The E1A N terminus (aa 1–29) of the highly oncogenic adenovirus type 12 harbours a trans-activation function not detectable in the non-oncogenic serotype 2

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Early region 1A (E1A) of adenoviruses (Ad) codes for potent activator and repressor molecules which are involved in the regulation of viral and cellular gene expression. Gene regulatory functions of E1A proteins are mainly located in their conserved regions (CR) 1 to 3. In addition to the CRs, specific amino acids (aa) of the N-terminal end play an important role in some gene regulatory functions. We describe here the identification and characterization of a novel trans-activation domain which is located in the non-conserved N-terminal end of Ad12 E1A, namely aa 1–29. Fusion of this region to the DNA-binding domain of the yeast transcription factor Gal4 generates a strong trans-activator which induces gene expression of reporter constructs in dependence on Gal4 DNA-binding sites. Furthermore, transient expression assays using the physiological E1A-responsive adenoviral E2 early promoter revealed that the N terminus is involved in its activation. The gene regulatory function of the N terminus is specific for E1A proteins of the highly oncogenic serotype Ad12, as the respective E1A N terminus of the non-oncogenic serotype Ad2 is unable to activate the expression of the reporter gene as Gal4 fusion protein. Moreover, deletion mutant analyses demonstrate that Ad12 E1A proteins carry three independently acting activation domains: (1) aa 1–29, (2) CR1 and (3) CR3.

Introduction

Protein products of the early transcription unit 1A (E1A) play an important role in the adenovirus (Ad) life cycle. They are necessary for the expression of all other adenovirus genes (Berk et al., 1979; Jones & Shenk, 1979; Nevins, 1981). In addition, they regulate the expression of specific cellular genes (reviewed in Rochette-Egly et al., 1990; Shenk & Flint, 1991). If expressed in primary rodent cells, E1A proteins are able to immortalize (Houweling et al., 1980; Ruley, 1983; Zerler et al., 1986) and, in combination with gene products of region E1B, are able to fully transform these cells (Jochensen et al., 1982; Byrd et al., 1988). Depending on the virus serotype used for transformation, the transformed cells either grow into tumours in immunocompetent, syngeneic rodents (e.g. serotype Ad12) or do not produce tumours (e.g. serotype Ad2) (reviewed in Williams et al., 1995). Genetic analyses have shown that the degree of oncogenicity is determined by the origin of the E1A gene (reviewed in Williams et al., 1995).

Region E1A of the highly oncogenic serotype 12 gives rise to six mRNAs (13S, 12S, 11S, 10S, 9–5S, 9S), from which five proteins are translated (266R, 235R, 106R, 52R, 53R; reviewed in Brockmann & Esche, 1995). The 266R protein contains three regions (CR1, CR2, CR3) which are highly conserved among different Ad serotypes. The 235R protein contains three regions (CR1, CR2, CR3) which are highly conserved among different Ad serotypes. The 235R protein is translated in the same reading frame as the 266R protein. It differs only in the lack of an internal stretch of 31 amino acids (aa) located in conserved region 3 (CR3) whose coding region is removed by splicing. The 106R, 52R and 53R proteins have the first 29 aa in common with both larger proteins. However, due to frame shifts caused by splicing events, they contain unique C-terminal ends. These three proteins do not carry any of the conserved regions (CR).

As E1A proteins do not bind sequence-specifically to DNA (Ferguson et al., 1985), they have to interact with cellular transcription factors to fulfil their gene regulatory functions. Domains which are responsible for most of these interactions are located in the CRs. For instance CR3, which is unique for the 266R protein, can serve as a molecular bridge between
molecule, indicating that the Ad12 E1A N terminus functions as an activator backbone (lacking CR1 and CR3) generates an activator 29 aa of Ad12 E1A in an Ad2 E1A

In addition to the CRs, the N terminus plays a crucial role in the gene regulatory functions of the Ad E1A proteins. Together with CR1, the N terminus binds to the p300 protein (Egan et al., 1988; Wang et al., 1993a). p300 is thought to participate in preventing the G0/G1 transition in the cell cycle, activate certain enhancers as co-factors, and stimulate specific differentiation pathways (Moran, 1993). Targeting of p300 (and of the highly related CREB-binding protein CBP) by the E1A proteins translated from the 13S and 12S mRNAs (Wang et al., 1993a, b) leads to the repression of its co-activator function, most probably by dissociating it from promoter-bound factors (Arany et al., 1995; Lundblad et al., 1995). Furthermore, the E1A N terminus binds to Dr1 (Byers Kraus et al., 1994), which is an inhibitor of the TATA-box-binding protein (Inostroza et al., 1992), and also mediates the association of the protein translated from the 12S mRNA with the transcription factor YY1 (Lewis et al., 1995). Disruption of the Dr1/TBP complex by E1A allows TBP to recruit the general transcription factor TFIIA and to form an active transcription initiation complex (Byers Kraus et al., 1994). In the case of YY1, the E1A N terminus dissociates an ATF/CREB-YY1 complex, thereby reversing YY1-mediated transcriptional repression of c-fos gene expression resulting in gene activation (Zhou & Engel, 1995). The functional importance of the E1A N terminus is further underlined by findings of Miller et al. (1995), who have shown that the N terminus of Ad5 E1A proteins (in combination with CR1) strongly inhibits yeast cell growth, arresting the cells in the G1 phase of the cell cycle.

The data described above were obtained by analysing the E1A N-terminal domain of the non-oncogenic serotypes 2 and 5, a region which is not conserved (except the arginine residue at aa position 2) among different Ad serotypes (Wang et al., 1993a). As the degree of Ad tumorigenicity is determined by the origin of the E1A region, such non-conserved regions might harbour distinct oncogenic determinants. Therefore, we have studied the transcriptional activity of the N terminus of Ad12 E1A proteins in transient expression assays and compared it with that of non-oncogenic Ad2 E1A. We have found that Ad12 E1A contains an independently acting trans-activation domain located between aa 1 and aa 29 which is involved in the activation of the adenoviral E2 early promoter. In contrast to Ad12 E1A, the E1A proteins of non-oncogenic Ad2 do not carry an activation domain in their N termini. Construction of a chimeric E1A protein consisting of the first 29 aa of Ad12 E1A in an Ad2 E1A trans-activation-deficient backbone (lacking CR1 and CR3) generates an activator molecule, indicating that the Ad12 E1A N terminus functions as an independently acting trans-activation module. Target-
gene activation mediated by the Ad12 E1A N terminus is, most probably, not dependent on a direct physical interaction with p300, as the arginine residue at aa position 2 and CR1, which are both essential for a physical association of E1A with p300 (Egan et al., 1988; Wang et al., 1993a), are not necessary for transcriptional activation.

**Methods**

- **Plasmids.** The cDNAs for the Ad12 E1A 266R and 235R wild-type proteins (Brockmann et al., 1990), as well as those for the 289R and 243R proteins of Ad2 E1A (a gift from W. Doerfler, Institute of Genetics, University of Cologne, Cologne, FRG) were cloned into the eukaryotic expression vector pBluescript (a gift from J. C. Doggett). Targeting of p300 was achieved by targeting the non-conserved regions of the Ad12 E1A proteins. The reporter construct G5–E1BTATA–CAT contains five Gal4 DNA-binding sites in front of the TATA-box. This reporter, as well as E1B19K–CAT, has been described previously (Lillie & Green, 1989). The selection of the E2 promoter (nt +40 to −108; Swamishathan & Thimmappaya, 1995) of Ad2, was kindly provided by C. Svensson, Department of Medical Immunology & Microbiology, Uppsala University, Sweden.

The correct reading frame of the fusion genes and mutants was confirmed by sequencing, using an automated laser fluorescent DNA sequencer (Pharmacia).

- **Cell culture, CAT assays and Western blot analysis.** HeLa-tk− cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum. For transient expression assays, 3.5 × 10^5 HeLa-tk− cells were seeded. After 24 h, cells were co-transfected by the lipofectamine method (Felgner & Ringold, 1989) with 0.2 μg of the reporter plasmid and equimolar amounts of the respective expression constructs. At 40 h after
transfection, whole cell extracts were prepared by the freeze and thaw procedure. The protein concentration of the extracts was determined using the Bradford method (Bradford, 1976). Equal amounts of protein were used to determine CAT enzyme activity as described previously (Gorman et al., 1982). CAT activity was quantified using an automatic TLC-linear analyser (Berthold, Bad Wildbad, FRG).

For Western blot analysis, 1×10⁶ HeLa-tk⁻ cells were seeded on 8.5 cm diameter tissue culture plates. After 24 h, cells were transfected by the lipofectamine method (Felgner & Ringold, 1989). Transfected cells were maintained for 24 h at 37 °C. Afterwards, cells were harvested, washed with PBS and lysed in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 2.0 μg/ml aprotinin and 2.0 μg/ml leupeptin in PBS) for 30 min on ice. Lysates were loaded on a Qiagen Shredder homogenizer column and the flow-through was used as crude cellular extract for Western blot analysis.

Samples of crude cellular extracts (100 μg) were separated by SDS–PAGE and blotted (Harlow & Lane, 1988) onto Hybond-C extra nitrocellulose membranes (Amersham). Detection of Gal4 or of the Gal4–E1A fusion proteins was performed using a polyclonal rabbit anti-Gal4 antibody raised against the DNA-binding domain of the yeast transcription factor (SC-577, Santa Cruz Biotechnology) as described by the manufacturer, or a polyclonal sheep anti-Ad12 E1A antiserum (a gift from P. H. Gallimore, CRC Institute for Cancer Studies, Edgbaston, Birmingham, UK). Enhanced chemiluminescence was carried out using the ECL Western blotting detection system (Amersham) as described by the manufacturer.

Results

Identification of a novel Ad12 E1A-specific trans-activation domain

In order to analyse functions of the non-conserved N-terminal end of Ad12 E1A proteins (aa 1–29), we constructed a Gal4 DNA-binding domain (Gal4) Ad12 E1A N terminus fusion protein (Gal4–N12) which is intended to be used in the yeast ‘two-hybrid’ system (Chevray & Nathans, 1992) to screen for interacting cellular factors. Surprisingly, Gal4–N12 induced lacZ gene expression in the absence of the Gal4 trans-activation domain fused to a hypothetical interacting partner (data not shown), indicating that this Ad12 E1A region harbours a trans-activation domain which is functional in yeast cells. To reproduce these results in a natural host cell system, we performed transient expression assays in HeLa-tk⁻ cells. Selection of Gal4 or of the Gal4–E1A fusion proteins was performed using a eukaryotic expression plasmid coding for Gal4–N12 (pJ3–N12) and a reporter construct containing five Gal4 DNA-binding sites in front of the Ad E1B TATA-box (G5–E1BTATA–CAT; Fig. 1a). Co-transfecting both plasmids in HeLa-tk⁻ cells results in a strong activation of CAT gene expression (260-fold; Fig. 1a). Similar results were obtained using a reporter construct carrying only one Gal4 recognition sequence (G1–E1BTATA–CAT). However, the activation in these experiments was considerably lower (data not shown).

Two sets of control experiments were performed to confirm that the trans-activation we measured was dependent on the Ad12 E1A N terminus and the Gal4 DNA-binding sites in the reporter construct. First, we co-transfected pJ3, which expresses the Gal4 DNA-binding domain but lacks the coding region for N12, with G5–E1BTATA–CAT in HeLa-tk⁻ cells.

As shown in Fig. 1(a), Gal4 failed to induce CAT gene expression. For the second control experiment, we co-transfected the basal CAT construct E1BTATA–CAT, which
lacks Gal4 DNA-binding sites, with p13–N12. Again, no induction of CAT gene expression was observed (Fig. 1b). Taken together, these data demonstrate that the activation of G5–E1BTATA–CAT is dependent on the Ad12 E1A N terminus as well as on the Gal4 recognition sequences of the reporter construct.

Because the E1A N termini are not conserved among different serotypes (except the R residue at aa position 2; Fig. 1c), it was interesting to investigate whether the respective region on non-ontogenic Ad2 E1A proteins carries an Ad12–E1A-comparable trans-activation function. We therefore transfected a Gal4 fusion construct expressing the N terminus of Ad2 E1A (aa 1–29) and the Gal4 DNA-binding domain (Gal4–N2) with G5–E1BTATA–CAT into HeLa-tk− cells. In contrast to Gal4–N12, Gal4–N2 had no trans-activation potential in these transient expression assays (Fig. 1a).

To make sure that the different trans-activation potentials we observed were not due to insufficient expression of Gal4–N2, we checked its concentration in transfected HeLa-tk− cells by Western blot analysis using an anti-Gal4 polyclonal antibody. These experiments revealed that Gal4–N2 and Gal4–N12 were expressed at comparable levels (see Fig. 5a, compare lane 3 with lane 4).

**Distinct differences in the trans-activation potentials of the Ad12 E1A 235R and Ad2 E1A 243R proteins**

In order to determine the transcriptional potentials of the E1A N termini in their natural contexts in full-length proteins, we constructed expression vectors coding for fusion proteins consisting of the Gal4 DNA-binding domain and the 13S- or 12S-derived Ad12 E1A (Gal4–266R, Gal4–235R) or Ad2 E1A (Gal4–289R, Gal4–243R) proteins. Both Gal4–266R and Gal4–289R were strong activators of CAT gene expression if co-transfected with G5–E1BTATA–CAT into HeLa-tk− cells (Gal4–266R, 67±3-fold; Gal4–289R, 42±3-fold: Fig. 2). This result is in contrast to data obtained by Liu & Green (1994), showing that the 289R protein of Ad2 does not activate CAT gene expression as Gal4 fusion protein from a reporter construct containing Gal4 DNA-binding sites in CHO cells. The reason for this discrepancy is not yet clear, but might be due to a cell-type-specific co-factor not present in CHO cells. However, the high activation factor we obtained in the transient expression assays might predominantly reflect the transcriptional activity of CR3, which was shown to trans-activate reporter gene expression as Gal4 fusion protein (Liu & Green, 1994).

Next we compared the trans-activation potentials of the 12S-generated proteins of both serotypes. In contrast to Gal4–235R (derived from Ad12 E1A), which was also a strong inducer of CAT gene expression (31±2-fold, Fig. 2), Gal4–243R (derived from Ad2 E1A) shows only a very low trans-activating potential (4±2-fold; Fig. 2). This difference was not due to a lower protein concentration of Gal4–243R in the transfected cells, as both fusion proteins were expressed at similar levels (compare Fig. 5a, lane 12 with Fig. 5b, lane 13).

The findings demonstrating that Gal4–N12, but neither Gal4–N2 nor Gal4–243R, functions as a strong activator led us to assume that aa 1–29 might be responsible for the trans-activating activity of the 235R protein. To confirm this hypothesis, we constructed the deletion mutant Gal4–ΔN/235R, which lacks the coding region for aa 1–29 (Fig. 3). To our surprise, deletion of the N terminus does not result in a decrease of the trans-activation potential of the 235R mutant compared to the 235R wild-type protein (Fig. 3). This result suggests that the 235R protein contains a second independently acting activation domain. To address this question, several deletion mutants were constructed and analysed in transient expression assays. Removal of aa 112–134 (Gal4–CS1/235R; this deletion corresponds to the main part of CR2 and the N-terminal part of the spacer region unique for Ad12 E1A) does not abolish the trans-activation capacity of the mutant protein (Fig. 3). This result is in agreement with data obtained using the mutant Gal4–CR2, in which the coding region for aa 106–123 of Ad12 E1A (spanning CR2) was fused to the Gal4 DNA-binding region. The resulting fusion protein showed no trans-activation potential (Fig. 3). Next, we deleted the coding region for CR1 (aa 39–79; Gal4–ΔCR1/235R) or the coding region for the N-terminal end plus CR1 (aa 1–79; Gal4–Δ11–79/235R). Whereas Gal4–ΔCR1/235R is a potent trans-activator, Gal4–Δ1–79/235R showed no trans-activation potential (Fig. 3) suggesting that aa 1–29 are responsible for the trans-activation properties of Gal4–ΔCR1/235R, and that CR1 might be the second activation domain. To confirm this assumption, we cloned the mutant Gal4–CR1 coding for a fusion protein consisting of the Gal4 DNA-binding domain and CR1 (aa 39–79) of Ad12 E1A. As expected, this mutant induces CAT gene expression from G5–E1BTATA–CAT very efficiently (Fig. 3). This result is in agreement with data published by Bondessen et al. (1994), demonstrating that CR1...
Trans-activation domains of Ad E1A proteins

Fig. 3. Trans-activation domains of the Ad12 E1A 235R protein are located in aa 1–29 and CR1. HeLa-tk− cells were cotransfected with the reporter plasmid G5–E1BTATA–CAT and expression plasmids coding either for the 235R full-length protein (Ad12 E1A), the 243R full-length protein (Ad2 E1A) or for Ad E1A mutants fused to Gal4, as indicated. Gal4–RG2 contains an amino acid exchange (R→G) at aa position 2 in Ad12 E1A. The hybrid Gal4–N12–C2 consists of the N terminus of Ad12 E1A and the C terminus of Ad2 E1A (243R); the hybrid Gal4–N2–C12 consists of the N terminus of Ad2 E1A and the C terminus of Ad12 E1A (235R); Gal4–∆1–79/235R lacks aa 1–79 of Ad2 E1A spanning the N terminus and CR1. CAT activity was determined 40 h after transfection. The activation factors represent the average of at least four independent experiments, one of which is shown. N, E1A N terminus (aa 1–29); CR, conserved region. Activation by Gal4 was set as 1. SD, standard deviation.

Interestingly, Gal4–N12, Gal4–CR1, the deletion mutant Gal4–∆N/235R lacking the N terminus and Gal4–∆CR1/235R lacking CR1 are stronger activators than the 235R wild-type protein, in which both trans-activation domains are present (Fig. 3). This might be due to a structural competition between two adjacent activation domains. In addition, both E1A domains are complexed by several cellular proteins if present in the same protein (e.g. p300; Yee & Branton, 1985; Harlow et al., 1986; Wang et al., 1993a). Such interactions might partially mask both activation domains in the 235R protein, resulting in a lower activation potential of the full-length protein compared to the mutants which carry only one of the respective domains and are, therefore, unable to bind these cellular factors. This hypothesis is supported by our observation that a mutant consisting of the N terminus and CR1 (Gal4 aa 1–79) shows a 6- to 7-fold lower trans-activation potential than either Gal4–N12 or Gal4–CR1 (data not shown).

One possibility of how the promoter-bound E1A N terminus might activate target-gene expression is by recruiting the cellular co-factor p300. Although CR1 is lacking in Gal4–N12, which should in principle prevent the formation of a complex between both proteins, we could not completely

Fig. 4. Amino acids 1–29 of Ad12 E1A are involved in the activation of the adenovirus E2 early promoter. (a) In the reporter construct E2–CAT, the expression of the CAT gene is driven by the adenoviral E2 early promoter (nt +40 to −108; Swaminathan & Thimmappaya, 1995). The binding sites for various cellular transcription factors are indicated. (b) HeLa-tk− cells were cotransfected with the reporter plasmid E2–CAT and expression plasmids coding for Gal4 fusion proteins carrying either the 235R Ad12 E1A protein or Ad12 E1A mutants as indicated. Gal4–∆N/235R lacks the Ad12 E1A N terminus (aa 1–29); Gal4–∆CR1/235R lacks CR1; and Gal4–∆1–79/235R lacks the N terminus as well as CR1. CAT activity was determined 40 h after transfection. The relative CAT activity represents the average of three independent experiments, one of which is shown. Activation by Gal4 was set as 1. SD, standard deviation.
Fig. 5. Western blot analysis of whole cell extracts prepared from Gal4+ or Gal4–E1A-transfected HeLa-tk− cells. Gal4–266R (lane 17) and Gal4–235 (lane 13) are full-length Ad12 E1A fusion proteins; Gal4–289R (lane 11) and Gal4–243R (lane 12) are full-length Ad2 E1A fusion proteins. Gal4 (lane 2) corresponds to the Gal4 DNA binding-domain; Gal4–N2 (lane 3) contains aa 1–29 of Ad2 E1A; Gal4–N12 (lane 4) contains aa 1–29 of Ad12 E1A. Gal4–CR1 (lane 5), Gal4–CR2 (lane 6), Gal4–RG2 (lane 7), Gal4–ΔN/235R (lane 14), Gal4–Δ1–79/235R (lane 15), Gal4–ΔCR1/235R (lane 16) and Gal4–CS1/235R (lane 18) are mutant proteins derived from Ad12 E1A; Gal4–Δ1–80/243R (lane 9) is a mutant protein derived from Ad2 E1A. The chimeric E1A proteins Gal4–N12–C2 (lane 8) and Gal4–N2–C12 (lane 10) consist of the N terminus (aa 1–29) of Ad12 E1A and the C-terminal end of Ad2 (aa 81–243; Gal4–N12–C2) or vice versa. For Western blots, 100 µg of crude cellular extracts were separated by SDS–PAGE and blotted onto Hybond-C extra nitrocellulose membranes. Detection of Gal4 or the Gal4–E1A fusion proteins was performed using a polyclonal rabbit anti-Gal4 antibody raised against the DNA-binding domain of the yeast transcription factor (a) or using a polyclonal sheep anti-Ad12 E1A antiserum (b). Antigen–antibody complexes were detected using the ECL Western blotting detection system. The arrowheads indicate the positions of Gal4 or the respective Gal4–E1A fusion proteins. Size markers are indicated.

rule out the possibility of a p300/Gal4–N12 interaction in our assays. Therefore, we constructed the point mutant Gal4–RG2, in which the R residue at position aa 2, which was shown to be essential for a physical interaction (Wang et al., 1993a, b), is substituted for a G residue. Transient expression assays revealed that Gal4–RG2 was as efficient at activating transcription from G5–E1BTATA–CAT as Gal4–N12 (189-fold; Fig. 3), suggesting that this trans-activation function of the Ad12 E1A N terminus is independent of a direct physical interaction with p300.

Finally, we wanted to confirm that aa 1–29 of Ad12 E1A behave like an independently acting protein domain, i.e. one which is able to induce a trans-activation potential if it is transferred into an amino acid backbone possessing no intrinsic activation capacity. We therefore constructed two chimeric mutants: (1) Gal4–N2–C12, expressing aa 1–29 of Ad2 E1A and aa 80–235 of Ad12 E1A, and (2) Gal4–N12–C2, expressing aa 1–29 of Ad12 E1A and aa 81–243 of Ad2 E1A. In comparison to Gal4–235R or Gal4–ΔCR1/235R, cotransfection of Gal4–N2–C12 with G5–E1BTATA–CAT into HeLa-tk− cells resulted in a total loss of CAT activity (Fig. 3). Transferring aa 1–29 of Ad12 E1A into the trans-activation-deficient Ad2 E1A backbone (Gal4–N12–C2) generates a trans-activator, even if its trans-activation potential is less than that observed using the Ad12 E1A N terminus in its natural context (Fig. 3). The relatively low trans-activation potential of Gal4–N12–C2 might be due to a suboptimal conformation of the chimeric protein. Both Gal4–N12–C2 and Gal4–N2–C12 were expressed at similar levels (see Fig. 5a, lanes 8 and 10).

In all cases in which induction of CAT gene expression through Ad12 E1A fusion mutants was observed, trans-activation was dependent on the Gal4 DNA-binding sites of
Amino acids 1–29 of Ad12 E1A are involved in the activation of the adeno viral E2 early promoter

To analyse whether aa 1–29 participate in the Ad12 E1A-mediated trans-activation of a physiological E1A-responsive promoter, we performed transient expression assays using a Gal4-independent reporter construct whose expression is driven by the adeno viral E2 early promoter (E2–CAT). The E2 early promoter contains two binding sites for the cellular transcription factor E2F and one ATF recognition sequence (Fig. 4a; reviewed in Swaminathan & Thimmapaya, 1995). All three cis-acting elements are critical for basal as well as E1A-mediated trans-activation. In addition, the TATA-box-like sequence of the major promoter [TBP(+1), Fig. 4a] is also essential for basal and E1A-dependent activation of the E2 early promoter. Both adenoviral E1A proteins, the 235R protein translated from the 125 mRNA, and the 266R protein translated from the 135 mRNA contribute to the stimulation of E2 early promoter activity (reviewed in Swaminathan & Thimmapaya, 1995). However, the 235R protein was chosen for our transient expression assays to be independent of the strong activation domain located in CR3 of the 266R protein (see Fig. 2).

As expected, co-transfection of E2–CAT with Gal4–235R into HeLa-tk− cells results in a 8-2-fold activation of CAT gene expression (Fig. 4b, compare lanes Gal4 and Gal4–235R). Removal of aa 1–29 from the 235R protein has only a slight effect on the induction of CAT gene expression from E2–CAT (Fig. 4b, lane Gal4–AN/235R), whereas deletion of CR1 leads to a 50% loss of the trans-activating activity of the mutant E1A protein (Fig. 4b, lane Gal4–ACR1/235R). However, if the N terminus and CR1 are deleted, the resulting 235R mutant protein is unable to activate CAT gene expression from E2–CAT (Fig. 4b, lane Gal4–ΔA1–79/235R), indicating that the trans-activation potential of Gal4–ΔCR1/235R is dependent on the N-terminal domain. On the other hand, neither Gal4–N12 nor Gal4–CR1 induces CAT gene expression from E2–CAT (data not shown), indicating that both E1A domains alone were unable to activate expression from this target promoter. Taken together, these data suggest that the N terminus (aa 1–29) of Ad12 E1A is involved in the activation of the E2 early promoter in transient expression assays.

Discussion

The 266R protein translated from the 135 mRNA of Ad12 E1A carries three trans-activation domains: CR3, CR1 and the N-terminal domain (aa 1–29); the latter is described for the first time in this paper. It is important to note that the trans-activating activity of all three domains is dependent on promoter-targeting mediated through the Gal4 DNA-binding domain in the E1A fusion proteins. No induction of CAT gene expression was obtained by co-transfecting the basal reporter construct E1B TATA–CAT with Gal4–266R or Gal4–235R (data not shown). This result is in contrast to data published by Wu et al. (1987) and Simon et al. (1988), who have shown that E1A of Ad2 and Ad5 can activate transcription from the adeno virus E1B TATA-box. The reasons for this discrepancy are presently unknown. However, other researchers were also unable to detect a trans-activation from E1B TATA–CAT using E1A wild-type proteins (Bondesson et al., 1994).

The mechanism by which the Ad12 E1A N terminus activates gene expression is not yet clear. The most simple explanation for our observation is that aa 1–29 contain an intrinsic activation domain which is targeted to the promoter of a responsive gene. Several different types of activation domains have been identified, and classified as glutamine-rich, proline-rich or acidic (Tjian & Maniatis, 1994). The N terminus has a net negative charge of six (under physiological conditions, taking into account the basic amino acids K, R and the acidic amino acids D, E; Fig. 1c), which might argue for an acidic activation domain. When recruited to the promoter region of a target gene, the acidic activation domain might then bind either to a co-factor like p300 or CBP, or directly to members of the basal transcription machinery to induce gene expression. Some of the Gal4–E1A mutant fusion proteins we have used in transient expression assays suggest that p300 might not be involved in trans-activation by the Ad12 E1A N terminus. Mutants lacking the binding sites for p300 (Gal4–RG2 and Gal4–ΔCR1/235R) are still capable of activating reporter gene expression. On the other hand, it is well known that distinct classes of activators bind to specific members of the basal transcription machinery like TBP, TBP-associated factors (TAFs) or general transcription factors (GTFs). Such specific interactions are required for transcriptional activation of target-gene expression through upstream binding factors (Chen et al., 1994), indicating that the N terminus of Ad12 E1A might induce transcriptional activation through this pathway. We are currently searching for cellular factors interacting specifically with the N terminus of Ad12 E1A in order to help us understand the mechanism of transcriptional activation.

Ma & Ptashne (1987) have shown that 1% of random E. coli genomic DNA fragments fused to the Gal4 DNA-binding domain were transcriptional activators in yeast. All of the activating sequences encoded by the E. coli DNA fragments have a net negative charge of at least one, although there was no strict correlation between net negative charge and activating capacity. From these studies, one might draw the conclusion that some acidic polypeptides might function as activators without physiological relevance. Interestingly, the N terminus of Ad2 E1A also has a negative charge (−4; Fig. 1c), but does not activate reporter gene expression in our
transient expression assays, suggesting that the respective region of Ad12 is not transcriptionally active solely because of its negative charge, and that other amino acids participate in the trans-activation function as well. Mutational analyses are in progress to define those Ad12 E1A amino acids.

The N-terminal trans-activation function is unique for E1A of the highly oncogenic serotype Ad12. Although its role in the productive infection is presently unknown, our data suggest that the N terminus is involved in the activation of the adenoviral E2 early promoter. In addition, our finding is also very interesting with respect to the observation that the degree of oncogenicity of adenoviruses is determined by the origin of the E1A gene products (for a review see Williams et al., 1995). Moreover, data of Jelinek et al. (1994) and Pereira et al. (1995) suggest that a function(s) located somewhere between aa 1–80 supports the process of tumorigenic transformation. Transformation assays using our chimeric E1A genes reintroduced into the natural adenovirus context will help us to understand to what extent the newly identified trans-activation domain is involved in the tumorigenic transformation of primary cells by Ad12.

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