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Transcription of the Transforming Growth Factor-β2 Gene Is Dependent on an E-box Located between an Essential cAMP Response Element/Activating Transcription Factor Motif and the TATA Box of the Gene*

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Transforming growth factor-β2 (TGF-β2) is an important regulator of cell proliferation and differentiation; however, its transcriptional regulation is not well understood. Here we report characterization of an essential E-box motif, positioned at −50/−45 between a previously described functional cAMP response element/activating transcription factor site and the TATA box of the human TGF-β2 promoter. By site-directed mutagenesis, we demonstrate that this E-box motif is necessary for the promoter activity, not only in differentiated cells derived from embryonal carcinoma cells, but also in choriocarcinoma cells and in MCF-7 breast carcinoma cells. We also demonstrate that the transcription factors USF1 and USF2 bind to this E-box motif *in vitro* when nuclear extracts from each of these cell lines are examined by gel retardation assays. Moreover, using a dominant-negative USF2 protein, we show that USF proteins are critical for TGF-β2 promoter activity *in vivo*. The importance of the E-box motif described in this study is supported by the presence of an E-box motif in the same position in the chicken TGF-β2 gene promoter.

Transforming growth factor-β2 (TGF-β2),[1] like other growth factors in the TGF-β family, is involved in the regulation of many different cellular functions, including cell proliferation and differentiation as well as production and maintenance of extracellular matrices (reviewed in Refs. 1 and 2). Through its multifaceted effects, TGF-β2 plays important regulatory roles in a host of biological events, from embryogenesis through repair processes, to regulation of the immune system. Hence, the regulation of TGF-β2 gene in various systems warrants detailed investigation. The studies presented here focus on the transcriptional regulation of the TGF-β2 gene in embryonal carcinoma (EC) cells and their differentiated counterparts, which represent a model system of early embryonic development (reviewed in Ref. 3). Given the importance of TGF-β2 production in implantation and in tumor malignancy (4–6), we extended our studies to two choriocarcinoma cell lines and a breast carcinoma cell line. Previous work demonstrated 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 (7–9). This region contains a functional CRE/ATF motif, which is indispensable for the positive effect of this promoter region in different cell types and capable of binding activating transcription factor 1 (ATF-1) *in vitro*. Recently, computerized sequence analysis demonstrated that the human TGF-β2 promoter also contains a CACGTG motif between −50 and −45, which conforms to the consensus sequence of an E-box motif, CANNTG (10). Interestingly, this E-box motif appears to be evolutionarily conserved, since the same CACGTG sequence is present in the chicken TGF-β2 promoter, and it is located in the same position relative to the similarly conserved CRE/ATF site and the TATA box of the chicken promoter (11). Thus, the putative E-box motif localizes to the previously identified positive regulatory region, which has been shown to be inactive in undifferentiated EC cells, but becomes active when EC cells are induced to differentiate, and, consequently, express TGF-β2 both at the RNA and protein levels. E-box motifs in other gene promoters have been shown to bind members of the bHLH-LZ family of transcription factors, including c-*myc* (12), Max (13), USF (14), or TFE3 (15) proteins, where the flanking nucleotides of the motif appear to provide for the discrimination in binding between different family members (16). Some of these transcription factors can act as either transactivators or repressors of gene expression, depending on the gene promoter or on their dimerization partner (17–21). Therefore, we examined whether the putative E-box motif in the TGF-β2 gene promoter is involved in negative and/or positive regulation of the gene in several cell types. Our results demonstrate that the −50/−45 E-box motif is critical for the positive effect of the −77/+63 regulatory region of the TGF-β2 gene promoter in differentiated cells derived from both murine and human EC cells. We also demonstrate that the transcription factors USF1 and USF2 are able to bind to this site *in vitro* before and after differentiation of EC cells. Importantly, similar observations were made in JAR and JEG-3 choriocarcinoma cells and MCF-7 breast cancer cells. Finally, a dominant-negative USF2-expression vector was used to demonstrate that USF transcription factors are utilized as positive transactivators of the TGF-β2 gene in differentiated cells derived from EC cells.

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[1] The abbreviations used are: TGF-β2, transforming growth factor-β2; CRE, cAMP response element; ATF-1, activating transcription factor 1; EC, embryonal carcinoma; dsODN, double-stranded oligodeoxynucleotide; LZ, leucine zipper; PCR, polymerase chain reaction.

EXPERIMENTAL PROCEDURES

Dulbecco's modified Eagle's medium was obtained from Life Technologies, Inc., and fetal bovine serum was obtained from HyClone (Logan, UT). All-*trans*-retinoic acid was purchased from Eastman Kodak Co. All other chemicals were purchased from Sigma, unless otherwise indicated.

Cell Culture and Differentiation of EC Cells—All cell lines were maintained and induced to differentiate as described previously, unless indicated otherwise (1, 8, 22, 23).

Preparation of Cell Extracts and Gel Mobility Shift Assay—Nuclear extracts were prepared as described previously (24) with slight modifications of the original method of Dignam *et al.* (25). Nuclear extracts were prepared in the presence of the protease inhibitors: pepstatin A, antipain, chymostatin, leupeptin (1 μg/ml), phenylmethylsulfonyl fluoride (1 mM), soybean trypsin inhibitor (20 μg/ml), benzamidine (2.5 mM), aprotinin (2.5 KIU/ml). Nuclear extracts of F9 EC cells and F9-differentiated cells also contained protein phosphatase inhibitors: $(NH_4)_2MoO_4$ (1 mM) and NaF (5 mM). The dialysis buffer contained the same protease inhibitors at a 10-fold lower final concentration. Protein concentrations were determined using the Pierce Micro BCA Protein Assay Reagent (Pierce). Gel mobility shift assays were based on the method of Fried and Crothers (26). The protocol and reaction conditions used in this report were the same as described previously (24), with the exception that all binding reactions contained $MgCl_2$ at a final concentration of 3 mM. In addition, binding reactions with JAR, JEG-3, and MCF-7 cell nuclear extracts were supplemented with 1.5 μl of 1 M NaCl and 1 μl of 0.25 M NaH_2PO_4 per 20-μl reaction mixture to promote binding of nuclear proteins. Reaction mixtures were incubated for 20 min at room temperature with F9 EC cell and F9-differentiated cell nuclear extracts, and for 40 min at room temperature with JAR, JEG-3, and MCF-7 cell nuclear extracts. The double-stranded oligodeoxynucleotide (dsODN) probes containing the wild type or mutant E-box motif were as follows (the putative transcription factor binding site is underlined): wild type E-box probe: 5′-ggcAGACACGTGGTT-3′, and its complement, 5′-ctgAACCACGTGTCT-3′; mutant E-box dsODN: 5′-ggcAGACGAGTGGTT-3′, and its complement, 5′-ctgAACCACTCGTCT-3′ (the mutations are double-underlined). When annealed, the probes had 5′ overhangs (shown in lowercase), which permitted radioactive labeling by Klenow fill-in reaction. The nonspecific DNA competitor used in the gel mobility shift assays was 0.05 μg/μl poly(dI)·poly(dC) (Pharmacia Biotech Inc.). For supershift analyses, the binding reactions were incubated overnight at 4 °C with the antibodies and the blocking peptides indicated, prior to the addition of the labeled probe. The USF1- and USF2-specific antibodies (catalog nos. sc-229 and sc-862, respectively) and their respective blocking peptides were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Nondenaturing 4% polyacrylamide gels were run at 4 °C in high ionic strength buffer (50 mM Tris base, 380 mM glycine, 2 mM EDTA).

Site-directed Mutagenesis of TGF-β2 Promoter/Reporter Gene Constructs—Site-directed mutagenesis of the −50/−45 E-box motif (numbering is according to Noma *et al.* (27)) was carried out using a slight modification of the PCR-based "megaprimer" method for mutagenesis, using the same cycle conditions as described previously (23). Primers for the first PCR were: Primer 1: 5′- CGCGCGTTGGCCGATTCA-3′, and the mutagenic primer annealing to the E-box motif (the mutated bases are in *lower case*): 5′-TCTCTCTGAACCACtcGTCTGCCTTC-3′. Primers for the second PCR were: the "megaprimer," which is the product of the first PCR, and Primer 2: 5′-GCCATTGGGATATATCAACGGTGGTA-3′. The mutant promoter fragment produced by the second PCR was cloned into the pGEM4-SVOCAT plasmid digested with *Pst*I and *Kpn*I. The entire promoter insert of the mutant clones was sequenced to verify the presence of the desired point mutations in the E-box motif, and to ensure that the *Pfu* polymerase did not introduce additional point mutations during PCR.

Transient Transfection Assay—F9-differentiated cells (day 3), JAR cells, and MCF-7 cells were transfected in monolayer by the calcium phosphate precipitation method (28) as described previously (23, 24). The normalization plasmid pCH110 (Pharmacia) contains the β-galactosidase reporter gene under the control of the SV-40 promoter. In some experiments (as indicated in the figure legends) the pCMVβg-normalizing plasmid was used, which contains the β-galactosidase reporter gene under the control of the CMV promoter (CLONTECH, Palo Alto CA). TGF-β2/CAT chimeric gene constructs were prepared as described previously (8, 23). The eukaryotic expression plasmid producing a dominant-negative USF2 protein was obtained from Dr. Michéle Sawadogo (University of Texas M.D. Anderson Cancer Center, Houston, TX) (29).

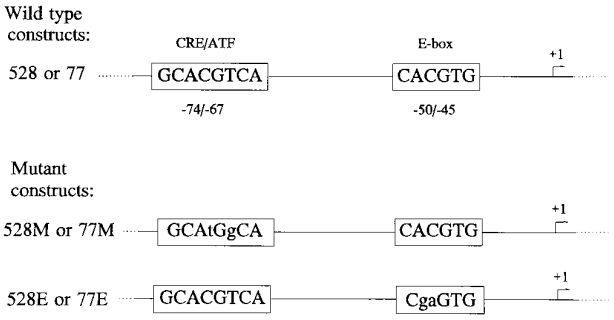

FIG. 1. **Point mutations introduced in the E-box motif and CRE/ATF site of the TGF-β2 promoter/reporter gene constructs.** The TGF-β2 promoter/reporter gene constructs were named pβ2-*n*, where "*n*" is the number of nucleotides upstream of the transcription start site (8). pβ2-528 (or "*528*") contained the −528/+63 fragment of the wild type human TGF-β2 promoter and the pβ2-77 construct (or "*77*") contained the −77/+63 fragment of the promoter. Mutant constructs 528M and 77M harbor point mutations in the CRE/ATF motif, whereas mutant constructs 528E and 77E harbor point mutations in the E-box motif (the mutations are in *lowercase*). *+1*, the transcription start site.

Plasmid DNA was purified by tip-500 columns (QIAGEN, Chatsworth, CA).

RESULTS

An E-box Motif Is Critical for the TGF-β2 Promoter Activity in Different Cell Lines—To determine the role of the −50/−45 putative E-box motif in TGF-β2 promoter activity, we employed site-directed mutagenesis to introduce point mutations in the E-box motif in our promoter/reporter gene constructs. The wild type E-box motif was changed to CGAGTG in the mutant constructs, since mutations in the CACGTG core E-box sequence have been shown to significantly inhibit binding of members of the bHLH-LZ family of transcription factors (30–32). One of the constructs that was selected for mutagenesis, pβ2-77, contains the −77/+63 fragment of the human TGF-β2 promoter. This region acts as a positive regulatory region of the TGF-β2 promoter in all cell types studied (7–9). We also introduced the same point mutations in the pβ2-528 construct, which contains 528 base pairs of the promoter region upstream from the transcription start site. This allowed us to test the function of the E-box in the context of a larger promoter region (Fig. 1; the point mutations are in lowercase). To test the effects of these changes, differentiated cells derived from the murine EC cell line, F9, or the human EC cell line, NT2/D1, were transiently transfected with the wild type or the mutant promoter/reporter gene constructs (Fig. 2, *A* and *B*). Activity of the pβ2-40 construct served as the base line, since this construct does not contain the E-box or the CRE/ATF site, and has a very low basal activity in all cell lines studied (7, 8). The results show clearly that mutations in the E-box motif significantly reduce TGF-β2 promoter activity in both cell lines, not only in the shorter construct, pβ2-77E, but also in the context of a larger promoter region, in the pβ2-528E construct. Similar results were obtained when the function of the E-box motif was studied in JAR choriocarcinoma cells (Fig. 2*C*), MCF-7 breast cancer cells (Fig. 2*D*), and JEG-3 choriocarcinoma cells (data not shown). In NT2/D1-differentiated cells, mutation of the E-box motif consistently resulted in slightly lower reduction of promoter activity than what was observed with the mutated CRE/ATF site, suggesting that cell-type specific differences may exist in utilization of these *cis*-regulatory elements. Nevertheless, the average reduction in promoter activity by the E-box mutation was in a similar range (60–80%) to that observed with mutations in the CRE/ATF motif in both constructs (9, 23), indicating that both *cis*-regulatory elements are func-

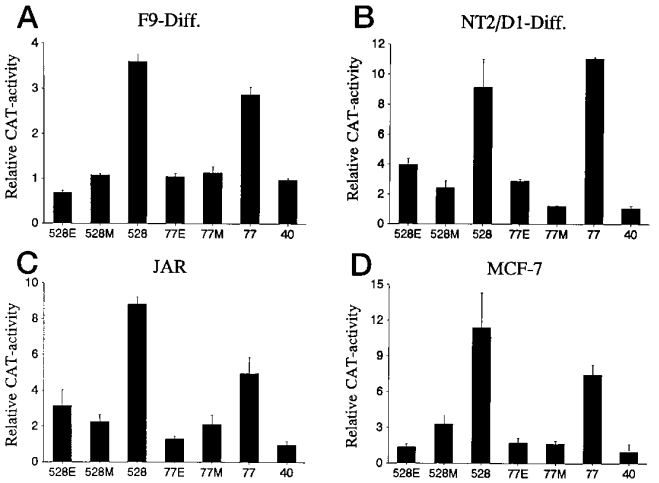

FIG. 2. **Functional analysis of the TGF-β2 E-box motif.** F9-differentiated cells (*A*), NT2/D1-differentiated cells (*B*), JAR choriocarcinoma cells (*C*), and MCF-7 breast carcinoma cells (*D*) were transfected in monolayer with the wild type and mutant TGF-β2 promoter/CAT plasmids together with the β-galactosidase normalizing plasmids. The normalizing plasmid was pCH110 in *A* and *B*, and pCMVβg in *C* and *D*. Plasmids pβ2-77M and pβ2-528M contain two point mutations within the CRE/ATF site of the promoter insert, whereas plasmids pβ2-77E and pβ2-528E contain two point mutations within the E-box motif. *Bars*, CAT activities of the plasmids relative to the activity of the pβ2-40 construct. CAT activities of pβ2-40 in F9-differentiated, NT2/D1-differentiated, JAR, and MCF-7 cells were 6702, 802, 372, and 1423 cpm, respectively. All experiments in this figure were repeated at least three times with similar results, using duplicate plates for each plasmid in *A* and *B*, and triplicate plates in *C* and *D*. Standard deviations (S.D.) are shown for these experiments; similar S.D. values were observed in parallel experiments.

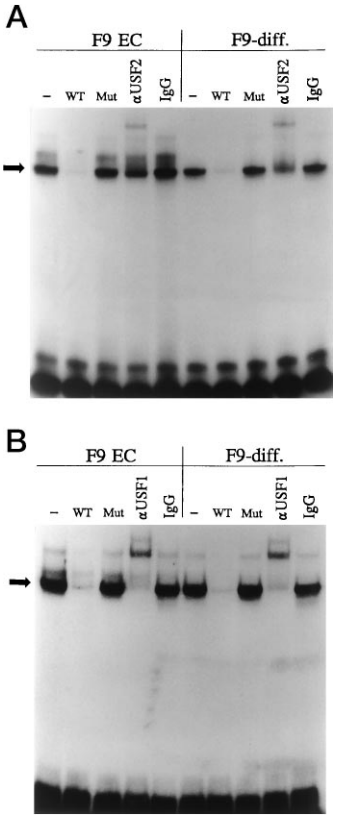

FIG. 3. **Gel mobility shift assay between the TGF-β2 E-box motif and nuclear extracts prepared from F9 EC cells and F9-differentiated cells.** Nuclear extracts were incubated with the ^{32}P-labeled wild type E-box probe, as described under "Experimental Procedures." Competition analysis of DNA-protein complex formation was performed using a 25-fold molar excess of either the unlabeled wild type dsODN (*WT*) or unlabeled mutated dsODN (*Mut*) in the lanes where indicated. The *arrow* indicates the position of the major DNA-protein complex. 3 μg of USF2-specific antibody was added to *lanes 4* and *9* in *A*, 3 μg of USF1-specific antibody was added to *lanes 4* and *9* in *B*, and 3 μg of rabbit IgG (negative control) was added to both *lanes 5* and *10* of *A* and *B*. The experiment was repeated with similar results.

tional and critical for the activity of the TGF-β2 promoter in the different cell types.

USF1 and USF2 Are Able to Bind to the TGF-β2 E-box Motif—Gel mobility shift analysis was employed to identify the transcription factors that bind to the TGF-β2 E-box motif *in vitro*. First, E-box-binding activities in nuclear extracts of EC cells and their differentiated counterparts were compared, since the promoter is inactive in the undifferentiated cells, but becomes active when the cells are induced to differentiate. F9 EC cell nuclear extracts formed one major and two minor DNA-protein complexes with the wild type E-box dsODN probe in the binding assays (Fig. 3, *A* and *B*). The minor complexes can be observed more readily in Fig. 3*B*. The intensities of the minor complexes varied between experiments and never approached the intensity of the major complex. F9-differentiated cell nuclear extracts also formed one major DNA-protein complex with the wild type E-box probe, plus a minor DNA-protein complex (Fig. 3, *A* and *B*). Both DNA-protein complexes formed with nuclear extracts of F9-differentiated cells co-migrated with two of the complexes formed by F9 EC cell nuclear extracts (Fig. 3, *A* and *B*). These complexes bound specifically to the E-box motif in the dsODN probe, since they were competed effectively with a 25-fold molar excess of the same, unlabeled dsODN containing the wild type E-box motif, but not with the same excess of the mutant dsODN harboring two critical point mutations in the E-box motif. Importantly, nuclear extracts prepared from PYS-2 cells, NT2/D1 EC cells, or NT2/D1-differentiated cells formed very similar DNA-protein complexes when incubated with the wild type E-box probe, with the exception that the minor complex migrating just above the major complex could be observed only with nuclear extracts prepared from F9 EC cells (data not shown). PYS-2 cells were included in this study, because these EC-derived differentiated cells also express TGF-β2. Finally, no DNA-protein complexes formed when nuclear extracts prepared from F9 EC cells or their differentiated cells were incubated with the labeled mutant dsODN as the probe (data not shown).

Interestingly, DNA binding by the major E-box binding protein was abolished by treatment with diamide, a commonly used oxidizing agent, but was unaffected by boiling the nuclear extracts for 10 min (data not shown). Since it has been shown that of the E-box binding bHLH-LZ transcription factors, USF proteins are both thermostable (33) and sensitive to oxidation (34), we tested whether USF1- and USF2-specific polyclonal antibodies could recognize the DNA-protein complexes formed between nuclear extracts and the E-box probe. A USF2-specific polyclonal antibody caused the formation of a supershifted DNA-protein complex both in F9 EC cell and F9-differentiated cell nuclear extracts (Fig. 3*A*). On the other hand, addition of USF1-specific antibody to the binding reaction resulted in the formation of two supershifted complexes both in F9 EC and F9-differentiated cell nuclear extracts (Fig. 3*B*). Neither antibodies recognized the minor DNA-protein complexes, since no change in their migration or intensities was observed. Although the supershift of the major complex by USF2-specific antibody appears to be incomplete (Fig. 3*A*, *lanes 4* and *9*), this is not due to inadequate amounts of the antibody, since dilution of the nuclear extracts did not result in a more complete supershift. Rather, it appears that the remaining E-box binding complex is likely to be formed by a homodimer of USF1, since

the USF1-specific antibody caused a nearly complete supershift of the major E-box binding complexes (Fig. 3*B*). It should also be noted, that the supershifted USF1 complex, which is the faster migrating of the two supershifted complexes in Fig. 3*B*, is clearly distinct from the minor complex observed in either F9 EC or F9-differentiated cell nuclear extracts, as demonstrated by their different mobilities in 8% polyacrylamide gel (data not shown). Furthermore, the slower migrating supershifted complex formed with USF1-specific antibody co-migrates with the single supershifted complex formed with USF2-specific antibody; thus, these complexes are likely to contain a heterodimer of USF1 and USF2. Both antibodies caused a decrease in the binding of the supershifted complexes, which is a frequently observed phenomenon in gel supershift reactions with mono- or polyclonal antibodies resulting from the recognition of epitopes in the DNA binding domain of transcription factors. Overall, our data suggest that both a heterodimer of USF1/USF2 and a homodimer of USF1 can bind to this E-box motif *in vitro*, but we cannot exclude the possibility that a small amount of USF2 homodimer binds to this site.

We also examined the E-box binding transcription factors produced by JAR cells and MCF-7 cells, because the E-box motif is critical for TGF-β2 promoter activity in these cell lines. Gel mobility shift analyses demonstrated that nuclear extracts prepared from MCF-7 cells form a very intense DNA-protein complex with the dsODN probe (Fig. 4*A*). Although in some experiments a minor complex appears to migrate just below the intense complex, the presence of this minor complex was quite variable, and, thus, does not appear to be significant. Nuclear extracts from JAR and JEG-3 cells also form an intense band, which appears to consist of two DNA-protein complexes that migrate very close to one another (Fig. 4*A*). The presence of the two complexes is readily apparent at shorter exposures of the autoradiogram (data not shown). In addition, both JAR and JEG-3 nuclear extracts form a second less intense complex that migrates faster than the main complex. The formation of this complex, like the one observed with extracts prepared from MCF-7 cells is variable. Importantly, each of the complexes observed with nuclear extracts from these three cell lines bound specifically to the E-box motif, as determined by competition analyses with the wild type and mutant E-box dsODNs. It is noteworthy that, although equal amounts of nuclear extracts (micrograms of protein) were used in the gel mobility shift assays, JEG-3 cell nuclear extracts appear to contain significantly less TGF-β2 E-box binding activity than the other two cell lines (Fig. 4*A*). Supershift analyses using USF1- and USF2-specific antibodies demonstrated the presence of both USF1 and USF2 in the DNA-protein complexes, although significantly more USF1 homodimer appears to bind to the E-box motif in JAR cell nuclear extracts, than in other cell nuclear extracts tested (Fig. 4*B*). In this regard, increasing the amount of the USF1 antibody in other experiments caused a nearly complete supershift of the DNA-protein complexes bound to the E-box motif; whereas addition of the USF2 antibody along with the USF1 antibody did not cause a supershift of the residual DNA-protein complexes (data not shown). It should also be noted that, despite the similarity of the USF proteins, the USF1- and USF2-specific antibodies do not crossreact. This is demonstrated by the fact that the supershift by USF2-specific antibody can be blocked only with a peptide fragment derived from USF2, but not with the blocking peptide derived from USF1 and *vice versa* (Fig. 4*B*). In this regard, the blocking peptides used in our gel shift studies are the same USF1 or USF2 peptides used to generate the respective antibodies.

USF Transcription Factors Are Required for TGF-β2 Promoter Activity in Vivo—Despite the binding of USF proteins to

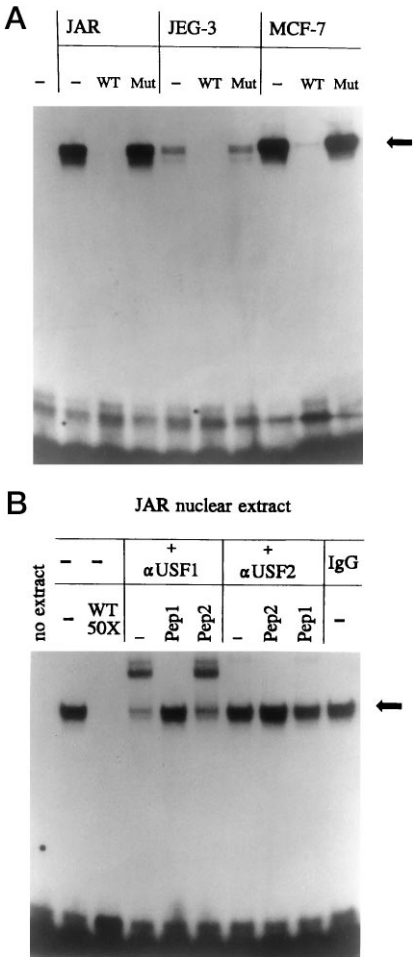

FIG. 4. **Gel mobility shift assay between the TGF-β2 E-box motif and nuclear extracts prepared from JAR and JEG-3 choriocarcinoma cells and MCF-7 breast carcinoma cells.** *A*, nuclear extracts were incubated with the ^{32}P-labeled wild type E-box probe, as described under "Experimental Procedures." Competition analysis of the DNA-protein complex formation was performed using a 50-fold molar excess of either the unlabeled wild type dsODN (*WT*) or unlabeled mutated dsODN (*Mut*) in the lanes where indicated. The *arrow* indicates the position of the major DNA-protein complex(es). *B*, JAR nuclear extract was incubated with the ^{32}P-labeled wild type E-box probe, as described under "Experimental Procedures." No extract was added to *lane 1*. A 50-fold molar excess of the unlabeled wild type dsODN was added to *lane 3*, 1 μg of USF1-specific antibody was added to *lanes 4–6*, and 1 μg of USF2-specific antibody was added to *lanes 7–9*. 10 μg of the blocking peptide derived from the C terminus of USF2 (*PEP2*) was added to *lanes 6* and *8*, and 10 μg of the blocking peptide derived from the C terminus of USF1 (*PEP1*) was added to *lanes 5* and *9*. The *arrow* indicates the position of the major DNA-protein complex(es). The experiment was repeated with similar results.

the TGF-β2 E-box motif *in vitro*, the possibility still remained that other bHLH-LZ proteins are responsible for the E-box-dependent promoter activity *in vivo*. Therefore, we employed a eukaryotic expression plasmid in transient transfection assays (psvUSF2ΔB) that expresses a murine USF2 mutant protein, which lacks the region required for DNA binding (amino acids 228–247) (29). Since specific DNA binding by hetero- or homodimers of USF proteins requires the presence of both proteins' DNA binding domains (14, 35, 36), the ectopically expressed mutant USF2 protein would effectively sequester wild type, endogenous USF1 and USF2 proteins in complexes that are unable to bind to E-box motifs. Cotransfection of plasmid psvUSF2ΔB (expressing mutant USF2) with the pβ2-77 promoter/reporter construct reduced promoter activity by approximately 60% in F9-differentiated cells (Fig. 5). This effect appears to be conveyed specifically through the E-box motif of the

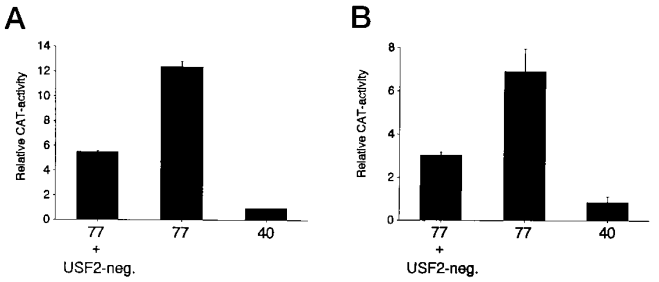

FIG. 5. **Effects of a dominant-negative USF2 protein on TGF-β2 promoter activity.** F9-differentiated cells were transfected in a monolayer with 5 μg of the pβ2-40 ("*40*") and pβ2-77 ("*77*") TGF-β2 promoter/CAT plasmids together with the β-galactosidase normalizing plasmids, pCH110 (*A*) or pCMVβg (*B*). 2 μg of the plasmid psvUSF2ΔB producing a dominant-negative USF2 protein was cotransfected with pβ2-77 where indicated (*USF2-neg.*). The amount of DNA transfected was kept at 22 μg for all samples by the addition of the parent plasmid, pSV5 (Stratagene, La Jolla, CA). *Bars*, CAT activities of the plasmids relative to the activity of the pβ2-40 construct. CAT activity of pβ2-40 was 373 and 289 cpm in *A* and *B*, respectively. The experiment was repeated several times with similar results, using duplicate plates for each plasmid. S.D. values are shown for this experiment; similar S.D. values were observed in parallel experiments.

promoter, since at the same time no change was observed in the activities of two different normalizing plasmids, pCH110 or pCMVβg (Fig. 5, *A* and *B*, respectively), which are under the control of the SV-40 promoter and the CMV promoter, respectively. This observation argues that USF transcription factors contribute directly to the activity of the TGF-β2 promoter in F9-differentiated cells. It should be noted that we observed an average differential of 7-fold between the CAT activity expressed by the pβ2-77 construct and the activity of the pβ2-40 construct when transfected into F9-differentiated cells (Fig. 5). However, in some experiments a smaller differential was observed (Fig. 2*A*). This variability is likely to result from several experimental conditions, particularly the extent of differentiation exhibited by the population of F9 EC cells treated with all-*trans*-retinoic acid. Nevertheless, the differences observed between the activities of the unmodified promoter/reporter gene constructs and their mutated counterparts were consistently larger (approximately 70%) than the standard deviations (<10%) observed between experiments.

DISCUSSION

Our studies identify a critical *cis*-regulatory element in the human TGF-β2 gene promoter, which conforms to the consensus binding site of the bHLH-LZ family of transcription factors. The intact E-box motif is required for TGF-β2 promoter activity in several cell types, including differentiated cells derived from murine and human EC cells (F9 EC and NT2/D1 EC, respectively), as well as two choriocarcinoma cell lines (JAR and JEG-3) and a breast carcinoma cell line (MCF-7). These observations, together with the fact that the E-box motif is conserved in a comparable region of the chicken TGF-β2 gene promoter, suggest that transcription factors binding to the E-box motif, in conjunction with other factors binding to a similarly conserved CRE/ATF motif nearby, play an important role in TGF-β2 gene expression in various systems. In this regard, our gel mobility shift analyses demonstrated that protein complexes containing USF1 and USF2 transcription factors are capable of binding to the TGF-β2 E-box motif in nuclear extracts of all cell types studied. Moreover, using a dominant-negative mutant of USF2 protein in F9-differentiated cells, we determined that binding of USF proteins to the E-box motif appears to be critical for the activity of the TGF-β2 promoter in cells. Interestingly, overexpression of USF1 or USF2 in the transfected F9-differentiated cells induced a general increase in transcription without a preferential increase in the expression of the TGF-β2 promoter/reporter gene constructs (data not shown). This suggests that USF1 and USF2 are not limiting for the expression of the TGF-β2 gene in F9-differentiated cells.

The data presented here also suggest that TGF-β2 E-box binding activities, comprised of USF1/USF2 heterodimers and USF1 homodimers, do not change dramatically upon differentiation of EC cells, similarly to CRE/ATF binding activities (9). The same observation was made with a murine (F9) and a human (NT2/D1) EC cell line and their differentiated cells and with the parietal endoderm-like PYS-2 cell line, which shares many characteristics with F9-differentiated cells. This was surprising, since the region of the TGF-β2 promoter containing these *cis*-regulatory elements is inactive in undifferentiated EC cells. One possible explanation is that, although DNA-binding ability of the USF complexes is unaffected by the differentiation status of the cells, they are unable to transactivate in the undifferentiated cells, perhaps due to their different state of phosphorylation. In this regard, it has been suggested that the transactivator domain of USF1 could be converted into an acidic activation domain upon phosphorylation of the multiple serine and threonine residues found in this region (37). However, treatment of F9 EC or F9-differentiated cell nuclear extracts with calf intestinal alkaline phosphatase does not appear to affect DNA binding, migration, or the supershift pattern of the USF complexes (data not shown). Nevertheless, without thorough analysis of the phosphorylation pattern of USF proteins, it cannot be excluded that TGF-β2 E-box binding USF-complexes have different transactivator abilities before and after differentiation of EC cells resulting from differential phosphorylation. On the other hand, it is also conceivable that USF complexes may not bind effectively to the TGF-β2 E-box motif before differentiation of EC cells due to chromatin structure, or methylation of the binding site. Alternatively, transcription factors that bind to other *cis*-regulatory elements in the TGF-β2 gene may interfere with the function of transcription factors that bind to the CRE/ATF motif and/or the E-box. Last, it is important to note that the E-box motif is positioned between an upstream CRE/ATF site and the downstream TATA box motif, and, based on the distance between them, all three motifs are positioned to face the same side of the DNA helix. This raises the possibility of direct or indirect interactions between the transcription factors binding to these *cis*-regulatory elements and the basal transcription machinery, which are necessary for the formation of an active preinitiation complex. In this regard, the leucine zipper (LZ) domain of several members of the bLZ and bHLH-LZ families of transcription factors have been shown to participate in various interactions with viral proteins or with other transcription factors (38–42). In addition, USF proteins have been shown to interact with TFIID binding to the TATA box motif (43). Thus, it is possible that the activity of the TGF-β2 promoter is regulated through modulation of these essential protein-protein interactions.

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