General repression of enhancer activity by the adenovirus-2 E1A proteins

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It has been shown previously that the adenovirus 2 (Ad2) E1A proteins repress activation of transcription by the SV40, polyomavirus and immunoglobulin gene enhancers. Here, we demonstrate that the repression of the SV40 enhancer is not specifically mediated by one of its constituent enhancers and/or proto-enhancers, but that each is subject to repression individually. This inhibitory effect of the E1A proteins is also observed with the AP-1 factor-binding enhancers from the polyomavirus and human metallothionein enhancers, and the MHC class I gene H-2K^b enhancer, which binds the KBF1/H2TF1/TC-IIB protein. Repression by the E1A gene products may, in fact, extend to all enhancer *trans*-activators, because the transcriptional activities of nuclear receptors (e.g., the estrogen and glucocorticoid receptors), of the yeast enhancer factor GAL4 expressed in HeLa cells, and of chimeric *trans*-activators (such as GAL-VP16) are all similarly inhibited. The E1A protein domains 2 and 3, including the acidic amino acid stretch that has been shown previously to be necessary for E1A-mediated *trans*-activation, are not required for repression. These results indicate that the amino-terminal region of the protein, which contains domain 1, plays a crucial role in repression, possibly by interfering in the transcriptional activation process at a step common to all *trans*-acting enhancer factors.

[Key Words: E1A; transcription; repression; enhancer activity]

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Differential splicing of the primary transcript of the human adenovirus-2 (Ad2) E1A early gene yields two overlapping mRNAs (see Fig. 1; Perricaudet et al. 1979). These 12S and 13S mRNAs encode two nuclear phosphoproteins, 243 and 289 amino acids in length (243R and 289R), respectively (Graham 1984), which share identical amino and carboxyl termini and differ in their primary sequence only by the presence of 46 amino acids that are unique to the 289R protein (Fig. 1).

The oncogenic E1A proteins have a pleiotropic effect on the regulation of eukaryotic gene transcription (for review, see Sassone-Corsi 1985; Berk 1986a,b; Jones et al. 1988). The 289R protein and, to a lesser extent, the 243R protein (Leff et al. 1984), activate transcription of the other adenovirus early genes and of a number of cellular genes (for reviews, see Berk 1986a,b; Grand 1987). On the other hand, the E1A proteins repress transcription from promoters that are linked to different viral and cellular enhancers, such as the SV40 enhancer (Borrelli et al. 1984; Velcich and Ziff 1985), the polyomavirus enhancer (Borrelli et al. 1984; Velcich et al. 1986), the IgH enhancer (Hen et al. 1985), the insulin enhancer (Stein and Ziff 1987), and the cytochrome P-450 gene enhancer (Sogawa et al. 1989). It was concluded from these studies that the enhancer, itself, was probably the target for the negative regulation by the E1A products. Furthermore, it

was shown that the E1A proteins repress the transcription of several cellular genes (K.A. Webster et al. 1988; Timmers et al. 1988, 1989; Young et al. 1989), but the target within the promoter region of these genes was not identified. The expression of class I major histocompatibility complex (MHC) antigens is also inhibited by the E1A proteins (Friedman and Ricciardi 1988), but in this case, the inhibition may not be only transcriptional (Vaessen et al. 1987; Meijer et al. 1989).

The various E1A functions have been mapped to three protein domains that are conserved among adenoviruses (see Fig. 1). One of these domains, domain 3 (amino acids 140–189), which is specific to the 289R protein, is sufficient for E1A-mediated transcriptional *trans*-activation (Lillie et al. 1986, 1987; Moran et al. 1986; M. Green et al. 1988; Jelsma et al. 1988; Fahnestock and Lewis 1989). On the other hand, domains 1 (amino acids 41–79) and 2 (amino acids 121–139), which are common to both the 289R and 243R proteins, appear to be required for both repression and/or oncogenic transformation but not for *trans*-activation, which suggested that transcriptional repression was important for transformation (Lillie et al. 1986, 1987; Schneider et al. 1987; Whyte et al. 1989). However, in other reports, enhancer repression could not be critically mapped to domains 1 and 2 (Kuppuswamy and Chinnadurai 1987; Velcich and Ziff 1988).

It is not known how transcription is activated by the E1A 289R protein. It does not appear to bind directly to DNA elements of the activated promoters (Ferguson et al. 1985); rather, it increases transcription by modifying

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E1A-mediated repression of enhancers

Figure 2. Organization of the SV40 promoter and structure of the different enhancers. (Top) The structure of the SV40 early promoter (Zenke et al. 1986), containing only one copy of the 72-bp sequence. The positions of the essential elements of the promoter, the TATA box, the 21-bp repeat element (21, 21, and 22), and the 72-bp enhancer element, are indicated, along with the coordinates of several restriction enzyme recognition sites. EES and LES indicate the positions of the early-early and late-early mRNA start sites, respectively. (Middle) The sequence of the late coding strand of the 72-bp element (indicated by vertical broken lines) and the 5'-flanking sequences. The different enhancers are boxed. (Bottom) The structure of the units containing the different enhancers (boxed) that were tetramerized and tested in this study for enhancer activity. For the SV40 enhancers, E and L designate the early and late SV40-coding strands, respectively. KB3 corresponds to a proto-enhancer element present in the promoter region of MHC class I H-2K^b gene (for references, see Kanno et al. 1989); Py-AP1 and MTII-AP1 correspond to AP1 factor-binding enhancers present in the polyomavirus enhancer (Imler et al. 1988, and references therein) and the human metallothionein IIA gene (W. Lee et al. 1987), respectively.

tory action can be extended to class D enhancers which, as monomers, possess enhancer activity independently (see Fromental et al. 1988), such as monomers of steroid hormone responsive elements that are activated by the corresponding nuclear receptor in the presence of the cognate hormone, and even to yeast upstream activator sequences (UASs), such as UAS_G, whose activity is mediated by the GAL4 *trans*-activator. Finally, we show that the acidic amino acid stretch that is located across the border of domains 2 and 3 of the E1A proteins is not important for enhancer repression, which indicates that

the amino-terminal region of the E1A proteins that contains domain 1 plays a crucial role in enhancer repression.

Results

Experimental design

The inhibitory action of the E1A gene products on enhancer activity was studied using transient transfection assays with human HeLa cells, mouse MPC11 plasm-

cytoma B cells, LMTK⁻ fibroblasts, and undifferentiated [F9(ND)] or retinoic acid-differentiated [F9(D)] F9 embryonal carcinoma cells. The reporter recombinants containing the various enhancers or proto-enhancers inserted in the promoter region of rabbit β -globin gene at either -109 (pG1 series) or -425 (pG2 series) (Fromental et al. 1988; N. Webster et al. 1988a,b; Kanno et al. 1989) were transfected together with the recombinant pE1ASV, which expresses both the 12S and 13S E1A mRNAs (Sassone-Corsi et al. 1983), and enhancer activity was determined by quantitative S1 nuclease analysis of RNA transcribed from the β -globin gene promoter (Glob+1 signal; see Figs. 3–6). A recombinant, pA56, containing the SV40 early promoter, but deleted for the enhancer sequence from coordinates 107–271 (see Fig. 2; Zenke et al. 1986), was cotransfected as a reference plasmid to correct for variations in transfection efficiency, as described previously (Fromental et al. 1988) (EES signal; see Figs. 3–6). The results were expressed as a percentage of transcription from the globin cap site (Glob+1), with 100% represented by either pG1B or pG2B, which contain the SV40 enhancer (with one copy of the 72-bp sequence; Fig. 2) inserted at position -109 (pG1B) or -425 (pG2B) of the β -globin gene promoter.

Alternatively, the results were expressed relative to pG1 or pG2 as the fold stimulation of transcription induced by the enhancer element. Control experiments were performed in all cases with the recombinant pE1A⁻ (Sassone-Corsi et al. 1983), which contains the E1A promoter but not the E1A-coding sequence, to check that the observed repression was not attributable

to competition between the promoters of the cotransfected recombinants (see, e.g., Figs. 4A and 5). Note that transcription from the isolated globin promoter in pG1 and pG2 was never affected by the E1A gene products (see Figs. 4A and 5; data not shown).

Individual SV40 proto-enhancers and related proto-enhancers are all repressed by E1A products in different cell types

As expected from a previous report (Borrelli et al. 1984), the activity of the SV40 enhancer in pG1B (where it causes a 400-fold increase in transcription) was progressively repressed when increasing amounts of pE1ASV were cotransfected into HeLa cells (Fig. 3A), whereas transcription from the reference plasmid pA56 was unaffected. The effect of the E1A gene products on the activity of the different SV40 enhancers was then studied using a pG1-based reporter gene containing tetramers of the various proto-enhancers previously characterized (see Figs. 2 and 3; Fromental et al. 1988; Kanno et al. 1989). The recombinants, 2GTIIC, GTIIC + GTI, TCII, Sph(II+I) (Fig. 3B–E), and SV40-P (Table 1) contain, in pG1, the tetramers of the respective synthetic oligonucleotides displayed in Figure 2 [note that in the case of Sph(II+I), the wild-type octamer enhancer, which is also present, is known to be inactive in HeLa cells (Davidson et al. 1986; Fromental et al. 1988)]. In all cases, similar profiles of repression by the E1A products were observed (Fig. 3). A summary of the results obtained with 1 μ g of cotransfected pE1ASV is given in Table 1, where the extent of repression is indicated by the bracketed

Figure 3. Repression by E1A proteins of the SV40 proto-enhancers cloned in pG1. HeLa cells were cotransfected with a constant amount (2 μ g) of pG1 recombinant containing the tetramerized proto-enhancers, as indicated, the reference plasmid pA56 (10 μ g), and varying amounts of the pE1ASV plasmid (0.01–5 μ g). For each pG1 recombinant, the results are represented as the ratio of β -globin transcription in the presence and absence of pE1ASV. At the top of each curve, the value in parentheses indicates the activity of each proto-enhancer as a percentage of the activity of the total SV40 enhancer in pG1B. In the case of pG1B, the value in parentheses (400 $\times$) indicates the amount of stimulation, compared with the enhancerless pG1 recombinant. The corresponding quantitative S1 nuclease analysis of cytoplasmic RNA is presented below. Protected fragments of 130–137 and 60 nucleotides correspond to mRNA initiated at the SV40 promoter early–early (EES of the reference plasmid pA56) and β -globin promoter (Glob+1) start sites, respectively. Repression by E1A proteins was tested on the activity of pG1B (A) and the proto-enhancers 2GT-IIC (B), GT-IIC + GT-I (C), TC-II (D), and Sph(II+I) (E). In the case of the proto-enhancer 2GT-IIC, the autoradiograph for 5 μ g pE1ASV is not shown. Transcription from the internal control of pA56 was not affected at any pE1ASV concentration.

Figure 4. Repression of the SV40 proto-enhancers cloned in pG2 by E1A proteins and their derivatives (A) or GAL-VP16 (B). (A) S1 nuclease analysis of cytoplasmic RNA extracted from HeLa cells cotransfected with 2 μ g of pG2, or pG2 recombinants containing either the 72-bp sequence of SV40 enhancer (lanes 1–4) or the tetramerized SV40 proto-enhancers [GT-IIIC + GT-I (lanes 9–12); 2GT-IIIC (lanes 13–16); TC-II (lanes 17–20) and Sph(II+I) (lanes 21–24)] in the absence (–) or in the presence (+) of pE1A[–] (1 μ g), pE1ASV (1 μ g), pE1ASV Δ (1 μ g) or GAL–E1A (AD) (5 μ g), as indicated. Transcription from the enhancerless SV40 early promoter in pA56 (EES signal) and from the enhancerless β -globin promoter in pG2 (lanes 5–8) was not affected by E1A proteins or their derivatives. EES and Glob +1 are as described in the legend to Fig. 3. (B) The experimental procedure is as described in A, except that each pG2 recombinant was cotransfected with (+) or without (–) 2 μ g of GAL–VP16. As a control and to check that the DBD (1–147) of GAL4 does not affect the β -globin transcription, GAL(1–147) was cotransfected with pG2B (lane 11). Note that transcription from the enhancerless SV40 early promoter present in the internal control pA56 (EES signal, lanes 2, 4, 6, 8, and 10), but not that from the enhancerless β -globin promoter in pG2 (lane 2; data not shown), was slightly repressed by GAL–VP16.

numbers. Because of tetramerized P element of the SV40 enhancer has no enhancer activity in HeLa cells (Table 1; Fromental 1989), we also tested the effect of the E1A gene products on two related tetramerized enhancers, the AP-1/PEA1-binding site (Immler et al. 1988, and references therein) of the polyomavirus enhancer, which is also known to be repressed by the E1A proteins (Borrelli et al. 1984; Velcich et al. 1986), and the ‘upstream’ AP-1-binding site of the human metallothionein IIA gene that is important for transcription in vivo (W. Lee et al. 1987) (see Py–AP1 and MTII–AP1 in Fig. 2). The enhancer activity of these tetramerized enhancers was also repressed by the E1A products (Table 1).

The stimulatory activity of the various SV40 tetra-

merized proto-enhancers was similarly inhibited by the E1A gene products when they were inserted ‘at a distance’ from the globin gene promoter in the pG2 recombinant (Fig. 4A; Table 2). Therefore, we conclude from the above results that the activity of all of the SV40 enhancers that are active in HeLa cells is repressed by the E1A proteins.

The possible repression of the SV40 cell-type-specific octamer proto-enhancer, which is active in lymphoid B cells (Davidson et al. 1986; Nomiyama et al. 1987; Fromental et al. 1988; Tanaka et al. 1988), was studied in MPC11 cells using a pG1-based recombinant containing a tetramer of the synthetic oligonucleotide Sph(II+I) + OCT M2 in which the Sph enhancers, but not the

Figure 5. Repression by E1A proteins of activation of transcription by the estrogen and glucocorticoid receptors. S1 nuclease analysis was performed with cytoplasmic RNA extracted from HeLa cells transfected with 2 μ g of the pG2 recombinants (as indicated) in the absence (–) or in the presence (+) of GAL(1–147) (5 μ g) and the different pE1ASV derivatives, pE1A[–] (1 μ g), pE1ASV (1 μ g), pE1ASV Δ (1 μ g), and GAL–E1A(AD) (5 μ g), as indicated. In each case, the ERE–pG2 estrogen reporter gene was cotransfected with the human estrogen receptor expression vector HEO (0.5 μ g) in the presence of E2 (10^{–8} M), whereas the GRE–pG2 glucocorticoid reporter gene recombinant was cotransfected with the glucocorticoid receptor expression vector HGO (1 μ g) in the presence of dexamethasone (DEX, 10^{–7} M). EES and Glob +1 are as described in the legend to Fig. 3.

Table 1. Repression by E1A proteins of the activity of the different proto-enhancers cloned in pG1, in the absence or in the presence of the tumor promoter TPA

Enhancer	Enhancer activity (relative to pG1B)			TPA + E1A
	Control	+ E1A	+ TPA	
pG1B	100	19[81]	135	29[79]
2GTIIIC	34	6[82]	34	6[82]
GTIIC + GTI	76	19[75]	76	19[75]
TCII	84	20[75]	160	38[76]
Sph(II + I)	30	4[87]	30	4[87]
SV40-P	0	0	0	0
Py-AP1	13	5[63]	174	60[66]
MTIIA-AP1	18	3[83]	76	40[47]
KB3	4	2[50]	56	22[61]

The enhancer activity of each recombinant (2 μ g), cotransfected in the presence or absence of TPA (100 ng/ml) with or without pE1ASV (1 μ g, + E1A), was determined by densitometric scanning of several exposures of autoradiograms similar to those shown in Fig. 3 from at least three different experiments. For each proto-enhancer, the average values ($\pm 5\%$) correspond to the relative transcription from the globin start site (Glob + 1), taking pG1B as 100%. Numbers in brackets indicate the percentage of enhancer activity inhibition by E1A proteins.

octamer enhanson, are mutated [Fromental et al. (1988); note that in the reciprocal oligonucleotide Sph(II+I) + OCT M1, the octamer enhanson, but not the Sph enhansons, is mutated; see Table 3]. The octamer enhanson [Sph(II+I) + OCT M2] was repressed in MPC11 cells to the same extent as the whole SV40 enhancer (pG1B) and the TC-II enhanson, which is also known to be active in lymphoid B cells (Kanno et al. 1989). As expected from our previous reports (Davidson et al. 1988; Fromental et al. 1988), the GT-II and GT-I enhansons were inactive in MPC11 cells. We note that in MPC11 cells, the SV40 'enhancerless' pA56 recombinant, which still contains the lymphoid cell-specific

purine box enhancer element (Pettersson and Schaffner 1987), was repressed by the E1A proteins (data not shown), which suggests that this latter enhanson may also be subject to E1A repression.

Finally, the inhibitory effect of the E1A products on the activity of the various SV40 enhansons was studied in the nonlymphoid mouse cell line LMTK⁻ (Table 3). A repression was clearly seen in all cases, although the magnitude of the inhibition (with 1 μ g of transfected pE1ASV) was lower than in HeLa or MPC11 cells, indicating that the E1A repression of the SV40 enhancer activity is not cell-specific, a conclusion that was further supported by the observation that the activity of the

Table 2. Repression by E1A proteins or by GAL-VP16 of the activity of the SV40 proto-enhancers cloned in pG2 and comparison with the repression of the activity of the human ER, GR, and GAL4 yeast activator

Enhancer	Enhancer activity (fold stimulation)			+ GAL-VP16
	Control	+ E1A		
pG2B	100	14[86]		46[54]
pG2	1	1		1
2GTIIIC-pG2	20	3[85]		ND
GTIIC + GTI-pG2	160	24[85]		16[90]
TCII-pG2	64	11[83]		30[53]
Sph(II + I)-pG2	26	5[80]		6[75]
ERE-pG2 + HEO	30	3[90]		ND
GRE-pG2 + HGO	25	2[92]		ND
UAS-pG2 + GAL4	37	4[89]		ND
UAS-pG2 + GAL-VP16	1055	160[85]		ND
UAS-pG2 + GAL-ER(EF)	45	4[91]		ND

Each pG2 recombinant (2 μ g) was transfected either alone or with pE1ASV (1 μ g, + E1A) or GAL-VP16 (2 μ g), as indicated (see also Fig. 4). ERE-pG2 and GRE-pG2 recombinants were transfected with the ER expression vector HEO (0.5 μ g) and the GR expression vector HGO (1 μ g), respectively, in the presence of E2 (10^{-8} M) or DEX (10^{-7} M) (see Fig. 5). UAS-pG2 was cotransfected with GAL4 (the expression vectors of GAL4, 1 μ g), GAL-VP16 (0.2 μ g), or GAL-ER(EF) (1 μ g; see Fig. 6). In the latter case, E2 (10^{-8} M) was added simultaneously. The results obtained (as in Table 1) are expressed as the extent of stimulation of transcription when compared with transcription from the enhancerless globin promoter present in pG2. Numbers in brackets indicate the percentage of enhancer activity inhibition by E1A proteins or by GAL-VP16. (ND) Not determined.

Table 3. Repression by E1A proteins of the activity of different proto-enhancers cloned in pG1 after transfection in MPC11 and LMTK⁻ cells

Enhancer	Enhancer activity (relative to pG1B)			
	MPC11		LMTK ⁻	
	Control	+ E1A	Control	+ E1A
pG1B	100	15[85]	100	43[57]
GTIIIC + GTI	0	0	12	5[59]
2GTIIIC	0	0	20	13[35]
TCII	210	39[81]	108	64[40]
Sph(II + I) + OCT M1	ND	ND	31	16[49]
Sph(II + I) + OCT M2	115	11[90]	ND	ND
KB3	172	43[75]	ND	ND

MPC11 cells were cotransfected with 3 μ g of the pG1 recombinants and 5 μ g of pE1ASV (+E1A), whereas LMTK⁻ cells were cotransfected with 2 μ g of the pG1 recombinants and 1 μ g of pE1ASV, as indicated. For each proto-enhancer, the average values ($\pm 10\%$) obtained (as in Table 1) correspond to the relative transcription from the globin start site (Glob + 1), taking pG1B as 100%. Numbers in brackets indicate the percentage of enhancer inhibition by E1A proteins.

same SV40 enhancer was also repressed in cotransfection experiments carried out in F9(ND) and F9(D) embryonal carcinoma cells (data not shown).

A variety of enhancer elements are repressed by the E1A proteins

The promoter region of MHC class I H-2K^b gene contains a proto-enhancer element whose sequence is related to the SV40 TC-II enhancer (Kanno et al. 1989, and references therein). The H-2K^b proto-enhancer (KB3 in Fig. 2) was tetramerized in pG1, and its activity was studied both in HeLa cells (Table 1), where it was low, and in MPC11 cells, where it was much higher (Table 3). In both cases, it was repressed by the E1A products.

A steroid hormone-responsive element belongs to class D enhancers (Fromental et al. 1988), in that it activates transcription as a monomer in the presence of its cognate liganded hormone receptor, which acts as an inducible enhancer factor (for review and references, see Green and Chambon 1988). ERE-pG2 and GRE-pG2, in which a synthetic estrogen-responsive element (ERE) and a synthetic glucocorticoid-responsive element (GRE) were inserted in pG2 at position -425 (see Materials and methods), are reporter genes for the estrogen receptor (ER) and the glucocorticoid receptor (GR). Cotransfection in HeLa cells of the ER expression vector HEO with ERE-pG2, and of the GR expression vector HGO with GRE-pG2, resulted in enhancement of transcription from the β -globin promoters (Fig. 5; Table 2). A strong repression of both the estrogen and glucocorticoid enhancer activity was brought about by cotransfection of 1 μ g of pE1ASV, but not pE1A⁻ (Fig. 5, lanes 13–15 and 19–21; Table 2).

Like higher eukaryote enhancers, the (UASs) of several yeast promoters are binding sites for *trans*-activating proteins, such as the yeast transcriptional activator GAL4, which interacts with the UAS_G element (for reviews, see Struhl 1987; Guarente 1988). Interestingly, GAL4 functions as an enhancer factor in HeLa cells, where it has been shown to efficiently stimulate tran-

scription from several higher eukaryote promoters to which the UAS_G element had been added (Kakidani and Ptashne 1988; N. Webster et al. 1988a). In UAS-pG2, the UAS_G element was inserted at position -425 upstream from the globin gene start site of pG2. Transfection of pE1ASV into HeLa cells, together with UAS-pG2 and a vector expressing the GAL4 protein (GAL4), resulted in a marked repression of GAL4 *trans*-activation (Fig. 6, cf. lanes 7 and 8), indicating clearly that the E1A proteins can repress the activity of the yeast *trans*-activator GAL4.

The repression function of the E1A gene products was further tested on two chimeric *trans*-activators whose

Figure 6. Repression by E1A proteins of the yeast activator GAL4 and the chimeric activators GAL-VP16 and GAL-ER(EF). S1 nuclease analysis was performed with cytoplasmic RNA extracted from HeLa cells transfected with 2 μ g of the UAS-pG2 reporter gene in the absence (lanes 5 and 6) or in the presence of vectors expressing the *trans*-activators GAL4 (1 μ g, lanes 7 and 8), GAL-VP16 (0.2 μ g, lanes 9 and 10), and GAL-ER(EF) (1 μ g, lanes 11 and 12), as indicated. The transfections were performed in the absence (-) or in the presence (+) of 1 μ g pE1ASV, as indicated. E2, (10^{-8} M) was added to cells transfected with GAL-ER(EF). EES and Glob + 1 are as described in the legend to Fig. 3.

DNA-binding domains (DBD) correspond to that of GAL4 (amino acids 1–147). In GAL–VP16 (Sadowski et al. 1988; Tora et al. 1989), the powerful acidic *trans*-activating domain (amino acids 411–491) of the herpesvirus VP16 protein is associated with GAL4[1–147]DBD. In GAL–ER(EF), the estradiol (E2)-inducible activating domain present in the ER region (EF), which also contains the hormone-binding domain, is associated with GAL4[1–147]DBD (N. Webster et al. 1988b; Tora et al. 1989). The enhancer activity of both chimeric *trans*-activators was markedly inhibited in HeLa cells when UAS–pG2 and pE1ASV were cotransfected with the GAL–VP16 and GAL–ER(EF) expression vectors (Fig. 6, lanes 9–12; Table 2).

Because the promoter of the parental vector that was used to express the estrogen and glucocorticoid receptors GAL4, GAL–VP16, and GAL–ER(EF) contains the SV40 enhancer (Breathnach and Harris 1983), it was important to check that the inhibition by the E1A products did not result from decreases in the amounts of *trans*-activators synthesized in the transfected HeLa cells. This was done in the case of the estrogen receptor (Fig. 7). The amount of receptor synthesized in the pE1ASV cotransfected cells was not decreased; in fact, we found that it was increased, using either a gel retardation assay with a ³²P-labeled ERE (Fig. 7A, cf. +E1A and –E1A) or Western immunoblotting (Fig. 7B, in which the upper band was nonspecific). The reason for this unexpected, but reproducible, increase in ER protein is unknown.

The magnitude of the E1A repression is not affected by treatment with the tumor promoter TPA

Phorbol esters, such as TPA, which activate the protein kinase C signal pathway (Nishizuka 1988), are known to stimulate the activity of a number of (but not all; see Table 1) proto-enhancers, notably in HeLa cells. Thus, the activity of the TC-II and H-2K^b enhancers is stimulated ~2-fold and ~14-fold, respectively (see Kanno et al. 1989), whereas that of the Py–AP1 enhancer (Imbler et al. 1988) is increased ~13-fold and that of the MTIIA–AP1 enhancer (Imagawa et al. 1987) is stimulated ~4-fold (Table 1). In all four cases, the magnitude of the E1A repression was not markedly affected by the TPA treatment. Moreover, it was apparently independent of the extent of the stimulation brought about by TPA (Table 1). In contrast to the report of Chiu et al. (1987), we did not find stimulation of the activity of the SV40 P element by TPA. The reason for this discrepancy is not known, but we note that in our case, the polymerized P element is flanked on its 5' side by the natural SV40 bases.

Enhancer repression does not require the acidic region of E1A proteins

Some transcriptional *trans*-activators, such as GAL4 and VP16, contain an acidic activating domain (for review, see Ptashne 1988). Moreover, it has been shown that the very potent acidic *trans*-activator GAL–VP16 (see above) can efficiently inhibit transcription from pro-

Figure 7. Gel retardation assay (A) and immunoblotting assay (B) of the ER in HeLa whole cell extracts. HeLa cells were cotransfected with ERE–pG2 (2 µg) and HEO (the vector expressing the estrogen receptor; 0.5 or 1 µg), in the absence (–) or in the presence (+) of pE1ASV (1 µg), as indicated. Whole cell extracts were prepared as described in Materials and methods and subjected to gel retardation (A) or immunoblotting (B). In A, the arrow indicates the localization of the band corresponding to the specific receptor complex. The control corresponds to an extract from HeLa cells cotransfected with ERE–pG2 and pE1ASV recombinants but not HEO. In B, proteins from whole cell extracts (2 µg) were resolved on a 10% polyacrylamide gel in the presence of SDS, electrotransferred to a nitrocellulose filter, and probed with an ER (F domain)-specific monoclonal antibody (Metzger et al. 1988). Filters were then incubated with rabbit anti-mouse antibodies, and the bands were visualized with ¹²⁵I-labeled protein A (Metzger et al. 1988). The arrow indicates the 67-kD ER. Note that the upper band, which is nonspecific, is present in the control lane.

motors lacking the cognate GAL4-binding sites (Gill and Ptashne 1988; Ptashne 1988). This phenomenon, called ‘squenching’, was reported (Gill and Ptashne 1988) to depend on the ‘strength’ of the acidic activating domain. Could such squenching account for repression by E1A? To test this hypothesis, we first studied the effect of GAL–VP16 expression on the activity of the SV40 enhancer and of its different enhancers using the pG2 series (Fig. 4B; Table 2), which does not carry a GAL4-binding site. It is clear that transfection into HeLa cells of 2 µg of a vector expressing GAL–VP16, together with reporter genes containing the whole SV40 enhancer (pG2B) or tetramers (in pG2) of either the GT-IIC + GT-I, the TC-II, or the Sph (II + I) proto-enhancers, resulted in repression of enhancer activity in all cases. Note here that the activity of the enhancerless SV40 promoter present in the pA56 reference recombinants (EES signal) was also reproducibly repressed by GAL–VP16, whereas no inhibition was seen for the globin promoter present in pG2 (Fig. 4B; data not shown).

The above results prompted us to investigate whether the acidic regions that are present between amino acids 126 and 148 of the 289R protein and amino acids 126

and 138 of the 243R protein (Fig. 1) could be responsible for the E1A repression of enhancer activity. We first constructed an expression vector, GAL-E1A(AD), in which the GAL4[1–147]DBD is fused with the *Clal*–*SmaI* fragment of the E1A gene (Fig. 1) which encodes the E1A amino acids 122–151. GAL-E1A(AD) did not repress the activity of the various SV40 enhancers in cotransfection experiments (Fig. 4A, lanes 4, 12, 16, 20, and 24; Fig. 5, lane 5), nor that of the ER and GR (Fig. 5, lanes 17 and 23). However, GAL-E1A(AD) could stimulate the expression of a reporter recombinant containing GAL4-binding sites (17M2-GLOB-CAT; see N. Webster et al. 1988b), but to a lesser extent than that achieved with GAL4 (approximately three to fourfold; data not shown).

To confirm that the acidic amino acid stretch present in the E1A proteins was not responsible for the enhancer repression, we constructed the vector pE1ASVΔ, in which the *Clal*–*SmaI* segment present in pE1ASV was deleted, resulting in the expression of a single E1A protein similar to the 289R protein, but deleted for amino acids 123–150, and therefore lacking the acidic amino acid stretch present in this region. The protein encoded in pE1ASVΔ repressed the activity of the whole SV40 enhancer, its different enhancers, and the ER and GR as efficiently as pE1ASV (Fig. 4A, lanes 3, 11, 15, 19, and 23; Fig. 5, lanes 4, 16, and 22). Thus, the acidic region of the E1A proteins does not appear to be essential for enhancer repression. However, it is important for E1A *trans*-activation, as suggested previously by Lillie and Green (1989). Indeed, pE1ASVΔ could not *trans*-activate the Ad2 E2 promoter under conditions where efficient *trans*-activation was readily achieved with pE1ASV (Fig. 8, cf. lanes 3 and 4).

Discussion

We have addressed the question of whether the repression of the SV40 enhancer by the E1A gene products can be ascribed specifically to one of its constituent proto-enhancers whose synergistic effects generate the overall enhancer activity. By studying enhancer elements separately, we demonstrate that they can all be repressed. The E1A gene products inhibit the activity of class A (GT-IIC, Sph-II, Sph-I), class B (GT-I), and class C (TC-II, octamer) enhancers (Tables 1–3; Figs. 3 and 4). Furthermore, our study clearly shows that the inhibitory effect of the E1A gene products on enhancer activity is not limited to the SV40 enhancers. The activities of the polyomavirus and human metallothionein enhancers, which bind the AP-1 factor, and the activity of the MHC class I gene enhancer H-2K^b, which binds the TC-IIB/KBF1/H2TF1 protein (Kimura et al. 1986; Baldwin and Sharp 1987, 1988; Macchi et al. 1989, and references therein), are also repressed by the E1A gene products. Moreover, our results demonstrate that repression by the E1A gene products is not restricted to enhancers composed of multiple proto-enhancers (e.g., those of SV40, polyomavirus, immunoglobulin, and insulin genes; for references, see introductory section) but can

also affect *trans*-activators binding to class D enhancers that possess enhancer activity independently (Fromental et al. 1988), for example, the glucocorticoid receptor and the estrogen receptor (Fig. 5; Table 2).

Our results suggest that the E1A gene products could repress all enhancer-binding *trans*-activator proteins. Thus, the activity of the yeast enhancer factor GAL4, when expressed in HeLa cells, as well as the activity of the chimeric *trans*-activators GAL-VP16 and GAL-ER(EF), are repressed by E1A. Interestingly, although both GAL4 and GAL-VP16 *trans*-activating domains contain acidic amino acid stretches (Kakidani and Ptashne 1988; Sadowski et al. 1988, and references therein; N. Webster et al. 1988a), the estrogen receptor *trans*-activating domain present in GAL-ER(EF) is not acidic and exhibits activating properties different from those of GAL4 or GAL-VP16 (Tora et al. 1989; Webster et al. 1989).

Our results indicate clearly that the inhibition of the activity of the ER by the E1A gene products is not attributable to a decrease in the amount of receptor synthesized from the cotransfected ER expression vector HEO. This is an important control result, because the promoter of the parental expression vector that was also used to express the glucocorticoid receptor, GAL4, GAL-VP16, and GAL-ER(EF), contains the SV40 enhancer, which may have resulted in decreases in the amounts of the *trans*-activators synthesized in HeLa cells cotransfected with the E1A expression vector. We cannot, however, perform similar control experiments in the case of the enhancer factors that mediate the activity of the SV40, polyomavirus, metallothionein, and H-2K^b enhancers. Thus, at the present time, we cannot exclude the fact that the inhibition of the activity of these enhancers reflects a repression of the transcription of the genes that encode their cognate factors.

How the E1A gene products can repress the activity of a wide variety of enhancers is not known. The E1A proteins do not appear to bind directly to DNA (Ferguson et al. 1985; Lillie and Green 1989, and references therein), and the sequence diversity of the repressed enhancers excludes the existence of a common DNA target for repression. Similarly, it is unlikely that the E1A proteins act by interacting directly with the various *trans*-activating factors that mediate the activity of the different enhancers. An induced phosphorylation of some *trans*-acting factors has recently been implicated in activation of transcription by E1A (Bagchi et al. 1989; Jones and Jones 1989; Raychaudhuri et al. 1989). One may speculate that such an E1A-induced phosphorylation may result in the repression of a different set of *trans*-activators or possible intermediary factor(s) mediating their activity (Fromental et al. 1988; Meyer et al. 1989; Tora et al. 1989). We note, however, that the phorbol ester (TPA)-induced activation of several enhancers, which is presumably mediated by protein kinase C, is not repressed by the E1A gene products (Table 1). Additional examples of induction of E1A-repressed promoters by phorbol esters and growth factors have been reported recently (Offringa et al. 1988; Timmers et al. 1989).

Alternatively, the E1A proteins may block the function of enhancer factors by binding to a protein that is necessary to mediate their activity. The repression function of the E1A gene products is present in both the 243R and 289R E1A proteins [e.g., see Borrelli et al. (1984); note that in all of the cases studied here the same repressions were observed by cotransfecting the expression vectors that encode either the 243R (vector pE1A12S) or the 289R (vector pE1A13S) proteins (C. Rochette-Egly, unpublished data)]. However, the 289R protein is a much better *trans*-activator than the 243R protein (for references, see introductory section). Therefore, it is unlikely that E1A repression results from the phenomenon referred to as squelching, that is, direct competition for a transcription factor present in limiting amount by an excess of an activating domain—an acidic domain such as that present in the VP16 protein (Gill and Ptashne 1988). Interestingly, although expression of the VP16 acidic domain present in GAL–VP16 can inhibit the activity of the SV40 enhancer and enhancers (Table 2; Fig. 4B; note that the TC-II enhancer appears to be less sensitive to VP16 than to E1A inhibition), deletion of the acidic amino acid stretch, which is located across the border of domain 2 and 3 of the E1A gene products (vector pE1ASVΔ, in which amino acids 123–150 are deleted; see Fig. 1), does not affect E1A repression. In contrast, the protein encoded by pE1ASVΔ has lost totally its *trans*-activating function (Fig. 8), in agreement with previous reports demonstrating the importance of E1A-conserved region 3 in E1A-induced activation of transcription (for references, see introduction section; legend to Fig. 1; Lillie and Green 1989). That, however, the acidic region of the 289R protein is neither a strong activator (data not shown) nor a squelcher of its own (Fig. 4A) is indicated by the results of transfection

experiments with GAL–E1A(AD), which encodes amino acids 122–151 of E1A.

Three nuclear proteins, 105K, 107K, and 300K, are known to bind to the E1A gene products (Egan et al. 1988, 1989; Whyte et al. 1988, 1989; Fig. 1). Two of them (107K and p105-RB) require E1A amino acids 121–127 for binding. The present results obtained with the expression vector pE1ASVΔ, in which the DNA sequences encoding amino acids 123–150 have been deleted (see above), indicate clearly that the E1A-conserved region 2 is dispensable for repression, in agreement with previous reports (Kuppuswamy and Chinnadurai 1987; Velcich and Ziff 1988; Jelsma et al. 1989; Sogawa et al. 1989). Because the conserved region 3 and exon 2 are also dispensable [see the preceding references and those in Fig. 1 and our unpublished data (C. Rochette-Egly) showing that a deletion of amino acids 121–223 does not affect the extent of repression], this repression could be attributable to the binding of the E1A gene products to the cellular 300K protein, thus blocking its activity. The function of the 300K protein is not known. Our present results suggest that it may be an essential common intermediary factor in the pathways mediating the activation function of the various enhancer-binding factors (see also Tora et al. 1989).

Materials and methods

Recombinant plasmids

The various individual motifs of the SV40 enhancer were chemically synthesized, tandemly tetramerized, and inserted into plasmids pG1 or pG2, containing the rabbit β -globin gene and its promoter region at positions –109 and –425, respectively, as described previously (Fromental et al. 1988; Kanno et al. 1989). The estrogen reporter gene ERE–pG2 was constructed by inserting in pG2 the synthetic oligonucleotide 5'-TCGAGCAAAGTCAGGTCACAGTGACCTGATCAAAT-3', flanked by *Xho*I and *Xba*I restriction enzymes sites in 5' and 3', respectively. The glucocorticoid reporter gene GRE–pG2 was constructed by inserting the *Xba*I–*Sac*I fragment of the PTG plasmid (Meyer et al. 1989) into the *Xba*I–*Sac*I site of the polylinker of pG2. The activation vectors HEO and HGO (corresponding to the eukaryotic expression vector pKCR2 expressing the human estrogen and glucocorticoid receptors, respectively) have been described previously (Breathnach and Harris 1983; Green et al. 1986; Kumar et al. 1987). The reporter gene UAS–pG2 and the GAL4 and GAL–ER(EF) expression vectors have been described (N. Webster et al. 1988a,b), as well as the GAL–VP16 expression vector (Tora et al. 1989).

The recombinant plasmids pE1ASV and pE1A[–] contained the entire E1A transcription unit (including the enhancer sequences) and only the E1A promoter region (Borrelli et al. 1984), respectively. pE1ASVΔ was constructed by excision of the *Cla*I–*Sma*I fragment (between nucleotides 916 and 1006) from the pE1ASV plasmid and religation by inserting a synthetic *Cla*I linker containing a *Xho*I site (5'-CGATCTCGAG-3'). GAL4–E1A(AD) was constructed first by cloning a synthetic *Cla*I–*Sma*I linker (5'-CGCTTCCCATCGATCTCTCCCGGGT-3') downstream of GAL4(1–147) between the *Cla*I–*Xho*I sites of pG4Mpoly (S. Green et al. 1988; Webster et al. 1989) and then inserting the *Cla*I–*Sma*I fragment of the pE1ASV plasmid.

Figure 8. Stimulation of adenovirus E1aE RNA synthesis by E1A proteins. The LS WTE1aE recombinant (Zajchowski et al. 1985) containing the E1aE sequences extending between –250 and +719 inserted in front of the rabbit β -globin sequence was cotransfected (5 μ g) with the internal control pA56 (10 μ g), either alone (lane 1) or together with 2 μ g of pE1A[–] (lane 2), 2 μ g of pE1ASV (lane 3), 2 μ g of pE1ASVΔ (lane 4), or 5 μ g of GAL–E1A(AD) (lane 5). Cytoplasmic RNA was analyzed by S1 nuclease mapping with the appropriate ³²P-5'-end-labeled single-stranded DNA probe. Specific transcripts initiated at the major E1aE start site, E1aE1, gave a protected fragment of 40 nucleotides.

Cell lines

HeLa cells were grown as described (Zenke et al. 1986). MPC11, LMTK⁻ and F9 cells were grown in Dulbecco's modified Eagle minimum essential medium (GIBCO), containing 10% fetal calf serum and 500 units penicillin, 400 µg gentamycin, and 100 µg streptomycin per milliliter. F9(D) cells were obtained by treatment of F9(ND) cells with retinoic acid (0.1 µM) for 4 days before transfection (Nomiya et al. 1987). TPA treatments after transfection were performed essentially as described by Imagawa et al. (1987).

DNA transfection and quantitative S1 nuclease analysis

HeLa, LMTK⁻, F9, and MPC11 cells were seeded and transfected as described by Fromental et al. (1988) and by Kanno et al. (1989). Calcium-phosphate-mediated transfections of all cell lines included the reference plasmid pA56 (Zenke et al. 1986; see Results), the appropriate derivatives of pG1 or pG2, pE1ASV, and plasmid carrier DNA to adjust the total quantity to 17 µg/9-cm dish.

Cytoplasmic RNA was isolated after lysing the cells with 0.5% NP-40 (Groudine et al. 1981), and 20 µg RNA was hybridized overnight at 68°C with an excess of single-stranded ³²P-5'-end-labeled probe (Zenke et al. 1986) in 10 mM PIPES (pH 6.5) and 400 mM NaCl. After a 2-hr S1 nuclease digestion at 25°C (200 U/64 µg of RNA), the protected fragments were analyzed by electrophoresis on an 8% denaturing polyacrylamide gel. Different exposures of the autoradiographs were densitometrically scanned. In all cases, the results corresponded to the average values obtained from at least three independent transfection experiments, using at least two different plasmid preparations.

Gel retardation assay for binding of ER to its ERE

Transfected cells were washed with chilled PBS, collected, and lysed by three successive freezings (at -80°C) and thawings in 20 mM Tris-HCl (pH 9.9), 25% glycerol, 0.1 mM EDTA, and 400 mM KCl (Kumar and Chambon 1988). After centrifugation at 8000g for 20 min at 4°C, the whole-cell extract (WCE) supernatant was collected and protein concentration determined by the Bradford assay (Bradford 1976). The supernatants were aliquoted, frozen in liquid nitrogen, and stored at -80°C.

For gel retardation assay, aliquots of proteins (5 µg) were mixed with an appropriate amount of poly[d(I-C)] (usually 5 µg) and incubated in a 20-µl reaction volume containing 15 mM HEPES (pH 7.9), 0.4 mM DTT, 0.2 mM EDTA, 80 mM KCl, and 4% glycerol for 15 min at 0°C. The binding reaction, initiated by adding a ³²P-5'-end-labeled synthetic oligonucleotide probe (4 × 10⁴ cpm; probe A in Kumar and Chambon 1988), was incubated at 25°C for 15 min. DNA-protein complexes were then separated at 20°C in a 5% nondenaturing polyacrylamide gel containing 6.6 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, and 1 mM EDTA at 25 mA with buffer recirculation.

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