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v-*src* Induction of the TIS10/PGS2 Prostaglandin Synthase Gene Is Mediated by an ATF/CRE Transcription Response Element

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We recently reported the cloning of a mitogen-inducible prostaglandin synthase gene, TIS10/PGS2. In addition to growth factors and tumor promoters, the v-*src* oncogene induces TIS10/PGS2 expression in 3T3 cells. Deletion analysis, using luciferase reporters, identifies a region between −80 and −40 nucleotides 5′ of the TIS10/PGS2 transcription start site that mediates pp60$^{v\text{-}src}$ induction in 3T3 cells. This region contains the sequence CGTCACGTG, which includes overlapping ATF/CRE (CGTCA) and E-box (CACGTG) sequences. Gel shift-oligonucleotide competition experiments with nuclear extracts from cells stably transfected with a temperature-sensitive v-*src* gene demonstrate that the CGTCACGTG sequence can bind proteins at both the ATF/CRE and E-box sequences. Dominant-negative CREB and Myc proteins that bind DNA, but do not transactivate, block v-*src* induction of a luciferase reporter driven by the first 80 nucleotides of the TIS10/PGS2 promoter. Mutational analysis distinguishes which TIS10/PGS2 *cis*-acting element mediates pp60$^{v\text{-}src}$ induction. E-box mutation has no effect on the fold induction in response to pp60$^{v\text{-}src}$. In contrast, ATF/CRE mutation attenuates the pp60$^{v\text{-}src}$ response. Antibody supershift and methylation interference experiments demonstrate that CREB and at least one other ATF transcription factor in these extracts bind to the TIS10/PGS2 ATF/CRE element. Expression of a dominant-negative *ras* gene also blocks TIS10/PGS2 induction by v-*src*. Our data suggest that Ras mediates pp60$^{v\text{-}src}$ activation of an ATF transcription factor, leading to induced TIS10/PGS2 expression via the ATF/CRE element of the TIS10/PGS2 promoter. This is the first description of v-*src* activation of gene expression via an ATF/CRE element.

Prostaglandin synthase (PGS) is the key enzyme in the synthesis of the prostanoids (prostaglandins, prostacyclins, and thromboxanes) from arachidonic acid (12). PGS converts arachidonic acid released from membrane stores to PGH_2, the precursor to all prostanoids. Prostanoids modulate many normal physiological processes, including cell division, the immune response, reproduction, and differentiation. Elevated prostanoids are also associated with pathophysiological states such as pain, fever, acute and chronic inflammation, atherosclerosis, and cancer. PGS1 (EC 1.4.99.1) has been well characterized biochemically, pharmacologically, and structurally (55).

We identified in Swiss 3T3 cells a mitogen-induced primary response gene, TIS10 (tetradecanoyl phorbol acetate-induced sequence 10 [35]) that encodes a second prostaglandin synthase (13, 29). Other laboratories have also identified PGS2 as a growth factor-inducible gene in murine fibroblasts (39, 47). TIS10/PGS2 induction by appropriate ligands has subsequently been demonstrated in endothelial cells (21), epithelial cells (22), monocytes and macrophages (58, 33), ovarian granulosa cells (51), osteoblasts (42), and neurons (62). The TIS10/PGS2 gene is also inducible in 3T3 cells by v-*src* (44, 39). Oncogenic transformation by retroviruses often results in the activation of genes whose transcription is induced in response to mitogens. Xie et al. (61) identified one of the cDNAs for a message induced in chicken embryo fibroblasts by activation of a temperature-sensitive v-*src* oncogene as an avian PGS.

pp60$^{v\text{-}src}$, the product of the Rous sarcoma virus v-*src* oncogene, was the first oncogenic protein whose biological activity, a protein tyrosine kinase, was identified (8). pp60$^{v\text{-}src}$ expression is sufficient to initiate and maintain oncogenic transformation (24). However, the mechanism by which pp60$^{v\text{-}src}$ transforms cells is still largely unknown. Several approaches, including identification of pp60$^{v\text{-}src}$ substrates, cloning of cDNAs for messages induced by pp60$^{v\text{-}src}$ expression, and identification of *cis*-responsive elements transcriptionally activated by pp60$^{v\text{-}src}$, have been taken to identify this mechanism. Gene expression can be induced by pp60$^{v\text{-}src}$ through different *cis*-acting regulatory elements, including AP-1 (5, 19, 48), the serum response element (43), the TATA box (2), and a unique *src*-responsive element (10).

Because (i) prostanoids are involved in so many normal and pathophysiological responses and (ii) pp60$^{v\text{-}src}$ is a potent oncogenic protein, the transcriptional regulation of the TIS10/PGS2 gene by pp60$^{v\text{-}src}$ is an important issue. We now report that the ATF/CRE element in the TIS10/PGS2 promoter (13) is essential for induction of TIS10/PGS2 by pp60$^{v\text{-}src}$. We also show that v-*src* induction occurs via a Ras-mediated signal transduction pathway.

MATERIALS AND METHODS

Cell culture. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum. UP1A1 cells, a BALB/c 3T3 cell line transfected with a temperature-sensitive v-*src* gene (37) (kindly provided by David Foster, Hunter College), and BALB/c 3T3 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Nuclear extract preparation. Nuclear extracts were prepared as described by Wu (60). UP1A1 and BALB/c 3T3 cells were grown to 80% confluence in 10% serum at 37°C, shifted to 0.5% serum medium, and then incubated at 39.5°C. After 24

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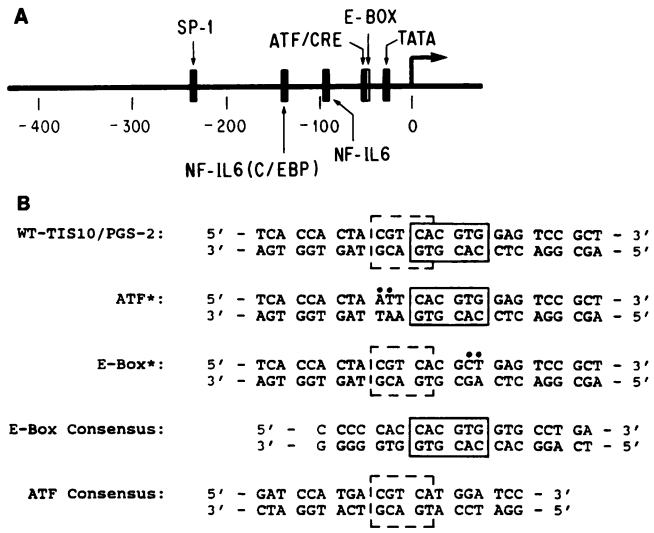

FIG. 1. The TIS10/PGS2 promoter. (A) Potential *cis*-acting transcriptional response elements of the TIS10/PGS2 gene. The sequence of the first kilobase of the TIS10/PGS2 gene has been reported previously (13). The diagram shows the potential response elements of the first 400 nucleotides of the TIS10/PGS2 gene, based on sequence similarities to consensus response elements. Distances are given as nucleotide positions relative to the start site of transcription. (B) The ATF/CRE and E-box region of the TIS10/PGS2 gene and related sequences used in subsequent experiments. WT-TIS10/PGS2 is the sequence of the TIS10/PGS2 gene between oligonucleotides −65 and −39. The solid box indicates the E-box sequence; the dashed box indicates the ATF/CRE core sequence. To create the ATF/CRE mutation, ATF*, the CG of the proximal portion of the ATF/CRE was changed to AT. To create the E-box mutation, E-box*, the TG of the distal portion of the E box was changed to CT. The E-box and ATF/CRE consensus sequences shown are those used in subsequent gel shift competition experiments.

h, half of the cells were shifted to the permissive temperature, 35°C, for 1 h prior to preparation of induced extracts. Noninduced nuclear extract was prepared from cells kept at 39.5°C. Protein concentrations were determined by the Bio-Rad Bradford assay.

Electrophoretic mobility gel shift assays. Gel shift assays were carried out essentially as described by Peterson et al. (41). Oligonucleotide G553 (5′-TCACCACTACGTCACGTGGAG TCCGCT-3′) from nucleotide −65 to −39 of the TIS10/PGS2 gene was end labeled with [^{32}P]ATP. The complementary oligonucleotide was annealed, and the resulting double-stranded DNA was purified. Each 20-μl binding reaction mixture contained 40,000 cpm of probe, 6 μg of nuclear extract, and 6 μg of poly(dI-dC) (Pharmacia). Competitors (Fig. 1) were added to the binding mixture containing poly(dI-dC) and nuclear extract 10 min before addition of the probe; incubation was continued for 15 min. For the supershift experiment, anti-CREB antibody was added to the poly(dI-dC) and nuclear extract 15 min before addition of the probe. Five percent nondenaturing acrylamide gel electrophoresis was performed in the cold at 20 mA for 2 to 3 h. Dried gels were exposed to X-ray film.

Methylation interference experiments. A 108-bp probe between nucleotides −28 and −135 of the TIS10/PGS2 promoter was prepared by PCR using ^{32}P-end-labeled primers. Two femtomoles of probe was used in 25-μl reaction mixtures containing 6 μg of nuclear extract and 8 μg of poly(dI-dC). For methylation interference of DNA binding, probes were methylated with 0.5% dimethyl sulfate for 12 min at 20°C. Competitions were done with a 400-fold excess of competitor DNA. Gel retardation bands were isolated, and DNA was extracted. DNA was analyzed for methylation interference by piperidine cleavage and separation of cleavage products on 10% polyacrylamide sequencing gels (50). Binding and methylation interference studies with recombinant CREB protein were done in a binding mixture containing 1.5 μg of recombinant protein and 5 μg of bovine serum albumin.

Plasmids. The v-*src* expression vector pMV-src and its empty vector, pEVX (28), were provided by David Foster (Hunter College). Dominant-negative Myc expression vectors D106 (56) and In373 (49) were gifts from Charles Sawyers (University of California, Los Angeles). Plasmids expressing wild-type CREB and dominant-negative CREB mutant M1 (18) were from Marc Montminy (Salk Institute). pZIP M17, an H-*ras* dominant-negative expression vector (7), was from Geoffrey Cooper (Harvard University). The pTIS10$_{-40}$ plasmid was constructed by direct cloning of two annealed oligonucleotides from −40 to +3 of the TIS10/PGS2 gene into the *Bgl*II and *Sal*I sites of the pXP2 luciferase expression vector. The pTIS10$_{-80}$ luciferase plasmid was made by cloning PCR-amplified DNA into *Bgl*II and *Sal*I sites of pXP2. PCR was carried out with pTIS10$_L$ (13) as the template, a 5′ oligonucleotide starting at position −80, and a 3′ oligonucleotide ending at +3. The −80WT, E-box*, and ATF* reporters were constructed by cloning the corresponding annealed double-stranded DNA (Fig. 1) into *Bam*HI and *Sal*I sites of the pTIS10$_{-40}$ construct. All constructs were confirmed by sequencing.

Transfections and luciferase assays. DNAs were purified by CsCl centrifugation. NIH 3T3 cells were grown to confluence in 100-mm-diameter dishes and split 1:12 onto 60-mm-diameter dishes (about 4 × 10^5 cells per dish). After 18 h of incubation in 5% CO_2 at 37°C, calcium phosphate DNA precipitates (13) were added. The DNA precipitates were made by mixing DNAs with 250 μl of 0.25 M CaCl and then adding 250 μl *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) phosphate buffer (pH 7.10) dropwise. The mixtures were allowed to precipitate for 10 to 15 min at room temperature before being added to cells. Five hours later, a 15% glycerol shock was performed and medium containing 0.5% serum was added. Cells were harvested 24 h later for luciferase assay. Three micrograms of reporter constructs and 1.5 μg of pMV-src or 1.5 μg of pEVX were used per plate unless otherwise indicated. Triplicate plates were used for all transfections. All experiments were repeated at least twice. Cells were broken by freeze-thaw lysis, and samples were centrifuged. Ten-microliter samples of the supernatants were mixed with 90 μl of 2× LAB buffer (200 mM K_2HPO_4 [pH 7.8], 10 mM, ATP, 2 mM dithiothreitol, 30 mM $MgSO_4$), and luciferase activity was measured in a Lumat luminometer (Berthold) as described previously (13). Luciferase activities were normalized to protein concentration.

RESULTS

A sequence between −80 and −40 nucleotides 5′ of the TIS10/PGS2 transcription start site is responsible for v-*src* induction. The endogenous TIS10/PGS2 gene is induced in 3T3 cells following expression of the v-*src* oncogene (44). In preliminary experiments, we cotransfected pTIS10$_S$ (the luciferase reporter construct containing 371 nucleotides of the TIS10/PGS2 promoter [13]) and either pMV-src, a plasmid expressing the v-*src* oncogene, or the corresponding empty vector, pEVX, into NIH 3T3 cells. Luciferase expression in the

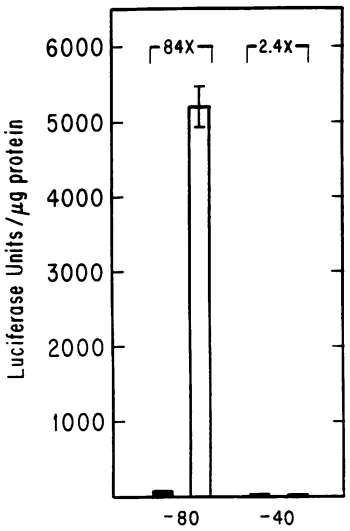

FIG. 2. The TIS10/PGS2 gene has a v-*src* response element between nucleotides −80 and −40 from the start site of transcription. Regions from −80 to +3 and from −40 to +3 of the TIS10/PGS2 promoter were fused to the luciferase reporter gene. NIH 3T3 cells were transfected with 3 μg of $pTIS10_{-80}$ (−80) or 3 μg of $pTIS10_{-40}$ (−40) along with either 1.5 μg of pMV-src, the plasmid that encodes the $pp60^{v\text{-}src}$ protein (open bars), or 1.5 μg of pEVX, the empty expression vector used to created plasmid pMV-src (solid bars). Cells were harvested and assayed for luciferase activity and protein as described in Materials and Methods. The fold inductions, calculated by dividing the luciferase activity in the presence of the v-*src* expression vector by the luciferase activity in the presence of the empty vector, are above columns for each pair of transfections. Three plates of cells were transfected and assayed for each experimental point. Values are averages ± standard deviations.

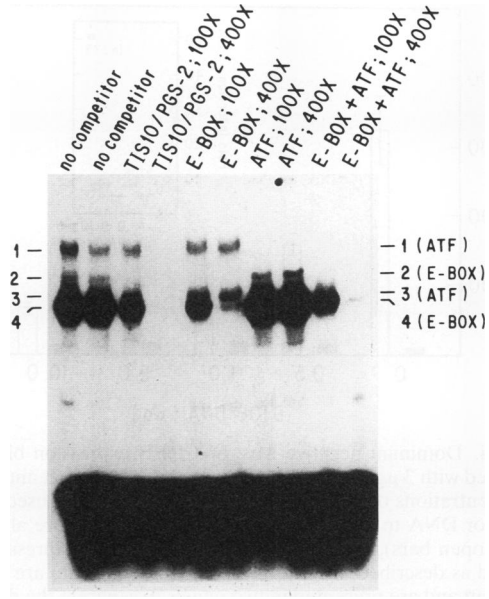

FIG. 3. The TIS10/PGS2 regulatory region has overlapping ATF/CRE- and E-box-binding sites. A 27-nucleotide probe extending from −65 to −39 of the TIS10/PGS2 gene was end labeled with ^{32}P and used as a probe. Nuclear extracts were prepared from UP1A1 cells grown at the permissive temperature. Gel shift experiments were performed as described in Materials and Methods. The sequences used for competition are presented in Fig. 1. Competition data are presented as fold excess of competitor oligonucleotides relative to the labeled probe and are in molar ratios.

presence of pMV-src was 40-fold greater than in the presence of pEVX (data not shown).

The TIS10/PGS2 regulatory region has several consensus sequences for transcriptional activation, including two potential NF-IL6 response elements and an Sp1 consensus sequence (Fig. 1). Further 3′, centered at 50 nucleotides from the transcription start site, are two overlapping consensus regulatory elements. The first sequence, the cyclic AMP (cAMP) response element or ATF/CRE, binds ATF/CREB transcription factors. The second sequence, the E box, binds basic helix-loop-helix transcription factors such as Myc, Max, Mad, and USF. We constructed additional TIS10/PGS2 promoter deletions fused to the luciferase reporter and cotransfected these constructs into NIH 3T3 cells along with either the pMV-src expression vector or the pEVX vector. For constructs between −371 and −80, the response to pMV-src cotransfection was in the range of 40- to 100-fold greater than that for cotransfection with pEVX. However, deletion to nucleotide −40 eliminates induction by $pp60^{v\text{-}src}$ (Fig. 2). We report both the enzymatic activities in luciferase units per microgram of protein as well as the ratios of luciferase activity in the presence of the v-*src* expression vector versus the empty vector. Readers can compare both luciferase specific activities and induction ratios.

The only known regulatory sequences located between −80 and −40 of the TIS10/PGS2 promoter are the overlapping ATF/CRE and E-box sequences. This nine-nucleotide sequence, CGTCACGTG, begins at nucleotide −56 and ends at −48. This region of the TIS10/PGS2 gene and the various constructs used in the following experiments are shown in Fig. 1.

The sequence between nucleotides −65 and −39 of the TIS10/PGS2 gene interacts with DNA-binding proteins that recognize ATF/CRE sequences and E-box sequences. UP1A1 is a BALB/c 3T3 cell line that has been stably transfected with a temperature-sensitive v-*src* gene (37). When UP1A1 cells are shifted to the permissive temperature, transcription from the endogenous TIS10/PGS2 gene is rapidly expressed. We used nuclear extracts from these cells to perform gel shift analyses. Nuclear extracts were prepared from UP1A1 cells grown at the permissive and nonpermissive temperatures, and gel shift analyses were performed with radiolabeled probes extending from −82 to −62 and from −65 to −39 of the TIS10/PGS2 promoter. No retardation of the probe extending from −82 to −62 was observed with nuclear extracts from cells grown at either temperature (data not shown). We observed four retarded bands with nuclear extracts from UP1A1 cells when the sequence from −65 to −39, containing the overlapping ATF/CRE and E-box regions of the TIS10/PGS2 gene, was used as a probe (Fig. 3). The presence of four bands is more clearly demonstrated by competition assays (described below). We saw no differences in the gel mobility patterns with extracts from cells grown at the permissive and nonpermissive temperatures and show only data obtained with extracts from the permissive temperature.

To identify which complexes are associated with the two putative DNA-binding sequences, we used unlabeled ATF/CRE and E-box consensus sequences (Fig. 1) as competitors. Addition of unlabeled E-box competitor eliminates complex 2 and complex 4. In contrast, the ATF/CRE consensus sequence competitor eliminates complexes 1 and 3. The homologous

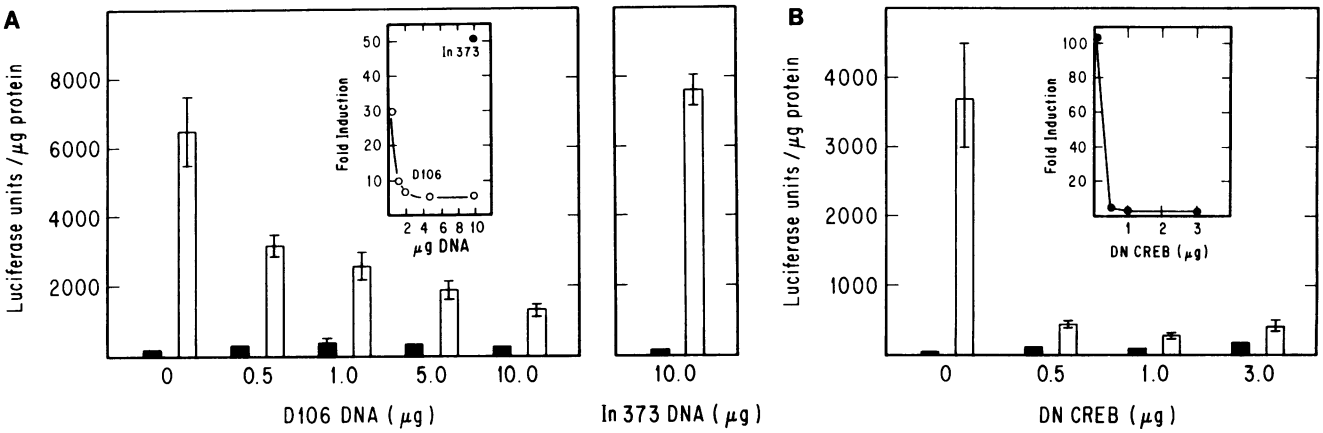

FIG. 4. Dominant-negative Myc or CREB expression blocks v-*src* induction of the pTIS10$_{-80}$ reporter construct. (A) NIH 3T3 cells were transfected with 3 μg of the pTIS10$_{-80}$ reporter construct and increasing amounts of the dominant-negative *myc* construct D106 or In 373. To keep the concentrations of DNA constant, the empty vectors used to create the two *myc* constructs were used to bring the total amount of competitor plus vector DNA to 10 μg. Triplicate plates of cells were also transfected with either 1.5 μg of pMV-src, the plasmid that encodes the pp60$^{v\text{-}src}$ protein (open bars), or 1.5 μg of pEVX, the empty expression vector of pMV-src (solid bars). Cells were harvested and luciferase activity was measured as described in Materials and Methods. Data are presented as described in the legend to Fig. 2. The data shown are all from a single experiment and are presented in two panels to separate the data for the two different Myc expression plasmids. The inset shows the data expressed as fold induction, i.e., luciferase activity observed in the presence of pMV-src divided by luciferase activity in the presence of pEVX. (B) NIH 3T3 cells were transfected with 3 μg of the pTIS10$_{-80}$ reporter construct and increasing amounts of the dominant-negative (DN) CREB construct M1. To keep the concentrations of DNA constant, the empty plasmid for this expression vector was used to bring the total amount of competitor plus vector DNA to 3 μg. Triplicate plates of cells were also transfected with either 1.5 μg of pMV-src, the plasmid that encodes the pp60$^{v\text{-}src}$ protein (open bars), or 1.5 μg of pEVX, the empty expression vector of pMV-src (solid bars).

TIS10/PGS2 sequence competes for binding of all four complexes. Simultaneous competition with the consensus ATF/CRE and E-box sequences also prevents formation of all four complexes. The data suggest (i) that complexes 1 and 3 observed in gel shift analysis with the TIS10/PGS2 promoter region between −65 and −39 are due to binding of nuclear proteins to the ATF/CRE sequence and (ii) that complexes 2 and 4 result from the binding of a nuclear protein(s) to the TIS10/PGS2 E box.

The sequence GTCAC within the overlapping ATF/CRE and E box is conserved in the mouse, rat, and human PGS2 promoters. Using a 32-bp probe from the TIS10 promoter centered on GTCAC but mutated in the ATF/CRE and E-box sites, we performed a gel retardation analysis with UP1A1 cell extract to determine whether another factor binds to the TIS10/PGS2 promoter by recognizing this sequence. We are unable to identify any novel factor which associates specifically with the conserved GTCAC TIS10/PGS2 promoter sequence.

The TIS10/PGS2 ATF/CRE and E-box elements are recognized by DNA-binding proteins with these specificities in vivo. To determine whether the −80 to −40 region of the TIS10/PGS2 gene can interact in cells with proteins that bind to the ATF/CRE and E-box elements, we used dominant-negative transcription factor mutants that recognize either the ATF/CRE or E-box sequence in cotransfection experiments. NIH 3T3 cells were transfected with (i) the pTIS10$_{-80}$ luciferase reporter containing the TIS10/PGS2 promoter sequence from −80 to +3, (ii) either the v-*src* expression vector or the corresponding empty vector, and (iii) increasing amounts of D106, an expression vector that encodes a Myc protein with an intact DNA-binding domain and an inactivated transactivation domain (49). Empty D106 expression vector was used to keep the DNA concentration constant. D106 dominant-negative Myc protein, which binds to E-box sequences, blocks nearly completely v-*src* induction of pTIS10$_{-80}$ (Fig. 4). The In373 plasmid encodes a Myc protein that is unable to bind to E-box sequences but has an intact transactivation domain (56). In373 Myc protein did not block v-*src* induction of pTIS10$_{-80}$, indicating that E-box binding by the dominant-negative D106 protein is required to block TIS10/PGS2 induction.

The dominant-negative CREB protein M1 is altered at a phosphorylation site in the transcriptional activation domain and cannot be converted to an active transcription factor by protein kinase A phosphorylation (18). However, the ATF/CRE-binding domain of the M1 mutant is intact. Cotransfection with the plasmid expressing the M1 dominant-negative CREB protein also blocks v-*src* activation of pTIS10$_{-80}$ (Fig. 4). We conclude that binding, in cells, of nontransactivating proteins to either the ATF/CRE element or the E-box element of the TIS10/PGS2 gene can block v-*src* induction of transcription.

TIS10/PGS2 promoter mutations demonstrate that the ATF/CRE element, but not the E box, is necessary for pp60$^{v\text{-}src}$ induction. It is possible that (i) binding of both an ATF/CREB transcription factor and an E-box transcription factor is necessary for TIS10/PGS2 induction by v-*src*, (ii) a protein(s) binding to only one or the other of these two elements is essential for induction of this gene, or (iii) no protein binding to this region is necessary for induction, but binding to this sequence interferes with induction. To distinguish among these possibilities we performed a mutational analysis.

Three luciferase reporter constructs were prepared. The WT (wild-type) construct maintains the nine-nucleotide ATF/CRE and E-box sequences and the surrounding TIS10/PGS2 sequences intact but incorporates the restriction sites used to make the other two constructs. In the E-box* construct, the last two bases of the TIS10/PGS2 E box are mutated (Fig. 1). In the ATF* construct, the first two bases of the TIS10/PGS2 ATF/CRE element are altered. These three constructs, as well as the pTIS10$_{-80}$ reporter construct, were transfected into NIH 3T3 cells along with either pMV-src or pEVX. Reconstruction of the wild-type TIS10/PGS2 reporter (the WT construct) with

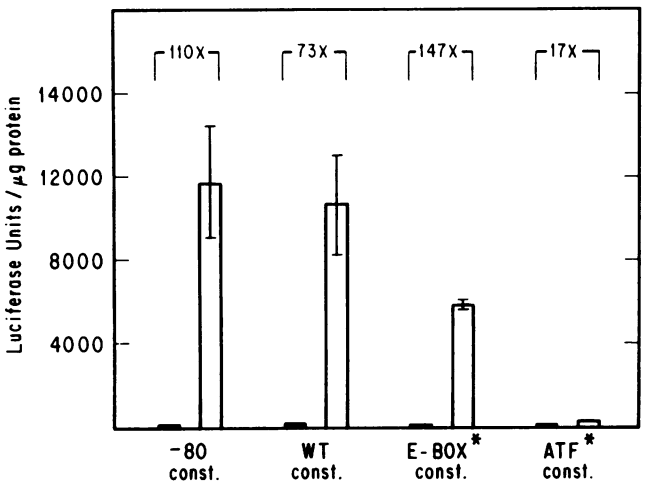

FIG. 5. Mutation of the E box of the TIS10/PGS2 promoter does not affect v-*src* induction, but mutation of the ATF/CRE element attenuates v-*src* induction. Promoter constructs with the mutations described in Fig. 1 were created with the luciferase expression vector. The WT construct (const.) has the same sequence as the −80 deletion construct, with the exception of an added *Sal*I restriction site in the front of the −40 promoter region to facilitate cloning (see Materials and Methods). The E-box* and ATF* constructs have the same nucleotide sequence as the WT construct, with the exception of the two nucleotide mutations shown in Fig. 1. Each of these reporter constructs (3 μg) was transfected into NIH 3T3 cells along with 1.5 μg of the v-*src* expression plasmid pMV-src (open bars) or its empty vector pEVX (solid bars). Triplicate plates were used for each assay. Luciferase assays were performed, and the data are presented as described in the legend to Fig. 2.

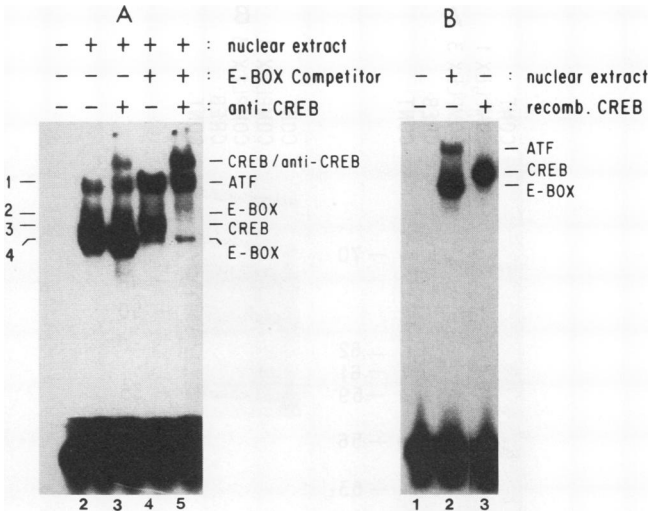

FIG. 6. The TIS10/PGS2 regulatory region forms complexes that contain CREB, a second member of the ATF family. (A) Nuclear extract, anti-CREB antibody (a gift from Marc Montminy), and the labeled 27-nucleotide probe from −65 to −39 of the TIS10/PGS2 regulatory region were incubated as described in Materials and Methods and then subjected to electrophoresis. In lanes 4 and 5, the E-box competitor (Fig. 1) was included at a 40-fold molar excess. The ATF and E-box designations for retarded complexes are based on the competition data of Fig. 3. The CREB and CREB/anti-CREB designations are based on the antibody supershift data shown here. (B) The TIS10/PGS2 probe was incubated either with nuclear extract or with recombinant CREB protein (the gift of Marc Montminy) prior to electrophoresis.

new cloning sites has no significant effect on basal expression, pp60$^{v\text{-}src}$-stimulated expression, or the fold induction in response to pp60$^{v\text{-}src}$ expression (Fig. 5). Although the absolute level of pp60$^{v\text{-}src}$-induced luciferase activity for the E-box mutant is slightly reduced (about twofold) in comparison with the WT construct, the basal level of luciferase activity is also reduced. The fold induction of pp60$^{v\text{-}src}$-induced luciferase activity for the E-box* mutant (147-fold) is actually increased over that observed with the WT construct (73-fold). In contrast, mutation of the TIS10/PGS2 ATF/CRE element extensively reduces induction by pp60$^{v\text{-}src}$. We conclude (i) that the TIS10/PGS2 E box is not required for regulation by pp60$^{v\text{-}src}$ and (ii) that the ATF/CRE sequence CGTCA at nucleotides −56 to −52 is essential for pp60$^{v\text{-}src}$-mediated TIS10/PGS2 induction.

A 27-nucleotide sequence identical to the TIS10/PGS2 promoter sequence between −64 to −38, placed in front of a minimal thymidine kinase promoter, was unable to confer pp60$^{v\text{-}src}$ inducibility to a luciferase reporter gene (data not shown). These data suggest the ATF/CRE element is necessary but not sufficient for induction by pp60$^{v\text{-}src}$.

CREB and a second ATF family member bind to the TIS10/PGS2 ATF/CRE element. There exists an extensive family of proteins that recognize ATF/CRE sequences (6, 20). To more completely characterize the proteins that bind to the TIS10/PGS2 ATF/CRE element, we used antibody supershift experiments. Two distinct retarded complexes (complex 1 and complex 3) are formed between the TIS10/PGS2 ATF/CRE element and nuclear extracts from UP1A1 cells (Fig. 3). CREB is the best-characterized member of the ATF family (6). Addition of anti-CREB antibody to the gel retardation reaction prior to electrophoresis causes a supershift of complex 3, the more rapidly migrating of the two ATF/CRE complexes (Fig. 6A). This supershift result is made particularly clear when the anti-CREB experiment is done in the presence of unlabeled E-box competitor, to eliminate complex 2 and complex 4 (Fig. 6B, lanes 4 and 5). We conclude that complex 3 contains CREB and that complex 1 contains another ATF family member(s). To confirm this hypothesis, we compared the complexes formed with this region of the TIS10/PGS2 promoter by UP1A1 nuclear extracts and by recombinant CREB protein (Fig. 6B). Recombinant CREB protein forms a complex that migrates similarly to complex 3 formed with nuclear extracts.

Methylation interference experiments define the contact points of the proteins in complexes 1 and 3 with the TIS10/PGS2 ATF/CRE region. To further characterize the ATF/CRE region of the TIS10/PGS2 gene, we determined which guanine residues, when methylated, interfere with gel retardation complex formation with UP1A1 nuclear extracts and with recombinant CREB protein. TIS10/PGS2 probes labeled on either the upper or lower strand with ^{32}P and subjected to limited methylation with dimethyl sulfate (see Materials and Methods) were incubated with UP1A1 nuclear extracts or with recombinant CREB protein and subjected to electrophoresis. The incubations also contained unlabeled E-box competitor for complex 2 and complex 4. Retarded complexes and free probe were identified by autoradiography, excised, subjected to piperidine cleavage, and subjected to electrophoresis.

Recombinant CREB protein is unable to interact with the probe in which guanines at positions −53 and −56 on the lower strand of the TIS10/PGS2 promoter are methylated (Fig. 7). These are the guanines in the lower strand of the consensus

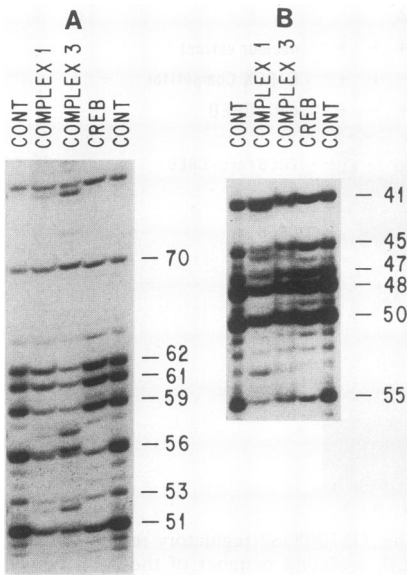

FIG. 7. The proteins present in complexes 1 and 3 formed by 3T3 cell nuclear extracts and the TIS10/PGS2 promoter make contacts with the guanines of the upper and lower strands of the ATF/CRE region. ^{32}P-labeled upper- and lower-strand probes were subjected to limited methylation with dimethyl sulfate, incubated with nuclear extracts from UP1A1 cells or with recombinant CREB protein in the presence of excess E-box competitor, and subjected to electrophoresis in a nondenaturing gel. Retarded complexes 1 and 3 (the retarded CREB complex) and free probe (CONT) were identified by autoradiography and cut from the gel. DNA was isolated, subjected to piperidine cleavage, and analyzed on a sequencing gel. Labeled lower-strand probe was used for panel A; labeled upper strand probe was used for panel B.

ATF/CRE element. Methylation of these guanines also interferes with formation of complex 1 and complex 3 from the UP1A1 extract. In addition, however, methylation of guanines at positions −51, −59, −61, and −62 on the lower strand results in interference of binding of the proteins present in complexes 1 and 3 from the nuclear extract. Methylation of guanine −55 on the upper strand, located in the consensus ATF/CRE element of the TIS10/PGS2 promoter, prevents binding of recombinant CREB protein and the proteins present in complexes 1 and 3. Methylation of guanine 45 or 47, both of which lie 3′ of the overlapping ATF/CRE and E-box sequences, also prevents protein(s) present in complex 1 from binding to the TIS10/PGS2 probe.

Dominant-negative Ras protein blocks v-*src* induction of the TIS10/PGS2 gene. Signal transduction mediated by pp60$^{v\text{-}src}$ and other cellular Src family kinases can be distally modulated by Ras (38, 54). To investigate whether the *ras* gene product might mediate v-*src* induction of the TIS10/PGS2 gene, we again used a dominant-negative strategy. We cotransfected increasing amounts of pZIPM17, a plasmid expressing a dominant-negative H-Ras protein, into NIH 3T3 cells along with constant amounts of pTIS10$_{-80}$ and either pMV-src or pEVX (Fig. 8). A dominant-negative CREB experiment was performed as a control. Dominant-negative Ras inhibits almost 90% of pp60$^{v\text{-}src}$-induced pTIS10$_{-80}$ activity, on the basis of total luciferase units per microgram of protein. Because the dominant-negative Ras protein also lowers the basal level of pTIS10$_{-80}$ expression, Ras inhibition plateaus at 63% when fold induction values are compared. These data suggest that pp60$^{v\text{-}src}$ activates TIS10/PGS2 gene expression through a Ras-dependent pathway.

DISCUSSION

Elevated prostaglandin production is one of many alterations in fibroblast phenotype resulting from Rous sarcoma virus transformation (3, 23). We demonstrate here that the ATF/CRE element of the TIS10/PGS2 gene, a mitogen-stimulated immediate-early (31) or primary-response (26) gene, is necessary for v-*src* induction in 3T3 cells. In the context of the TIS10/PGS2 regulatory region, the ATF/CRE element confers strong inducibility by v-*src*. Our data suggest that an ATF or ATF-related transcription factor(s) is activated by pp60$^{v\text{-}src}$. Dominant-negative Ras expression blocks v-*src* in-

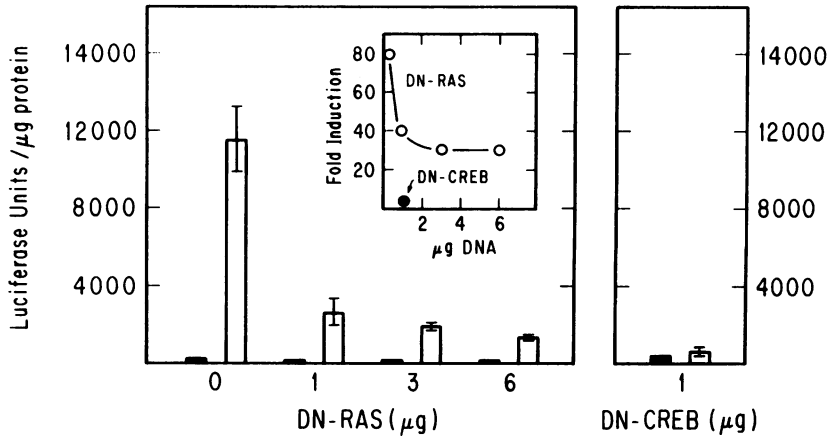

FIG. 8. Dominant-negative Ras expression blocks v-*src* induction of the pTIS10$_{-80}$ reporter construct. NIH 3T3 cells were transfected with 3 μg of the pTIS10$_{-80}$ reporter construct and increasing amounts of the dominant-negative Ras construct pZIPM17. To keep the amount of DNA constant, the empty plasmid for the Ras expression vector was used to bring the amount of competitor plus vector DNA to 6 μg. Triplicate plates of cells were also transfected with either 1.5 μg of the v-*src* expression plasmid pMV-src (open bars) or its empty vector pEVX (solid bars). Cells transfected with the dominant-negative CREB expression vector M1 were included in this experiment for comparison. Luciferase assays were performed, and data are presented as described in the legend to Fig. 2.

duction of a TIS10/PGS2-luciferase reporter. The simplest interpretation consistent with these data is that Ras mediates the $pp60^{v\text{-}src}$-initiated activation of an ATF factor that binds to the ATF/CRE element of the TIS10/PGS2 promoter and stimulates transcription.

$pp60^{v\text{-}src}$ is reported to modulate gene expression through serum response elements of the egr1/TIS8 gene (43), a dyad symmetry element and the Sis-inducible factor-responsive element of the c-*fos* gene (16), the CCAAT and TATAA elements of the *junB* gene (2), and a *src*-responsive unit of the 9E3/CEF-4 gene (10). The collagenase, tumor growth factor β1, and stromolysin genes are activated relatively late after $pp60^{v\text{-}src}$ expression and require synthesis of intervening proteins. Moreover, AP-1 sites confer responsiveness to v-*src* expression for all of these genes (5, 19, 48). ATF/CRE mediation is a previously unreported mechanism for v-*src* induction of gene expression.

Contrary to its primary-response induction in fibroblasts (29, 39, 47), TIS10/PGS2 induction in granulosa cells is dependent on protein synthesis (53). A C/EBPβ-binding sequence in the promoter of the rat PGS2 gene has been identified as a key element in gonadotrophin regulation; the sequence 5′-TTAT GCAAT-3′ located at nucleotides −140 to −132 in the rat PGS2 gene promoter contributes to transcriptional regulation by pituitary hormones and forskolin (51, 52). Mutation of the C/EBP sequence reduces forskolin induction of a rat promoter-reporter construct by 50% (52), suggesting that other regions of the rat PGS2 gene may also contribute to regulation by forskolin and pituitary hormones.

The ATF/CRE element is present in a number of viral and eukaryotic promoters (6, 64). About 10 ATF/CREB family members have been cloned. Thus, the ATF/CRE sequence is a widely used regulatory element that confers complex transcriptional regulatory features on many genes. The transactivation, selectivity, and specificity of ATF/CREB transcription factors are achieved in many ways; they exhibit great functional diversity, through the formation of heterodimers and homodimers (6, 20, 32, 64). ATF proteins are activated by several signal transduction pathways. CREB (18), CREM (14), and ATF-1 (45) are transcriptionally activated by cAMP-mediated protein kinase A phosphorylation. A p120 ATF protein is phosphorylated by protein kinase C (1). ATF/CREB proteins can interact with other nuclear proteins to modulate activity; ATF/CREB proteins form complexes with E1a (36) and Jun (50). Through alternative splicing, one gene can encode both ATF activators and antagonists (15). Alterations in the ATF/CRE consensus sequence and adjacent sequences can have profound effects on target gene expression (11, 20).

Antibody supershift experiments demonstrate that both CREB and at least one other ATF protein can bind to the TIS10/PGS2 ATF/CRE element. We could not restore, with a wild-type CREB expression vector, $pp60^{v\text{-}src}$-dependent $pTIS10_{-80}$ induction to NIH 3T3 cells expressing a dominant-negative CREB protein (data not shown). Thus, CREB may not be the ATF transcription factor mediating TIS10/PGS2 induction by v-*src*. Alternatively, CREB may be the $pp60^{v\text{-}src}$-activated transcription factor, but production of excess CREB may squelch transcriptional activation by competing for a limiting coactivator necessary for interaction with the transcription initiation complex.

We see no differences in gel shift patterns with extracts from cells grown at the permissive and nonpermissive temperatures for expression of functional $pp60^{v\text{-}src}$, suggesting that $pp60^{v\text{-}src}$ induces TIS10/PGS2 by activating a transcription factor(s) without changing its DNA binding. This mechanism is true for several transcription factors, including CREB itself (27). Methylation interference experiments demonstrate that proteins present in nuclear extracts contact an extended region of the TIS10/PGS2 sequence compared with recombinant CREB. This may be due to an ATF protein that makes contact over a larger DNA sequence than does CREB. Alternatively, auxiliary factors may form complexes with ATF proteins at the TIS10/PGS2 ATF/CRE element, creating additional contact sites.

The 5′-CGTCACGTG-3′ sequence in the TIS10/PGS2 gene may allow for increased regulatory complexity by the overlap of the ATF/CRE element and the E box. Our mutational data suggest that the TIS10/PGS2 E box is not involved in v-*src* induction. Antibodies to Myc do not supershift the complexes formed between the TIS10/PGS2 E box and UP1A1 nuclear extracts (data not shown). However, the E box is a core element that is recognized by a wide range of basic helix-loop-helix homodimeric and heterodimeric transcription factors. E-box elements and cell-type-specific basic helix-loop-helix dimers that modulate expression regulate the acetylcholine receptor (4), tyrosine hydroxylase (17, 63), and calcitonin (40) genes. Cell-type-specific E-box-binding proteins may be involved in the modulation of TIS10/PGS2 expression in specific cell types, e.g., myoblasts or neurons. The E box might also serve as a site of cell-type-specific repression, preventing TIS10/PGS2 expression. An E-box-binding protein that can repress transcription has recently been described (34). In this regard, it is interesting that differentiation-deficient murine myoblast cell lines are lacking MyoD (a basic helix-loop-helix protein that binds to the E box) and synthesize prostaglandin E_2, while the parental myoblast cells express MyoD and do not produce prostaglandin (46). Moreover, MyoD overexpression in differentiation-deficient myoblast cell lines suppresses prostaglandin E_2 production (59).

The TIS10/PGS2 gene can be activated by a variety of extracellular stimuli, including platelet-derived growth factor (PDGF), colony-stimulating factor, serum, endotoxin, and interleukins. $pp60^{v\text{-}src}$ and other cellular Src family tyrosine kinases are thought to be involved in signal transduction pathways initiated by several of these ligands, including PDGF (30), colony-stimulating factor (9), and interleukin-2 (25). Microinjection of kinase-deficient, dominant-negative mutants of Src and Fyn and microinjection of antibodies to Src, Fyn, or Yes demonstrate that the cellular Src family kinases play a required role in PDGF-stimulated DNA synthesis in NIH 3T3 cells (57). It seems likely that $pp60^{v\text{-}src}$ is also involved in the signal transduction pathways leading from at least some of these ligands to TIS10/PGS2 gene transcription. Dominant-negative CREB blocks serum and PDGF induction of the $pTIS10_S$ reporter construct (extending from nucleotide −371; data not shown). No serum response elements or Sis-inducible elements are present in the TIS10/PGS2 promoter region that confers responsiveness of luciferase constructs to these agents (13). It seems likely, therefore, that the TIS10/PGS2 ATF/CRE element also plays a role in the induction of transcription of this gene by several ligands.

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