JOURNAL OF VIROLOGY, June 1996, p. 3844–3851
0022-538X/96/$04.00+0

Vol. 70, No. 6

Adenovirus E4 Open Reading Frame 4 Protein Autoregulates E4 Transcription by Inhibiting E1A Transactivation of the E4 Promoter

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Received 27 November 1995/Accepted 20 March 1996

Here we show that the adenovirus early region 4 (E4) open reading frame 4 (ORF4) protein autoregulates its own transcription by inhibiting adenovirus E1A-induced activation of E4 transcription both in transient transfection experiments and during lytic virus growth. The inhibitory activity of E4-ORF4 was selective for E1A-CR3-dependent transactivation and had no effect on CR1 transactivation. The inhibitory activity of E4-ORF4 was relieved by okadaic acid treatment, which inhibits the cellular protein phosphatase 2A (PP2A), suggesting that E4-ORF4 controls the phosphorylated status of transcription factors important for E4 promoter activity. This conclusion agrees with previous demonstrations that E4-ORF4 associates with PP2A and causes a partial dephosphorylation of certain transcription factors, including E1A (U. Müller, T. Kleinberger, and T. Shenk, J. Virol. 66:5869–5878, 1992; T. Kleinberger and T. Shenk, J. Virol. 67:7556–7560, 1993). However, our results indicate that dephosphorylation of E1A itself might not be the primary target for E4-ORF4. Instead, the E4-ORF4–PP2A complex appears to work by dephosphorylation of multiple cellular transcription factors that are involved in E1A transactivation of the E4 promoter.

The adenovirus E1A gene encodes a family of structurally related proteins that are required to activate all viral early promoters during lytic virus growth. The E1A region produces two major proteins of 289 and 243 amino acids (E1A-289R and E1A-243R). Both proteins have identical amino- and carboxy termini but differ in that the smaller protein lacks an internal 46-amino-acid stretch. The E1A gene encodes three amino acid sequences that are well conserved among E1A proteins from different serotypes, designated conserved region (CR) 1, CR2, and CR3 (17). Genetic studies have shown that these domains play a crucial role in the multiple activities ascribed to E1A in transcriptional control and transformation (reviewed in references 3 and 6).

E1A activates transcription through several different mechanisms which involve all three conserved domains (reviewed in reference 1). However, the classical E1A transcription activation domain is contained within CR3 and is therefore unique to the E1A-289R protein. This domain has been shown to participate in transcription activation by physically interacting with both basal and upstream binding transcription factors, as well as to stimulate transcription by inducing phosphorylation of certain cellular transcription factors, such as E4F, E2F, and TFIIIC (reviewed in reference 1).

Studies of the adenovirus type 5 E4 promoter sequence have identified two binding sites for the cellular transcription factor E4F that are of critical importance for E4 activity during a lytic virus infection. The DNA binding activity of E4F is stimulated by E1A with kinetics that coincide with E4 promoter activation in vivo (32, 33). Mechanistically, this promoter activation has been suggested to work through an E1A-induced increase in the DNA binding activity of E4F through a phosphorylation of E4F (31). E1A activation of the E4F transcription factor requires the CR3 domain plus one of two interchangeable elements in the nonconserved carboxy-terminal exon, designated auxiliary region (AR) 1 and AR2 (5). The E4 promoter also contains binding sites for members of the activating transcription factor (ATF) family (12), of which some are E1A inducible through an alternative pathway to E1A-mediated phosphorylation (21, 23). However, the major pathway for E1A activation of the E4 promoter in HeLa cells appears to work through the E4F transcription factor (5, 32, 33).

The E4 transcription unit is complex and codes for a minimum of seven different protein products, called open reading frames (ORFs) (10, 37). E4 proteins are required for lytic virus growth, because they provide functions that facilitate viral DNA replication and accumulation of nuclear and cytoplasmic RNA derived from the major late transcription unit (13, 39). Biological functions have been ascribed to four E4 proteins. E4-ORF3 and E4-ORF6 play important roles in alternative splicing of the tripartite leader during lytic virus growth (28, 29). They appear to have redundant activities during infection, and expression of either one seems to be sufficient to support an essentially wild-type virus infection (7, 15). The E4-ORF6/7 hybrid protein augments transcription of the E2 gene by facilitating cooperative binding of transcription factor E2F to the E2 promoter (16, 24, 27). Of specific interest for this study, the E4-ORF4 protein has been shown to form a complex with the serine- and threonine-specific protein phosphatase 2A (PP2A) by binding to its B subunit (18). The E4-ORF4–PP2A complex reduces E1A and cyclic AMP-mediated induction of AP1 activity in virus-infected cells (25). This reduction in transcription factor activity correlates with an increased accumulation of underphosphorylated forms of E1A and c-Fos. The effect of E4-ORF4 appears to be specific, since other phosphoproteins, like E1B-55K and JunB, are not affected (25). Collectively,

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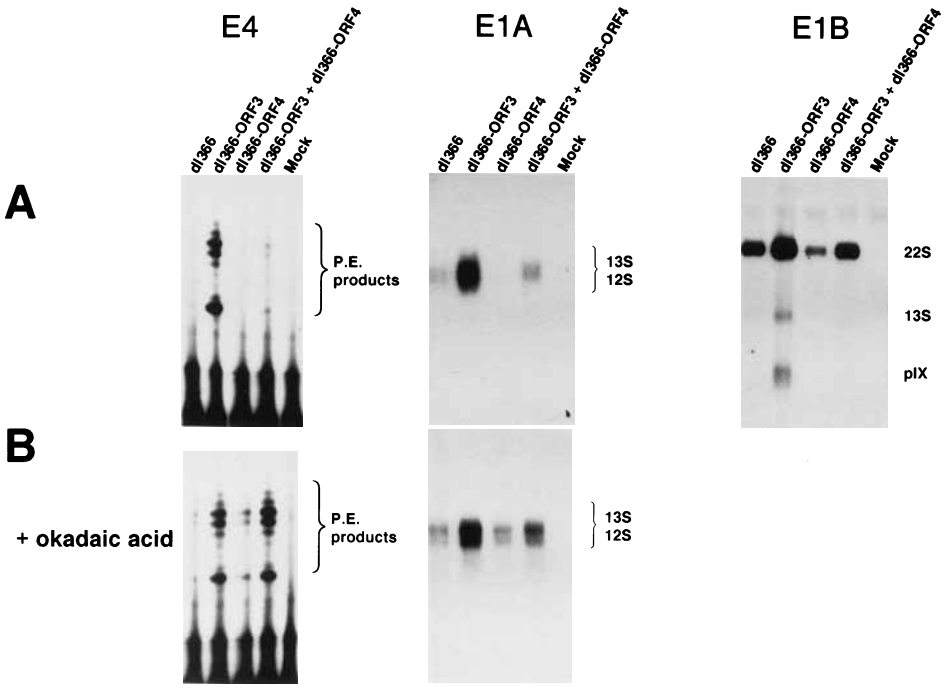

FIG. 1. E4-ORF4 inhibits activation of the E4 and the E1A promoter during adenovirus infection. (A) HeLa cells were infected with H5dl366, H5dl366-ORF3, or H5dl366-ORF4 or double-infected with H5dl366-ORF3 and H5dl366-ORF4 as described in Materials and Methods. Infected cells were maintained in media containing Ara-C (11) and cytoplasmic RNA was prepared 24 h postinfection. (B) A duplicate set of infected and Ara-C-treated plates was also treated with 100 nM okadaic acid for 12 h before harvest. E4 mRNA expression was assayed by primer extension, and E1A and E1B mRNA expression was assayed by Northern (RNA) blot analysis. P.E. products designate primer extension products corresponding to the multiple cap sites in the E4 promoter (2). The positions of the E1A 13S and 12S and the E1B 22S and 13S mRNAs are indicated. pIX designates the polypeptide IX mRNA.

available results suggest that the E4-ORF4–PP2A complex directly controls the phosphorylated status of some transcription factors.

Since E1A transactivation of several viral promoters has been suggested to work through an induced phosphorylation of cellular transcription factors (reviewed in reference 1), it seemed possible that the E4-ORF4 protein would regulate certain early viral promoters through the PP2A dephosphorylation pathway. In agreement with this hypothesis, E4-ORF4 inhibited E1A and E4 mRNA expression during lytic virus growth but had no or minor effects on E1B mRNA expression under similar conditions. The autoregulatory effect of E4-ORF4 on E4 promoter activity was studied further. We show that the adenovirus type 2 E4-ORF4 protein inhibits the capacity of E1A to activate the E4 promoter also in a transient transfection assay. The inhibitory activity of E4-ORF4 was almost completely abolished by okadaic acid, which inhibits PP2A, suggesting that E4-ORF4 inhibited E1A transactivation through dephosphorylation of a critical factor required for E4 promoter activity. E4-ORF4 repression was specific for CR3-specific transactivation. The E1A-289R protein is heavily phosphorylated at multiple serines in vivo (9). However, our results suggest that the inhibitory activity of E4-ORF4 does not correlate with a change in the phosphorylated state of E1A-CR3, suggesting that the primary target of E4-ORF4 is not E1A. More likely, E4-ORF4 inhibits E1A transactivation of the E4 promoter by causing a dephosphorylation of several upstream and/or basal transcription factor(s).

MATERIALS AND METHODS

Plasmid DNA. Plasmid pML005 (which expresses E1A) is a derivative of pKGO-007SVRI (36), which contains nucleotides 1 to 1773 of the adenovirus type 2 genome (4).

The Gal4-E1A fusion proteins described in this investigation contain amino acids 1 to 147 of the *Saccharomyces cerevisiae* Gal4 transcription factor and are derivatives of Gal4-E1A (here designated Gal-C13Xoff), which has been previously described (19). The nomenclature used to name the plasmids is as follows. R and C indicate that the junction between the Gal4 region and the adenovirus type 2 E1A sequences are at the *Rsa*I site at nucleotide position 636 (R) or the *Cla*I site at position 917 (C). Boff, Aoff, Doff, and Xoff indicate that a translational stop codon was introduced after the *Bsp*EI (position 825), *Acc*I (position 1106), *Dde*I (position 1241), and *Xba*I (position 1336) restriction sites, respectively. Gal-C13Doff and Gal-RBoff have been described previously (4). Gal-C13Aoff was constructed by replacing a fragment between *Dra*III (position 952) and *Xba*I (position 1336) of Gal-C13Xoff with the corresponding fragment from pKGO-007AX̂ (20). Gal-C13DoffA172 was constructed by PCR amplification with designed oligonucleotide primers. It is identical to Gal-C13Doff except that it has a point mutation converting serine 172 to an alanine. The cytomegalovirus plasmids expressing the different E4 ORFs have previously been described (29). The reporter plasmids E4CAT and G5E1BCAT have been described previously (19).

Virus infection. In Fig. 1, subconfluent monolayers of HeLa cells grown on 6-cm-diameter plates were infected at a total multiplicity of infection of 100 fluorescence-forming units (FFU) per cell (30) with the following virus combinations: lanes dl366, 100 FFU of H5dl366; lanes dl366-ORF3, 50 FFU H5dl366 plus 50 FFU H5dl366-ORF3; lanes dl366-ORF4, 50 FFU H5dl366 plus 50 FFU H5dl366-ORF4; and lanes dl366-ORF3 + dl366-ORF4, 50 FFU H5dl366-ORF3 plus 50 FFU H5dl366-ORF4. At the time of infection, 1-β-D-arabinofuranosylcytosine (Ara-C) was added to all infections at a concentration of 20 μg/ml, and an additional 20 μg of Ara-C per ml was added every 8 h (11). The infections shown in panel B were similarly treated and also contained 100 nM okadaic acid that was added at 12 h postinfection. Total cytoplasmic RNA was prepared from all plates 24 h postinfection by IsoB–Nonidet P-40 treatment and phenol-chloroform extraction (34).

Primer extension reaction. Ten micrograms of cytoplasmic RNA was incubated at 65°C for 90 min with a 5′ end-labelled 18-nucleotide-long primer (5′-ACACCACTCGACACGGCA-3′) complementary to nucleotides +23 to +40, relative to the major cap nucleotide in the E4 promoter. After incubation, the samples were allowed to slowly cool to room temperature before avian myeloblastosis virus reverse transcriptase was added. The primer extension reaction was carried out at 42°C for 1 h in a buffer containing 25 mM Tris (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.15 mM deoxynucleotide triphosphates, 0.3 mM EDTA, and 5 U of avian myeloblastosis virus reverse

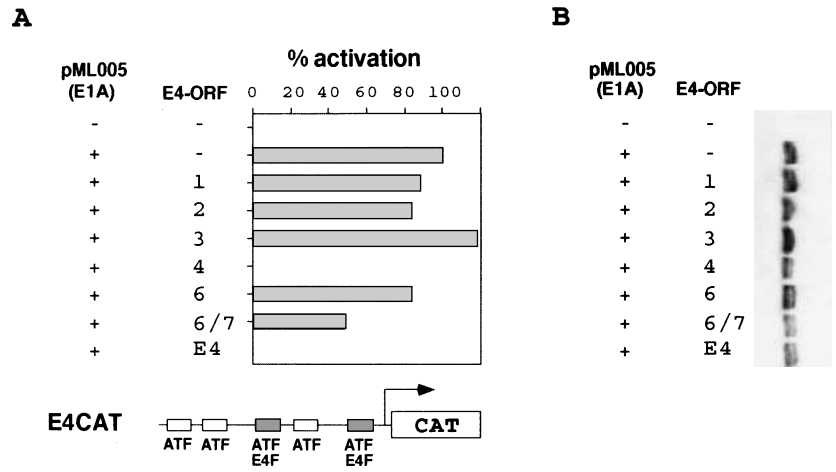

FIG. 2. E4-ORF4 inhibits E1A-mediated activation of the E4 promoter in transient transfection experiments. (A) Diagram showing the effects of different E4 ORFs on pML005 (which encodes E1A) activation of the E4 promoter. pML005 activation of the E4 promoter in the absence of E4 ORFs was set as 100%. Relative CAT activities represent the mean values from at least three independent experiments. (B) Western blot analysis showing the steady-state levels of E1A proteins in cells cotransfected with the different E4 ORFs.

transcriptase. The primer extension reaction was terminated by RNase A digestion followed by phenol-chloroform extraction. The cDNA product was concentrated by ethanol precipitation and analyzed on an 8% acrylamide–7 M urea gel.

RNA blot analysis. 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 ^{32}P-labelled E1A (adenovirus type 2, bp 1 to 1336)- or E1B (adenovirus type 2, bp 3322 to 3931)-specific probes at 42°C for 18 h. The hybridization solution contained 50% formamide, 5× Denhardt's solution, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10% dextran sulfate, and 1% sodium dodecyl sulfate (SDS). After hybridization, the filter was washed two times for 15 min at room temperature in 2× SSC before autoradiography.

Transfections and reporter gene analysis. Cells were maintained in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% newborn calf serum. One hour before transfection, the medium was replaced with DMEM containing 10% fetal calf serum. Cells were transfected by the calcium phosphate coprecipitation technique (40) with a total of 15 μg of DNA per 60-mm-diameter culture dish. The standard transfection cocktail contained 3 μg of reporter plasmid, 0.5 to 5 μg of E1A or Gal4-E1A activator plasmid, 0.25 to 2 μg of the indicated E4-ORF plasmid, and carrier DNA. Ten hours posttransfection, cells were shocked by treatment with 15% glycerol in HBS buffer (160 mM NaCl, 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.1], and 0.75 mM NH_2PO_4) for 3 min and then continued to grow on DMEM supplemented with 10% fetal calf serum. Forty-eight hours posttransfection, cells were harvested and lysed by freeze-thawing. When so indicated in the figure, cells were incubated with 100 nM okadaic acid for 12 h before harvest. Chloramphenicol acetyltransferase (CAT) assays were performed as previously described (35). Quantitative results were obtained by PhosphorImager scanning with the Image Quant program (Molecular Dynamics).

Protein expression analysis. Approximately 43 h posttransfection, cells were incubated either in phosphate-free Spinner medium supplemented with 2% fetal calf serum, 20 mM HEPES (pH 7.0), and 1.8 mM $CaCl_2$ or in methionine-free DMEM for 45 min. Cells were labelled with 250 μCi of phosphorus 32 per 60-mm-diameter dish for 5 h or 200 μCi of [^{35}S]methionine per 60-cm-diameter dish for 3.5 h. Cells were harvested and lysed by IsoB–Nonidet P-40 treatment (10 mM Tris [pH 7.9], 150 mM NaCl, 1.5 mM $MgCl_2$, 0.65% Nonidet P-40, 5 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, protease inhibitors). Immunoprecipitation of ^{32}P- or ^{35}S-labelled extracts was performed as described previously (5) with a mouse monoclonal anti-Gal antibody (Promega). Immunoprecipitated proteins were analyzed on an SDS–12% polyacrylamide gel. The Western blot (immunoblot) shown in Fig. 2B was hybridized to primary antibody M73 diluted 1:2,000 (14), and enhanced chemiluminescence was carried out with an enhanced chemiluminescence Western blotting system as described by the manufacturer (Amersham).

RESULTS

Adenovirus E4-ORF4 represses E1A and E4 mRNA expression during lytic virus growth. To study the effect of E4-ORF4 on early viral gene expression, we compared the mRNA profile in HeLa cells infected with E4 deletion mutant viruses H5dl366 (E4$^-$), H5dl366-ORF3 (encoding only E4-ORF3), or H5dl366-ORF4 (encoding only E4-ORF4) (15), or a mixture of viruses. Infections were done in the presence of Ara-C to prevent virus infection from entering the late phase of the infectious cycle (11). As shown in Fig. 1A, expression of E4-ORF3 (H5dl366-ORF3) resulted in a significant stimulation of mRNA expression from the E1A and E4 transcription units. The stimulatory effect of E4-ORF3 on E1B mRNA expression was moderate. In H5dl366-ORF4-infected cells, by contrast, the low levels of E1A and E4 mRNA expression were drastically reduced compared with those of the control (H5dl366). Again, the effect was much smaller on E1B mRNA expression (reduced to approximately 50%). Thus, E4-ORF3 and E4-ORF4 expression has opposite effects on E1A and E4 mRNA accumulation. E4-ORF3 stimulated E1A and E4 mRNA expression, possibly through its positive effect on pre-mRNA splicing (28, 29), whereas E4-ORF4 expression resulted in a repression of E1A and E4 mRNA accumulation. Coinfection with H5dl366-ORF3 and H5dl366-ORF4 resulted in a severe impairment of the E4 and E1A mRNA levels seen in H5dl366-ORF3-infected cells, while no or only minor negative effects were seen on E1B mRNA expression. Taken together, these results show that the adenovirus E4-ORF4 protein almost completely annulled the stimulatory effect of E4-ORF3 on E1A and E4 mRNA accumulation.

Since E4-ORF4 activates PP2A (18), these results indicate that viral promoters regulated by phosphorylation may be specific targets for E4-ORF4 repression. To test this hypothesis, we treated E4 mutant virus-infected cells with okadaic acid, which is a characterized inhibitor of PP2A (8). As shown in Fig. 1B, okadaic acid treatment resulted in high levels of E1A and E4 mRNA expression in H5dl366-ORF4-infected cells. Furthermore, okadaic acid partially (E1A) or completely (E4) relieved the repressive effect of E4-ORF4 on E4-ORF3-activated E1A and E4 mRNA accumulation.

Collectively, these results suggest that many, and potentially all, viral promoters regulated by phosphorylation and dephosphorylation are targets for E4-ORF4.

E4-ORF4 inhibits E1A transactivation of the E4 promoter in a transient transfection assay. Since E1A transactivation of the viral E4 promoter has been shown to work through an induced phosphorylation of the cellular transcription factor

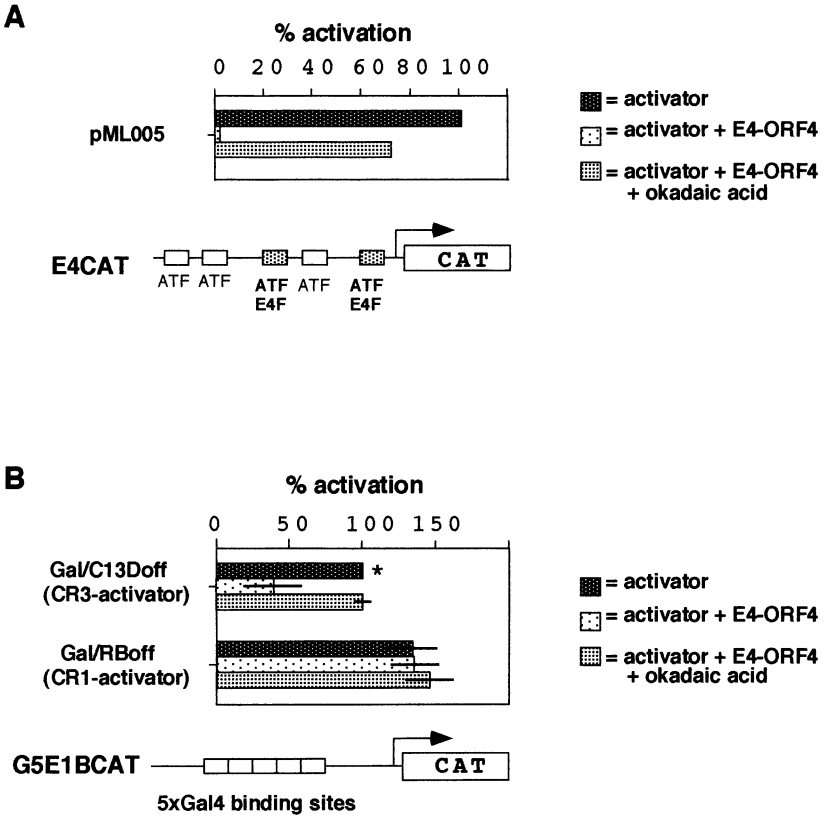

FIG. 3. Okadaic acid counteracts E4-ORF4-mediated inhibition of E1A-CR3-dependent transcription activation. (A) Diagram showing the relative CAT activities of E1A activation in the absence of E4-ORF4 and okadaic acid (set as 100%). A schematic diagram of the reporter construct, E4CAT, is shown at the bottom. (B) Diagram showing the relative CAT activities of Gal4-CR3 (Gal-C13Doff) and Gal4-CR1 (Gal-RBoff) activation in the absence or presence of E4-ORF4 and okadaic acid. In all experiments Gal-C13Doff transactivation of G5E1BCAT was set as 100% (*). Bars show the experimental variations relative to Gal-C13Doff in the different experiments analyzed. A schematic diagram of the reporter construct, G5E1BCAT, is shown at the bottom. Relative CAT activities represent the mean values from at least three independent experiments.

E4F (31), we decided to further investigate the effect of E4-ORF4 on E4 promoter activity. To test the effect of individual adenovirus type 2 E4 proteins on E1A-mediated transactivation of the viral E4 promoter, we cotransfected pML005 (encoding E1A) and cytomegalovirus expression vectors, making various combinations of E4 proteins with a reporter construct of the E4 promoter coupled to the CAT gene (E4-CAT). pML005 activates this promoter approximately 20 times (set to 100% in Fig. 2A). Cotransfection of pCMV-E4 (which makes all E4 proteins) completely eliminated E1A transactivation of the E4 promoter (Fig. 2A), demonstrating that an E4 product indeed counteracts E1A transactivation of this promoter. Interestingly, cotransfection of E4-ORF4 was sufficient to efficiently inhibit the E1A-mediated transactivation of the E4 promoter. In comparison, none of the other E4 ORFs had any major effect, although it is noteworthy that E4-ORF6/7 reduced E1A transactivation to about 50% that of the control.

Since E4-ORF4 reduces E1A expression during lytic virus growth (Fig. 1A), it was important to demonstrate that the inhibitory activity of E4-ORF4 was not due to an inhibition of E1A expression in transfected cells. For this experiment, cell extracts from the same transfections shown in Fig. 2A were subjected to Western blot analysis using the anti-E1A monoclonal antibody M73 (14). As shown in Fig. 2B, E4-ORF4 expression did not result in any major decrease in the level of E1A protein expression compared with the E1A protein expression of other E4 ORFs. The slight reduction in the steady-state concentration of E1A in E4-ORF4-cotransfected cells is not enough to account for the efficient E4-ORF4-dependent shutoff of E1A transactivation of E4 transcription (Fig. 2A).

Okadaic acid blocks the repressive activity of E4-ORF4 on E4 transcription. To determine whether the observed E4-ORF4-dependent inhibition of E1A activation of the E4 promoter worked through the PP2A phosphatase pathway, we treated transfected cells with okadaic acid. As shown in Fig. 3A, okadaic acid treatment almost completely reversed the inhibitory effect of E4-ORF4 on E1A transactivation of the E4 promoter. This result suggests that the E4-ORF4–PP2A complex mediates the E4-ORF4-induced inhibition of E4 transcription.

The CR3 domain is the target for E4-ORF4 inhibition of transcription. Several studies have shown that the 49 amino acids constituting CR3 represent an essential domain for E1A-mediated transcription activation. In itself, CR3 encodes a powerful activating region when fused to the DNA binding domain of Gal4 and when assayed on a promoter containing Gal4 binding sites (19). We have recently shown that a region comprising CR1 (amino acids 28 to 90) functions, at least as efficiently as CR3, as a transcription activator when assayed in the context of a Gal4 fusion protein (4).

To identify the target domain in E1A susceptible to E4-ORF4 repression, we cotransfected Gal-C13Doff (CR3 activator) or Gal-RBoff (CR1 activator) together with the G5E1 BCAT reporter construct containing five Gal4 binding sites

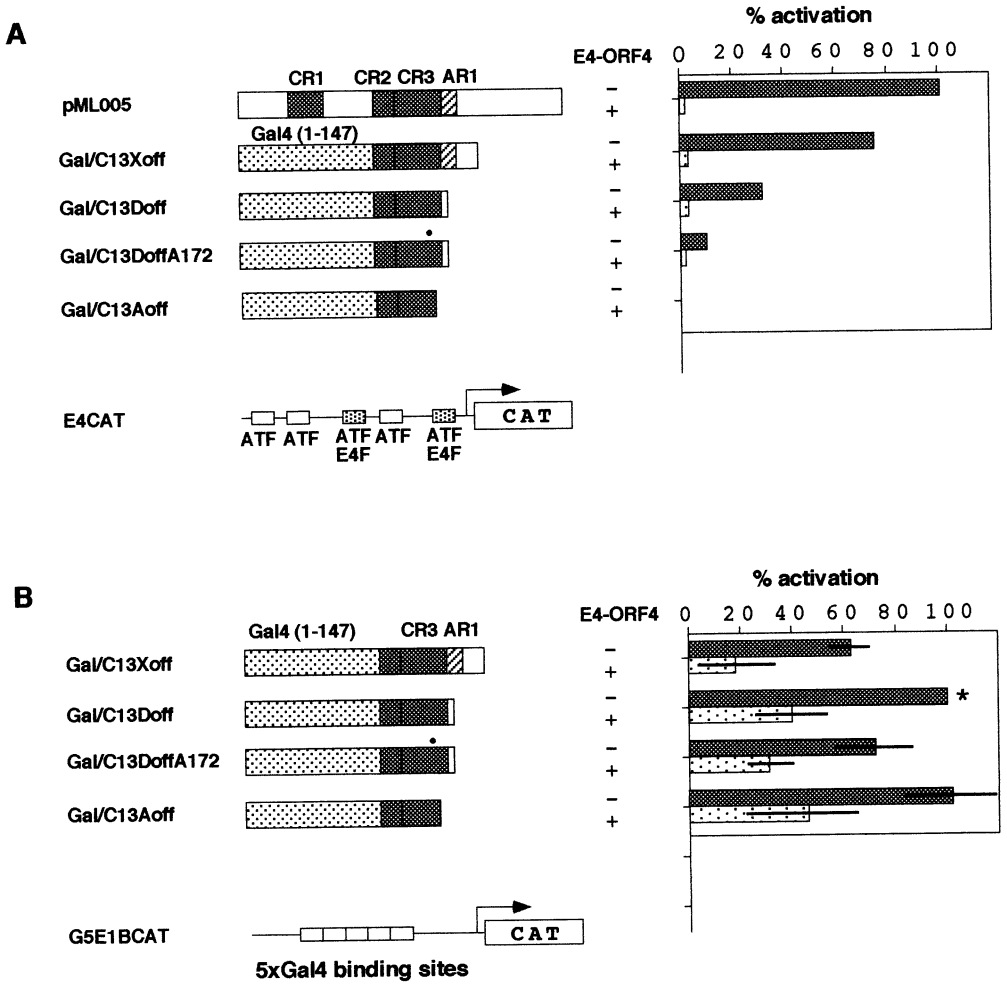

FIG. 4. Serines 172, 185, and 188 in adenovirus type 2 CR3 are not essential targets for E4-ORF4-mediated inhibition of E1A transactivation. (A) Schematic representations of adenovirus type 2 E1A and the Gal4-E1A mutants are shown to the left. A diagram of the relative CAT activities in percentages of E1A activation (pML005) in the absence of E4-ORF4 (set as 100%) is shown to the right. A schematic diagram of the reporter construct, E4CAT, is shown at the bottom. (B) Schematic representations of the Gal4-E1A mutants are shown to the left. A diagram of the relative CAT activities in percentages of Gal-C13Doff activation in the absence of E4-ORF4 is shown to the right. In all experiments, Gal-C13Doff transactivation of G5E1BCAT was set as 100% (*). Bars show the experimental variations relative to Gal-C13Doff in the different experiments analyzed. A schematic diagram of the reporter construct, G5E1BCAT, is shown at the bottom. In both panels, relative CAT activities represent the mean values from at least three independent experiments.

(19), with or without E4-ORF4 and okadaic acid. Figure 3B shows that while Gal-C13Doff-mediated transactivation was repressed by E4-ORF4, Gal-RBoff-mediated transactivation was completely unaffected. Furthermore, the E4-ORF4 repression of Gal-C13Doff was completely annulled by okadaic acid treatment (Fig. 3B). Collectively, these data suggest that E4-ORF4 specifically targets CR3-dependent transactivation without having any effect on CR1 transactivation. This hypothesis was further confirmed by demonstrating that CR1 and/or CR2 mutants of E1A-289R were as sensitive as the wild-type protein to E4-ORF4 repression (data not shown).

Sequences in adenovirus type 2 CR3 required for E4-ORF4 repression. The E1A proteins are heavily phosphorylated in vivo (9). However, mutational analyses have not identified any residues that are of critical importance for E1A function. PP2A is a serine and threonine phosphatase (26). The CR3 domain contains four serines and two threonines, which therefore are potential targets for E4-ORF4–PP2A action. Previous studies have shown that mutations of serines 172, 185, and 188 severely impair E1A transactivation of the adenovirus E3 promoter, while serine 156 and threonines 164 and 178 are less critical (38). It is not known whether any of these residues are phosphorylated in vivo. In this study we tested the significance of serines 172, 185, and 188 for E4-ORF4-induced inhibition of E1A transactivation. Mutation of serine 172 to alanine (Gal-C13DoffA172) impaired Gal4-E1A activation of the E4 promoter (Fig. 4A) and, to a lesser extent, G5E1BCAT transactivation (Fig. 4B). However, Gal-C13DoffA172-activated E4CAT or G5E1BCAT transcription was still inhibited by E4-ORF4 expression (Fig. 4) almost to the same extent as that of the parental protein (Gal-C13Doff). Similarly, deletion of the carboxy-terminal region of CR3 (including serines 185 and 188, by which mutant Gal-C13Aoff was created) resulted in a protein that was still repressed by E4-ORF4 when assayed on the G5E1BCAT reporter (Fig. 4B). As expected, Gal-C13Aoff was unable to activate the wild-type E4 promoter (Fig. 4A). The highly conserved carboxy terminus of CR3 contributes a promoter targeting signal that appears to be required to recruit E1A to the promoter (19, 21, 23). Okadaic acid also reversed

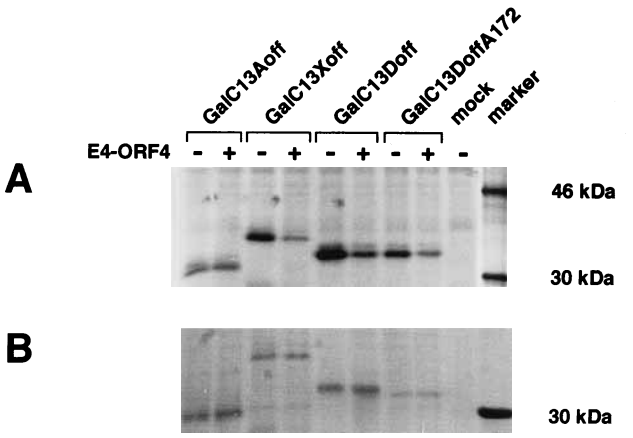

FIG. 5. In vivo phosphorylation of Gal4-E1A fusion proteins in the presence or absence of E4-ORF4. (A) In vivo ^{32}P-labelled proteins were immunoprecipitated with a mouse monoclonal anti-Gal antibody (Promega). (B) Immunoprecipitation of ^{35}S-labelled extracts with the anti-Gal4 monoclonal antibody (Promega) showing the level of Gal4-E1A fusion protein expression. Proteins were analyzed on an SDS–12% polyacrylamide gel.

the inhibitory effect of E4-ORF4 on Gal-E1A fusion protein activation of the wild-type E4 promoter (data not shown).

Collectively, these results suggest that serines 172, 185, and 188, which are of critical importance for efficient adenovirus type 2 E1A activation of E3 and E4 transcription, are not essential targets for E4-ORF4 when assayed on a Gal4 reporter plasmid. Furthermore, as a CR2 deletion mutant of E1A-289R is as sensitive as the wild-type protein to E4-ORF4-mediated repression (data not shown), our results indicate that amino acids 141 to 182 in the E1A-289R protein are sufficient for E4-ORF4-induced inhibition of Gal4-E1A transactivation.

E4-ORF4 inhibition of Gal4-E1A transactivation does not work through a dephosphorylation of CR3. To study what effect E4-ORF4 had on the phosphorylated status of E1A, HeLa cells transfected with various Gal4-E1A fusion proteins were labelled with [^{32}P]orthophosphate before immunoprecipitation with an anti-Gal4 monoclonal antibody. As shown in Fig. 5A, coexpression of E4-ORF4 resulted in accumulation of hypophosphorylated forms of the Gal-C13Xoff, Gal-C13Doff, and Gal-C13DoffA172 proteins. However, E4-ORF4 expression did not change the phosphorylated status of Gal-C13Aoff. Immunoprecipitation of ^{35}S-labelled extracts demonstrated that E4-ORF4 expression did not have an adverse effect on Gal4-E1A fusion protein expression (Fig. 5B). Since Gal-C13Aoff transactivation of the G5E1BCAT reporter is efficiently repressed by E4-ORF4 (Fig. 4B), these results suggest that dephosphorylation of CR3 itself might not be the primary target for E4-ORF4.

DISCUSSION

In this report we show that the E4-ORF4 protein autoregulates its own expression by efficiently inhibiting E1A-induced E4 transcription both in transient transfection experiments and during lytic virus growth. The E4-ORF4 protein has been shown to associate with the cellular serine-threonine phosphatase PP2A (18). The complex possesses phosphatase activity typical of PP2A and causes a decrease in the level of transcription, probably through dephosphorylation of some selective transcription factors (25). Here we show that E4-ORF4-mediated inhibition of E1A-induced E4 transcription was reversed by okadaic acid treatment. Taken together with previous results (18, 25), our results suggest that the E4-ORF4–PP2A complex mediates the E4-ORF4-induced repression of E4 transcription.

Using Gal4 fusion proteins, we show that E4-ORF4–PP2A repression of transcription was specific for CR3-dependent transactivation without having any effect on the CR1 transactivator function (4) (Fig. 3B). The experiments further corroborate our previous conclusion that the Gal4-CR1 and Gal4-CR3 transactivations work through different cellular targets (4). Most likely the difference between Gal4-CR1 and Gal4-CR3 reflects different mechanisms by which they activate transcription. They probably interact with different cellular coactivator proteins or basal transcription factors, some of which are sensitive to dephosphorylation (CR3-dependent transactivation).

The E4-ORF4–PP2A complex may theoretically exert its negative effect on E1A-induced transcription through different effector proteins, for example, E1A itself, a cellular transcription factor targeted by E1A, or more indirectly through a protein kinase required to phosphorylate components of the transcriptional machinery. To begin to answer questions concerning the effects of the E4-ORF4–PP2A complex, we analyzed the phosphorylated states and transactivation properties of some variant Gal4-E1A CR3 fusion proteins (Fig. 4 and 5). Our aim was to determine whether hypophosphorylation of CR3 correlated with the inhibitory activity of E4-ORF4 on Gal4-E1A transactivation (25). E4-ORF4 cotransfection affects the phosphorylated status of most Gal4-CR3 fusion proteins (Fig. 5A) without having any negative effects on Gal4-CR3 fusion protein expression (Fig. 5B). However, we found one mutant (Gal-C13Aoff) with particularly interesting properties. Although Gal-C13Aoff transactivation of the G5E 1BCAT reporter was efficiently repressed by E4-ORF4 cotransfection (Fig. 4B), we could not detect any negative effects of E4-ORF4 on the phosphorylated status of the Gal-C13Aoff protein (Fig. 5A). On the basis of this observation, we suggest that dephosphorylation of E1A itself might not be the primary target for E4-ORF4 in controlling CR3 transactivation of a Gal reporter plasmid. In addition, our results suggest that residues 183 to 192 are of critical importance for E4-ORF4-induced dephosphorylation of CR3 (compare Gal-C13Doff and Gal-C13Aoff) (Fig. 5), possibly by being subjected to E4-ORF4–PP2A-induced dephosphorylation. Alternatively, the highly conserved C-terminal part of CR3 (17) may function as a targeting signal for binding of the E4-ORF4–PP2A complex to E1A.

So what is the primary target of the E4-ORF4–PP2A complex? Current data suggest that both cellular transcription factors ATF-2 and E4F are individually able to mediate E1A transactivation of the E4 promoter (21, 33). At the outset of these experiments we suspected that E4F might be the primary target of E4-ORF4-induced dephosphorylation. This suspicion was based on the previous observation that E1A stimulates E4F transcription factor activity by an induced phosphorylation (31). However, this simplistic solution does not seem to be entirely true. As shown here (Fig. 4A), both Gal-C13Xoff (with AR1) and Gal-CDoff (without AR1) were repressed by E4-ORF4 coexpression. We have previously shown that AR1 is selectively required for E1A-induced E4F but not ATF-2 transcription activation (5). Therefore, these results suggest that E1A-induced activation of the E4 promoter through both the E4F and ATF-2 pathways is sensitive to dephosphorylation by the E4-ORF4–PP2A complex. In this respect it is interesting that the transactivation capacity of ATF-2 is phosphorylation dependent (22). Furthermore, the observation that the activation of a Gal4 reporter plasmid by Gal4-CR3 fusion proteins is

also repressed by E4-ORF4 (Fig. 4B) suggests that a coactivator or a basal transcription factor specific for CR3-dependent transactivation (Fig. 3B) is subjected to E4-ORF4–PP2A dephosphorylation. However, it is noteworthy that the inhibitory activity level of E4-ORF4 was lower on the Gal4 reporter (Fig. 4B) than that of the wild-type E4 promoter (Fig. 4A), suggesting that its repressive activity works mainly through upstream binding transcription factors, like E4F and ATF-2. Collectively, our data suggest that multiple transcription factors involved in E1A-induced transactivation of the E4 promoter are subjected to inhibition by E4-ORF4.

Next to nothing is known about the function of AR1 in the CR3-dependent activation of E4F. Here we show that, similar to the results of our experiments using mutant E1A proteins (5), the Gal4 derivative Gal-C13Xoff (which contains AR1) activates the wild-type E4 promoter more efficiently than mutant Gal-C13Doff (which lacks AR sequences) (Fig. 4A). The effects are smaller with the Gal4 derivatives than those of E1A genes (5), although the tendency to have an AR dependence was the same. In contrast, the same Gal4 derivatives do not show any AR dependence for activation of transcription on the synthetic G5E1BCAT reporter plasmid (Fig. 4B). In fact, AR1 appears to be inhibitory under these conditions (compare Gal-C13Xoff and Gal-C13Doff) (Fig. 4B). These results are consistent with the hypothesis that AR1 is part of a protein-protein interaction surface in the E1A-289R activation of E4F. The promoter localization signal required for E1A-induced activation of cellular ATF-2 includes a highly conserved amino acid sequence in the C-terminal half of CR3 which mediates protein-protein contact between E1A and ATF-2 (19, 21). It is noteworthy that AR1 is located immediately downstream of this conserved sequence. Thus, an artificial DNA binding domain, like Gal4, could be predicted to obviate the need for an E1A-protein interaction surface normally provided by the C-terminal part of CR3 plus the AR elements in the context of the wild-type E1A protein. We have previously shown that E1A activation of E4F, but not of ATF-2, is AR1 dependent (5). Thus, E1A interaction with E4F may require a more extended protein interaction domain than ATF-2 transactivation. It is not known whether the potential E1A interaction with E4F results in a recruitment of E1A to the E4 promoter, similar to what has been proposed for E1A activation of ATF-2 (19, 21), or whether such an interaction helps an E1A-associated protein kinase to phosphorylate E4F.

The inhibitory activity of E4-ORF4 was not restricted to E4 transcription. As shown in Fig. 1A, E4-ORF4 also repressed E1A transcription during lytic virus growth. Most likely, E4-ORF4 will reverse E1A activation of many promoters that are subjected to control at the level of phosphorylation.

ACKNOWLEDGMENTS

We are grateful to P. Hearing and M. Green for kind gifts of E4 mutant viruses and plasmids, respectively. We also thank Catharina Svensson for helpful comments on the manuscript.

This work was supported by the Swedish Cancer Society.

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