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Two functions encoded by adenovirus early region 1A are responsible for the activation and repression of the DNA-binding protein gene

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Human adenovirus early region 1A (E1A) gene products differentially regulate the expression of early region 2A (E2A) encoding the DNA-binding protein (DBP). In a microinjection system, plasmids containing the DBP gene associated with both its early (map coordinate 75) and late (coordinate 72) promoters, or only with the early promoter, are inefficiently expressed, and the presence of E1A DNA is required for full expression. In contrast, the E2A plasmid in which the DBP gene is associated solely with its late promoter, efficiently produces DBP, the synthesis of which is significantly inhibited by an E1A gene product. To identify which of the E1A products is responsible for either activation or repression of DBP gene expression, two E1A mutants (Ad5*hr*1 and Ad2/5pm975) have been tested in the microinjection system in the presence of different DBP plasmids containing either one or both promoters. The results obtained indicate that the product encoded by the E1A 13S mRNA is responsible for the stimulation of DBP produced from the early promoter and that the 12S mRNA codes for the product which represses the synthesis of DBP from the late promoter. These results were confirmed using clones in which the E2A early or late promoter was associated to the chloramphenicol acetyltransferase (CAT) gene and assayed for CAT activity after cell transfection in the absence or in the presence of wild-type or mutant E1A plasmids, and we have also shown that this promoter-dependent regulation is reflected in the relative amount of specific DBP mRNA.

Key words: adenovirus/microinjection/regulation/transcription

Introduction

It is known that early region 1A of adenovirus encodes multifunctional products. Studies utilizing adenovirus type 2 (Ad2) and 5 (Ad5) mutated in this region have indicated that these proteins are required for transformation of rat embryo cells, establishment of primary cells in culture, replication of viral DNA and for the expression of the other early regions (Harrison *et al.*, 1977; Jones and Shenk, 1979a, 1979b; Berk *et al.*, 1979; Houweling *et al.*, 1980; Carlock and Jones, 1981; Nevins, 1981; Montell *et al.*, 1982; Rossini, 1983; Ruley, 1983).

Two mRNAs (12S and 13S), resulting from differential splicing during RNA processing, are transcribed from early region 1A at early times during infection (Berk and Sharp, 1978; Chow *et al.*, 1979; Kitchingman and Westphal, 1980). They code for proteins of 243 and 289 amino acids, respectively (Perricaudet *et al.*, 1979) which have their amino- and carboxy-terminal portions in common, and differ in 46 amino acids unique to the larger product. A major functional role has been assigned to the product encoded by the 13S E1A mRNA in the activation of transcription of other early regions (Berk *et al.*, 1979; Carlock and Jones, 1981; Ricciardi *et al.*, 1981; Montell *et al.*, 1982; Leff *et al.*, 1984; Ferguson *et al.*, 1984).

We have explored one aspect of adenoviral early gene regulation, the interaction of early region 1A with early region 2A that encodes the 72 kd DNA-binding protien (DBP). The E2A gene is transcribed from different promoters at different times during the infectious cycle. At early times, the majority of transcripts have 5' ends at coordinate 75. At late times the DBP mRNA is transcribed primarily from its late promoter located at coordinate 72 (Westphal and Lai, 1977; Chow *et al.*, 1979). Results recently presented have revealed a dual opposite effect of the E1A gene products in the regulation of DBP gene expression. It was shown that, in a microinjection system, an E1A product(s) stimulates the synthesis of DBP transcribed from the early promoter and inhibits the expression of DBP from its late promoter (Rossini, 1983).

As a tool to dissect E1A functions and to assign to each of the two mRNA products a possible role in the regulation of DBP gene expression, we have used the E1A plasmid derived from two different mutant viruses: Ad5*hr*1 and Ad2/5pm975. The Ad5 mutant *hr*1 has a single base pair deletion which results in a reading frameshift and termination of the protein coded by the 13S mRNA while the protein coded by the 12S mRNA is not affected (Esche *et al.*, 1980; Ricciardi *et al.*, 1981). The Ad2/5pm975 has a single base transversion at nucleotide 975 which eliminates the 12S mRNA splicing but leaves the 13S mRNA and its product unchanged (Montell *et al.*, 1982). The experiments described here indicate that the 13S mRNA product is responsible for the activation of the E2A early promoter and

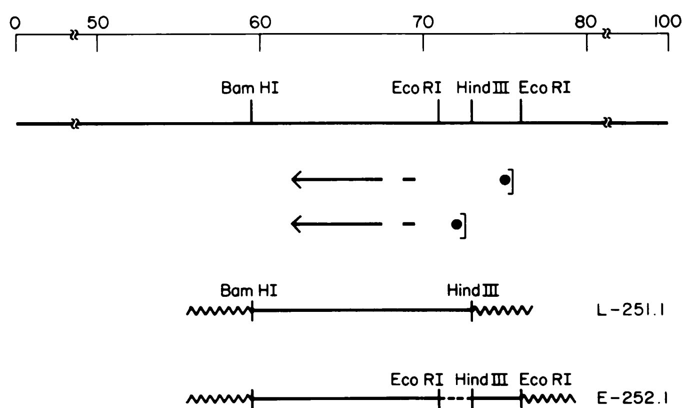

Fig. 1. Plasmids containing the DBP gene associated with its early or late promoter. A simplified depiction of transcripts from early region 2A is shown. At early times during infection, the majority of mRNAs have 5' ends at map coordinate 75 and at late times at coordinate 72. The plasmid L-251.1 contains sequences encoding the DBP late transcription unit. The plasmid E-252.1 contains sequences comprising the DBP early transcription unit which has been deleted in the late promoter region between coordinate 70.7 and 72.8. The construction of these plasmids is described in Materials and methods.

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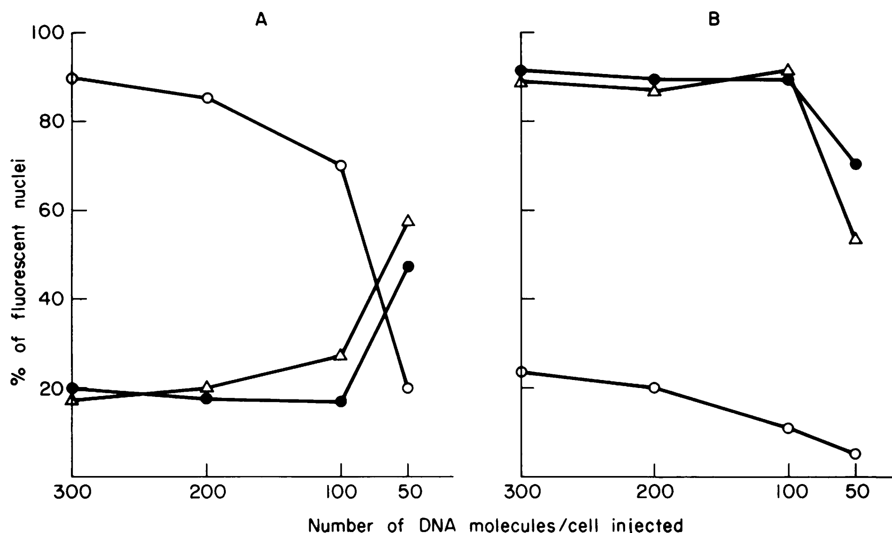

Fig. 2. Effect of gene dosage on the expression of the DBP gene. Nuclear microinjection of thymidine kinase-deficient hamster cells, TK$^-$ ts13 (Jonak and Baserga, 1980) were performed according to Graessmann *et al.* (1980). Cells were cultured as previously described (Rossini, 1983). About 10^{-11} ml of plasmid DNAs suspended in 10 mM Tris-HCl pH 7.2 at a given concentration were injected. 20 h after microinjection, cells were fixed and stained with antibody specific for the 72 kd DBP as described in Materials and methods. The ordinate indicates the percentage of nuclei positive for the E2A DNA binding protein. The abscissa indicates the number of DNA molecules injected. (**Panel A**) ○——○ L-251.1. (E2A-late promoter); ●——● L-251.1 and HE4 (E1A), the concentration of which was kept constant at 200 copies/cell; △——△ L-251.1 at a constant concentration of 200 copies/cell and HE4 as indicated in the abscissa. (**Panel B**) ○——○ E-252.1 (E2A-early promoter); ●——● E-252.1 and HE4 (E1A), the concentration of which was kept constant at 200 copies/cell; △——△ E-252.1 at a constant concentration of 200 copies/cell and HE4 as indicated in the abscissa.

the product of the 12S mRNA causes repression of the E2A late promoter.

Results

Expression of DBP by microinjection analysis

Effect of gene dosage. Two E2A recombinants, L-251.1 and E-252.1 (Figure 1), containing the DBP gene associated respectively with its late or early promoter were injected in varying amounts into Tk$^-$ts13 nuclei to analyze the effect of gene dosage on the expression and regulation of the DBP gene. These are recombinants which contain the complete promoter, coding sequence and polyadenylation for the synthesis of DBP. Thus, we directly analyzed the presence of DBP by immunofluorescence of the injected cells.

The results of such experiments are shown in Figure 2. The percentage of cells producing DBP after injection of the late promoter clone, L-251.1, was found to be high ($\sim$85%) for most of the DNA concentrations tested (Figure 2, panel A), decreasing to $\sim$20% only when 50 copies/cell were injected and being undectable at lower concentrations. In contrast, the expression of DBP after injection of the E2A early promoter clone E-252.1 was very poor with a maximum of 20–30% positive cells when injected with 300 molecules/cell (Figure 2, panel B). The presence of E1A DNA (HE4, coordinate: 0–4.3; Stow, 1981) at a concentration of 200 copies/cell, led to an increase in the percentage of positive cells for all the concentrations of E-252.1 injected. Consistent with our previous finding, a decrease in the number of cells producing DBP was observed after microinjection of different concentrations of L-251.1 in the presence of E1A DNA. Surprisingly, the presence of E1A DNA positively affects the percentage of cells producing DBP after injection of 50 copies/cell of L-251.1 with an increase from 20% to $\sim$50%. Injection of pBR322 DNA does not affect the expression of DBP (data not shown), suggesting a rather specific action. This stimulatory effect could be a result of an unbalanced ratio (1:4) of the two genes, since it is eliminated when the amount of E1A DNA is reduced (100 and 50 molecules/cell; data not shown). The inhibitory and stimulatory effects of the E1A plasmid were equally effective at E1A DNA concentrations of 300, 200 and 100 molecules/cell, slightly decreasing at a concentration of 50 molecules/cell and virtually absent at a concentration of 25 copies/cell.

Effect of E1A mutant plasmids. To define which of the E1A products is responsible for the activation and/or repression of the DBP gene, two E1A mutant plasmids derived from Ad5*hr*1 and Ad2/5pm975 viruses, were used in the microinjection system.

The E1A mutant plasmid, phr1, (derived from Ad5*hr*1 virus) in which the mutation affects the 13S mRNA product, but not the product of the 12S mRNA (Esche *et al.*, 1980 Ricciardi *et al.*, 1981) was co-injected with the DBP early promoter clone (E-252.1; Figure 3, panel A) or with the DBP late promoter clone (L-251.1; Figure 3, panel B). As shown in Figure 3A, when compared with the wild-type (HE4) plasmid, the mutant has lost the ability to stimulate the synthesis of DBP. Only 15–20% of the injected nuclei were positive, reflecting the basal level observed in the absence of E1A DNA.

However, phr1 has maintained the wild-type property of repressing DBP production from the late promoter (Figure 3, panel B). The percentage of nuclei positive for DBP decreases from 80% to 15%. These results suggest that the E1A 12S mRNA product is sufficient for the repression of DBP synthesis from the late promoter but is not necessary for the stimulation of the DBP early promoter.

Panels A and B of Figure 3 also show the results of experiments in which a second E1A plasmid (pEK pm975, derived from Ad5/2pm975) was tested for its effect on DBP expression. The pEK pm975 mutant produces only the 13S mRNA but not the 12S mRNA (Montell *et al.*, 1982). When this plasmid was co-injected with the E2A early promoter clone the same extent of stimulation of DBP production as the wild-type E1A plasmid was observed (Figure 3, panel A). Co-injection of pEKpm975 with the E2A late promoter clone had no effect on the production of

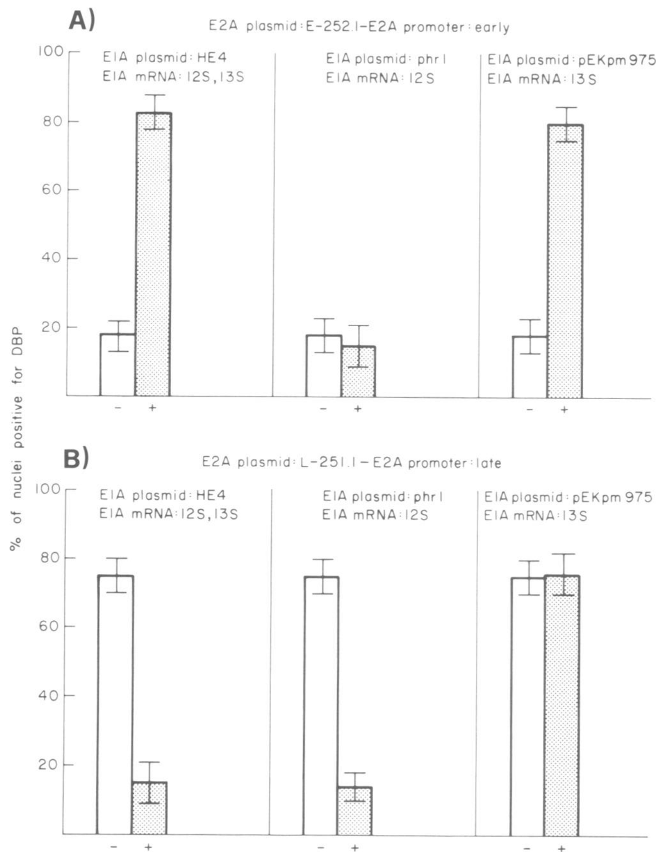

Fig. 3. Effect of E1A mutations on the expression of E2A plasmids containing the DBP gene associated with the early or the late promoter. The E2A plasmids E-252.1 (**top panel**) or L-251.1 (**bottom panel**) described in the text were injected into Tk^{-}ts13 cell nuclei in the absence (open bar) or in the presence (stippled bar) of the E1A wild-type or mutant plasmids as indicated in the figure. The wild type mRNAs and proteins produced by each E1A plasmid are also indicated. About 200 copies/cell of each plasmid was injected. The ordinate indicates the percentage of nuclei that stain positively for the E2A DNA binding protein. The average percentage of positive nuclei and standard deviations (arrow bar) were calculated from five to 10 separate experiments.

DBP from the late promoter (Figure 3, panel B).

These results suggest that the product of the 13S mRNA but not the protein encoded by the 12S mRNA is responsible for the activation of the DBP synthesis from its early promoter.

The results obtained with phr1 and pEKpm975 were reproduced using cDNA clones (constructed by Drs. Brad Zerler and Elizabeth Moran, Cold Spring Harbor Laboratory) containing the E1A 12S and 13S coding regions, respectively (data not shown), suggesting that this effect is not due to the point mutations themselves, but to the presence of specific E1A translation products.

DBP mRNA in transfected cells

The results obtained by microinjection suggest that the role of the 12S mRNA product is to repress the DBP gene expression from its late promoter. To determine whether this repression is exerted at the transcriptional or post-transcriptional level, we measured the relative amounts of DBP mRNA synthesized from the E2A late promoter in HeLa cells transfected with L-251.1 plasmid in the absence or presence of wild-type or mutant E1A plasmid DNAs. Specific DBP mRNA was identified by hybridization of total mRNA from transfected cells to a complementary RNA prepared *in vitro* by SP6 promoter-dependent transcription of the 5′ end leader sequences of the E2A late transcription unit (Figure 4). The resulting hybrid should yield an RNase-resistant fragment of 67 nucleotides in length (Baker *et al.*, 1979; Baker and Ziff, 1981; Kruijer *et al.*, 1981). The results of the experiment shown in Figure 5 indicate that cells transfected with L-251.1 alone (lane 1) synthesize amounts of DBP-specific mRNA which are comparable with that made in the presence of the E1A mutant defective for the generation of the 12S mRNA (pEKpm975, lane 3). In contrast, production of the DBP late transcript is significantly decreased in cells co-transfected with the wild-type E1A plasmid HE4 (lane 2) or with its mutant, phr1 (lane 4), which produces only an intact 12S mRNA product. As internal control, equal amounts of the same RNA preparation used to analyze the E2A transcripts were hybridized with the E1A probe, SP1AXO (Figure 4). The 162 nucleotide RNA fragment unique to the E1A 12S mRNA is not generated in the case of pEKpm975-transfected cell RNA. The relative amount of the predicted E1A-specific 107, 162, and 300 nucleotide fragments is similar in RNA samples extracted from cells transfected either with the wild-type or mutant E1A plasmids. Despite the frameshift mutation in phr1, a 13S message is synthesized in cells transfected with this mutant (lane 7), as already shown in Ad5*hr*1-infected cells (Berk *et al.*, 1979).

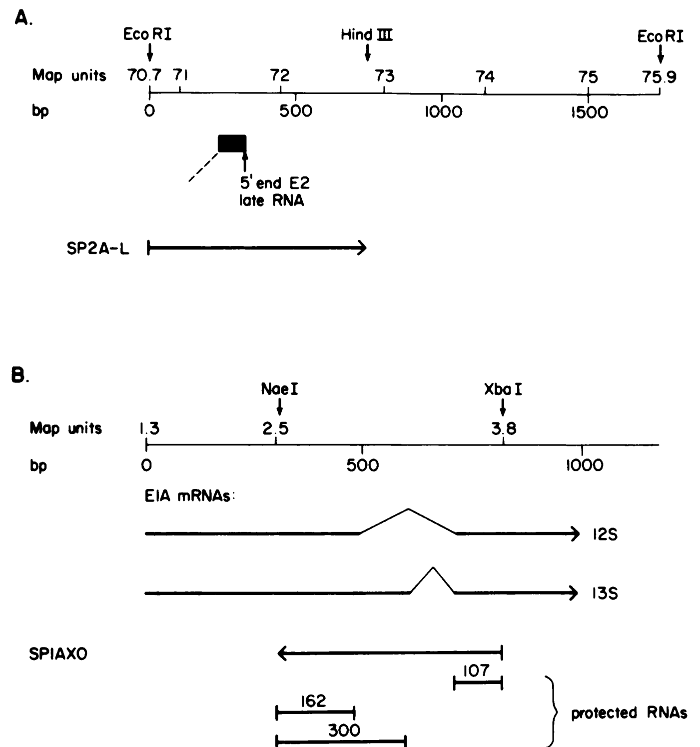

Fig. 4. SP6 probes used to analyze the mRNAs transcribed from early region 1A and 2A. The plasmid was constructed as described in Materials and methods. Adenovirus-2 *Eco*RI-*Hind*III fragment containing the 5′ end leader sequences of the E2A late transcription units was inserted into plasmid containing the bacteriophage SP6 promoter. This plasmid was used as templates for SP6 polymerase-mediated run-off transcription to generate the RNA probe SP2A-L, as shown. The plasmid SP1AXO was obtained from E.Ruley. The *Nae*I-run off product protects the regions of the E1A 12S and 13S mRNAs indicated.

These results strongly suggest that the repressed levels of DBP mRNA synthesized from the E2A late promoter in the presence of E1A is the consequence of expression of the 12S mRNA product.

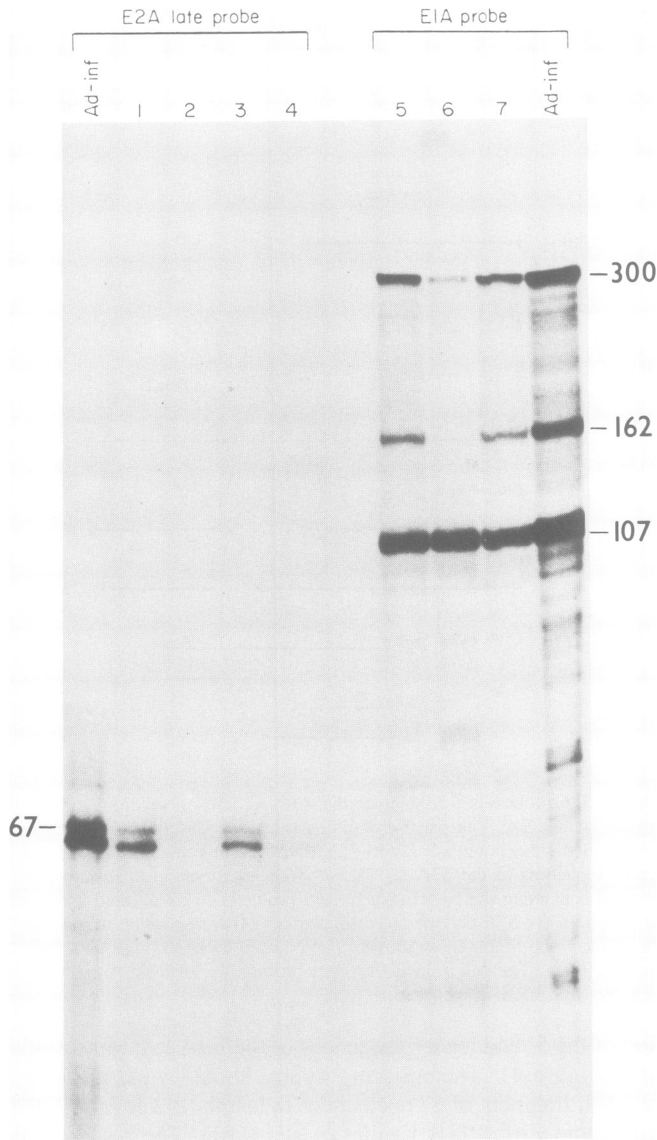

Fig. 5. Effect of E1A mutants on DBP mRNA transcribed from the E2A late promoter in transfected cells. Transfection was performed with calcium phosphate precipitates containing 5 μg of L-251.1 and 25 μg of either pBR322 (**lane 1**), HE4 (**lanes 2, 5**), pEKpm975 (**lanes 3** and **6**), or phr1 (**lanes 4** and **7**). This E2A:E1A ratio was found to be the most effective in achieving either the activation of the E2A early promoter plasmid or the repression of the E2A late promoter clone (data not shown). All precipitates were normalized to 30 μg DNA using pBR322 DNA. At 36 h post-transfection, mRNA was isolated and hybridized to 5 x 10^5 c.p.m. of either the SP2A-L probe (**lanes 1–4**) or the SP1AXO probe (**lanes 5–7**) and RNase treated as described in Materials and methods. Hybridization to adenovirus-infected cell RNA (**lanes 1** and **5**) was performed to provide a positive control marker. The protected RNAs were denatured and electrophoresed on an 8 M urea-8% polyacrylamide gel and subjected to autoradiography. The protected RNAs co-migrate with the adenovirus-infected cell RNA marker. The 107, 162 and 300 nucleotides corresponding to the E1A mRNAs (see text) are indicated.

Expression of chloramphenicol acetyltransferase coding sequences from the E2A promoters

To determine if the sequences responsive to E1A regulation are confined to the E2A promoter regions, we carried out transfection experiments using plasmids containing the E2A early or late promoter associated with the bacterial chloramphenicol acetyltransferase (CAT) gene. The expression of the chimeric gene

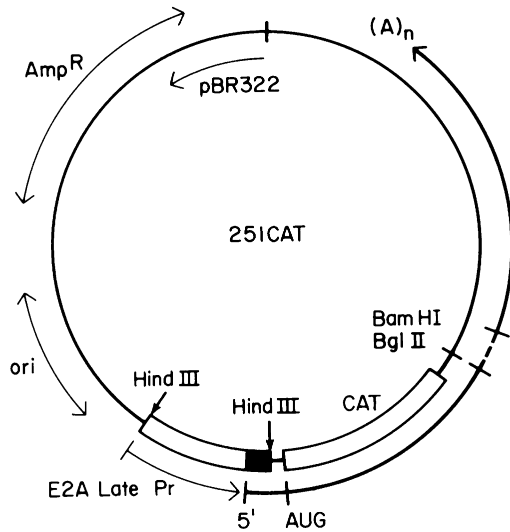

Fig. 6. A plasmid containing the CAT gene linked to the E2A late promoter. In the plasmid shown, 251CAT, a 420-bp fragment comprising a major portion of the E2A late leader and 5′-flanking promoter region was inserted into *Hind*III site of pSVOCAT. The CAT gene in pSVOCAT is associated with the 3′-flanking sequences which contain the SV40 small t intron and polyadenylation signals (Gorman *et al.*, 1982). The construction of 251-CAT plasmid is described in Materials and methods.

can be readily measured by determining the proportion of chloramphenicol converted to the acetylated form (CAT activity). The E2A early promoter-CAT clone (p2CAT) was obtained from Dr. N.Jones (Weeks and Jones, 1983). The E2A late promoter-CAT chimeric plasmid, 251-CAT, is described in Figure 6. Both plasmids contain, fused to the CAT gene, the transcriptional initiation site and a major portion of the 5′-untranslated leader sequence. HeLa cells were transfected with these plasmids in the absence or presence of plasmid containing wild-type or mutant E1A regions. Forty-eight hours after transfection, cell extracts were prepared and assayed for CAT activity as described in Materials and methods.

The results of these experiments are shown in Figure 7. HeLa cells transfected with the E2A early promoter-CAT chimeric plasmid (p2CAT) show a barely detectable level of CAT activity (lane 1). This activity was elevated 10- to 20-fold in cells co-transfected with the same CAT plasmid in combination with either the wild-type (HE4) or the mutant (pEKpm975) E1A plasmids (lanes 2 and 3) both producing the 13S mRNA product. The comparison between lanes 1 and 4 shows that the amount of CAT activity is equally low in cells transfected with the p2CAT plasmid alone or in combination with the mutant plasmid phr1 which codes for the 12S mRNA protein. HeLa cells transfected with the E2A late promoter-CAT chimeric plasmid (251-CAT) show high levels of CAT activity (lane 5). Co-transfection with the same plasmid in combination with the E1A wild-type (HE4) or phr1 mutant plasmids results in a 10- to 15-fold reduction of the CAT activity (lanes 6 and 8). The CAT activity was not altered in the extracts of cells transfected with the 251-CAT and the E1A pEKpm975 mutant plasmids (lane 7).

These results show that the fusion of the E2A early and late promoter regions to the structural sequences of the CAT gene renders the chimeric gene susceptible to the E1A-mediated regulation. We conclude that the sequences responsive to the positive or negative control exerted by the 12S and 13S mRNA products are located at the 5′ end of the DBP early and late transcription units.

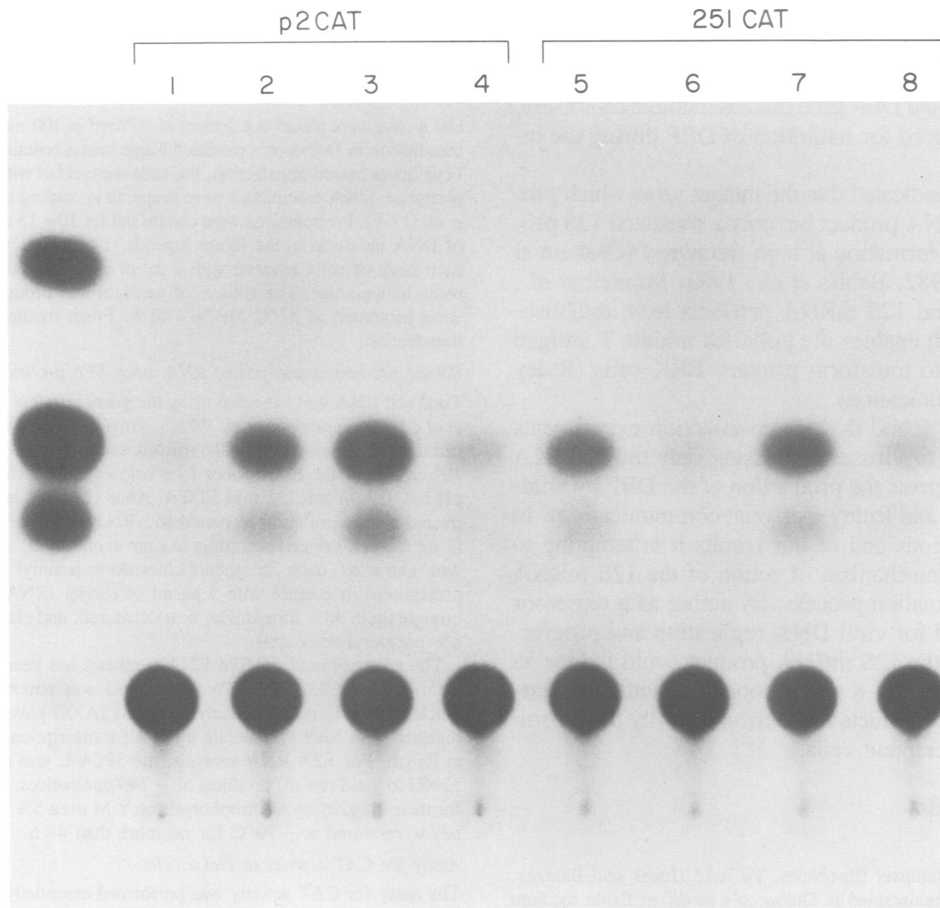

Fig. 7. CAT assay of cells transfected with the E2A early and late promoter CAT plasmids. HeLa cells were transfected with p2ACAT or 251CAT, in the absence or presence of the wild-type or mutant E1A plasmids. The calcium phosphate-DNA precipitate contained 20 μg/plate of the CAT plasmids and an equimolar amount of the E1A plasmid DNAs (40 μg/plate). For transfections of CAT plasmid alone, pBR322 DNA was substituted for the E1A. After 48 h, cell extracts were assayed for CAT activity and analyzed by t.l.c. as described in Materials and methods. **Lanes 1−4** represent transfections with p2CAT; **lanes 5−8**, transfections with 251CAT. **Lanes 1** and **5**, CAT plasmids alone; **lanes 2** and **6**, co-transfections with HE4; **lanes 3** and **7**, co-transfections with pEKpm975; **lanes 4** and **8**, co-transfections with phr1. The ^{14}C spots in the autoradiograph corresponding to the acetylated forms of choramphenicol were cut out of the silica gel and counted. C.p.m. in **lanes 1−8**: **1**, 1709; **2**, 19288; **3**, 39780; **4**, 4324, **5**, 18033, **6**, 2208; **7**, 23031; **8**, 3768.

Discussion

Activation of adenovirus early transcription units has been described as being a function of the product encoded by the E1A 13S mRNA (Berk *et al.*, 1979; Carlock and Jones, 1981; Ricciardi *et al.*, 1981; Montell *et al.*, 1982; Leff *et al.*, 1984; Ferguson *et al.*, 1984), while the role of the product encoded by the 12S mRNA is still the object of controversy.

In this report we have analyzed in detail the interaction of E1A with the DBP gene in an attempt to clarify an earlier observation suggesting that E1A gene products had different effects in a system in which the E2A early and late promoters could be tested separately. It was found that E1A could activate the expression of DBP from its early promoter and repress the synthesis of DBP transcribed from its late promoter (Rossini, 1983).

We have shown here that E1A repression of the DBP late promoter is a function encoded only by the 12S mRNA. This negative control is promoter-dependent and exerted only on the late and not the early DBP promoter and is reflected in changes of the relative amounts of DBP-specific mRNA, strongly suggesting a control at the transcriptional level. Repression of transcription by E1A has recently been observed in other systems, such as the SV40 early promoter, although this activity seems to be carried out by both 13 and 12S mRNA products (Velcich and Ziff, personal communication).

The mechanism by which E1A gene products regulate viral gene expression is still the subject of speculation. It has been suggested that a cellular factor is involved in the regulation of adenoviral early gene expression. This cellular product would prevent the accumulation of early viral mRNAs and the function of E1A product(s) would be to inactivate it, resulting in an activation of the early viral genes (Nevins, 1981). Since the difference between the two E1A products resides in 46 amino acids unique to the larger protein which is encoded by the 13S mRNA, this portion might contain an interaction site for the cellular factor. If this was the case, the inhibitory function could reside in a domain common to the two products and the extra 46 amino acids in the 13S mRNA protein would confer the activating function to this product. The choice between activation and repression could also be determined by specific sequences and/or DNA conformation at the promoter regions. Recent results obtained with deletion mutants in the DBP late promoter region suggest that a region of the DNA between nucleotide 87 and 51 upstream of the late initiation site is necessary for E1A repression (Guilfoyle, Osheroff and Rossini, in preparation). DNA sequences responsive to E1A activation have not been found in the early promoter region (Imperiale and Nevins, 1984; Guilfoyle and Rossini, unpublished) suggesting that repression of the DBP late promoter by the 12S mRNA product is specific.

The characterization of a new function for the E1A 12S mRNA product is important for the understanding of the regulation of gene expression of adenoviruses. Additional investigations are required to explain why the DBP gene utilizes multiple promoters, if and when there is a need for inhibition of DBP during the infectious cycle.

Several reports have indicated that the mutant virus which produces an intact 12S mRNA product but only a truncated 13S product, can induce focus formation at high frequency (Graham *et al.*, 1978; Ho *et al.*, 1982; Babiss *et al.*, 1983; Montell *et al.*, 1984). Both the 13S and 12S mRNA products have establishment functions and both enables the polyoma middle T antigen and T24Ha-*ras* genes to transform primary BRK cells (Ruley *et al.*, personal communication).

Recently it has been found that, in co-infection experiments in HeLa cells, Ad5*hr*1 or viruses expressing only the 12S E1A RNA, were able to suppress the production of the DBP by wild-type virus (Grodzicker and Ruley, personal communication). In view of these observations and of our results it is tempting to speculate on a second mechanism of action of the 12S mRNA product in the transformation process. By acting as a repressor of DBP that is required for viral DNA replication and progression to the late phase, the 12S mRNA product could induce an abortive infection. Thereafter it would cooperate with other early viral or cellular gene products to determine a fully transformed phenotype of the recipient cells.

Materials and methods

Cell culture

Thymidine kinase deficient hamster fibroblasts, Tk^{-}ts13 (Jonak and Baserga, 1980) and HeLa cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% calf or fetal calf serum, respectively.

Plasmids

Plasmid L-251.1 containing the late promoter was constructed by inserting the Ad2 *Bam*HI-*Hin*dIII fragment from coordinates 59.5 to 72.8 into the respective sites in pBR322.

Plasmid E-252.1 containing only the early promoter, was constructed by insertion of the Ad2 *Hin*dIII-*Eco*RI fragment (coordinates: 72.8 – 75.9) upstream of the *Bam*HI-*Eco*RI fragment (coordinates: 59.5 – 70.70) cloned in pBR322 at the *Bam*HI and *Hin*dIII sites. The vector was modified by introducing the *Hin*dIII linker at the *Eco*RI site (coordinate: 70.7) to facilitate the construction.

The plasmid p2CAT was obtained from Dr. N.Jones (Weeks and Jones, 1982). For the construction of the 251-CAT plasmid, the L-251.1 plasmid was digested with *Kpn*I (coordinate: 71.9) and treated briefly with *Bal*31 nuclease to remove the presumptive donor splice junction of the E2A late 5′ leader sequence. *Hin*dIII linkers were ligated to the blunt ends. Subsequent cleavage with *Hin*dIII released an insert also containing 420 bp of the E2A late promoter region up to the *Hin*dIII site located at map coordinate 72.8. The deleted fragments were purified and inserted into the *Hin*dIII site of pSVOCAT (Gorman *et al.*, 1982) in both orientations. The isolate used in this report contains the promoter in the correct orientation to drive expression of the CAT gene. As expected, 251-CAT contains a small deletion (5 – 10 nucleotides) at the 3′ end of the E2A late leader the extent of which has not been determined precisely. In pSVOCAT reside the CAT gene, SV40 small t intron, and the SV40 early polyadenylation site.

The SP2A-L plasmid was constructed by inserting the Ad2 *Eco*RI-*Hin*dIII fragment (coordinates: 70.7 – 72.8) into the SP6-5, a plasmid in which a 'polylinker' is inserted eight nucleotides downstream of the first base of the SP6 transcript, is cloned in the opposite orientation with respect to the SP6 promoter.

Plasmid DNAs were prepared according to Birnboim and Doly (1979) and purified by cesium chloride-ethydium bromide equilibrium gradient centrifugation (Radloff *et al.*, 1967).

Microinjection and immunofluorescence

Nuclear microinjection was performed into Tk^{-}ts13 cells according to Graessmann *et al.* (1980). Tk^{-}ts13 cells were cultured in the presence of glass slides (22 x 30 mm) numbered and marked with circles. A different DNA sample was injected into each circle. About 10^{-11} ml of plasmid DNA suspended in 10 mM Tris-HCl pH 7.2 at a given concentration was injected.

Twenty hours after microinjection, Tk^{-}ts13 cells were fixed for 15 min with methanol at −20°C. Ad2 72-kd DNA binding protein was visualized in microinjected cells by indirect immunofuorescence using hamster anti-72-kd serum (C783, provided by A.Lewis) as the first antibody, and fluorescein-isothiocyanate-labelled goat anti-hamster globulin as the second antibody.

DNA transfection

HeLa cells were plated at a density of $10^4/cm^2$ in 100 mm plates on the day before transfection in Dulbecco's modified Eagle media containing 10% fetal calf serum. Four hours before transfection, the cells were refed with fresh medium. Calcium phosphate-DNA precipitates were prepared according to the procedure of Wigler *et al.* (1978). Precipitations were carried out for 10 – 15 min and contained amounts of DNA indicated in the figure legends. 1 ml of the precipitates was added to each plate of cells covered with 2 ml of medium and incubated for 15 min at room temperature. The volume of medium was brought to 10 ml and the cells were incubated at 37°C for 36 – 48 h. Fresh medium was added 24 h post-transfection.

RNAse protection analysis of RNA using SP6 probes

Total cell RNA was extracted using the guanidium/hot phenol method (Chirgwin *et al.*, 1979; Feramisco *et al.*, 1982). Poly$(A)^+$ RNA was selected by oligo(dT)-cellulose chromatography. The mRNA samples were hybridized with 0.5 – 1 x 10^6 c.p.m. of the SP6 probes (see below) in 80% formamide, 40 mM Pipes pH 6.4, 0.4 M NaCl, 1 mM EDTA. After 12 – 16 h at 45°C, the reactions were treated with a mixture of pancreatic RNase (40 μg/ml) and T1 ribonuclease (2 μg/ml) and stopped according to Zinn *et al.* (1983). The RNase-resistant RNA was extracted once in phenol-chloroform-isoamyl alcohol (1:1:0.04), and precipitated in ethanol with 5 μg/ml of carrier tRNA. The RNA pellets were brought up in 90% formamide, heat denatured, and electrophoresed on 8 M urea 8% polyacrylamide gels.

The protocol used for SP6 RNA synthesis has been described previously by Zinn *et al.* (1983). The SP6 polymerase was purchased from New England Nuclear. For E1A RNA analysis the SP1AXO plasmid described above was digested with *Nae*I to generate a run-off transcription probe of 584 nucleotides in length. For E2A RNA analysis, the SP2A-L was digested with *Hin*dIII and *Eco*RI to yield run-off products of ~740 nucleotides. The probes were checked for their integrity by electrophoresis on 8 M urea-5% polyacrylamide gels. Probes were stored at −70°C for no more than 48 h.

Assay for CAT activity in HeLa cells

The assay for CAT activity was performed essentially as described by Gorman *et al.* (1982) using some minor modifications. The washed cell pellets were suspended in 100 μl of 0.25 M Tris (pH 8.7) and sonicated briefly. The sonicated cells were centrifuged for 15 min at 4°C in an Eppendorf Microfuge. The entire supernatant was assayed for CAT activity by the addition of 1 μCi of $[^{14}C]$chloramphenicol (40 mCi/mmol, New England Nuclear Corp.), 0.5 μl of 40 mM acetyl coenzyme A, and 100 μl of water. The reactions were carried out for 60 min at 37°C and then extracted with 1 ml of ethyl acetate. The organic layer was dried, taken up in 20 μl of ethyl acetate, and spotted onto silica gel t.l.c. plates (Eastman Kodak Co., no. 13179). The plates were developed in chloroform-methanol (95:5).

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