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Transcriptional Regulation of the Human Tumor Suppressor p14^{ARF} by E2F1, E2F2, E2F3, and Sp1-like Factors

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The human ARF/INK4a locus encodes two cell cycle inhibitors, p16^{INK4a} and p14^{ARF}, by using separate promoters. A variety of mitogenic stimuli upregulate ARF but a direct modulation at the transcriptional level has been reported only for E2F-1. We show here that the ARF promoter is strongly responsive also to E2F2 and E2F3, thus providing a strong support to their suggested role in the induction of apoptosis. Through the usage of both deletion mutants and/or site-directed mutants, we surprisingly found that none of the four putative E2F consensus sites is strictly necessary for the upregulation of ARF expression, as a minimal deletion mutant, lacking all the putative E2F binding sites, is still transactivated by E2F. Moreover, our data suggest that the ARF promoter is regulated by E2F through both direct binding to the promoter sequences and indirectly, probably by being tethered to the ARF promoter by Sp1-like factors.

Key Words: **E2F; human p14ARF; tumor suppressor; Sp1-like factors; apoptosis.**

The ARF/INK4a locus, one of the most frequently disrupted loci in human cancer (1, 2), encodes two cell cycle inhibitors, p16^{INK4a} and p14^{ARF}, by using separate promoters (3). The p16 protein specifically inhibits the ability of cyclin D/CDK4 or CDK6 complexes to phosphorylate the retinoblastoma protein (4, 5). Ectopic ARF expression stabilizes p53 and induces p53-responsive genes, Mdm2 among them. ARF can physically interact with Mdm2 and its binding blocks both Mdm2-induced p53 degradation and transactivational silencing (6–9). p53 is a homotetrameric transcription factor induced by DNA damage or by inappropriate mitogenic signaling, and its activation and accumulation, largely through protein stabilization, can trigger cell-cycle arrest or apoptosis (10–12). A variety of mitogenic stimuli including E1A, myc, oncogenic ras, V-Abl, and E2F upregulate ARF, leading to p53 stabilization (13–17).

The human ARF promoter is a CpG island containing numerous Sp1 binding sites and, although at a low frequency, can be silenced by DNA methylation (18). At present, there are few studies which directly address the mechanisms regulating human ARF expression. For instance, it has been reported by the use of adenoviral vectors expressing the five members of the E2F family of transcription factors that ARF mRNA levels were elevated after expression of both E2F1 and E2F2 (19), but a direct modulation at the transcriptional level has been reported only for E2F-1 (17, 18). On the other hand, despite the fact that myc induces ARF to accumulate very rapidly, it is presently unclear whether myc activates the ARF promoter directly (14).

To gain a better understanding of the regulation of the human ARF promoter, we performed a series of cotransfection experiments, using expression vectors encoding for the different members of the E2F family, myc and oncogenic ras. Our data indicate that only E2F1, E2F-2, and E2F-3 are able to upregulate the ARF promoter. Moreover, through the usage of both deletion mutants and/or site-directed mutants, we surprisingly found that none of the E2F consensus sites is strictly necessary for the upregulation of ARF expression, as a deletion mutant lacking all the putative E2F binding sites is still transactivated by E2F.

MATERIALS AND METHODS

Cell culture and transfection. NIH3T3, Saos2, C33A, and Hela cell lines were maintained in DMEM, supplemented with 10% FCS.

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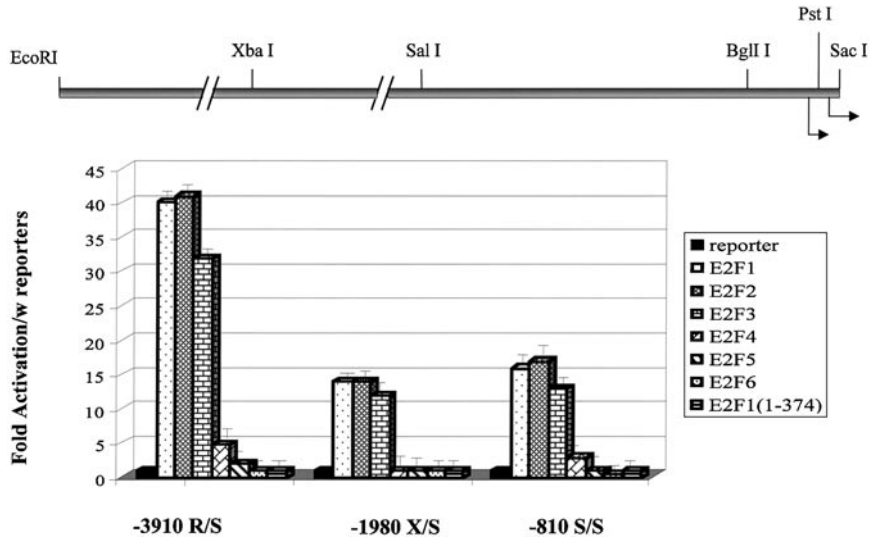

FIG. 1. Regulation of the ARF promoter by E2F family members. The −3910R/S, the −1980X/S and the −810S/S reporters were cotransfected with 10 μg of expression vectors coding for the six members of the E2F family and for the E2F(1–374) mutant. The results of triplicate transfections are reported as the mean fold activation with each of the effectors (activity with effector/activity with empty expression vector). Values presented were normalized with an internal control as described under Materials and Methods. Standard deviations are shown by vertical bars.

CAT assays were as described (20). The pCMV-βgal plasmid (1.5 μg) was used to normalize CAT values for transfection efficiency.

DNA library screening. A WI38 genomic library was screened (3×10^5 plaques) using as probe the p14ARF exon 1β generated by PCR using the LA33 and LA34 primers and Hela genomic DNA as template. The isolated phage clone (P1) was analyzed by restriction enzyme analysis, Southern blot and DNA sequencing of relevant regions.

Plasmids. The pRc-CMV encoding E2F1, E2F2, E2F3, E2F4, E2F5, E2F6, E2F1 (1–374), the PRc-CMV-DP1 and the reporter plasmid pE2F$_4$CAT were obtained from K. Helin. Expression vectors for myc, ras and reporter plasmids pB11 and pG5-83CAT were gifts from, respectively, B. Vogelstein, G. Del Sal (originally from A. Levine), B. Amati, and B. Majello. alfa 6 was already described (20). A *Bam*HI–*Hin*dIII fragment was retrieved from pPAC-E2F1(113–120) clone (a gift of J. C. Azizkhan), filled-in and cloned in the *Hin*dIII filled-in site of pRc-CMV vector to give the pCMVE2F1(113–120). An *Eco*RI/*Sac*I fragment from the P1 phage was cloned in the same sites of pGEM3: from this clone we derived the −3910R/S and the −1980X/S clones by blunting the *Eco*RI/*Sac*I or the *Xba*I/*Sac*I fragments and cloning them in the SmaI site of pCAT-O and the −810 S/S, by blunting the 3′ end of a *Sal*I/*Sac*I fragment and cloning in the *Sal*I/*Hin*dIII filled site of pCAT-0. From this last clone a *Sal*I/*Pst*I fragment was cloned in the SalI/PstI sites of pCAT0 to give the −810S/P clone.

To construct the −338S/Pm clone we amplified a fragment from the −810S/P clone with the sense primer 450SPF (overlapping the putative E2Fa binding site) and the antisense primer CAT located within the CAT gene. The amplification product was digested with *Sal*I and *Pst*I and cloned in the same sites in pCAT-O. To obtain the −810S/Pm clone, the PCR fragment amplified with the 450SPR primer (designed on the putative E2Fa binding site) and the PR19DF primer, encompassing the 5′ end of the *Sal*I/*Pst*I fragment from the −810S/P construct, was digested with *Sal*I and cloned in the SalI site of the −338S/Pm. To obtain the −226B/P clone a *Bgl*II/*Pst*I fragment from the −338S/Pm plasmid was inserted in pCAT-O digested *Sma*/*Pst*I, after blunting the *Bgl*II end. The −144B/Pm clone was created by PCR starting from the −810S/P, using the BSSHIIF primer overlapping the putative E2Fc binding site and the CATprimer. The PCR product was digested with *Bam*HI/*Pst*I and cloned in the same sites of pCAT-O.

Oligonucleotides. LA33, 5′-CGCGCCTGCGGGGCGGAGATG-3′; LA34, 5′-CGGACTTTTCGAGGGCCTTTC-3′; PR19DF, 5′-GAGTCGACAGCTCCGGCAGC-3′; 450SPR, 5′-TTGTCGACCCTGTGTGCGCCCC-3′; 450SPF, 5′-GGGTCGACCCGTGGCGGTAGGCGGG-3′; BSSHIIF, 5′-GAGGATCCGTGCGCGCCTGCGGGG-3′; OLIGO1, 5′-TGGCACACAGGGCGGGAAAGTGGCGGTAGGCGGGAGG-3′; OLIGO1m, 5′-TGGCACACAGGGTCGACCCGTGGCGGTAGGCGGGAGG-3′; OLIGO2, 5′-TGGGGCGCGCTCAGGGAAGGCGGGTGCGCGCCTGCGG-3′; OLIGO2m, 5′-TGGTTGCGAATTCCCGGGGATCCGTGCGCGCCTGCGG-3′; OLIGOE2F1, 5′-GATCCATTTAAGTTTCGCGCCCTTTCTCAAA-3′; OLIGOE2F1m, 5′-GATCCATTTAAGTTTCGATCCCTTTCTCAAA-3′; OLIGOSp1, 5′-CCCGGGGCGCACGGCTCTATAAATACGAGTGCGCGGCGGGCCGGGCGAGAGCGTAGTGGAGGAGGCGCGGTTGTGAGTA-3′.

Nuclear extracts and EMSA. Nuclear extracts were prepared from subconfluent C33A cells transfected with pCMV-E2F1 and pCMV-DP1 plasmids as described (21). EMSA were performed as described (21).

For the supershift experiment, polyclonal antibodies against E2F1 were used (C20-Santa Cruz Biotechnology).

RESULTS

Only E2F1, E2F2, and E2F3 Are Able to Transactivate the ARF Promoter

We have isolated genomic sequences encompassing the p14^{ARF}-specific exon 1β and created a reporter plasmid in which the expression of the CAT gene was regulated by an approximately 4-kb fragment ending at a Sac I site, located 26 bp upstream from the ATG initiation codon for p14ARF (−3910R/S; Fig. 1). Starting from this fragment, 5′-end deletions were made by

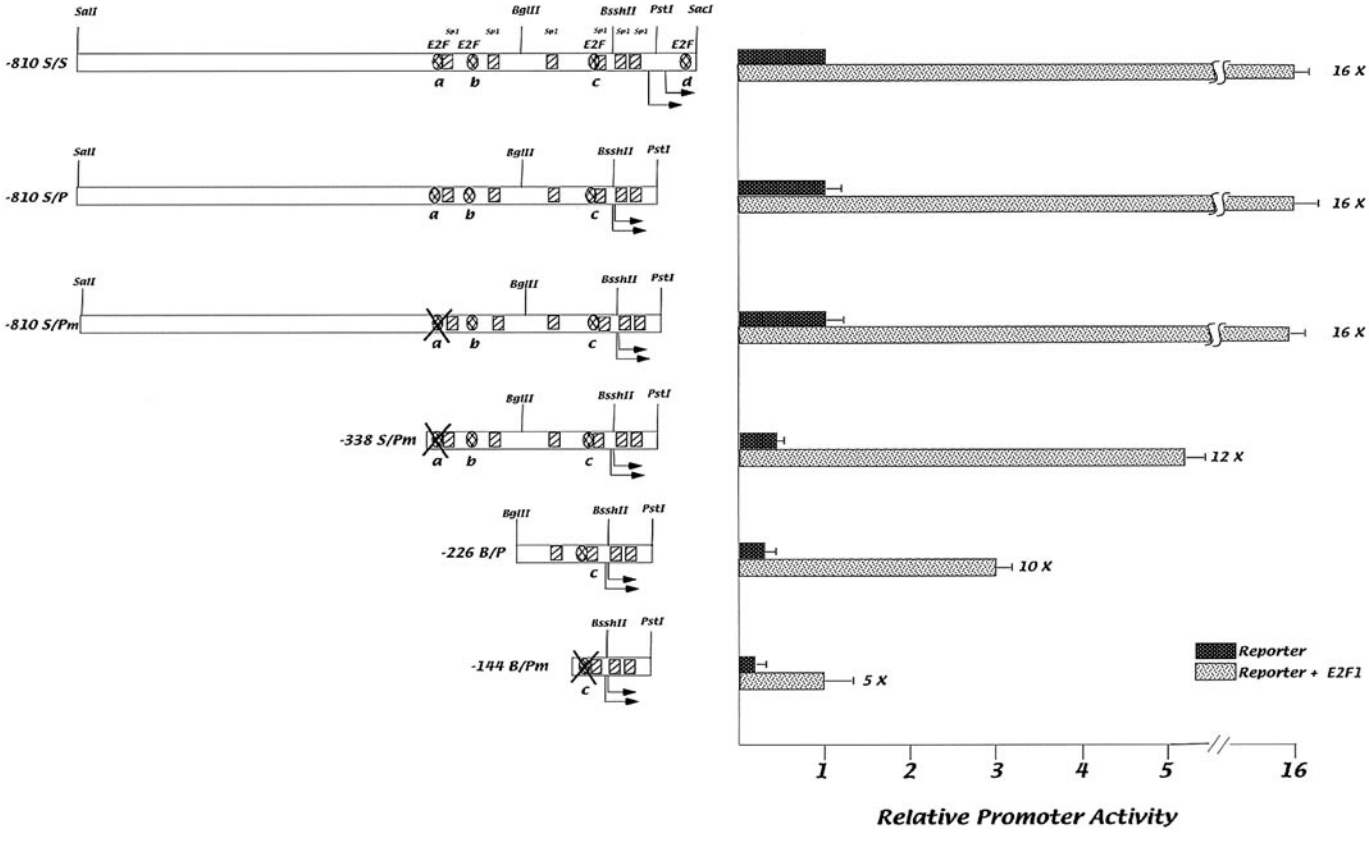

FIG. 2. E2F1 is able to transactivate also a minimal ARF promoter. On the left are schematically represented the promoter constructs utilized. The putative E2F (E2Fa, b, c, and d) and Sp1 binding sites are indicated. Each of the ARF promoter constructs was cotransfected with an equal amount of pCMVE2F1 or empty parental expression vector. CAT activity was normalized to that of the −810S/S reporter.

using naturally occurring restriction sites, thus constructing the −1980X/S and the −810S/S reporter plasmids (all numbering is relative to the ATG initiation codon). These reporters were transfected in NIH3T3 cells and in human cell lines not expressing p53, i.e., Hela, Saos2, and C33A cell lines. As the highest levels of CAT expression were obtained in C33A cells (data not shown), we choose this cell line for the successive experiments.

The three reporter plasmids were cotransfected with expression vectors encoding myc, RasVal12, the six different known members of the E2F-family and a mutated form of E2F-1, E2F1(1–374), lacking the activation domain.

The results of three independent experiments (Fig. 1 and data not shown) clearly indicate that only E2F1, E2F2, and E2F3 are able to upregulate all the reporter clones, with E2F1 and E2F2 working at higher efficiency. Coexpression of DP1, a heterodimer partner of the E2F factors did not affect the relative promoter activation properties of the E2F proteins (data not shown). As expected E2F6, which lacks a transactivation domain, does not substantially affect the level of expression of the reporter plasmids. The lack of activation obtained with the mutant E2F1(1–374), however, strongly suggests that the expression of E2F1, E2F-2, and E2F-3 results in a specific transcriptional activation of the human ARF promoter.

The E2F Binding Sites in the ARF Promoter Are Not Necessary for E2F Transactivation

Figure 2 schematically shows the location of potential binding sites for the Sp1 and E2F transcription factors in the −810S/S fragment (18). Also indicated are two presumed transcription start sites (22, 23).

We first constructed a 3′ deletion mutant ending at a *Pst*I site (−810S/P), thus lacking a 3′ 65-bp fragment bearing the E2Fd site. Interestingly, the basal level of expression did not change (Fig. 2) and the deleted promoter fragment was transactivated by E2F1 to the same extent as the longer promoter fragment (−810S/S). We then mutated, by site-directed mutagenesis, the distal E2Fa site (−810S/Pm). Again, the responsiveness of the double-mutated promoter to E2F factors did not change, suggesting that neither of the two E2F consensus sites play an essential role in E2F1 mediated transactivation. However, progressive deletions up to −338 (−338S/Pm), −226 (−226B/P, lacking the E2Fb consensus box) and −144 (−144B/P construct,

bearing a mutated E2Fc site) resulted in a progressive decline of the response to E2F-1. Interestingly, the last construct, lacking all the E2F-consensus sites, is still transactivated 4- to 5-fold by E2F-1. Similar results were obtained with E2F-2 and E2F-3 transcription factors (data not shown).

The E2F Factors Transactivate the ARF Promoter through Both Direct and Indirect Binding

It is relevant to note that progressive deletions resulted also in a progressive decrease in the level of basal transcription, as already described (18), suggesting that the deleted sequences, bearing consensus sites for Sp1-like factors, could play a role in mediating both basal and E2F-dependent transcriptional activity.

The observation that E2F1, E2F2, and E2F3 are able to transactivate a minimal ARF promoter lacking all the E2F consensus sites, raises the possibility that E2F factors transactivate the ARF promoter through both direct and indirect effects. To explore this point we followed different approaches. First, we wanted to confirm the authenticity of the potential E2F-binding sites E2F a, b, and c by EMSA (Fig. 3). The incubation of a radiolabeled oligonucleotide (Oligo1, Fig. 3A) carrying the two E2F binding sites a and b with nuclear extracts prepared from E2F and DP1 cotransfected C33A cell line, led to the formation of three specific protein–DNA complexes. Competition experiments were performed with an excess of unlabeled oligonucleotides that carry respectively a wild-type E2F-binding site or a mutated version of the E2F-binding site. Incubation of C33A cell extracts with a 100× molar excess of intact but not mutated E2F-binding site abolished the formation of DNA–protein complexes I and III (Fig. 3A, lanes 3 and 4). To ensure that these protein-DNA complexes were made up of human E2F protein, protein-DNA-binding assay was incubated with antibodies against E2F-1. As shown in Fig. 3A (lane 5) inclusion of this antiserum resulted in a supershifted protein–DNA complex.

As inspection of the Oligo1 sequence revealed the presence of a Sp1 consensus site partially overlapping the E2Fa site, we used a 100× molar excess of an unlabeled oligonucleotide bearing two Sp1 sites in competition experiments (Fig. 3A, lane 6). The results clearly show that complex II as well as complex I were eliminated, indicating a binding of a Sp1-like factor and suggesting that E2F and Sp1-like factors are involved in the formation of the same DNA–protein complex.

Similar experiments performed with a radiolabeled oligonucleotide (Oligo1m, Fig. 3A) carrying the mutated version of the E2Fa site, did not result in the formation of E2F or Sp1-DNA specific complexes. In fact, only a very faint band was detected (lane 7) that was not competed by a 100× molar excess of intact E2F-binding site (lane 8), mutated E2F-binding site (lane 9), and Sp1-binding sites (lane 10).

Similar EMSA experiments performed with a radiolabeled oligonucleotide carrying the E2Fc site (Fig. 3B, Oligo2) or with an oligonucleotide carrying a mutated version of the E2Fc site (Fig. 3B, Oligo2m) both resulted in the formation of specific complexes that were competed for only by a 50× (lanes 3 and 9) and 100× molar excess (lanes 4 and 10) of the Sp1 unlabeled competitor but not by 200× excess of the E2F unlabeled competitor (lanes 5 and 11).

It has been described that E2F1 and Sp1 act synergistically in activating the DHFR promoter (24). When these factors are transiently cotransfected on a DHFR promoter containing only Sp1 sites, E2F1 can superactivate Sp1-dependent transcription, conversely Sp1 can transactivate the DHFR transcription through E2Fs sites.

Two observations render Sp1 as a good candidate for the role of E2F tethering factor on ARF promoter: (a) the −144B/Pm plasmid, the shorter clone transactivated by E2F still contains various Sp1 binding sites; (b) only E2F1, E2F2, and E2F3, though neither E2F4 nor E2F5 are able to interact with Sp1.

To investigate if Sp1 and E2F1 cooperate to activate ARF transcription we have analyzed the transcriptional effect of the E2F1 mutant, E2F1(113–120), which activates E2F-dependent transcription to approximately the same extent as does the wild-type E2F1, but is unable to functionally interact with Sp1 (24). The results (Fig. 4) clearly show that the −810S/P reporter was transactivated to the same extent by both E2F vectors, while the −144B/Pm reporter was efficiently upregulated exclusively by the wild-type E2F-1 factor, suggesting that interaction with Sp1 is necessary to gain activation by E2F factors.

To exclude the possibility of a non-specific effect of E2F-factors on a minimal promoter, in our experimental conditions, we cotransfected various minimal CAT-promoter constructs with E2F-1 expression vector. In particular, we used three plasmids bearing respectively: (a) the minimal promoter derived from the LTR of a human endogenous provirus ERV9 (20), bearing one Sp1-site, a TATA box and an Inr-like sequence, (b) the minimal promoter of the early region of SV40, carrying six Sp1 sites and a TATA box (25), and (c) the minimal promoter from HIV, containing three Sp1 sites, a TATA box and an Inr-like sequence (25). The results (Fig. 5) clearly indicated that E2F-1 specifically upregulates only the −144B/Pm construct derived from the ARF promoter.

Taken together, these results strongly suggest that E2F factors are able to activate the ARF promoter not only by direct binding but also through an indirect way, i.e., probably by being tethered to the promoter by Sp1-like factors.

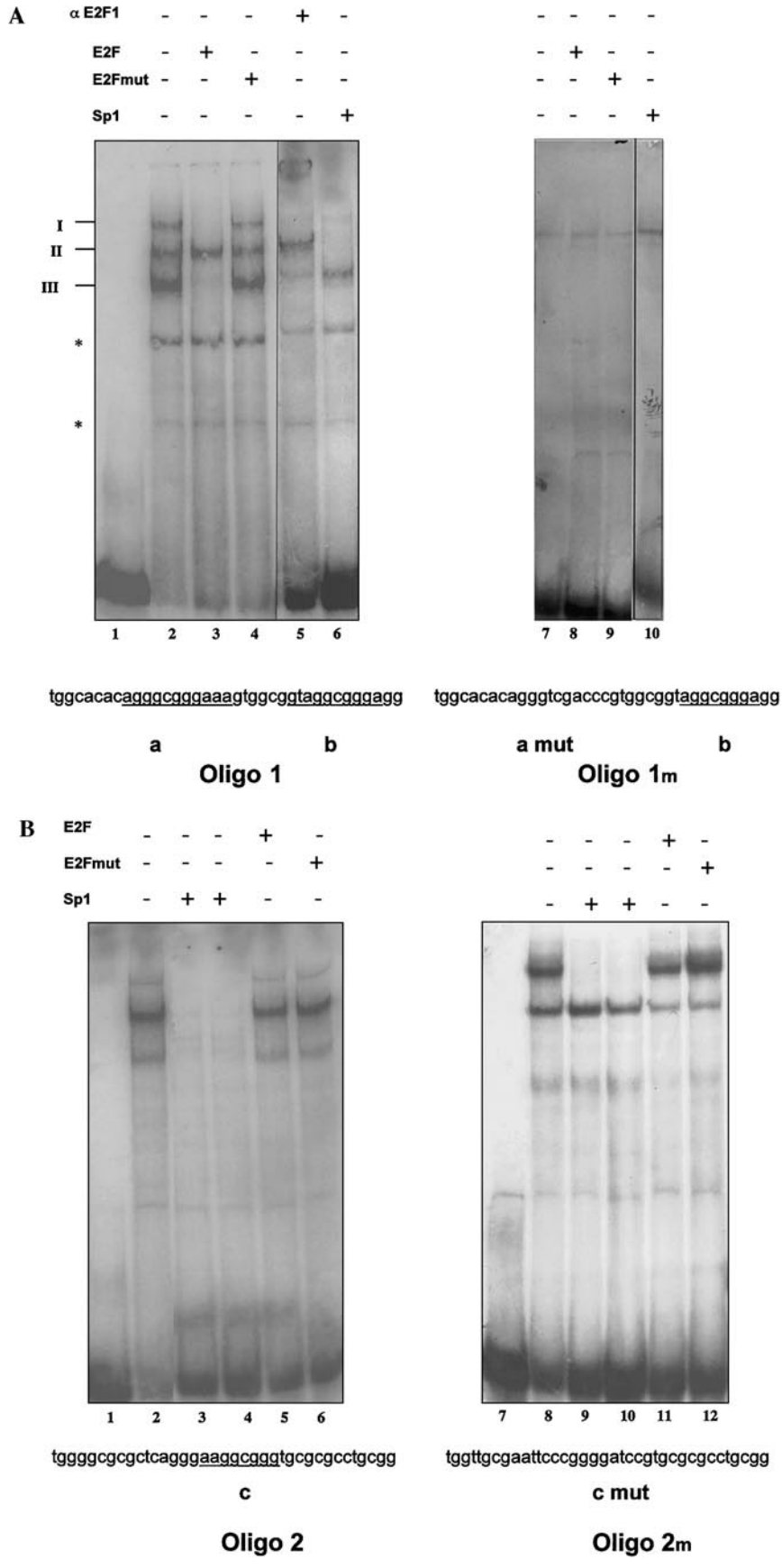

FIG. 3. Protein–DNA binding assays. The oligonucleotides utilized in the experiments are shown on the bottom of each panel. Competitors and antibody used are as indicated. Lane 1 in A and lanes 1 and 7 in B are controls without the nuclear extracts. * indicate nonspecific binding.

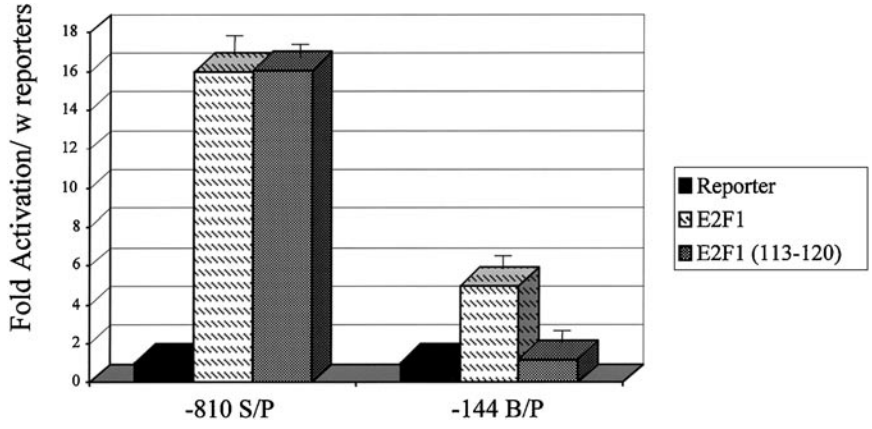

FIG. 4. Transcriptional activity of the mutant E2F1(113–120). The indicated reporter plasmids were cotransfected with 10 μg of the wild-type E2F1or the mutant E2F1(113–120) expression vectors. Results of triplicate transfections are reported.

DISCUSSION

Expression of the majority of the known E2F target genes is regulated during cell growth, and they are activated just before S-phase (26). A notable exception is the p19/p14 ARF gene, whose expression is not cell growth regulated but nevertheless is controlled by E2F (17, 18, 27).

In addition to cell proliferation, the overexpression of E2F can trigger cells to undergo apoptosis through both p53-dependent and p53-independent mechanisms (19, 28). Importantly, although there is evidence to suggest that apoptosis can be triggered by the ectopic expression of E2F1, E2F2 and E2F3 (29, 30) it is believed to be a specific property of E2F1.

In this paper we demonstrate that the promoter of the p14ARF tumor suppressor is upregulated almost at the same level by three of the members of E2F family, so providing a strong support to the suggested role of these two members of the E2F family in the induction of apoptosis. Intriguingly, it has been reported very recently a role for p19ARF in the destabilization of E2F1, E2F2, and E2F3, suggesting the existence of a negative feedback loop that might be relevant to the interrelationship of ARF and E2F1, E2F2, and E2F3 (31).

E2F factors have been found to act as either a transcriptional repressor or activator of responsive genes, depending on the growth state or cell cycle stage of the cells (26). These genes vary with respect to the position and number of Sp1 and E2F sites in their promoters, suggesting that Sp1-E2F interactions may be a factor in their differential regulation. Consistently with this view, it has been demonstrated that E2F1, E2F2 and E2F3 are able to physically interact with Sp1 and, moreover, that functional interactions between E2F-1 and Sp1 already occurs (24, 32), at least in the context of some promoters.

The mutational analysis of ARF promoter that we describe in this paper clearly shows that an ARF promoter fragment lacking all the putative E2F-consensus

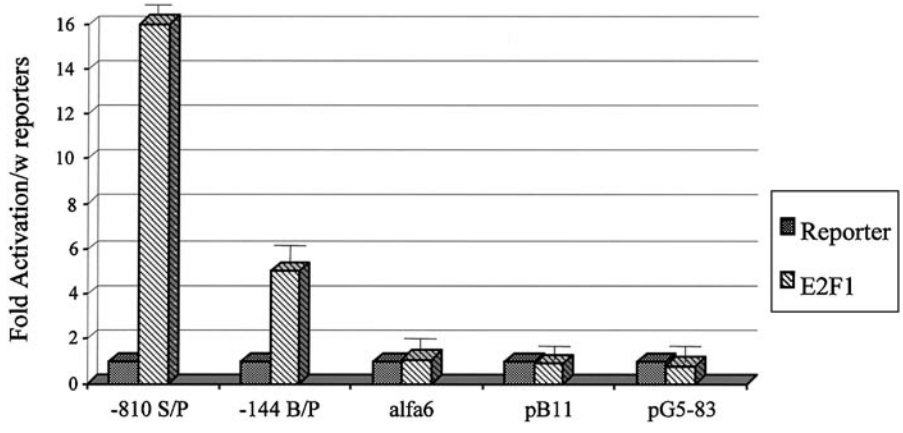

FIG. 5. Transcriptional activity of E2F1 on various minimal promoters-CAT constructs. The indicated reporter plasmids were cotransfected with 10 μg of the wild-type expression vector. The results of triplicate transfections are reported.

sites and carrying only Sp1 sites is still significantly transactivated by E2F1, E2F2, and E2F3. EMSA experiments indicate, on the other hand, that only the distal E2Fa consensus site is able to specifically bind E2F1 *in vitro*. Moreover, our experiments also show that binding of Sp1-like factors occurs at many sites in the promoter and that, at least in one case, E2F and Sp1-like factors seem to be involved in the formation of the same DNA–protein complex.

Accordingly, however, the mutant E2F1(113–120), which cannot interact with Sp1, was able to transactivate a promoter fragment bearing the E2Fa site but failed to significantly transactivate the ARF minimal promoter, strongly suggesting that activation of the minimal promoter by E2F factors occurs through interaction with Sp1-like factors.

Taken together these results suggest that the ARF promoter is regulated by E2F through both a direct binding to promoter sequences and indirectly, probably by being tethered to the ARF promoter by Sp1-like factors.

A possible scenario is that at physiological conditions the relative abundance of E2F and Sp1-like factors is such to keep ARF expression at low levels. A mitogenic stimulus enhances E2F expression which strongly activate ARF transcription by using both a direct and a Sp1-mediated binding to the ARF promoter.

Future investigation using more specific experimental *in vivo* approaches could help to clarify the contribution of E2F and Sp1-like factors in transcription of the $p14^{ARF}$ gene in its natural setting, given the important role of chromatin structure in the regulation of gene expression.

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