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Adenovirus E3-early promoter: sequences required for activation by E1A

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ABSTRACT

To identify sequences within the adenovirus-5 E3 promoter necessary for E1A trans-activation, a series of promoter deletion mutants were constructed and analysed. A region between positions -82 and -105 was shown to be critical both for E1A induced expression as well as uninduced expression. The importance of this region was confirmed by constructing hybrid promoters consisting of E3 and Herpes simplex virus thymidine kinase sequences. The E1A insensitive tk promoter could be converted to an E1A sensitive promoter by replacing sequences upstream of position -79 with the corresponding region of the E3 promoter. This critical region of the E3 promoter contains a sequence 5′ AGATGACTA3′ which is also present in important upstream regions of the E2A and E4 promoters.

INTRODUCTION

The expression of the Adenovirus type 5 (Ad5) genome in infected cells is highly regulated with different genes being expressed at different times of the productive cycle. A number of the Ad5 genes are expressed during the early phase of the productive cycle, before the onset of viral DNA replication (1,2). The first such gene to be expressed is the 'immediate-early' E1A gene located at the extreme left-hand end of the viral genome. A product encoded by this gene is subsequently required for the efficient expression of the other early transcriptional units (E1B, E2A, E3 and E4; 3,4). This stimulation of early gene expression by E1A is due to an increase in transcription initiation (5) and is known to involve control sequences located within the 5′ flanking region of the regulated genes (6-8). Thus, fusion of early gene promoter sequences to a variety of structural sequences results in chimeric genes inducible by E1A. We have shown previously that a chimeric gene containing the upstream promoter region of the early E3 gene fused to sequences encoding the enzyme chloramphenicol acetyltransferase (CAT) is expressed at least 10 times more efficiently in the presence of E1A gene product (6); the E1A product can be supplied by cotransfection with the intact E1A gene (6), by microinjection of purified E1A protein (9) or by transfecting

into a particular cell line (293 cells) that contains and expresses an integrated copy of the E1A gene (6).

The primary transcript of E1A gives rise at early times of infection to two overlapping mRNAs, 12s and 13s in size, by differential splicing (1,2). The two messages share common 5′ and 3′ termini but differ by the extent of internal sequences removed. The two polypeptides encoded by the messages are 243 and 289 amino acids in length and are identical except for the additional 46 residues encoded by the unique portion of the 13s mRNA. It is not clear which polypeptide is responsible for the transcriptional activation although most of the existing evidence suggests that it is predominantly a function of the 289AA protein. Viral mutants have been isolated that contain specific alterations within the E1A sequences that differentially alter the 13s and 12s mRNAs. Mutants that synthesise normal 12s mRNA but altered or no 13s mRNA exhibit a regulatory defect (3, 10-13) whereas mutants that synthesise normal 13s mRNA but altered or no 12s mRNA regulate early gene expression normally (13,14). A number of studies using a HeLa cell transient expression system and plasmids encoding the E1A region give similar results (8,15). However, there is some evidence suggesting that the 243AA protein is also capable of transcriptional activation (13,16), and indeed the 243AA protein synthesized in and purified from E. coli can activate early gene expression when microinjected into mammalian cells (Ferguson, B., Kripple, B., Westphal, H., Andrisani, O., Jones, N.C. and Rosenberg, M., in preparation). The reason for the apparently conflicting results is not presently clear.

To gain further insight into the mechanism of E1A induced transcriptional activation, we have examined the sequences in the E3 promoter region required for this activation to take place. Removal of sequences upstream of position -105 had no effect on E1A activation whereas removal of additional sequences to position -82 resulted in very low expression levels both in the presence and absence of E1A. The results indicate that sequences between these two positions are important for normal induction. Additional support for this conclusion was provided by constructing chimeric promoters consisting of sequences from the E3 promoter and the herpes simplex virus thymidine kinase (tk) promoter. Exchange of sequences upstream of position -79 of the tk promoter with analogous sequences from the E3 promoter resulted in a hybrid that was activated by E1A.

MATERIALS AND METHODS

Cells

HeLa cells were maintained as monolayer cultures is Dulbecco modified

Eagle medium containing 10% fetal calf serum.

Construction of plasmids.

The plasmid p3CAT which contains the E3 promoter fused to the chloramphenicol acetyl-transferase (CAT) coding sequence was constructed by cloning the Ad5 623bp Sau3A restriction fragment containing the E2A and E3 cap sites and upstream sequences into the BamHl site of pCATB' (6) (Figure 1). E3CAT alleles carrying deletions in the E3 upstream promoter sequences were constructed as follows. Fragments extending from the Sau3A cleavage site at position +62 to either the Ddel, BstNl or Rsal sites at positions -82, -105 and -122 respectively were isolated following separation on a 6% polyacrylamide gel. The protruding 5' ends of each fragment were filled in with DNA polymerase (Klenow fragment), blunt-end ligated to synthetic BamHl linkers and cloned into the unique BamHl site of pCATB'. Orientation of the inserts was determined by restriction endonuclease site mapping. In the resulting plasmids pCAT•Ddel, pCAT•BstNl and pCAT•Rsal the orientation of the insert is such that CAT gene expression is controlled by E3 promoter sequences. The plasmid pCAT•BstXl was constructed by cleaving p3CAT with Clal and BstXl, removing the protruding termini with Sl nuclease and religating. The resulting plasmid thus contained E3 promoter sequences extending from the Sau3A site at +62 to the BstXl site at -127.

Plasmids containing E3/tk chimeric promoters fused to CAT coding sequences are diagrammatically shown in Figure 2 and constructed as follows. Derivatives of the linker-scanning (LS) mutants (17) were prepared by substituting the tk coding sequence with CAT coding sequence; this was accomplished by replacing the BglII-Sall fragment from the LS mutants that contains the tk coding region with the BamHl-Sall fragment from pCATB' (Fig 1) containing CAT sequences. The LS mutants were kindly provided by S. McKnight. The two LS derivatives that were used for subsequent constructions were LS-115/-105 CAT and LS-84/-74 CAT; the two contain unique BamHl restriction sites at positions -100 and -79 respectively. Transcription from the LS-115/-105 promoter is equally efficient as transcription from the wild-type promoter and we therefore used LS-115/-105 CAT to estimate expression from the natural intact tk promoter. The chimeric E3/TK promoters were initially assembled in the plasmid pUC8. The 623 bp fragment from the Ad5 genome that contained the E2A and E3 promoters (see Fig. 1) is cleaved once by the enzyme Ddel at position -82 relative to the E3 transcriptional start. The two fragments that result from such a digestion were cloned into the Smal site of pUC8. For the construction of E3/TK CAT-1 the BamHl-Sall fragment from LS-84/-74 CAT

(containing the promoter sequences up to -80 and the entire CAT coding region) was cloned into the BamHI-SalI restriction sites of the pUC8 derivative that contained the -82 to -560 E3 promoter sequences; this placed the -79 sequence of the tk promoter adjacent to the -82 sequence of the E3 promoter. The chimeric promoter was subsequently moved into pCATB' utilizing the EcoR1 restriction site at -230. The chimeric promoter in pE3/TK CAT-2 was constructed by cloning the HindIII-BamHl fragment from LS-84/-74 CAT (containing the tk promoter sequences from -79 to -490) into the HindIII-BamHl sites of the pUC8 derivative containing the -82 to +65 E3 promoter sequences so that the -79 sequence of the tk promoter was adjacent to the -82 sequence of the E3 promoter. The chimeric promoter was moved into pCATB' using the HindIII site at -490.

The plasmids pE3/TKCAT-3 and pE3/TKCAT-4 shown in Figure 3 are derivatives of pLS-115/-105 CAT that contain promoter sequences at the 5' or 3' end of the chimeric gene. pE3/TKCAT-3 contains the tk promoter sequences up to position -110; these promoter sequences are followed upstream by E3 promoter sequences extending from -82 to -250. This construct was made essentially as described for pE3/TKCAT-1 except that the BamHl-SalI fragment cloned into the pUC8 derivative originated from pLS-115/-105CAT instead of pLS-84/-74CAT. pE3/TKCAT-4 is a derivative of pLS-115/-105 CAT that contains E3 promoter sequences at the 3' end of the CAT coding region. It was constructed by cloning the HindIII-SalI fragment of pLS-115/-105 CAT into the pUC8 derivative containing the -82 to -560 sequences of the E3 promoter. The chimeric gene plus 3' flanking E3 promoter sequences were moved into pBR322 by cloning the HindIII-SmaI fragment (HindIII site is at -490 of the promoter and the Sma 1 site at -250 of the E3 promoter sequences) into the HindIII-NruI sites of pBR322.

The plasmids pLS-115/-105 CAT-1 and -2 (Figure 4) were constructed by moving the HindIII-SalI fragment of pLS-115/-105 CAT containing the TK-CAT chimeric gene into pBR327 and pUC8 respectively.

CAT expression in transfected cells.

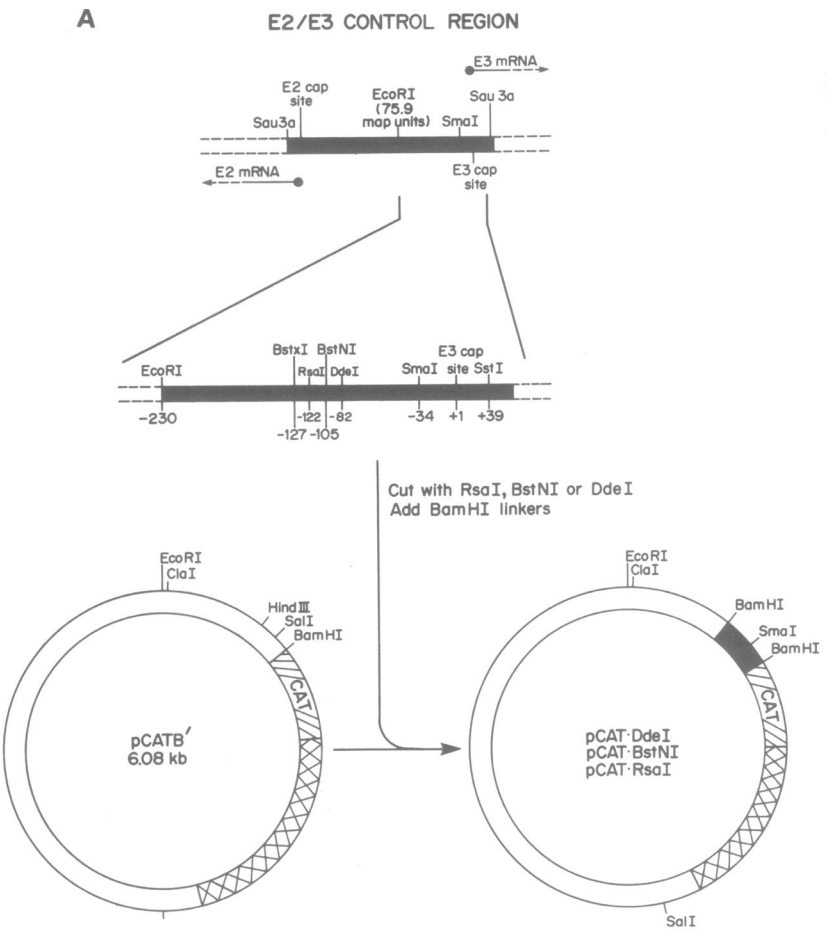
The expression of the CAT gene in HeLa cells transfected with the indicated plasmids with or without the E1A containing plasmid pJOLC3 was determined as described previously (6). Each 100-mm plate of cells was transfected with 10µg of E3CAT plasmid and 20µg of either pBR322 (-E1A) or pJOLC3 (+E1A;6). A number of different experiments using different preparations of plasmid DNAs were performed with essentially the same results.

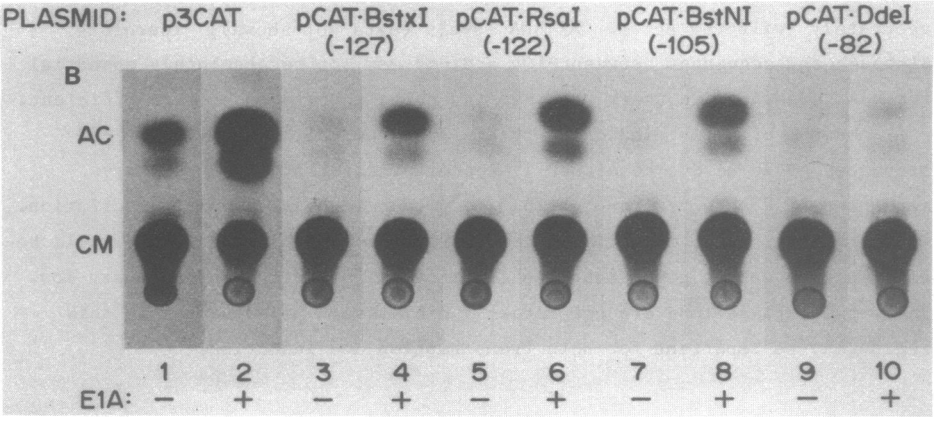
RESULTS

Construction and characterization of pE3CAT mutants with deletions in the E3 promoter sequences.

Fusion of sequences from the 5′ end of the E3 gene to the coding region specifying the CAT enzyme, results in a chimeric construct that is sensitive to E1A(6); in the presence of E1A, expression of E3CAT is 10-40 times higher than in it's absence. To examine the role of these E3 sequences in E1A induction in greater detail we constructed a series of E3CAT alleles containing deletions within the E3 promoter region. For the constructions we made use of restriction endonuclease cleavage sites at positions -82, -105, -122, and -127 relative to the transcriptional start site (Figure 1A). Fragments extending from position +65 to each of these sites were inserted at the 5′end of the CAT coding sequences in the plasmid pCATB′ (Figure 1A). The plasmids were introduced into HeLa cells in the presence and absence of a second plasmid containing the E1A gene and CAT expression measured 40-50 hours later. The results are shown in Figure 1B. Removal of sequence upstream from nucleotide -105 had little effect on normal induction by E1A; with constructs having deletion endpoints at -105 (Figure 1B, lanes 7,8), -122 (lanes 5,6) and -127 (lanes 3,4) expression was at least 10 fold higher in the presence of E1A (Figure 1B, lanes 9,10). However, deletion of sequences between positions -105 and -82 resulted in close to background levels of CAT activity both in the presence and absence of E1A. In some experiments a small induction of CAT activity by E1A was seen with pCAT•Ddel. However, the induction was never found to be more than two-fold and was not reproducible. S1 mapping using a uniformally labelled probe that detected CAT coding sequence confirmed that the CAT activities detected with each of the various constructs in the presence and absence of E1A accurately reflected actual CAT RNA levels (data not shown). Therefore although the sequences between -105 and -82 may not be absolutely essential for E1A induction, in their presence induction appears to be more efficient.

Absence of normal induction by E1A upon removal of the -105 to -82 sequences could be due to either 1) the elimination of E1A regulatory sequences or 2) greater than 15-fold reduction in basal level transcription. In this latter case, induction of transcription by E1A of 5-10 fold would be difficult to detect given that the level of CAT activity would be very low. These two explanations are not mutually exclusive; the sequences in this region may be important for both transcription and induction.


A
E2/E3 CONTROL REGION
E3 mRNA
E2 cap site
EcoRI (75.9 map units)
Sau 3a
Sau3a
SmaI
E2 mRNA
E3 cap site
BstxI BstNI
EcoRI
RsaI DdeI
E3 cap
SmaI site SstI
-230
-122 -82
-127 -105
-34 +1 +39
Cut with RsaI, BstNI or DdeI
Add BamHI linkers
EcoRI
ClaI
HindIII
SalI
BamHI
CAT
pCATB'
6.08 kb
pCAT·DdeI
pCAT·BstNI
pCAT·RsaI
SmaI
SalI

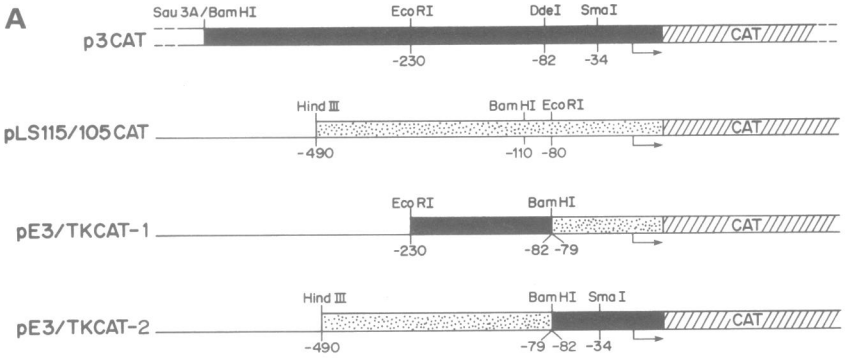

PLASMID: p3CAT
pCAT·BstxI (-127)
pCAT·RsaI (-122)
pCAT·BstNI (-105)
pCAT·DdeI (-82)
B
AC
CM
1 2 3 4 5 6 7 8 9 10
E1A: − + − + − + − + − +

Construction and characterization of chimeric promoters consisting of E3 and tk promoter sequences.

To further explore the role of the E3 sequences in E1A induction, we constructed chimeric promoters containing the upstream regions of the E3 gene and the HSV tk gene. A hybrid tk promoter/CAT gene in pBR322 is not induced by E1A as measured in transient expression assays (Fig. 2B, lanes 3,4). In addition, we have shown previously that E1A does not influence the ability of the intact tk gene to convert tk^- cells to a tk^+ phenotype (6). We have examined whether sequences within the tk promoter can be exchanged with positionally analogous sequences of the E3 promoter resulting in E1A inducibility. Two of the chimeric promoters constructed are shown in Fig. 2A. The hybrid promoter in E3/TKCAT-1 consists of E3 sequences from -230 to -82 and tk sequences from -79 to the transcriptional start site. In contrast, the hybrid promoter in E3/TKCAT-2 contains tk promoter sequences from -490 to -79 and the E3 sequences from -82 to the transcription start site. Both plasmids contain CAT as the test gene. E3/TKCAT-1 is induced by E1A although the level of induction was not as great as that seen with the intact E3 promoter (approximately 5-fold compared to 10-fold, Fig. 2B, lanes 5,6). In contrast, although basal level expression in plasmid E3/TKCAT-2 was detectable, induction by E1A was never found to be more than 1.5 fold in a number of separate experiments with different plasmid preparations (lanes 7,8). The results therefore confirm that sequences within the '-100' region of the E3 promoter contribute significantly to the sensitivity of that promoter to E1A.

Imperiale et al. (18) utilizing a CAT gene under the control of adenovirus E2 promoter mutants have determined that the sequences required for E1A induced expression map between 21 and 79 nucleotides upstream of the transcriptional initiation site. In addition they suggest that these

Figure 1. Transcriptional properties of E3CAT gene alleles carrying deletions in upstream promoter sequences. A, The Ad5 623bp fragment containing both the E2A and E3 cap sites and upstream sequences is shown (solid bar). This fragment contains all the information necessary for transcription and E1A trans-activation from both cap sites. Below is shown in greater detail the position of the restriction endonuclease cleavage sites within the E3 promoter region that were utilized to construct the E3CAT alleles. Fragments extending from the Sau3A cleavage site at position +62 to either the DdeI, BstNI or RsaI sites at positions -82, -105 and -122 respectively were inserted into the BamHI site of the plasmid pCATB' (6). CAT coding sequences are depicted by the striped bar and 3' eukaryotic control sequences by the hatched bar. B, Relative expression of CAT in HeLa cells transfected with the various E3CAT alleles in the absence (-) or presence (+) of the E1A containing plasmid pJ0LC3. The position of chloramphenicol (CM) and its monoacetylated forms (AC) is shown.



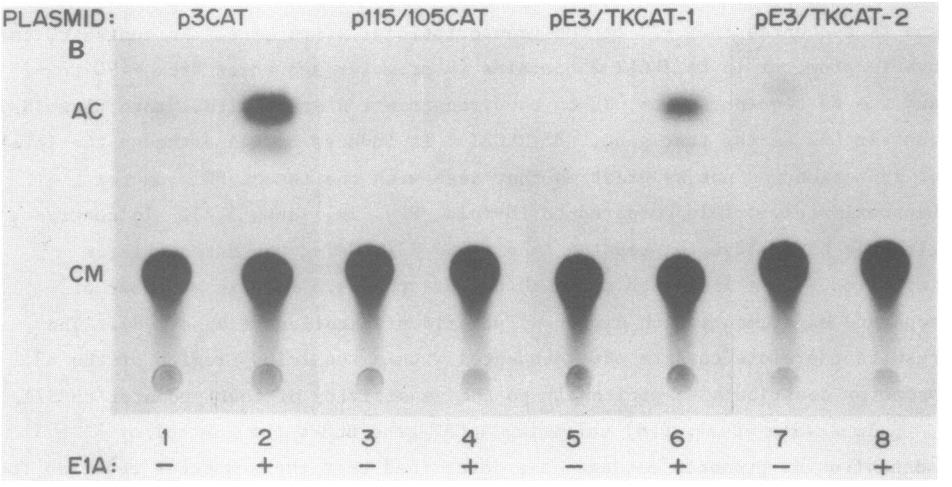


Figure 2. Transcriptional properties of genes containing the CAT coding sequence fused to chimeric E3/TK promoters. A, Structure of the chimeric promoters in pE3/TKCAT-1 and 2. Solid bars represent E3 promoter sequences, stippled bars tk promoter sequences and striped bars CAT coding sequences. All the constructs are derivatives of pCATB' (see Fig. 1). Also shown are the promoters in p3CAT (Fig. 1) and pLS-115/-105 CAT. B, Expression of the CAT gene in HeLa cells transfected with pE3/TKCAT-1 and -2 in the presence and absence of E1A.

sequences can confer inducibility to a heterologous promoter at a distance and independent of orientation. We have asked whether the E3 promoter sequences operate in a similar fashion. The plasmid pE3/TKCAT-3 contains the entire functional tk promoter region (from the transcriptional start site to position -110) fused to the CAT coding sequences. Upstream of the promoter sequences are E3 sequences extending from position -82 to -250; thus the E3 -82 to -105

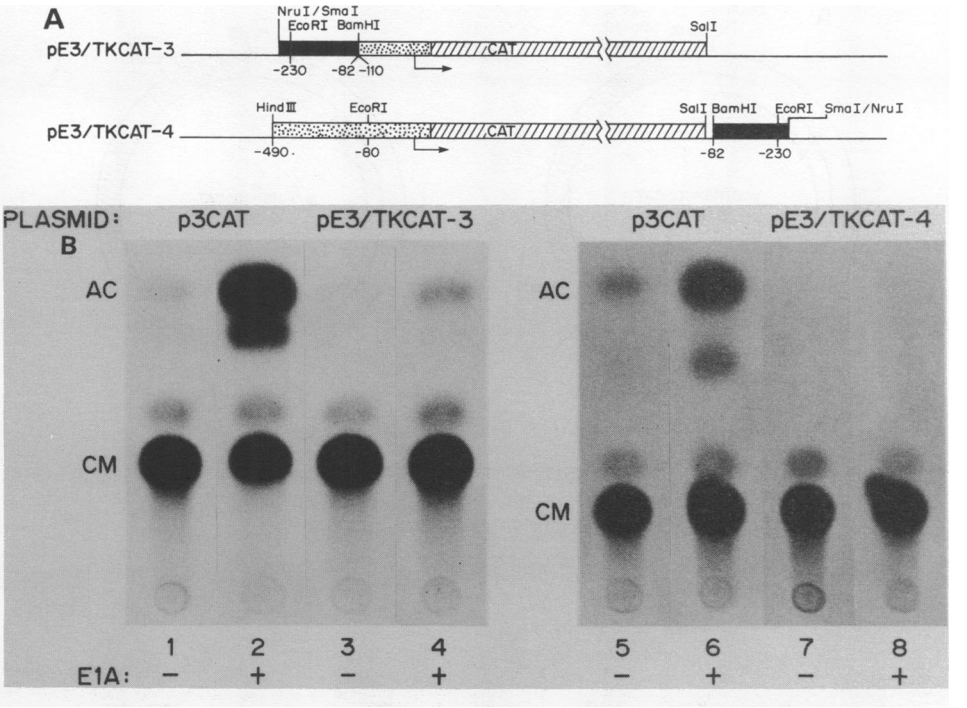


Figure 3. A, The structure of pE3/TKCAT-3 and pE3/TKCAT-4; both are derivatives of pLS-115/-105 CAT that contain E3 promoter sequences at the 5′ or 3′ end of the chimeric gene. B, Expression of the CAT gene in HeLa cells transfected with pE3/TKCAT-3 and -4 in the presence and absence of E1A.

sequences are displaced with respect to the transcriptional start site, being approximatey 30 nucleotides further upstream. CAT expression from this construct is induced by E1A but approximately two-fold less well than from pTKCAT-1. In contrast, placing the E3 promoter sequences at the 3′end of the TKCAT gene as in pE3/TKCAT-4, did not result in E1A trans-activation. We conclude from these results that the E3 promoter sequences can not render E1A sensitivity in a position independent manner.

Interference of E1A transcriptional activation by plasmid sequences.

During the course of these experiments we observed to our great surprise that if the TKCAT gene was transferred from pBR322 to either the plasmid pUC8 or pBR327 expression from the tk promoter was induced signficantly by E1A (Fig. 4). Plasmid copy numbers were found to be the same in the presence and absence of E1A (data not shown). The results suggest that sequences present in pBR322 but not the other two vectors interfere with transcriptional

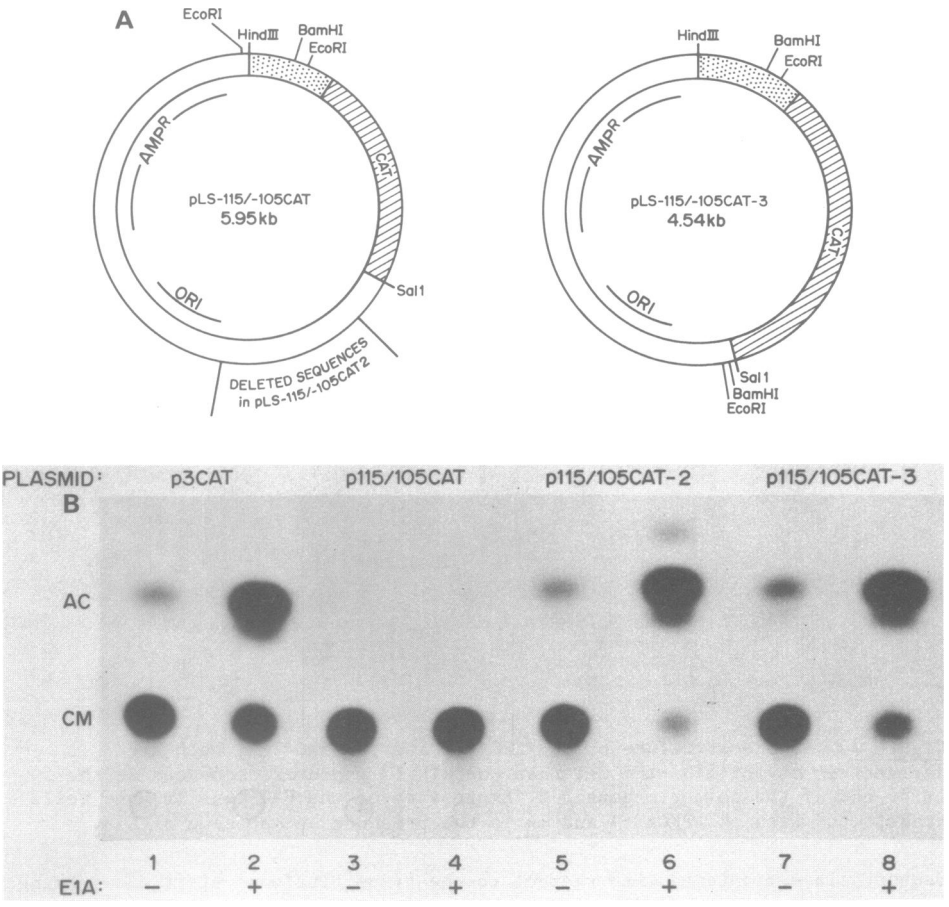


Figure 4. Expression of pLS-115/-105CAT in different plasmid backgrounds. A. Structure of the TKCAT gene in pBR322 (pLS-115/-105CAT), pBR327 (pLS-115/-105 CAT-2) and pUC8 (pLS-115/-105 CAT-3). B. CAT expression in HeLa cells transfected with the indicated plasmids in the presence and absence of E1A.

activation by E1A. The common sequences missing in pUC8 and pBR327 but present in pBR322 extend from the EcoRI1 restriction site at nucleotide position 1442 to the PvuII restriction site at 2066. It should be emphasized at this point that all the constructions described in the previous sections (Figs. 1-3) were in pBR322 indicating that transcriptional activation from the E3 promoter and the hybrid promoter in pE3/TKCAT-1 could take place even in the presence of these interfering plasmid sequences. We subsequently retested other promoters we had categorized as being insensitive to E1A induction after

5'-AGATGAC-TA	E3 -96 to -88
5'-AGATGACGTA	E2 -79 to -70
5'-AG-TGACGTA	E4 -170 to -162
5'-AGA-GACG	E3 -118 to -112

Figure 5. Comparison of upstream regions of the E3, E2A and E4 promoters. The position of the sequences with respect to the transcription initiation sites is indicated.

removal of these specific plasmid sequences (by transferring the gene to different vectors). For instance, a chimeric gene containing the CAT coding sequence fused to the promoter from the rat cholecystokinin gene (19) can be transcriptionally activated by E1A when cloned into the pUC8 vector but not pBR322. The Ad5 $1\underline{V}a_2$ gene promoter fused to the CAT structural sequences gives similar results. The interference of E1A action by these plasmid sequences is therefore not specific to the tk promoter. What distinguishes the Ad5 early gene promoters is their ability to show transcriptional activation even in the presence of these plasmid sequences.

DISCUSSION

We previously showed that fusion of the upstream region of the E3 gene to the CAT coding sequence resulted in an E3CAT chimeric gene whose expression was induced 10-40 fold in the presence of E1A (6). We have extended these studies to define more precisely the sequence elements required for this induction to take place. Analysis of E3 promoter deletions suggest that a critical element is located between positions -105 and -82 relative to the transcriptional start site; deletion of these sequences significantly lowered promoter efficiency both in the presence and absence of E1A. The level of expression in the presence of E1A was greater that 10-fold lower than the level obtained with the intact promoter. In contrast, deletion of sequences upstream of position -105 had little effect on expression and a normal induction ratio with E1A was obtained. The importance of the -105 to -82 region was confirmed by constructing chimeric promoters consisting of E3 and HSV tk sequences. Expression of the TKCAT gene present in the plasmid pBR322 was not induced by E1A. However by substituting the tk promoter sequences upstream of position -79 with similarily positioned sequences of the E3 promoter resulted in a hybrid that was induced by E1A. The results suggest that the differences in sensitivity of the tk and E3 promoters to E1A induction was due, at least in part, to differences in the upstream `-100' region.

Studies with other early gene promoters have also demonstrated a role of upstream promoter sequences in E1A induced expression. A critical element for

transcription from the E2A promoter both in the presence and absence of E1A is located between positions -79 and -59 (18). Deletion of these sequences inactivates the promoter. In addition point mutations in this region and specifically between positions -82 to -66, reduce promoter efficiency 9-12 fold (20). The E4 promoter also appears to have a critical upstream element although in this case it is situated between positions -158 and -179 (8). Therefore, the exact position of these critical elements can vary and any model to explain E1A activation has to accomodate this positional flexibility. A comparison of the critical upstream regions of the E2A, E3 and E4 promoters reveals an interesting conservation of sequence (Fig. 5; 8). The absolute importance of these conserved regions within the E3 and E4 promoters will depend upon the isolation of mutants with single base-pair changes in this region. This detailed analysis has been carried out with the E2A promoter and indeed this conserved region is essential for efficient promoter activity (20).

Leff et al. (personal communication) have also constructed and characterized a series of E3 promoter deletion mutants. Their results suggest that an internal deletion between positions -111 and -59 has no effect on promoter utilization, a result in apparent conflict with the data we have presented in this report. However, examination of the sequences just upstream of position -111 reveals a sequence homologous to the conserved regions described above (Figure 5); this sequence is located between positions -118 and -112. Therefore in the E3 promoter two copies of the conserved region is present and we would suggest that both can serve as regulatory elements. Thus, deletion of one or the other has minimal effect on transcription from the E3 promoter whereas deletion of both (as in pDde•CAT) drastically reduces promoter efficiency. Within the tk promoter, there are two positions where sequences similar to the conserved region are found (between nucleotides -57 to -66 and -110 to -116). However, in both cases these sequences are dispensible for efficient promoter function in microinjected Xenopus oocytes (17).

The sequences located between -105 and -82 are clearly important for efficient E1A trans-activation and efficient basal transcription of the E3 gene. However, whether the sequences reponsible for uninduced and induced transcription can be uncoupled cannot be determined with the present constructs. A very detailed analysis of the E2A promoter, where almost every nucleotide of the promoter was mutated in a series of linker-scanning mutants, did not reveal any sequence that was specifically and uniquely required for

E1A trans-activation (20). All mutations that affected the induced level of expression also affected uninduced expression. These results suggest that uninduced and induced transcriptional signals cannot be uncoupled and argues against E1A acting via specific recognition signals. Support for this conclusion comes from two other observations. (1) Transcription of a number of non-viral genes can be activated by E1A when they are newly introduced into mammalian cells (21,22). (2) Transcription of the adenovirus early genes can be activated by regulatory products encoded by the pseudorabies (23) and herpes simplex virus genomes (Haley, K. and Jones, N., unpublished). These studies make it unlikely that E1A acts directly upon a specific nucleotide sequence. What is the role of this region of the E3 promoter in trans-activation? Additional studies are required to approach this important question. The results presented here and elsewhere do imply a close link between uninduced and induced transcription. It is possible that the -82 to -105 region of the E3 promoter is recognized by a specific transcriptional factor and that in the presence of E1A either the effective concentration of this factor is increased or the interaction of the factor and this region is enhanced.

Expression of the TKCAT gene in the plasmid pBR322 is not increased by E1A. In contrast however, E1A does induce expression if the gene is in different plasmid backgrounds (pUC8 or pBR327). The results suggest that specific plasmid sequences can interfere with promoter use. The nature of this interference is not known but it does caution against concluding that a promoter is unaffected by the action of the E1A gene based on the negative results of a single plasmid construct.

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