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Promoter trans-activation of protooncogenes c-*fos* and c-*myc*, but not c-Ha-*ras*, by products of adenovirus early region 1A

(cellular oncogenes/transcription/adenovirus type 2/regulated gene expression)

PAOLO SASSONE-CORSI AND EMILIANA BORRELLI

Molecular Biology and Virology Laboratory, The Salk Institute, P.O. Box 85800, San Diego, CA 92138

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ABSTRACT **The E1A (early region 1A) oncogene products of adenovirus type 2 trans-activate the other early viral transcription units, as well as some cellular promoters. Using a short-term cotransfection assay in murine NIH 3T3 fibroblasts, we show that c-*fos* and c-*myc* promoter activities are stimulated by the E1A proteins, whereas c-Ha-*ras* transcription is not affected. The product of E1A 13S mRNA is responsible for the trans-activation, whereas the 12S mRNA product has no effect. Analysis of the c-*fos* promoter sequences required for the E1A stimulation shows that responsive sequences are located between positions −402 and −240 upstream of the transcription initiation site. This same region also contains the c-*fos* serum-responsive element. Furthermore, transcription of the endogenous c-*fos* gene in HeLa cells is increased after E1A transfection.**

The E1A (early region 1A) oncogene products of adenovirus type 2 (Ad2) have been implicated in the transcriptional activation of the other early viral genes during infection (1, 2). Some cellular genes, such as those encoding β-tubulin and the heat shock proteins (3, 4), are also transcriptionally activated by the E1A proteins. In addition to the trans-activation properties, E1A proteins can specifically repress the activity of several viral and cellular transcriptional enhancers (5–7). These pleiotropic characteristics of the E1A products suggest that the role that they play in cellular metabolism during adenovirus infection must be complex.

The E1A proteins have immortalizing properties as well as the ability to transform primary cells in conjunction with other oncogenes, such as E1B (the natural adenovirus complementary oncogene) and *ras* (8). Two mRNA forms, 12S and 13S, are produced by the E1A transcription unit, early in infection. Mutational analysis indicates that the E1A transforming activity requires the 12S product, which is also responsible for the enhancer-repressor function (9). Additional evidence suggests that both products may be more potent in inducing complete transformation (10). The 13S product, on the other hand, appears to be principally responsible for the trans-activation function. The mechanism by which the E1A products elicit their functions is not totally understood but appears to involve a response of a distinct cellular factor(s) that might interact with the responsive promoter. To better understand the role played by the E1A products, we tested their potential for regulating the transcription of genes thought to influence cellular proliferation, differentiation, and metabolism. In this communication we show that the E1A products trans-activate the expression of c-*fos* and c-*myc* protooncogenes, whereas the c-Ha-*ras* promoter activity is not altered. Both c-*myc* and c-*fos* are nuclear proteins themselves, presumably acting at the level of transcription. The c-Ha-*ras* p21 gene product, on the other hand, has homology to GTP-binding proteins and probably acts at the plasma-membrane level. The tumorigenic potential of c-Ha-*ras* is most frequently correlated with a mutation changing the amino acid encoded by codon 12 and not with enhanced levels of the germ-line gene product. At a more general level these results suggest that nuclear regulatory proteins may interact to form an interactive cascade or network of control used to integrate complex functions by coordinately altering the expression of genes.

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MATERIALS AND METHODS

Plasmids. The recombinants used in this study have already been described. pE1ASV (11) is a pBR322-based plasmid containing the entire Ad2 E1A transcription unit (0–4.5 map units) linked to the simian virus 40 poly(A)-addition signal (coordinates 2469–2604); pE1A12S and pE1A13S (12) are equivalent to pE1ASV except that the E1A coding sequence has been replaced by the cDNAs corresponding to the 12S and 13S mRNAs; pE1AG (12) contains the complete E1A promoter linked to a rabbit β-globin gene fragment between positions −9 and +1650 (13). FC3 (14) contains the human c-*fos* promoter (positions −711 to +42) linked to the bacterial chloramphenicol acetyltransferase (CAT) gene. c-*myc*-CAT contains the human c-*myc* promoter (15) (positions −2325 to +36) linked to the CAT gene. prasCAT1 (16) contains the human c-Ha-*ras* promoter (positions −420 to +130) linked to the CAT gene. The deletion mutants of the c-*fos* promoter used in this study are described in the legend to Fig. 3.

Tissue Culture and DNA-Mediated Transfer. Murine NIH 3T3 fibroblasts and human HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells at 70% confluence were transfected by the calcium phosphate coprecipitation technique (5) and exposed to the precipitate for 12–16 hr. When less than 20 μg of specific DNA was used per 10-cm culture dish, pUC19 plasmid DNA was added to give 20 μg of total DNA.

Transcription Assays. After DNA transfection, the cells were harvested and CAT assays (17) or RNA-protection analyses (18) were performed as described. RNA-protection analysis was performed by hybridizing 30 μg of total cellular RNA to a ^{32}P-labeled RNA probe complementary to the human c-*fos* *Bss*HII (−100)–*Sph* I (+973) region (14, 19). The protected fragment, after RNase digestion of the hybrid, is 297 bases long.

RESULTS

Trans-Activation of c-*fos* and c-*myc* Promoters by the Ad2 E1A Products. A cotransfection assay was used to investigate the effect of E1A products on transcription from c-*fos*, c-*myc*, and c-Ha-*ras* promoters. This approach consists of the cotransfection of specific amounts of a reporter plasmid DNA and of a recombinant bearing the E1A transcription unit. The

Abbreviations: Ad2, adenovirus type 2; CAT, chloramphenicol acetyltransferase; E1A, early region 1A.

E1A recombinant used in this study, pE1ASV, has been described (5, 11) and consists of the Ad2 sequences from map unit 0 to 4.5 linked to the simian virus 40 poly(A)-addition signal.

In the present studies the promoters from c-*fos*, c-*myc*, and c-Ha-*ras* genes were linked to a reporter gene encoding CAT. To test the functional capability of the expressed E1A products as positive transcriptional factors, we performed CAT assays with cell extracts obtained after cotransfection with the various reporter constructs. The relative activity of the three cellular oncogene promoters after short-term transfection of 5 μg of recombinant DNA in mouse 3T3 fibroblasts is shown in Fig. 1 (lanes 1). When 4 μg and 10 μg of the pE1ASV recombinant were cotransfected with FC3 and c-*myc*-CAT, enhancement of the transcription from these two promoters was observed (Fig. 1 *A* and *B*, lanes 2 and 3; Table 1). On the other hand, no effect on the c-Ha-*ras* promoter activity was detectable (Fig. 1*C*, lanes 2 and 3). To determine if one or both E1A products are responsible for the observed trans-activation, we performed the same experiment with recombinants individually expressing the 12S and 13S gene products (12). These plasmids are analogous to pE1ASV, but instead of the total genomic adenoviral coding sequence, the cDNAs correspondent to the 12S and 13S mRNAs are linked to the E1A promoter (pE1A12S and pE1A13S, respectively). As shown in Fig. 1, the 13S product is responsible for the trans-activation activity on c-*fos* and c-*myc* promoters (lanes 6 and 7 in *A* and *B*). Again, no effect on the transcriptional activity of the c-Ha-*ras* promoter was observed (lanes 6 and 7 in *C*). The extent of trans-activation by pE1A13S is comparable to the one observed using the pE1ASV plasmid (Table 1). Interestingly, when pE1A12S was cotransfected with FC3 or c-*myc*-CAT, a very weak, but reproducible, trans-activation was detected (Table 1).

To rule out the possibility that the increased transcription from the c-*fos* and c-*myc* promoters could be due to the titration of cellular repressors by the cotransfected E1A promoter, we performed the same experiment using the pE1AG recombinant. pE1AG (12) contains the rabbit β-globin coding sequence linked to the E1A promoter; therefore, it would compete for cellular trans-acting factors as well as pE1ASV. As shown in Fig. 1 (lanes 8 and 9), there was no effect on the activity of the cellular oncogene promoters after cotransfection with pE1AG. Finally, it could be argued that the E1A coding sequence contains some cryptic promoter elements, as has been suggested (20), and thus be the cause of the titration of potential repressor molecules acting on the c-*fos* and c-*myc* promoters. Therefore, we transfected the protooncogene–CAT gene constructs with 5 μg of "promoterless" cDNA subclones bearing the 12S and 13S coding sequences. As expected, no effect on the transcriptional activity of the protooncogene promoters was detectable (Fig. 2). These results confirm that the observed regulation is a consequence of a trans-acting product from the E1A gene.

Sequences Required for c-*fos* Promoter Trans-Activation by E1A Products. To identify promoter sequences mediating the trans-activation by the E1A products, a series of experiments was performed using a set of c-*fos* promoter deletion mutants. The c-*fos* promoter is well characterized (14, 19, 21) and is thus ideal for this approach. The c-*fos* promoter contains a canonical "TATA box" at position −30, an upstream promoter element, and a serum-inducible enhancer element (indicated as E in Fig. 3) located between positions −332 and −276 (14, 19). The c-*fos* promoter deletion mutants used here are linked to the CAT gene and have been characterized (14). Fig. 3 *Upper Left* shows the transcriptional activity of a variety of promoter deletion mutants. The endpoints of the deletions are indicated in Fig. 3 *Lower*.

When equal amounts of pE1ASV were cotransfected with the various deletion mutants, the observed trans-activation activity was significantly higher for FC3 and FC4 than for FC7, FC10, and FC11 (see Fig. 3 *Upper Right* and legend for quantification). Note that the c-*fos* transcriptional enhancer is present between the endpoints of the FC4 and FC7 deletions. These experiments indicate that the sequences required for most of the c-*fos* trans-activation by E1A products are located between positions −402 and −240.

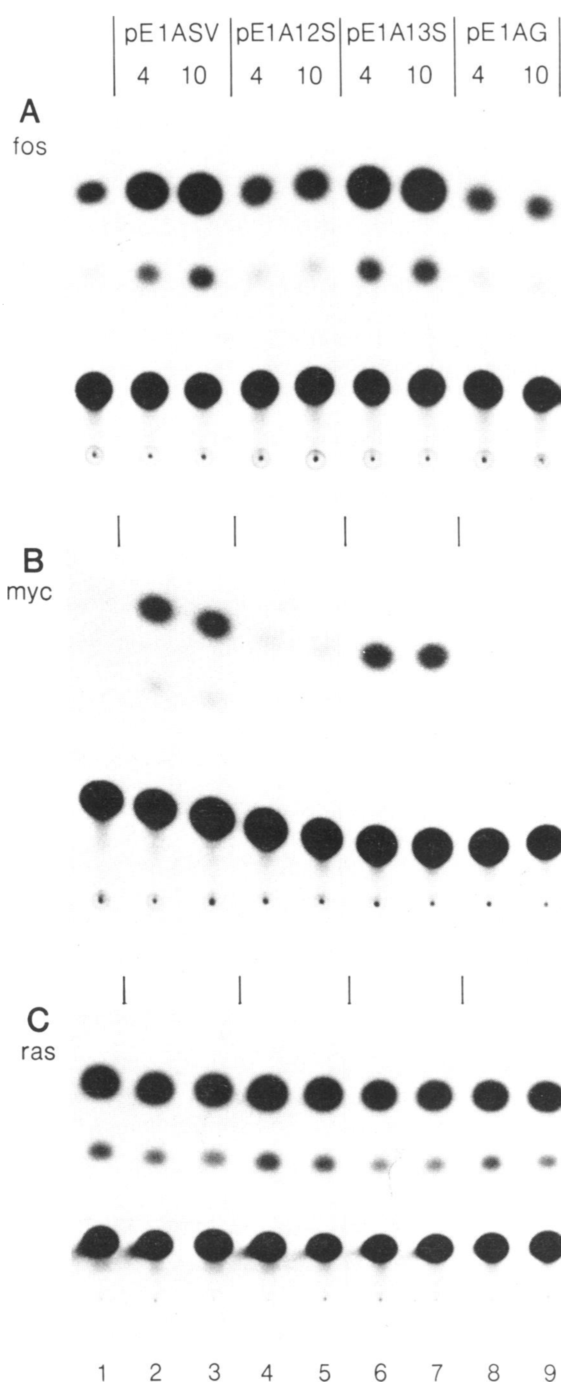

FIG. 1. Trans-activation of c-*fos* and c-*myc* promoters. NIH 3T3 fibroblasts were transfected with 5 μg of one of the protooncogene promoter–CAT gene recombinant plasmids: FC3 (*A*), c-*myc*-CAT (*B*), or prasCAT1 (*C*). Basal promoter activities are shown in lane 1; CAT activity after cotransfection with 4 and 10 μg of pE1ASV (lanes 2 and 3), pE1A12S (lanes 4 and 5), pE1A13S (lanes 6 and 7), and pE1AG (lanes 8 and 9) are shown in the indicated lanes. The CAT assay (17) monitors acetylation of [^{14}C]chloramphenicol in cell extracts; [^{14}C]chloramphenicol and its products are separated by thin-layer chromatography and visualized by autoradiography.

Endogenous c-*fos* Is Also Trans-Activated by the E1A

Table 1. Trans-activation of protooncogene promoter–CAT gene recombinants

Promoter	Relative transcription (CAT expression)		
	E1A	12S	13S
c-*fos*	7.3	1.9	7.2
c-*myc*	13.4	2.8	14.1
c-Ha-*ras*	1.3	1.4	1.0

The level of trans-activation by E1A products was measured by densitometric scanning of autoradiograms as shown in Fig. 1. Values are averages of 3–4 experiments and indicate the extent of trans-activation with respect to the basal level (1.0) of each oncogene–CAT gene construct.

Products. The experiments reported in the previous sections demonstrate that the E1A products trans-activate the c-*myc* and c-*fos* promoters when they are introduced into the cells as episomes. We therefore tested whether transcription of the endogenous c-*fos* gene could be activated by the E1A products. We transfected pE1ASV or pE1AG into HeLa cells and probed for c-*fos* RNA by hybridization with a ^{32}P-labeled single-stranded RNA probe complementary to human c-*fos* positions −100 to +973 (18). The specific c-*fos* protected fragment (297 bases long) was detectable also in nontransfected HeLa cells, representing the c-*fos* RNA basal level (Fig. 4, lane 1). When 5 and 15 μg of pE1ASV DNA were transfected, the c-*fos* RNA level increased about 7-fold with respect to the basal level (compare lanes 2 and 3 to lane 1). This increase was induced by the E1A products, because transfection of 5 or 15 μg of pE1AG DNA did not elicit the same transcriptional enhancement (lanes 4 and 5). This result indicates that the trans-activating function of the E1A proteins can affect transcription of c-*fos* in its natural genomic environment.

DISCUSSION

Protooncogenes, such as c-*fos* and c-*myc*, are considered to play an important role in cellular proliferation and differentiation (22, 23), and thus it is critical to understand mechanisms influencing their primary expression.

Our results show that in a cotransfection system, the E1A gene products trans-activate the c-*fos* and c-*myc* promoters, whereas the c-Ha-*ras* promoter activity is not altered. In this respect it is noteworthy that the differential effect of the E1A

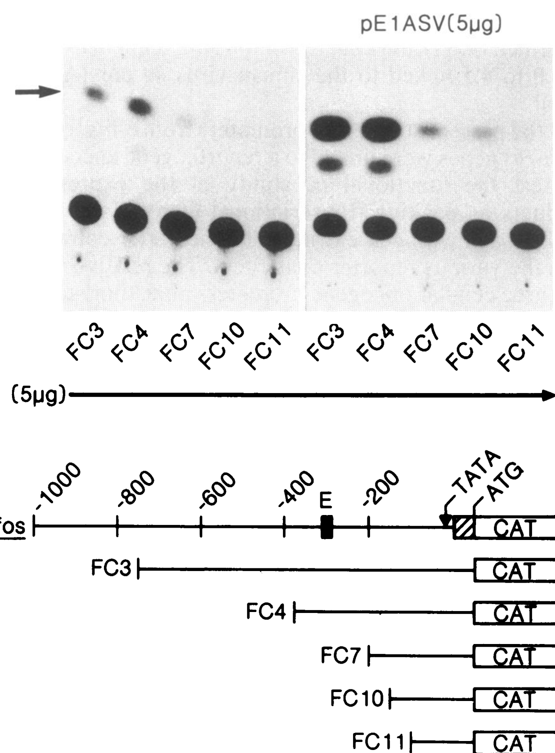

FIG. 3. c-*fos* sequence requirement for E1A trans-activation. The c-*fos* promoter deletion mutants used in this study have been described (14). The 3′ endpoints of the deletions map at positions −711 (FC3), −402 (FC4), −240 (FC7), −124 (FC10), and −64 (FC11). The 5′ border is +42 for all the recombinants and the CAT gene was linked at this position. Black box labeled E represents the serum-responsive enhancer. (*Upper Left*) Transcriptional activity after transfection of 5 μg of the various deletion plasmid DNAs. (*Upper Right*) Trans-activation was analyzed by cotransfection of 5 μg of pE1ASV DNA. The extent of trans-activation for the deletion mutants is as follows: FC3, 7.3; FC4, 7.1; FC7, 2.8; FC10, 2.1; FC11, 1.5. These values are the average of several experiments.

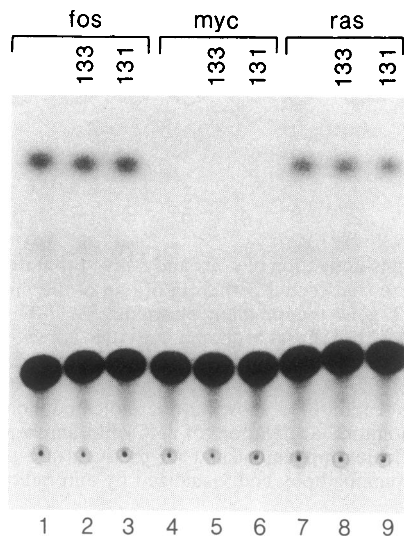

FIG. 2. The basal transcriptional level of c-*fos*, c-*myc*, and c-Ha-*ras* is not affected by cotransfection with promoterless E1A recombinants. Plasmids 133 and 131 contain the cDNAs equivalent to the 12S and 13S mRNAs (12). The basal CAT level after transfection of 5 μg of FC3 (lane 1), c-*myc*-CAT (lane 4), and prasCAT1 (lane 7) is shown. Cotransfection of 5 μg of 133 (lanes 2, 5, and 8) or 131 (lanes 3, 6, and 9) plasmid DNA does not affect transcriptional activity of the protooncogene promoters.

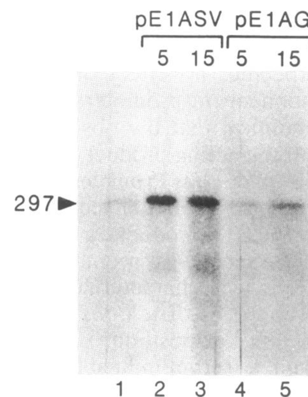

FIG. 4. Trans-activation of endogenous c-*fos*. The c-*fos* RNA basal level in HeLa cells is shown in lane 1, after hybridization of 30 μg of total cellular RNA with a human c-*fos*-specific RNA probe followed by digestion with RNase, electrophoresis, and autoradiography. The protected fragment is 297 bases long. Five and 15 μg of pE1ASV (lanes 2 and 3) or pE1AG (lanes 4 and 5) were transfected into HeLa cells and the c-*fos* RNA levels were analyzed.

products on various promoters appears to be independent from the basal transcriptional activity of the different promoters. The trans-activation also affects transcription of the endogenous c-*fos*, indicating that the observed effect is not limited to the episomal conformation of the transfected protooncogene promoters. The extent of transcriptional activation is significant, being 7- to 8-fold for the c-*fos* promoter and 10- to 15-fold for the c-*myc* promoter (Table 1). Previous analyses of other early adenoviral promoters (24–26) failed in the search for specific E1A-responsive sequences. In all cases, however, a segment of the promoter, generally bearing upstream regulatory elements, would be necessary in order to obtain efficient trans-activation (27, 28). We analyzed several deletion mutants of the c-*fos* promoter to determine the boundaries of the E1A-responsive element (Fig. 3). The results indicate that the region most critical for trans-activation by E1A products is located between positions −402 and −240 with respect to the transcription start site, the same region that contains the serum-dependent c-*fos* enhancer element (19). It is worth noting, in this respect, that in several cases the E1A products repress the enhancer-mediated transcription (5–7). On the other hand, recent data indicate that the E1A repressor activity could be cell-specific (29) and that, in the case of the adenovirus E2 early promoter, the E1A products have a trans-activating function on the enhancer element (30). In any case, it is clear that in the case of the c-*fos* promoter, the E1A products show a positive regulation and do not trans-repress the serum-dependent enhancer activity.

An interesting aspect of the results reported here is that the E1A products do not stimulate the c-Ha-*ras* promoter activity. This could be a common feature of all housekeeping promoters, since the adenosine deaminase promoter also is unaffected by the E1A products (unpublished data). These results indicate that the previously described complementation of E1A and *ras* in a transforming assay (8) is probably not based on a direct trans-activation mechanism.

The results reported in this paper indicate that nuclear transforming proteins are involved in a complex network that includes the specific activation (or inactivation) of cellular genes and that, in particular, the trans-activation of some oncogenes could be a step in a cascade of molecular events required for transformation by the adenovirus E1A proteins.

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