Transcriptional Regulation of the Transforming Growth Factor-β2 Promoter by cAMP-responsive Element-binding Protein (CREB) and Activating Transcription Factor-1 (ATF-1) Is Modulated by Protein Kinases and the Coactivators p300 and CREB-binding Protein*

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Transcription of the transforming growth factor-β2 (TGF-β2) gene is dependent on a cAMP-response element/activating transcription factor (CRE/ATF) site that is bound by CREB and ATF-1 as well as an E-box motif that is bound by upstream stimulating factors 1 and 2 (USF1 and USF2). To identify additional factors involved in the expression of the TGF-β2 gene, we employed F9 embryonal carcinoma (EC) cells, which express TGF-β2 only after the cells differentiate. We show that overexpression of the transcription factors, CREB, ATF-1, USF1, and USF2 dramatically increases TGF-β2 promoter activity in F9-differentiated cells. We further show that the coactivators p300 and CBP up-regulate the TGF-β2 promoter when CREB and ATF-1 are expressed in conjunction with protein kinases that phosphorylate CREB on serine 133 and ATF-1 on serine 63. Importantly, we identify the presence of serine 133-phosphorylated CREB in the nucleus of F9-differentiated cells but not in the nucleus of F9 EC cells. This phosphorylated form is present in whole cell extracts of both the parental and differentiated cells, suggesting that nuclear accumulation of serine 133-phosphorylated CREB is regulated during differentiation of F9 EC cells and is likely to play an important role in the activation of the TGF-β2 gene.

Transforming growth factor-β2 (TGF-β2) is a member of the TGF-β superfamily, which represents a growing number of structurally related yet functionally distinct polypeptides involved in the regulation of a broad range of cellular events, including cell growth and differentiation as well as tissue morphogenesis (reviewed in Refs. 1–3). A subset of this superfamily are the TGF-βs, which consists of at least five genes encoding distinct proteins in vertebrates (referred to as TGF-β1–5). TGF-β1–3 are the mammalian homologs and share approximately 70% amino acid sequence homology. Despite this high sequence homology, each isoform has a distinct temporal and spatial pattern of development (4–8). Interestingly, TGF-β1, -β2 and -β3 null mice show distinctive developmental defects with little to no phenotypic overlap. TGF-β1 knockout mice die shortly after birth due to multifocal inflammatory disease (9). TGF-β2-null mice exhibit perinatal lethality with a wide range of developmental defects including cardiac, craniofacial, limb, lung, spinal column, eye, inner ear, and urogenital defects (10). Mice that lack the TGF-β3 isoform die within 20 h after birth due to delayed pulmonary development and defective palatogenesis (11). Thus, it appears that there are numerous non-compensated functions between each of the TGF-β isoforms. Furthermore, striking differences exist between the 5′-flanking regions of each gene, suggesting that differences in isoform expression may be mediated through tissue-specific gene transcription (12–16). TGF-β2 first appears in the preimplantation blastocyst (4, 17) and continues into adulthood (18), where it seems to play important roles in a host of biological functions including extracellular matrix production, wound healing, and regulation of the immune system (1–3). The studies presented here focus on the transcriptional regulation of the TGF-β2 gene in embryonal carcinoma (EC) cells and EC-differentiated cells, which represent an in vitro model system of early embryonic development. In the current study, F9 EC cells were utilized. These cells resemble biochemically and morphologically the inner cell mass of the early mouse embryo and under normal culture conditions exhibit very limited spontaneous differentiation (19). Treatment of F9 EC cells with retinoic acid (RA) induces differentiation toward an extraembryonic endoderm-like phenotype (20).

Utilizing the F9 EC model system, previous work by this laboratory and others have demonstrated that TGF-β2 is activated at both the RNA and protein levels when F9 cells are induced to differentiate with RA (21, 22). Important for the modulation of the TGF-β2 gene during the differentiation of F9 EC cells is the presence of a critical positive regulatory region in the TGF-β2 gene promoter, localized between −77 and +63, where +1 is the transcription start site (23, 24). Within this positive regulatory region are two cis-regulatory elements: a CRE/ATF site positioned at −74 to −67 (23, 24) and an E-box motif located between −50 and −45 (25). The nucleotide sequence of and spacing between both of these sites are evolutionarily conserved in the human, chicken, and mouse promoters.
The catalytic subunit of protein kinase A (PKA) and calmodulin demonstrate that the activity of CREB and ATF-1 is enhanced by we demonstrate that ATF-1, CREB, USF1, and USF2 function are poorly understood, they are involved in the regulation of a canonical CACGTG core DNA sequence (termed an E-box) proteins that are ubiquitously expressed and bind as dimers to promoter in F9-differentiated cells had not been elucidated.

USF1 and USF2 are basic/helix loop helix/leucine zipper proteins that are ubiquitously expressed and bind as dimers to a canonical CACGTG core DNA sequence (termed an E-box) (29–32). Although the biological functions of the USF proteins are poorly understood, they are involved in the regulation of a wide variety of genes including α1(I) collagen (33), the CYP1A1 gene (34), and the human immunodeficiency virus type 1 long terminal repeat (35). Recent findings have identified USF1 as a phosphoprotein (36), which may contribute to its regulation. Additionally, USF1 proteins do not appear to interact with nucleosomes that are associated with histone H1 (37), but they preferentially bind nucleosomal DNA highly acetylated on histone H4 (38).

The CRE/ATF transcription factors, ATF-1 and CREB have been well characterized, exhibit approximately 70% homology overall, and are over 90% homologous within their DNA binding, dimerization, and kinase-inducible domains (39). The kinase-inducible domain contains multiple phosphorylation sites that are phosphorylated by a number of different kinases (reviewed in Refs. 39 and 40). Within this domain, specific phosphorylation at serine 63 of ATF-1 or at serine 133 of CREB induces a stable interaction with the coactivators p300 and the CREB-binding protein, CBP (41–44). p300 and CBP are structurally similar, functionally redundant proteins (45, 46) essential for proper development (47–49). These proteins function as co-activators by interacting with a wide range of transcription factors, including MyoD (50), NF-κB (51, 52), signal transducer and activator of transcription 1 and 2 (53, 54), Smad3 (55), retinoid X receptor/retinoic acid receptor (56), ER (57), and YY1 (58), which appear to target p300/CBP to specific promoters and bridge transcription factors to the basal transcriptional machinery by binding TFIIB (42), TBP (59, 60), and RNA polymerase II (61, 62). Additionally, p300/CBP have been shown to acetylate histones (H2A, H2B, H3, and H4) intrinsically (63, 64) and through their association with p300/CBP-associated factor (65, 66). Acetylation of histones is thought to destabilize nucleosomes and facilitate access of regulatory factors to DNA (reviewed in Refs. 67 and 68). p300/CBP have also been shown to acetylate several transcription factors including p53 (69), NF-Y (70), and GATA-1 (71), which influences their binding to DNA.

The primary purpose of this study was to understand how the TGF-β2 gene is regulated in F9-differentiated cells. First, we demonstrate that ATF-1, CREB, USF1, and USF2 function in vivo to up-regulate TGF-β2 promoter activity. We also demonstrate that the activity of CREB and ATF-1 is enhanced by the catalytic subunit of protein kinase A (PKA) and calmodulin kinase IV (CaMKIV). Additionally, since phosphorylation of CREB at serine 133 and ATF-1 at serine 63 by these kinases has been shown previously to recruit p300 and CBP (41–44), we examined the ability of p300 and CBP to modulate TGF-β2 promoter activity. We determined that p300 and CBP augment TGF-β2 promoter activity when expressed in conjunction with CREB or ATF-1 and the catalytic subunit of PKA or constitutively active CaMKIV. This activation is dependent on an intact CRE/ATF site but not an intact E-box. We also show that the serine 133-phosphorylated form of CREB is present in the nucleus of F9-differentiated cells but not in the nucleus of F9 EC cells. Importantly, this phosphorylated form is present in whole cell extracts of both the parental and differentiated cells, suggesting that nuclear accumulation of serine 133-phosphorylated CREB is regulated during differentiation of F9 EC cells. In view of the findings presented here, we propose that accumulation of serine 133-phosphorylated CREB in the nucleus of F9-differentiated cells allows for recruitment of p300/ CBP to the TGF-β2 promoter, and this is likely to play an important role in the activation of the TGF-β2 gene when EC cells differentiate.

Experimental Procedures

Materials—Dulbeco’s modified Eagle’s medium HG-21 and Ham’s F-12 were purchased from Life Technologies, Inc. Fetal bovine serum was obtained from HyClone (Logan, UT). All-trans-RA was purchased from Acros Organics, a division of Fisher. All other chemicals, including protease and phosphatase inhibitors, gelatin, and dibutyryl cAMP were purchased from Sigma, unless otherwise indicated.

Cell Culture and Differentiation of EC Cells—F9 EC cells were maintained in Dulbeco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and grown on tissue culture dishes coated with 0.1% gelatin. When cellular extracts were prepared, differentiation of F9 EC cells was induced by a 4-day treatment with 5 μM RA. When differentiated cells were used in transfection studies, they were treated for 3 days with 5 μM RA before transfection. Stock cultures and all experimental cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2.

Transient Transfection Assay—F9-differentiated cells were transfected by the calcium phosphate precipitation method as modified by our laboratory (29). The plasmids utilized in each study are modified by our laboratory (23). The plasmids utilized in each study. The p300-CHA, p53, or p53/CREB plasmid was transfected into each study and the p300/CREB plasmid was transfected in conjunction with p53 or p53/CREB plasmid.

Cloning and expression of the plasmids p300-CHA, p53, or p53/CREB plasmid were performed by Dr. Richard Goodman. The expression plasmid for p300, pCMVβ300-CHA, was obtained from Dr. Richard Davidson and contains a β300 cDNA insert from nucleotides 1134–8329 with a C-terminal HA tag cloned into the pCI vector (78). The pSRE4 plasmid was provided by Dr.

2 P. Wilder and A. Rizzino, unpublished observations.
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Steven K. Hanks and contains the cDNA for the human PKA type α catalytic subunit driven by the CMV promoter in the pcD vector (79). pRSV-CaMKII(1–290) contains a cDNA insert that contains residues 1–290 of CaMKII and codes for a constitutively active form of the kinase (80). pRSV-Mouse CaMKIV(1–313) contains residues 1–313 of the mouse cDNA of CaMKIV and produces a constitutively active form of the kinase (80). The CaM kinase expression plasmids were provided by Dr. Richard Maurer. All plasmids were purified by Qiagen (Chatsworth, CA) tip-500 columns.

Preparation of Nuclear and Whole Cell Extracts—Nuclear extracts of F9 EC cells and F9-differentiated cells (4-day treatment with RA) were prepared as described previously (25) with minor modifications described here. All buffers were supplemented with the following protease and phosphatase inhibitors: 2.5 kallikrein-inactivating units/ml aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, 20 μg/ml soybean trypsin inhibitor, 2.5 mM benzamide, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml chymostatin, 2 μg/ml pepstatin A, 1 mM sodium molybdate, 2 mM sodium vanadate, and 5 mM sodium fluoride. Nuclear extracts were not subjected to dialysis, but following nuclear extraction, they were stored at −80 °C. Whole cell lysates from F9 EC and F9-differentiated cells (4-day treatment with RA) were prepared in the following manner. Cells were washed twice in ice-cold PBS and scraped into lysis buffer containing 10 mM Tris, pH 7.6, 150 mM NaCl and 1% Triton X-100 supplemented with the following protease and phosphatase inhibitors: 2.5 kallikrein-inactivating units/ml aprotinin, 0.2 mM phenylmethylsulfonyl fluoride, 20 μg/ml soybean trypsin inhibitor, 2.5 mM benzamide, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml chymostatin, 2 μg/ml pepstatin A, 1 mM sodium molybdate, 2 mM sodium vanadate, and 5 mM sodium fluoride. Cells were lysed on ice for 30 min followed by centrifugation at 6000 × g for 2 min. Supernatants were stored at −80 °C.

Western Blot Analysis—For Western blot analysis, the PhosphoPlus CREB (Ser133) antibody kit from New England Biolabs (Beverly, MA) was used. Briefly, 20 μg of nuclear or whole cell extracts were fractionated by 14% SDS-polyacrylamide electrophoresis and transferred to an Immobilon-P (polyvinylidine difluoride) membrane (Millipore Corp., Bedford, MA). Following transfer, membranes were washed briefly in 1 × Tris-buffered saline (TBS), pH 7.6, and then blocked for 1 h at room temperature in blocking buffer (1 × TBS, 0.1% Tween 20, 5% (w/v) nonfat dry milk). The membranes were washed three times for 5 min each in TBS/T (1 × TBS, 0.1% Tween 20) and then incubated with a 1:1000 dilution of either CREB antibody or P-CREB antibody in primary antibody dilution buffer (1 × TBS, 0.1% Tween 20, 5% bovine serum albumin) overnight at 4 °C. Membranes were again washed three times with TBS/T and then incubated with a 1:2000 dilution of the horseradish peroxidase-conjugated secondary antibody in blocking buffer for 1 h at room temperature. Following three washes with TBS/T, the horseradish peroxidase-conjugated secondary antibody was detected using LumiGLO (New England Biolabs) as specified by the manufacturer and exposed to x-ray film. Protein expression was quantitated using a densitometer.

RESULTS

The Transcription Factors ATF-1, CREB, USF1, and USF2 Stimulate the TGF-β2 Promoter in F9-differentiated Cells—As previously reported, transcription of the TGF-β2 gene is dependent on a CRE/ATF element located from −74 to −67 and an E-box motif between −50 and −45 relative to the transcription start site (Fig. 1) (29–35). Electrophoretic gel mobility shift assay analysis determined that CREB and ATF-1 bind to the CRE/ATF site in the TGF-β2 promoter (Ref. 27 and data not shown) and USF1 and USF2 bind to the E-box motif (25). We have also shown that transient transfection of a dominant negative USF plasmid decreases (by approximately 80%) the activity of the TGF-β2 promoter/reporter gene construct, pβ2–77E (Fig. 2A). The pβ2–77 construct contains the TGF-β2 promoter region located between −77 and +63 in relationship to the transcription start site and drives the expression of the CAT reporter gene (Fig. 1). Prior to the current study, the function of the E-box-binding proteins and the CRE/ATF transcription factors at the TGF-β2 promoter had not been elucidated. Therefore, we initially transiently transfected expression plasmids for USF1, USF2, ATF-1, and CREB with pβ2–77 into F9-differentiated cells. When expression plasmids for either USF1 or USF2 were co-transfected with pβ2–77 into F9-differentiated cells, we observed a 60–80-fold activation of pβ2–77 with USF1 and a 20-fold stimulation with USF2 (Fig. 2A). When both USF1 and USF2 were transfected in equal amounts, the USF2 expression plasmid appeared stronger. From these studies, it appears that USF1 homodimers activate the TGF-β2 promoter more strongly than USF2 homodimers and that USF1/USF2 heterodimers have a transactivation potential that corresponds to USF2. As expected, when USF1 and USF2 were expressed with the pβ2–40 construct, which contains only the TATA box of the TGF-β2 promoter, no stimulation was observed (data not shown).

When expression plasmids for ATF-1 and CREB were co-transfected with pβ2–77 into F9-differentiated cells, we observed a 60–80-fold activation of the TGF-β2 promoter/reporter gene constructs were named pβ2–n, where n represents the number of nucleotides upstream of the transcription start site. pβ2–77 contains the −77/+63 fragment of the human TGF-β2 promoter. The mutant construct pβ2–77E harbors a two-base pair mutation (shown in lowercase type) in the CRE/ATF site. The mutant construct pβ2–77E contains a two-base pair mutation (shown in lowercase type) in the E-box motif.

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80, 82), protein kinase C (83), and PKA (84) are able to phosphorylate CREB and ATF-1 at these specific serine residues. Since PKA activity increases with F9 differentiation (85, 86) and because we have observed that the addition of cAMP to F9-differentiated cells induces at least a 3-fold increase in the expression of the pβ2–77 construct, it is possible that PKA plays a role in the regulation of the TGF-β2 promoter. To test this possibility more directly, we examined the ability of PKA to modulate the activity of CREB at the TGF-β2 promoter in F9-differentiated cells. In these experiments, suboptimal concentrations of the expression plasmid for CREB were used. When the catalytic subunit of PKA (cPKA) was co-transfected with CREB into F9-differentiated cells, a dose-dependent increase (up to 5-fold) in the expression of the pβ2–77 was observed (Fig. 3). The cPKA expression plasmid on its own did not alter the expression of pβ2–77, which argues that the transcriptional activation observed was not due to general effects on transcription but was specific to CREB. Additionally, expression of pCREB in the presence of a serine 133 to alanine 133 mutant of CREB (CREBM1) had little to no effect on the expression of pβ2–77 (data not shown).

Expression of the Catalytic Subunit of PKA in Conjunction with CREB Targets p300 and CBP to the CRE/ATF Site within the TGF-β2 Promoter—It is well documented that phosphorylation ofCREB at serine 133 and ATF-1 at serine 63 allows for recruitment of the coactivators p300 and CBP (41–43). Therefore, since PKA increased CREB activation of the TGF-β2 promoter, we examined the ability of p300 and CBP to modulate the expression of the pβ2–77 construct. Utilizing transient transfection assays, expression plasmids for p300 or CBP were co-transfected with expression plasmids for CREB and cPKA. We observed that transfection of p300 or CBP in the presence of cPKA and CREB increased pβ2–77 expression (3–4-fold) over the expression observed with CREB transfected with pCPKA alone (Fig. 4). In the presence of CREB, pCPKA, and p300 or CBP, we observed an overall 40-fold increase in the expression of pβ2–77. When pCPKA was not included or when a serine 133 to alanine 133 mutant of CREB (CREBM1) was used, p300 and CBP did not affect the expression of the TGF-β2 promoter/reporter construct (data not shown). The induction that we observed in TGF-β2 promoter activity by p300 and cPKA is specific to CREB (and ATF-1). Although ATF-2 has been shown to interact with p300 at other promoters (87), p300 did not modulate the activity of pβ2–77 when transfected with ATF-2 in the presence or absence of cPKA (Fig. 5 and data not shown). Similarly, p300 did not increase the response of pβ2–77 to USF1 in the presence or absence of cPKA (Fig. 5 and data not shown).

We also did not observe transactivation when CREB, cPKA, and CBP were expressed with either pβ2–40 (data not shown) or pβ2–77C (Fig. 6). Mutation of the CRE/ATF site typically reduces the expression of the TGF-β2 promoter/reporter construct by 60–80% (23, 24). A similar reduction in expression of the pβ2–77C construct was observed in this experiment. However, for more direct comparison, expression of both pβ2–77 and pβ2–77C were set to 1.0 (Fig. 6). Overall, we observed a 17-fold increase in the level of expression of pβ2–77 and only a 1.8-fold increase in the level of expression of pβ2–77C (Fig. 6). These results argue that activation by CREB; CREB and cPKA; and CREB, cPKA, and CBP is dependent on an intact CRE/ATF site.

Although the E-box is essential for optimal expression of the TGF-β2 gene, we observed that modulation of the TGF-β2 promoter by p300/CBP was not dependent on an intact E-box motif. As shown previously, when the E-box is mutated at two critical base pairs (see Fig. 1), expression of the mutant con-
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FIG. 3. Expression of the catalytic subunit of PKA in conjunction with CREB increases the activity of pβ2–77. F9-differentiated cells were transfected in monolayer with 10 μg of pβ2–77 together with 2 μg of the pCH110 normalization plasmid. One μg of pRc/RSV-CREB was co-transfected with increasing amounts (shown in μg) of the catalytic subunit of PKA (pSKG4) as indicated (CREB or cPKA). The amount of DNA was kept constant at 23 μg using the null plasmid, pUC-19. The bars represent the CAT activities relative to the activity of pβ2–77 alone (1423 cpm). This experiment was performed in duplicate and repeated at least three times with similar results. S.D. values are shown.

FIG. 4. CBP and p300 enhance the expression of pβ2–77. F9-differentiated cells were transfected with 10 μg of pβ2–77 and 2 μg of the pCH110 normalizing plasmid. 0.5 μg of pRc/RSV-CREB (CREB) and 0.5 μg of pSKG4 (cPKA) were co-transfected as indicated. Increasing amounts (shown in μg) of either pCMVp300-CHA (p300) or pRc/RSV-mCBP.HA.RK (CBP) were added as shown. The total amount of DNA was kept constant at 23 μg using the null plasmid, pUC-19. The bars represent the CAT activities relative to the activity of pβ2–77. The CAT activity of the pβ2–77 construct was 1435 cpm for this experiment, which was performed in duplicate at least five times with similar results. S.D. values are shown.

FIG. 5. Expression of p300 and CBP are unable to modulate ATF-2 and USF1 activity at the TGF-β2 promoter. F9-differentiated cells were transfected with 10 μg of pβ2–77, 1 μg of pECATF-2 (ATF-2) or pSVUSF1-pN3 (USF1), 0.5 μg of pSKG4 (cPKA), 10 μg of pCMV/p300-CHA (p300), and 2 μg of the pCH110 normalizing plasmid. The total amount of DNA was kept constant at 23.5 μg using the pUC-19 plasmid. The bars represent CAT activities relative to the activity of pβ2–77 alone (5760 cpm). The experiment was performed in duplicate at least three times with similar results. S.D. values are shown.

pβ2–77E, is reduced approximately 80% (see Fig. 1) (25). We observed a similar reduction (approximately 80%) in expression of pβ2–77E for this study. Again, for a more direct comparison, the expression of pβ2–77 and pβ2–77E was set to 1.0 (Fig. 7). Regardless of whether we utilized pβ2–77 or pβ2–77E, titration of p300 with ATF-1 and cPKA increased expression 4-fold above the expression of ATF-1 and cPKA, and increased expression of the TGF-β2 promoter/reporter constructs approximately 25-fold overall (Fig. 7). We observed similar results with CBP and with the substitution of CREB for ATF-1 (data not shown).

p300 Stimulates TGF-β2 Promoter Activity in the Presence of Constitutively Active CaMKIV but Not CaMKII—Similar to PKA, CaMKIV can phosphorylate CREB on serine 133 and ATF-1 on serine 63. Also like PKA, the activity of CaMKIV increases with CaM kinases increases with differentiation of F9 EC cells (88), and this increase in activity may play a role in the activation of the TGF-β2 promoter. Therefore, we repeated the previous studies using an expression plasmid for constitutively active CaMKIV in place of the catalytic subunit of PKA. We observed that transfection of CaMKIV with CREB and p300 stimulated pβ2–77 activity (Fig. 8). Hence, both PKA and CaMKIV can regulate TGF-β2 promoter activity. On the other hand, expression of constitutively active CaMKII had little or no effect on the ability of CREB to activate pβ2–77 expression (Fig. 8). This is in agreement with previous observations made for other promoters (80, 82). CaMKII has been shown to phosphorylate CREB on serine 133 and serine 142 and phosphorylate ATF-1 on serine 63 and serine 72. It has been proposed that this dual phosphorylation of CREB and ATF-1 blocks their activation (82) and interferes with their ability to interact with p300 or CBP (80, 82).

CREB Is Phosphorylated at Serine 133 in the Nucleus of F9-differentiated Cells but Not in the Nucleus of F9 EC Cells—As we have shown, the addition of cPKA or CaMKIV with CREB and p300 or CBP increases the expression of the
TGF-β2 promoter in F9-differentiated cells where the gene is normally expressed. Since phosphorylation of CREB at serine 133 and ATF-1 at serine 63 is important for recruitment of p300 and CBP (42–44), it is possible that these phosphorylated forms are not present in the parental cells where TGF-β2 is not expressed. To test this possibility, we examined the phosphorylation status of CREB and ATF-1 in F9 EC cells and in F9-differentiated cells utilizing Western blot analysis, employing antibodies that specifically recognize CREB and ATF-1 phosphorylated at serine 133 and serine 63, respectively. We analyzed both whole cell and nuclear extracts for the presence of the phosphorylated forms of CREB or ATF-1 (Fig. 9). In whole cell extracts prepared from both F9 EC cells and F9-differentiated cells, CREB was phosphorylated at serine 133 (Fig. 9A). Densitometry analysis comparing phospho-CREB to the total level of CREB showed no significant difference in the level of phosphorylation between the whole cell extracts of F9 EC and F9-differentiated cells. However, when the same experiment was performed using nuclear extracts from F9 EC and F9-differentiated cells, phosphorylation of CREB at serine 133 was detected only in nuclear extracts prepared from F9-differentiated cells. There was no apparent change in the total amount of CREB between nuclear extract preparations of F9 EC and F9-differentiated cells. Thus, it appears that the serine 133-phosphorylated form of CREB accumulates in the nucleus only upon differentiation of F9 EC cells. Although we have
shown previously that ATF-1 is present in F9 EC cells and in F9-differentiated cells and can bind in vitro to the TGF-β2 CRE/ATF site, we did not detect serine 63-phosphorylated ATF-1 in either F9 EC or F9-differentiated extracts. It does not appear that our assay or nuclear extract preparation excluded serine 63-phosphorylated ATF-1, since we detected a significant amount of serine 63-phosphorylated ATF-1 in U87MG nuclear extracts where the TGF-β2 promoter is highly expressed (data not shown).

**DISCUSSION**

In this study, we sought to identify factors involved in the transcriptional regulation of the TGF-β2 gene. Previous studies utilizing the somatostatin and vasoactive intestinal peptide promoters in F9-differentiated cells showed that expression of CREB or ATF-1 on their own was unable to activate these promoters (77, 84, 89). Only in the presence of cAMP or the catalytic subunit of PKA were ATF-1 and CREB able to activate the SS and VIP promoters. Unlike these previous reports, our studies show that overexpression of CREB and ATF-1 significantly induces TGF-β2 promoter activity in F9-differentiated cells. We demonstrate that USF1 and USF2 are also able to stimulate TGF-β2 promoter activity in the differentiated cells. Although relatively little is known about the regulation of USFs, the functions of ATF-1 and CREB are tightly regulated by phosphorylation. Transcriptional activation of multiple promoters is enhanced by phosphorylation of ATF-1 at serine 63 and CREB at serine 133, as is their affinity for co-activators p300 and CBP (41, 42, 44, 82, 90). Consistent with these studies, we demonstrate that expression of the catalytic subunit of PKA with CREB or ATF-1 stimulates TGF-β2 promoter activity in F9-differentiated cells. We also observed that expression of cPKA or CaMKIV in conjunction with ATF-1 or CREB is required for the further stimulation of TGF-β2 promoter activity by the co-activators p300 and CBP. The action of these factors requires the TGF-β2 CRE/ATF site, since we do not observe activation of the TGF-β2 promoter with CREB, cPKA, or CBP when the CRE/ATF site is mutated (Fig. 6). Additionally, p300/CBP appear to function specifically through ATF-1 and CREB, since p300 was unable to modulate promoter activity through ATF-2. This was somewhat surprising, since ATF-2 has been shown to interact with p300, and this interaction potentiates transactivation of the c-Jun promoter (87). We also demonstrate that unlike another E-box binding protein, MyoD, which has been shown to interact with p300 and mediate several muscle-specific genes (50), USF1 does not invoke a p300 response at the TGF-β2 promoter.

It is interesting that ATF-1 and CREB appear to promote activation of the TGF-β2 promoter equally. Since ATF-1 and CREB are ubiquitous proteins, it is possible that depending on tissue type and cellular environment, either CREB or ATF-1 may regulate the TGF-β2 promoter. In some cases, it is possible that they function coordinately, since CREB and ATF-1 can form heterodimers. Although they are highly homologous, distinct differences are found in their N-terminal and kinase-inducible domains. ATF-1 and CREB are extremely divergent in their N-terminal regions, which may play a role in their regulation or subcellular localization (39, 91). In addition, ATF-1 lacks a GSK-3 kinase phosphorylation site in its kinase-inducible domain, whereas this site is present in CREB and appears to promote phosphorylation of CREB at serine 133 (92). Whether these differences play a role in the in vivo interaction with p300/CBP at specific promoters has yet to be determined.

We also examined various protein kinases that have been shown to phosphorylate CREB and ATF-1 at serine 133 and serine 63, respectively, and whose activities are increased upon differentiation of F9 EC cells (85, 86, 88). The protein kinase that we examined initially was PKA. Although PKA is expressed at the protein level by both F9 EC and F9-differentiated cells, PKA is not activated by cAMP in F9 EC cells (89). Upon differentiation, PKA is activated and becomes responsive to cAMP (85, 86, 89). As in the case of the transcription factors CREB and ATF-1 and the co-activators p300 and CBP, PKA and CaMKIV appear to be functionally redundant in their ability to activate the TGF-β2 promoter. Similar to PKA (Fig. 4), only when we express constitutively active CaMKIV with CREB, p300 (Fig. 8), and CBP (data not shown) are we able to activate the TGF-β2 promoter. However, as in the case of previous reports for other promoters (80, 82), CaMKII had little or no effect on the ability of CREB and p300 to modulate TGF-β2 promoter activity.

One of the most intriguing findings reported in this study is that the serine 133-phosphorylated form of CREB is present in the nucleus of F9-differentiated cells but is not detected in the nucleus of F9 EC cells. Therefore, as summarized in Fig. 10, our data suggest that localization of serine 133-phosphorylated CREB to the nucleus upon differentiation may be an important control pathway for the activation of the TGF-β2 gene through recruitment of p300/CBP. Multiple mechanisms may account for the presence of serine 133-phosphorylated CREB in the nucleus of F9-differentiated cells, but not in the nucleus of F9 EC cells. Although serine 133-phosphorylated CREB is present in the whole cell extracts of both F9 EC cells and F9-differentiated cells, it was not determined whether CREB is differentially phosphorylated on other residues in F9 EC cells and F9-differentiated cells. Phosphorylation of CREB at serine 133 is important for transactivation, but it is possible, if not likely, that phosphorylation of CREB on additional sites regulates its
location within the cell. Thus, it is possible that phosphorylation or dephosphorylation at a particular residue(s) allows for nuclear import or nuclear export of serine 133-phosphorylated CREB as occurs with the transcription factor NF-AT4 (93). Nuclear import of NF-AT4 is stimulated by the activation of the intracellular calcium phosphatase, calcineurin, which dephosphorylates masking residues around the nuclear localization signal of NF-AT4. In turn, rephosphorylation of NF-ATF4 at these sites stimulates its export from the nucleus.

It is also possible that (de)phosphorylation of CREB at particular residues may allow for interaction with cytoplasmic anchoring proteins that retain serine 133-phosphorylated CREB in the cytoplasm of F9 EC cells. Such a mechanism exists for the transcription factors, NF-κB and Dorsal, which are retained in the cytoplasm as inactive complexes by their anchoring proteins IκB (94, 95) and Cactus (96), respectively. Specific phosphorylation of NF-κB and Dorsal plays a critical role in their release from the cytoplasmic anchoring proteins as well as their translocation to the nucleus and subsequent activation of NF-κB- and Dorsal-responsive genes. A similar mechanism may exist for CREB. Another possibility is that the protein kinase responsible for accumulation of serine 133-phosphorylated CREB in the nucleus may not be active in F9 EC cells. Multiple kinase activities are up-regulated during F9 differentiation, including PKA, protein kinase C, and CaM kinases (85, 86, 88, 97–99). Some of these kinases phosphorylate CREB not only on serine 133 but also on other residues (100–102), which again may play important roles in the proper cellular localization of CREB. It is possible that one or more of these mechanisms may be responsible for the presence of serine 133-phosphorylated CREB in the nucleus of F9-differentiated cells, and an in depth analysis will be needed to distinguish between them.

Finally, it is noteworthy that genes such as c-jun (87), FGFR-3 (103), tissue plasminogen activator (104), and the TGF-β type II receptor (105) also contain CRE sites and turn on with RA-induced differentiation of EC cells. Like TGF-β, their promoters contain additional cis-regulatory elements that provide specificity and ensure that the correct gene is activated at the precise time. The nuclear localization of serine 133-phosphorylated CREB may be one step in the coordinate regulation of multiple genes during differentiation. Hence, understanding how the TGF-β2 gene is activated at the level of transcription will not only further our knowledge of this gene but may also provide important clues into the expression of many genes regulated by differentiation.

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Transcriptional Regulation of the Transforming Growth Factor-β2 Promoter by cAMP-responsive Element-binding Protein (CREB) and Activating Transcription Factor-1 (ATF-1) Is Modulated by Protein Kinases and the Coactivators p300 and CREB-binding Protein

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