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Mutational analysis of the adenovirus E1a gene: the role of transcriptional regulation in transformation

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To determine whether the transcription regulatory activities of the adenoviral E1a gene play a role in its ability to transform primary cells we have constructed an extensive series of mutations within the E1a gene. The mutants have been characterized for their ability to transactivate the adenoviral early promoters, repress the transcriptional stimulation of the polyoma virus enhancer, establish primary baby rat kidney cells in culture and cooperate with the activated Ha-*ras* oncogene in morphologically transforming these cells. The mutant phenotypes reveal that: (i) the two transcription regulatory activities of E1a are separable since essential protein domains map within different regions of the protein; (ii) transactivation is unlikely to contribute significantly to E1a-mediated transformation since several isolated mutants lost the ability to transactivate but were neverthless efficient at transformation; and (iii) both establishment and oncogene cooperation are linked to enhancer repression suggesting that E1a transforms cells by the repression of a cellular enhancer.

Key words: adenovirus E1a/enhancer repression/transactivation/cell establishment/oncogene cooperation

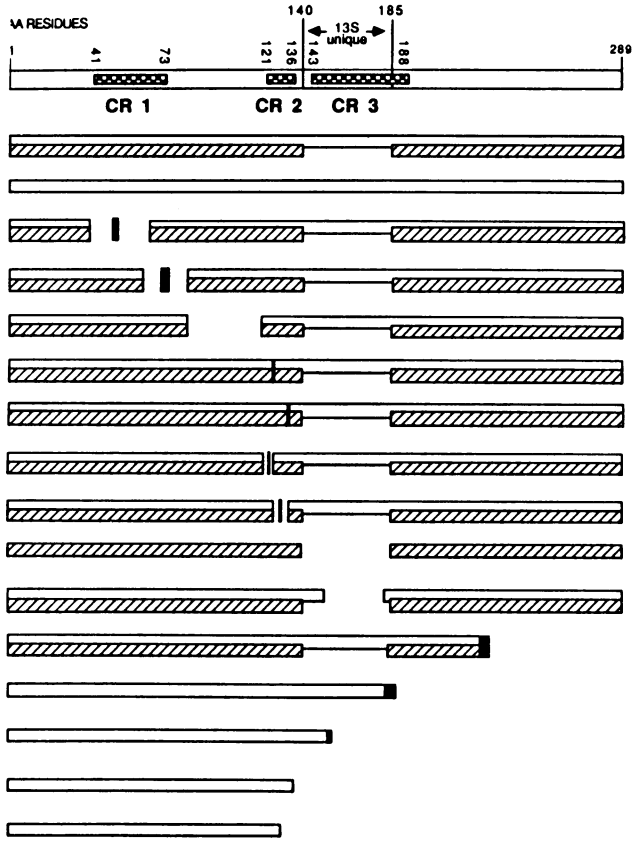
Introduction

The E1a gene of the human adenoviruses is required by the virus for both efficient growth in permissive cells and oncogenic transformation of primary rodent cells. During early times of infection and in transformed cells the E1a region expresses two overlapping mRNAs (12S and 13S) that differ only by the amount of internal splicing (Berk and Sharp, 1978; Chow *et al.*, 1979; Perricaudet *et al.*, 1979; Kitchingman and Westphal, 1980). The major protein products from these two transcripts are nuclear phosphoproteins of 243 and 289 amino acid residues, respectively, and are identical except for an internal 46 amino acid residues missing from the product of the 12S mRNA. At late times of infection, the E1 region produces a third mRNA (9S) encoding a putative 54-amino acid protein.

Transformation of primary cells by adenovirus involves the concerted action of both the E1a and adjacent E1b genes. The exact role of each gene in the transformation process remains unclear. However, E1a is known to be a member of a group of oncogenes capable of extending the growth potential of primary cells enabling their establishment as permanent cell lines (Jones and Shenk, 1979; Houweling *et al.*, 1980; Ruley, 1983; Zerler *et al.*, 1986). The resulting cells however, do not possess a fully transformed phenotype and are non-tumorigenic. To produce cells with a fully transformed phenotype, E1a normally cooperates with its viral partner E1b (van der Eb *et al.*, 1977; Graham *et al.*, 1978; Chinnaduria, 1983), but it can also cooperate with a number of other viral and cellular oncogenes (Ruley, 1983; Haley *et al.*, 1984; Zerler *et al.*, 1986). For example, E1a will cooperate with the activated *ras* oncogene to fully transform baby rat kidney (BRK) cells. The cooperative function that E1a provides is thought to be establishment although some studies suggest that additional functions may also be provided.

In addition to its transforming activity, E1a is also capable of both transactivating and repressing the transcription of a variety of unrelated viral and cellular genes. For example, during infection E1a is responsible for transactivating the expression of the other early adenovirus genes E1b, E2a, E3 and E4 (Berk *et al.*, 1979; Jones and Shenk, 1979; Nevins, 1981; for review, see Berk, 1986). in the absence of E1a, early gene expression is very inefficient and the virus fails to propagate. Additionally, both the human Hsp70 (Nevins, 1982; Kao and Nevins, 1983; Wu *et al.*, 1986) and β tubulin (Stein and Ziff, 1984) genes have been shown to be transactivated in an E1a-dependent manner and it is possible that other endogenous genes are similarly transactivated but not yet recognized. The mechanism for transactivation is unclear, but it is known to occur at the level of transcriptional initiation (Nevins, 1981). Recent reports suggest that E1a may increase the effective concentration of a normal cellular transcription factor (Kovesdi *et al.*, 1986). Repression by E1a involves *cis*-acting promoter elements called enhancers. These elements are required for the efficient expression of some viral and cellular genes, and can also stimulate transcription from many heterologous promoters (for review, see Serfling *et al.*, 1985). E1a proteins have been shown to interfere with the transcriptional stimulation of the viral SV40, polyoma and adenoviral E1a enhancers (Borrelli *et al.*, 1984; Velcich and Ziff, 1985), as well as the cellular immunoglobulin heavy chain (Hen *et al.*, 1985) and insulin enhancers (Stein and Ziff, 1987).

Are the transcriptional regulatory activities of E1a important to its role in cell transformation? It is clear from a number of other studies that transformation can result from the aberrant expression of specific endogenous cellular genes (for review, see Weinberg, 1985). Since E1a has been shown to be capable of both transactivating and repressing particular cellular genes, either activity could function in altering the growth regulation of cells resulting in establishment and/or cooperation. In order to approach this important question we have undertaken an extensive mutational analysis of the E1a gene. A variety of single amino acid substitutions, small in-frame deletions and truncations have been introduced into the gene and the resulting mutants assayed for their ability to transactivate the adenovirus early promoters, repress the polyoma enhancer, establish primary BRK cells and cooperate with the activated *ras* gene in morphologically transforming these cells. By correlating the various activities of the different mutants, three general conclusions can be made. Firstly, the two regulatory activities of E1a are separable since mutants have been isolated which can transactivate but fail to repress and vice versa. Secondly, transactivation is unlikely to play any major role in transformation by E1a since many mutants defective



PLASMID	VIRUS	CONSERVED REGION AFFECTED	DELETION → SUBSTITUTION
pCE	dl309	-	NONE
JN20 (13S)	dl975	-	NONE
G5/3	dl526	1	38-65 → Ser, Ser, Ile, Glu, Val
G3NX	-	1	64-86 → Ser, Ser, Arg, Ser
GNC	dl527	-	86-120
PM933	pm933	2	125, His → Leu
PM957	pm957	2	133, Asp → Val
GCX	dl528	2	121-125 → Val
G3/2	dl529	2	125-133 → Leu
JF12(12S)	dl520	3	140-185
GXmA	-	3	152-182
GCE-R	-	-	223-289 → Ser
pMX (13S)	dl530	3	185-289 → Ser, Arg, Leu, Gln, Ser, Cys, Val
SmaX (13S)	-	3	151-289 → Leu
S-Stop (13S)	-	2,3	135-289
X-Stop (13S)	-	2,3	127-289

Fig. 1. Description of the mutant E1a genes. The open box at the top of the figure represents the 13S cDNA coding sequence that encodes the E1a 289-amino acid product. The relative position of the 12S donor and 13S donor/acceptor splice junctions are indicated by the vertical bars. Residues that are highly conserved between the human Ad5, 7 and 12 serotypes (van Ormondt *et al.*, 1980; Kimelman *et al.*, 1985) are indicated by the checkered box ▦. The three regions of conserved residues are indicated as CR1, CR2 and CR3. Below are indicated the nature of the alterations in each of the mutants studied and the nature of the predicted proteins they encode. □ denotes the predicted product of the 13S message and ▨ of the 12S message. Deleted residues are denoted as gaps and substituted residues as filled in boxes ■. The exact nature of the deleted and substituted residues are described together with the conserved regions that are altered. Where appropriate the name of the reconstructed virus form of the mutant plasmid is indicated.

for transformation were found nevertheless to transactivate at wild-type levels. Thirdly, a good correlation was found between the ability of E1a to repress transcription and its ability to transform. Therefore, the possibility exists that the mechanism whereby E1a deregulates cellular growth (i.e. transformation) may be that of transcriptional repression of a cellular gene.

Results

Construction of E1a mutants

Mutants of E1a containing small in-frame deletions, truncations or single base-pair changes were constructed in order to investigate the relationship between the transcriptional regulation activities of E1a and transformation, and to identify domains of the protein critical for function. A comparison of the predicted E1a amino acid sequence of a number of different serotypes reveals three regions or domains that are relatively highly conserved (50−70%) (van Ormondt *et al.*, 1980; Kimelman *et al.*, 1985). The residues between these domains are poorly conserved (5−10%). As shown in Figure 1, two of the conserved regions (CR1 and CR2) are within the common 5′ exon of the 12S and 13S messages and the third (CR3) is mostly unique to the 13S message. None of the domains are within the sequences that encode the 9S message. The conservation seen in each conserved region suggests some importance for E1a function. The series of mutants described in this report contain alterations in each of these regions enabling their importance to be directly tested.

The mutants analysed in this study are shown in Figure 1. G5/3 and G3NX contain in-frame deletions removing amino acids 38−65 and 64−86, respectively. Taken together these deletions remove all the residues that comprise the CR1 region. A number of mutants have specific alterations within CR2. The mutants PM933 and PM957 contain single base alterations within CR2 that not only result in single amino acid changes at residues 125 (His to Leu) and 133 (Asp to Val), respectively, but also create new restriction endonuclease cleavage sites that were subsequently exploited to give the deletions GCX and G3/2, and the truncations S-stop and X-stop. Together the two deletions GCX and G3/2 remove all but three residues of the CR2 region. This region is rich in acidic residues and shows some homology within the *myc* oncogene product (Ralston and Bishop, 1983) and polyoma large T protein (Stabel *et al.*, 1985). Most of the unconserved residues between CR1 and CR2 are removed in the mutant GNC. The majority of CR3 is unique to the 13S messange and is therefore removed in the 12S cDNA clone JF12 and partially removed in GXmA, which contains an in-frame deletion removing residues 152−183. The truncations SmaX and pMX contain small inserts at nucleotide positions 1010 and 1110, respectively that alter the translational reading frame resulting in premature termination of translation. SmaX encodes a cDNA product containing the normal amino-terminal 150 amino acids with a single abnormal residue added at its carboxy terminus. In the case of

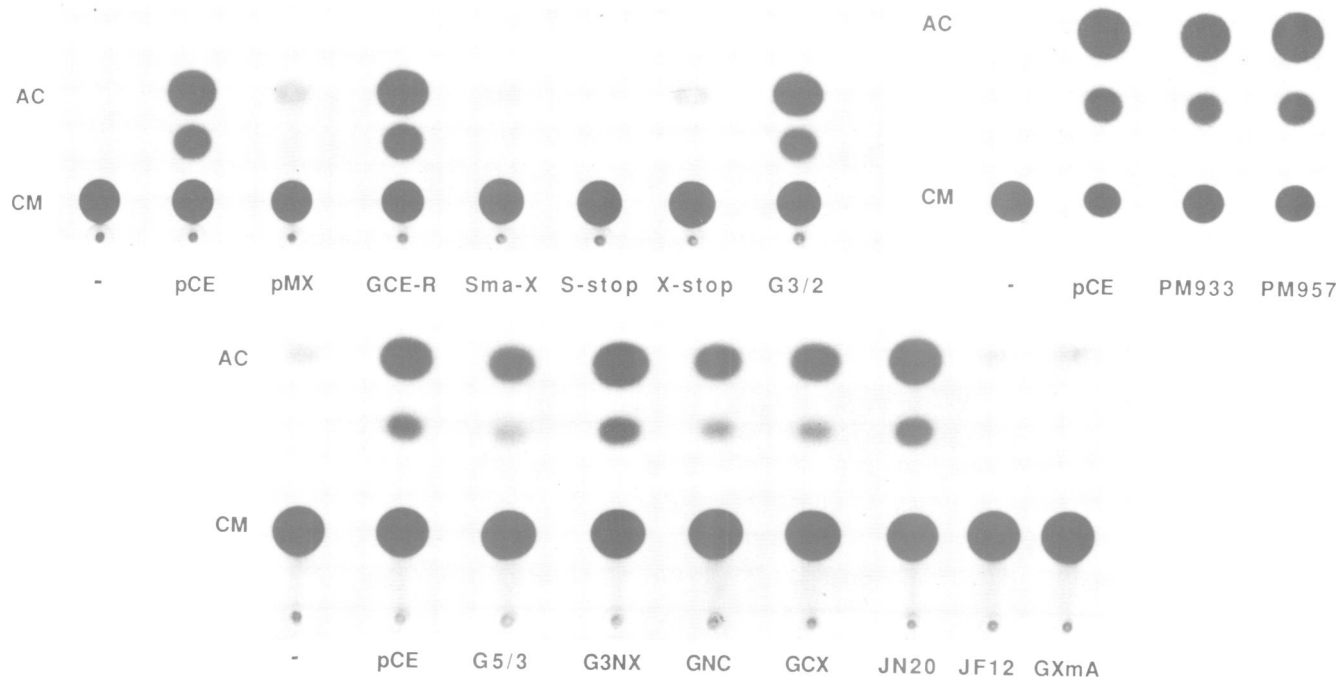


Fig. 2. Relative expression of E3-CAT plasmid in HeLa cells transfected in the absence (−) or presence of mutant E1a alleles. The position of chloramphenicol (CM) and its mono and diacetylated forms (AC) are shown.

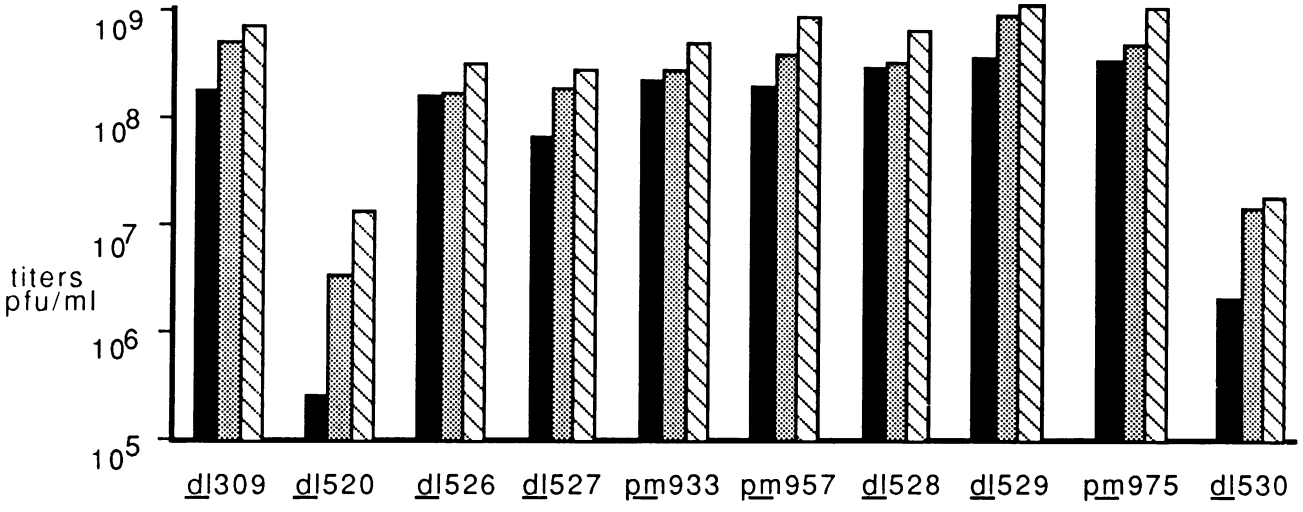


Fig. 3. Growth of mutant virus within HeLa cells was measured as described in Materials and methods. Cells were infected at m.o.i. of 1 ■, 10 ▩ and 100 ▨ p.f.u./cell. Virus progeny were harvested 24 h post-infection and titred on 293 cells. Titres are expressed as p.f.u./ml.

pMX, all of the 13S 5′ exon sequences are present but none of the 3′ exon sequences. In addition there is a point mutation at position 975 (Montell *et al.*, 1982) that prevents 12S message production, thus allowing the functional importance of the 13S 5′ exon sequences alone to be assessed. The carboxy-terminal 66 amino acids are removed in the mutant GCE-R.

Transactivation of the early gene promoters by the mutants

The ability of the E1a mutants to transactivate viral early gene expression was assayed by two methods. The first was to test their ability to transactivate expression of chimeric test genes in transient expression assays (Weeks and Jones, 1983). The second was to reconstruct viruses containing the various E1a alterations and to measure their ability to propagate in HeLa cells as the virus's ability to grow is dependent upon E1a transactivation of the early genes.

Results on the transactivation of the E3CAT gene contained in the plasmid pKCAT23 (Weeks and Jones, 1983) following transfection into HeLa cells are shown in Figure 2. Transfection of HeLa cells with pKCAT23 alone results in very low levels of CAT activity. Co-transfection of cells with pKCAT23 and a wild-type E1a plasmid (pCE) increases CAT activity >10-fold. Surprisingly, all mutants containing in-frame deletions within the 5′ exon common to the 12S and 13S messages were capable of activating E3CAT expression to a level similar to that seen with wild-type E1a (G5/3, G3NX, GNC, GCX, G3/2). This indicates

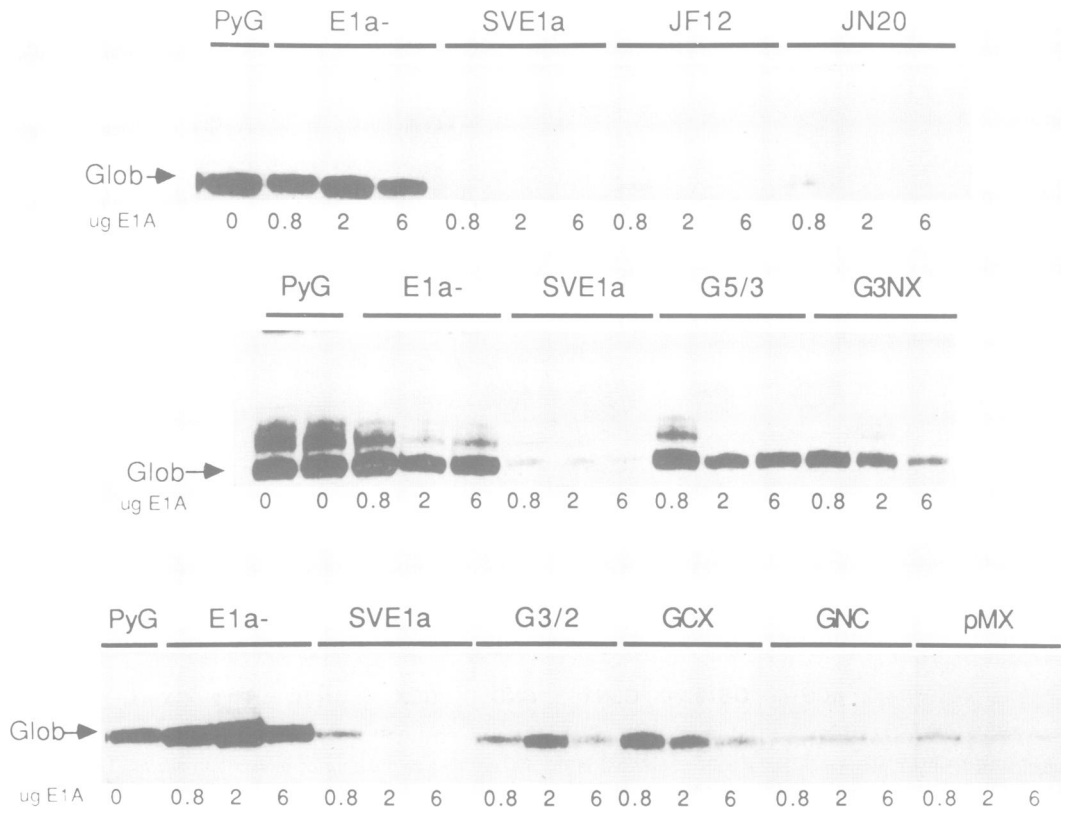


Fig. 4. Repression of the polyoma enhancer. S1 analysis is described in Materials and methods. PyG refers to HeLa cells transfected with $p\beta(244^+)\beta$ plus carrier only. The mutants co-transfected along with $p\beta(244^+)\beta$ are indicated above each group of three lanes. The amount of co-transfected E1a DNA per plate is indicated below each lane. The protected band of 205 bp is indicated.

that the residues from position 38 to 133, which include all of CR1 and most of CR2, play no crucial role in the transactivation function of E1a. In contrast, however, CR3 is critically important. Removal of all or most of this region as in JF12, GXmA, SmaX, S-stop and X-stop results in complete loss of transactivation activity. The loss of activity in pMX, which contains all of the unique 13S residues suggests that some residues within the second exon may also be important for transactivation. If so these would have to be in the 5′ portion of this exon since deletion of residues from 223 to 289 (GCE-R) has no effect on transactivation. CR3 extends into the second exon by four residues and it is tempting to speculate that it is the lack of these residues in pMX which results in loss of transactivation function. Indeed, missense mutations which alter residue 185 which is partly encoded by the first exon and partly by the second exon are also found to have lost the transactivation function (Glenn and Ricciardi, 1985; Lillie *et al.*, 1986). The ability of the mutants to stimulate expression of a E2CAT plasmid in a transient expression assay has also been studied and the results obtained were identical (data not shown).

Several of the mutants were reconstructed into virus and propagated on 293 cells which contain an integrated copy of the E1a gene and thus complement growth of all E1a mutant viruses. Growth assays were then performed to determine the ability of the mutant viruses to propagate within actively growing HeLa cells, thus indirectly measuring E1a's ability to transactivate all the early genes. HeLa cells were infected with the mutants at multiplicities of infection (m.o.i.) of 1, 10 and 100 plaque forming units (p.f.u.)/cell. Forty-eight hours post-infection the cells were harvested and viral progeny titered on 293 cells. As shown in Figure 3, all the mutants except dl520 and dl530 grew with wild-type (dl309) efficiencies at all m.o.i.s. Growth of dl520 and dl530 was at least 100- to 1000-fold lower than wild-type. These results are in complete agreement with those from the transient CAT assays described above and confirm that the first exon residues between 38 and 133 are not critical for transactivation. In addition, we infected HeLa cells containing an integrated E3-CAT gene (HeLa SV-T cells) with each of the viral mutants and 24 h later assayed CAT levels. Elevated levels of CAT enzyme were detected in cells infected with wild-type and all mutant viruses except dl520 and dl530, thus confirming the conclusions made from the viral growth assays above (data not shown).

It was important to show that the mutations we introduced into E1a did not affect either expression of the gene or stability of the product. Thus we infected both BRK and HeLa cells at a m.o.i. of 25 p.f.u./cell and harvested the cells 24 h post-infection. Lysates were produced and assayed for the presence of E1a protein by Western blot analysis. Similar levels of E1a protein were found in all cases except for dl530 (pMX) which was ~5-fold lower than wild-type (data not shown).

In summary, both the viral growth data and the transient assays demonstrate that of the three conserved regions of E1a only CR3 is critical for transactivation. Mutations in either CR1, CR2 or indeed the intervening non-conserved regions have very little effect.

Repression of the polyoma enhancer

In addition to transactivation, E1a is also capable of repressing the stimulatory action of a variety of enhancers (Borrelli *et al.*,

1984; Hen *et al.*, 1985; Velcich and Ziff, 1985). Thus genes whose expression is dependent on any of these enhancers will be repressed by E1a. We have assayed the ability of the mutant E1a plasmids to repress the function of the polyoma enhancer contained in the plasmid pβ(244$^+$)β (Borrelli *et al.*, 1984). This plasmid contains two copies of the rabbit β-globin gene linked to a fragment of the polyoma genome that contains the enhancer sequences. Efficient expression of the globin genes in this construct is dependent upon the enhancer elements and consequently globin RNA levels reflect enhancer activity. Repression of this enhancer by E1a was assessed by S1 mapping of globin RNA following co-transfection of HeLa cells with the globin- and E1a-containing DNAs. As seen in Figure 4, transfection of cells with pβ(244$^+$)β alone (termed PyG in figure) results in efficient expression of the globin gene as demonstrated by the readily detectable S1-protected band of 205 bp. However, when co-transfected together with SVE1A (Borrelli *et al.*, 1984) which directs the synthesis of both the 243 and 289 amino acid products, globin RNA levels are drastically reduced. The reduction is at least 10-fold when 10 μg of the pβ(244$^+$)β plasmid are transfected into cells together with as little as 0.8 μg of E1a plasmid. This repression is also seen when the co-transfecting plasmid directs the synthesis of 12S (JF12) or 13S (JN20) message only, although the 13S cDNA plasmid appears to be slightly less efficient. To ensure that our observed repression was not simply due to the E1a enhancer/promoter competing for cellular factors, the plasmid pE1a$^-$ (Sassone-Corsi *et al.*, 1983) containing the E1a enhancer/promoter but lacking most of the coding sequences was co-transfected together with pβ(244$^+$)β. This construct had very little effect on globin expression emphasizing that our observed repression is dependent upon synthesis of E1a protein.

Increasing concentrations of mutant plasmid were transfected into HeLa cells together with 10 μg of pβ(244$^+$)β in order to investigate which regions of E1a were critical for repression activity. One critical region was found to be CR1. The mutant G5/3 failed to display repression at any of the co-transfecting concentrations of mutant plasmid, whereas G3NX showed virtually no repression at the low co-transfecting concentrations and only slight repression when 6 μg of mutant plasmid was used. This lack of repression was consistently found in a number of experiments that employed different preparations of mutant plasmid DNAs. Amino acid residues between positions 38 and 86 are therefore critical for repression. Residues located within the CR2 region also appear to be important for efficient repression, although less so than CR1 residues. Some repression was detected with the in-frame deletion mutants GCX and G3/2, although the repression was clearly much less efficient than with wild-type E1a. Even when the cells were co-transfected with 6 μg of mutant E1a plasmid, repression was not as severe as that obtained with 0.8 μg wild-type plasmid. Again this decreased efficiency was consistently found with different plasmid preparations. The non-conserved residues in between CR1 and CR2 that are removed in GNC are not important for repression since this plasmid decreased expression of the co-transfected globin gene almost as efficiently as SVE1A. The CR3 residues unique to the 13S message are clearly dispensible for repression since the 12S cDNA construct (JF12) represses efficiently. Second exon residues, including those that are contained within the CR3 region and which are important for transactivation, are also dispensible. As shown in Figure 4, the mutant pMX retained its ability to repress the polyoma enhancer. In most experiments we performed, including the one shown, repression by pMX was very efficient. However, in the occasional experiment using this particular mutant, repression was found to be weak. We do not know the cause of this variability. We also measured E1a RNA levels in cells transfected with the different mutants and found the levels to be similar in all cases (data not shown). The differences in repression activity therefore are not due to differences in expression of the mutant genes. We conclude from these repression studies that: (i) the domains essential for repression are within the 5′ exon of the 12S product; (2) transactivation and repression can be uncoupled; (iii) residues within the CR1 region are critical for repression; and (iv) residues within CR2 contribute to the effiency of repression although low levels of repression can still take place in their absence.

Table I. Focus formation on primary BRK cells.

Plasmid	No selection (average foci number/plate)		G418 selection (average number of colonies/plate)	
	ras		*ras*	
	−	+	−	+
pBR322	0	0	0	0
pCE	36	56	16	40
JN20	1	7	3	7
PM933	18	37	23	25
PM957	7	13	2	10
G5/3	0	0	0	0
G3NX	0	9	0	10
GNC	8	14	10	13
GCX	0	0	<1	<1
G3/2	0	0	<1	<1
GXmA	28	44	15	16
GCE-R	40	66	9	51
pMX	0	50	0	44
SmaX	0	15	0	14
S-stop	0	3	0	4
X-stop	0	0	0	0

Numbers refer to the average number of foci per plate from at least four different experiments. Assays as described in Materials and methods.

Transformation of baby rat kidney cells

The transforming potential of the mutant E1a genes was tested by measuring their ability to establish BRK cells and to cooperate with the activated *ras* gene to fully morphologically transform these cells. For the establishment assay, primary BRK cells from 3- to 6-day-old rats were transfected with mutant E1a DNAs alone, or together with the plasmid pSV2Neo (Southern and Berg, 1980) which can confer G418 drug resistance to mammalian cells. The transfected cells were maintained in either normal or G418-containing medium for 3−4 weeks and then examined for the presence of partially transformed foci or G418-resistant colonies. Upon establishment by E1a, these cells have not only acquired the property of extended growth potential *in vitro*, but are also partially morphologically altered. They form densely packed foci that can be distinguished from the background of non-growing, non-transformed cells. Upon selection with the drug G418, the presence of transfected and established cells was readily seen since the untransfected background cells whose presence might affect growth and detection of the E1a-containing cells, were killed. Representative foci or colonies were picked, expanded into mass culture and maintained for several months. For the cooperation assay, BRK cells were co-transfected with E1a and the plasmid EJ6.6 (Shih and Weinberg, 1982). This plasmid contains the Ha-*ras* oncogene from the EJ/T24 human bladder carcinoma cell line. Again in some experiments pSV2Neo was included in the

Table II. Summary of E1a mutant phenotypes. Mutants are grouped by phenotype and conserved region affected[a]

Plasmid	Conserved region affected	Transactivation of adenoviral early promoters	Repression of polyoma enhancer	Establishment	Cooperation with Ha-*ras*
pCE, GNC	–	+	+	+	+
JF12, GXmA	3	–	+	+	+[a]
pMX, SmaX	3	–	+[b]	–	+
PM933, PM957	2	+	ND[c]	↓	↓
GCX, G3/2	2	+	↓	–	–
G5/3	1	+	–	–	–
G3NX	1	+	↓	–	↓

[a]12S cDNA [JF12] reference for *ras* cooperation and establishment (Haley *et al.*, 1984).
[b]No data for SmaX in the repression assay.
[c]No data.

transfection and the cells subsequently selected for G418 resistance. The foci or colonies produced from such cooperative transfections contained cells that were morphologically *ras* like, in that they were rapidly growing, rounded and refractile.

The results of the cooperation assay shown in Table I demonstrate that sequences within the CR1 and CR2 regions but not CR3 are critical for this function of E1a. Mutants which have alterations in CR2 (which shares homology with other *ras* cooperating oncogenes) all have reduced capacities for cooperation. The two point mutants PM933 and PM957, both were slightly less efficient at cooperation. Interestingly, the more highly conserved aspartic acid residue at 133 when changed produced a greater effect (5-fold) than the less conserved histidine at 125 (2-fold). Furthermore, the small in-frame deletion mutants G3/2 and GCX were completely defective for cooperation indicating the critical role of CR2 in cooperation. Sequences within CR1 are also important for the cooperation function especially the highly conserved sequences between residues 41 and 62 which are deleted in mutant G5/3. This mutant was completely cooperation defective. Deletion of the neighbouring residues 64–86 reduced the efficiency of cooperation 6-fold. The large in-frame deletion GNC (86–120) produced numerous foci indicating that these residues are not critical. In contrast, mutants with alterations in CR3, such as GXmA, cooperated efficiently and therefore agree with previous reports which showed that cooperation could be accomplished by the 12S cDNA (Haley *et al.*, 1984; Moran *et al.*, 1986b; Zerler *et al.*, 1986). Since the truncation mutants GCE-R, pMX and, to a lesser extent, SmaX all cooperated efficiently, second exon residues appear to be unimportant. Foci produced from cooperation with pMX and SmaX were usually much larger in size than those produced with wild-type E1a. In addition the cells differed in morphology being more epithelial in appearance, less rounded and more adherent. S-stop which truncates at residue 133 also retained some capacity to cooperate with *ras* although at a much lower efficiency but further truncation to residue 126 (X-stop) resulted in no detectable cooperation.

The abilities of the mutants to establish BRK cells in most cases mirrored the results obtained for cooperation. All mutants which failed to cooperate (G5/3, GCX, G3/2 and X-stop) also failed to establish; likewise most of the mutants with slightly reduced cooperation efficiency (GNC, PM933 and PM957) had similar reductions in their abilities to establish. However, a novel class of mutants exist which retain their ability to cooperate but completely fail to establish. Included in this group are pMX, SmaX and G3NX. No established colonies could be detected even when drug selection was utilized.

Discussion

We have constructed and analysed a number of mutants with alterations within the E1a coding region in order to investigate the interrelationship between the ability of this gene to transform and its ability to regulate gene transcription. Our results suggest that it is transcriptional repression rather than transcriptional activation that is important for transformation (Table II).

The transactivation and transformation activities of E1a are clearly separable. This was first indicated by studies showing that the 12S cDNA sequences of E1a were fully capable of establishing primary rat cells and cooperating with the activated *ras* gene to effect full morphological transformation (Haley *et al.*, 1984; Zerler *et al.*, 1986). However, many studies have shown that these sequences, either following transfection or viral infection, were unable to transactivate the viral early genes or the human heat-shock gene (Carlock and Jones, 1981; Montell *et al.*, 1982; Svensson and Akusjarvi, 1984; Winberg and Shenk, 1984; Lillie *et al.*, 1986; Moran *et al.*, 1986a; Wu *et al.*, 1986). This initial indication is now strongly supported by the characterization of many additional mutants described in this report that are phenotypically either transformation positive, transactivation negative or conversely transformation negative, transactivation positive. Mutants of the first phenotype all have alterations within the unique region of the 13S message which is critically important for transactivation but which can be removed without affecting establishment or cooperation. Mutants of the latter phenotype fall into two classes, those that have alterations within the conserved region between residues 121 and 136 (CR2) and those that have alterations within the conserved region between residues 41 and 62 (CR1). Deletion of either region results in the complete loss of both establishment and cooperation activities, but has little effect on the ability of the gene to transactivate. Other mutants with alterations in the CR2 region have been isolated and characterized. Four mutants with single amino acid alterations within CR2 have been described (Lillie *et al.*, 1986; Zerler *et al.*, 1986); all four had similar phenotypes to the ones described here. Our results demonstrate that mutations in two different domains of the Ela coding region result in reduced transformation activities without affecting transactivation and therefore considerably strengthens the argument that these two activities are not linked.

Ela and *myc* share the ability to establish primary cells and cooperate with the *ras* gene (Land *et al.*, 1983; Ruley, 1983). In addition, Ralston and Bishop (1983) have suggested that the products of these two genes also show a certain degree of

homology, although more recently the existence or significance of this homology has been severely questioned (McLachlan and Boswell, 1985). Nevertheless, it is intriguing to note that as Ralston and Bishop originally indicated there is a stretch of residues in the *myc* product that very closely resembles the highly conserved E1a residues located in the CR2 region between positions 132 and 138. Since removal of these sequences in E1a results in loss of its transforming function it would be of interest to determine whether the removal of these sequences in the *myc* gene would also result in loss of transformation.

Recent reports have linked E1a's ability to interfere with enhancer activation with its ability to establish primary cells and cooperate with other oncogenes (Velcich and Ziff, 1985; Lillie *et al.*, 1986). In our analysis of mutant E1a products there are no examples in which a mutant can establish primary cells without also being capable of repressing the test enhancer. Likewise, in cases where the capacity to transform primary cells has been negatively affected, the capacity to repress has also decreased. The clearest examples of this are with the mutants that have deletions in CR1 (mutants G5/3 and G3NX). In the case of G5/3, both the transforming (establishment and *ras* cooperation) and enhancer repression activities are completely lost. For G3NX, although the establishment activity is lost the mutant can still cooperate with *ras* at a reduced frequency. We also detected some capacity for enhancer repression but only with the higher concentrations of E1a plasmid DNA and even then the degree of repression was much less than that seen with wild-type. Thus the transformation defects of these mutants are paralleled by defects in enhancer repression. The correlation between these two activities of E1a is not as good with mutants containing alterations in the CR2 region. Both G3/2 and GCX are completely defective for establishment and *ras* cooperation but nevertheless have some capacity to repress the polyoma enhancer. Although the efficiency of repression is lower than with wild-type, some repression was seen even at the lowest E1a plasmid concentrations used. These results differ slightly from those recently published by Lillie *et al.* (1986). They described two mutants that have single amino acid alterations within the CR2 region. These mutants were found to be severely defective both in their ability to transform and to repress enhancers leading the authors to speculate that enhancer repression is a key activity of E1a in transformation. The discrepancy in the CR2 domain's involvement in enhancer repression between these results and our own might be due to several differences between the assays employed in the two reports. The cell lines, the methods used to introduce the test DNA into the cells and the particular enhancers studied all differed. For example, they measured the ability of the E1a mutants to repress both the SV40 enhancer and the cellular immunoglobulin enhancer, whereas in our study we have measured the repression of the polyoma enhancer only. It is possible that the different enhancers vary in their sensitivity to E1a repression, the polyoma enhancer being more sensitive. If this was the case it would not be surprising to identify mutants of E1a that retained some ability to repress the polyoma enhancer but not the SV40 and immunoglobulin enhancers.

By considering all the available data the case for a close relationship between E1a's ability to repress and transform becomes strong. However the importance of this relationship rests upon the validity of comparing the assays used for measuring each activity. To measure transformation activity a long-term assay involving the integration of E1a sequences into the cellular genome is used, whereas transcriptional regulation has been assayed transiently following introduction of the DNA into the cell by transfection or infection. In addition the cells used in each assay are different. Therefore some degree of caution should be exercised when considering these correlations.

The concept that repression of a gene or genes might result in uncontrolled growth is clearly not new. An increasing body of evidence supports the idea that cell growth is controlled not only by endogenous stimulators but also by endogenous proliferation inhibitors (for review, see Wang and Hsu, 1986). The loss of such an inhibitory force may be just as important for cell transformation and the development of malignancies as is the activation of stimulatory forces. If one or more of the genes that encode such inhibitors are controlled by E1a-sensitive enhancer elements, then in the presence of E1a the expression of these genes would be depressed and cell growth no longer held in check. It would be of obvious interest to determine whether other genes capable of the establishment of primary cells could also interfere with enhancer activity. The proposed role of E1a in cell establishment might represent a unique mode of action amongst the different nuclear located oncogenes. Alternatively, the antagonism of proliferation inhibitors may represent a more general function of such oncogenes.

It was surprising to find that the majority of sequences within the common first and second exons of the 12S and 13S messages are not critically important for transcriptional activation. Amino acid residues 41−136 and 223−289 can be removed without affecting the ability of E1a to transactivate expression of the E3 or E2 promoters in co-transfection experiments or the virus's ability to propagate efficiently in HeLa cells. Why then have the domains CR1 and CR2 within the E1a gene been so strongly conserved when their importance for transactivation and propagation seems minimal? Adenovirus has evolved to propagate efficiently in host organisms where the majority of cells it encounters are quiescent in nature. Expression of the E1a gene is known to stimulate DNA replication and division of quiescent cells (Braithwaite *et al.*, 1981; Stabel *et al.*, 1985), which presumably is advantageous to viral replication (Spindler *et al.*, 1985). It is reasonable to assume that this deregulation of quiescent cell growth is a manifestation of the same activity that results in cell establishment. Since the conserved regions within the first exon are critical for establishment, it would then follow that these regions are also important for efficient viral growth in quiescent cells. If true, the present results predict that the maintenance of the quiescent state is due in part to the expression of gene(s) controlled by enhancer element(s) sensitive to E1a repression. We are testing this hypothesis by examining the ability of the different mutants to induce DNA synthesis and for growth in quiescent cells.

Materials and methods

Construction of mutants

JF12 and JN20 contain the 12S and 13S cDNAs, respectively (Haley *et al.*, 1984). Plasmid pCE results from cloning the SV40 *Hin*dIII enhancer fragment into JOID (Haley *et al.*, 1984), which contains the adenovirus sequences 1−2804. All genomic mutants have pCE as their parent plasmid. Single base substitutions were engineered at base pairs 933 (A to T) and 957 (A to T) by standard oligonucleotide mutagenesis (Zoller and Smith, 1983) into the phage M13Gori (Hines and Ray, 1980). The *Cla*I−*Xba*I fragment of pCE was replaced by the corresponding mutagenized fragment from M13Gori to yield the mutant plasmids PM933 and pM957. The base-pair changes in PM933 and PM957 create unique *Xho*I and *Sal*I sites, respectively. GCX was made by cutting PM933 at its unique *Cla*I (919) and *Xho*I (933) sites, filling in with Klenow and religating. G3/2 was made by combining the *Eco*RI (0)−*Xho*I (933) fragment of PM933 and the *Eco*RI−*Sal*I vector fragment of PM957. GNC was constructed by removing the base pairs

between the *Nae*I (813) and the filled-in *Cla*I sites of pCE. The base pairs between 1011 and 1108 were removed from pCE to make the mutant GXmA by cutting at *Xma*I (1008) and *Acc*I (1108), filling in and religating. The truncation mutant pMX was constructed by replacing the *Sst*II (350)–*Sma*I fragment of XAF (Haley *et al.*, 1984) by the analogous fragment from PM975 (Montell *et al.*, 1982). SmaX was constructed by inserting an *Xba*I linker into the *Sma*I site of JN20 creating an in-frame termination codon. S-stop was produced by cutting PM957 with *Sal*I and *Xba*I, filling in and religating, thus producing a frame shift and immediate truncation. X-stop was made in an analogous fashion using PM933 cut with *Xho*I and *Xba*I. The *Xho*I linker scanner mutants pSVXL105 and pSVXL3 (gifts from E.Ziff; Smith *et al.*, 1985) were used to construct G5/3 and G3NX. For G3NX, the E1a-containing *Pst*I/*Eco*RI fragment of pSVXL3 was moved into PUC 8, cut at *Nae*I and a *Bgl*II linker added (10-mer). This intermediate was cut with *Xho*I and *Bgl*II, filled in and religated to correct the reading frame. The *Eco*RI–*Cla*I fragment of this intermediate was then moved into pCE to give G3NX. G5/3 was made by combining the *Eco*RI/*Xho*I fragment of pSVXL105 and *Eco*RI/*Xho*I vector fragment of pSVXL3. The resulting construct was cut with *Xho*I, filled in and religated to correct the reading frame. The *Eco*RI/*Cla*I fragment from this intermediate was moved into pCE.

The pβ(244$^+$)β plasmid contains the rabbit β-globin gene flanked by the *Bcl*I/*Pvu*II fragment of polyoma containing the entire polyoma enhancer (Borrelli *et al.*, 1984). EJ6.6 contains the Ha-*ras* gene isolated from T24 human bladder carcinoma cells (Shih and Weinberg, 1982). The plasmid E1a contains E1a transcription sequences −500 to +130 (Sassone-Corsi *et al.*, 1983). SVE1a contains the left-most 1570 bp of Ad-2 (pE1ASV in Sassone-Corsi *et al.*, 1983).

Cells and viruses

Cultures of HeLa, 293 and BRK cells were grown in Dulbecco modified Eagle's medium supplemented with 10% fetal calf serum. Mutant viruses were constructed by the method of Stow (1981) into Ad5dl309 and propagated in 293 cells. Infections and titre assays were performed as previously described.

Transformation of primary baby rat kidney cells

Primary BRK cells were prepared by trypsination of kidneys from 3-day-old Fisher rats. Cells were plated onto 60-mm dishes and transfected the next day (day 2) by the DNA–calcium phosphate co-precipitation method of Wigler *et al.* (1978) at 20–40% cell confluency. Transfection cocktail contained 3 μg E1a plasmid plus or minus 3 μg EJ6.6 plasmid. Four micrograms of the plasmid pSV2Neo (Southern and Berg, 1980) was included for assays utilizing G418 selection. When the cells reached confluency (usually day 3) they were passed onto 100-mm dishes. Drug selection was added after passage at 200 μg/ml. Colonies were fixed, stained and counted 3–4 weeks later.

Transient expression assays of chloramphenicol acetyltransferase activity

The expression of the E3-CAT gene in HeLa cells transfected with or without E1a-containing plasmids was determined as described previously (Weeks and Jones, 1983). Each 100-mm plate of HeLa cells was transfected by the Wigler *et al.* (1978) method with 5 μg E3-CAT and plus or minus 10 μg of an E1a-containing plasmid. A number of different experiments utilizing several preparations of plasmid DNA were performed with essentially the same results.

Viral growth assay

HeLa cells were infected at a m.o.i. of 1, 10 and 100 p.f.u./cell. Viral progeny were harvested 48 h post-infection and titred on 293 cells. Plaques were visually counted on day 7.

Transient expression of E1a-mediated polyoma enhancer repression

HeLa cells were transfected with 10 μg pβ(244$^+$)β (Borrelli *et al.*, 1984) together with either 0.8, 2.0 or 6.0 μg E1a-containing plasmid and enough carrier (pBR322 or M13 RF form) to make the amount of DNA per transfection 16 μg/plate (10 cm) and RNA was harvested 48 h post-transfection. RNA (10 μg) was hybridized to 1 pmol ^{32}P end-labelled single-stranded probe in 15 μl of a buffer containing 400 mM NaCl, 10 mM Pipes pH 6.5 and 1 mM Na_2-EDTA at 68°C for 10–20 h. The hybridization reaction was diluted with 300 μl of cold S1 digestion buffer (0.3 M NaCl, 30 mM sodium acetate pH 4.7 and 2 mM zinc acetate) and the mixture incubated at 25°C for 2 h in the presence of 2000 units (Miles) S1 nuclease. The RNA/DNA duplexes were ethanol precipitated and redissolved in loading buffer, electrophoresed through 8% urea acrylamide gels and detected by autoradiography.

The single-stranded probes used for this study were either 3′ end-labelled at position +292 and derived from a *Nco*I fragment (+288 to +611) spanning the second splice donor within the rabbit β-globin coding sequences, or 5′ end-labelled at position +139 and derived from a *Bst*NI–*Pst*I fragment (+137 to −100) spanning the globin cap-site. The S1 nuclease protected products were therefore 139 bp using the 5′ end-labelled probe or 205 bp using the 3′ end-labelled probe. Both probes gave identical results.

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