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The E4 promoter of adenovirus type 2 contains an E1A dependent cis-acting element

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ABSTRACT

To study how the E1A polypeptides of adenovirus type 2 regulate transcription, we have constructed chimeric plasmids containing the bacterial gene encoding chloramphenicol acetyl transferase (CAT) under the control of either the wild type or the deleted E4 promoter of adenovirus type 2. Our previous results showed that promoter sequences located upstream from position -158, as measured from the cap site, are essential to the transactivation process. From a new set of deletion mutants, we now show that two regions, located between positions -239 and -218 and between positions -179 and -158, are involved in the E1A transactivation process. The deletion of only one of them does not significantly alter the E1A induction process compared with the wild type. Moreover, we show that these two regions lie within a DNA fragment which possesses the properties of an E1A-inducible "enhancer-like" element. In addition, the DNA fragment which contains this enhancer element is also able to confer the E1A inducibility to a heterologous promoter.

INTRODUCTION

The expression of the adenovirus type 2 (Ad2) genome in infected cells is highly regulated. The expression of the immediate early gene E1A, located at the very left-end of the viral genome (0 - 4.5 m.u.), regulates the transcription of the other early viral regions E1B, E2, E3 and E4 (1-6). In addition, the transcription of two endogenous cellular genes coding for a human heat shock-like protein (7-8) and for βtubulin (9) is stimulated by the action of the E1A products. The E1A region encodes two polypeptides, p243 and p289, which have identical N and C terminal ends, but differ by the presence of an internal stretch of 46 amino acids in the larger protein (10). Whereas the p289 polypeptide efficiently stimulates transcription from the early viral promoters (11-

13), the role of the p243 in this process is less clear, and conflicting results have been obtained (14-15).

Several mechanisms have been proposed for an E1A transactivation of adenovirus promoters. The E1A products might inactivate a host cell protein that inhibits early adenovirus transcription (16). On the other hand, they might catalyse the assembly of stable transcriptional complexes (17) ; it has been suggested that E1A proteins modify the level of one or several intracellular components which would allow the RNA polymerase II to efficiently recognize the E1A-sensitive promoters. The concentrations of these components could be regulated during cell differentiation and could also depend on the cell line (18). This cellular participation in the control of early adenovirus transcription makes it difficult to identify the E1A-sensitive sequences within promoters, since the deletion of such sequences alters the levels of transcription both in the absence and in the presence of E1A.

To obtain further insight into the mechanism of the E1A-mediated transactivation, we have deleted the upstream sequences of the E4 promoter by mutagenesis and have examined the effects of these deletions. Thus, two critical regions between positions -239/-218 and between positions -179/-158 relative to the E4 transcription initiation sites have been identified. Moreover, our results show that the sequences between -256 and -89 contain an E1A-inducible enhancer-like element. The insertion of these sequences in cis-position relative to a heterologous promoter renders it E1A-inducible.

MATERIALS AND METHODS

Cells

The adenovirus-transformed human embryo cell line (line 293-31) was provided by J.C. Nicolas (Hôpital Trousseau, Paris) and has been previously described (19). Monolayer cultures of HeLa and line 293 cells were maintained in Dulbecco's modified Eagle medium containing 10 % fetal calf serum.

Materials

Restriction enzymes, T4 DNA ligase, Escherichia coli DNA polymerase I (Klenow fragment) and Bal-31 nuclease were

purchased from New England Biolabs and Amersham.

(^{32}-P)dATP (800 Ci/mmole) and (^{14}C) chloramphenicol (53 mCi/mmole) were obtained from Amersham.

Preparation of plasmid DNAs

Plasmid DNAs were prepared according to the method of Birnboim and Doly (20) followed by two cesium chloride-ethidium bromide equilibrium gradient centrifugations.

Transfections

Transfections and CAT assays were carried out as previously described (13).

Constructions of plasmids

Constructions of pE4-CAT and its Bal-31 derivatives pE4-CAT(-X), where X refers to the last nucleotide still present in the different deleted promoters, have been previously described (13).

Plasmid pE4-CATΔ has been obtained by inserting the EcoRI-AhaIII restriction fragment of pE4-CAT into the EcoRI restriction site of pE4-CAT(-158) after filling in the sticky ends with DNA polymerase I (large fragment) (Fig.1). The Bal-31 pE4-CAT(-XΔ) derivatives were subsequently obtained as described (13). Briefly, pE4-CATΔ DNA was linearized with EcoRV, mildly digested with Bal-31 and the Bal-31-NcoI DNA fragments were reinserted between the SmaI and NcoI restriction sites of pE4-CAT (Fig.1).

Plasmids -89R5', -89R'5', -89R3' and -89R'3' have been constructed by inserting the FnuDII restriction fragment mapping between positions -256 and -89 in the E4 promoter, either into the EcoRI, or into the BamHI restriction site of pE4-CAT(-89) in both orientations after filling in the sticky ends with DNA polymerase I (large fragment) (Fig.3).

Plasmid dlpSV1-CAT was obtained by inserting the SphI-HindIII (128-5171) restriction fragment of SV40 between the SmaI and HindIII restriction sites of pHP34-CAT (13) after blunt ending the SphI sticky end with DNA polymerase I (large fragment).

Plasmids dlpSV1-CAT R5', dlpSV1-CAT R'5', dlpSV1-CAT R3' and dlpSV1-CAT R'3' were constructed by inserting the FnuDII restriction fragment mapping between position -256 and -89 in

the E4 promoter, either into the EcoRI or into the BamHI restriction site of dlpSV1-CAT in both orientations (Fig.5) after filling in the sticky ends with DNA polymerase I (large fragment).

Plasmid pE1A (Ad2) contains the SacI-D restriction fragment (0-5 m.u.) of Ad2 cloned between the EcoRI and BamHI restriction sites of pML2 (13).

M13 sequencing strategy

The HindIII-EcoRI restriction fragments of pE4-CATΔBal-31 deletion mutants were subcloned into the derivative mp19 of M13 bacteriophage and sequenced (21-22).

Analysis of in vivo transcripts

Total RNAs were extracted 40-48 h after calcium phosphate transfection as previously described (23).

Single-stranded probe for mapping mRNA transcribed from the E4 promoter was prepared from the M13 clone DNA. A restriction fragment mapping between EcoRI and PvuII of pE4-CAT (Fig.1) was inserted between EcoRI and SmaI of mp19. A complementary strand was synthesized by using Klenow DNA polymerase from an end-labeled oligonucleotide localised in the CAT gene : 5'CCATTTTAGCTTCCTTA3'. After digestion with EcoRI, the probe was separated from its complementary cold strand on a denaturing polyacrylamide gel. Total RNAs were hybridized with an excess of the appropriate single strand M13 probe and digested with S1 nuclease (Worthington) as previously described (24). RNA-protected probe fragments were separated by electrophoresis through 10 % polyacrylamide urea gels.

RESULTS

Construction of plasmid pE4-CATΔ and its biological activity

We have already shown that a critical promoter element is located between positions -179 and -158 (13). Moreover, this regulatory element could be detected when sequences mapping upstream from -179 were also deleted. In order to establish the role, if any, of the sequences located upstream from -179, we constructed an internal deletion mutant of the E4 promoter, pE4-CATΔ (Fig.1), which lacks the sequences mapping between positions -179 and -158 (Fig.2). The transcriptional activity

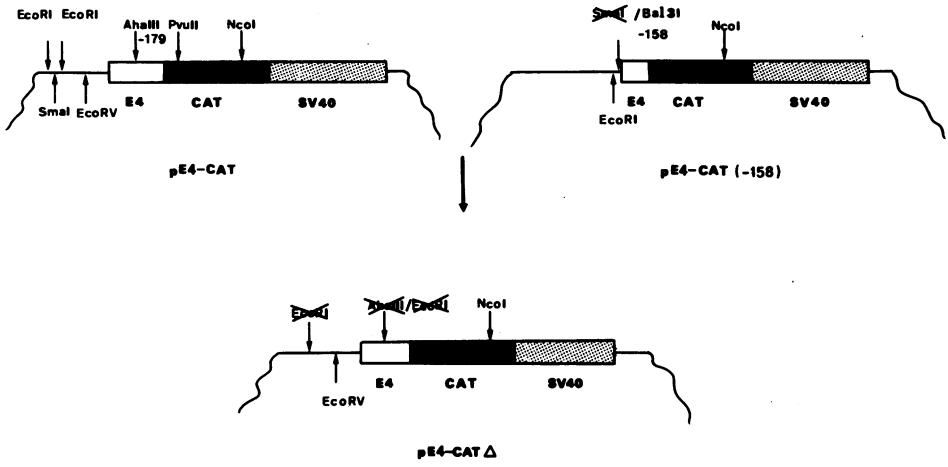

Figure 1 : Schematic drawing showing the construction of the plasmid pE4-CATΔ from pE4-CAT and pE4-CAT(-158). Coding CAT region, SV40 T antigen mRNA processing signals and Ad2 E4 sequences are respectively described by black ■, dotted ▩ and white ▭ areas . Only restriction sites using for the construction are indicated.

of pE4-CATΔ was then tested by transient expression assay with 293 or HeLa cells and compared with the activities of wild type pE4-CAT and pE4-CAT(-158) (Fig.1). The pE4-CAT(-158) mutant deleted between positions -329 and -158 relative to the E4 transcription initiation site, showed a tenfold reduced activity as compared to pE4-CAT (13). Surprisingly, the transcriptional activities of pE4-CATΔ and pE4-CAT were found to be identical (Table 1). It was thus concluded that promoter sequences mapping between positions -179 and -158 are not essential for E1A transactivation when sequences upstream from position -179 are conserved. This result suggested that there is another sequence between positions -329 and -179 which can functionally replace the -179 to -158 region previously identified.

Construction of pE4-CATΔ Bal-31 mutants and their biological activities

In order to localize this upper region, we have progressively deleted in a 5' to 3' direction the pE4-CATΔ mutant beginning from position -329.

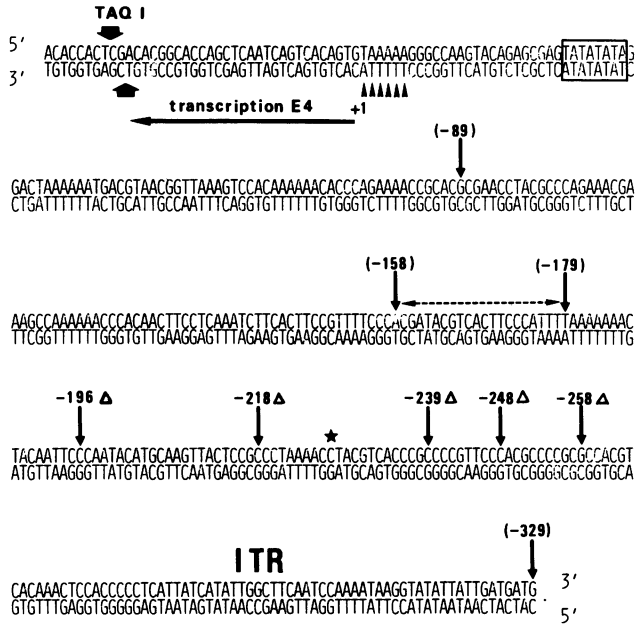

Figure 2 : Nucleotide sequence of the very right-end of the Ad2 genome. The last nucleotide of the ITR is indicated by a star symbol (*). The "TATA" homology is boxed. The cap sites of the E4 mRNAs are also shown and the major one referred as position +1. The location of the restriction site Taq I used to construct pE4-CAT is also shown.
Δ indicates the sequence deleted in pE4-CATΔ. Vertical arrows refer to the last nucleotide present in different deleted mutants.

Figure 2 shows the nucleotide sequence surrounding the transcription start sites of the E4 region (25). A library of deletion mutants was constructed from plasmid pE4-CATΔ by sequential digestion with Bal-31. These deletion mutants were extensively analysed by restriction enzyme digestion and sequencing. Plasmids pE4-CATΔ deletion mutants which retain 258, 248, 239, 218 and 196 bp were isolated (Fig.2) and their transcriptional activities were tested by CAT assay.

Transcriptional activities were not affected by the deletion of sequences mapping upstream from position -239 compared to pE4-CAT or pE4-CATΔ activities (Table 1). However, an additional deletion of the adjacent region mapping between -239 and -218 resulted in an 8-10 fold drop in transacti-

TABLE 1

Plasmid	Relative expression		
	Hela cells		293 cells
	Adenovirus -	E1A gene +	
pE4-CAT	11	100	100
pE4-CAT Δ	15	109	90
-258 Δ	20	90	85
-248 Δ	15	128	96
-239 Δ	20	80	65
-218 Δ	15	7	8
-196 Δ	6	7	11
pE4-CAT(-158)	12	17	7

Table 1 : Relative expression in HeLa and 293 cells of the pE4-CAT plasmid and of the upstream deletion mutants.

vation by E1A (Table 1). The transcriptional activities of pE4-CAT(-218Δ) and pE4-CAT(-196Δ) were in fact similar to the pE4-CAT(-158) activity. These results show that in the absence of the -179 to -158 region, the E4 promoter is still E1A transactivated due to the existence of a functionally equivalent region located between -239 and -218.

Detection of an activator element in the E4 promoter

To understand the mechanism of E1A-mediated transactivation, we attempted to restore the activity of a non functional deleted E4 promoter by the insertion of a DNA fragment containing the two previously identified regulatory regions. Thus, the FnuDII restriction fragment mapping between positions -256 and -89 (Fig.2) has been inserted into the pE4-CAT(-89), either upstream or downstream, and in both orientations relative to the CAT gene (Fig.3). The resulting plasmids -89R5', -89R'5', -89R3' and -89R'3' were tested for their transcriptional activities by CAT assay (Table 2) and S1 mapping (Fig.4). As reported before (13), the expression of the

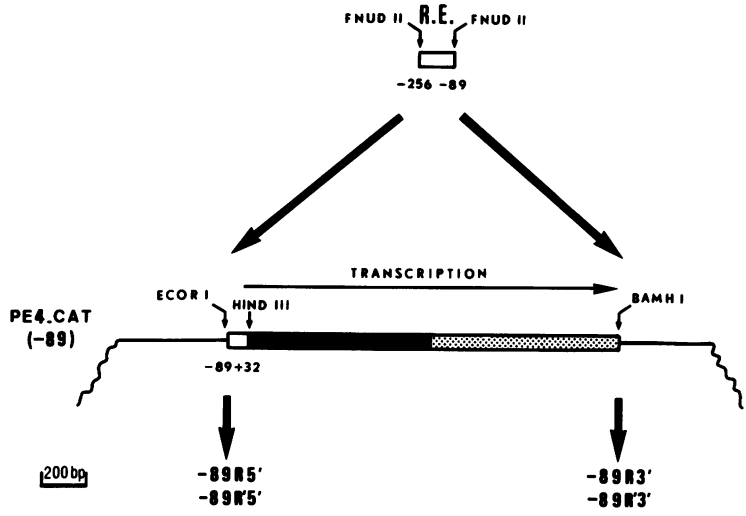

Figure 3 : Schematic drawing showing the construction of -89R5', -89R'5', -89R3' and -89R'3' plasmids. The FnuDII fragment (-256, -89) has been inserted into EcoRI or BamHI site of pE4-CAT(-89). Coding CAT region, SV40 T antigen mRNA processing signals and Ad2 E4 sequences are respectively described by black ■, dotted ▩ and white ▭ areas. Only restriction sites using for the construction are indicated.

TABLE 2

Plasmid	Relative expression		
	Hela Cells		293
	Adenovirus E1A gene		
	-	+	
pE4-CAT	13	100	100
pE4-CAT(-89)	1	0,5	0,5
-89R5'	9,5	105	90
-89R'5'	0,5	25	35
-89R3'	0,8	3,9	12,7
-89R'3'	0,7	2,3	10,3

Table 2 : Relative expression in Hela and 293 cells of pE4-CAT, pE4-CAT(-89), -89R5', -89R'5', -89R3' and -89R'3' plasmids.

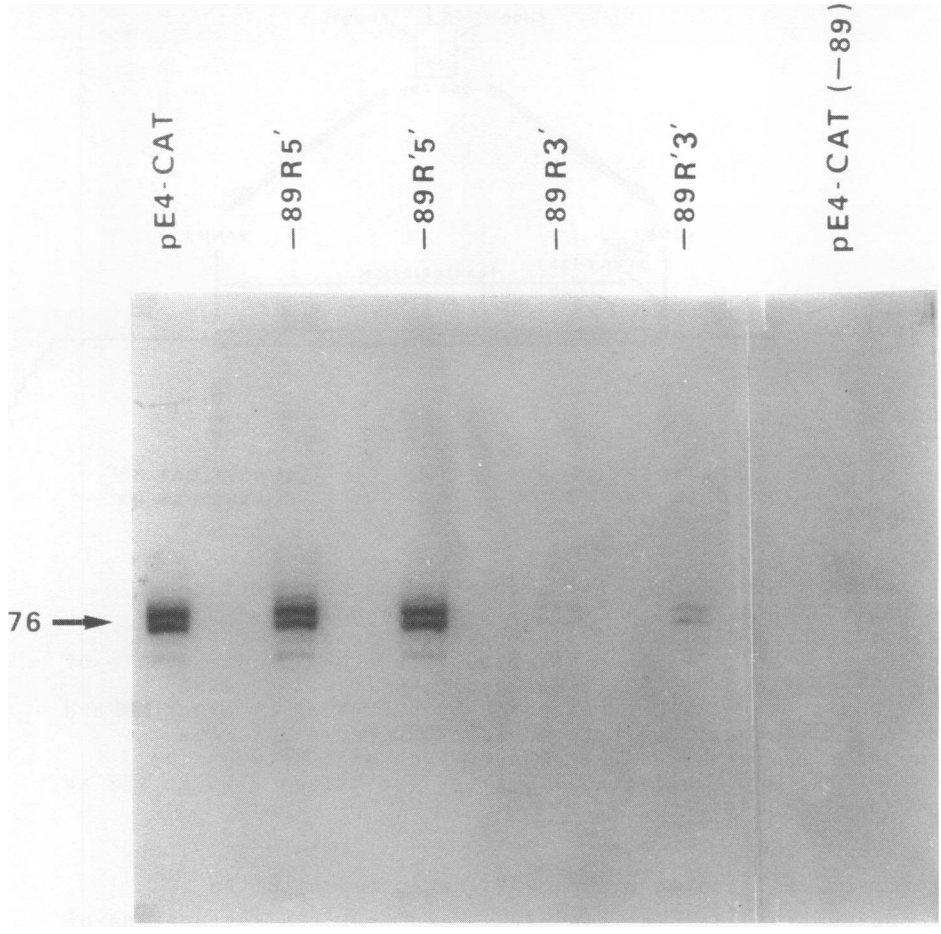

Figure 4 : SI-nuclease mapping of mRNA synthesized from pE4-CAT, -89R5', -89R'5', -89R3', -89R'3' and pE4-CAT(-89) plasmids. The position of the labeled probe protected by the E4 transcript is indicated.

pE4-CAT(-89) is less than 1 % of the wild type pE4-CAT level. The insertion of the -256 to -89 sequence in 5' position relative to the cap sites restores a strong activity when compared with the -89 mutant level. This restoration is nearly independent of orientation with respect to the transcriptional start sites. Although insertion in the 3' position relative to the CAT gene leads to a weaker recovery, the -256 to -89 regulatory element remains active over a large distance.

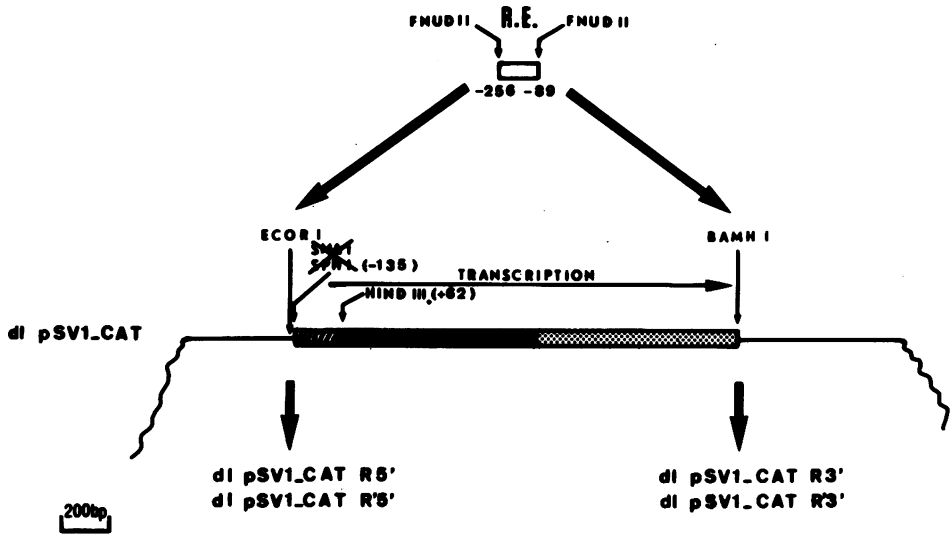

Figure 5 : Schematic drawing showing the construction of dlpSV1-CAT R5', dlpSV1-CAT R'5', dlpSV1-CAT R3' and dlpSV1-CAT R'3' plasmids. The FnuDII fragment (-256, -89) of E4 promoter, has been inserted into EcoRI or BamHI site of dlpSV1-CAT. Coding CAT region and SV40 T antigen mRNA processing signals are respectively described by black ■ and dotted ▪▪▪ areas. The hatched area ▨ represents the enhancerless early SV40 promoter which maps between the SphI (128) and HindIII (5171) sites (positions -135 to +62 as measured from the cap sites).

Moreover, S1 mapping experiments performed with the reconstructed plasmids clearly showed that the restoration of CAT activity was essentially due to RNA initiated at the norm E4 cap sites (Fig. 4).

Thus, the E1A dependent cis-action of the -256 to -89 DNA fragment can occur when this fragment is moved from its normal location or when its orientation is reversed.

The E4 -256 to -89 DNA fragment can confer E1A inducibility to a foreign promoter

In order to know if the sequences involved in the E1A transactivation of the E4 promoter could also function when located near a foreign promoter, we inserted the -256 to -89 E4 fragment upstream from the SV40 early gene promoter, previously deleted of its own "enhancer" element. Thus, we first deleted

TABLE 3

Plasmids	Relative expression		
	Hela cells		293 cells
	Adenovirus E1A gene		
	-	+	
dlpSV1-CAT	3	1,5	0,01
dlpSV1-CAT R5'	4	100	100
dlpSV1-CAT R'5'	4	50	110
dlpSV1-CAT R3'	4	7	48
dlpSV1-CAT R'3'	1,5	6	23

Table 3 : Relative expression in Hela and 293 cells of dlpSV1-CAT, dlpSV1-CAT R5', dlpSV1-CAT R'5', dlpSV1-CAT R3' and dlpSV1-CAT R'3' plasmids.

the 72 base pair repeats from the SV40 early gene promoter (dlpSV1-CAT) and then replaced them with the -256 to -89 DNA fragment of the E4 promoter in either orientation with respect to the initiation start sites (dlpSV1-CAT R5' and dlpSV1-CAT R'5') (Fig.5).

As was expected, CAT dosage shows that the enhancerless early gene promoter (dlpSV1-CAT) has no detectable activity in 293 or in HeLa cells. The insertion of the -256 to -89 E4 DNA fragment in the 5' position with respect to the start sites of dlpSV1-CAT restores promoter activity in the presence of E1A (Table 3), whatever the orientation. This result shows that the -256 to -89 E4 DNA fragment is self-sufficient to confer the E1A dependent cis-action to a foreign promoter. Moreover, transfection of plasmids containing the E4 regulatory element inserted in the 3' position compared with the CAT gene of dlpSV1-CAT (dlpSV1-CAT R3' and dlpSV1-CAT R'3') (Fig.5) shows that the E1A-dependent cis-action of the E4 promoter element is partially preserved even when located a long distance from the foreign promoter (Table 3).

Thus, the -256 to -89 E4 DNA fragment possesses E1A dependent "enhancer" element properties which are also conserved when this regulatory element flanks a foreign promoter.

DISCUSSION

We have previously shown that transcriptional activity of a chimeric gene resulting from the fusion of the CAT coding sequence to the E4 promoter is increased 10-50 times in the presence of E1A during a transient expression assay (13). Progressive deletion in a 5' to 3' direction of the 5' flanking region upstream from the cap sites suggested that critical regulatory elements for E4 transcription in the presence of the E1A gene are located upstream from position -158 (13). In this paper, we describe studies to more precisely define the sequence elements involved in the induction process. We have identified two E1A sensitive regions which are located between positions -239 to -218 and -179 to -158. It is very interesting to note that these two regions possess the same sequence of 8 nucleotides : 5' GTGACGTA 3'. A very similar sequence is also present once in upstream elements critical for the activity of E2 and E3 promoters in presence of the E1A gene (13-26). This suggests that this type of sequence might play an important role in the E1A transactivation of these promoters. Whereas these regulatory sequences are found in a single copy between positions -79 and -70 of the E2 promoter (27-31) and between positions -105 and -82 of the E3 promoter (26), they were found duplicated and much further upstream in the E4 promoter. However, deletion of only one regulatory element does not affect transcriptional activity from the E4 promoter. Are both regions simultaneously involved in the E1A induction of the wild type E4 promoter ? It is difficult to answer this question since they were both discovered as a single copy. Nevertheless, the duplication might compensate the large distance between the regulatory elements and the E4 TATA box.

It is important to note that none of our deletion mutants can express CAT activity in the absence of the E1A products. This observation seems to distinguish the E4 promoter from the E2 promoter since in the latter case Jalinot and Kedinger have been able to isolate constitutive mutants and to identify two putative negative control regions (32).

Our results also show that the action of the two regulatory elements is not strictly dependent on their location

relative to the E4 TATA box, although the insertion in a 3' position relative to the CAT gene leads to a weaker activity. Thus, the upstream sequences mapping between positions -256 and -89 contain a similar element to those that define an enhancer element. However, this enhancer element is efficiently used only in the presence of E1A. These results recall those described for the adenovirus E2 promoter since, in that case, the sequences located between -262 and -21 positions also possess the properties of an E1A inducible enhancer element (27-28). It suggests that the same mechanism might be used for the E1A transactivation of the E2 and E4 promoters.

The insertion of the E4 enhancer element in cis-position relative to the enhancerless SV40 early promoter can confer E1A inducibility to this promoter. Thus, the properties of the E4 enhancer element also exist when this regulatory element flanks a foreign promoter. On the other hand, it is known that the E1A products normally repress the wild type SV40 early promoter by a negative regulation of the 72 bp repeats (33-35). It appears that the type of enhancer element which precedes this promoter determines its behavior in the presence of E1A.

The mechanism of E1A regulation is not clearly understood. In fact, a large spectrum of genes can be activated by E1A. The transcription of a number of endogenous cellular genes, like those coding for a heat-shock-like protein (7) and β tubulin (9) can be activated by E1A. In addition, transcription of the adenovirus early genes can be activated by regulatory products encoded by the pseudorabies (36), herpes simplex (37), and human T-cell leukemia viruses (38). Does the transactivation induced by these unrelated products take place through the enhancer element ? Additional experiments are clearly required to answer this question.

How can the E1A polypeptide(s) have both, an activator, and a repressor action on enhancer elements ? An interesting observation is the behavior of the E1A-induced adenovirus promoters and the SV40 early promoter in F9 embryonal carcinoma cells during retinoic acid-induced differentiation. F9 stem cells, which constitutively express a heat shock protein allow adenovirus early gene expression in the absence of E1A (18)

whereas they restrict the SV40 early gene expression (35,39). Upon differentiation, the cells become restrictive for adenovirus early viral gene expression which then requires the E1A gene product(s) (18) and become permissive for the SV40 early gene expression (35). Thus, the state of the cells, undifferentiated or differentiated, governs the regulation for these promoters. Although it has not been directly shown that the gene expression under the control of adenovirus early promoter in F9 stem cells results from a permissive state for their enhancer elements, we would like to propose that positive and negative regulators may control the viral enhancers in the embryonal carcinoma cells. This series of results seems to indicate that E1A products do not act directly upon upstream regulatory elements but probably regulate the expression level of cellular factors which modify the stability of transcriptional complexes.

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