MOLECULAR AND CELLULAR BIOLOGY, Aug. 1994, p. 5242–5248
0270-7306/94/$04.00+0

Vol. 14, No. 8

The Immediate-Early Gene *Egr-1* Regulates the Activity of the Thymidine Kinase Promoter at the G_0-to-G_1 Transition of the Cell Cycle

GYONGYI MOLNAR,* ANNE CROZAT,† AND ARTHUR B. PARDEE

Division of Cell Growth and Regulation, Dana-Farber Cancer Institute, Boston, Massachusetts 02115

Received 18 October 1993/Returned for modification 23 November 1993/Accepted 23 May 1994

Production of thymidine kinase (TK) protein parallels the onset of DNA synthesis during the cell cycle. This process is regulated at transcriptional, posttranscriptional, and translational levels to cause a 40- to 50-fold increase in cytosolic enzymatic activity as cells progress from G_1 to S phase. Transcriptional activation of the mouse TK gene through the cell cycle is dependent upon previously characterized *cis* elements of the proximal promoter, called MT1, MT2, and MT3, that bind at least two different complexes: TKE during the transition of cells from quiescence (G_0) to G_1, and Yi later at the G_1/S boundary. To identify the transcription factors involved in this regulation, we screened a mouse fibroblast cDNA expression library with a labeled MT3 oligonucleotide probe and isolated a clone that encodes Egr-1, an immediate-early transcription factor, whose expression is regulated by serum or growth factors during the G_0-to-G_1 transition when cells reenter the cell cycle. Electrophoretic mobility shift assays demonstrate that Egr-1 is involved in the TKE complex that binds to the MT3 element and that expression of Egr-1 induces transcription of a mouse TK-chloramphenicol acetyltransferase reporter in transient transfections. These results suggest a role for Egr-1 in regulating expression of the TK gene at the G_0-to-G_1 transition.

Thymidine kinase (TK) is a cytosolic enzyme that catalyzes the phosphorylation of thymidine to form dTMP, a precursor of DNA. Like other genes encoding enzymes that participate in the biosynthesis of DNA, the TK gene is regulated at transcriptional, posttranscriptional, and translational levels to ensure a large increase in TK activity when cells enter S phase (10, 11, 18, 19, 21, 23, 39). Regulation of the TK gene is complex, and its mechanism depends on the physiological state of the cells (cycling cells versus quiescent cells and transformed cells versus nontransformed cells). We have focused on transcriptional regulation of the mouse TK gene as a model for the progression of cultured cells through the cell cycle, particularly during the critical transition between the G_1 and S phases. It is likely that the same mechanisms that activate TK transcription also participate in controlling the expression of other genes and DNA replication during the G_1-to-S transition; therefore, TK is a useful model for studying regulatory events in the cell cycle (for a review, see reference 32).

We have characterized *cis*-regulatory sequences located between −174 and +159 bp of the mouse TK translation start site that are essential and sufficient to confer efficient and serum-responsive expression of a reporter gene (15). By DNase footprint analysis, we identified three distinct regions within this sequence, called MT1, MT2, and MT3, that can bind nuclear factors (11). Electrophoretic mobility shift assays (EMSAs) demonstrate the presence of at least two cell cycle-regulated DNA-binding protein complexes that interact with those sequences: TKE in early G_1 and in normal cells, Yi at the G_1-to-S transition (11, 12). In transformed cells, which have partially lost the ability to control their cell cycle, Yi binding activity remains present in G_0 after serum starvation and is stable across the cell cycle (4), suggesting a role for the Yi complex in normal growth regulation. To further characterize these binding activities and generate molecular probes that would allow analysis of regulatory events important in the progress of a cell through the cell cycle, we proceeded to direct-expression cloning of cDNAs encoding proteins capable of binding the MT sites.

Agents that stimulate quiescent cells (G_0) to reenter the cell cycle, such as serum and growth factors, start with the induction of RNA of immediate-early response genes (5, 25) without the need for de novo protein synthesis. Most of these genes encode DNA-binding proteins (2, 3, 7, 24, 26) and include members of the c-*fos*, c-*jun*, c-*myc*, and Egr families (for a review, see reference 41).

Egr-1 (also known as NGF1-A, TIS8, Krox-24, and Zif268) expression is rapidly induced with *fos*-like kinetics by diverse mitogens during the G_0-to-G_1 transition in several cell types (27, 41). Members of the Egr family contain three zinc finger DNA-binding domains with similarity to the DNA-binding domain of transcription factor Sp1 (22). Egr-1 can activate transcription by high-affinity binding to the regulatory element GCG(G/T)GGGCG (EGR) in a zinc-dependent manner (6, 7, 28). This recognition element has been identified by educated guesses and demonstrated by using synthetic oligonucleotides, but only a few physiologically relevant target genes of Egr-1 are actually known (1, 6, 7, 13, 20, 28).

We report here that Egr-1 binds specifically to the MT3 site of the mouse TK promoter at the G_0-to-G_1 transition of the cell cycle, and it induces TK-chloramphenicol acetyltransferase (CAT) reporter activity in transient transfections. The involvement of Egr-1 in transcriptional regulation of the mouse TK gene documents a physiologically relevant target gene for Egr-1 and provides additional information on the mechanism involved in G_0/G_1 activation of the TK gene.

* Corresponding author. Mailing address: Division of Cell Growth and Regulation, Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115. Phone: (617) 632-4685. Fax: (617) 632-4680.

† Present address: Population Council, New York, N.Y.

TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence
MT1	TCG AGT CTT CGT CCC GCC CCC TTT
	CA GAA GCA GGG CGG GGG AAA AGC T
MT2	TCG AGA GTT CGC GGG CAA ATG
	CT CAA GCG CCC GTT TAC AGC T
MT3	TCG ACA CCC ACG GAC TCT CGG TGC T
	GT GGG TGC CTG AGA GCC ACG AAG CT
EGR	AGC TTC GCG GGG GCG AGG A
	AG CGC CCC CGC TCC TTC GA

MATERIALS AND METHODS

Oligonucleotides. Sequences of the oligonucleotides used are listed in Table 1. The probe used for library screening was a concatemer of the MT3 double-stranded oligonucleotide, random labeled by nick translation (Bethesda Research Laboratories kit). DNA probes for EMSAs were end labeled by filling in the 4-base overhangs with the Klenow fragment, including [α-^{32}P]dCTP.

Library screening. A lambda gt11 cDNA expression library from mouse 3T3-L1 cells (gift of B. Spiegelman) was screened with the concatemer ^{32}P-labeled MT3 oligonucleotide as a ligand according to the published protocol (37).

Inserts were subcloned into pBluescript KS+ (Stratagene) and sequenced by the dideoxynucleotide chain termination method (35) with a Sequenase kit (U.S. Biochemicals).

Expression vectors and reporter constructs. Glutathione-*S*-transferase (GST) fusion plasmids were made by subcloning into a pGEX vector either the full-length *Eco*RI insert of clone 17 to produce pGEX/17 or a *Stu*I fragment from the endogenous *Stu*I site (nucleotide 922 of Egr-1 [41]) to the 3′ end of clone 17 (nucleotide 1552 [41]) to form pGEX/17Stu, encoding the DNA-binding domain of Egr-1.

pCMVEgr-1 and pCMVETTL expression vectors were a generous gift from V. Sukhatme (20). pCMVEgr-1 contains the entire coding sequence of Egr-1 under the control of the cytomegalovirus promoter. pCMVETTL contains a terminator codon after serine 170 of pCMVEgr-1.

Construction of the pmTK-CAT reporter construct has been described elsewhere (previously called pACATm[−174]A) (15). Briefly, pmTK-CAT contains the mouse wild-type TK promoter sequence located between −174 and +159 bp of the translation start site and inserted into the promoterless vector pBLCAT3 (29) modified as described previously (14). pmutTK-CAT has a 16-bp deletion and 4-bp substitution that completely removes the MT3 recognition element so that in the final sequence the *Eco*RI recognition site, GAATTC, corresponds to the original position −41 to −20 bp of the promoter.

Bacterial expression of GST fusion proteins. Expression and recovery of purified GST fusion proteins by adsorption to glutathione-Sepharose were performed as described previously (38). The relevant pGEX expression vectors were transformed into *Escherichia coli* JM109 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 to 4 h. For affinity purification of the corresponding fusion proteins, bacteria lysates were bound to glutathione-Sepharose, eluted in a 50 mM Tris buffer (pH 8.0) containing 10 mM reduced glutathione, and stored at −70°C in the same buffer containing 10% glycerol.

Southwestern (DNA-protein) blot analysis. Transformed bacteria induced with IPTG were pelleted, lysed in Laemmli buffer, and separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis, and proteins were electroblotted onto nitrocellulose membranes. Blots were blocked for 1 h on ice in TNE60 (10 mM Tris [pH 7.5], 60 mM NaCl, 1 mM EDTA) containing 1 mM dithiothreitol, 10 μg of salmon sperm DNA per ml, and 5% nonfat milk. Binding reactions were performed for 1.5 h at room temperature and then for 20 min on ice in TNE60–2 mM dithiothreitol–2 μg of poly(dI-dC) per ml with an MT3 double-stranded multimerized oligonucleotide as a probe. Before autoradiography, filters were washed in TNE60 three times for 10 min on ice.

Preparation of nuclear extracts. Nuclear extracts were prepared as described previously (36).

EMSA. DNA binding assays were performed with labeled double-stranded oligonucleotides (MT3 or EGR) essentially as described previously (4). Excesses of nonlabeled oligonucleotides (MT1, MT2, MT3, and EGR) were added as competitors in binding reactions as indicated. Complexes were resolved on a 4% nondenaturing polyacrylamide gel.

For antibody perturbation experiments, 1 μl of a 1/10 dilution of the corresponding polyclonal antibodies or normal rabbit serum was added to the nuclear extract for 15 min at room temperature prior to the addition of the binding mixture. From the affinity-purified Egr-1 antibody, 1 μl per reaction was used under the same conditions as described above.

Egr-1-specific antiserum was a gift from V. Sukhatme, and the affinity-purified antibody was from F. Rauscher III. C-12 antiserum, raised against a different MT3-binding protein, and TS antiserum (9) were used as nonspecific sera in these experiments.

Cell lines and culture. Mouse BALB/c3T3 clone A31 cells, benzo[a]pyrene-transformed BALB/c3T3 clone BPA31 cells, and monkey kidney CV1 cells were maintained in an atmosphere of 10% CO_2 at 37°C in Dulbecco's modified Eagle's medium containing 10% bovine calf serum and 4 mM L-glutamine.

As indicated, A31 and BPA31 cells were brought to quiescence by serum deprivation in the presence of 0.4 and 0.2% calf serum for 60 and 84 h, respectively, as previously described (4).

Cell transfections. CV1 cells were transfected by the calcium phosphate-DNA coprecipitation method (31). Transfection efficiency was monitored with a *lacZ* expression vector under the control of the β-actin promoter. Typically, 2 μg of pmTK-CAT or pmutTK-CAT reporter plasmids, 1 to 10 μg of pCMVEgr-1 or pCMVETTL expression vectors, and 2 μg of *lacZ* construct were used. Total DNA was completed with 10 μg of salmon sperm DNA. Cells were incubated with the DNA precipitate for 16 h and glycerol shocked. Following glycerol shock, transfected CV1 cells were starved for 24 h (0.4% calf serum) before being harvested.

Chloramphenicol acetyltransferase (CAT) assays were performed as described previously (31).

β-Galactosidase activity in the same cellular extracts was determined by using *o*-nitrophenyl-galactopyranoside (ONPG) as the substrate, essentially as described previously (31).

RESULTS

Isolation of a cDNA clone encoding an MT3-binding protein. To identify recombinant clones that encode proteins capable of binding to the mouse TK MT3 element, a lambda gt11 3T3 fibroblast cDNA library was screened with a ^{32}P-labeled double-stranded oligonucleotide containing concatemers of the MT3 oligonucleotide. Several recombinant clones that reproducibly bound the multimeric MT3 probe were isolated. One of them, clone 17, contained an insert of about 1.5 kb. DNA sequence analysis of this insert disclosed that it

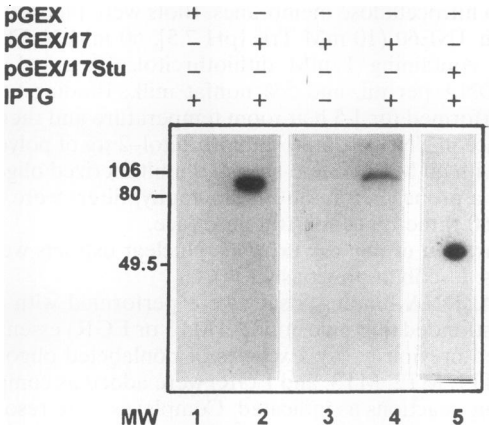

FIG. 1. Egr-1 binds the MT3 element in a Southwestern blot assay. Shown is a Southwestern blot of the cloned bacterially expressed Egr-1 GST fusion protein (pGEX/17) and a truncated version of the fusion protein containing only the DNA-binding domain (pGEX/17Stu). The blot was probed with a ^{32}P-labeled double-stranded MT3 oligonucleotide. The Egr-1-containing fusion protein (lane 2) as well as the Egr-1 DNA-binding domain (lane 5), but not the GST-encoding parental plasmid (lane 1), produces a fusion protein capable of binding the MT3 element. Binding activity is dependent upon induction of the transformed bacteria by IPTG (lane 3, no IPTG; lane 4, 1 mM IPTG in the culture medium).

encodes an uninterrupted open reading frame continuous with the β-galactosidase gene of the cloning vector. Data bank comparison showed a perfect identity with the murine transcription factor Egr-1 (8, 27, 41) between nucleotides 235 and 1552 of the *Egr-1* nucleotide sequence (41), including the three zinc fingers of the DNA-binding domain.

Sequence-specific DNA binding of Egr-1 to the MT3 element in vitro and in vivo. To evaluate the binding of Egr-1 to the promoter of the mouse TK gene, we expressed in bacteria, as GST fusions, either the full-length clone 17 (pGEX/17) or a more soluble 243-amino-acid peptide corresponding to the DNA-binding domain of Egr-1 (822 to 1552), spanning nucleotides 587 to 1317 of the clone 17 sequence (pGEX/17Stu). Lysates from bacteria transformed with pGEX/17 or pGEX/17Stu expression vectors contained a protein that is inducible by IPTG (Fig. 1, compare lanes 3 and 4) and recognized by the MT3 probe in a Southwestern blot (Fig. 1, lanes 2, 4, and 5). Lysates from control bacteria, expressing GST alone, were not recognized by the MT3 probe under similar conditions (Fig. 1, lane 1).

Fusion protein purified from lysates of bacteria transformed with the pGEX/17Stu expression vector bound ^{32}P-labeled MT3 in an EMSA with high affinity and specificity (Fig. 2A). Binding to MT3 was abolished by an excess of the homologous unlabeled MT3 oligonucleotide (Fig. 2A, lanes 7 and 8) but not by the same excess of the unrelated oligonucleotide MT2 (lanes 5 and 6). MT1, which contains a perfect SP1 consensus binding site, exhibited low affinity for the Egr-1 DNA-binding domain (Fig. 2A, lanes 3 and 4).

We compared the affinity of Egr-1 toward the MT3 site with its affinity toward an established Egr-1 binding site (EGR) (6, 7, 28). Equal amounts of the affinity-purified Egr-1 binding domain were bound to the MT3 probe and inhibited by increasing amounts of unlabeled MT3 or EGR oligonucleotides (Fig. 2B). Under these conditions, about 4 μmol of MT3 was necessary to inhibit 50% of Egr-1 DNA binding activity, whereas only about 1 μmol of the EGR oligonucleotide was required to inhibit it at the same level. Thus, the bacterially expressed Egr-1 zinc finger domain of clone 17 has fourfold-greater affinity for the EGR consensus recognition site than for MT3.

Following demonstration of high-affinity binding of Egr-1 to the MT3 element in vitro, we wanted to know if Egr-1 participates in the MT3-binding complex present in serum-stimulated nuclear extracts. BPA31 fibroblasts were synchronized in G_0 and stimulated with serum. Nuclear extracts were prepared from cells at various time points following serum stimulation, and extracts were assayed for the presence of proteins capable of binding the MT3 element and the EGR site. DNA binding activity detected with both probes and of indistinguishable low mobility was induced by serum stimulation of the cells (Fig. 3). This activity comigrates as does the previously described TKE complex in A31 cells (11). TKE DNA binding activity, absent in serum-starved cells, was detected after as little as 30 min of serum stimulation (Fig. 3, lanes 3 and 9), peaked at 1 h, and visibly declined within 4 h. A similar activation pattern was found when A31 nuclear extract was applied (data not shown). These kinetics are consistent with those described for the induction of Egr-1 in serum-stimulated fibroblasts (6). In addition to the TKE band, nuclear extracts contain an MT3-binding Yi complex which is stable across the cell cycle in transformed BPA31 cells, as already described (4). The nature of the constitutive band detected with the EGR probe is not known.

The relationship of serum-induced TKE activity to Egr-1 was studied by using an antiserum that specifically recognizes Egr-1. Addition of this antiserum to the gel shift reaction led to elimination of serum-stimulated Egr-1 binding to the EGR probe (Fig. 4A, lane 2). We observed similar inhibition of serum-stimulated TKE binding activity to MT3 by this antiserum (Fig. 4B, lane 2, and 4C, lanes 2 and 5). Addition of antisera to unrelated proteins did not eliminate the TKE band, attesting to the specificity of the assay just as retention of the Yi band in the presence of any antiserum does. Inhibition of TKE and Egr-1 DNA binding by the Egr-1 antiserum is consistent with the antiserum having been raised against almost the entire protein and has been shown previously to interfere with the DNA binding activity of Egr-1 in EMSAs (6). In addition, the migration of the native Egr-1-specific band is similar to that of the purified DNA-binding domain of Egr-1 (Fig. 2). The small difference is probably derived from the molecular weight difference of native and truncated forms of Egr-1. We observed a nonspecific increase in TKE and Egr-1 complexes in the presence of the different antisera tested (Fig. 4A; 4B, lanes 3 to 5; and 4C, lanes 1 and 4), as well as the appearance of an additional complex of lower mobility with the MT3 probe. This phenomenon is reproducible, and we assume that the weak binding of Egr-1 to the MT3 element observed in vitro in the absence of a certain minimal protein concentration in the assay might also be compensated for in vivo by the protein concentration in the nucleus. The additional complex formed in the presence of MT3 might be a reflection of a nonspecific MT3 binding activity present in the rabbit serum. These results demonstrate that an activity indistinguishable in its mobility and immunological reactivity from Egr-1 participates in formation of the serum-stimulated TKE complex on the MT3 site of the TK gene.

Egr-1 activates the mouse TK promoter through the MT3 element. Having demonstrated Egr-1 binding to the MT3 element, we then asked whether Egr-1 could activate transcription of a reporter gene driven by the MT3-containing TK promoter sequence, previously shown to be serum responsive (15). Cotransfection of an Egr-1 expression plasmid with the

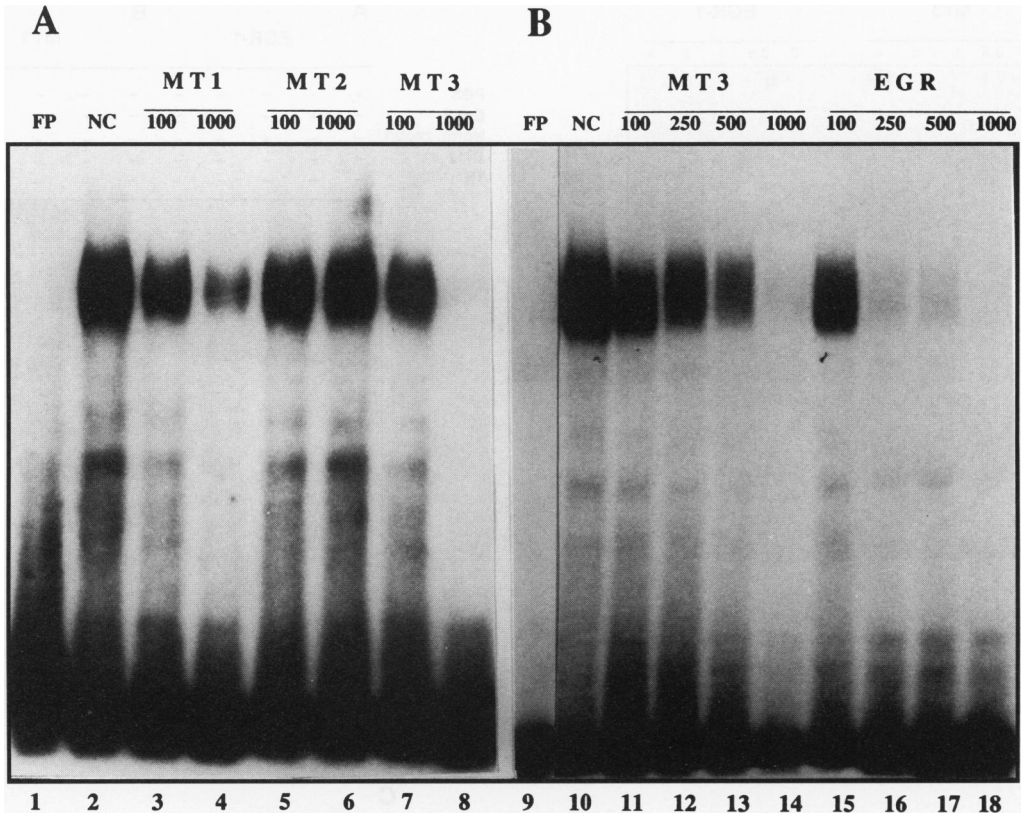

FIG. 2. Sequence-specific DNA binding of Egr-1 to the MT3 element. Gel retardation of labeled MT3 oligonucleotide by the bacterially expressed DNA-binding domain of clone 17 was determined. Purified bacterially expressed GST fusion protein containing a 243-amino-acid fragment spanning the DNA-binding domain of Egr-1 was used to shift a ^{32}P-labeled MT3 probe in nondenaturing 4% polyacrylamide gel electrophoresis. (A) Competition by unlabeled oligonucleotides. The identity of the competitor oligonucleotide and its molar excess over the MT3 probe (60 fM) are indicated above each lane. Lanes 1 and 9, free probe (FP); lanes 2 and 10, no competitor DNA (NC). (B) Comparison of the affinity of Egr-1 for MT3 with its affinity for a consensus EGR site. The same amounts of protein as in panel A were inhibited by the indicated molar excesses of EGR or MT3 oligonucleotide for binding to the MT3 probe.

pmTK-CAT reporter led to sixfold stimulation of reporter gene activity (Fig. 5A). A control expression plasmid encoding a truncated version of Egr-1 (pCMVETTL) led to no increase in reporter gene activity. To demonstrate that activation of the TK promoter by Egr-1 was dependent on the MT3 binding site, we utilized a reporter plasmid in which the MT3 site had been destroyed by site-directed in vitro mutagenesis. CAT activity driven by the mutant reporter plasmid was not increased by cotransfection of the Egr-1 expression plasmid (Fig. 5B). Activation of the TK promoter by Egr-1 was much stronger in serum-starved cells than in cells maintained in the presence of serum after transfection (data not shown). The difference can be explained by the higher basal activity of the reporter plasmid under serum-rich conditions. This observation is consistent with the possibility that other factors, such as Yi, present in cells growing in serum can obscure the effect of exogenous Egr-1 directed by the expression plasmid.

DISCUSSION

Cell cycle-dependent regulation of the mouse TK gene correlates with changes in the binding of nuclear proteins to three *cis*-acting DNA elements: MT1, MT2, and MT3 (11). At least two distinct DNA-binding complexes have been shown to assemble on these regulatory elements. The TKE complex, induced early upon serum stimulation, binds exclusively to the MT3 site, and Yi complexes that are induced later upon growth stimulation bind all three elements (11, 12). Using the MT3 sequence as a probe, we isolated a cDNA clone and found that it encodes the known DNA-binding protein Egr-1. We have shown that bacterially expressed Egr-1 is capable of binding specifically to the MT3 element in vitro, that antiserum directed against Egr-1 interferes with the DNA binding activity of the TKE complex in nuclear extracts from serum-stimulated cells, and that Egr-1 activates a reporter gene driven by the TK promoter in a transient cotransfection assay. Activation by Egr-1 is dependent on the presence of intact MT3 in the reporter gene. These observations led us to conclude that Egr-1, or a functionally and structurally related protein, participates in formation of the previously characterized G_0/G_1 cell cycle-dependent DNA binding activity TKE.

At least two distinct signals have been hypothesized to regulate the reentry of cells into the cell cycle and progression of normal cells through the cycle. The first, governing the transition of cells from quiescence, or G_0, to G_1, is the induction of competence genes, including many immediate-early genes. This occurs in response to certain exogenous growth factors, and while it is insufficient in and of itself to promote progression through the cell cycle, this signal is necessary to bring cells to a state of competence for responding to later stimuli (34). A second signal, responding to additional growth factors, including IGF-1 (43), and presumably involving

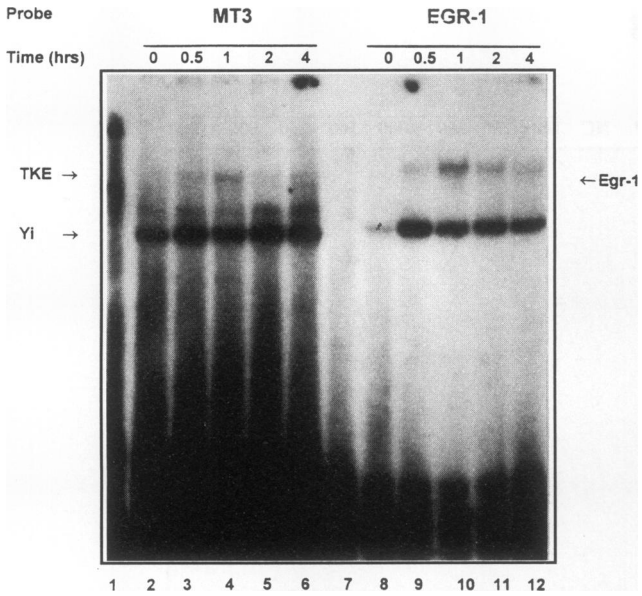

FIG. 3. TKE and Egr-1 binding activity are indistinguishable at the exit from G_0 to the early G_1 phase of the cell cycle. Nuclear extracts from BPA31 cells harvested at the indicated time points after serum stimulation (10% fetal calf serum) were used in gel retardation assays to shift the MT3 probe (lanes 1 to 6) or the consensus EGR site (lanes 7 to 12). Positions of migration of the TKE-Egr-1 complex and the Yi DNA-binding complex are indicated by arrows on the sides.

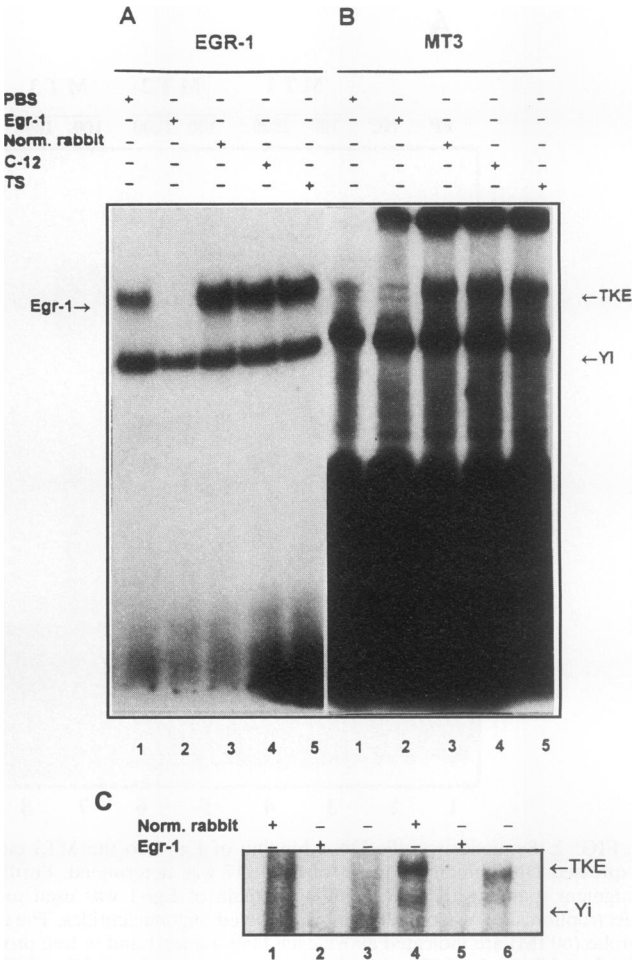

FIG. 4. Immunological identity between TKE and Egr-1. Extracts from early-G_1-phase BPA31 (A, B, and C, lanes 4, 5, and 6) and A31 (C, lanes 1, 2, and 3) cells were used in a gel retardation assay to shift the consensus EGR-binding site (A) or the MT3 element (B and C). One microliter of 1/10 diluted rabbit antiserum to Egr-1 or three unrelated control sera (normal rabbit [Norm. rabbit], C-12, and TS) was added to the assay mix. Egr-1 antiserum, but none of the control antisera or the addition of phosphate-buffered saline (PBS), interferes with Egr-1 binding to the EGR probe (A) and with TKE binding to the MT3 site (B). A similar effect was observed with 1 μl of affinity-purified Egr-1 antibody in the assay (C). Individual DNA-protein complexes are identified by arrows.

the Yi complex, is required to carry competent cells through the final stages of G_1 and across the G_1/S boundary (15). Our data suggest that Egr-1 may be a competence factor acting in early G_1 and may be relevant to the ultimate activation of the TK gene later in the G_1/S phase.

This new Egr-1 recognition sequence, MT3, matches only loosely the previously identified EGR consensus sequence, and in vitro Egr-1 protein has lower affinity for MT3 than for other previously defined EGR-binding sites. The sequences to which Egr-1 binds were initially identified by anticipating that the promoter of the Egr-1 gene itself might have binding sites for this protein. This turned out to be the case, and once these prototypical sequences were identified, a number of potential EGR-binding sites in the promoters of other genes were found by nucleotide homology search and confirmed by DNA binding assays. This information was used to establish a consensus high-affinity binding sequence for Egr-1: GCG (G/C/T)GG GCG (6, 7, 28). However, in addition to the Egr-1 gene itself, to date only two target genes that are regulated by Egr-1 in vivo have been identified: the rat cardiac α-MHC gene through the binding site GTG GGG GTG (20) and the murine adenosine deaminase gene through the sequence GCG TGG GCG (1). The Wilms' tumor gene product, WT1, has been shown to be a member of the Egr family and can also bind the EGR consensus site (30, 33). WT1 binds to several *cis* elements of the IGF-II promoter and acts as a transcriptional inhibitor, probably by interfering with the action of Egr-1 (13). Binding sites for WT1 in the IGF-II promoter are of two kinds. High-affinity binding can be seen at sites that resemble the Egr-1 recognition elements (GCG GGG GCG), and lower-affinity binding can be seen at other GC-rich sequences in the promoter. It seems likely, therefore, that Egr-1 could also bind to nonclassic sequences with lower affinity. Such a nontypical recognition site (TCCTCCTCCTCCTC) was also reported in the promoter of the platelet-derived growth factor A chain (42). Although a detailed mutagenesis study has not yet been reported, compilation of data from the literature shows that Egr-1 can bind to many sequences and a loose consensus element, G(C/A/T)G (G/T/C)GG G(C/T)G, can thus be defined. In this context, the MT3 site (G AGT CCG TGG GTG) becomes a fairly good Egr-1 binding site and is consistent with the relative promiscuity of Egr-1 binding reported so far.

Egr-1 is believed to be involved in both proliferation and differentiation signaling. However, most target genes identified to date are implicated in a differentiation pathway. The Egr-1 gene is induced during neuronal differentiation of PC12 cells (6), the rat cardiac α-MCH gene binds Egr-1 in a model system of cardiac myocyte differentiation (20), and the IGF-II gene is involved in the differentiation of multipotent kidney blastemal cells (13). In contrast, the TK gene represents an identification

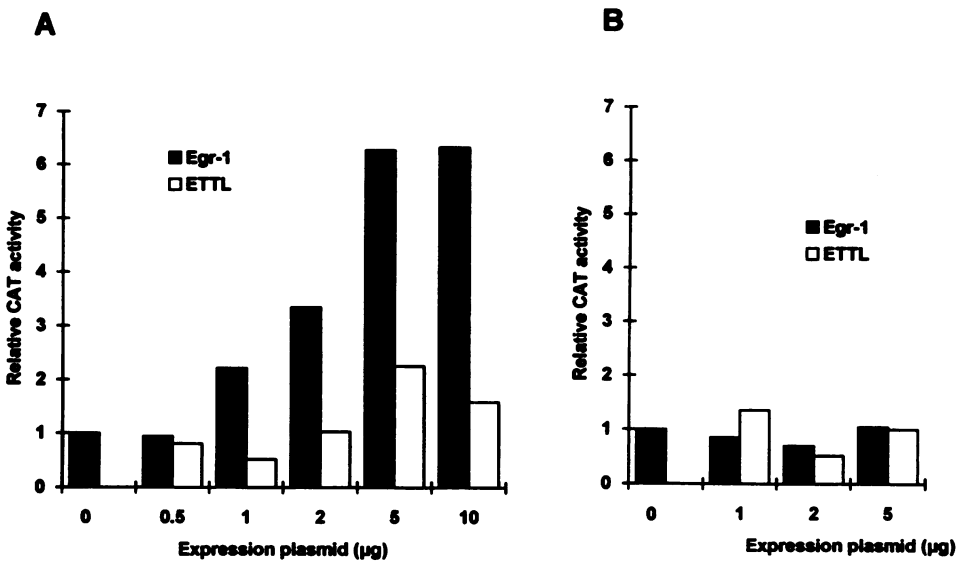

FIG. 5. An Egr-1 expression vector activates the TK promoter in transient transfections. Wild-type mouse TK promoter (A) or a mutated form in which MT3 has been replaced by an *Eco*RI recognition site (B) was linked to the promoterless pBLCAT3 to give pmTKCAT and pmutTKCAT, respectively. Two micrograms of each construct was transiently cotransfected in CV1 cells together with the indicated concentrations of pCMVEgr-1 or pCMVETTL expression vectors, and cells were serum starved for 24 h before harvest for CAT activity. CAT activities are reported as increases above the basal activity of TK-CAT reporter genes transfected in the absence of an expression vector.

of a growth-related target gene for Egr-1. We speculate that the lower affinity of Egr-1 binding to MT3 than to the classic EGR site (Fig. 2B) may be functionally compensated for in vivo by the presence of other factors specific to the G_0/G_1 phase or by interaction with other constitutive binding factors, such as the nearby Sp1 (see below). This mode of cooperation between several factors might allow tighter regulation of the TK gene and its timely expression along the proliferation pathway. The existence of factors modifying the activity of Egr-1 is suggested by our observation that Egr-1-mediated activation of the TK reporter gene could be seen only in serum-starved cells and not in a population of serum-fed cycling cells (data not shown).

The TKE binding complex appears early and transiently on the MT3 element of the TK promoter at passage between G_0 and G_1, when quiescent cells are induced to proliferate, and it is replaced later by Yi complexes (11, 12). The Yi binding activity of 3T3 cells is absent in G_0, is low throughout G_1, increases sharply as the cells cross the G_1/S boundary, and remains elevated through the S and G_2 phases, showing a close correlation with the G_1/S-specific induction of TK gene expression. This course of induction suggests that the Yi complex may play a more important role in TK gene activation than the TKE complex and is consistent with the observation that the TK gene is also activated at the G_1/S boundary of actively dividing cells, i.e., in the absence of Egr-1 induction. Furthermore, in a detailed dissection of the TK promoter, we have found that mutations and deletions of the MT3 site produce less pronounced effects on overall reporter gene activity than mutations that affect the MT2 site, a site that does not bind TKE. However, the G_0 and early G_1 levels of expression of mutant MT3-containing reporter genes were slightly elevated and exhibited an earlier time of induction after serum release relative to the wild-type TK promoter (reference 16 and unpublished observations).

Egr-1 is a bifunctional regulatory protein. Amino acids 281 to 314 are able to repress transcription on a heterologous DNA-binding domain of GAL4, while the N-terminal domain is a potential activator of Egr-1. It is conceivable, therefore, that in the context of the normal TK gene and under conditions of entry from quiescence into the cell cycle, Egr-1 delays TK expression until the G_1/S boundary, at a time at which the Yi complex is fully induced. Induction of the TK-based reporter gene observed in our transient transfection assays might then be explained by the limited supply of an additional factor(s) necessary for appropriate function of Egr-1. An alternative hypothesis is that Egr-1 functions to alter the regional structure of chromatin in the TK gene and to prepare the TK promoter for further activation at the G_1-to-S transition. In this case, the role of Egr-1 in the proliferation pathway would be more as a G_0-to-G_1 transition factor than as an activator of transcription per se.

ACKNOWLEDGMENTS

We thank Vikas Sukhatme for providing the pCMVEgr-1 and pCMVETTL vectors as well as the Egr-1 polyclonal antiserum and Judith Fridovich-Keil for the CAT reporter genes used in this study.

This work was supported by a grant from The Council for Tobacco Research.

REFERENCES

1. **Ackerman, S. L., A. G. Minden, G. T. Williams, C. Bobonis, and C. Y. Yetung.** 1991. Functional significance of an overlapping consensus binding motif for Sp1 and Zif268 in the murine adenosine deaminase gene promoter. Proc. Natl. Acad. Sci. USA **88**:7523–7527.
2. **Angel, P., E. A. Allegretto, S. T. Okino, K. Hattori, W. J. Boyle, T. Hunter, and M. Karin.** 1988. Oncogene *jun* encodes a sequence-specific trans-activator similar to AP-1. Nature (London) **332**:166–171.
3. **Bohmann, D., T. J. Bos, A. Admon, T. Nishimura, P. K. Vogt, and R. Tjian.** 1987. Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science **238**:1386–1392.
4. **Bradley, D. W., Q. P. Dou, J. L. Fridovich-Keil, and A. B. Pardee.**

1990. Transformed and nontransformed cells differ in stability and cell cycle regulation of a binding activity to the murine thymidine kinase promoter. Proc. Natl. Acad. Sci. USA **87**:9310–9314.
5. **Bravo, R.** 1989. Growth factor inducible genes in fibroblasts, p. 324–343. *In* A. Habenicht (ed.), Growth factors, differentiation factors and cytokines. Springer-Verlag, Heidelberg, Germany.
6. **Cao, X., R. A. Koski, A. Gashler, M. McKiernan, C. F. Morris, R. Gaffney, R. V. Hay, and V. P. Sukhatme.** 1990. Identification and characterization of the Egr-1 gene product, a DNA-binding zinc finger protein induced by differentiation and growth signals. Mol. Cell. Biol. **10**:1931–1939.
7. **Christy, B., and D. Nathans.** 1989. DNA binding site of the growth factor-inducible protein Zif268. Proc. Natl. Acad. Sci. USA **86**: 8737–8741.
8. **Christy, B. A., L. F. Lau, and D. Nathans.** 1988. A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with zinc finger sequences. Proc. Natl. Acad. Sci. USA **85**:7857–7861.
9. **Chu, E., D. M. Koeller, J. L. Casey, J. C. Drake, B. A. Chabner, P. C. Elwood, S. Zinn, and C. J. Allegra.** 1991. Autoregulation of human thymidylate synthase messenger RNA translation by thymidylate synthase. Proc. Natl. Acad. Sci. USA **88**:8977–8981.
10. **Coppock, D. L., and A. B. Pardee.** 1987. Control of thymidine kinase mRNA during the cell cycle. Mol. Cell. Biol. **7**:2925–2932.
11. **Dou, Q. P., J. L. Fridovich-Keil, and A. B. Pardee.** 1991. Inducible proteins binding to the murine thymidine kinase promoter in late G1/S phase. Proc. Natl. Acad. Sci. USA **88**:1157–1161.
12. **Dou, Q. P., P. J. Markell, and A. B. Pardee.** 1992. Thymidine kinase transcription is regulated at G1/S phase by a complex that contains retinoblastoma-like protein and a cdc2 kinase. Proc. Natl. Acad. Sci. USA **89**:3256–3260.
13. **Drummond, I. A., S. L. Madden, P. Rohwer-Nutter, G. I. Bell, V. P. Sukhatme, and S. J. Rauscher III.** 1992. Repression of the insulin-like growth factor II gene by the Wilms' tumor supressor WT1. Science **257**:674–678.
14. **Fridovich-Keil, J. L., J. M. Gudas, I. B. Bryan, and A. B. Pardee.** 1991. Improved expression vectors for eukaryotic promoter-enhanced studies. BioTechniques **11**:572–579.
15. **Fridovich-Keil, J. L., J. M. Gudas, Q. P. Dou, I. Bouvard, and A. B. Pardee.** 1991. Growth-responsive expression from the murine thymidine kinase promoter: genetic analysis of DNA sequences. Cell Growth Differ. **2**:67–76.
16. **Fridovich-Keil, J. L., P. J. Markell, J. M. Gudas, and A. B. Pardee.** 1993. DNA sequences required for serum responsive regulation of expression from the mouse thymidine kinase promoter. Cell Growth Differ. **4**:679–687.
17. **Gashler, A. L., S. Swaminathan, and V. P. Sukhatme.** 1993. A novel repression module, an extensive activation domain, and a bipartite nuclear localization signal defined in the immediate-early transcription factor Egr-1. Mol. Cell. Biol. **13**:4556–4571.
18. **Gross, M. K., and G. F. Merrill.** 1989. Thymidine kinase synthesis is repressed in nonreplicating muscle cells by a translational mechanism that does not affect the polysomal distribution of thymidine kinase mRNA. Proc. Natl. Acad. Sci. USA **86**:4987–4991.
19. **Gudas, J. M., G. B. Knight, and A. B. Pardee.** 1988. Nuclear post-transcriptional processing of thymidine kinase mRNA at the onset of DNA synthesis. Proc. Natl. Acad. Sci. USA **85**:4705–4709.
20. **Gupta, M. P., M. Gupta, R. Zak, and V. P. Sukhatme.** 1991. Egr-1, a serum-inducible zinc finger protein, regulates transcription of the rat cardiac alpha-myosin heavy chain gene. J. Biol. Chem. **266**:12813–12816.
21. **Ito, M., and S. E. Conrad.** 1990. Independent regulation of thymidine kinase mRNA and enzyme levels in serum-stimulated cells. J. Biol. Chem. **265**:6954–6960.
22. **Kadonaga, J. T., K. R. Carner, F. R. Masiarz, and R. Tjian.** 1987. Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell **51**:1079–1090.
23. **Kauffman, M. G., and T. J. Kelly.** 1991. Cell cycle regulation of thymidine kinase: residues near the carboxyl terminus are essential for the specific degradation of the enzyme at mitosis. Mol. Cell. Biol. **11**:2538–2546.
24. **Landschulz, W. H., P. F. Johnson, and S. L. McKnight.** 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240**:1759–1764.
25. **Lau, L. F., and D. Nathans.** 1991. Genes induced by serum growth factors, p. 165–202. *In* P. Cohen and J. G. Foulkes (ed.), Molecular aspects of cellular regulation, vol. 6. Hormonal regulation of gene transcription. Elsevier, Amsterdam.
26. **Lee, W., A. Haslinger, M. Karin, and R. Tjian.** 1987. Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. Nature (London) **325**:368–372.
27. **Lemaire, P., O. Relevant, R. Bravo, and P. Charnay.** 1988. Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. Proc. Natl. Acad. Sci. USA **85**:4691–4695.
28. **Lemaire, P., C. Vesque, J. Schmitt, H. Stunnenberg, R. Frank, and P. Charnay.** 1990. The serum-inducible mouse gene *Krox-24* encodes a sequence specific transcriptional activator. Mol. Cell. Biol. **10**:3456–3467.
29. **Luckow, B., and G. Schutz.** 1987. CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. Nucleic Acids Res. **15**:5490.
30. **Madden, S. L., D. M. Cook, J. F. Morris, A. Gashler, V. P. Sukhatme, and F. J. Rauscher.** 1991. Transcriptional repression mediated by the WT1 Wilms' tumor gene product. Science **253**:1550–1553.
31. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Pardee, A. B.** 1989. G1 events and regulation of cell proliferation. Science **246**:603–608.
33. **Rauscher, F. J., J. F. Morris, O. E. Tournay, D. M. Cook, and T. Curran.** 1990. Binding of the Wilms' tumor locus zinc finger protein to the Egr-1 consensus sequence. Science **250**:1259–1262.
34. **Rollins, B. J., and C. D. Stiles.** 1989. Serum inducible genes. Adv. Cancer Res. **53**:1–31.
35. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
36. **Schreiber, E., P. Matthias, M. M. Muller, and W. Schaffner.** 1989. Rapid detection of octamer binding proteins with mini-extracts, prepared from a small number of cells. Nucleic Acids Res. **17**:6419.
37. **Singh, H., J. H. LeBowitz, A. S. Baldwin, and P. A. Sharp.** 1988. Molecular cloning of an enhancer binding protein: isolation by screening of an expression library with a recognition site DNA. Cell **52**:415–423.
38. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione-*S*-transferase. Gene **67**:31–40.
39. **Stuart, P., M. Ito, C. Stewart, and S. E. Conrad.** 1985. Induction of cellular thymidine kinase occurs at the mRNA level. Mol. Cell. Biol. **5**:1490–1497.
40. **Sukhatme, V. P.** 1990. Early transcriptional events in cell growth: the Egr family. J. Am. Soc. Nephrol. **1**:859–866.
41. **Sukhatme, V. P., X. Cao, L. C. Chang, C. H. Tsai-Morris, D. Stamenkovich, P. C. P. Ferreira, D. R. Cohen, S. A. Edwards, T. B. Shows, T. Curran, M. M. Le Beau, and E. D. Adamson.** 1988. A zinc finger-encoded gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell **53**:37–43.
42. **Wang, Z. Y., and T. F. Deuel.** 1992. An S1 nuclease-sensitive homopurine/homopyrimidine domain in the PDGF A-chain promoter contains a novel binding site for the growth factor-inducible protein Egr-1. Biochem. Biophys. Res. Commun. **188**:433–439.
43. **Yang, H. C., and A. B. Pardee.** 1986. Insulin-like growth factor 1 regulation of transcription and replicating enzyme induction necessary for DNA synthesis. J. Cell. Physiol. **127**:410–416.