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Adenovirus E4 Open Reading Frame 4-Induced Dephosphorylation Inhibits E1A Activation of the E2 Promoter and E2F-1-Mediated Transactivation Independently of the Retinoblastoma Tumor Suppressor Protein

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Previous studies have shown that the cell cycle-regulated E2F transcription factor is subjected to both positive and negative control by phosphorylation. Here we show that in transient transfection experiments, adenovirus E1A activation of the viral E2 promoter is abrogated by coexpression of the viral E4 open reading frame 4 (E4-ORF4) protein. This effect does not to require the retinoblastoma protein that previously has been shown to regulate E2F activity. The inhibitory activity of E4-ORF4 appears to be specific because E4-ORF4 had little effect on, for example, E4-ORF6/7 transactivation of the E2 promoter. We further show that the repressive effect of E4-ORF4 on E2 transcription works mainly through the E2F DNA-binding sites in the E2 promoter. In agreement with this, we find that E4-ORF4 inhibits E2F-1/DP-1-mediated transactivation. We also show that E4-ORF4 inhibits E2 mRNA expression during virus growth. E4-ORF4 has previously been shown to bind to and activate the cellular protein phosphatase 2A. The inhibitory effect of E4-ORF4 was relieved by okadaic acid, which inhibits protein phosphatase 2A activity, suggesting that E4-ORF4 represses E2 transcription by inducing transcription factor dephosphorylation. Interestingly, E4-ORF4 did not inhibit the transactivation capacity of a Gal4-E2F hybrid protein. Instead, E4-ORF4 expression appears to result in reduced stability of E2F/DNA complexes.

INTRODUCTION

The E2F family of transcription factors are important regulators of the cell cycle, controlling the transcription of genes needed for DNA synthesis (reviewed in La Thangue, 1994). They form heterodimeric complexes consisting of one of six E2F proteins (E2F-1–6) and one of two DP proteins (DP-1 or -2) (reviewed in Dyson, 1998). The activity of these complexes, here collectively referred to as E2F, is controlled by several mechanisms. First, binding of the retinoblastoma tumor suppressor protein (pRB) and its relatives p107 and p130 to E2F transcription factors results in active repression of transcription (reviewed in Dynlacht, 1997). The pRB/E2F association is cell cycle regulated and controlled by cyclin-dependent kinases such that pRB phosphorylation dissociates the inhibitory complex, resulting in a release of active E2F (reviewed in Weinberg, 1995) Second, the activity of E2F is directly controlled by phosphorylation. Cyclin A and its associated kinase bind to E2F-1 and phosphorylates its partner DP-1, which results in loss of DNA binding (Dynlacht et al., 1994; Krek *et al.,* 1994) Third, the stability of E2F proteins is controlled by ubiquitin-dependent protein degradation, which is prevented by pRB and adenovirus E1A and E1B proteins (Campanero and Flemington, 1997; Hateboer *et al.,* 1996; Hofmann *et al.,* 1996).

Adenovirus has been a valuable tool in elucidating the functions of E2F proteins (Nevins, 1992). E2F was first identified as a transcription factor required for adenovirus E2 promoter activity (Kovesdi *et al.,* 1986b). The activity of the E2 promoter increases during virus infection concomitant with an increased binding of E2F to the E2 promoter (Kovesdi *et al.,* 1986a; Reichel *et al.,* 1988). Increased E2F activity can be attributed to several virus-induced changes. Adenovirus E1A binds to pRB (Whyte *et al.,* 1988), thereby dissociating the inhibitory pRB/E2F complex. However, E1A also activates transcription by alternative mechanisms (reviewed in Akusjärvi, 1993). Thus evidence has been published suggesting that E1A might induce phosphorylation of E2F, resulting in increased binding of E2F to the E2 promoter (Bagchi *et al.,* 1989). Also, adenovirus encodes the E4-ORF6/7 protein that binds to E2F and induces cooperative binding of E2F transcription factors to the two inverted E2F binding sites in the viral E2 promoter (reviewed in Lam and La Thangue, 1994).

To address the role of phosphorylation in E1A transactivation, we investigated the effect of the adenovirus E4-ORF4 protein on E2 promoter activity and E2F-1-mediated transactivation. This viral protein binds to and activates the serine- and threonine-specific protein phosphatase 2A (PP2A) (Kleinberger and Shenk, 1993).

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We have previously shown that the E4-ORF4 protein inhibits E1A activation of the adenovirus E4 promoter through PP2A (Bondesson *et al.,* 1996). Here we extend these studies and show that E4-ORF4 regulates E2F transactivation of the viral E2 promoter in transient transfection experiments and E2 mRNA expression during virus growth. To be able to study E1A and E2F phosphorylation-dependent activation independently of E2F–pRB association, the transient transfection experiments were performed in HeLa cells, which express the human papilloma virus (HPV) E7 protein that binds and inactivates pRB (Dyson *et al.,* 1989), and in SAOS-2 cells that do not express a functional pRB (Shew *et al.,* 1990). We found that efficient E1A activation of the E2 promoter requires E2F binding sites in the E2 promoter and further show that the E4-ORF4-PP2A protein complex inhibits E1A activation of the E2 promoter as well as E2F-1/DP-1-mediated transactivation through PP2A. However, E4-ORF4 does not affect the transactivation capacity of E2F-1. Mechanistically, E4-ORF4 appears to destabilize E2F/DNA interaction.

RESULTS

The adenovirus E4-ORF4 protein inhibits E1A but not E4-ORF6/7 transactivation of the E2 promoter

We have previously shown that the E4-ORF4-PP2A complex prevents E1A activation of the E4 promoter (Bondesson *et al.,* 1996). Because the E2 promoter similarly has been suggested to be regulated through E1A-induced phosphorylation of E2F (Bagchi *et al.,* 1989), we tested the effect of the viral E4-ORF4 protein on E2 promoter activity in HeLa cells. As shown in Fig. 1, E4-ORF4 had a small stimulatory effect on E2 promoter activity in the absence of E1A (lanes 1 and 2). In contrast, E4-ORF4 coexpression resulted in a significant inhibition of E1A transactivation of the E2 promoter (lanes 3 and 4). These observations are in line with the hypothesis that E2F phosphorylation can have both a negative and a positive effect on E2F activity (see *Discussion*). Importantly, E4-ORF4 coexpression did not affect the level of E1A protein expression (Bondesson *et al.,* 1996; and data not shown). Treatment of the cells with okadaic acid, which inhibits PP2A, annulled the repressive effect of E4-ORF4 on E1A transactivation (lanes 4 and 5), suggesting that the effect of E4-ORF4 requires recruitment of PP2A.

The E4-ORF6/7 protein binds to E2F/DP heterodimers and stabilizes binding to the inverted E2F-binding sites present in the E2 promoter. Figure 1, lanes 6 and 7, shows that in contrast to E1A-mediated activation, E4-ORF4 had little effect on E4-ORF6/7 transactivation. We conclude that the effect of E4-ORF4 is selective in that it dramatically inhibits E1A activation of the E2 promoter while only modestly affecting the E2 transactivation capacity of the E4-ORF6/7 protein.

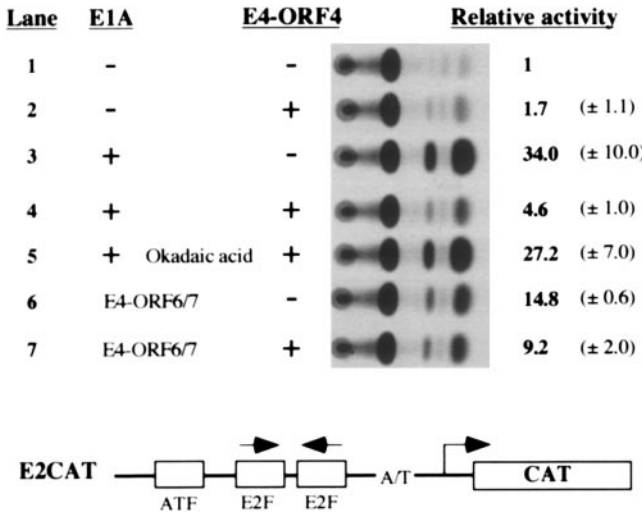

FIG. 1. The adenovirus E4-ORF4 protein inhibits E1A but not E4-ORF6/7 transactivation of the E2 promoter. The E2CAT reporter plasmid (bottom) was cotransfected together with an E4-ORF4 expressing plasmid (+) or the CMV promoter backbone plasmid (−) into HeLa cells. Where indicated, a wild-type E1A expression plasmid or an E4-ORF6/7 expression plasmid was included. In lane 6, 100 nM okadaic acid was added to the growth medium approximately 12 h before harvest. Shown is a representative CAT assay and the mean values calculated form at least three independent experiments with the standard deviations shown within brackets.

E4-ORF4 works through the E2F-binding sites in the E2 promoter

The adenovirus E2 promoter consists of a single ATF-binding site and two inverted E2F-binding sites (see Fig. 1). To determine which factor mediates E1A induction and E4-ORF4 inhibition of E2 transcription, we compared the E2(−ATF)CAT and E2(−E2F)CAT promoter derivatives, that lack the ATF or E2F sites, respectively. As shown in Fig. 2, E2(−ATF)CAT was efficiently activated by E1A, a stimulation that was inhibited by E4-ORF4 coexpression. Okadaic acid treatment of transfected cells abolished completely the inhibitory effect of E4-ORF4 on E1A transactivation of E2(−ATF)CAT. In contrast, E2(−E2F)CAT did not respond significantly to either E1A or E4-ORF4 expression. Thus in HeLa cells, the E2 promoter is activated by E1A mainly through the E2F-binding sites, which also are major targets of the E4-ORF4 protein.

E2F-1/DP-1 transactivation is inhibited by E4-ORF4

The results obtained with E2(−ATF)CAT suggested that E2F is the primary target of E1A activation. We therefore determined the effect of E1A on an E2F4CAT reporter plasmid containing four tandem E2F binding sites. As shown in Fig. 3A, E1A activation of this reporter was modest, only ~4-fold (lanes 1 and 2). Cotransfection of E2F-1 and DP-1 efficiently activated E2F4CAT (lanes 1 and 3), but inclusion of E1A resulted in a minor stimula-

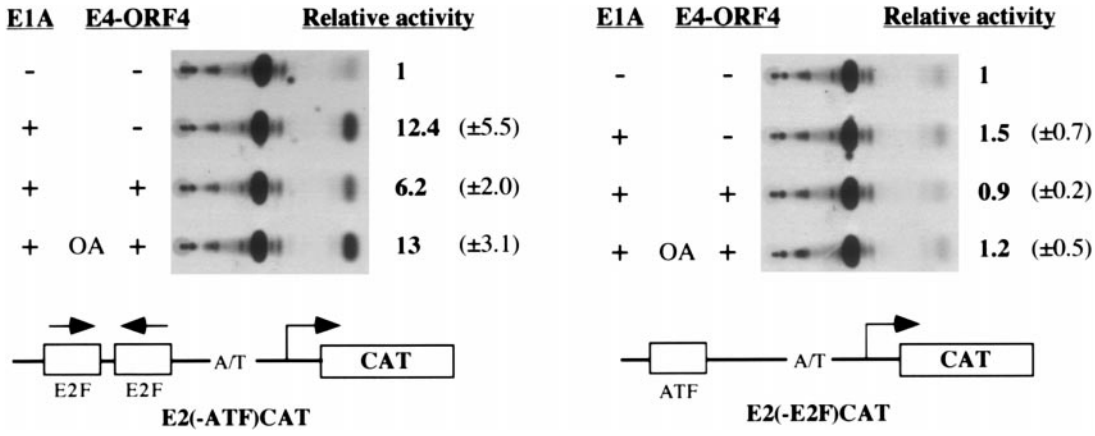

FIG. 2. E1A activation and E4-ORF4 inhibition of E2 transcription requires the E2F binding sites in the E2 promoter. The reporter plasmids E2(−ATF)CAT and E2(−E2F)CAT, lacking the ATF or E2F sites, respectively (schematically shown at the bottom of the figure), were transfected together with the E4-ORF4 expression plasmid (+) or the empty CMV promoter control plasmid (−) into HeLa cells. An E1A-expressing plasmid (+) was included when so indicated. OA indicates that okadaic acid was added to the growth medium ~12 h before harvest. Representative CAT assays are shown with quantifications based on three independent experiments, with the standard deviations within brackets.

tory effect on transcription (lanes 3 and 4). The effect of E1A expression on E2F4CAT contrasts sharply with that of E2(−ATF)CAT, which is activated ~12-fold by E1A (Fig. 2). These results might be related to the fact that the E2F-binding sites in the E2 promoter are inverted (Fig. 1), whereas they are parallel in the E2F4CAT reporter (Fig. 3A). Thus the organization of E2F sites in the promoter may be crucial for E1A transactivation.

Although E4-ORF4 did not repress basal E2 promoter activity (Fig. 1, lanes 1 and 2), it greatly impaired the ability of E2F-1/DP-1 to activate E2F4CAT (Fig. 3B, lanes 2 and 4), without affecting the levels of E2F-1 protein expression (data not shown). Treatment of the cells with okadaic acid stimulated E2F-1/DP-1 transactivation by 4-fold (Fig. 3B, lanes 2 and 3). Cotransfection of E4-ORF4, which activates PP2A dephosphorylation of target proteins, resulted in a 5-fold reduction in E2F4CAT activity (Fig. 3B; lanes 2 and 4). Inclusion of okadaic acid not only annulled the inhibitory activity of E4-ORF4 but also stimulated E2F4CAT expression (Fig. 3B; lanes 4 and 5), suggesting that the effect of E4-ORF4 is due to its recruitment of PP2A. Collectively, these results suggest that phosphorylation is important for E2F function. Thus the addition of okadaic acid results in a stimulation of E2F4CAT expression due to inhibition of phosphatases in the cell. In this model, E4-ORF4 cotransfection results in an increased recruitment of PP2A and thus a dramatic reduction in E2FCAT expression. We conclude that the transactivation capacity of E2F-1 is dependent on phosphorylation, an activity that can be regulated by the E4-ORF4 protein.

Gal4-E2F-1 transactivation is refractory to E4-ORF4 inhibition

To determine whether E4-ORF4 works by inactivating the transactivation domain of E2F-1, a fusion protein between the Gal4 DNA-binding domain and E2F-1 (Gal4-E2F-1) was transfected together with the G5E1BCAT reporter plasmid containing five Gal4-binding sites (Fig. 4). In agreement with our previous results (Bondesson *et al.,* 1996), E4-ORF4 inhibited Gal4-E1A CR3 transactivation in this type of assay (lanes 1 and 2). In contrast, Gal4-E2F-1-dependent transactivation was unaffected by E4-ORF4 coexpression (lanes 3 and 4). Thus we conclude that the repressive effect of E4-ORF4 on E2F-dependent transcription does not occur through inactivation of the transactivation capacity of the E2F-1 activation domain.

E4-ORF4 destabilizes the DNA-binding capacity of E2F-1/DP-1 heterodimers

Because the transactivation capacity of the E2F-1 effector domain was unaffected by E4-ORF4, we tested the DNA-binding ability of E2F-1/DP-1 in the presence of E4-ORF4 by gel-shift analysis. As shown in Fig. 5A, almost no specific E2F-binding complexes with a single E2F site probe were observed in mock transfected cells (lane 1). Transfection of E2F-1/DP-1 resulted in a major complex and a minor more slowly migrating complex (lane 2). Because the majority of pRB is expected to be inactivated in HeLa cells (Dyson *et al.,* 1989), the major complex most likely represent free E2F. Both complexes were supershifted with an E2F-1-specific antibody (lane 3) or an anti-HA tag antibody recognizing DP-1 (lane 4) but not by normal rabbit serum (lane 5). The complexes were refractory to the inclusion of a 100-fold excess of mutant competitor oligonucleotide (lane 6) but almost completely abolished by wild-type competitor (lane 7). Importantly, neither E4-ORF4 (lane 8) nor E1A (lane 9) cotransfection affected the DNA-binding capacity of E2F-1/DP-1 in this type of binding assay.

We therefore compared the stability of the E2F-DNA complexes in the absence and presence of E4-ORF4. For

this experiment, we preincubated extracts prepared from E2F-1/DP-1 or E2F-1/DP-1 + E4-ORF4 cotransfected cells with the E2F probe for 15 min and then included wild-type competitor oligonucleotide for increasing amounts of time. As shown in Fig. 5B, the dissociation rate of E2F-1/DP-1 from DNA increased ~2-fold in the presence of E4-ORF4. Thus it is possible that the repressive effect of E4-ORF4 on E2F-1/DP-1 transactivation in our assay system (Fig. 3A) results, at least in part, from a reduced stability of E2F binding to DNA.

Inhibition of E2F transactivation by E4-ORF4 occurs in the absence of pRB

All experiments thus far were performed in HeLa cells, which are believed to contain inactivated pRB, due to the expression of the integrated HPV-E7 gene product (Dyson *et al.,* 1989). Still, it is possible that a small fraction of pRB remains active in HeLa cells. Because pRB dephosphorylation would be expected to inhibit E2 transcription by sequestering E2F into an inhibitory complex, it became important to determine whether the inhibitory effect of E4-ORF4 on E2F-1/DP-1 transactivation functioned in the absence of pRB. As shown in Fig. 6, coexpression of E4-ORF4 with E2F-1/DP-1 also resulted in a significant reduction in E2F transactivation capacity in SAOS-2 cells that do not express a functional pRB (Shew *et al.,* 1990). Thus E4-ORF4 represses E2F transactivation in the absence of pRB. As expected, cotransfection of pRB also repressed E2F-1/DP-1 transactivation. Interestingly, the inhibitory effect of pRB was not significantly enhanced by E4-ORF4 in SAOS-2 cells. This result may be important because it implies that the E4-ORF4-PP2A complex does not affect the phosphorylated status of pRB. In agreement with this possibility, we were unable to detect a change in pRB phosphorylation in the presence of E4-ORF4 (data not shown). Taken together, our results suggest that the inhibitory effect of E4-ORF4 on E2F transactivation works independently of pRB.

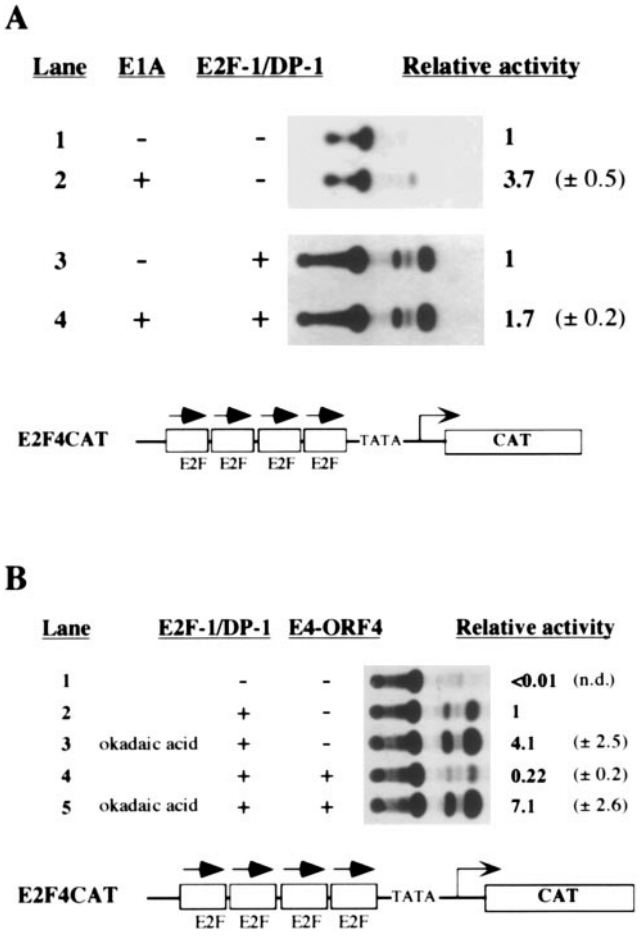

FIG. 3. E4-ORF4 inhibits E2F-1/DP-1-mediated transactivation. HeLa cells were transfected with the E2F4CAT reporter plasmid in the absence or presence of E2F-1 and DP-1 expression plasmids. Inclusion of E4-ORF4 is indicated by a plus sign. In all transfections, an empty CMV promoter control plasmid was included to give equal amount of CMV promoter-containing plasmids. (A) E1A-expressing plasmid (pML005) or the pMLΔE1A control plasmid (lanes 1 and 3) was additionally included. (B) In lanes 3 and 5, okadaic acid was added to the growth medium ~12 h before harvest. (Bottom) Schematic of the E2F4CAT reporter plasmid. n.d., not detectable. Representative CAT assays and quantifications based on at least three independent experiments are shown, with the standard deviations within brackets.

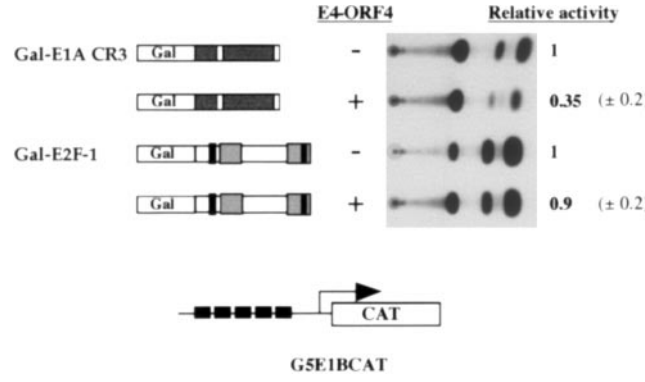

FIG. 4. Transactivation by a Gal4-E2F-1 fusion protein is unaffected by the E4-ORF4 protein. Plasmids expressing fusion proteins between the Gal4 DNA-binding domain and CR3 (Gal4-E1A CR3) or full-length E2F-1 (Gal4-E2F-1) were transfected into HeLa cells together with the G5E1BCAT reporter plasmid containing five Gal4-binding sites. In addition, empty CMV promoter control (−) or E4-ORF4-expressing (+) plasmids were included. Shown is a representative CAT assay and the mean values calculated from at least three experiments, with the standard deviations within brackets. A schematic representation of the Gal4 fusion proteins is shown to the left, and the reporter plasmid is at the bottom.

E4-ORF4 inhibits E2 mRNA expression in virus-infected cells

To determine whether the effect of E4-ORF4 on E2 transcription also extended to a viral infection, we compared E2 mRNA expression in HeLa cells infected with deletion mutant viruses expressing single E4 proteins (Huang and Hearing, 1989). We used H5dl366 (E4$^-$), H5dl366-ORF3 (encoding E4-ORF3), H5dl366-ORF4 (en-

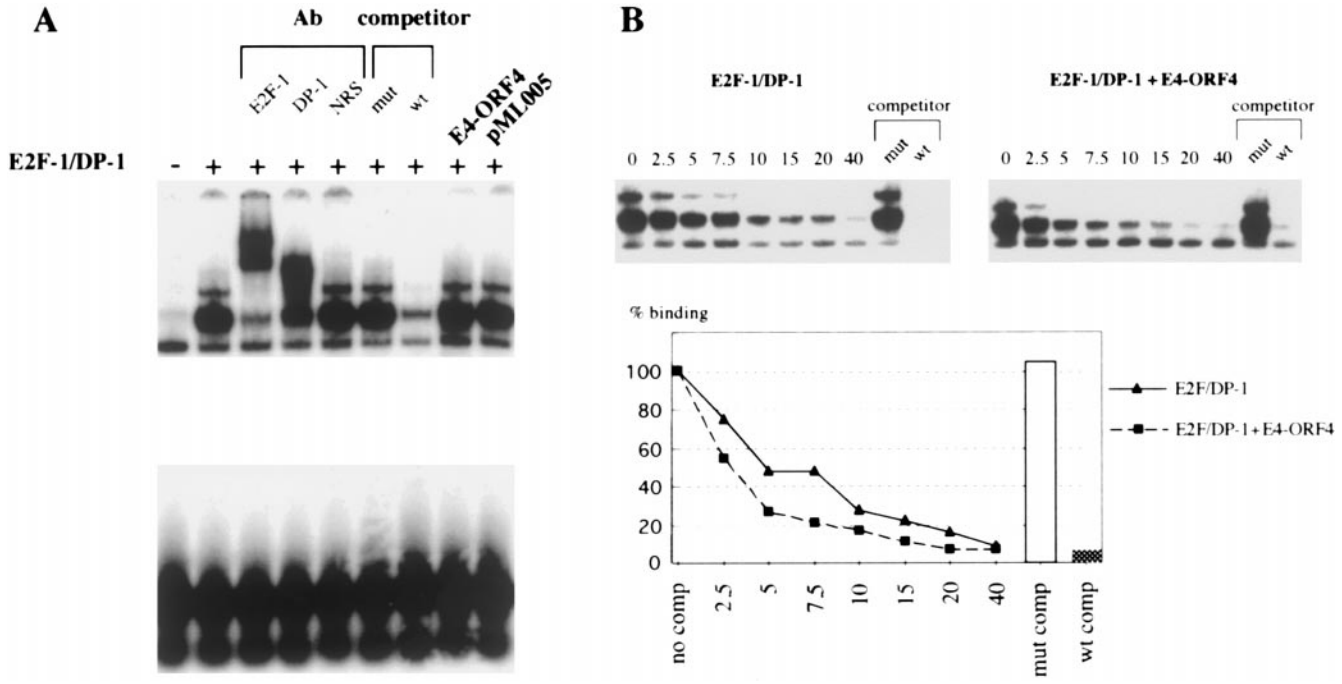

FIG. 5. E4-ORF4 destabilizes E2F binding to DNA. (A) Extracts from mock, E2F-1/DP-1, E2F-1/DP-1 + E4-ORF4, and E2F-1/DP-1 + E1A (pML005) transfected HeLa cells were tested for DNA binding to a single-site E2F oligonucleotide. Antibodies specific for E2F-1 (sc-193; Santa Cruz), HA-tagged DP-1 (12CA5), or normal rabbit serum (NRS) were included where indicated. When so indicated, a 100-fold excess of mutant (mut) or wild-type (wt) unlabeled oligonucleotide was included. (B) Extracts of E2F-1/DP-1 or E2F-1/DP-1 + E4-ORF4 transfected HeLa cells were incubated with the single-site E2F probe for 15 min, after which a 100-fold excess of unlabeled oligonucleotide was included for increasing amounts of time. As a control, a 100-fold excess of unlabeled mutant or wild-type oligonucleotide was included before the addition of extract in the last two lanes of each panel. A quantification of the experiment is shown at the bottom. Similar results were obtained in two independent experiments with different batches of extracts.

coding E4-ORF4), or a mixture of both viruses. As shown in Fig. 7A, infection with H5dl366-ORF3 resulted in a significant stimulation of E2 mRNA expression (compare lanes 1 and 2). This finding is in line with the previous demonstration that E4-ORF3 expression is sufficient to support an essentially wild-type infection (Bridge and Ketner, 1989; Huang and Hearing, 1989). In contrast, infection with H5dl366-ORF4 resulted in a dramatic re-

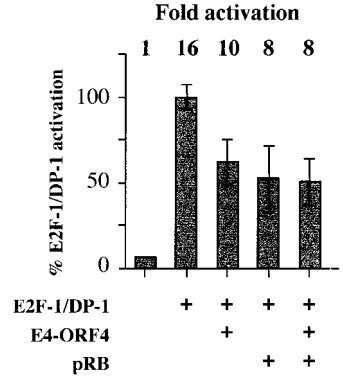

FIG. 6. E4-ORF4 inhibits E2F-1/DP-1-mediated transactivation in the absence of pRB. Levels of CAT activity in SAOS-2 cells cotransfected with the E2F4CAT reporter and the E2F-1/DP-1 activator plasmids were assayed. Where indicated, E4-ORF4 and/or pRB expression plasmids were included. Relative CAT activities, based on the mean value from at least three independent experiments, are given in percentage of E2F-1/DP-1 activation (set as 100%). Error bars show the standard deviation. The fold activation, relative to basal reporter activity, is shown at the top.

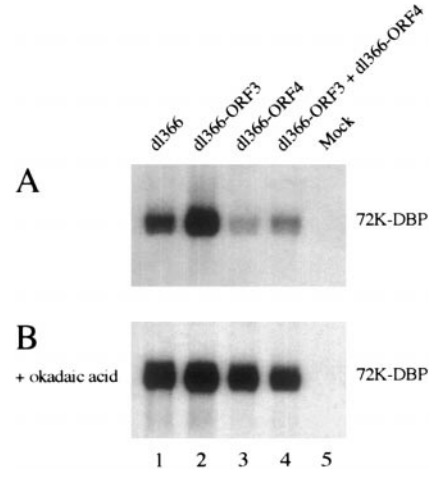

FIG. 7. E4-ORF4 inhibits E2 mRNA expression during an adenovirus infection. (A) HeLa cells were infected with E4 mutant viruses as described in the text. Infected cells were maintained in medium containing ara-C to block virus DNA replication, and cytoplasmic RNA was prepared 24 h postinfection. E2A mRNA expression was assayed by Northern (RNA) blot analysis. (B) A duplicate set of infected cells was treated with 100 nM okadaic acid for 12 h before harvest and RNA analysis.

duction in E2 mRNA accumulation (compare lanes 1 and 3). Interestingly, the negative effect of E4-ORF4 on E2 expression was dominant over the positive effect of E4-ORF3. Thus coinfection with H5dl366-ORF3 and H5dl366-ORF4 resulted in a severe impairment of E2 mRNA expression (compare lanes 2 and 4). Importantly, treatment of mutant virus infected cells with okadaic acid nullified the inhibitory effect of E4-ORF4 on E2 mRNA accumulation (Fig. 7B).

Collectively, these results strengthen the conclusions drawn from the transient transfection experiments by suggesting that E4-ORF4 also functions as an inhibitory protein of E2 transcription during virus growth. In combination with our previous results (Bondesson *et al.,* 1996), we conclude that E4-ORF4 regulates mRNA expression from multiple viral early promoters (E1A, E1B, E2, and E4). Interestingly, the effects vary slightly on individual promoters, probably reflecting the target specificity of E4-ORF4 (Bondesson *et al.,* 1996). In a more extensive study, determining the effect of individual E4 proteins on E2 transcription and virus replication, Medghalchi *et al.* (1997) presented evidence suggesting that E4-ORF4 inhibits E2 mRNA expression by directly reducing the rate of E2 transcription.

DISCUSSION

The E2F transcription factor is an important cell cycle regulator, controlling the expression of genes active in S-phase (La Thangue, 1994). It is subjected to regulation by several mechanisms. For example, binding of pRB to E2F-1, -2, or -3 and the pRB related proteins p107 and p130 to E2F-4 and -5 results in repression of transcription (Dynlacht, 1997). This interaction is controlled by phosphorylation such that pRB phosphorylation prevents the interaction (Weinberg, 1995). E2F itself is subjected to a complex regulation with both positive and negative signals brought about by phosphorylation (see introductory paragraphs).

To further investigate the possible contribution of phosphorylation in the control of E2F activity in the absence of pRB, we investigated the effect of the E4-ORF4 protein on E2 transcription in HeLa and SAOS-2 cells. E4-ORF4 binds to the serine/threonine phosphatase PP2A (Kleinberger and Shenk, 1993) and activates dephosphorylation of specific target proteins. Thus the E4-ORF4-PP2A complex reduces junB transcription (Kleinberger and Shenk, 1993), E1A- and cAMP-mediated induction of AP-1 activity (Muller, Kleinberger, and Shenk, 1992), and E1A activation of the viral E4 promoter (Bondesson *et al.,* 1996). In our assay system, E4-ORF4 dramatically reduced E1A activation of the E2 promoter (Fig. 1). The inhibition of E1A activation was reversed by okadaic acid, suggesting that E4-ORF4-PP2A-induced dephosphorylation of a target protein or proteins is the cause of this effect. Because E1A activation is primarily mediated through the E2F sites in the E2 promoter (Fig. 2), E4-ORF4 could affect either E2F or E1A activity. We have previously shown that E1A itself does not appear to be the primary target of E4-ORF4 (Bondesson *et al.,* 1996). We therefore favor a model in which E4-ORF4-PP2A-induced dephosphorylation reduces the activity of E2F.

Consistent with this model, E2F-1/DP-1-mediated activation of the E2F4CAT reporter plasmid was impaired by E4-ORF4 coexpression (Fig. 3B). The treatment of E2F-1/DP-1-transfected HeLa cells with okadaic acid stimulated transactivation, suggesting that E2F-1/DP-1 activity in our experimental system is positively regulated by phosphorylation (Fig. 3B). Interestingly, the stability of the E2F-1/DP-1 DNA complex appears to be reduced by E4-ORF4 (Fig. 5B), suggesting that phosphorylation is important for stable E2F binding to DNA. Because the transactivation capacity of the E2F-1 activation domain was unaffected by E4-ORF4 (Fig. 4), we suggest that the ~2-fold decrease in the stability of the E2F/DNA complex induced by E4-ORF4 could at least in part explain the inhibitory effect of E4-ORF4 on E2F-1-mediated transactivation. An interesting experiment to be done is to determine whether okadaic acid treatment would reverse the inhibitory effect of E4-ORF4 on E2F DNA stability. Because transcription reinitiation appears to be a rate-limiting step *in vivo* (Ho *et al.,* 1996), stable binding of E2F to DNA could be important for efficient reassembly of the preinitiation complex.

We have not been able to demonstrate a change in the total phosphorylated status of either E2F-1 or DP-1 in transfected cells (data not shown), suggesting that the E4-ORF4-PP2A complex does not cause a massive dephosphorylation of E2F. More likely, the E4-ORF4 -PP2A complex is specific, causing a dephosphorylation of one or a few amino acids important for E2F function. However, the failure to detect a change in E2F phosphorylation raises the possibility that the effects we observe are indirect due to E4-ORF4 dephosphorylation of another factor or factors involved in E2 transcription.

Altoik *et al.* (1997) recently showed that PPAR-induced phosphorylation of DP-1 inhibited the transactivation capacity of E2F. In this context, PP2A-induced dephosphorylation stimulated E2F DNA binding. We observe the opposite effect, namely, that E4-ORF4-PP2A-induced dephosphorylation inactivates E2F transactivation and potentially DNA binding. However, it should be noted that both studies suggest a significance of the phosphorylated status of E2F for its function. The difference in results may arise from differences in target specificity. Thus E1A and PPAR may induce phosphorylation of different residues in E2F, with one causing activation (E1A) and the other resulting in inhibition (PPAR). In both cases, PP2A-induced dephosphorylation reverses the effects. Alternatively, there might be cell type-specific differences in E2F function because in our experimental

system, inhibition of phosphatases by okadaic acid treatment activated E2F in the absence of E4-ORF4 (Fig. 3B, lane 3).

Recent studies have shown that E4-ORF4 can induce apoptosis (Lavoie *et al.,* 1998; Marcellus *et al.,* 1998; Shtrichman and Kleinberger, 1998). It is therefore important to point out that we did not detect a decrease in cell survival in our short-term transient transfection experiments (data not shown). Furthermore, because E4-ORF4 had little effect on E4-ORF6/7 transactivation of the E2 promoter (Fig. 1) and Gal4-E2F-1 transactivation of the G5E1BCAT reporter plasmid (Fig. 4) it appears unlikely that the effects that we observe are due to E4-ORF4-induced apoptosis. In fact, E4-ORF4 slightly activates basal transcription from the E2 promoter (Fig. 1). The inhibition of E1A and E2F transactivation by E4-ORF4 therefore is probably not caused simply by cell death under our experimental conditions.

Previous data have shown that phosphorylation of pRB is important to relieve its inhibitory effect on E2F transcription factor activity. Here we present data suggesting that phosphorylation also is important in E1A activation of the E2 promoter as well as E2F transactivation in the absence of pRB. Collectively, our data suggest that the viral E4-ORF4 protein regulates E2 promoter activity by controlling the phosphorylated status of E2F or a downstream target. In agreement with this, E2 expression is reduced by E4-ORF4 during lytic virus growth (Fig. 7) (Medghalchi *et al.,* 1997).

MATERIALS AND METHODS

Plasmid DNA

pML005 (which expresses E1A) contains nucleotides 1–1773 of the adenovirus type 2 genome (Bondesson *et al.,* 1994). pMLΔE1A is a derivative of pML005 that lacks most of the E1A coding sequence, except for the 18 amino-terminal amino acids. These plasmids were kindly provided by Dr. Catharina Svensson. pCMVDP-1 (Helin *et al.,* 1993) (containing an HA epitope tag), pCMVE2F-1 (Helin *et al.,* 1993), pSGRB (kindly provided by W. G. Kaelin), the E4-ORF4- and E4-ORF6/7-expressing plasmids, and the CMV promoter backbone plasmid (Ohman *et al.,* 1993) have all been described previously. Gal-E2F-1 was constructed by transfer of the E2F-1 coding sequence into the pSG424 plasmid (encoding Gal4 (1–147) (Sadowski *et al.,* 1988). Gal-CDoff (here designated Gal-E1A CR3) has been described previously (Bondesson *et al.,* 1994). The reporter plasmids E2CAT (p2CAT in Weeks and Jones, 1983), E2(−ATF)CAT, E2(−E2F)CAT [referred to as pE2(−80/70)CAT and pE2(−64/60, −45/36)CAT in Loeken and Brady, 1989], E2F4CAT (Helin *et al.,* 1993), and G5E1BCAT (Lillie and Green, 1989) have all been described previously.

Transfection and reporter gene analysis

Subconfluent HeLa monolayer cells were grown and transfected according to the calcium phosphate coprecipitation technique as described previously (Bondesson *et al.,* 1996). Transfections were supplemented with either pUC19 or salmon sperm DNA up to a total of 12–15 μg of DNA/60-mm dish. In Figs. 1 and 2 5 μg of E2CAT reporter plasmid or its derivatives E2(−ATF)CAT and E2(−E2F)CAT was transfected together with 2 μg of E1A-expressing plasmid and 1 μg of empty CMV vector or E4-expressing plasmids. In Fig. 3 100 ng of pCMVE2F-1 and 100 ng of pCMVDP-1 were cotransfected with 3 μg of E2F4CAT reporter. Where indicated, 2 μg of E1A-expressing plasmid and/or 1 μg of CMV promoter or E4-ORF4 expression plasmids was included. In Fig. 4 3 μg of the G5E1BCAT reporter was transfected together with 200 ng of Gal4-E1A CR3 or 500 ng of Gal4-E2F-1 and 1 μg of CMV promoter or E4-ORF4-expressing plasmids. Where indicated, the cells were treated with 100 nM okadaic acid (Boehringer) for ~12 h before harvesting. Cell extracts were prepared by freeze-thawing. The protein concentration was determined according to the Bradford method (Ausubel *et al.,* 1987), and extracts equalized for protein concentration were assayed for CAT activity as described previously (Bondesson *et al.,* 1996).

Subconfluent SAOS-2 cells were grown on 35-mm petri dishes and transfected using the FuGENE 6 transfection reagent (Boehringer). Here 1 μg of E2F4CAT reporter plasmid was cotransfected with 0.1 μg of pCMVE2F-1 and pCMVDP-1 together with 1.0 μg of the E4-ORF4 expression plasmid. An empty CMV expression plasmid was used as a control to give an equal amount of CMV promoter-containing plasmids in each transfection. Where indicated, 0.5 μg of a pSGRB was included. At 48 h posttransfection, the cells were lysed and analyzed as described above. All experiments were performed at least three times. Quantitative results were obtained with PhosphorImager (Bio-Rad) scanning.

Gel retardation assays

Gel retardation assays were performed essentially as described previously (Helin and Harlow, 1994). HeLa cells grown on 10-cm tissue culture plates were transfected with 8 μg of pCMVE2F-1 and pCMVDP-1 plasmids, 4 μg of empty CMV vector, E4-ORF4 or E1A expression plasmids, and whole-cell extracts prepared as described by Helin and Harlow (1994). Then 5-μg portions of extract were added to a 10-μl mix consisting of binding buffer (Helin and Harlow, 1994), 2 μg of sonicated salmon sperm DNA, 5% glycerol, and 0.3 ng of a 5′-^{32}P-end-labeled probe and incubated for 30 min at room temperature. The samples were run on a 4% polyacrylamide gel in 0.25× TEB at 4°C and 180 V for 2–3 h. Where indicated, a 100-fold excess of wild-type or mutant

oligonucleotide was added before the extract. Then 1 μl of antibody specific for E2F-1 (sc-193; Santa Cruz), the HA epitope tag in DP-1 (12CA5), or normal rabbit serum was added after 15 min of preincubation when so indicated.

In the off-rate experiment, extract was incubated with probe for 15 min at room temperature, after which a 100-fold excess of cold wild-type oligonucleotide was added, and incubation continued for the indicated amount of time (Fig. 5B).

The wild-type and mutant oligonucleotides containing a single E2F site have been described (Helin *et al.,* 1992).

Virus infection and RNA blot analysis

Subconfluent monolayers of HeLa cells were grown on 6-cm plates and infected at a multiplicity of 100 fluorescence-forming units (FFU)/cell with the following combinations of E4 mutant viruses (Huang and Hearing, 1989): lane 1, 100 FFU H5dl366; lane 2, 50 FFU H5dl366-ORF3 plus 50 FFU H5dl366; lane 3, 50 FFU H5dl366-ORF4 plus 50 FFU H5dl366; lane 4, 50 FFU H5dl366-ORF3 plus 50 FFU H5dl366-ORF4; and lane 5, mock infected. At the time of infection, 1-β-D-arabinofuranosylcytosine (ara-C) was added at a concentration of 20 μg/ml, and an additional 20 μg/ml ara-C was added every 8 h. The infections shown in Fig. 7B also contained 100 nM okadaic acid that was added 12 h postinfection. Total cytoplasmic RNA was prepared 24 h postinfection by IsoB/Nonidet P-40 treatment and phenol–chloroform extraction (Svensson and Akusjärvi, 1984). Five micrograms of cytoplasmic RNA from each infection was electrophoresed in a 2% agarose gel containing 1.8% formaldehyde, transferred to a nylon filter, and hybridized to a ^{32}P-labeled E2 probe (Ad2 bp 23304–24890; detecting the gene for the E2A 72K-DBP) at 42°C for 18 h. The hybridization solution contained 50% formamide, 5× Denhardt's solution, 5× SSC, 10% dextran sulfate, and 1% SDS. After hybridization, the filter was washed 2 times for 15 min at room temperature in 2× SSC before autoradiography.

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