Requirement of the adenovirus E1A transformation domain 1 for inhibition of PC12 cell neuronal differentiation

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Expression of the adenovirus early gene E1A inhibits the nerve growth factor (NGF)-induced differentiation of PC12 pheochromocytoma cells. Expression of the 12S form of E1A, which lacks the transcription activation region, also inhibited PC12 cell differentiation in a manner similar to the wild-type gene. Three cellular proteins—the retinoblastoma susceptibility gene product referred to as 105(Rb)-, 107-, and 300-kDa proteins—stably interacted with the different E1A polypeptides. Analysis of the association of these cellular proteins with mutant E1A polypeptides demonstrated that a functional domain 1, which is minimally involved in the association of the 300-kDa protein with E1A, was sufficient to inhibit neuronal differentiation. Deletion of transformation domain 2, which encodes sequences necessary for the binding of the 105(Rb)- and 107-kDa proteins, did not influence the ability of the mutant E1A polypeptide to inhibit PC12 cell differentiation. E1A was also shown to alter the expression of mRNAs for the early response genes *c-fos*, *c-myc*, *egr-1*, and *c-jun* and their regulation in response to NGF. In clones expressing either 12S or 13S E1A, NGF stimulation of *c-fos* and *c-myc* was repressed. In contrast, basal mRNA levels for *c-jun* and *egr-1* were constitutively elevated and not significantly affected further by challenge with NGF. Simply expressing *c-jun* by gene transfer, however, did not mimic the action of E1A because constitutively expressing *c-jun* clones differentiated in response to NGF. Thus, expression of the E1A polypeptide disrupts NGF

control of early transcription events that have been shown to be critical for PC12 cell neuronal differentiation.

Introduction

PC12 pheochromocytoma cells respond to nerve growth factor (NGF) with the onset of a neuronal differentiation program involving the acquisition of a sympathetic neuron-like phenotype (Greene and Tischler, 1976). Binding of NGF to its receptor results in the activation of multiple protein kinase pathways (Heasley and Johnson, 1989a,b), which converge in the nucleus to alter the control of gene transcription and cell growth leading to the differentiated phenotype (Cho *et al.*, 1989; Herschmann, 1989). Included in the events rapidly stimulated by NGF in PC12 cells is the increased transcription and expression of several early response genes, including *c-fos*, *c-myc*, *egr-1*, and *c-jun* (Greenberg *et al.*, 1985; Ito *et al.*, 1989; Wu *et al.*, 1989).

Previously, it was shown that the constitutive expression of either *c-myc* or *c-fos* using gene transfection inhibited NGF-induced PC12 cell differentiation (Maruyama *et al.*, 1987; Ito *et al.*, 1989), apparently because of repression of transcriptional events required for initiation of the neuronal differentiation program. During the course of these studies, we also found that the adenovirus E1A protein, which, like *c-myc*, can immortalize primary cells (Ruley *et al.*, 1984), also inhibited PC12 cell differentiation in response to NGF. The E1A protein has the ability to regulate transcription from a wide variety of viral and eukaryotic promoters (Berk, 1986; Bagchi *et al.*, 1990), and it interacts with nuclear proteins involved in regulation of the cell cycle (Cooper and Whyte, 1989; Whyte *et al.*, 1989). Detailed deletion and point mutation analysis of the E1A protein has clearly discriminated the transcription activation region from the cell cycle regulatory domains (Moran *et al.*, 1986; Moran and Zerler, 1988). The cell cycle control

domains encoded within the 12S form of E1A have been shown to bind up to 10 different proteins of 28, 40, 50, 60, 80, 90, 105(Rb), 107, 130, and 300 kDa (Harlow *et al.*, 1986). Of these, the 105(Rb)-, 107-, and 300-kDa proteins are the most prominent cellular proteins that interact with E1A. It has also recently been shown that the regions of E1A involved in binding the 105(Rb)-, 107-, and 300-kDa cellular proteins are the same regions required for cell transformation (Whyte *et al.*, 1989). In this report, we show that an E1A mutant that binds the 300-kDa protein, but lacks the ability to bind the 105(Rb)- and 107-kDa proteins, is able to inhibit PC12 cell neuronal differentiation.

Results

Influence of the adenovirus E1A proteins on PC12 cell phenotype

The adenovirus E1A gene products inhibit NGF-induced differentiation of PC12 pheochromocytoma cells. Figure 1 shows the neurite extension response elicited by NGF plus forskolin in PC12 cell clones expressing the 12S or 13S forms of E1A. Control cells transfected with the expression plasmid lacking E1A sequences (neo-3) have a rounded, loosely adherent morphology. In regular growth medium, the E1A-expressing cells exhibit a flattened morphology and tightly adhere to the substratum. On incubation in the presence of NGF plus forskolin for 4 d, the control neo-3 clone extends neurites that are several times the length of the PC12 cell body. PC12 cell clones transfected with the E1A gene, which encodes both the 12S and 13S E1A forms (E1A-11), or with the E1A 12S (E1A12S-11) or 13S (E1A13S-9) cDNAs fail to extend neurites in response to NGF plus forskolin. The morphological phenotype is similar for PC12 clones transfected with the E1A gene or 12S and 13S E1A cDNAs. The difference between 12S and 13S E1A gene products is the absence of the transcription activation region in the 12S form (Berk, 1986; Moran *et al.*, 1986). Thus, the transcription activation region of E1A is not required for inhibition of PC12 cell differentiation, suggesting that the E1A regions required for transformation and immortalization are responsible for inhibition of neurite extension in response to NGF plus forskolin.

Immunoprecipitation analysis of wild-type and deletion mutants of E1A and associated cellular proteins

Domains involved in transformation functions of the E1A polypeptide have been previously

defined by deletion mapping. Within the E1A polypeptide, amino acids 1–76 (domain 1) and 121–127 (domain 2) are required for transformation (Moran *et al.*, 1986; Moran and Zerler, 1988; Whyte *et al.*, 1989). The transformation regions encoded in the E1A polypeptide have also been shown to be required for stable interaction with the 105(Rb)-, 107-, and 300-kDa proteins (Whyte *et al.*, 1989). The sites required for stable 300-kDa protein association with E1A map to amino acids 1–76 of domain 1. The site required for stable interaction with the 107-kDa protein resides within E1A residues 121–127 within domain 2. Stable interaction of the 105(Rb) protein with E1A requires amino acid residues 30–60 in domain 1 and 121–127 of domain 2.

We have expressed three previously described (Moran and Zerler, 1988) E1A deletion mutants in PC12 cells that disrupt the sequences in domains 1 and 2 that affect the binding of the 105(Rb)-, 107-, and 300-kDa proteins (Figure 2C). The deletion mutants PSdl and CXdl, respectively, lack amino acids 23–107 and 121–150. Thus, it is predicted that PSdl will have lost its ability to interact with the 300- and 105(Rb)-kDa proteins, whereas CXdl should have normal binding to the 300-kDa protein but altered interactions with 105(Rb)- and 107-kDa proteins. A third mutant, NCdl, deletes amino acids 86–120, a sequence that does not overlap with the regions defined for E1A interaction with the 105(Rb)-, 107-, and 300-kDa proteins.

Figure 2A shows immunoprecipitations of the ³⁵[S]amino acid-labeled E1A polypeptides as well as the cellular proteins that coprecipitate from the PC12 clones transfected with the different E1A mutants. As a control, immunoprecipitated proteins from adenovirus-5-transformed 293 cells are also shown (Harlow *et al.*, 1986). Immunoprecipitates of the 293 cells clearly showed the 12S and 13S E1A polypeptides (40–60 kDa) and the major 105(Rb)-, 107-, and 300-kDa-associated proteins. In immunoprecipitates of PC12 cell transfectants, the level of E1A expression is significantly lower than that observed in 293 cells, but the appropriate E1A polypeptides are readily detected (Figure 2A). Heterogeneity of the 12S and 13S forms of the E1A polypeptides is evident in the immunoprecipitates from PC12 clones transfected with the E1A gene or the 12S and 13S cDNAs. The heterogeneity of the E1A polypeptides is well documented and results from processing and posttranslational modifications (Harlow *et al.*, 1985).

Figure 1. Influence of wild-type E1A polypeptides on differentiation of PC12 cells. PC12 clones that express neo-resistance only, genomic E1A, or constructs that yield only the 12S or 13S form of E1A were cultured in the presence or absence of 100ng/ml NGF and 20 μ M forskolin for 4 d. The results are representative of six genomic E1A clones, three 12S E1A, and three 13S E1A clones.

Figure 2. Immunoprecipitation of E1A and associated cellular proteins from PC12 clones expressing wild-type and mutant E1A polypeptides. (A) Transfected PC12 clones were labeled with [35 S] amino acids. The E1A polypeptides and associated cellular proteins in the lysates were then immunoprecipitated with anti-E1A antibody coupled to agarose beads, washed, and separated on 8% SDS polyacrylamide gels (see Methods). The results represent two independent experiments, shown as two panels. The left panel was exposed to film for 16 h, whereas the right panel was exposed to film for 60 h, except the lane from 293 cells, which was for 16 h. Black arrows indicate positions of the various wild-type and mutant E1A polypeptides; white arrows designate the 105(Rb)- and 107-kDa polypeptides. (B) Human 293 cells and PC12 clones expressing neo-resistance only (neo-6), genomic E1A (E1A-11), or the 12S form of E1A (E1A12S.11) were lysed at 0°C in 1 ml of 250 mM

The wild-type, 12S, 13S, and NCdl deletion mutant polypeptides expressed in PC12 cells all bind the 105(Rb)-, 107-, and 300-kDa proteins, as measured by coimmunoprecipitation from PC12 extracts using anti-E1A antibody (Figure 2A). Relative to the migration of the 105(Rb) protein from 293 cells, the PC12 105(Rb) protein migrates slightly faster on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gels. This is evident in E1A complexes from ^{35}S -methionine-labeled cells (Figure 2A) as well as in 105(Rb) immunoblots of E1A complexes from unlabeled cells (Figure 2B). Figure 2A also demonstrates that the PSdl mutant did not bind the 105(Rb)- or 300-kDa proteins but did bind the 107-kDa protein. The PSdl mutant is not stably expressed at high levels in PC12 cells, but clone PSdl-34 has approximately the same level of expression of the mutant E1A polypeptide as is observed for the 12S and 13S polypeptides in the wild-type E1A-11 clone. Similar levels of the 107-kDa protein are also coimmunoprecipitated with the E1A-11 and PSdl-34 clones, indicating that PSdl is a functional E1A mutant polypeptide retaining a subset of its binding properties with cellular proteins. In this regard, PSdl clones 9, 17, and 34 are respectively expressed at increasing levels and bind a corresponding amount of 107-kDa protein. In contrast to the PSdl mutant, the CXdl polypeptide bound the 300-kDa protein but not the 105(Rb)- or 107-kDa proteins (Figure 2A). Thus, the PSdl and CXdl mutants can be used to discriminate functional properties of the domain 1 and domain 2 sequences required for transformation by the E1A polypeptide.

Phenotype of PC12 cells expressing E1A deletion mutants

The phenotypic consequence of the E1A mutants on PC12 cell differentiation is shown in Figure 3. The NCdl mutant binds the 105(Rb)-, 107-, and 300-kDa proteins and inhibits NGF-plus-forskolin-induced differentiation. In contrast, the PSdl mutant in which association with

the 105(Rb)- and 300-kDa polypeptides is disrupted (Figure 2) does not inhibit differentiation. The CXdl mutant polypeptide inhibited neuronal differentiation in response to NGF plus forskolin, similarly to E1A12S, E1A13S, and NCdl expression in PC12 cells.

Cumulatively, the findings indicate that E1A domain 1, which binds the 300-kDa protein, is required for inhibition of NGF-induced neuronal differentiation. Loss of interaction with the 105(Rb)- and 107-kDa proteins (CXdl mutant) did not influence the ability of the E1A deletion mutant to disrupt neurite extension. It should be noted that additional proteins of 28, 40, 50, 60, 80, 90, and 130 kDa have been shown to coimmunoprecipitate with E1A in 293 cells (Harlow *et al.*, 1986). The functional significance of the interaction of these proteins with E1A on cell phenotype remains obscure. Our immunoprecipitation analysis of E1A from PC12 cells did not reveal the reproducible association of any cellular proteins with the different E1A polypeptides other than the 105(Rb)-, 107-, and 300-kDa proteins. This may be related to the low abundance of the other cellular proteins reported to interact with E1A or the failure to resolve them on one-dimensional gels. Nonetheless, the findings clearly define E1A domain 1 as essential for inhibition of NGF-plus-forskolin-induced neuronal differentiation. Loss of domain 2 function, as defined by loss of 105(Rb)- and 107-kDa binding, results in an E1A mutant that retains 300-kDa protein binding and inhibition of PC12 cell differentiation.

Altered early response gene expression in E1A-expressing clones

Although the E1A transcription activating region unique to the 13S form is not required for inhibition of differentiation (Figure 1), the complexing of the 105(Rb)-, 107-, and 300-kDa and possibly other cellular proteins could still alter the regulation of gene expression in PC12 cells. This hypothesis was based on the predicted role of the cellular proteins that have functions al-

NaCl, 50 mM HEPES (pH 7.0), 0.1% NP-40. Clarified extracts containing equal protein were incubated (16 h, 4°C) with anti-E1A agarose, and the bound complexes were washed as described (see Methods). The bound proteins were eluted with 100 μl of SDS sample buffer lacking β -mercaptoethanol. The eluted samples were then supplemented with reducing agent (5% final), boiled, and resolved on an 8% SDS polyacrylamide gel. After transfer of the proteins to nitrocellulose, the filter was probed with a monoclonal Rb antibody. Bound monoclonal antibodies were visualized by incubating with rabbit anti-mouse secondary antibodies and then [^{125}I] protein A. Autoradiography was for 16 h. The white arrows indicate the position of the 105(Rb) polypeptide that specifically complexed with E1A. (C) The schematic representation of the E1A polypeptides is based on information from previous reports (Moran *et al.*, 1986; Moran and Zerler, 1988; Whyte *et al.*, 1989).

Figure 3. Influence of E1A deletion mutants on PC12 differentiation. PC12 clones expressing the various E1A deletion mutants were cultured for 4 d in the absence or presence of 100ng/ml NGF and 20 μ M forskolin. The results are representative of six NCdl clones, six PSdl clones, and three CXdl clones.

tered by interaction with E1A and that are thought to be involved in the control of cell cycle progression and normal growth control (Moran and Zerler, 1988; Cooper and Whyte, 1989; Weinberg, 1989; Whyte *et al.*, 1989). For this reason, the regulation of several early-response genes that have expression controlled by growth factors (Greenberg *et al.*, 1985; Ito *et al.*, 1989; Wu *et al.*, 1989) was determined in E1A-expressing PC12 cells. Figure 4A shows that E1A dramatically alters the regulation of mRNA levels for the early-response genes *c-fos*, *c-myc*, *c-jun*, and *egr-1*. In control PC12

cells not expressing E1A (neo-3 and 6), NGF induces an increase in mRNA levels for the early response genes. However, in E1A-expressing clones, the regulation of early response gene mRNA levels by NGF is differentially influenced. The increase in *c-fos* and *c-myc* mRNA levels resulting from NGF stimulation is significantly repressed in E1A-expressing cells. For *c-jun* and *egr-1*, mRNA levels are significantly increased in resting cells; and challenge with NGF has a limited influence on *egr-1* and essentially no effect on *c-jun*. The level of *c-jun* in resting E1A transfected clones was, in fact, similar to

Figure 4. Early-response gene expression in E1A-transfected PC12 clones. (A) duplicate 100-mm dishes of the various PC12 clones were pretreated for 1 h (37°C) with 10 μ g/ml cycloheximide, except for analysis of *c-myc*, for which it was omitted. Subsequently, the cells were incubated for 1 h (*c-fos*, *c-jun*, and *egr-1*) or 2 h (*c-myc*) with or without 50ng/ml NGF. Total RNA was isolated (see Methods) and 10 μ g was analyzed by Northern blots for *c-fos*, *c-myc*, *c-jun*, and *egr-1* mRNA. The experiment shown is representative of five or six independent experiments and six independently derived E1A clones. (B) Clones expressing either the 12S or 13S form of E1A were treated as in panel A and RNA was analyzed for expression of *c-jun* and *egr-1*.

that observed with NGF stimulation of control cells.

The alteration in early-response gene mRNA levels in PC12 cells was independent of the transcription activation region of the E1A polypeptide. Figure 4B shows that the 12S and 13S forms of E1A, where the 12S polypeptide lacks the transcription activation region, have similar influences on *c-jun* and *egr-1* mRNA levels relative to control cells. Thus, E1A is able to disrupt the normal NGF-stimulated transcription of several early-response genes, the appropriate regulation of which is known to be critical for

the onset of PC12 neuronal differentiation (Maruyama *et al.*, 1987; Ito *et al.*, 1989).

The ability of constitutively expressed *c-jun* to form heterodimers with *c-fos* and to down-regulate *c-fos* transcription (Sassone-Corsi *et al.*, 1989) might account for the inability of NGF to induce differentiation in E1A-expressing clones. This seemed possible because inhibition of *c-fos* expression in other cell types has been shown to inhibit growth factor-stimulated, transcription-dependent events (Holt *et al.*, 1986; Nishikura and Murray, 1987). To test this possibility, we isolated stable PC12 clones trans-

fectected with a *c-jun* expression vector that constitutively expressed *c-jun*. Figure 5 shows that, unlike E1A, expression of *c-jun* alone did not influence the ability of NGF plus forskolin to induce PC12 cell differentiation. This indicates that the effect of *c-jun* on PC12 cell differentiation must be significantly different than that for *c-fos* or *c-myc*, because constitutive expression of these proto-oncogenes, unlike *c-jun*, inhibits PC12 cell differentiation (Maruyama *et al.*, 1987; Ito *et al.*, 1989).

Discussion

We have shown that NGF-induced PC12 cell differentiation is inhibited by the adenovirus E1A polypeptides. The proteins that interact with E1A have been shown in other cell types to be

involved in cell cycle control (Ludlow *et al.*, 1990), and ablation of their expression or function may lead to a transformed phenotype (Weinberg, 1989; Bookstein *et al.*, 1990). Perturbation of the function of cellular proteins involved in cell-cycle control would be predicted to have a major influence on PC12 cell differentiation because accumulation of PC12 cells at early G1 in the cell cycle is a consequence of NGF treatment (Rudkin *et al.*, 1989). Similar to the requirements for fibroblast transformation by E1A (Whyte *et al.*, 1989), there is a strong correlation between the interaction of E1A with the 300-kDa protein and inhibition of PC12 cell differentiation. Expression of the CXdl mutant resulted in interaction with the 300-kDa protein, but not with the 105(Rb)- or 107-kDa proteins, and inhibited PC12 differentiation in response

Figure 5. Influence of constitutive *c-jun* expression on PC12 differentiation. (A) PC12 cells were transfected with pRSVc-*jun*, and seven clones that stably expressed the transfected cDNA were identified by Northern analysis. Illustrated are the relative levels of *c-jun* mRNA in total RNA (10 μ g) from a clone expressing neo-resistance only, an E1A-expressing clone, and a representative *c-jun* clone that had not been treated with either cycloheximide or NGF so that basal *c-jun* levels could be appropriately assessed. Lane 1, E1A-11; lane 2, neo-3; lane 3, RSVc-*jun*-30. (B) Neo-6 and RSVc-*jun*-30 were cultured for 4 d in growth medium with or without 100ng/ml NGF and 20 μ M forskolin. The differentiation response observed in RSVc-*jun*-30 is representative of all seven independently derived clones.

to NGF. In baby rat kidney cells, the CXdl mutant can effectively stimulate DNA synthesis, but cannot induce the fully transformed phenotype, as measured by focus formation (Moran and Zerler, 1988). Thus, the requirements for E1A-mediated inhibition of PC12 cell differentiation are similar to the requirements for mitogenic responses of fibroblasts.

Consistent with a role for the transformation domain of E1A in inhibition of PC12 cell differentiation was the clear demonstration that the transcription activation domain was not involved in the E1A-induced phenotype. However, the interaction of E1A with resident PC12 proteins dramatically disrupts the normal control of the four early-response genes (*c-fos*, *c-myc*, *c-jun*, and *egr-1*) we examined. This result, in combination with the findings that stable transfection of *c-myc* (Maruyama *et al.*, 1987) or *c-fos* (Ito *et al.*, 1989) inhibits NGF-induced PC12 cell differentiation, indicates that the correct temporal induction and transient expression of these early-response genes are at least partially controlled by the proteins interacting with E1A.

Expression of E1A differentially altered the resting levels and repressed the ability of NGF to induce early-response gene mRNAs. The promoter/enhancer regions of these early response genes contain numerous DNA sequence motifs, which are differentially regulated by various cellular transmodulating factors in response to a variety of signals. It seems less likely that specific complexes generated by interaction of E1A with cellular proteins are individually responsible for the observed changes in early-response gene expression. We favor the possibility that these changes in gene expression are due to accumulated events that lead to general alterations in cell cycle control. In support of this, the Rb gene product was recently shown to act as a negative regulator of *c-fos* transcription by interacting directly with a DNA sequence element located 90 base pairs upstream of the transcription start site (Robbins *et al.*, 1990). In our system, simple attenuation of 105(Rb) activity by binding to transfected E1A did not enhance expression of *c-fos* (compare Figures 2 and 4). Thus, many additional factors must participate in the changes in *c-fos* control presented here.

A candidate for the multiple effects of E1A on early-response gene regulation is the E2F transcription factor, which is known to bind to other proteins, creating inactive complexes that E1A can dissociate (Bagchi *et al.*, 1990). Modulation of available E2F has been postulated to

regulate transcription of appropriate genes. The interaction of E1A with the E2F-containing complex does not require the E1A domain 3 but is dependent on domain 2 (Bagchi *et al.*, 1990). The E1A construct CXdl deletes domain 2, binds the 300-kDa protein, and inhibits PC12 cell differentiation. These observations indicate that E2F interaction with E1A is not responsible for the differentiation-inhibited phenotype but might participate in the altered control of the early-response genes. Experiments are now being undertaken to specifically address this question.

The genes that have been shown to be regulated by NGF are, in general, also regulated by the mitogenic growth factor, epidermal growth factor (Greenberg *et al.*, 1985). Interestingly, expression of *c-myc* or E1A causes NGF to behave as a weak mitogen, stimulating growth of PC12 cells (Maruyama *et al.*, 1987). This observation demonstrates that the NGF receptor-stimulated signal cascade can elicit either differentiation and growth arrest or proliferation, depending on the expression of specific genes and the physiological state of PC12 cells. This is consistent with the finding that fibroblast growth factor also induces PC12 cell differentiation (Rydel and Greene, 1987), even though the response of most cells expressing the fibroblast growth factor receptor is enhanced DNA synthesis and proliferation. The finding that E1A alters the response to a hormonal or growth factor stimulus indicates that a major control point for phenotypic differentiation versus growth involves cell cycle regulatory proteins.

The specific influence of E1A on the function of 105(Rb)-, 107-, and 300-kDa nuclear regulatory proteins also explains the inhibition of cyclic AMP (cAMP) responses in E1A-expressing PC12 cell clones. Wild-type PC12 cells transiently extend short neurites in response to cAMP analogues or agonists, such as forskolin, which stimulate cAMP synthesis (Richter-Landsberg and Jastorff, 1986). The cAMP-dependent protein kinase is also required for NGF induction of voltage-dependent Na⁺ channels in PC12 cells (Kalman *et al.*, 1990). The loss of these responses in E1A-expressing clones (Maruyama *et al.*, 1987 and unpublished observations), even though the cAMP-dependent protein kinase activity is similar to that in wild-type cells, indicates that control of cellular proteins that associate with E1A is necessary not only for growth factor signaling but for cAMP-mediated responses as well. The E1A-induced

perturbation of the protein kinase signals entering the nucleus to regulate cell phenotype is thus a probable mechanism for inhibition.

Methods

Cell culture

PC12 pheochromocytoma cells were cultured at 37°C in Dulbecco's modified Eagle's medium containing 5% heat-inactivated horse serum, 5% fetal bovine serum, 5% OptiMEM-1 (GIBCO, Grand Island, NY), 100 U/ml penicillin and 100 µg/ml streptomycin. Transfected clones were grown in the same medium supplemented with 250 µg/ml G-418.

Expression plasmids

p1Aneo is a pUC18 derivative that contains adenovirus 5 E1A sequences 1–1834 as well as the Tn5 neo^r gene under the control of the SV40 early promoter (Maruyama *et al.*, 1987). pE1A12S and pE1A13S express the 12S and 13S forms of E1A (Leff *et al.*, 1984), respectively, and were generously provided by Drs. Claude Keding and Pierre Chambon (Institut de Chimie Biologique, Strasbourg, France). pE1A-NCdl, pE1A-CXdl, and pE1A-PSdl are deletion mutants of E1A generated by Moran *et al.* (Moran *et al.*, 1986; Moran and Zerler, 1988) and were gifts of Dr. James Cook (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). pRSVc-*jun* contains a 1.8-kbp *c-jun* cDNA under the control of Rous sarcoma virus long terminal repeat with 3' SV40 splice and polyadenylation sequences (gift from Drs. Robert Chiu and Michael Karin, University of California, San Diego).

DNA-mediated gene transfection

PC12 cells were suspended and washed with ice-cold phosphate-buffered saline (PBS) and suspended in the same buffer to a final concentration of $1-3 \times 10^7$ /ml. Aliquots (0.8 ml) of the cell suspension were mixed in an electroporation cuvette (4-mm electrode gap) with plasmid DNA (10–25 µg) and incubated on ice for 10 min. For plasmids lacking a selectable marker, 1 µg of pCW-1 (Woon *et al.*, 1988) was cotransfected with the various E1A expression plasmids. The cells were electroporated (0.2 kV, 960 µF) in a BioRad (Richmond, CA) Gene Pulser and incubated on ice for another 10 min. The cells were then diluted into growth medium and seeded onto 10-cm dishes ($\sim 1-5 \times 10^6$ cells/dish) that had been previously coated with poly-L-lysine. Twenty-four hours later, the cells were placed in medium containing 250 µg/ml active G-418. After 3–4 wk, resistant colonies were picked, clonally expanded, and screened for expression by Northern analysis.

RNA isolation and Northern blot analysis

PC12 transfectants were treated for 1 h at 37°C with 10 µg/ml cycloheximide to superinduce the subsequent expression of *c-fos*, *c-jun*, and *egr-1* (Greenberg *et al.*, 1986; Wu *et al.*, 1989) and then incubated for 1 h in the presence or absence of 50 ng/ml NGF. For analysis of *c-myc* mRNA, the cycloheximide treatment was excluded because it has been reported to diminish levels of this mRNA species (Greenberg *et al.*, 1986). Total RNA was isolated by modification of a previously published procedure (Segerson *et al.*, 1987). Briefly, attached cells from a 10-cm dish were rinsed with ice-cold PBS and dissolved in 1 ml of 6M urea, 3M LiCl. The samples were collected into tubes, sonicated briefly to shear genomic DNA, and incubated at 4°C for ≥ 72

h. The precipitated RNA was collected by centrifugation (5 min in a microfuge) and dissolved in 10 mM tris(hydroxymethyl)aminomethane (Tris)-Cl (pH 8.0), 5 mM EDTA, 0.1% SDS. After one extraction with phenol:chloroform (1:1) and one with chloroform, the RNA was precipitated with ethanol at –20°C. Total RNA (10 µg) was chromatographed on 1.2% agarose gels containing 6.7% (vol/vol) formaldehyde, electrophoretically transferred to Zetabind filters (CUNO, Meriden, CT) and immobilized to the filters with a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Radiolabeled DNA probes were hybridized to the filters at a concentration of 2×10^6 cpm/ml and were prepared by random priming of a 3 kbp *Nco* I-*Xho* I fragment of the human *c-fos* gene, a full-length *c-jun* cDNA, murine *egr-1* (gift from Dr. V. Sukhatmi, University of Chicago), or a full-length murine *c-myc* cDNA.

Immunoprecipitation of E1A polypeptides

The various E1A-expressing PC12 clones were seeded into poly-L-lysine-coated 100-mm dishes at $\sim 5 \times 10^6$ cells per dish. Two to 3 d later, the cells were labeled in 6 ml of methionine-free Dulbecco's modified Eagle's medium containing 1 mCi Trans³⁵S-label (ICN, Radiochemicals, Irvine, CA) for 4 h. The attached cells were then washed with ice-cold PBS and lysed (30 min at 0°C) in 1 ml of 250 mM NaCl, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.0), 0.1% NP-40. After centrifugation ($100,000 \times g$ for 15 min), the samples were adjusted for equal radioactivity and incubated for 16 h at 4°C with anti-E1A monoclonal antibody M73 (Harlow *et al.*, 1985) immobilized to agarose beads (Oncogene Science, Manhasset, NY). The immune complexes were washed once with lysis buffer containing 1M NaCl and then three or four times with regular lysis buffer. The immune complexes were dissolved in SDS sample buffer and chromatographed on 8% polyacrylamide gels, which were subsequently prepared for autoradiography by immersion in Autofluor (National Diagnostics, Manville, NJ).

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