Analysis of pRb Family Binding Regions of Adenovirus Type 12 E1A

Hisaki KOSAWA[1)]*, Yukiharu SAWADA[1)], Takayuki TAKAHASHI[1)], Masanobu SHINDOH[2)] and Kei FUJINAGA[1)]

[1)] Department of Molecular Biology, Cancer Research Institute, Sapporo Medical University School of Medicine, South 1, West 17, Central Ward, Sapporo 060, Japan,

[2)] Department of Oral Pathology, Hokkaido University, School of Dentistry, North 15, West 7, North Ward, Sapporo 060, Japan.

Key words: Adenovirus, E1A, p107, p300, Transformation

ABSTRACT

Adenovirus early 1A (E1A) products can form complexes with cellular proteins including the pRb tumor suppressor, pRb-related p107 and p130, and p300 proteins related to the CREB-binding protein (CBP). Within the E1A sequence, CR2 and CR1 mediate interaction with pRb family proteins, and the amino terminus and CR1 are involved in association with p300 protein. These interactions are essential for the transforming activity of E1A proteins of various adenovirus serotypes. Since E1A of highly oncogenic adenovirus type 12 (Ad12) have some differences in transformation efficiency and oncogenicity from non-oncogenic Ad5 E1A, we have analyzed the role of CR1 and CR2 sequences of Ad12 E1A in transformation and pRb family binding using specific mutations. On the basis of the characteristic phenotypes of some CR1 mutations we suggest a modification of the previously proposed consensus regarding CR1 sequence for pRb binding. Combinations of CR1 and CR2 mutations revealed different roles for nonconserved residues within the conserved pRb binding motif LXCXE of CR2, and interaction of CR1 and CR2 of Ad12 E1A through specific amino acid residue is suggested. These structural features of Ad12 E1A proteins may explain at least in part the functional differences from Ad5 E1A proteins.

INTRODUCTION

Early region 1A (E1A) proteins of human adenoviruses, when infected in human cells, promote viral replication by activating viral and cellular genes to stimulate cellular machinery of DNA synthesis (1). In rodent cells infection is

* To whom requests for reprints should be addressed

generally abortive, but in combination with early region 1B (E1B), E1A can induce cell transformation and tumor formation in susceptible animals (2). In the case of adenovirus type 5 (Ad5), the E1A gene produces major transcripts of 12S and 13S that encode proteins of 243 and 289 residues (243R and 289R) respectively, that are identical except for 46 internal residues (3). Three regions of E1A proteins that are conserved in most adenoviruses, termed CR1, CR2 and CR3, are responsible for both activation and repression of viral and cellular genes (4-8). CR3, which comprises the unique portion of 289R, activates gene expression through interaction with transcription factors but is dispensable for cellular DNA synthesis and transformation (6, 7, 9-12). CR1, CR2 and the amino terminus are required for induction of cellular DNA synthesis and transformation (6, 7, 10). These portions of the E1A protein are involved in a complex formation with a series of cellular proteins (13, 14). The amino terminus and CR1 can bind p300 (15, 16), a member of CREB-binding protein (CBP) family (17). CR2 and CR1 are required for the binding of pRb, p107 and p130, which share homology in the pocket region that interacts directly with E1A proteins (16, 18-22). Analysis of E1A deletion mutants indicated that defects in regions involved in the complex formation with p300 and pRb-related proteins affect both induction of DNA synthesis and cell transformation (10, 23, 24).

CR2 of Ad5 E1A proteins contains a motif *Leu*-Thr-*Cys*-His-*Glu* (LXCXE, where X can represent any amino acid) found also in oncoproteins of other DNA tumor viruses and cellular proteins that bind pRb. These include large T antigens of simian virus 40 (SV40) and polyoma virus, and human papillomavirus (HPV) E7 protein (25-27) as well as certain class of cellular proteins. This sequence is believed to represent the primary binding site in these proteins and alteration of Cys or Glu in this motif of E1A proteins has been found to abrogate E1A mediated transforming activity (6, 28, 29). However, binding is also affected by mutations within CR1 (16, 19) and it has been suggested that the residues Glu-X-X-X-Leu-X-Glu/Asp-Leu-X-(X)-Leu could play a role (21, 27). Sequences within CR1 were involved in interactions with p300, but residues within the amino terminus of Ad5 E1A proteins are critical to form complexes with p300 (19, 24, 30).

pRb forms complexes with transcription factor E2F and thus inhibits expression of genes under the control of E2F that are required for DNA synthesis (31-33). E1A proteins disrupt such complexes to release active E2F allowing cells to enter S phase (34, 35). p300 can work as a coactivator and mediate upstream regulatory functions to the basal transcription complexes formed on the promoter proximal site (17). E1A binds p300 and may repress expression of certain genes by modulating p300 functions (36, 37). Recent studies have suggested that p300

and pRb-related proteins can form complexes on the same E1A molecules simultaneously, allowing functional interactions between them to exert full transformation activity (38, 39).

In the present study, we have analyzed CR1 and CR2 of the E1A protein of highly oncogenic Ad12 by introducing specific mutations. Conserved residues in CR1 were shown to be required for efficient transformation. Mutations that alter Ad12 specific residues to Ad5 specific ones, when introduced singly in CR1 or CR2, had no or only a slightly enhancing effect on transformation. When these were introduced in combination, one in CR1 and another in CR2, the effects on transformation diverged between the CR2 mutations. These results suggest that CR1 and CR2 of Ad12 E1A proteins interact through specific amino acid residues.

MATERIALS AND METHODS

Cell culture

Monolayer cultures of rat 3Y1 (40), primary rat embryonic fibroblast (REF), 293 human embryonic kidney cells transformed with Ad5 sequences and HeLa cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 10 % fetal bovine serum (FBS). YH1, a clone of transformed 3Y1 cells induced by Ad12 E1A plasmid DNA, was grown in DME for suspension culture supplemented with 0.1 mM $CaCl_2$ and 10 % FBS. Primary baby rat kidney (BRK) cells were prepared from Fischer rats at 5 days old and cultured in DME supplemented with 10 % FBS. Soft agar culture was made by plating 1×10^4 cells resuspended in 10 ml of DME containing 10 % FBS and 0.3 % agar noble (DIFCO) over a 0.6 % agar medium layer.

Plasmid DNA

p12C3 and p5XhoC contain the E1 and the flanking sequences of Ad12 and Ad5 DNA respectively, cloned on pBR322 or its derivative pML2. p5A12 is a derivative of p5XhoC with E1A sequence replaced by Ad12 E1A (41). p5A12-NI carries a chimeric E1A sequence composed of Ad12 first exon and intron and Ad5 second exon sequences in place of Ad5 E1A of p5XhoC (42, 43). pE2ACAT is a reporter plasmid where the expression of chloramphenicol acetyltransferase is controlled by the E2A promoter sequence of Ad2 (44). pCGorf6/7 was constructed by inserting Ad2 E4 orf6/7 cDNA (45, recloned by D. Ornelles) between cytomegalovirus enhancer/promoter and rabbit β-globin splicing and poly(A) addition sequences of pCMVβ plasmid vector.

Construction of mutants

Substitution and insertion of single amino acid were introduced into the E1A

Table 1 *Synthesized oligonucleotide primers for mutagenesis, PCR and sequencing. Underlines indicate nucleotides substituted for mutagenesis and/or introduction of restriction sites.*

1.	in38Es	5'-TGA TCT TTA TGA GGT TCC GTC TC-3'
2.	Y42Hs	5'-CGT CTC TTC ACG AAC TG-3'
3.	L108Ts	5'-TGG ATT TAA CGT GCT ACG-3'
4.	Y110Hs	5'-TAT TGT GCC ACG TG-3'
5.	S40Ks	5'-TAT GTT CCG AAG CTT TAC GAA C-3'
6.	S40Ka	5'-TCG TAA AGC TTC GGA ACA TA-3'
7.	LY44,45KLs	5'-CTT TAC GAA AAG CTT GAT CTT GAT-3'
8.	LY44,45KLa	5'-AGA TCA AGC TTT TCG TAA AG-3'
9.	ED54,55KLs	5'-CTG CCG GTA AGC TTA ATA ATG AAC-3'
10.	ED54,55KLa	5'-ATT ATT AAG CTT ACC GGC AG-3'
11.	12A-480s	5'-AGC TCA TTT TCA CGG ATC CAA AAT GAG AAC TGA AA-3'
12.	12A-560s	5'-AGC ATT TGG TGG ACA ACT-3'
13.	12A-760s	5'-CCT GTC TGT GAG CCT ATT-3'
14.	12A-760a	5'-AAT AGG CTC ACA GAC AGG-3'
15.	5A-1579a	5'-GCG TTA ACC ACT CGA GCA AT-3'

gene by site-directed mutagenesis using synthetic oligonucleotides (1-4 in Table 1) as primers for *in vitro* DNA synthesis by the procedure of Kunkel *et al.* (46). Using *E. coli* strains that lack dUTPase (*Dut*) and Uracil-DNA glycosylase (*Ung*), single stranded plasmid DNA with dU substitution in place of dT was prepared and used as templates for *in vitro* DNA synthesis. Plasmid DNAs with introduced mutations were recovered through selective replication in Dut^+ Ung^+ cells. Double mutations were constructed by combining both CR1 mutations with each CR2 mutation through polymerase chain reaction (PCR). CR1 and CR2 mutations were recovered separately using primer pairs of 11 and 14 or 13 and 15 shown in Table 1, and hybridized with each other at the short overlapping sequence derived from primers 13 and 14, and elongated by Klenow fragment of E. coli DNA polymerase I to prepare the templates for the second PCR reactions. Mutations that replace two consecutive amino acid residues for lysine-leucine (KL) were introduced by ligation of PCR products after cleavage with HindIII whose restriction site was included in primers 5-10 in Table 1. All the mutant E1A genes were confirmed by nucleotide sequencing.

Transformation

3Y1 cells were transfected by the calcium phosphate co-precipitation method at 20-30 % confluency one day after plating. Transfection mixture contained 2 μg of mutant or parental E1 plasmid or vector plasmid DNA per five 6 cm dishes. Cells were fed with DME supplemented with 5 % FBS for the first 3 days

and then with DME for suspension culture supplemented with 0.1 mM $CaCl_2$ and 5 % FBS. At four weeks transformed foci were counted after staining with Giemsa's solution. Primary cultures of BRK cells were also transfected with mutant or parental E1 plasmid or vector plasmid DNA with or without activated human H-*ras* plasmid and fed with DME supplemented with 5 % FBS for 3 weeks before staining.

Immunoprecipitation

Cells were labeled with [^{35}S] methionine and [^{35}S] cysteine (110 mCi/ml, Amersham) for 3 hrs and lysed in lysis buffer composed of 20 mM sodium phosphate (pH 7.0), 250 mM NaCl, 5 mM EDTA, 30 mM pyrophosphate, 0.1 mM sodium vanadate, 10 mM sodium fluoride, 5 mM DTT, 0.1 % NP40, 1 mM PMSF, 0.2 TIU/ml aprotinine and 1 μg/ml pepstatin A. Aliquots (3×10^7 cpm) of the lysates were precleared with protein-A agarose beads (Pierce), and incubated with M73 monoclonal antibody (47, Oncogene Science) for 90 min at 4°C. Immune complexes were collected on protein-A agarose beads, washed and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were detected by fluorography.

Protein binding assay

GST-E1A fusion vectors were constructed by cloning Asp718-PvuII fragments of wt and mutant Ad12 E1A DNAs into pGT-1, a derivative of pGEX-2T (Pharmacia). GST fusions of the subregion of wt and mutant Ad12 E1A proteins were produced in *E. coli* in the presence of 0.6 mM IPTG and purified essentially as described (48). Five μg of GST or GST fusion proteins were incubated with pre-cleared extract of labeled YH1 or REF cells prepared as above for 90 min at 4 °C. Protein complexes were collected on Glutathione Sepharose beads, washed and subjected to SDS-PAGE.

Transient CAT expression assay

Transcriptional regulatory activity of mutant E1A genes was assayed on HeLa cells using pE2ACAT as a reporter gene. Two μg of pE2ACAT and 2 μg of mutant or parental p5A12-NI with or without 0.5 μg of pCGorf6/7 were transfected by the calcium phosphate co-precipitation method. CAT activity expressed in transfected cells was assayed essentially as described previously (49).

RESULTS

CR1 and CR2 mutations

Mutations introduced to CR1 and CR2 regions in this study fall under two

classes. One class of mutations has substituted conserved amino acid residues with unrelated ones as in S40K and LY44,45KL. They alter the conserved residues within the CR1 sequence required for binding to the pRb family proteins and are expected to lack the binding activity and transforming potential. ED54,55KL also alters the conserved residues in the center of CR1, but has not been implicated in pRb binding or p300 binding. Another class includes mutations that alter amino acid residues specific to Ad12 E1A into those specific to Ad5 E1A proteins as in Y42H, L108T and Y110H. The pRb binding domain of CR1 of Ad5 E1A includes an acidic amino acid at the position four residues N-terminal to the conserved leucine. in38E adds a glutamic acid at the corresponding position of Ad12 E1A as shown in Fig. 1. The mutations of the second class were used to construct CR1-CR2 double mutations.

Conserved residues in CR1 are essential for transformation

p5XhoC (Ad5 E1) induced transformed foci very efficiently on 3Y1 cells as compared with p12C3 (Ad12 E1) and p5A12 (Ad12 E1A and Ad5 E1B) as shown in Fig. 2. p5A12-NI, the parental plasmid with an Ad5/Ad12 chimeric E1A gene

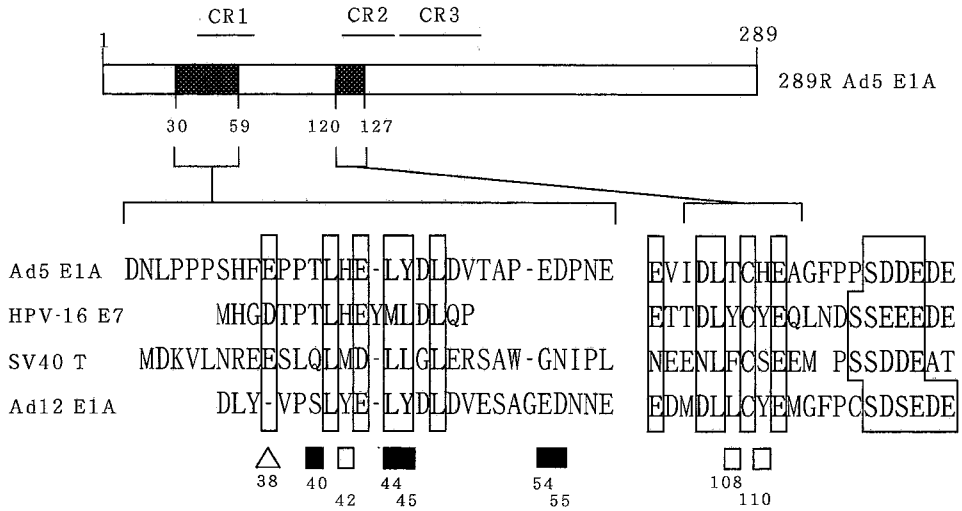

Fig 1. Structure of E1A protein and pRb binding regions.

Structure of Ad5 E1A 289R protein is shown on the top with the positions of three conserved regions CR1, CR2 and CR3. Amino acid sequences of portions of CR1 and CR2 (residues 30-59 and around 120-127) are shown on the bottom, aligned with corresponding regions of HPV-16 E7, SV40 large T antigen and Ad12 E1A. The positions of insertion mutation (△), mutations in conserved or semiconserved residues (■) and those replacing nonconserved residues for Ad5-specific amino acids (□) are shown under the sequence of Ad12 E1A.

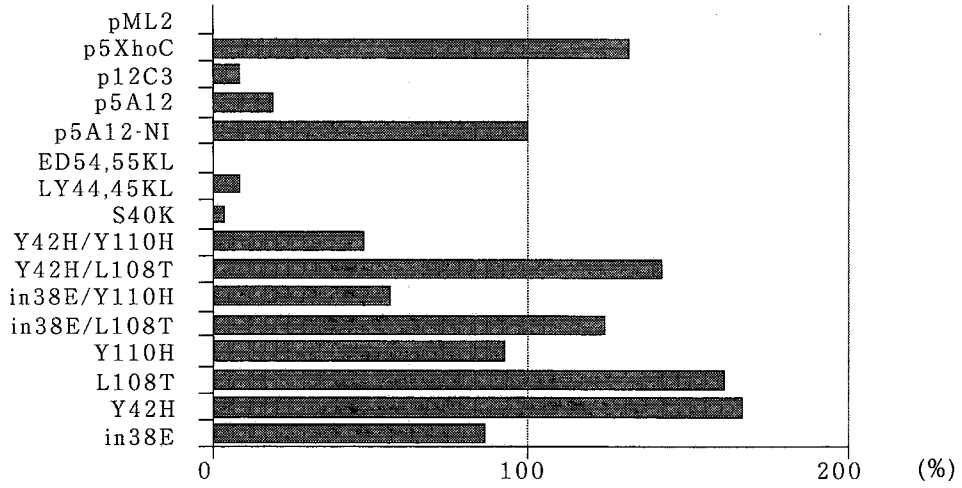

Fig 2. Transforming activity of mutant E1A genes.
Transforming activity was assayed as focus formation efficiency on 3Y1 cells, and the average of three experiments is shown as a percentage of that of the parental p5A12-NI. All the mutations were introduced into Ad12 E1A moiety of Ad12/Ad5 chimeric E1A proteins encoded by p5A12-NI (see text). pML2, vector control. p5XhoC encodes Ad5 E1A and E1B; p12C3, Ad12 E1A and E1B; p5A12, Ad12 E1A and Ad5 E1B.

used for mutagenesis in this study, showed relatively high transformation efficiency between p5XhoC and p5A12 (42). Two mutations, S40K and LY44, 45KL, induced foci only at highly reduced efficiencies (Fig. 2), indicating that the amino acid residues 40S, 44L and 45Y in the CR1 region of Ad12 E1A proteins are essential to the transformation function. Another mutation ED54,55KL completely impaired the transformation activity, suggesting an indispensable role of this conserved dipeptide to the integrity of the transforming function.

Transformation activity of allogenotypic mutations

in38E and Y110H showed transformation efficiencies comparable to that of the parental p5A12-NI (Fig. 2). These results indicate that an acidic residue conserved in the preceding region to the pRb binding sequence is not required for the transformation function in the context of the Ad12 E1A protein sequence and that a tyrosine to histidine alteration at the position 110 is tolerable to the function. In contrast, Y42H and L108T showed enhanced efficiencies as compared to the parental construct and even to p5XhoC (Fig. 2). When these mutations, Y42H in CR1 and L108T in CR2, were combined into a construct of double mutation, Y42H/L108T, the transformation efficiency was kept higher than that of

p5A12-NI. Thus, the low transformation activity of Ad12 E1A can be attributed in part to the sequence specificity at those positions. When L108T was combined to in38E, the resulting double mutation in38E/L108T also kept a high efficiency comparable to that of p5A12-NI. To the contrary, when Y110H was combined to CR1 mutants, both of the resulting double mutations in38E/Y110H and Y42H/Y110H showed reduced transformation efficiencies; approximately half as much as that of p5A12-NI (Fig. 2). Since each of the single mutations had enhanced or at least comparable transformation efficiencies to that of p5A12-NI, the results indicate incompatibility of those double mutations to the functional integrity of the E1A proteins. Primary BRK cells did not develop any transformed colonies after the transfection of mutant and parental plasmids, except p5XhoC (data not shown). It seems likely that the allogenotypic mutations of Ad5 specific amino acid residues are not enough to activate the low potential of Ad12 E1A in immortalization and/or transformation of primary BRK cells.

Growth in soft agar medium

Six independent foci induced by each of the mutant genes as well as wild type genes were isolated in two separate experiments. Most cell clones grew into continuous cell lines, but so did a few of those induced by S40K and LY44,45KL. The latter cells did not grow in soft agar culture, while cells induced by Y42H and L108T had the potential to form colonies (Fig. 3). Cells induced by other mutants and the parental plasmid showed variable potential to grow in the medium depending on the individual clones.

Expression of mutant E1A proteins in transformed cells

To analyze expression levels of mutant E1A proteins, cells from independently transformed foci were grown and labeled. Since all the mutations were introduced on the chimeric E1A protein NI that has the C-terminal sequence from Ad5 E1A, the mutant E1A proteins can be detected by immunoprecipitation with M73 monoclonal antibody. The results, shown in Fig. 4, indicate that the mutant E1A proteins are expressed in relevant cell lines at detectable levels, except in three cell lines. Among the E1A-interacting proteins, p300 and p107 were detected as co-immunoprecipitated proteins from transformed cells induced by p5XhoC and p5A12-NI. These E1A-interacting proteins were also detected in cells transformed by mutant E1A genes such as in38E, Y42H, L108T, in38E/L108T, Y42H/L108T and Y42H/Y110H. In the case of cells transformed by Y110H, in38E/Y110H and S40K, the relative levels of p107 as compared with those of p300 were reduced, indicating that the bindings of pRb family proteins are affected by these mutations. The LY44,45KL mutation also appears to have

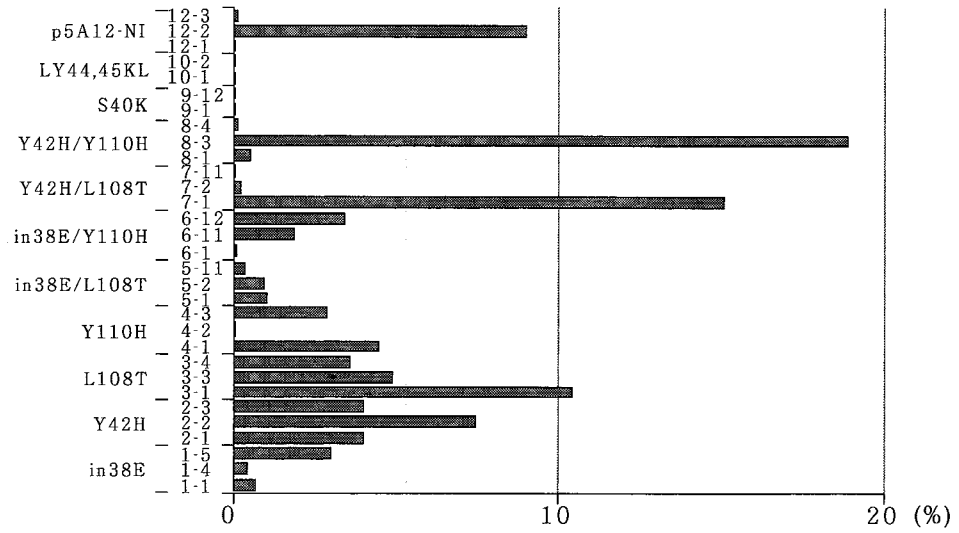

Fig 3. Growth of transformed cells in soft agar medium.
Efficiency of colony formation of transformed cells in soft agar medium is shown by the percentage fraction of cells grown per cells plated.

impaired the activity of interaction with those cellular proteins, though the level of E1A expression itself is low.

In vitro protein interaction

To confirm the protein binding of mutant E1A proteins including ED54,55KL that produced no transformed foci, *in vitro* protein binding assay was carried out using GST-E1A fusion proteins purified from induced *E. coli*. Since E1A proteins are known to interact with many cellular proteins *in vitro*, a portion of E1A proteins encompassing CR1 and CR2 regions was fused to GST as described in Materials and Methods. Among the single mutations, Y42H showed enhanced binding to p107 as compared with wild type and other single mutations (Fig. 5). The enhanced p107 binding of Y42H was retained by double mutations Y42H/L108T and Y42H/Y110H. S40K and LY44,45KL were severely defective in p107 binding *in vitro*. The *in vitro* binding to pRb was not so evident as that to p107, but showed a similar tendency of binding levels. The *in vitro* binding of E1A fusion proteins to p130 was not clear enough to be identified. All the single mutations except S40K exhibited slightly (2～3×) reduced levels of p300 binding while double mutations reduced more (5～6×) as compared with the wild type fusion protein. ED54,55KL reduced the p300 binding severely while the p107 binding was comparable to that of the wild type fusion protein. Interestingly, the

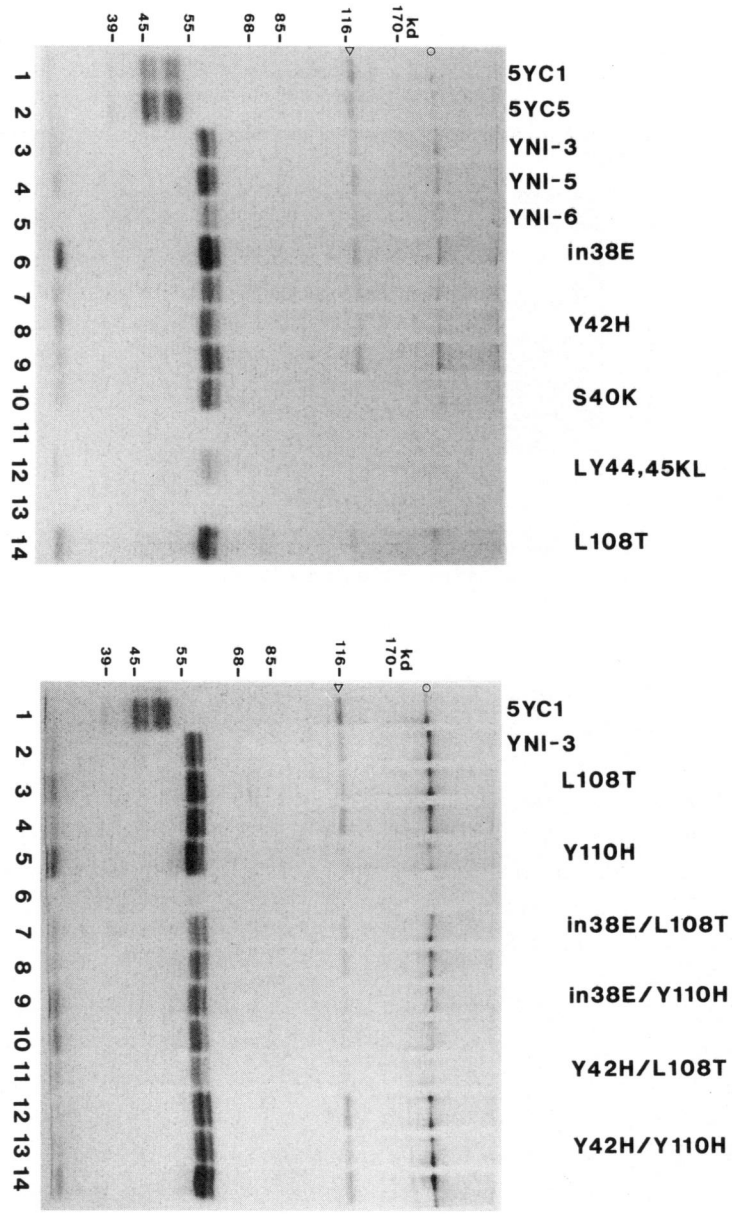

Fig 4. Expression of E1A proteins in transformed cell clones.

Mutant and parental chimeric NI proteins as well as Ad5 E1A proteins were detected by immunoprecipitation with M73 monoclonal antibody from [^{35}S]-labeled extracts of transformed cells induced by the relevant E1A clones indicated on the top, followed by SDS-PAGE and fluorography. ○ indicates p300 and △ depicts p107.

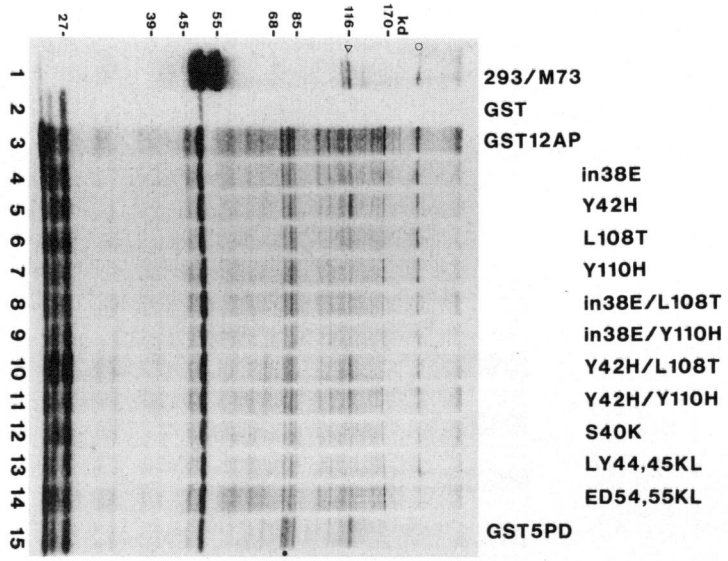

Fig 5. In vitro binding of mutant E1A proteins with cellular proteins.

Portions of wild type and mutant Ad12 E1A proteins containing CR1 and CR2 were fused to GST, expressed in and purified from *E. coli* and used as protein probes to detect interacting cellular proteins in [^{35}S]-labeled rat cell extracts. GST fusion of the corresponding Ad5 E1A portion was used in lane 15. A tentative p74 is marked by the dot on the right. Lane 1 includes immunoprecipitation of Ad5 E1A protein complexes from 293 cells. Proteins were separated on SDS-PAGE and visualized by fluorography. p300 and p107 are indicated as in Fig. 4.

corresponding portion of Ad5 E1A protein (CR1 through CR2) fused to GST moiety did not bind a detectable level of p300 while it bound a high level of p107 as shown in Fig. 5. Another point that is different between Ad12 and Ad5 E1A proteins is binding qualities to a protein of approximately 74 Kd. The tentative p74 bound specifically to Ad5 E1A (CR1+CR2) but not apparently to Ad12 E1A (CR1+CR2). A feasible band of the tentative p74 was able to be seen only with Y42H/L108T fusion among all the mutant Ad12 E1A fusions. All the wild type and mutant Ad12 E1A fusions bound to a protein of approximately 75 Kd to which Ad5 E1A also bound faintly (Fig. 5, lane 15).

Activation of E2F-dependent promoter by CR1 and CR2 mutants

To analyze the ability of E1A mutants to interact with pRb family proteins, they were assayed for their ability to transactivate the transcription from an E2F-dependent E2A promoter. The CAT activity is very low without E1A expression and is enhanced when E1A proteins are expressed, as shown in Fig. 6.

The chimeric E1A NI further stimulated the CAT expression more than did wild type Ad5 or Ad12 E1A as shown previously using adenovirus E3 promoter (42). S40K and LY44,45KL mutations reduced the transactivating potential significantly and ED54,55KL reduced the activity only slightly. These three mutations show good correlation between *in vitro* protein binding and transactivation. Four sin-

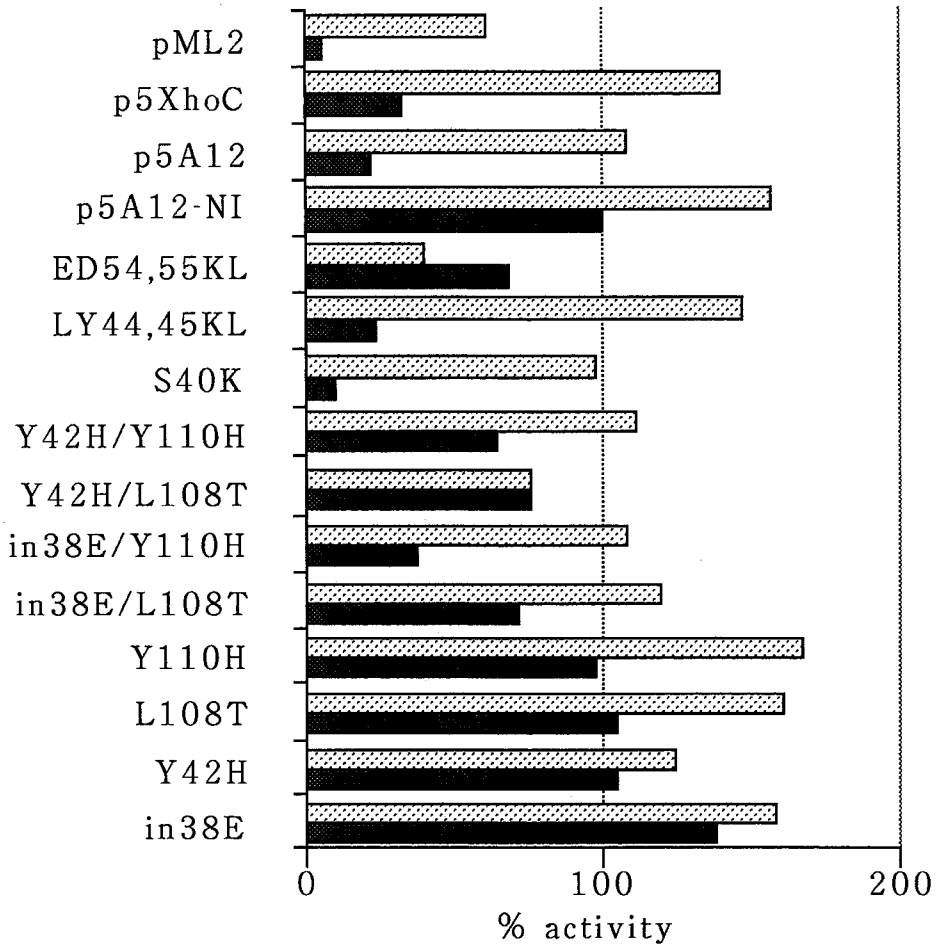

Fig 6. Analysis of E2F-dependent transcription induced by E1A.
CAT activity expressed from pE2ACAT reporter plasmid in the presence of mutant or parental E1 plasmids was assayed as described in Materials and Methods. The results shown are averages of three experiments, each in the absence (■) or presence (□) of the E4 orf6/7 expression plasmid.

gle mutations have the same potential to stimulate the E2A promoter as the parental NI gene regardless of the enhanced binding of Y42H to p107 *in vitro* shown above. Four double mutations also showed similar levels of transactivation, which are slightly less than that of the parental NI gene. In the presence of E4 orf6/7 proteins that bind E2F and stabilize E2F-DNA complexes on the E2A promoter (50), the CAT activity was generally enhanced (Fig. 6). The results show that E4 orf6/7 proteins may recruit E2F on the promoter without E1A proteins and confirm that E1A enhances the E2F activation of the E2A promoter. The reason why ED54,55KL reduced the E2F activation in the presence of E4 orf6/7 is not known.

DISCUSSION

Among the CR1 mutations, LY44,45KL reduced the transforming activity and p107/pRb binding, confirming the essential role of the conserved residues in Ad12 E1A. S40K also showed the importance of 40S in Ad12 E1A, though the position is not implicated in the proposed consensus (27). Interestingly, the large T antigen of polyoma virus with Arg at the corresponding position (27) has no transforming activity. An acidic amino acid appears 3 residues amino terminal to conserved Leu in oncoproteins of most DNA tumor viruses (Fig. 1, 27) and is implicated in the consensus motif (27). But it is not required in Ad12 E1A, since in38E had no effect on transformation or p107/pRb binding (Fig. 5). Thus, the consensus CR1 motif for pRb family binding may be modified as X-L-X-E/D-L-X-X-L, where the first X is neither of the basic residues.

Our results of *in vitro* protein binding experiment clearly showed that the CR1 portion of Ad12 E1A has capacity to bind p300 *in vitro* (Fig. 5). In contrast, the corresponding portion of Ad5 E1A showed no detectable binding of p300 (Fig. 5) consistent with the previous report that p300 binding to Ad5 E1A protein is mainly dependent on the amino terminal region but not on CR1 (30). This aspect of Ad12 E1A may contribute to the transforming activity, by compensating for the less binding to pRb in rat cells as compared with Ad5 E1A. A CR1 mutation ED54,55KL, which alters two consecutive acidic amino acids conserved among adenoviruses, but not in HPV E7 or SV40 large T, impaired transforming activity completely. This mutation reduces binding to p300 but affects only slightly p107 binding. Thus, 54E and 55D are not included in the pRb binding domain, but are probably included in the p300 binding domain of CR1 of Ad12 E1A.

The LXCXE motif for pRb binding in CR2 has been analyzed extensively by a series of mutations (29). Since it is conserved in oncogene products of most DNA tumor viruses (Fig. 1, 27), it is reasonable to assume comparable roles for

those tightly conserved residues in CR2 of Ad12 E1A. As for non-conserved residues represented by X in the motif, the fourth site between C and E can be variable amino acids except acidic ones, as seen in oncoproteins of various DNA tumor viruses (27) and as shown by mutational analysis (29, 51). The second site between L and C seems to allow any amino acids except Pro, but may have some preference for Thr and Phe (27, 29, 51). In this regard, our results showing that L108T is an up mutation and Y110H is neutral are quite reasonable. Thus, 108L as well as 42Y may explain in part the lower transforming activity of Ad12 E1A as compared to that of Ad5 E1A.

The nature of double mutations described in this paper is interesting. L108T is an up mutation and keeps its transforming activity when combined to either CR1 mutation, in38E or Y42H. Thus the amino acid residue at position 108 of Ad12 E1A is independent from structural alteration in CR1. In contrast, Y110H has wt level of transformation activity, but when combined with CR1 mutations, in38E and Y42H, both double mutations reduced their transforming activity to approximately half of that of parental p5A12-NI. For the full transformation activity, Y110H is not compatible with either CR1 mutation, in38E or Y42H, which themselves are acceptable in the context of Ad12 E1A or rather an up mutation. Thus, our results suggest close contact between CR1 and CR2 through a specific amino acid residue. When a mutation or small deletion is introduced in CR1 or CR2 that affects either pRb or p300 binding, it may also affect the efficiency of binding to the other protein because of structural perturbation mediated through the interaction between CR1 and CR2.

ACKNOWLEDGMENTS

The authors thank D. Ornelles and T. Shenk for supplying us with plasmids. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. SHENK T. Adenoviridae: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM, editors. Fields Virology. Philadelphia, Lippincott-Raven Publishers, 1996, 2111-2148.
2. RICCIARDI RP. Transformation and tumorigenesis mediated by the adenovirus E1A and E1B oncogenes. In: Barbanti-Brodano G, Bendinelli M, Friedman H, editors. DNA tumor viruses: oncogenic mechanisms. New York, Plenum Press, 1995, 195-225.
3. PERRICAUDET M, AKUSJÄRVI G, VIRTANEN A, PETTERSSON U. Structure of two spliced mRNAs from the transforming region of human subgroup C adenoviruses. Nature 1979, 281: 694-696.

4. HEN R, BORRELLI E, CHAMBON P. Repression of the immunoglobulin heavy chain enhancer by the adenovirus-2 E1A products. Science 1985, 230: 1391-1394.
5. VELCICH A, ZIFF E. Adenovirus E1a proteins repress transcription from the SV40 early promoter. Cell 1985, 40: 705-716.
6. LILLIE JW, GREEN M, GREEN MR. An adenovirus E1a protein region required for transformation and transcriptional repression. Cell 1986, 46: 1043-1051.
7. SCHNEIDER JF, FISHER F, GODING CR, JONES NC. Mutational analysis of the adenovirus E1a gene: the role of transcriptional regulation in transformation. EMBO J 1987, 6: 2053-2060.
8. YU D, SUEN T-C, YAN D-H, CHANG L-S, HUNG M-C. Transcriptional repression of the neu protooncogene by the adenovirus 5 E1A gene products. Proc Natl Acad Sci U S A 1990, 87: 4499-4503.
9. FLINT J, SHENK T. Adenovirus E1A protein: paradigm viral transactivator. Annu Rev Genet 1989, 23: 141-161.
10. MORAN E, MATHEWS MB. Multiple functional domains in the adenovirus E1A gene. Cell 1987, 48: 177-178.
11. LILLIE JW, GREEN MR. Transcription activation by the adenovirus. Nature 1989, 338: 39-44.
12. LEE WS, KAO CC, BRYANT GO, LIU X, BERK AJ. Adenovirus E1A activation domain binds the basic repeat in the TATA box transcription factor. Cell 1991, 67: 365-376.
13. YEE S-P, BRANTON PE. Detection of cellular proteins associated with human adenovirus type 5 early region 1A polypeptides. Virology 1985, 147: 142-153.
14. HARLOW E, WHYTE P, FRANZA BR Jr, SCHLEY C. Association of adenovirus early-region 1A proteins with cellular polypeptides. Mol Cell Biol 1986, 6: 1579-1589.
15. EGAN C, BAYLEY ST, BRANTON PE. Binding of the *Rb1* protein to E1A products is required for adenovirus transformation. Oncogene 1989, 4: 383-388.
16. WHYTE P, WILLIAMSON NM, HARLOW E. Cellular targets for transformation by the adenovirus E1A proteins. Cell 1989, 56: 67-75.
17. ECKNER R, EWEN ME, NEWSOME D, GERDES M, DECAPRIO JA, LAWRENCE JB, LIVINGSTON DM. Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. Genes Dev 1994, 8: 869-884.
18. WHYTE P, BUCHKOVICH KJ, HOROWITZ JM, FRIEND SH, RAYBUCK M, WEINBERG RA, HARLOW E. Association between an oncogene and antioncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature 1988, 334: 124-129.
19. EGAN C, JELSMA TN, HOWE JA, BAYLEY ST, FERGUSON B, BRANTON PE. Mapping of cellular protein-binding sites on the products of early-region 1A of human adenovirus type 5. Mol Cell Biol 1988, 8: 3955-3959.
20. EWEN ME, XING Y, LAWRENCE JB, LIVINGSTON DM. Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. Cell 1991, 66: 1155-1164.

21. DYSON N, GUIDA P, MCCALL C, HARLOW E. Adenovirus E1A makes two distinct contacts with the retinoblastoma protein. J Virol 1992, 66: 4606-4611.
22. LI Y, GRAHAM C, LACY S, DUNCAN AMV, WHYTE P. The adenovirus E1A-associated 130-kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. Genes Dev 1993, 7: 2366-2377.
23. STEIN RW, CORRIGAN M, YACIUK P, WHELAN J, MORAN E. Analysis of E1A-mediated growth regulation functions: binding of the 300-kilodalton cellular product correlates with E1A enhancer repression function and DNA synthesis-inducing activity. J Virol 1990, 64: 4421-4427.
24. HOWE JA, MYMRYK JS, EGAN C, BRANTON PE, BAYLEY ST. Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis. Proc Natl Acad Sci U S A 1990, 87: 5883-5887.
25. DECAPRIO JA, LUDLOW JW, FIGGE J, SHEW J-Y, HUANG C-M, LEE W-H, MARSILIO E, PAUCHA E, LIVINGSTON DM. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell 1988, 54: 275-283.
26. MÜNGER K, WERNESS BA, DYSON N, PHELPS WC, HARLOW E, HOWLEY PM. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J 1989, 8: 4099-4105.
27. DYSON N, BERNARDS R, FRIEND SH, GOODING LR, HASSELL JA, MAJOR EO, PIPAS JM, VANDYKE T, HARLOW E. Large T antigens of many polyomaviruses are able to form complexes with the retinoblastoma protein. J Virol 1990, 64: 1353-1356.
28. MORAN E, ZERLER B, HARRISON TM, MATHEWS MB. Identification of separate domains in the adenovirus E1A gene for immortalization activity and the activation of virus early genes. Mol Cell Biol 1986, 6: 3470-3480.
29. CORBEIL HB, BRANTON PE. Functional importance of complex formation between the retinoblastoma tumor suppressor family and adenovirus E1A proteins as determined by mutational analysis of E1A conserved region 2. J Virol 1994, 68: 6697-6709.
30. WANG H-GH, RIKITAKE Y, CARTER MC, YACIUK P, ABRAHAM SE, ZERLER B, MORAN E. Identification of specific adenovirus E1A N-terminal residues critical to the binding of cellular proteins and the control of cell growth. J Virol 1993, 67: 476-488.
31. HIEBERT SW, CHELLAPAN SP, HOROWITZ JM, NEVINS JR. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev 1992, 6: 177-185.
32. FLEMINGTON EK, SPECK SH, KAELIN WG Jr. E2F-1-mediated transactivation is inhibited by complex formation with the retinoblastoma susceptibility gene product. Proc Natl Acad Sci U S A 1993, 90: 6914-6918.
33. HELIN K, HARLOW E, FATTAEY A. Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. Mol Cell Biol 1993, 13: 6501-6508.
34. RAYCHAUDHURI P, BAGCHI S, DEVOTO SH, KRAUS VB, MORAN E, NEVINS JR. Domains of the adenovirus E1A protein required for oncogenic activity are also required for dissociation of E2F transcription factor complexes. Genes Dev 1991, 5: 1200-1211.

35. BANDARA LR, LATHANGUE NB. Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature 1991, 351: 494-497.
36. ARANY Z, NEWSOME D, OLDREAD E, LIVINGSTON DM, ECKNER R. A family of transcriptional adaptor proteins targeted by the E1A oncoprotein. Nature 1995, 374: 81-84.
37. LUNDBLAD JR, KWOK RPS, LAURANCE ME, HARTER ML, GOODMAN RH. Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP. Nature 1995, 374: 85-88.
38. BARBEAU D, CHARBONNEAU R, WHALEN SG, BAYLEY ST, BRANTON PE. Functional interactions within adenovirus E1A protein complexes. Oncogene 1994, 9: 359-373.
39. WANG H-GH, MORAN E, YACIUK P. E1A promotes association between p300 and pRB in multimeric complexes required for normal biological activity. J Virol 1995, 69: 7917-7924.
40. KIMURA G, ITAGAKI A, SUMMERS J. Rat cell line 3Y1 and its virogenic polyoma- and SV40-transformed derivatives. Int J Cancer 1975, 15: 694-706.
41. SAWADA Y, FÖHRING B, SHENK TE, RASKA K Jr. Tumorigenicity of adenovirus-transformed cells: region E1A of adenovirus 12 confers resistance to natural killer cells. Virology 1985, 147: 413-421.
42. HAMAMATSU C, SAWADA Y. Functional analyses of human adenovirus types 5 and 12 chimeric early region 1A recombinants. Sapporo Med J 1989, 58: 99-109.
43. SAWADA Y, RASKOVA J, FUJINAGA K, RASKA K Jr. Identification of functional domains of adenovirus tumor-specific transplantation antigens in types 5 and 12 by viable viruses carrying chimeric E1A genes. Int J Cancer 1994, 57: 598-603.
44. MURTHY SCS, BHAT GP, THIMMAPPAYA B. Adenovirus EIIA early promoter: transcriptional control elements and induction by the viral pre-early E1A gene, which appears to be sequence independent. Proc Natl Acad Sci U S A 1985, 82: 2230-2234.
45. FREYER GA, KATOH Y, ROBERTS RJ. Characterization of the major mRNAs from adenovirus 2 early region 4 by cDNA cloning and sequencing. Nucleic Acids Res 1984, 12: 3503-3519.
46. KUNKEL TA, ROBERTS JD, ZAKOUR RA. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol 1987, 154: 367-382.
47. HARLOW E, FRANZA B, SCHLEY C. Monoclonal antibodies specific for adenovirus early region 1A proteins: extensive heterogeneity in early region 1A products. J Virol 1985, 55: 533-546.
48. SMITH DB, JOHNSON KS. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene 1988, 67: 31-40.
49. GORMAN CM, MOFFAT LF, HOWARD BH. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 1982, 2: 1044-1051.
50. HUANG MM, HEARING P. Adenovirus early region 4 encodes two gene products with redundant effects in lytic infection. J Virol 1989, 63: 2605-2615.
51. WANG ZX, GERMINO FJ. Mutational analysis of the conserved region 2 site of adenovirus E1A and its effect on binding to the retinoblastoma gene product: use of the "double-tagging" assay. Proc Natl Acad Sci U S A 1995, 92: 4631-4635.