JOURNAL OF VIROLOGY, Sept. 1996, p. 5852–5859 Vol. 70, No. 9
0022-538X/96/$04.00+0

The CR1 and CR3 Domains of the Adenovirus Type 5 E1A Proteins Can Independently Mediate Activation of ATF-2

MONIQUE C. A. DUYNDAM, HANS VAN DAM, ALEX J. VAN DER EB, AND ALT ZANTEMA*

Laboratory for Molecular Carcinogenesis, Sylvius Laboratory, Leiden University, 2333 AL Leiden, The Netherlands

Received 11 January 1996/Accepted 9 May 1996

The adenovirus 12S E1A protein can stimulate the activity of the c-*jun* promoter through a conserved region 1 (CR1)-dependent mechanism. The effect is mediated by two AP-1/ATF-like elements, jun1 and jun2, that preferentially bind c-Jun–ATF-2 heterodimers. In this study, we show that the ATF-2 component of the c-Jun–ATF-2 heterodimer is the primary target for 12S E1A: 12S E1A can enhance the transactivating activity of the N terminus of ATF-2 when fused to a heterologous DNA-binding domain, whereas the transactivating activity of the c-Jun N terminus is not significantly affected. Activation of the ATF-2 N terminus by 12S E1A is dependent on CR1. In the context of the 13S E1A protein, CR1 and CR3 can both contribute to activation of ATF-2, and their relative contributions are dependent on the cell type. In contrast to activation of ATF-2 by stress-inducing agents, CR1-dependent activation of ATF-2 was found not to depend strictly on the presence of threonines 69 and 71 in the N terminus of ATF-2, which are targets for phosphorylation by stress-activated protein kinases (SAPKs). In agreement with this observation, we did not observe phosphorylation of threonines 69 and 71 or constitutively enhanced SAPK activity in E1A- plus E1B-transformed cell lines. These data suggest that CR1-dependent activation of ATF-2 by 12S E1A does not require phosphorylation of threonines 69 and 71 by SAPK.

The protein products of the early region 1A (E1A) of human adenoviruses (Ad) play an essential role in Ad-induced transformation of cells. Two E1A proteins are translated from the differentially spliced 13 and 12S mRNAs. The E1A proteins of Ad contain three regions, designated CR1, CR2, and CR3, that are highly conserved and contain important functional domains. CR1 and CR2 are present in both the 13S and the 12S products, whereas CR3 is unique to the 13S product. In addition to the three conserved regions, the N termini of the proteins each encode an important functional domain. The E1A proteins are believed to induce transformation by modulating the activity of various regulatory transcription factors. Binding of CR1 and CR2 to the retinoblastoma gene product pRb, p107, or p130 leads to activation of members of the E2F family of transcription factors, which play a key role in regulation of cell cycle control genes (5, 6, 10, 38, 43). The N terminus and CR1 of the E1A proteins are involved in binding p300 and cyclic AMP response element-binding protein (CREB)-binding protein (CBP), two proteins that have recently been identified as coactivators of the transcription factors CREB, c-Jun, and c-Fos. Binding of these coactivators to E1A leads to inhibition of the transactivating activities of these transcription factors (3, 4, 7, 11, 14, 27, 33, 46).

CR3 contains a strong transactivation domain, which is essential for the activation of the other adenoviral early genes. It encompasses a region interacting with sequence-specific activator proteins (2, 31) and one interacting with the TATA-binding protein TBP (21, 29, 53). These two types of interactions may lead to activation of the transcription-initiation complex. Many adenoviral early genes contain binding sites for members of the activating transcription factor (ATF) family, suggesting that E1A enhances the transactivation potential of ATF proteins (42). Two related members of this family, ATF-2 and ATFa, have indeed been shown to mediate CR3-dependent activation of transcription (9, 15, 30, 31, 34). CR3 interacts with the leucine zipper of ATF-2 and ATFa (9, 31), which is believed to induce a conformational change resulting in exposure of their N-terminal activation domains. These domains may be masked in the full-length ATF-2 and ATFa proteins, since these proteins have been found to be transcriptionally inert in several studies (9, 15, 31). However, transcriptional activity of full-length ATF-2 has also been reported (35). The transactivation domains of ATF-2 and ATFa efficiently stimulate transcription when fused to DNA-binding domains of Gal4 and c-Myb (1, 9, 15, 31, 35). The transactivation domain of ATF-2 can also be activated by E1A via a mechanism that is independent of the leucine zipper region, since it can still be activated in a CR3-dependent manner when fused to the DNA-binding domain of Gal4 (15). This zipper-independent activation may involve phosphorylation of two threonine residues in the N terminus of ATF-2, Thr-69 and Thr-71, since these residues are crucial for activation (17, 32). ATF-2 can also be activated in a phosphorylation-dependent manner by serum, UV irradiation, interleukin 1, and genotoxic stress (17, 32, 51). These agents activate members of the family of proline-directed stress-activated protein kinases (SAPKs) that phosphorylate Thr-69 and Thr-71 of ATF-2 (12, 17, 28, 32, 51). The ATF-2 SAPKs (p46 and p54) are highly related (but not identical) to the Jun kinases p46 and p54, which also belong to the family of SAPKs and phosphorylate c-Jun in response to cellular stress (20, 28, 51). At present, it is not known whether the SAPKs are also activated by E1A.

The 12S E1A product (12S E1A) can also activate transcription, although generally to a lesser extent than the 13S product (23, 36, 44, 48, 49, 54). One of the targets of 12S E1A is the c-*jun* promoter, which is stimulated by 12S E1A in a CR1-dependent manner (25, 26, 48, 49). This activation is mediated by two AP-1/ATF-like elements, called jun1 and jun2, which preferentially bind c-Jun–ATF-2 heterodimers (48, 49). Expression of c-*jun* is enhanced in Ad-transformed cell lines, indicating that c-Jun–ATF-2 heterodimers are constitutively

* Corresponding author. Mailing address: Laboratory for Molecular Carcinogenesis, Sylvius Laboratory, Leiden University, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands. Phone: 31-71-5276113. Fax: 31-71-5276284.

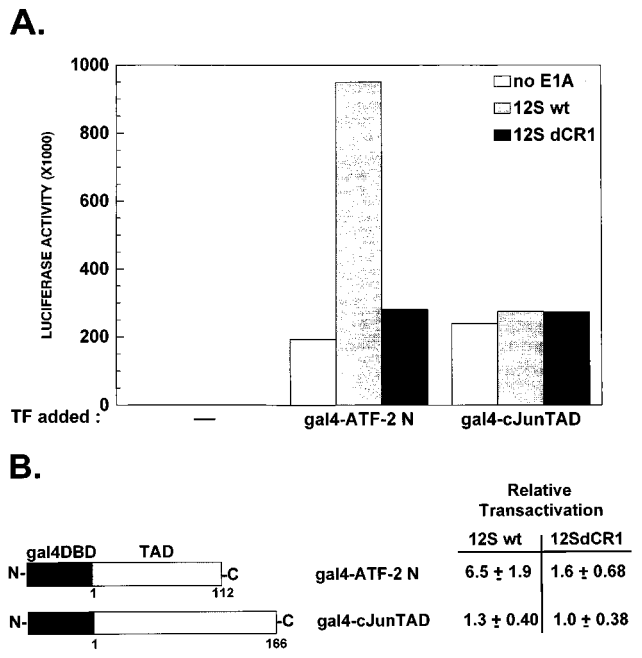

FIG. 1. Activation of the N terminus of ATF-2 by 12S E1A is dependent on CR1. (A) F9 cells were transiently transfected with 1 μg of 5×Gal4E4-luciferase plasmid together with 1 μg of the indicated Gal4 fusion expression vectors in the absence or presence of an expression vector (0.25 μg) encoding either wt 12S E1A or 12S E1A with a deletion in CR1 and 2.25 μg of pUC18 carrier DNA. Transfection efficiency was monitored by including 0.5 μg of pCMVLacZ per transfection (Materials and Methods). TF, transcription factor. (B) The Gal4 fusion constructs. Numbers indicate amino acid positions in the wt ATF-2 and c-Jun proteins. The relative transactivation values represent Gal4–ATF-2- or Gal4–c-Jun-induced promoter activity in the presence of E1A relative to the activity in the absence of E1A. The values for 12S wt and 12SdCR1 are the means of six and three independent experiments, respectively. The results of one of them are shown in panel A. DBD, DNA-binding domain; TAD, transactivation domain.

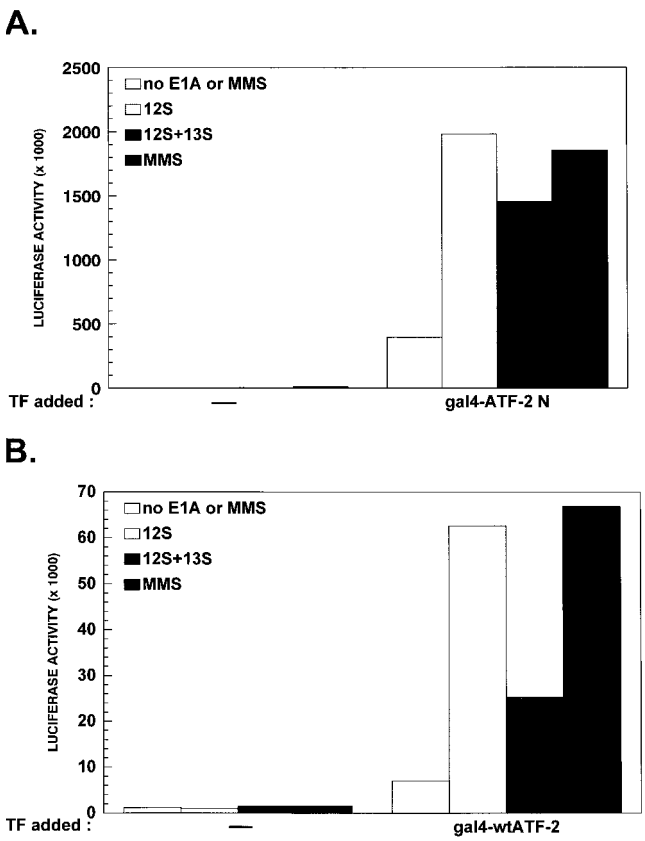

FIG. 2. 12S E1A, wt E1A, and MMS are equally potent activators of Gal4–ATF-2 in F9 cells. F9 cells were transiently transfected with 1 μg of 5×Gal4E4-luciferase plasmid together with 1 μg of Gal4 fusion expression vectors containing either the N terminus of ATF-2 (A) or the full-length ATF-2 protein (B) in the absence or presence of an expression vector (0.25 μg) encoding 12S E1A or wtE1A (12S + 13S) and 2.25 μg of pUC18 carrier DNA. In the case of MMS, cells were treated with 1 mM MMS at 16 h after transfection for an additional 6 h. Two independent experiments gave similar results; results of one of them are shown. Standard deviations, <20%. TF, transcription factor.

activated in transformed cells. This suggests the possibility that c-Jun–ATF-2 transcription factors may play a role in transformation. Activation of c-Jun–ATF-2 may be due to activation of the c-Jun protein via phosphorylation of Ser-63 and Ser-73 and/or due to activation of ATF-2 (18, 48).

To examine which component of the c-Jun–ATF-2 heterodimer is the main target of 12S E1A, we have analyzed the effects of 12S E1A on the transactivating potential of Gal4–ATF-2 and Gal4–c-Jun fusion proteins. Our results show that 12S E1A can efficiently activate Gal4–ATF-2 through a CR1-dependent mechanism. Activation requires the intactness of the N-terminal zinc finger structure of ATF-2 but is not strictly dependent on the presence of Thr-69 and Thr-71. Furthermore, Thr-69 and Thr-71 do not appear to be phosphorylated in Ad-transformed cells, nor did we measure enhanced SAPK activity. Our data suggest that CR1-dependent activation differs from CR3-dependent activation in the requirement for Thr-69 and Thr-71 and that CR1-dependent activation may not involve phosphorylation of these residues by SAPKs.

MATERIALS AND METHODS

Plasmid constructs. RSVGal4-ATF-2 N, T69 + T71, and S90 constructs were cloned by exchanging the *Sal*I-*Xba*I fragment of pRSVGal4-d9-ATF-2 (51) for the *Sal*I-*Xba*I fragments of pMLVGal4-ATF-2 (codons 19 to 505) (47) and pMLVGal4-ATF-2 T69 + T71 and pMLVGal4-ATF-2 S90 (32), respectively. To construct pRSVGal4-ATF-2 N, pmE, pmA, and pm27/32, the *Sal*I-*Xba*I fragment of pRSVGal4-d9-ATF-2 was exchanged for the *Sal*I-*Xba*I fragments of pMLVGal4-ATF-2, pMLVGal4-ATF-2pmE, pMLVGal4-ATF-2pmA, and pMLVGal4-ATF-2pm27/32, respectively (15). To construct pRSVGal4-wtATF-2 (codons 19 to 505), lacking the first 18 codons for ATF-2, the *Sal*I-*Xba*I fragment of pRSV-Gal4-wtATF-2 (51) was replaced by the *Sal*I-*Xba*I fragment of pMLVGal4ATF-2 (codons 19 to 505). The Gal4-cJunTAD construct has been described previously (37). The Gal4 reporter construct 5×Gal4E4-luciferase was kindly provided by K. Livingston and N. Jones. The E1A plasmids expressing wild-type (wt) 12S (pJF12), 12S dCR1 (pG5/3 JF12), E1Awt (pHINDIIIG), and E1AdCR1 (pG5/3) have been described previously (40, 49). The bacterial expression vectors pGEX-ATF-2 N and pGEX-cJun N(1-166) have also been described previously (51).

Cell culture, transfections, and luciferase assay. Primary human embryonic retinoblasts (HER cells) and their Ad type 5 (Ad5) E1-transformed derivatives were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml). HeLa, primary baby rat kidney (BRK), simian virus 40 (SV40)-transformed BRK, and E1-transformed BRK cells were kept in DMEM–10% fetal calf serum–penicillin (100 μg/ml)–streptomycin (100 μg/ml). F9 cells were grown in F12-DMEM (1:1) supplemented with 10% fetal calf serum, penicillin (100 μg/ml), streptomycin (100 μg/ml), and 0.1 mM β-mercaptoethanol. F9 cells and HeLa cells were transfected on 3-cm-diameter dishes by the calcium phosphate precipitation method (16). After a 6-h incubation, the cells were washed and refed with culture medium. Sixteen hours after transfection, the cells were lysed in 250 μl of lysis reagent (25 mM Tris-phosphate [pH 7.8], 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′*N*′-tetraacetic acid, 10% glycerol, 1% Triton X-100), and luciferase activity was measured according to the manufacturer's protocol (Promega). To correct for transfection efficiency, in some cases, 0.5 μg of pCMVLacZ was cotransfected and β-galactosidase activity was determined as described by Sambrook et al. (39).

Labeling and immunoprecipitation. For in vivo phosphorylation analysis, cells were incubated in medium deficient for sodium phosphate and labeled for 3 h in

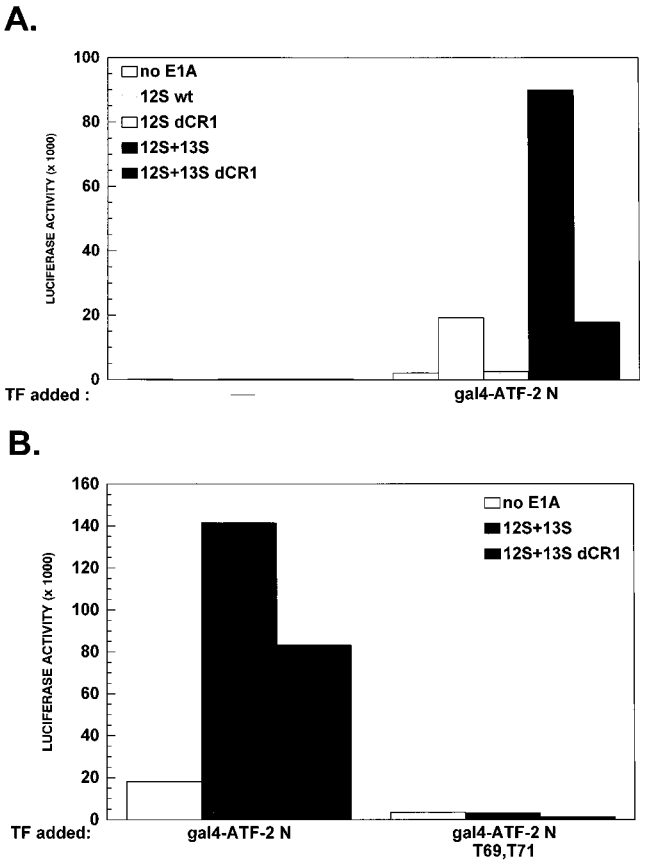

FIG. 3. CR3-dependent activation is the dominant transactivating activity on ATF-2 in HeLa cells and is dependent on threonines 69 and 71 of ATF-2. (A) HeLa cells were transiently transfected with 1 μg of 5×Gal4-E4-luciferase plasmid together with 1 μg of a Gal4–ATF-2 N expression vector in the absence or presence of an expression vector (0.25 μg) encoding either 12S E1A, 12S E1A with a deletion in CR1 (dCR1), wtE1A (12S + 13S), or E1A (12S + 13S) with a deletion in CR1 and 2.25 μg of pUC18 carrier DNA. Three independent experiments were performed with similar outcomes. Results of one of them are shown. Standard deviations, <25%. TF, transcription factor. (B) HeLa cells were transiently transfected as described above. The Gal4 fusion proteins tested contain either the N terminus of ATF-2 or a mutant version in which the threonines at positions 69 and 71 were replaced by alanines (see Fig. 4A). Three independent experiments were performed with similar outcomes. Results of one of them are shown. Standard deviations, <25%.

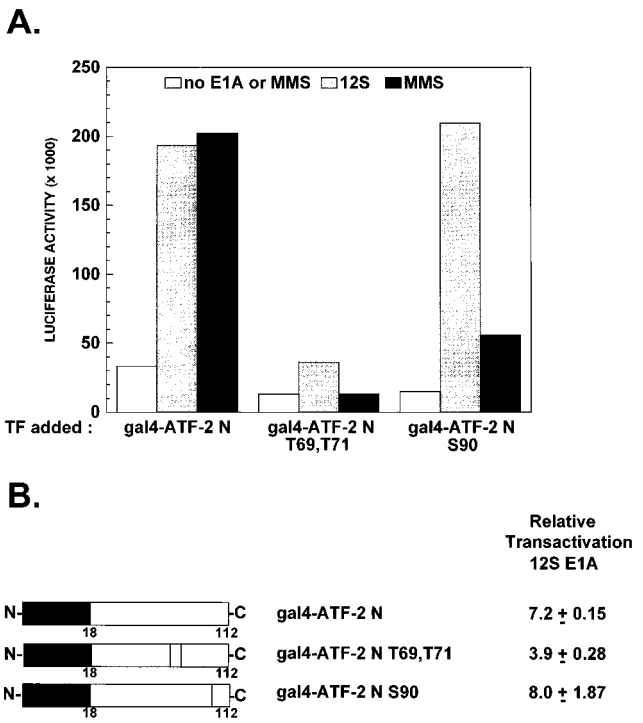

FIG. 4. Threonines 69 and 71 of ATF-2 are not strictly required for transactivation of ATF-2 by 12S E1A. (A) F9 cells were transiently transfected with 1 μg of 5×Gal4-E4-luciferase plasmid together with 1 μg of the indicated Gal4 fusion expression vectors in the absence or presence of an expression vector (0.25 μg) encoding wt 12S E1A and 2.25 μg of pUC18 carrier DNA. Transfection efficiency was monitored by including 0.5 μg of pCMVLacZ per transfection (Materials and Methods). (B) The Gal4 fusion constructs. Numbers indicate amino acid positions in the wt ATF-2 protein. In the ATF-2 mutants, threonines at positions 69 and 71 and a serine at position 90 have been replaced by alanines. Standard deviations, <20%. TF, transcription factor. Relative transactivation figures represent Gal4–ATF-2-induced promoter activity in the presence of E1A relative to the activity of the Gal4-luciferase in the absence of E1A. Values are the means of three different experiments; results of one of them are shown.

the presence of 1 mCi of $^{32}P_i$. Subsequently, the cells were lysed in RIPA-1 buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Nonidet P-40, 1% sodium deoxycholate, 200 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, and 0.5 mM trypsin inhibitor), and the lysates were cleared with normal rabbit antiserum bound to protein A beads. c-Jun and ATF-2 proteins were immunoprecipitated with affinity-purified polyclonal antibodies raised against the DNA-binding domain of c-Jun (Pep-1; Oncogene Science) and the C terminus of ATF-2 (C19; Santa Cruz), respectively. The immunoprecipitates were resolved on SDS–8% (ATF-2 immunoprecipitates) or SDS–10% (c-Jun immunoprecipitates) polyacrylamide gels.

Protein kinase assay. Cells were washed twice with phosphate-buffered saline (PBS) (4°C) and detached from the plate with a rubber policeman in PBS. After centrifugation, the cells were resuspended in 1 ml of kinase buffer consisting of 1.5 mM sodium ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (Na-EGTA), 50 mM β-glycerophosphate (pH 7.3), 1 mM dithiothreitol, 1 mM Na_3VO_4, 200 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, and 1 μM leupeptin and homogenized by using an ultra-turrax at maximum speed (twice for 1 min each).

For solid-state phosphorylation assays, 200 μg of whole-cell extract was incubated for 1 h at 4°C with 5 μg of glutathione *S*-transferase (GST), GST–ATF-2 N, or GST–c-Jun N fusion proteins coupled to glutathione-Sepharose beads. Subsequently, the beads were washed once with 0.2 M NaCl in kinase buffer and twice with kinase buffer and incubated for 90 min at 30°C in 50 μl of kinase buffer containing 25 μM ATP, 100 μM $MgCl_2$, and 5 μM [γ-^{32}P]ATP (>5,000 Ci/mmol). The reaction was stopped by the addition of 25 μl of 3× Laemmli buffer, after which the samples were boiled for 5 min and resolved on a 12% polyacrylamide gel. Preparation of the GST–ATF-2 N (amino acids 19 to 96 of ATF-2) and GST–c-Jun N (amino acids 1 to 166) fusion proteins were performed as described previously (45).

For total kinase assays, 200 μg of whole-cell extract was incubated with 5 μg of GST, GST–ATF-2 N, or GST–c-Jun N in kinase buffer containing 1 μM [γ-^{32}P]ATP (>5,000 Ci/mmol), 5 μM ATP, and 20 μM $MgCl_2$ for 90 min at 30°C. After the phosphorylation reaction, the samples were incubated for 1 h at 4°C with glutathione-Sepharose beads. The beads were washed three times with kinase buffer and boiled for 5 min in 1× Laemmli buffer, and the proteins were resolved on a 12% polyacrylamide gel.

RESULTS

12S E1A activates the N terminus of ATF-2 through the CR1 domain. We have shown previously that 12S E1A can activate binding sites for c-Jun–ATF-2 heterodimers in a CR1-dependent manner in a variety of cell types, including F9 and HeLa cells (47–49). To determine whether the transactivation domain of both or one of these proteins can be activated by 12S E1A when fused to a heterologous DNA-binding domain, we performed transient-transfection experiments with F9 and HeLa cells. A luciferase reporter construct containing five Gal4 binding sites in front of a TATA box was cotransfected with expression vectors of fusion proteins containing the *Saccharomyces cerevisiae* Gal4 DNA-binding domain and the N-terminal domains of ATF-2 or c-Jun (Fig. 1). In F9 cells,

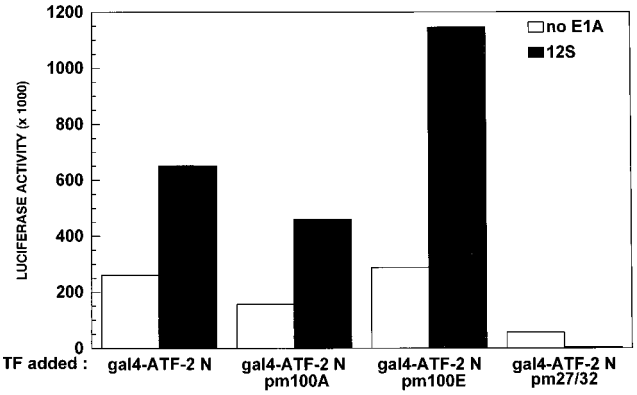

FIG. 5. The zinc finger structure in the N terminus of ATF-2 is essential for transactivation by 12S E1A. F9 cells were transiently transfected with 1 μg of 5×Gal4E4-luciferase plasmid together with 1 μg of the indicated Gal4 fusion expression vectors in the absence or presence of an expression vector (0.25 μg) encoding wt 12S E1A and 2.25 μg of pUC18 carrier DNA. Transfection efficiency was monitored by including 0.5 μg of pCMVLacZ per transfection (Materials and Methods). Three independent experiments were performed with similar outcomes; results of one are shown. Standard deviations, <25%. TF, transcription factor.

cotransfection of a construct expressing 12S E1A activated the N terminus of ATF-2 sixfold, whereas the N terminus of c-Jun was not significantly activated (Fig. 1). Also in HeLa cells, we found that E1A preferentially activates the N terminus of ATF-2 (data not shown). Similar differences were found when a larger or smaller amount of the E1A plasmid was cotransfected. Therefore, we conclude that ATF-2 is the primary target for 12S E1A in both F9 and HeLa cells.

Previous studies with HeLa and CHO cells have demonstrated that the transactivation potential of ATF-2 is strongly enhanced by 13S E1A in a CR3-dependent manner. Compared with the activation by 13S E1A, the effect of 12S E1A was insignificant in these studies (15, 30). In addition to E1A, genotoxic agents such as UV and methyl methanesulfonate (MMS) have also been found to enhance ATF-2 activity. Activation by these agents is mediated through phosphorylation by activated SAPKs (17, 32, 51). To compare the effects of 12S E1A, wtE1A (12S + 13S), and MMS, we tested these agents in parallel. Interestingly, 12S E1A, wtE1A, and MMS were found to be equally potent activators of the ATF-2 N terminus in F9 cells (Fig. 2A). A Gal4 fusion protein containing the full-length ATF-2 protein was also activated to a similar extent by MMS and 12S E1A, and activation by wtE1A was even lower (Fig. 2B). These results indicate that CR3 does not play a major role in the activation of ATF-2 in F9 cells. To analyze the effects of 12S E1A and wtE1A on the activity of ATF-2 in another cell type, we performed similar experiments with HeLa cells. In agreement with results of others (15), wtE1A was a much more potent activator of the N terminus of ATF-2 than 12S E1A in HeLa cells (Fig. 3A). Under the experimental conditions used, transactivation by wtE1A and 12S E1A was about 20- and 5-fold, respectively (Fig. 3A). To verify whether activation of the N terminus of ATF-2 by E1A reflects the activation of the c-*jun* promoter, which is dependent on CR1, we examined the effect of CR1 mutants of E1A. Deletion of CR1 of 12S E1A completely abolished transactivation of the N terminus of ATF-2 in F9 (Fig. 1) and HeLa (Fig. 3A) cells. In addition, wtE1A with a deletion in CR1 showed reduced activation of the N terminus of ATF-2 in HeLa cells (Fig. 3A). Differences in transactivation capacities of the different E1A proteins were not due to differences in expression levels, as was verified by Western blot (immunoblot) analysis (data not shown). In addition, none of the different E1A proteins affected the activity of the Rous sarcoma virus promoter that drives the expression of the Gal4 fusion proteins, both in HeLa and in F9 cells. We conclude that both the CR1 and the CR3 domains of E1A contribute to activation of ATF-2. The relative contributions of these domains to transactivation by wtE1A are apparently dependent on the cell type.

CR1-dependent activation is not strictly dependent on threonines 69 and 71 and requires an intact N-terminal zinc finger structure of ATF-2. Previous studies performed with CHO cells (17, 32) have shown that CR3-dependent activation of Gal4–ATF-2 requires the presence of Thr-69 and Thr-71, located in the N terminus of ATF-2. These residues were also found to be essential for the transactivating activity of the ATF-2 N terminus in the absence of E1A (32) and for the activation of ATF-2 by genotoxic agents (51). As shown in Fig. 3B, also in HeLa cells, a Gal4 fusion protein containing the N-terminal ATF-2 domain in which Thr-69 and Thr-71 were replaced by alanines was unable to stimulate transcription, in either the absence or the presence of wtE1A. We next determined whether Thr-69 and Thr-71 also play a role in transactivation of ATF-2 by 12S E1A in F9 cells. As a control, we studied the effects of MMS. As shown in Fig. 4A, the transactivating activity of the fusion protein with mutations of Thr-69 and Thr-71 in the absence of E1A was lower than the activity of the fusion protein containing the wt N terminus of ATF-2. As expected, mutation of Thr-69 and Thr-71 led to a complete loss of the inducibility of the N terminus of ATF-2 by MMS. In contrast, 12S E1A could still activate the mutant, although to a lesser extent (fourfold; Fig. 4B). We also examined the role of Ser-90, another phosphorylation site. Mutation of Ser-90 into an alanine did not affect transactivation by 12S E1A, whereas the response to MMS was reduced (Fig. 4A). These results indicate that, in contrast to their role in CR3-dependent activation, Thr-69 and Thr-71 do not play a major role in activation of ATF-2 via CR1 and that CR3- and CR1-dependent activations of ATF-2 are thus mediated by different mechanisms.

In addition to Thr-69, Thr-71, and Ser-90, the N terminus of ATF-2 contains a serine residue at position 100, which is a potential phosphorylation site for protein kinase A or casein kinase II. Furthermore, the N terminus contains two cysteines (Cys-27 and Cys-32) and two histidines (His-41 and His-45) that have the potential to form a single metal-binding finger, which has been found to be essential for CR3-dependent activation of ATF-2 (15, 35). To test whether these motifs are important for 12S E1A-dependent transactivation, we used Gal4 fusion constructs in which Cys-27 and Cys-32 were replaced by alanines (pm27/32) or in which Ser-100 was replaced by alanine (pm100A) or glutamic acid (pm100E) (15). As shown in Fig. 5, mutation of the serine residue at position 100 did not affect 12S E1A-dependent transactivation. In contrast, disruption of the zinc finger motif reduced the basal activity of the ATF-2 N terminus and completely abolished the enhancement of ATF-2 activity by 12S E1A. In fact, the activity of the zinc finger mutant was even inhibited by 12S E1A. Thus, in contrast to Thr-69 and Thr-71, the zinc finger structure is essential for transactivation by both 12S and 13S E1A.

Hyperphosphorylated forms of ATF-2 are not detected in E1A plus E1B-transformed cells. Activation of SAPKs leads to phosphorylation of ATF-2 on Thr-69 and Thr-71, which can be detected by a mobility shift in SDS-PAGE. ATF-2 proteins with mutations in Thr-69 and Thr-71 do not show this mobility shift (17). As these threonine residues are essential for the full transactivation activity of ATF-2, also in the presence of E1A

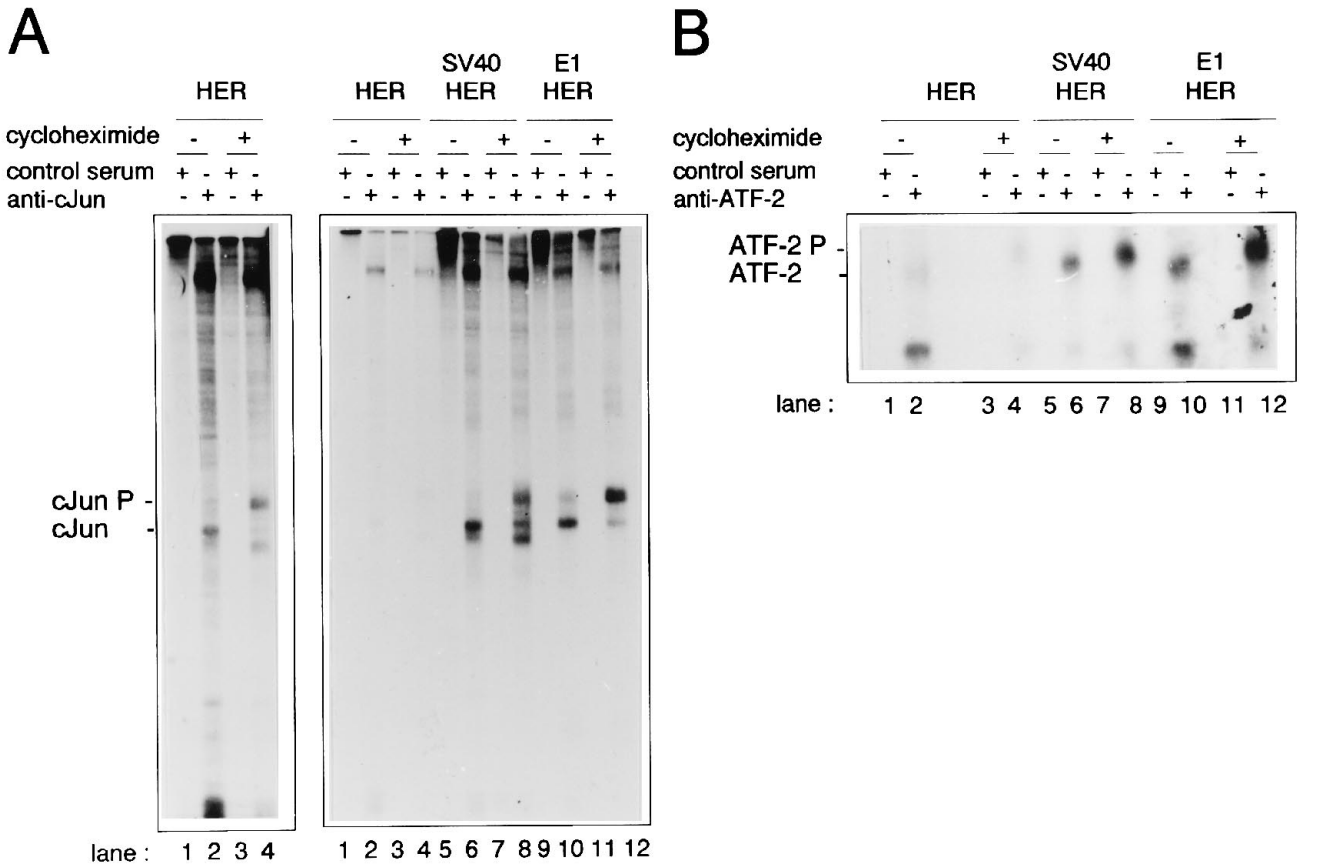

FIG. 6. c-Jun and ATF-2 are both hyperphosphorylated upon cycloheximide treatment, whereas only c-Jun is hyperphosphorylated in E1A plus E1B-transformed HER cells. Untransformed HER cells (lanes 1 to 4), SV40-transformed HER cells (lanes 5 to 8), and E1A plus E1B-transformed HER cells (lanes 9 to 12) were labeled for 3 h with [^{32}P]phosphate. Cycloheximide (200 μM) was added for the last 90 min (lanes 3, 4, 7, 8, 11, and 12). Lysates were incubated with nonimmune serum or a c-Jun-specific antiserum (A) and with nonimmune serum or an ATF-2-specific antiserum (B). Immunocomplexes were resolved by SDS-PAGE and visualized by autoradiography. The leftmost four lanes in panel A are a longer exposure of lanes 1 to 4 of the right lanes in panel A.

(Fig. 4), it is possible that E1A stimulates the activity of SAPK or other kinases acting on ATF-2. Induction of SAPK activity also leads to a mobility shift of c-Jun via phosphorylation of Ser-63 and Ser-73 (12, 13, 20, 28, 37). E1A plus E1B-transformed cell lines contain the same modified forms of c-Jun in which Ser-63 and Ser-73 are hyperphosphorylated. These hyperphosphorylated forms of c-Jun were not detected in cells transformed by other DNA tumor viruses, such as SV40 (18, 19) (Fig. 6A). To examine whether the phosphorylation pattern of ATF-2 is also altered in E1A plus E1B-transformed cells, we compared the electrophoretic mobilities of ATF-2 isolated from primary, SV40- and E1-transformed HER cells. As a control, we also analyzed ATF-2 isolated from cells in which SAPK activity was induced by cycloheximide treatment. In all cell lines, both ATF-2 and c-Jun migrate with decreased electrophoretic mobility upon cycloheximide treatment (Fig. 6A, lanes 4, 8, and 12; Fig. 6B, lanes 4, 8, and 12). Although hyperphosphorylated forms of c-Jun can clearly be detected in the untreated E1A plus E1B-transformed HER cells (Fig. 6A, lane 10), the mobility of ATF-2 in these cells is essentially the same as that in primary HER cells and in the SV40-transformed HER cells (Fig. 6B, lanes 2, 6, and 10). The increased phosphorylation signal of ATF-2 in SV40- and E1-transformed HER cells is most likely due to the elevated ATF-2 protein levels in these cells (data not shown). Similar results are obtained with E1A plus E1B-transformed cells derived from primary BRK cells (data not shown). Thus, although Thr-69 and Thr-71 are required for complete activation of ATF-2 by E1A in transient-transfection assays, these sites do not appear to be differently phosphorylated in E1A plus E1B-transformed cell lines.

SAPKs are not constitutively activated in E1A plus E1B-transformed BRK cells. To further investigate the role of kinases in the activation of c-Jun and/or ATF-2 by E1A, we examined whether c-Jun- and/or ATF-2-associated kinase activity is increased in E1A plus E1B-transformed cell lines. Cell extracts of primary BRK cells and SV40- and E1-transformed BRK cells were incubated with GST proteins containing the transactivation domains of ATF-2 (amino acids 1 to 109) and c-Jun (amino acids 1 to 166) bound to glutathione-agarose beads. After extensive washing, a solid-state kinase assay was performed. As a control, SAPK activity was induced in these cell lines by cycloheximide treatment. Figure 7A shows that a cycloheximide-inducible kinase could be purified with GST–ATF-2 and GST–c-Jun proteins in all cell lines (lanes 5, 6, 11, 12, 17, and 18). The phosphorylation signal was specific for c-Jun and ATF-2, since no signal could be detected when the GST protein was used. E1A plus E1B-transformed cells did not contain a constitutively enhanced c-Jun- and ATF-2-associated kinase activity compared with those of the other two cell types, although we could detect a basal kinase activity in all three cell types. In fact, this basal kinase activity was even

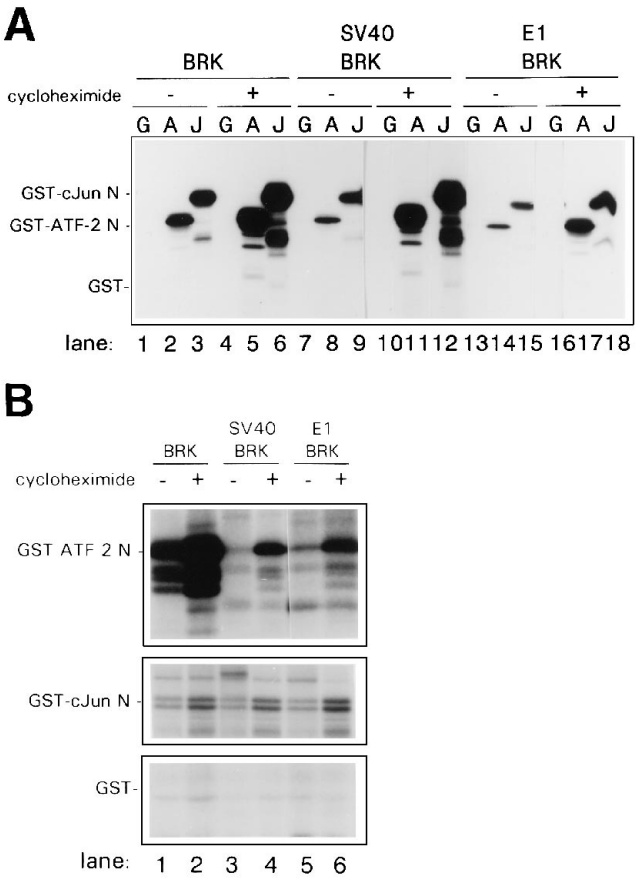

FIG. 7. Constitutively activated c-Jun and/or ATF-2 protein kinase cannot be detected in E1A plus E1B-transformed BRK cells. (A) Samples (200 μg) of whole-cell extracts of untreated and cycloheximide-treated primary BRK cells and BRK cells transformed by SV40 or by E1A plus E1B were incubated with GST (G), GST–ATF-2 N (A), or GST–c-Jun N (J) proteins coupled to glutathione-Sepharose beads for 1 h. The beads were washed extensively, and a solid-state phosphorylation assay was performed. After 90 min, the proteins were resolved by SDS-PAGE. The migration positions of GST, GST–ATF-2 N, and GST–c-Jun N proteins are indicated. (B) Samples (200 μg) of whole-cell extract of untreated and cycloheximide-treated primary BRK cells and BRK cells transformed by SV40 or E1A plus E1B were incubated with 5 μg of GST, GST–ATF-2 N, and GST–c-Jun N in kinase buffer containing [γ-^{32}P]ATP at 30°C for 90 min. After the phosphorylation reaction, the extracts were incubated with glutathione-Sepharose beads for 1 h at 4°C. The beads were washed, and the proteins were resolved by SDS-PAGE. The migratory positions of GST, GST–ATF-2 N, and GST–c-Jun N proteins are indicated.

somewhat higher in the primary BRK cells. However, it is possible that activated c-Jun- and ATF-2-directed kinases are present in E1A plus E1B-transformed cells but bind only very weakly to the N termini of c-Jun and ATF-2 and hence could not be purified and detected in the experiment whose results are shown in Fig. 7A. To test this possibility, we incubated soluble GST–ATF-2 and GST–c-Jun proteins with whole-cell extracts in kinase buffer containing [^{32}P]ATP. After the phosphorylation reaction, the GST proteins were isolated by the addition of glutathione-agarose beads. As can be seen in Fig. 7B, increased phosphorylation of ATF-2 and c-Jun is observed only after cycloheximide treatment. These data strongly suggest that the SAPKs and/or other kinases acting on the c-Jun and ATF-2 N termini are not activated to a significant extent in E1A plus E1B-transformed cells. Combined with the observations that 12S E1A-induced enhancement of the transactivating activity of ATF-2 is partially independent of Thr-69 and Thr-71 in the transient-transfection experiments and the absence of hyperphosphorylated forms of ATF-2 in E1A plus E1B-transformed cell lines, these results indicate that stimulation of ATF-2 activity by 12S E1A does not involve phosphorylation but employs a different mechanism.

DISCUSSION

It has been well documented that E1A can stimulate c-Jun–ATF-2 binding sites in a CR1-dependent manner in HeLa and F9 cells (25, 26, 47–49). Ad-transformed cells contain hyperphosphorylated forms of c-Jun, which are caused by enhanced phosphorylation of Ser-63 and Ser-73, located in the transactivation domain (18). This suggests that activation of c-Jun–ATF-2 heterodimers may be mediated by phosphorylation of the c-Jun N terminus. However, c-Jun is not expressed in F9 cells, which do contain ATF-2. The experiments in this study were performed to determine whether both c-Jun and ATF-2 are targets of 12S E1A. It was found that in F9 cells, the 12S E1A protein enhances the activity of the N terminus of ATF-2 fused to Gal4 about sixfold, whereas the activity of the N terminus of c-Jun fused to Gal4 was not significantly enhanced. It should be noted, however, that in experiments showing a relatively high level of induction of the N terminus of ATF-2, the N terminus of c-Jun was induced about twofold. Previous studies had already shown that activation of the N terminus of c-Jun fused to GHF1 by 12S E1A is weak in F9 cells (18). In HeLa cells, we found that 12S E1A also preferentially activates ATF-2. Thus, ATF-2 and not c-Jun is the primary target of 12S E1A in both F9 and HeLa cells.

Activation of ATF-2 via CR3 of E1A has been well established. Our data demonstrate that activation of ATF-2 can also be obtained by a CR1-dependent mechanism. Deletion of CR1 completely abolished activation by 12S E1A in F9 cells. Under our conditions, the CR3 domain does not seem to contribute significantly to activation in F9 cells, since the transactivation capacities of 12S E1A and wtE1A were approximately equal. This might be due to the presence of an E1A-like activity that has been found to be present in these cells and which has a function similar to that of 13S E1A (22). In normal, non-embryonal-carcinoma cells, 13S E1A has been shown to function as a bridging factor between transcription factors and components of the transcription-initiation complex (8, 41). In agreement with the idea of a 13S E1A-like activity acting on ATF-2 in F9 cells, we found that the full-length ATF-2 fusion protein, which is transcriptionally inactive in HeLa cells, is active in F9 cells (Fig. 2B and data not shown). Also in agreement with other studies is our finding that wtE1A is a much more potent activator than 12S E1A in HeLa and CHO cells. Deletion of CR1 in the context of the 12S E1A protein completely abolished activation of the ATF-2 N terminus in HeLa cells but only reduced activation of ATF-2 in the context of the 12S plus 13S E1A protein. Thus, CR1- and CR3-dependent effects can act independently of each other on ATF-2 and may even act synergistically. In this respect, it is interesting that synergistic cooperation between CR1 and CR3 has also been observed in transactivation of viral early genes (53).

Recent studies have shown that CR3-dependent activation of the N terminus of ATF-2 requires the presence of two threonine residues, Thr-69 and Thr-71, which are potential targets for phosphorylation by SAPKs (17, 32). Genotoxic agents, such as MMS, which activate SAPK, also induce ATF-2 via Thr-69 and Thr-71 (32, 51). In our transient-transfection assays, we found that activation of ATF-2 by MMS was strictly dependent on Thr-69 and Thr-71, whereas E1A-dependent activation via CR1 was still significant if these amino acid

residues were mutated. This suggests that different mechanisms are involved. So far, it is not clear whether the SAPKs play a role in the E1A-dependent activation of ATF-2. Our findings that Ad-transformed cell lines do not contain increased SAPK activity and that ATF-2 is not hyperphosphorylated in these cells argue against the involvement of SAPKs in activation of either ATF-2 or c-Jun. This implies that a different kinase or inhibition of a phosphatase must be responsible for hyperphosphorylation of the transactivation domain of c-Jun in these cell lines.

The CR1-dependent activation of ATF-2 may involve one or more of the cellular proteins that bind to E1A. The proteins binding to the CR1 and CR2 domains of E1A do not appear to be involved, since deletion of the CR2 domain had no effect on transactivation of ATF-2 by wtE1A (data not shown). This excludes a role in CR1-dependent activation of the direct interaction between E1A and pRb, a known activator of ATF-2 (24). This renders p300 and CBP the most likely candidates for mediating CR1-dependent activation of ATF-2, because binding of these proteins to E1A does not require CR2 but only CR1 and the N terminus. Moreover, these proteins act as coactivators and are known to bind and stimulate the activity of the transcription factors c-Jun, c-Fos, and CREB (3, 4, 7, 33, 46). One can envision a model in which 12S E1A is present in a complex with ATF-2, a p300-like coactivator, and possibly proteins of the basal transcription machinery. This is supported by the observation that 12S E1A has been found to interact with ATFa and TBP, although not as strongly as 13S E1A (9, 21). Since we found the zinc finger structure of ATF-2 to be essential for E1A-dependent activation, this postulated complex may require the intactness of the metal-binding structure. In addition to its participation in a protein complex, 12S E1A may induce or target a kinase to phosphorylate a coactivator, which may then become activated. E1A-dependent phosphorylation of p300 has been observed in two independent studies (26, 52). In fact, certain experiments suggest that differently phosphorylated forms of p300 are present in complexes that bind to the jun2 element of the c-*jun* promoter (26). This observation suggests that 12S E1A activates the c-*jun* promoter by phosphorylation of p300. We are currently investigating whether CR1-dependent activation of ATF-2 involves p300, CBP, or similar coactivators.

ACKNOWLEDGMENTS

We are grateful to Nic Jones and Peter Angel for plasmid constructs and to H. van Ormondt for help in preparing the manuscript.

This work was supported by the Netherlands Organization for Scientific Research (NWO) through the Foundation of Chemical Research in the Netherlands (SON) (M.C.A.D.) and by a fellowship of the Royal Netherlands Academy of Arts and Sciences (H.V.D.).

REFERENCES

1. **Abdel-Hafiz, H. A.-M., C.-Y. Chen, T. Marcell, D. J. Kroll, and J. P. Hoeffler.** 1993. Structural determinants outside of the leucine zipper influence the interactions of CREB and ATF-2: interaction of CREB with ATF-2 blocks E1A-ATF-2 complex formation. Oncogene **8:**1161–1174.
2. **Agoff, S. N., and B. Wu.** 1994. CBF mediates adenovirus E1A trans-activation by interaction at the C-terminal promoter targeting domain of conserved region 3. Oncogene **9:**3707–3711.
3. **Arany, Z., D. Newsome, E. Oldread, D. M. Livingston, and R. Eckner.** 1995. A family of transcriptional adapter proteins targeted by the E1A oncoprotein. Nature (London) **374:**81–84.
4. **Arias, J., A. S. Alberts, P. Brindle, F. X. Claret, T. Smeal, M. Karin, J. Feramisco, and M. Montminy.** 1994. Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. Nature (London) **370:** 226–229.
5. **Bagchi, S., P. Raychaudhuri, and J. R. Nevins.** 1990. Adenovirus can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A transactivation. Cell **62:**659–669.
6. **Bandara, L. R., and N. B. La Thangue.** 1991. Adenovirus E1A prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature (London) **351:**494–497.
7. **Bannister, A. J., and T. Kouzarides.** 1995. CBP-induced stimulation of c-Fos activity is abrogated by E1A. EMBO J. **14:**4758–4762.
8. **Berkenstam, B., M. del Mar Vivanco Ruiz, D. Barettino, M. Horikoshi, and H. G. Stunnenberg.** 1992. Cooperativity in transactivation between retinoic acid receptor and TFIID requires an activity analogous to E1A. Cell **69:**401–412.
9. **Chatton, B., J. L. Bocco, M. Gaire, C. Hauss, B. Reimund, J. Goetz, and C. Kedinger.** 1993. Transcriptional activation by the adenovirus larger E1A product is mediated by members of the cellular transcription factor ATF family which can directly associate with E1A. Mol. Cell. Biol. **13:** 561–570.
10. **Chellapan, S. P., S. Heibert, M. Mudryj, J. M. Horowitz, and J. R. Nevins.** 1991. The E2F transcription factor is a cellular target for the RB protein. Cell **65:**1053–1061.
11. **Chrivia, J. C., R. P. S. Kwok, N. Lamb, M. Hagiwara, M. R. Montmini, and R. H. Goodman.** 1993. Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature (London) **365:**855–859.
12. **Derijard, B., M. Hibi, I.-H. Wu, T. Barret, B. Su, T. Deng, M. Karin, and R. Davis.** 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell **76:**1025–1037.
13. **Devery, Y., R. A. Gottlieb, T. Smeal, and M. Karin.** 1992. The mammalian ultraviolet response is triggered by activation of src tyrosine kinases. Cell **71:**1081–1091.
14. **Eckner, R., M. E. Ewen, D. Newsome, M. Gerdes, J. A. DeCaprio, J. B. Lawrence, and D. M. Livingston.** 1994. Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kDa protein (p300) reveals a protein with properties of a transcriptional adapter. Genes Dev. **8:**869–884.
15. **Flint, K. J., and N. C. Jones.** 1991. Differential regulation of three members of the ATF/CREB family of DNA-binding proteins. Oncogene **6:**2019–2026.
16. **Graham, F. L., and A. J. van der Eb.** 1973. Transformation of rat cells by DNA of human adenovirus 5. Virology **54:**536–539.
17. **Gupta, S., D. Campbell, B. Derijard, and R. J. Davis.** 1995. Transcription factor ATF-2 regulation by the JNK signal transduction pathway. Science **267:**389–393.
18. **Hagmeijer, B. M., H. Konig, I. Herr, R. Offringa, A. Zantema, A. J. van der Eb, P. Herrlich, and P. Angel.** 1993. Adenovirus E1A negatively and positively modulates transcription of AP-1 dependent genes by dimer-specific regulation of the DNA binding and transactivation activities of Jun. EMBO J. **12:**3559–3572.
19. **Hagmeijer, B. M., and A. Zantema.** Unpublished data.
20. **Hibi, M., A. Lin, T. Smeal, A. Minden, and M. Karin.** 1993. Identification of an oncoprotein and UV responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev. **7:**2135–2148.
21. **Horikoshi, N., K. M. Maguire, A. Kralli, E. Maldonado, D. Reinberg, and R. Weinman.** 1991. Direct interaction between adenovirus E1A protein and the TATA box-binding transcription factor IID. Proc. Natl. Acad. Sci. USA **88:**5124–5128.
22. **Imperiale, M. J., H. T. Kao, L. T. Feldman, J. R. Nevins, and S. Strickland.** 1984. Common control of the heat shock gene and early adenovirus genes: evidence for a cellular E1A-like activity. Mol. Cell. Biol. **4:**867–874.
23. **Kaddurah-Daouk, R., J. W. Lillie, G. H. Daouk, M. R. Green, R. Kingston, and P. Schimmel.** 1990. Induction of a cellular enzyme for energy metabolism by transforming domains of adenovirus E1A. Mol. Cell. Biol. **10:**1476–1483.
24. **Kim, S. J., S. Wagner, F. Liu, M. A. Reilly, P. D. Robbins, and M. R. Green.** 1992. Retinoblastoma gene product activates expression of the human TGF-β2 gene through transcription factor ATF-2. Nature (London) **358:** 331–334.
25. **Kitabayashi, I., R. Chiu, G. Gachelin, and K. Yokoyama.** 1991. E1A dependent up-regulation of c-jun/AP-1 activity. Nucleic Acids Res. **19:**649–655.
26. **Kitabayashi, I., R. Eckner, Z. Arany, R. Chiu, R. Gachelin, D. Livingston, and K. Yokoyama.** 1995. Phosphorylation of the adenovirus E1A-associated 300 KDa protein in response to retinoic acid and E1A during differentiation of F9 cells. EMBO J. **14:**3496–3509.
27. **Kwok, R. P. S., J. C. C. Lundblad, J. P. Richards, H. P. Bachinger, R. G. Brennan, S. G. E. Roberts, M. R. Green, and R. H. Goodman.** 1994. Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature (London) **370:**223–226.
28. **Kyriakis, J. M., P. Banerjee, E. Nikolaki, T. Dai, E. A. Rubie, M. F. Ahmad, J. Avruch, and J. R. Woodgett.** 1994. The stress-activated protein kinase subfamily of c-Jun kinases. Nature (London) **369:**156–160.
29. **Lee, W. S., C. C. Kao, G. O. Bryant, X. Liu, and A. J. Berk.** 1991. Adenovirus E1A activation domain binds the basic repeat in the TATA box transcription factor. Cell **67:**365–376.
30. **Liu, F., and M. R. Green.** 1990. A specific member of the ATF transcription factor family can mediate transcription activation by the adenovirus E1A protein. Cell **61:**1217–1224.
31. **Liu, F., and M. R. Green.** 1994. Promoter targeting by adenovirus E1A

through interaction with different cellular DNA-binding domains. Nature (London) **368:**520–525.
32. **Livingston, C., G. Patel, and N. Jones.** 1995. ATF-2 contains a phosphorylation-dependent transcriptional activation domain. EMBO J. **14:**1785–1797.
33. **Lundblad, J. R., R. P. S. Kwok, M. E. Laurance, M. L. Harter, and R. Eckner.** 1995. Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional coactivator CBP. Nature (London) **374:**85–88.
34. **Maekawa, T., S. Matsuda, J.-I. Fujisawa, M. Yoshida, and S. Ishii.** 1991. Cyclic AMP response element-binding protein, CRE-BP1, mediates the E1A-induced but not the Tax-induced trans-activation. Oncogene **6:**627–632.
35. **Matsuda, S., T. Maekawa, and S. Ishii.** 1991. Identification of the functional domains of the transcriptional regulator CRE-BP1. J. Biol. Chem. **266:** 18188–18193.
36. **Morris, G. F., and M. B. Mathews.** 1991. The adenovirus E1A transforming protein activates the proliferating cell nuclear antigen promoter via an activating transcription factor site. J. Virol. **65:**6397–6406.
37. **Radler-Pohl, A., C. Sachsenmaier, S. Gebel, H.-P. Auer, J. T. Bruder, U. Rapp, P. Angel, H. J. Rahmsdorf, and P. Herrlich.** 1993. UV-induced activation of AP-1 involves obligatory extranuclear steps including Raf-1 kinase. EMBO J. **12:**1005–1012.
38. **Raychaudhuri, P., S. Bagchi, S. H. Devoto, V. B. Kraus, E. Moran, and J. R. Nevins.** 1991. Domains of the adenovirus E1A protein required for oncogenic activity are also required for dissociation of E2F transcription factor complexes. Genes Dev. **5:**1200–1211.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. **Schneider, J. F., F. Fisher, C. R. Goding, and N. C. Jones.** 1987. Mutational analysis of the adenovirus E1A gene: the role of transcriptional regulation in transformation. EMBO J. **6:**2053–2060.
41. **Schöler, H. R., T. Ciesolka, and P. Gruss.** 1991. A nexus between Oct4 and E1A: implications for gene regulation in embryonic stem cells. Cell **66:**291–304.
42. **Shenk, T., and J. Flint.** 1991. Transcriptional and transforming activities of the adenovirus E1A proteins. Adv. Cancer Res. **57:**47–85.
43. **Shirodkar, S., M. Ewen, J. A. DeCaprio, J. Moran, D. M. Livingston, and T. Chittenden.** 1992. The transcription factor E2F interacts with the retinoblastoma gene product and a p107-cyclin A complex in a cell cycle-regulated manner. Cell **68:**157–166.
44. **Simon, M. C., K. Kitchener, H. T. Kao, E. Hickey, L. Weber, R. Voellmy, N. Heintz, and J. R. Nevins.** 1987. Selective induction of human heat shock gene transcription by the adenovirus E1A gene products, including the 12S E1A product. Mol. Cell. Biol. **7:**2884–2890.
45. **Smith, S. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione *S*-transferase. Gene **67:**31–40.
46. **Smits, P. H. M.** 1995. Unpublished data.
47. **van Dam, H.** 1994. Unpublished data.
48. **van Dam, H., M. C. A. Duyndam, R. Rottier, A. Bosch, L. de Vries-Smits, P. Herrlich, A. Zantema, P. Angel, and A. J. van der Eb.** 1993. Heterodimer formation of cJun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus E1A protein. EMBO J. **12:**479–487.
49. **van Dam, H., R. Offringa, I. Meijer, B. Stein, A. M. Smits, P. Herrlich, J. L. Bos, and A. J. van der Eb.** 1990. Differential effects of the adenovirus E1A oncogene on members of the AP-1 transcription factor family. Mol. Cell. Biol. **10:**5857–5864.
50. **van Dam, H., and A. J. van der Eb.** 1994. Regulation of AP-1 activity by adenovirus E1A: dissection of dimers containing Jun, Fos and ATF/CREB members, p. 261–273. *In* P. E. Angel and P. A. Herrlich (ed.), The Fos and Jun families of transcription factors. CRC Press, Boca Raton, Fla.
51. **van Dam, H., D. Wilhelm, I. Herr, A. Steffen, P. Herrlich, and P. Angel.** ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-*jun* induction in response to genotoxic agents. EMBO J. **14:**1798–1811.
52. **Wang, H.-G. H., Y. Rikitake, M. C. Carter, P. Yaciuk, S. E. Abraham, B. Zerler, and E. Moran.** 1993. Identification of specific adenovirus E1A N-terminal residues critical to the binding of cellular proteins and to the control of cell growth. J. Virol. **67:**476–488.
53. **Wong, K. W., and E. B. Ziff.** 1994. Complementary functions of E1A conserved region 1 cooperate with conserved region 3 to activate adenovirus serotype 5 early promoters. J. Virol. **68:**4910–4920.
54. **Wu, L., D. S. E. Rosser, M. C. Schmidt, and A. J. Berk.** 1987. A TATA box implicated in E1A transcriptional activation of a simple adenovirus 2 promoter. Nature (London) **326:**512–515.