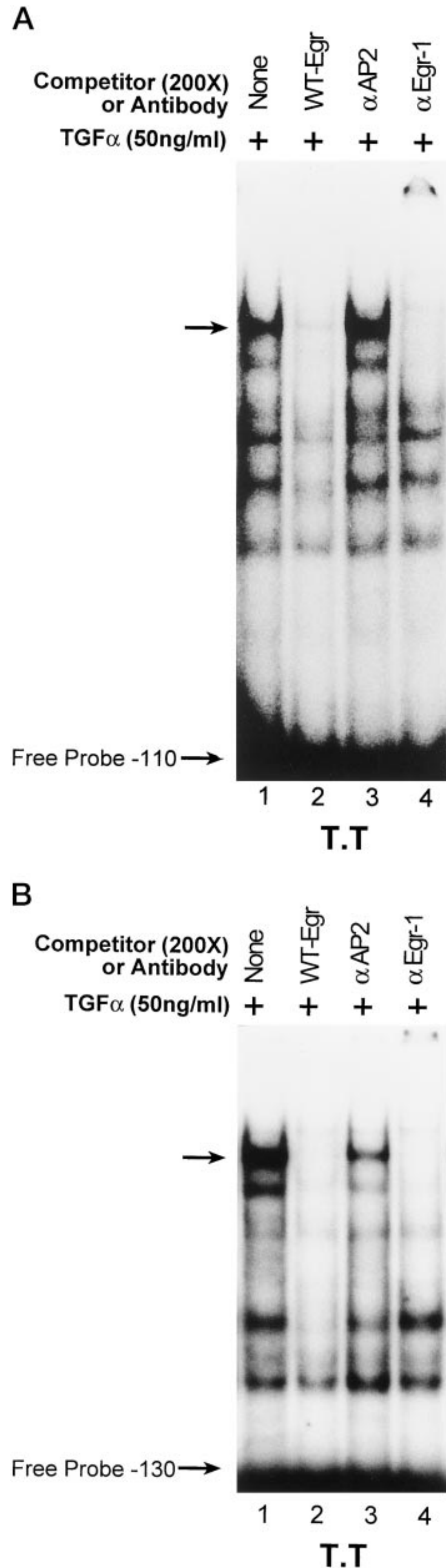


FIG. 6. **EMSA showing that the Egr complex is competed away by unlabeled Egr oligonucleotide and anti-Egr-1 antibody but not by anti-AP2 antibody.** Nuclear extracts from TGFα-treated T.T cells were incubated with indicated antibodies at a 1:200 dilution for 15 min at room temperature before ^{32}P radioactively labeled oligonucleotide −110 (*A*) or −130 (*B*) was added into the EMSA reactions. Identical results were obtained with TE-1 nuclear extracts with both −110 and −130 ^{32}P radioactively labeled oligonucleotide probes (data not shown). Although it is not shown, Sp1 antibody did not supershift any complexes.

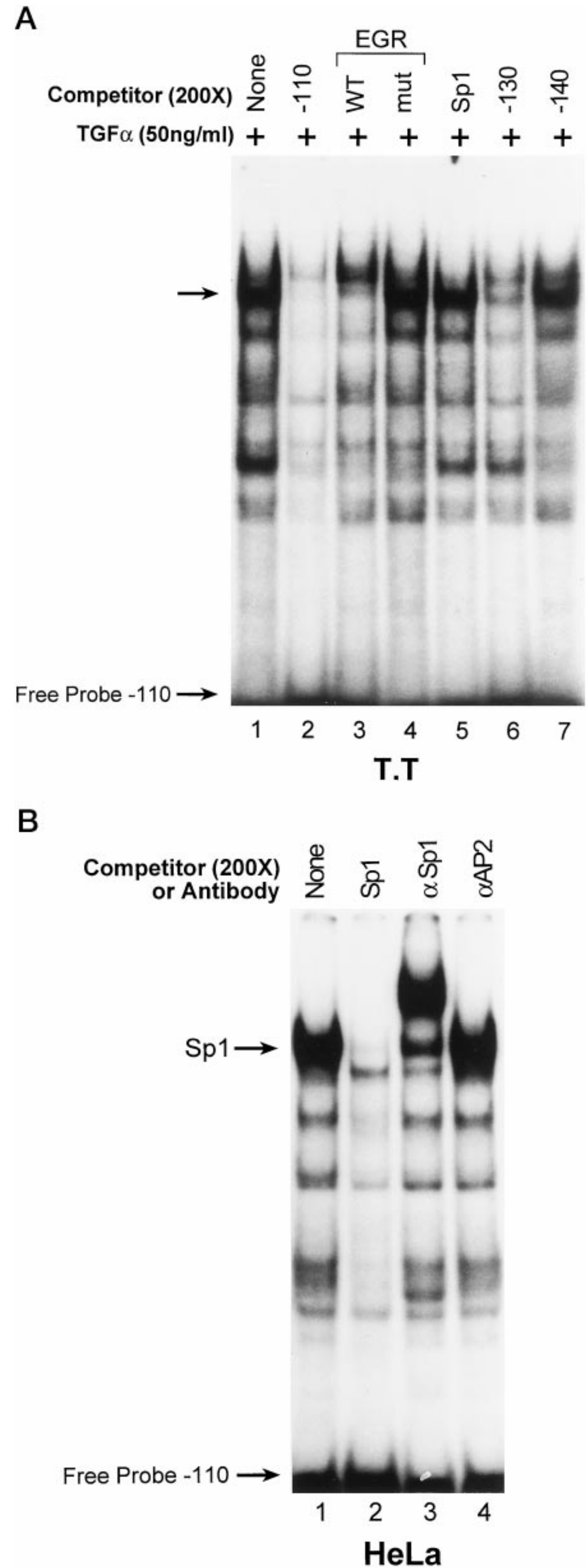


FIG. 7. **EMSA showing interaction of the G+C-rich element (−110 oligonucleotide) in the cyclin D1 promoter with Sp1 does not affect binding of Egr-1.** *A*, nuclear extracts from TGFα-treated T.T cells were incubated with ^{32}P radioactively labeled oligonucleotide −110 for competition experiments with 200-fold molar excess of indicated unlabeled oligonucleotides. A double-stranded Sp1 oligonucleotide, 5′-GATCGATCGGGCGGGGCGATC-3′, contains the Sp1 consensus motif. Identical results were observed with TGFα-treated TE-1 cells (data not shown). *B*, a competition experiment and immune-supershift assays were done using nuclear extracts from HeLa cells with ^{32}P radioactively labeled oligonucleotide −110. The Sp1 competitor oligonucleotide was in 200-fold molar excess, and the indicated antibodies were used at a 1:200 dilution.

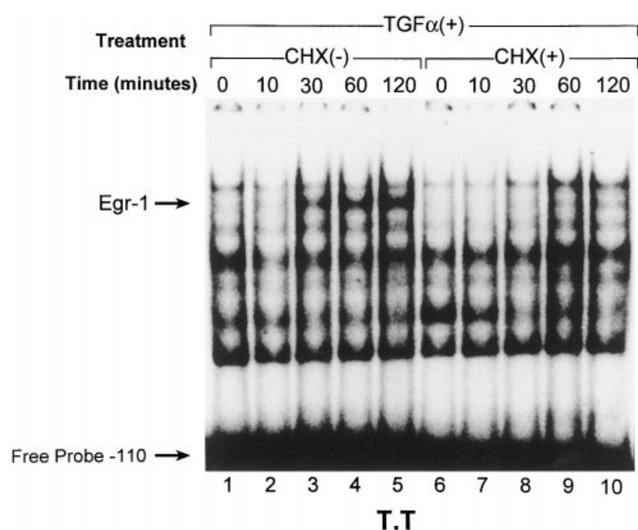


FIG. 8. **Effect of cycloheximide on the TGFα-induced Egr binding activity to the −110 oligonucleotide.** To inhibit *de novo* protein synthesis, T.T cells were pretreated with 10 μg/ml cycloheximide (in serum-free medium) for 30 min, and fresh serum free medium was exchanged at time 0. Cells were then maintained in the absence or presence of cycloheximide (re-added at time 0). Then, all cells were treated with TGFα (50 ng/ml), and nuclear extracts were prepared at times 0, 10, 30, 60, and 120 min. EMSA was performed with the ^{32}P radioactively labeled −110 oligonucleotide.

activities, including the Egr complex in nuclear extracts from TGFα-treated T.T cells (Fig. 7*A, lane 5*) or TGFα-treated TE-1 cells (data not shown). Because esophageal squamous cancer cell lines have virtually undetectable levels of Sp1 (44), we used the human cervix epithelioid carcinoma cell line HeLa, which is known to express high levels of Sp1. This was done to determine whether Sp1 could competitively bind to the same motif as Egr-1 in the −110 oligonucleotide probe. Competition and immune-supershift experiments shown in Fig. 7*B* demonstrated that unlabeled Sp1 oligonucleotide did not effect the Egr-1/DNA complex (Fig. 7*B, lane 2*), and anti-Sp1 antibody did not abolish or enhance the Egr-1 binding activity (Fig. 7*C, lane 3*). The Sp1 complex also migrated more slowly than the Egr-1 complex. An anti-AP2 antibody was used as a control for antibody specificity, and it showed no effect on the binding activities for the oligonucleotide −110 probe. Thus, Sp1 is present in low levels in esophageal squamous epithelial cells and does not interfere with Egr binding to the −110 oligonucleotide probe. Moreover, although it is present in greater abundance in HeLa cells, Sp1 still does not interfere with Egr binding to the −110 oligonucleotide probe.

Induction of the Egr-1 Binding Activity Requires de Novo Protein Synthesis—We observed that very weak Egr binding activity was detectable in cells growing in medium with 0.1% fetal calf serum (Fig. 5*B, lanes 1* and *6*). The Egr binding activity was consistently enhanced to a detectable level upon TGFα stimulation within 4 h after treatment. To investigate whether this enhancement required *de novo* protein synthesis, cycloheximide was used. All cells were pretreated with cycloheximide (10 μg/ml) for 30 min and then maintained either in the absence of cycloheximide or with the re-addition of cycloheximide at time 0. All cells were treated with TGFα at time 0. Fig. 8 shows that the Egr complex did not appear upon TGFα treatment in the continued presence of cycloheximide (Fig. 8, *lanes 6–10*). In contrast, the Egr complex appeared at 30 min in the absence of cycloheximide after TGFα stimulation (Fig. 8, *lanes 1–5*). These data are consistent with the notion that TGFα-mediated induction of the Egr complex requires *de novo* protein synthesis. Although these experiments were performed with the −110 oligonucleotide because of greater Egr binding affinity, we might predict a similar result with the EMSA employing the ^{32}P radioactively labeled −130 oligonucleotide.

DISCUSSION

Cyclin D1 is important in the progression of the cell cycle through G_1 phase, in part through the phosphorylation of pRB (7, 8). Cyclin D1 is frequently overexpressed in squamous epithelial cell cancers of the head/neck and esophagus (11–15). Mechanisms responsible for cyclin D1 overexpression in malignant squamous epithelial cells include gene amplification and transcriptional induction by mitogens; the latter is also present in normal squamous epithelial cells. A key mitogenic peptide involved in squamous epithelial proliferation is TGFα, which is believed to be more physiologically relevant in these tissues than epidermal growth factor, although both bind the same epidermal growth factor receptor (30, 44). We have found that TGFα induces cyclin D1 mRNA by 4 h after treatment, consistent with other reports that cyclin D1 mRNA expression is stimulated by a number of different mitogens in other systems (22, 36–38). The degree of induction of cyclin D1 mRNA by different mitogens appears to be consistently 3–4-fold. The induction of cyclin D1 mRNA by TGFα appears to correlate with cyclin D1 protein overexpression during the same time period. It has recently been demonstrated that cyclin D1 protein turnover is regulated by ubiquitination and proteasomal degradation (39). We postulate that although cyclin D1 protein is induced by TGFα stimulation, in the absence of TGFα (serum-free medium) or with TGFα depletion (time point 24 h), there is rapid cyclin D1 protein degradation. However, during the time period of cyclin D1 protein induction (4–8 h after TGFα stimulation) there is an association of increased cdk6 kinase activity, and in the time period immediatly thereafter (8–12 h after TGFα stimulation), there is an overall shortening of the G_1 phase and more cells in the S phase. The time lag would presumably involve, in part, activation of the cyclin E/cdk2 complex, which is well documented (1, 5). The apparent preferential activation of cdk6 found by us and others is interesting because it may be a mechanism more evident in squamous epithelial cells of the oral-esophageal tract (40). Overall, the induction of cyclin D1 mRNA and protein with a corresponding increase in cdk6 kinase activity appear to be physiologically relevant.

It is important to emphasize that the regulation of cyclin D1 is complex, involving transcriptional, posttranscriptional, and posttranslational mechanisms (5). The focus of our study has been the transcriptional regulation of cyclin D1 expression by TGFα in human esophageal squamous carcinoma cell lines employing serial cyclin D1 promoter deletion constructs fused to the luciferase reporter gene. TGFα appears to affect multiple *cis*-regulatory elements, positive and negative, within the cyclin D1 promoter. A key region appears to reside within nucleotides −144 to −104 of the cyclin D1 promoter, although clearly other positive and negative *cis*-regulatory elements may be also critical.

EMSAs demonstrated that Egr binds two independent Egr motifs centered at −130 and −110 nucleotides of the cyclin D1 promoter, although Egr appears to have stronger affinity for the −110 oligonucleotide. Specific Egr-1 binding for its cognate DNA motif was established by competition experiments with wild-type and mutant Egr oligonucleotides, as well as immune-supershift assays; the latter were performed with an anti-Egr-1 antibody. Furthermore, *de novo* protein synthesis is required. Interestingly, the Egr motif at −110 overlaps an Sp1 consensus motif. However, EMSAs indicated that Sp1 does not compete away Egr-1 DNA binding activity with the −110 oligonucleotide. According to our previous observation that less DNA bind-

ing activity of the Sp1 protein is present in cells of squamous epithelial origin from the head/neck and esophagus (45) and the observation that Sp1 has much weaker immunoreactivity in the upper gastrointestinal squamous epithelia (46), it appears that the Sp1 transcriptional factor does not play a central role in contributing to TGFα-mediated cyclin D1 promoter activity in these squamous epithelial cells. We observed an absence of dual interplay between Egr-1 and Sp1, which is different from what has been described in other promoters that have overlapping Egr and Sp1 motifs, including the proximal platelet-derived growth factor A chain promoter (47), the human interleukin 2 gene promoter (48), and the rat phenylethanolamine *N*-methyltransferase gene promoter (49). This has important implications regarding how Egr-1 and Sp1 may or may not cooperate with each other, depending upon the mitogenic stimulus and the cell type.

Cyclin D1 promoter basal activity is complicated, as was observed previously (23, 25, 26), and involves multiple *cis*-regulatory elements and *trans*-acting nuclear factors. Mitogenic stimulation of the cyclin D1 promoter is likely mediated through the induction of different nuclear transcriptional factors that in turn interact with different *cis*-regulatory elements, as demonstrated with *ras*, c-*myc*, c-*jun*, and serum. The *cis*-regulatory elements within the cyclin D1 promoter that are affected by different signal transduction pathways may vary depending upon the cell type and tissue context.

TGFα, a potent mitogen of epithelial cells, especially of the squamous type, is critical in mediating cell proliferation. Multiple genes are likely affected by TGFα-mediated intracellular signal transduction pathways (for example, cyclin D1 and cdk6) (50, 51). Its induction of cyclin D1 mRNA can in part be explained and now appreciated through a novel finding that the Egr-1 transcriptional factor is induced with subsequent binding to two different motifs between −144 and −104 of the cyclin D1 promoter. Our results would also appear to be the first demonstration that a transcriptional factor, Egr-1, is induced in response to a mitogenic stimulus of the cyclin D1 promoter. The complex interplay between Egr-1 and other transcriptional factors (52) in the transcriptional regulation of the cyclin D1 promoter upon TGFα stimulation, as well as the signal transduction pathway from TGFα to Egr-1, is currently under investigation.

Acknowledgments—We thank Drs. V. Sukhatme, D. Rhoads, and P. Hinds for helpful discussions.

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