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The E4 transcriptional unit of Ad2: far upstream sequences are required for its transactivation by E1A

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ABSTRACT

We have investigated the effect of the E1A polypeptides of adenovirus 2 on the transcription of the viral E4 region. For this purpose, we have fused the promoter region of the E4 gene to the bacterial gene coding for chloramphenicol acetyl transferase. We have found that transcription from the E4 promoter is increased at least 20 fold in the presence of the E1A region. We have also found that the largest E1A polypeptide is the regulating factor, whereas the shortest has no apparent effect. Deletion of sequences upstream from position -158, as measured from the cap site, reduces the efficiency of the transcription in the presence of E1A by more than 15 fold. An important regulatory domain lies between positions -158 and -179. This domain contains the sequence 5' GGGAAGTGAC 3' which is homologous to the E1A enhancer core sequence. Another similar sequence is also present at position -149.

INTRODUCTION

Adenovirus type 2 (Ad2) has been extensively used as a model to study eukaryotic gene expression. Virus multiplication in permissive cells can be divided into two phases separated by the onset of viral DNA replication (1). mRNAs are transcribed from regions E1-E4 during the early phase (2,3). The E1 region is subdivided into two regions, E1A and E1B, which possess their own promoters. The E1A region is transcribed immediately after infection into two spliced overlapping mRNAs, 12S and 13S (4,5). These mRNAs encode polypeptides 243 (P243) and 289 (P289) amino acids long respectively, which have identical amino- and carboxy-terminal ends, their only difference being a deletion of 46 internal amino acids (6). The E1A region is required for transcriptional activation of the other early regions (2,3,7) ; in its absence, early mRNAs can be processed normally in the cell but the time-course of their synthesis is much delayed (7,8). A mutant virus, unable to mature the 12S mRNA, but which nevertheless synthesizes an authentic P289, grows as well as the wild type virus, suggesting that neither the 12S mRNA nor its polypeptide product are essential for the growth of the virus in tissue culture (9,10).

The mechanism by which transcription of the early regions is activated is poorly understood. Two mechanisms have been proposed : the E1A product might inactivate an unstable host cell protein that inhibits early adenovirus transcription (7) or it might catalyse the assembly of stable transcriptional complexes (8). In order to study the way by which E1A regulates transcription, we fused the promoter of the E4 region to the bacterial gene which encodes chloramphenicol acetyltransferase (CAT), and then measured the transient expression of CAT activity after DNA mediated transfection. We find that the E4 promoter possesses a low basal activity which can be considerably stimulated by the E1A polypeptide P289. This transactivation process can be inhibited without significantly altering the basal transcriptional activity by deleting sequences upstream from position -158. We find that the region mapping between positions -158 and -179 plays a key role in the regulation.

MATERIALS AND METHODS

Cells

All cells were maintained as monolayer cultures in Dulbecco modified Eagle medium containing 10 % foetal calf serum.

Materials

Restriction enzymes, T4 DNA ligase, Escherichia coli DNA polymerase I (large fragment) and Bal 31 nuclease were purchased from New England Biolabs and Amersham. (^{32}P) dATP (800 Ci/mmole) and (^{14}C) chloramphenicol (40 mCi/mmole) were obtained from New England Nuclear.

Preparation of plasmid DNAs

Plasmid DNAs were prepared according to the method of Birnboim and Doly (11) followed by two cesium chloride-ethidium bromide equilibrium gradient centrifugations.

Construction of plasmids

Clone CE4 (Ad2) was constructed by inserting the EcoRI C restriction fragment (89.7-100 map units) of Ad2 between the EcoRI and PvuII restriction sites of plasmid pvΔ (Figure 1). Plasmid pvΔ is a derivative of pML2 (12) which contains a PvuII restriction site between the EcoRI and the ClaI restriction sites (Perricaudet, unpublished). Plasmid pHP34-CAT contains the CAT coding HindIII-BamHI restriction fragment of pSV2 CAT (13) between the HindIII and BamHI restriction sites of plasmid pHP34 (14) (Figure 1). The resulting pHP34-CAT plasmid harbours the CAT gene downstream from a unique SmaI restriction site (Figure 1).

Plasmid pE1A (Ad2) was prepared by cloning the SacI D restriction

fragment (0-5.0 map units) of Ad2 between the EcoRI and BamHI restriction sites of pML2 (Figure 2), after filling in the sticky ends with DNA polymerase I (large fragment). Both restriction sites EcoRI and BamHI were regenerated after the cloning.

Construction of deletion mutants

To construct 5' deletion mutants, pE4-CAT DNA was linearized with EcoRV and incubated at 30°C with 6 units of Bal31 in 400 µl of 600 mM NaCl - 12 mM $CaCl_2$ - 20 mM Tris-HCl pH 8.0 - 1 mM EDTA. At 3 minutes intervals 40 µl aliquots were removed, the reaction stopped with 20 mM EGTA and the DNA precipitated with ethanol. Samples were digested with NcoI and the Bal31 - NcoI fragments were inserted between the SmaI and NcoI restriction sites of pHP34-CAT.

M13 sequencing strategy

The HindIII-EcoRI restriction fragments of Bal31 deletion mutants were subcloned into the derivative mp9 of M13 bacteriophage and sequenced (15, 16).

Eukaryotic cell transfection and CAT assay

Transfections were carried out by using calcium phosphate (17). Four hours before the addition of DNA, cells were refed with fresh medium containing 10 % foetal calf serum. Plasmid DNA (20 µg) and high molecular weight salmon sperm DNA (10 µg) were suspended in 937.5 µl of a Hepes buffered saline (HeBS) containing (grams per liter) : NaCl, 8.0 ; KCl, 0.37 ; $Na_2HPO_4.2H_2O$, 0.125 ; glucose, 1.0 ; Hepes (N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid), 5.0 (pH 7.05). Next, 62.5 µl of 2M $CaCl_2$ was then added and mixed by bubbling with air for about 15 seconds. The precipitate was allowed to form at room temperature for 30 minutes and was then added directly to the medium on a 100 mm plate of cells at 30 % confluence. After 24 hrs incubation at 37°C, the medium was replaced with fresh medium containing 10 % foetal calf serum. Cell extracts were made 40 hrs after transfection and CAT activity measured essentially as described (13). Briefly, the cells were harvested 40 hrs after transfection and cell extracts were incubated with (^{14}C) chloramphenicol and 4 mM acetyl-CoA in 250 mM Tris-HCl (pH 7.8), under conditions in which the reaction was linear. After analysis by ascending thin layer chromatography, the rates of conversion were determined by scintillation counting.

RESULTS

Construction of plasmid pE4-CAT and its biological activity in 293 cells

The strategy we used to put the CAT gene under the control of the E4 promoter is depicted in Figure 1. Plasmid pE4-CAT was constructed by subcloning into the ClaI restriction site of pHP34-CAT (Figure 1) the TaqI restriction fragment of CE4 (Ad2) which spans the junction between the 5' flanking region of the E4 gene and the plasmid. The resulting plasmid, designated pE4-CAT, harbours 361 nucleotides from the very right end of the Ad2 genome which contains, in addition to the E4 promoter, the 103 nucleotide inverted terminal repeat (ITR) of the adenovirus genome.

The expression of the CAT gene has been tested by introducing the pE4-CAT DNA into 293 cells (18-20), an adenovirus transformed human cell line, as a calcium phosphate precipitate. Under these conditions, transfection with the pE4-CAT DNA results in the detection of CAT activity whereas no activity can be detected with the promoterless plasmid pHP34 CAT used as a control (data not shown). The expression of the CAT gene from the chimeric plasmid shows that the cloned E4 promoter is biologically active.

The E4 promoter is activated by the E1A 13S mRNA protein product

In order to investigate whether the transcription from the E4 promoter can be directly activated by the E1A polypeptides, we constructed

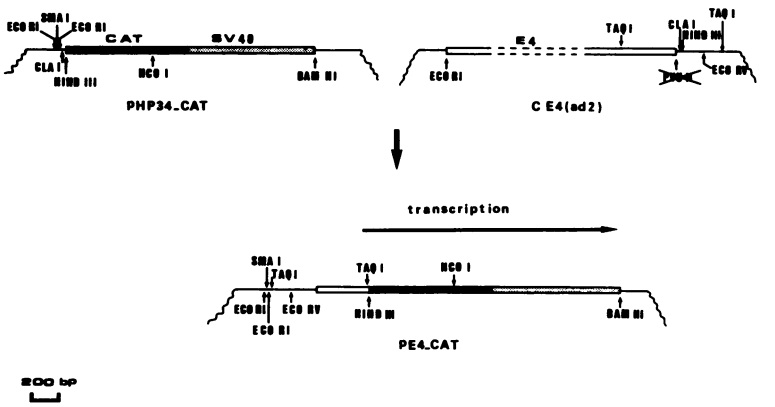

Figure 1. Schematic drawing showing the construction of the plasmid pE4-CAT. Coding CAT region, SV40 T antigen mRNA processing signals and Ad2 E4 sequences are respectively described by black, dotted and white areas. Only the restriction sites of interest for the construction of pE4-CAT and its Bal31 deleted derivatives are indicated. The sense of transcription in pE4-CAT is indicated by an horizontal arrow. Plasmid vector is represented by a thin line.

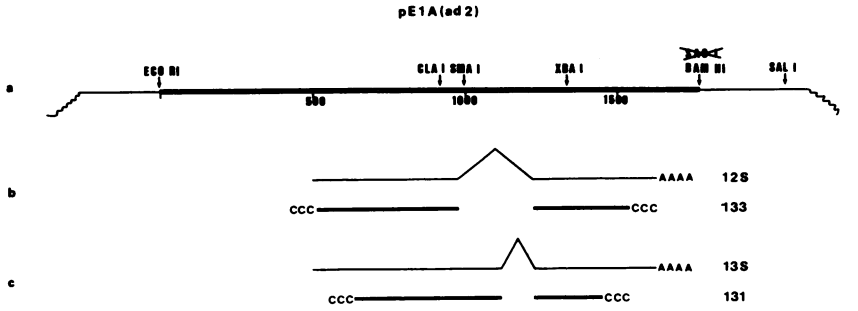

Figure 2. Schematic drawing of the clone pE1A (Ad2). The structure of the two cDNA clones 133 and 131 (6) which are derived from the two spliced 12S and 13S mRNAs transcribed from this region is also shown. Restriction sites used to construct the intronless E1A regions are indicated. Plasmid vector is represented by a thin line.

recombinant plasmids harbouring either the genomic E1A region or chimeric E1A regions which lack the intervening sequences that are removed during the maturation of the 12S and 13S mRNAs (Figure 2). This was achieved by replacing a genomic E1A fragment with a corresponding but shorter fragment derived from clones 131 and 133 (6) which contain a cDNA copy of the 13S and 12S mRNAs respectively. The SmaI-XbaI restriction fragment from clone 131 and the ClaI-XbaI restriction fragment from clone 133 were used to construct plasmids pE1A-13S and pE1A-12S which lack the 13S and 12S mRNA introns respectively (Figure 2). Transfection experiments with pE4-CAT

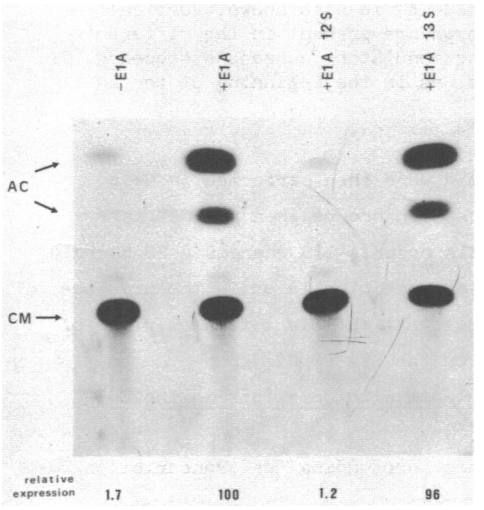

Figure 3. Relative expression of CAT in Hela cells transfected with pE4-CAT in the absence or in the presence of E1A, E1A 12S and E1A 13S. The value for the transfection in the presence of E1A is arbitrarily set to 100.

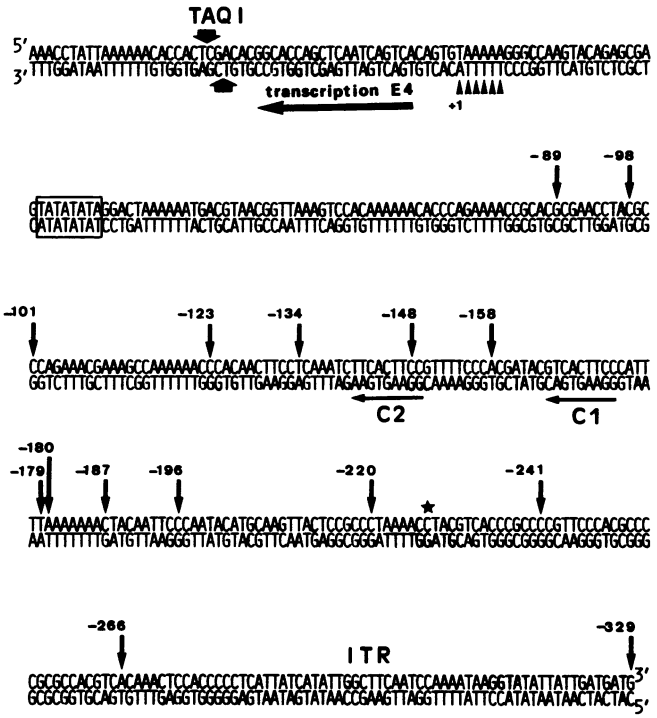

Figure 4. Nucleotide sequence of the very right end of the Ad2 genome (21). The last nucleotide of the ITR is indicated by a star symbol (*). The "TATA" homology which is a part of the E4 promoter is boxed. The cap sites (31) of the E4 mRNAs are indicated. Numbers are referred to the A residue which is used as the major cap site (31). The position of the restriction site TaqI which was used to construct pE4-CAT is also shown. Vertical arrows refer to the last nucleotides which are present in the different deleted promoters. The two copies of the modulatory consensus sequence (C1, C2) are delineated by horizontal arrows, as is the beginning of the E4 transcript.

alone or in combination with each plasmid were then performed in Hela cells. A strong and comparable CAT expression occurs when pE4-CAT is introduced into the cell with either pE1A or pE1A-13S whereas a 20-50 fold weaker CAT activity is observed in the absence of pE1A or in the presence of pE1A-12S (Figure 3). This result shows that efficient transcription of the E4 region is exclusively dependent on the E1A P289.

Construction and characterization of mutants with deletions in the E4 promoter

Figure 4 shows the nucleotide sequence surrounding the transcription

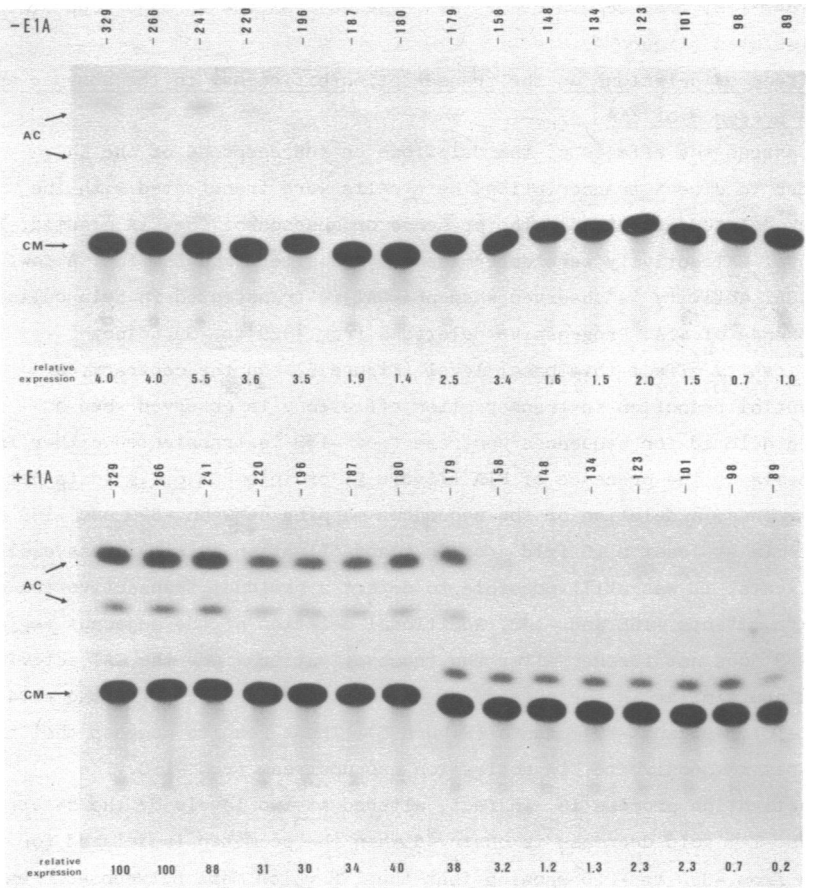

Figure 5. Relative expression of CAT in Hela cells transfected with pE4-CAT (-329) and its deleted derivatives -266 to -89 in the absence or in the presence of pE1A (Ad2). The value for the wild type gene (-329) transfected in the presence of E1A is arbitrarily set to 100. The faint spot migrating in front of CM is an impurity present in the chloramphenicol batch.

start sites of the E4 region (21). A library of deletion mutants was constructed from plasmid pE4-CAT by sequential digestion with Ba131. Plasmid pE4-CAT was cleaved with EcoRV, mildly digested with Ba131 and then digested with NcoI. The shortened Ba131 - NcoI fragments were then cloned between the SmaI and NcoI restriction sites of pHP34-CAT. A series of deletion mutants was thus isolated, and extensively analyzed by restriction enzyme digestion and sequencing.

Deletion mutants which retain 266, 241, 220, 196, 187, 180, 179, 158,

148, 134, 123, 101, 98 and 89 bp (as measured from the E4 major cap site) were isolated (Figure 4).

The effect of deletions on the transcription efficiency in the absence and in the presence of E1A

To assess the effects of the deletions on the response of the E4 promoter to direct transcription, Hela cells were transfected with the various deleted plasmids in the presence or absence of the E1A plasmid. The levels of CAT activity were determined 40 hrs after transfection. A low basal CAT activity is observed when pE4-CAT is transfected in Hela cells in the absence of E1A. Progressive deletions from -329 to -89 do not significantly affect this basal level (Figure 5). On the contrary, a substantial reduction in transcription efficiency is observed when a plasmid deleted for sequences upstream from -158 is transfected either into Hela cells in the presence of E1A (Figure 5) or into 293 cells (data not shwon). Thus, a deletion of the sequences mapping between -329 and -158 results in at least a 15 fold drop in transactivation by E1A. In several experiments, it was still possible to detect a residual transactivation by E1A with mutants -158 and -148. Additional deletion of the adjacent region -134 -89 does not further alter the transactivation since the CAT activity levels are similar in the absence or in the presence of E1A for the -134, -123, -101, -98 and -89 mutants (Figure 5). These results suggest that the sequences responsive to E1A activation map upstream from -158. The transactivation process is, in fact, altered at two levels by the deletion. A modest 2-3 fold decrease is observed when the promoter is deleted for the region from -329 to -220 showing that the ITR which maps between -329 and -227 participates in the regulation. An additional deletion of the adjacent -179 -158 region results in a further 8-10 fold reduction, suggesting that this region contains an essential regulatory element.

DISCUSSION

The mechanism of activation of the early genes of adenovirus by E1A is unknown. In order to study this regulatory process, we have first investigated whether such a control also exists when cloned viral genes are introduced into the cell by transfection. Such a procedure isolates genes from their natural viral environment, and thus limits the number of viral gene products in the cell. To this end, we have put the CAT gene under the control of the E4 promoter of Ad2 and have studied the response of the promoter to the E1A regulator by measuring transient expression of CAT activity. We have found that transfections performed in the presence of E1A

resulted in at least a 20 fold increase in the level of CAT activity as compared to the basal level detected in its absence. These results are in agreement with those recently published by Weeks and Jones (22) who showed that transcription of the HSV thymidine kinase gene put under the control of the E4 promoter of Ad2 was controlled by E1A. It seems likely that the transactivation by E1A of the cloned E4 promoter occurs by an identical mechanism to that which is observed during infection. If we assume that the level of CAT activity correlates with the concentration of the mRNAs, it is likely that the action of E1A takes place at the level of the initiation of transcription rather than at later stages such as processing, stability or transport of the mRNAs. These experiments with cloned DNAs exclude the possibility of an activation in cascade by intermediate viral products such as the 72K polypeptide encoded by the E2A region (23).

The correct processing in cells of the intronless E1A plasmids (24) suggests that they can be used to faithfully direct the synthesis of each E1A polypeptide. Whereas both polypetides P243 and P289 are able to activate transcription from the E2A and E3 early promoters (25), the results we have obtained with the intronless Ad2 E1A plasmids implicate only the polypeptide P289 as the regulatory agent of the E4 promoter. This result is in agreement with the recent report of Svensson et al (26) and suggests a different susceptibility of these promoters to the E1A polypeptides.

In order to narrow down the regulatory region of the E4 promoter responsible for E1A inducibility, we have constructed deletion mutants in a 5' to 3' direction which reduced the 5' flanking region upstream from the cap site. We have found that sequences located very far upstream from the cap site are necessary to keep the transactivation process at its maximum level. Progressive deletions of the region mapping between -329 and -220 result in a modest decrease of the transcription efficiency. Thus, the loss of this region, which spans the ITR, lowers the CAT level about to 2-3 fold as compared with the undeleted promoter (position -329). An additional deletion of the short adjacent region which maps between positions -179 and -158 has a more drastic effect, causing an 8-10 fold drop in transcription as compared to the -179 deletion mutant. It is striking that this region contains a sequence, C1, (Figure 4) homologous to the E1A enhancer core sequence (27). Interestingly, another similar sequence, C2, maps between -149 and -141 (Figure 4). Each sequence lies within two almost perfectly duplicated elements, 25 bp and 23 bp, starting respectively at positions

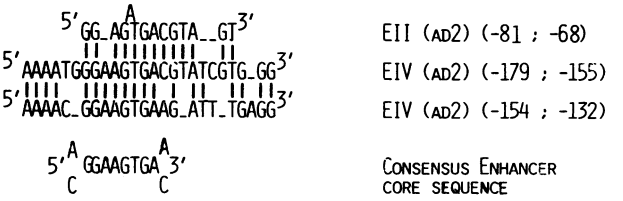

Figure 6. Comparison of the 5' distal regions of the E2A and E4 early promoters. The sequences are aligned according to maximal homology with the consensus enhancer core sequence proposed by Hearing and Shenk (27). The positions of the sequences with respect to the transcription initiation sites are indicated.

-179 and -154 (Figure 6). In several experiments, a residual transactivation could still be detected with mutants -158 and -148. It is possible that both copies, C1 and C2, are required for full regulation.

The sequences which are essential to the transactivation process of the E4 promoter appear to be further from the "TATA" homology than those of the E2A early promoter since no more than 79 nucleotides upstream from the cap site are required for regulation of this latter gene (28,29). However, since transcription from both promoters is dependent on E1A, it may be that some degree of homology exists within their regulatory regions. To emphasize any homology, we have compared the 5' distal sequences which allow these promoters to keep their E1A regulation. Thus, we have found a very well conserved sequence between the -80 -67 region of the E2A early promoter and both -154 -132 and -179 -155 regions of the E4 promoter (Figure 6). Interestingly, the conserved sequence spans the consensus E1A enhancer core sequence, and strengthens the hypothesis that this peculiar domain plays a key role in the regulation of the E4 promoter. The presence of two copies of the consensus enhancer core sequence within the E1A sensitive region of the E4 promoter suggests the interesting possibility that this region functions as an E1A dependent enhancer. Enhancer elements have been already defined within the E1A and E2A early promoters (27,29). However, it is unknown whether these elements function in an E1A dependent manner. Thus if the E4 promoter also contains an enhancer element as suggested by the sequence data, then it might be that activation of transcription of viral genes occurs via an E1A mediated interaction of the RNA polymerase II with specific enhancer sequences.

The role played by the -329 -220 region of E4 promoter is difficult to interpret since the deletion of these sequences results in a modest

decrease in transcription efficiency. Possibly, this region has evolved to perfect the regulatory action of the -179 -134 domain. Sharp has recently shown that transcription of the E4 gene is efficient in a whole cell extract which does not contain the E1A polypeptides (30). However, it is severely hampered when the promoter is deleted for sequences upstream from -140. Thus, it is likely that the same sequences are required for both *in vitro* transcription and transactivation by E1A *in vivo*. Such a requirement is reminiscent of the results obtained by Elkaim for the E2A early promoter (28).

The E4 promoter is poorly recognized in the cell in the absence of E1A, suggesting that a cell block prevents it from functioning properly. This block would be suppressed by E1A *in vivo* whereas it would not take place *in vitro*. The cell might operate by blocking the site of interaction of RNA polymerase II with the promoter. This site might be located upstream from -140 since a deletion of these distal sequences prevents E1A transactivation *in vivo* and efficient transcription *in vitro*. Nevins has proposed that a cellular repressor (7) might constitute such a block.

In any case, the efficiencies of the transcription from the E4 deleted promoters, as measured in the absence or the presence of E1A (Figure 5) exclude the possibility that repression takes place at a site upstream from the promoter. If the E4 promoter contains an enhancer modulatory sequence, then the repressor might directly act at the level of this enhancer. Alternatively, the DNA might be packaged in a chromatin structure which would make the promoter inoperative ; E1A would then modify this structure.

Clearly, isolation of additionnal mutants within the regulatory domain -179 -134 as well as its displacement both in orientation and position should allow us to clarify our understanding of E1A activation.

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