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E2F4-RB and E2F4-p107 complexes suppress gene expression by transforming growth factor β through E2F binding sites

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ABSTRACT **Transforming growth factor β (TGF-β) causes growth arrest in most cell types. TGF-β induces hypophosphorylation of retinoblastoma susceptibility gene 1 product (RB), which sequesters E2F factors needed for progression into S phase of the cell cycle, thereby leading to cell cycle arrest at G_1. It is possible, however, that the E2F-RB complex induced by TGF-β may bind to E2F sites and suppress expression of specific genes whose promoters contain E2F binding sites. We show here that TGF-β treatment of HaCaT cells induced the formation of E2F4-RB and E2F4-p107 complexes, which are capable of binding to E2F sites. Disruption of their binding to DNA with mutation in the E2F sites did not change the expression from promoters of *E2F1*, *B-myb*, or *HsORC1* genes in cycling HaCaT cells. However, the same mutation stimulated 5- to 6-fold higher expression from all three promoters in cells treated with TGF-β. These results suggest that E2F binding sites play an essential role in the transcription repression of these genes under TGF-β treatment. Consistent with their repression of TGF-β-induced gene expression, introduction of E2F sites into the promoter of cyclin-dependent kinase inhibitor $p15^{INK4B}$ gene effectively inhibited its induction by TGF-β. Experiments utilizing Gal4-RB and Gal4-p107 chimeric constructs demonstrated that either RB or p107 could directly repress TGF-β induction of $p15^{INK4B}$ gene when tethered to $p15^{INK4B}$ promoter through Gal4 DNA binding sites. Therefore, E2F functions to bring RB and p107 to E2F sites and represses gene expression by TGF-β. These results define a specific function for E2F4-RB and E2F4-p107 complexes in gene repression under TGF-β treatment, which may constitute an integral part of the TGF-β-induced growth arrest program.**

Transforming growth factor β (TGF-β) represents a large family of cytokines that are involved in the regulation of growth, differentiation, and morphogenesis in a wide range of cell types (1–4). TGF-β causes growth inhibition in most cell types of epithelial and lymphoid origins by inducing hypophosphorylation of retinoblastoma susceptibility gene 1 product (RB) (5). Hypophosphorylated RB then sequesters active transcription factors, such as E2F transcription factors, which are needed for progression into the S phase of the cell cycle, thereby leading to growth arrest at G_1. However, it has become clear that E2F factors also play an equally, if not more, important role in the transcription repression of genes whose promoters contain E2F binding sites. For example, E2F binding sites present on the mouse *B-myb* promoter do not participate in the transcription activation of *B-myb* gene and instead play a dominant role in transcription repression in serum-starved quiescent (G_0) cells, because mutation of the E2F sites caused constitutive expression of B-myb gene (6). Moreover, serum stimulation of quiescent cells rendered E2F binding sites present on the B-myb promoter unoccupied by E2F factors, suggesting a de-repression mechanism by serum stimulation (7). E2F factor-mediated transcription repression is probably achieved through the factors' association with the RB family of pocket proteins, which act as dominant repressors (6, 8–10). Consistent with this notion, RB was shown to physically associate with specific transcription factors, such as c-myc, Elf-1, and PU.1, and block their interaction with the basal transcription machinery, therefore selectively repressing their transcription activation potentials (11).

To determine any roles that E2F factors may play in a TGF-β-induced growth arrest process, we investigated the effect of TGF-β on the transcription regulation of three promoters that contain E2F binding sites, namely the E2F1 (12), B-myb (6), and HsORC1 (human origin recognition complex 1) (13) promoters. We found that TGF-β specifically induced the formation of DNA-bound E2F4-RB and E2F4-p107 complexes in human keratinocyte HaCaT cells. Mutation in E2F sites had little effect on the expression from these promoters in cycling HaCaT cells. In the presence of TGF-β, however, the same mutation caused 5- to 6-fold higher expression from all three promoters, suggesting that E2F binding sites served an important function in the repression of these promoters after cells were treated with TGF-β and arrested at G_1. Furthermore, introduction of E2F sites into the promoter of the cyclin-dependent kinase inhibitor $p15^{INK4B}$ gene effectively inhibited its induction by TGF-β. Similar transcription inhibition could be mimicked by coexpression of the Gal4-RB or Gal4-p107 chimeric molecules with a luciferase reporter construct driven by a modified $p15^{INK4B}$ promoter that contains the Gal4 DNA binding sites. Our results suggest that one of the primary TGF-β effects is the induction of E2F4-RB and E2F4-p107 complexes, which subsequently act, through E2F binding sites, to negatively regulate promoters of specific genes, such as *E2F1*, *B-myb*, and *HsORC1*, whose expression could potentially interfere with the TGF-β-induced growth arrest program.

MATERIALS AND METHODS

Materials. Oligonucleotides were synthesized in the Oligonucleotide Core Facility at Duke University Medical Center (Durham, NC). Sequences of the E2F and mE2F oligonucleotides are shown in Fig. 4*A*. Yeast Gal4 oligonucleotides have the sequence of (5′-CGG AGG ACT GTC CTC CG-3′). Human TGF-β1 was a generous gift from Amgen. Polyclonal antibodies against E2F1(SC-193X), E2F4(SC-866X), and p107(SC-318X) were purchased from Santa Cruz Biotechnology. Monoclonal antibody against RB (XZ77) was a generous gift from T. Van Dyke (University of North Carolina, Chapel Hill).

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Abbreviations: TGF-β, transforming growth factor β; RB, retinoblastoma susceptibility gene 1 product; *HsORC1*, human origin recognition complex 1; EMSA, electrophoretic mobility-shift assay.

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Plasmids. Oligonucleotides corresponding to both strands of the hamster DHFR E2F binding sites were annealed and inserted into the *Pvu*II site in p15P113-luc (14) construct to create p15+1×E2F-luc. Mutant E2F binding sites were introduced similarly to create p15+1×mE2F-luc. E2F and mE2F oligonucleotides were first phosphorylated by T4 polynucleotide kinase in the presence of 2 mM ATP and concatemerized with T4 DNA ligase for 10 min at room temperature before they were inserted into p15P113-luc to obtain p15+2×E2F-luc and p15+2×mE2F-luc. All constructs were confirmed by dideoxynucleotide sequencing. E2F1-luc, mE2F1-luc, HsORC1-luc, and mHsORC1-luc were generous gifts from K. Ohtani and J. R. Nevins (12). B-myb-luc and mB-myb-luc were generous gifts from D. Johnson (Duke University Medical Center). Gal4 vector pSG147, Gal4-RB, Gal4-RB706, and Gal4-RB592 were generous gifts from P. Robbins (University of Pittsburgh) (15). Gal4-mRbΔp34 and Gal4-p107Δ133 were generous gifts from R. Bremner (Toronto Western Hospital) (16).

Electrophoretic Mobility-Shift Assay (EMSA). Dignam nuclear extracts were prepared as described (17). Gel mobility shift assay was carried out in a 10-μl reaction that contained 20 mM Hepes (pH 7.9), 40 mM KCl, 6 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.15% BSA, and 0.1 mg/ml sonicated salmon sperm (Sigma). Five micrograms of total proteins were added to each reaction before the addition of competitors, as indicated above the figures. Probe (1 ng) was added last and was labeled by T4 polynucleotide kinase according to the standard method (18).

RNase Protection Assay. Total cytoplasmic RNA was isolated from equal numbers of untreated HaCaT cells as well as from cells treated with 100 pM human TGF-β1 for 12 hr. Total cytoplasmic RNA was used in an RNase protection assay using the *Sal*I–*Bgl*II fragment of the human E2F1 cDNA as a riboprobe. Glyceraldehyde-3-phosphate dehydrogenase riboprobe was included in the same reaction as control. For RNase protection assay, we used RPA II RNase Protection Assay Kit from Ambion (Austin, TX) and followed protocol supplied by the manufacturer.

Cell Culture and Transfection. Human HaCaT cells were grown in MEM supplemented with 10% fetal bovine serum. Transient transfections were carried out with the standard DEAE-Dextran method and the luciferase activities measured as described previously (14).

RESULTS

TGF-β Treatment Induces DNA-Bound E2F4-RB and E2F4-p107 Complexes. To test the effect of TGF-β on the formation of DNA-bound E2F complexes, nuclear extracts were prepared from HaCaT cells treated with human TGF-β1 for either 0, 4, 8, or 12 hr. These nuclear extracts were used in an EMSA using an oligonucleotide probe representing the E2F binding sites from the hamster DHFR promoter (sequences are shown in Fig. 4*A*). As shown in Fig. 1*A*, three major complexes were detected by EMSA. Two slower migrating complexes were gradually induced by TGF-β treatment, whereas the fastest migrating complex gradually diminished. We routinely observed the broad banding pattern for the two slower migrating complexes, and they were better resolved only when one of the complexes was absent (Fig. 1*B*, lanes 7 and 8). All three complexes were specific to the E2F probe used because they were readily competed away by an excess of unlabeled E2F oligonucleotides (Fig. 1*B*, lane 2), but not by an excess of mutant E2F oligonucleotides (Fig. 1*B*, lane 3), or the yeast Gal4 oligonucleotides (Fig. 1*B*, lane 4). Polyclonal antibodies raised against human E2F4 abolished all three complexes and caused the formation of "supershifted" complexes (Fig. 1*B*, lane 6), indicating that all three complexes contained E2F4. On the contrary, antiserum against E2F1 had no effect on any of the complexes (Fig. 1*B*, lane 5). Addition of a monoclonal antibody against human RB to the EMSA reaction specifically abolished the second slowest migrating complex (Fig. 1*B*, lane 7), indicating that this complex also contained RB in addition to E2F4. Addition of antibody against RB in the EMSA reaction also increased the DNA-bound E2F4 most

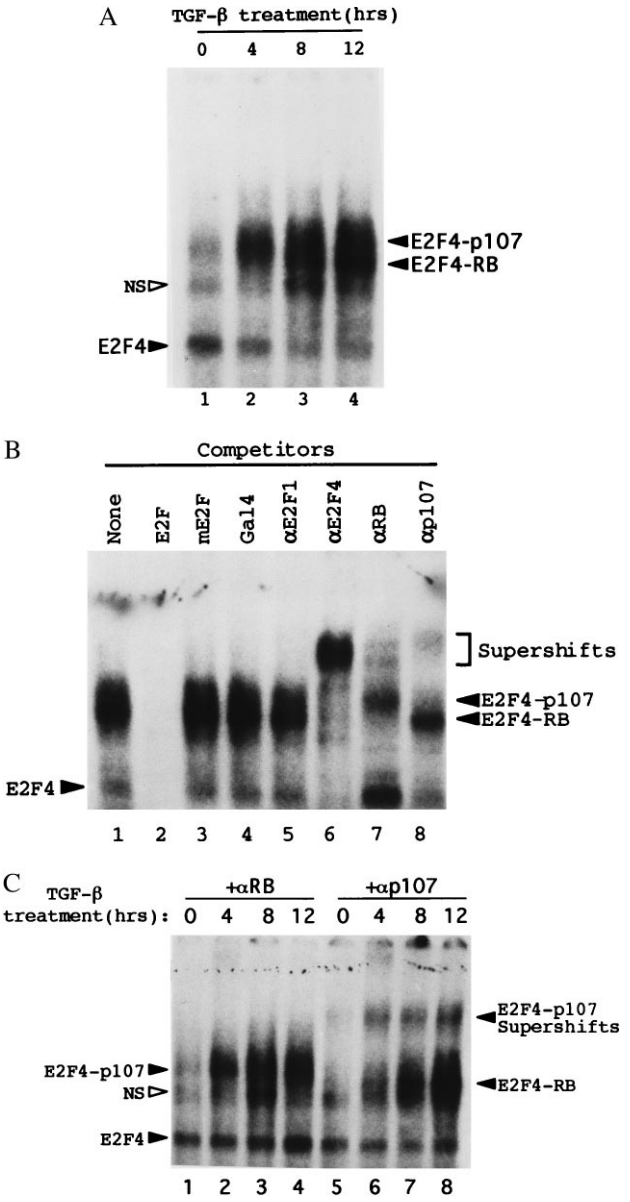

FIG. 1. TGF-β induces the formation of E2F4-RB and E2F4-p107 complexes. (*A*) Nuclear extracts were prepared from HaCaT cells after they were treated with human TGF-β1 for 0, 4, 8, or 12 hr. Extracts were used in an EMSA using the hamster DHFR E2F binding sites as a probe. Three specific complexes are indicated as E2F4, E2F-RB, and E2F4-p107 (see *B*). NS, nonspecific complex. (*B*) Nuclear extract from the 8-hr time point was used in EMSA as described in *A*. Twentyfold excess of nonradiolabeled oligonucleotides or 1 μl of specific antibodies were included as competitors in EMSA reactions as indicated above each lane: 1, no competitor; 2, E2F oligonucleotides; 3, mutant E2F oligonucleotides; 4, Gal4 oligonucleotides; 5, polyclonal antibody against E2F1; 6, polyclonal antibody against E2F4; 7, monoclonal antibody against RB; 8, polyclonal antibody against p107. Three specific complexes as well as the supershifted complexes are indicated. (*C*) EMSA was performed exactly as in *A*, except that the monoclonal antibody against RB (lanes 1–4) or polyclonal antibodies against p107 (lanes 5–8) were included in the gel shift reactions. Three specific complexes are indicated, as are the supershifted complexes caused by the addition of antiserum against p107. NS, nonspecific complex.

likely due to the release of RB from the DNA-E2F4-RB complex following the antibody recognition of RB protein (Fig. 1*B*, lane 7). Similarly, antiserum against human p107 abolished the slowest migrating complex, indicating that it contained p107 (Fig. 1*B*, lane 8). Unlike RB, antibody recognition of p107 caused the formation of "supershifted" complexes without increasing the DNA-E2F4 complex (Fig. 1*B*, lane 8).

The time-dependent induction of E2F4-p107 complex was more apparent when the experiment shown in Fig. 1*A* was repeated with the inclusion of antibody against RB in the EMSA reaction (Fig. 1*C*, lanes 1–4). In the presence of RB antibody, DNA-E2F4 complex increased slightly over the time course of TGF-β treatment, even though DNA-E2F4-p107 complex did increase significantly (Fig. 1*C*, lanes 1–4). It is possible that, following the antibody recognition of RB, more DNA-E2F4 complexes were released from the otherwise increasing amount of DNA-E2F4-RB complexes over the time course of TGF-β treatment. Similarly, when the same experiment was repeated with antiserum against p107 included in the EMSA reaction, the induction of the E2F4-RB complex was more obvious (Fig. 1*C*, lanes 5–8). At the same time, the supershifted E2F4-p107 complexes also increased over the time course of TGF-β treatment, indicating that the slowest migrating band was indeed E2F4-p107 (Fig. 1*C*, lanes 5–8). None of the complexes observed contained cyclin A, because addition of antiserum against cyclin A did not affect their formation (data not shown). Taken together, these results demonstrated that TGF-β specifically induced the formation of DNA-bound E2F4-RB and E2F4-p107 complexes at the expense of free E2F4 in HaCaT cells.

Mutation on E2F Sites Relieves Transcription Repression in Cells Treated with TGF-β. We next investigated whether E2F4-RB and E2F4-p107 complexes induced by TGF-β play any roles in the regulation of genes whose promoters contain E2F binding sites. We chose to study three such cellular genes: *E2F1*, *B-myb*, and *HsORC1*. All three genes are required for the progression into the S phase of cell cycle, and their promoters contain functional E2F binding sites that function primarily to repress gene expression in serum-starved quiescent (G_0) cells (6, 12, 13). Moreover, E2F1 gene expression was regulated by TGF-β treatment. The steady-state E2F1 mRNA levels decreased significantly in HaCaT cells treated with TGF-β (Fig. 2). Similar down-regulation of E2F1 mRNA has been reported in the mink lung epithelial cells, and the ectopic expression of E2F1 could override TGF-β-mediated growth arrest of mink lung epithelial cells (19). For each of these three genes, we obtained luciferase reporter constructs containing either the wild-type or mutant promoters, which have their functional E2F sites mutated (Fig. 3*A*). When these constructs were transfected transiently into the cycling HaCaT cells, mutation of E2F sites did not change significantly the expression from all three promoters (Fig. 3*B*). However, in the presence of TGF-β treatment, the same mutation on the E2F sites caused 5- to 6-fold of increase in gene expression from all three promoters when compared with their wild-type counterparts (Fig. 3*B*). These results suggested that E2F binding sites were required for the transcription repression of these promoters when cells were treated with TGF-β, most probably through the TGF-β-induced E2F4-RB and E2F4-p107 complexes binding to the E2F sites.

E2F Binding Sites Inhibit TGF-β-Mediated Promoter Induction of *p15^{INK4B}* Gene. If E2F binding sites indeed function to suppress TGF-β-mediated gene expression, we would expect the exogenously introduced E2F binding sites to inhibit the genes that are normally induced by TGF-β, such as the cyclin-dependent kinase inhibitor *p15^{INK4B}* gene (14). Oligonucleotides representing the wild-type or mutant E2F binding sites from the hamster DHFR promoter were introduced into the p15^{INK4B} promoter linked to a luciferase reporter gene

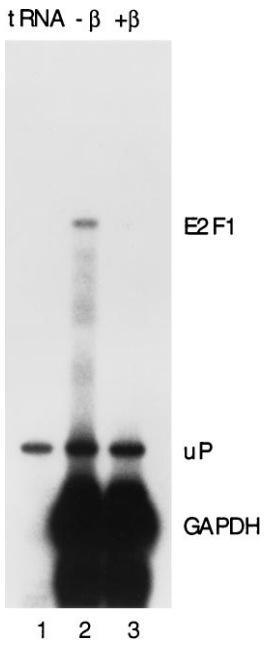

FIG. 2. TGF-β reduces steady-state E2F1 mRNA levels in HaCaT cells. Total cytoplasmic RNA was isolated from HaCaT cells (lane 2, $-\beta$) or cells treated with TGF-β1 for 12 hr (lane 3, $+\beta$). Both preparations of RNA were used in an RNase protection assay with a uniformly labeled human E2F1 riboprobe. The protected fragment by E2F1 mRNA is indicated. Also indicated is the protected fragment representing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Lane 1, tRNA control. uP, undigested GAPDH riboprobe.

(Fig. 4*A*). These constructs were transiently transfected into HaCaT cells and assayed for their relative luciferase activities in the absence or presence of TGF-β treatment. As shown in Fig. 4*B*, introduction of one copy of E2F binding site into the p15^{INK4B} promoter significantly reduced its induction by TGF-β (Fig. 4*B*, p15+1xE2F-luc), whereas a mutant E2F site had no effect (Fig. 4*B*, p15+1xmE2F-luc). Two copies of E2F binding sites completely abolished TGF-β induction of p15^{INK4B} promoter (Fig. 4*B*, p15+2xE2F-luc). In contrast, two copies of the mutant E2F binding sites reduced TGF-β induction by only 2-fold (Fig. 4*B*, p15+2xmE2F-luc), probably due to the increased spacing between the essential Sp1 binding site and the transcription initiation site (14). These results strongly suggest that E2F binding sites, most likely through the action of E2F4-RB and E2F4-p107 complexes, can indeed function to actively repress gene expression induced by TGF-β treatment.

Promoter-Bound RB or p107 Inhibits TGF-β-Mediated Induction of p15^{INK4B} Promoter. One potential mechanism for the active repression of transcription by the family of E2F-RB complexes involves blocking of interaction between enhancer-binding proteins and the basal transcription machinery (11). To test whether promoter-bound RB or p107 can directly repress transcription activation by TGF-β, thus mimicking the function of E2F4-RB and E2F4-p107, we introduced one copy of yeast Gal4 DNA binding sites into the same location of p15^{INK4B} promoter where E2F binding sites were previously inserted (Fig. 5*A*). Introduction of Gal4 binding sites alone did not affect either the uninduced or TGF-β-induced expression from the modified p15^{INK4B} promoter (data not shown). However, Cotransfection of the Gal4-RB expression plasmid, which has the Gal4 DNA binding domain covalently linked to RB (15), reduced specifically the TGF-β-mediated induction of p15^{INK4B} promoter in a dosage-dependent manner without affecting the uninduced expression (Fig. 5*B*). Two constructs that harbor the naturally occurring RB mutations, Gal4-RB592 and Gal4-RB706 (15) (Fig. 5*A*), failed to repress TGF-β-induced gene expression from the p15^{INK4B} promoter

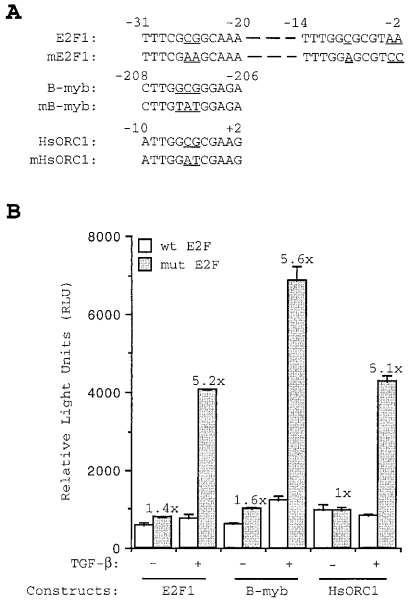

FIG. 3. Mutation of E2F binding sites relieves transcription repression in cells treated with TGF-β. (*A*) Promoters of the human *E2F1*, mouse *B-myb*, and human *HsORC1* genes containing either the wild-type or mutant E2F sites were placed in front of the luciferase reporter gene to create E2F1-luc, mE2F1-luc, B-myb-luc, mB-myb-luc, HsORC1-luc, and mHsORC1-luc. The wild-type and mutant E2F binding sites for these three genes are shown. Numbers indicate nucleotide positions relative to their respective transcription start sites, except in B-myb and mB-myb, where numbers indicate their relative positions to the translation start site (ATG). (*B*) Constructs shown in *A* were transiently transfected into human HaCaT cells and treated with or without 100 pM human TGF-β1 for 24 hr. Relative light units were measured after lysates were prepared from these cells. Induction folds by the mutation of E2F sites are shown. Error bars = SD of duplicates.

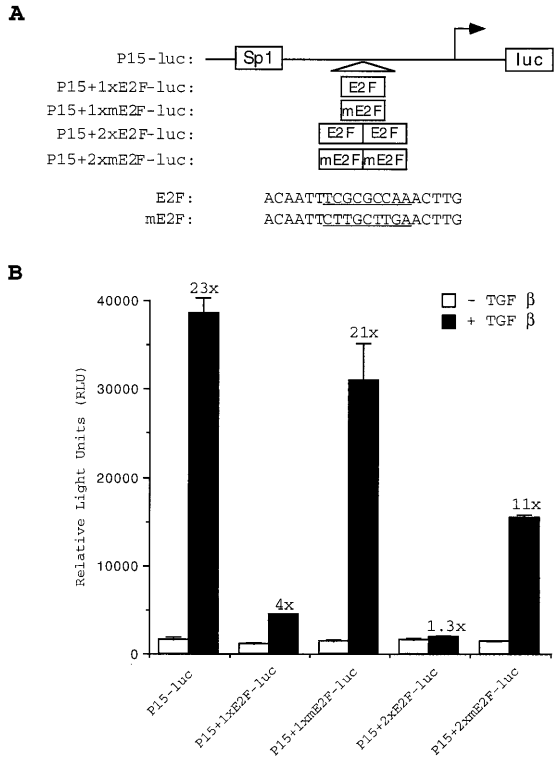

FIG. 4. Introduction of E2F binding sites into the $p15^{INK4B}$ promoter suppresses its induction by TGF-β. (*A*) E2F binding sites from the hamster DHFR promoter were introduced into the minimal inducible $p15^{INK4B}$ promoter construct, p15P113-luc (14), to create p15+1×E2F-luc. The mutant E2F binding sites were inserted into the same position to create p15+1×mE2F-luc. Two copies of the same wild-type or mutant E2F binding sites were similarly introduced to create p15+2×E2F-luc and p15+2×mE2F-luc. Nucleotide sequences for the wild-type and mutant E2F binding sites are underlined. (*B*) Constructs shown in *A* were transiently transfected into HaCaT cells, and their relative light units were measured after cells were treated with or without 100 pM human TGF-β1 for 24 hr. Induction folds by TGF-β are shown. Error bars = SD of duplicates.

(Fig. 5*C*, Gal4-RB592 and Gal4-RB706), suggesting that a functional RB is required for the effective repression of TGF-β-mediated gene expression. Although both RB592 and RB706 have lost their ability to bind to E2F factors and therefore could not be recruited to the promoter via E2F, they nevertheless also lost their function as transcription repressors against TGF-β-induced gene expression. We also used these two mutant RB constructs as transfection controls for the transcription repression by Gal4-RB and Gal4-p107. It is possible that the loss of both their ability to bind to E2F factors and their ability to repress gene expression by TGF-β contribute dually to their loss of tumor-suppression activities. Interestingly, a phosphorylation-deficient mouse RB construct, Gal4-mRbΔp34, which has all eight potential phosphorylation sites in RB mutated and thus constitutively binds E2F factors (16), completely abolished TGF-β induction of $p15^{INK4B}$ promoter (Fig. 5*C*, Gal4-mRBΔp34). This result suggests that the hypophosphorylated RB, which is the binding partner of E2F factors, represents the active form of RB for the repression of TGF-β-mediated gene induction. Similar results were obtained for the Gal4-p107 chimeric construct (Fig. 5*C*, Gal4-p107Δ133; data not shown). Therefore, RB or p107 can directly repress TGF-β-mediated gene expression when bound to the promoter, consistent with our results that TGF-β specifically induces E2F4-RB and E2F4-p107 complexes to repress gene expression when cells are treated with TGF-β.

DISCUSSION

Promoters of many cellular genes contain E2F binding sites that contribute to their transcription regulation. They include genes required for DNA synthesis (*DHFR*, thymidine kinase, DNA pol-α, *HsORC1*) and genes regulating cell proliferation (*E2F1*, *c-myc*, *N-myc*, *B-myb*, *Rb*, *cdc2*) (9, 20). For most of these genes, E2F binding sites are necessary but not sufficient for their cell cycle regulation, suggesting that additional transcription factors may be required. E2F site can function either as an enhancer or a repressor depending on the promoter context within which it resides. For example, mutations on E2F sites in N-myc and DHFR promoters led to a >80% reduction in promoter activities (21, 22), whereas similar mutations resulted in a 3- to 10-fold increase in the activities of the B-myb, E2F1, and HsORC1 promoters (6, 12, 13, 23). It is important to point out that those studies of transcription activation or repression caused by mutation of E2F sites in specific promoters were mostly carried out in the serum-starved, quiescent G_0 cells, which were subsequently induced to reenter cell cycle by the addition of serum. In contrast, mutation of E2F sites has very little effect on promoter activities in cycling cells (ref. 20; see also Fig. 3*B*).

In certain cell types, TGF-β causes hypophosphorylation of RB and possibly p107, which, in turn, sequester free E2F factors and consequently block the entry to S phase of the cell cycle (5). In this report, we extended previous findings by showing that TGF-β induces the formation of E2F4-RB and E2F4-p107 complexes, which are capable of binding to E2F sites, at the expense of free E2F4 in HaCaT cells (Fig. 1*A*). The consequence of the induced E2F4-RB and E2F4-p107 complexes was manifested in the repression of promoters from three cellular genes: *E2F1*, *B-myb*, and *HsORC1*. While E2F

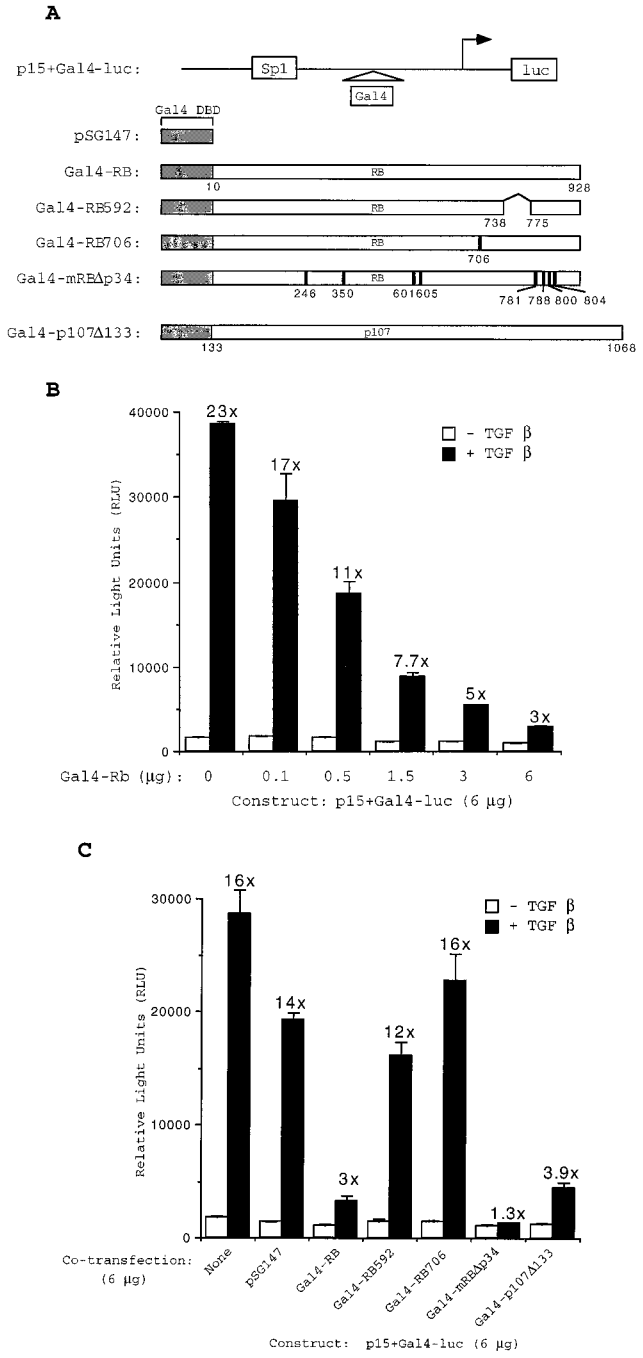

FIG. 5. Promoter-bound RB and p107 suppress TGF-β-mediated gene expression from $p15^{INK4B}$ promoter. (*A*) Gal4 DNA binding sites were introduced into the p15-luc construct to obtain p15+Gal4-luc. Various Gal4 fusion constructs are shown. pSG147, vector plasmid containing the amino acids 1–147 of the yeast Gal4 DNA binding domain (Gal4 DBD); Gal4-RB, fusion protein construct between the Gal4 DNA binding domain and the human RB missing the first 10 amino acids; Gal4-RB592, same as Gal4-RB but containing deletions from amino acids 738–775; Gal4-RB706, same as Gal4-RB but with a point mutation at amino acid 706; Gal4-mRBΔp34, fusion protein construct between the Gal4 DNA binding domain and the mouse RB homologue, which has all eight potential phosphorylation sites (at amino acids 246, 350, 601, 605, 781, 788, 800, and 804) mutated; Gal4-p107Δ133, fusion protein construct between the Gal4 DNA binding domain and the human p107 from amino acids 133–1,068. Solid vertical bars denote specific amino acid mutations. (*B*) p15+Gal4-luc construct was cotransfected with increasing amounts of Gal4-RB DNA, as indicated, into HaCaT cells, and their luciferase activity was assayed as described for Fig. 3*B*. Induction folds by TGF-β are indicated. Error bars = SD of duplicates. (*C*) p15+Gal4-luc construct was cotransfected with different Gal4 fusion constructs, as indicated, and luciferase activities were assayed as described for Fig. 3*B*. Induction folds by TGF-β are indicated. Error bars = SD of duplicates.

binding sites have been implicated previously in the transcription repression of these genes in quiescent cells (6, 12, 13), our results demonstrated that E2F sites also function to repress promoter expression from these genes in cells treated with TGF-β, most probably through the action of the induced E2F4-RB and E2F4-p107 complexes. Moreover, mutation of E2F sites resulted in elevated expression from all three promoters in the presence of TGF-β treatment, whereas the same mutation had little effect in the cycling cells when cells were not treated with TGF-β. These results indicate that E2F4-RB and E2F4-p107 complexes are necessary for transcription repression of genes, such as *E2F1*, *B-myb*, and *HsORC1*, whose expression could potentially override TGF-β-induced growth arrest. Loss of E2F-mediated transcription repression could result from mutations on E2F or RB family proteins that disrupt interactions between E2F and RB proteins, or their binding to E2F sites. Such mutations will likely abolish TGF-β-mediated growth arrest programs and cause excess growth and proliferation, which may eventually lead to tumor formation. This scenario is consistent with the recent observation that adult E2F1 null mice developed multiple types of tumors (24, 25). On the other hand, disruption of the TGF-β signal transduction pathway may abrogate the induction of E2F-RB and E2F-p107 complexes by TGF-β and thus demolish their function as transcription repressors, which could also contribute to excessive cellular growth and proliferation. Indeed, most cancer cells lose their responsiveness to TGF-β signaling and fail to arrest at G_1 phase of the cell cycle upon TGF-β treatment.

Five members of the E2F family of transcription factors, E2F1 to E2F5, have been identified so far. In most cell types, E2F1 preferentially binds to RB, whereas E2F4 tends to bind to RB family members p107 and p130 (26–32), although recent studies have shown that E2F4 also binds to RB (33, 34). Our results indicate that E2F4 is the predominant E2F factor in HaCaT cells that is capable of binding to RB as well as to p107 (Fig. 1*B*). Unlike E2F1, which is activated near the G_1/S boundary, E2F4 is constitutively expressed and its protein level remains constant throughout the cell cycle (26). This feature may set E2F4 apart from other members of the E2F family and make E2F4 an ideal candidate for RB- and p107-mediated transcription repression, including the G_1 arrest induced by TGF-β. It is possible that E2F-RB and E2F-p107 complexes that contain E2F factors other than E2F4, such as E2F1, are present in certain cell types to perform similar functions. A determination of the exact roles played by E2F4 in transcription repression awaits further investigation and the generation of appropriate animal models.

At present, the precise mechanism by which promoter-bound RB and p107 repress TGF-β-induced gene expression is not known. RB and p107 may repress the TGF-β-mediated expression by blocking the cross-talk between an effector(s) of TGF-β signaling pathway and the basal transcription machinery. Alternatively, RB and p107 may interact directly with the basal transcription machinery such that they compete for basal transcription factors with TBP or TFIIB, because there are significant sequence homologies between the A pocket domain of RB and TBP, as well as between the B pocket domain and TFIIB, respectively (35). Our results favor the first model because promoter-tethered RB and p107 do not affect the uninduced expression and only specifically inhibit TGF-β-induced expression (Fig. 5*C*). Such a model is also consistent with the observation made by Weintraub *et al.* (11) that promoter-tethered RB failed to block Sp1-mediated transcription activation per se. The second model would predict that RB

or p107 should suppress the uninduced as well as TGF-β-induced expression.

Finally, our results also provide one molecular basis for the differential promoter activation by TGF-β. DNA binding motifs for the transcription factor Sp1 have been implicated in the transcription activation of *p15^{INK4B}* and *p21(Cip1/WAF1)* genes by TGF-β (14, 36). However, the mechanism by which the specific and selective induction of CKI genes by TGF-β through Sp1 binding motifs remains unknown, especially in light of the fact that promoters of many cellular genes contain Sp1 binding sites. Many of the E2F site-containing genes, including *E2F1* (12), *B-myb* (6), and *HsORC1* (13), also contain Sp1 binding sites in close proximity of the E2F binding sites. It is reasonable to suggest that the functional Sp1 sites present on these promoters mediate the observed gene activation by TGF-β after E2F sites were mutated (Fig. 3*B*), since canonical Sp1 binding sites alone are able to support TGF-β-induced gene expression (J.-M.L. and X.-F.W., unpublished data). TGF-β-induced E2F4-RB and E2F4-p107 complexes consequently could block the interaction between Sp1-associated factor(s) and the basal transcription machinery and actively repress the potential induction of these genes by TGF-β, thus providing a mechanism by which the differential promoter activation by TGF-β could be achieved.

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