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The Adenovirus E1A 12S Product Displays Functional Redundancy in Activating the Human Proliferating Cell Nuclear Antigen Promoter

CHITRA KANNABIRAN,† GILBERT F. MORRIS, CLAUDE LABRIE, AND MICHAEL B. MATHEWS*

Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724

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The adenovirus E1A 243R oncoprotein stimulates expression from the promoter of the human proliferating cell nuclear antigen (PCNA). To gain insight into the mechanism of activation, we analyzed deletion and point mutations of the 243R protein for their abilities to activate PCNA promoter-directed reporter gene expression upon cotransfection into HeLa cells. Large deletions that in combination span the entire protein severely impaired the ability of E1A 243R to induce PCNA expression. Smaller deletions and specific point mutations that target specific E1A-binding proteins were less deleterious to PCNA induction. The data suggest that E1A activates transcription of the PCNA gene by multiple mechanisms and that, of the known 243R-associated proteins, p300 and p107-cyclin A can mediate the response while p105-RB does not appear to participate. Presumably, the functional redundancy ensures that 243R can activate expression of this essential DNA replication protein regardless of cell type and physiological conditions.

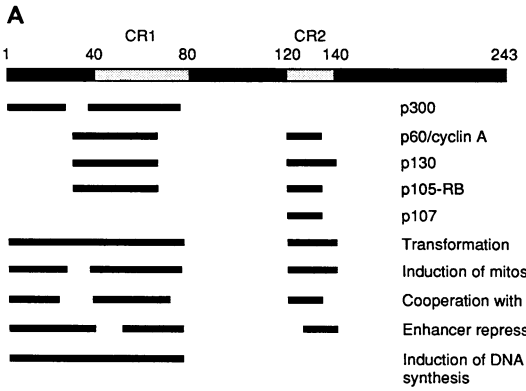
The adenovirus E1A gene can immortalize primary cells (27), and it can cooperate with the adenovirus E1B gene or other oncogenes such as Ha-*ras* and polyomavirus middle T antigen (60, 80) to transform them. Differential splicing of the E1A transcript produces a number of E1A mRNAs, of which the 13S and 12S species predominate (7, 16, 35). The 13S mRNA encodes a protein of 289 amino acids (289R protein), while the 12S mRNA produces a protein 243 amino acids in length (243R protein). Two regions conserved among adenovirus serotypes (conserved region 1 [CR1] and conserved region 2 [CR2]) are common to both 289R and 243R proteins, while a third conserved region (conserved region 3 [CR3]) is unique to the 289R protein (19, 43, 52). All three conserved regions lie within the first exon; CR2 and CR3 are contiguous and separated from CR1, which is equidistant from CR2 and the protein's N terminus (43). Transactivation of viral early genes, a function attributed to E1A, is generally associated with CR3 of the E1A 13S product (6, 19). An exception to this is the E2 promoter, which can be transactivated through CR1 and CR2 (2, 36, 50, 79).

The mechanisms underlying the effects of the 243R protein on gene expression are not well understood. It can both activate and repress transcription (2, 39, 49, 50, 53, 65, 73, 74), and it apparently brings about these effects by a distinct mechanism that requires interactions with a number of cellular factors that directly or indirectly affect transcriptional activity. The segments of the 243R protein that are associated with its several biological and biochemical functions are diagrammed in Fig. 1A. The transcriptional repression function of the E1A 12S product correlates with its ability to bind the cellular protein p300 (69), but CR2 function may also be required (39). Transactivation of viral and cellular genes via the cellular transcription factor E2F is associated with binding of the 243R protein to p105-RB as well as to p107 and cyclin A. A number of cellular genes that are growth regulated contain E2F sites (8, 51, 72). While a causal connection between these transcriptional effects of the 12S product and transformation has not been established, the ability to transform cells in cooperation with other oncogenes correlates with binding to these cellular proteins.

The 243R protein is also capable of inducing DNA synthesis and mitosis in quiescent cells (43, 61, 67). The proliferating cell nuclear antigen (PCNA) is a DNA replication protein. It functions in cellular DNA replication as an auxiliary factor for DNA polymerase δ (10, 56, 57), and it also functions in DNA repair (64). It is a highly conserved protein (41) essential for DNA replication in *Saccharomyces cerevisiae* (5), frog (82), and mammalian (30, 40) cells. PCNA synthesis is growth regulated (1, 31, 42), although only slight variations in synthesis occur in cycling human HeLa (47) and Chinese hamster ovary (CHO) cells (40). PCNA mRNA levels are induced by serum stimulation of quiescent cells (31, 67) or by adenovirus infection (11, 46). Induction of PCNA synthesis in response to serum appears to occur at both transcriptional and posttranscriptional levels (13). It is not clear whether both types of regulation occur in the response of the PCNA gene to adenovirus infection, but a virus expressing only the E1A 12S product can induce PCNA expression in baby rat kidney (BRK) cells (81), and the transcriptional response of the PCNA promoter to the 12S product has been demonstrated in HeLa cells by using a transient expression system (49). This response is mediated by a site that exhibits homology to the consensus DNA sequence for ATF/CREB binding located about 50 nucleotides upstream of the cap site (37, 49).

The 12S product mediates other biological effects that are part of its immortalizing activity, such as the induction of DNA synthesis in quiescent cells (33, 68) and maintenance of cellular proliferation (27, 60). Initial efforts to map PCNA induction and correlate it with other biological effects of

* Corresponding author.
† Present address: Department of Biochemistry, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, NJ 08854.



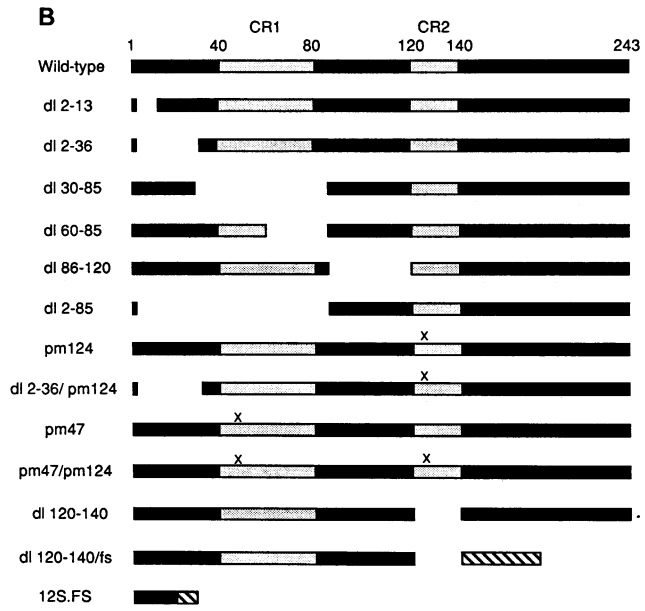


FIG. 1. Schematic of the E1A 12S gene product and mutants. (A) Functional domains. At the top is a bar representation of 243R. The conserved regions (CR1 and CR2) are stippled, and the nonconserved regions are solid. Numbers denote the amino acid residues at the boundaries of the conserved regions and the ends of the protein. The lines below correspond to the regions of 243R associated with binding the indicated proteins in vitro or possessing the indicated biological activity (adapted from Ruley [61]). (B) Mutants of 243R. At the top, the wild-type protein is represented as in panel A. The names of the mutants are shown on the left, deleted amino acids are indicated by gaps, and point mutations are marked by X. Hatched boxes refer to regions that are predicted to be translated out of frame.

243R revealed that *pm*928, a point mutation at amino acid 124 in CR2 that is transformation defective, did not prevent PCNA induction by adenovirus (81). A more detailed analysis of the E1A gene in virus-infected BRK cells suggested that PCNA induction may involve multiple pathways, because induction of PCNA synthesis was not abrogated by a number of E1A deletions which together encompassed the entire first exon (32). Interpretation of the latter results is complicated by the presence of the 13S product which can activate PCNA through CR3 by a mechanism independent of the 12S product. Further, in a virus infection, it is difficult to separate the effects of other viral genes from those due to the 12S product itself.

In this study, we examined the domains of the 12S product required for PCNA induction in HeLa cells by cotransfecting a 12S expression vector with a PCNA promoter-driven chloramphenicol acetyltransferase (CAT) reporter gene. Such an analysis allows transcriptional induction to be correlated with the binding of previously identified cellular proteins (Fig. 1A) that can modulate transcriptional activity. We found that sequences throughout the E1A protein are required for full stimulation of expression of the PCNA promoter in HeLa cells. This result indicates that more than one of the known E1A-associated proteins mediates activation of PCNA expression by 243R and suggests that more than one pathway is involved. Although we cannot exclude the involvement of an unidentified 243R-associated protein(s), the partial activity of 12S mutants with alterations in the N terminus suggests that binding to the cellular protein p300 contributes to activation of the PCNA-CAT reporter. Furthermore, the contribution of CR2 to activation of PCNA-CAT by 243R appears to occur via binding of the p107-cyclin A complex, since point mutants which impair binding of the other CR2-associated proteins are not impaired for PCNA-CAT induction. On the other hand, the retinoblastoma susceptibility gene protein, p105-RB, does not seem to play a role in this response.

MATERIALS AND METHODS

Plasmids. The PCNA-CAT constructs, −1265 to +62 PCNA-CAT and −87 to +62 PCNA-CAT (48), the E1A and E1B constructs, pCMV12S, pCMV12S.FS, pCMV13S, and pCMV19K (49, 77), and pON260, a plasmid expressing the β-galactosidase gene under the control of the cytomegalovirus (CMV) promoter (66), were described previously. For simplicity, in this report we refer to the mutants by the amino acid residues deleted or mutagenized (original names in brackets): *dl*2-13 [NTdl598] (78), *dl*2-36 [p12S(2-36)] (69), *dl*30-85 [dl646N] (78), *dl*60-85 [dl 739N] (78), *dl*2-85 [NTdl814] (78), *dl*86-120 [NCdl] (45), *pm*124 [*pm*928] (45); *dl*2-36/*pm*124 [928 (2-36)] (75), *pm*47/*pm*124 [YH47.928] (76), and *pm*47 [YH.47] (76). E1A mutants (courtesy of E. Moran and E. Harlow) were subcloned into pCMV12S by using polymerase chain reaction (PCR) methods with the following primers: 5′-CCCCGAATTCACTCTTGAGTGCCAGCGAG TA-3′ (primer A), 5′-GCATTCTCTAGACACAGGTGATG T-3′ (primer B), and 5′-TTGTACCGGAGGTGATCGATCT TAC-3′ (primer C). The upstream primer (A) is complementary to sequences beginning at the transcriptional start site of E1A at nucleotide 499, with an *Eco*RI site included at the 5′ end for cloning purposes. The downstream primers are complementary to sequences spanning the *Xba*I site in the second exon (B) or to the *Cla*I site in the first exon (C). The *Xba*I site was used for mutants *dl*2-36, *pm*124, *dl*2-36/*pm*124, and *dl*86-120, which were all originally in a 12S background. The *Cla*I site was used for constructs *dl*2-13, *dl*30-85, and *dl*60-85 to convert them into a 12S background, since these plasmids were in a background containing both 12S and 13S sequences. PCR products were digested with *Eco*RI and either *Cla*I or *Xba*I and subcloned into pCMV12S digested with *Eco*RI and either *Xba*I or *Cla*I as appropriate. Mutant *pm*47/*pm*124 was constructed by subcloning the *Pvu*II-*Xba*I fragment from the parental plasmid into pCMV12S digested with *Pvu*II and *Xba*I. Deletions in CR2 were made by ligating the *Eco*RI-*Cla*I fragment of pCMV12S, filled in at the *Cla*I site, into *Eco*RI-*Acc*I-digested pCMV13S, filled in at the *Acc*I site. The resulting constructs, *dl*120-140 and *dl*120-140/fs, had the following junction sequences:

*dl*120-140	ATC Ile	GAC Asp	AGT Ser
*dl*120-140/fs	ATC Ile	GCT Ala	ACA Thr

Fragments obtained by PCR were subcloned into pUC118 and pUC119 and sequenced by using single-stranded DNA templates to confirm the absence of PCR artifacts.

Transfection assays. Monolayer cultures of HeLa cells (ATCC CCL2) were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum and 100 μg of penicillin and streptomycin per ml. Cells at about 50% confluency in 6-cm plates were transfected by the calcium phosphate coprecipitation method as described previously (25), except that the glycerol shock was omitted. The precipitate was left on the cells for about 16 h, and then the monolayers were rinsed with phosphate-buffered saline and fresh medium was added. Each transfection mix contained 5 μg of PCNA-CAT reporter, 0.5 μg each of pCMV12S, pCMV19K, and pON260, and salmon sperm DNA to a total of 20 μg.

CAT and β-galactosidase assays. Cells were harvested at 48 h posttransfection and assayed for CAT (23, 25) and β-galactosidase (24) activities. Results are expressed as ratios of CAT activity to β-galactosidase activity.

Immunoprecipitation. Cells were labeled for 4 h with [^{35}S]methionine at 24 h posttransfection and were harvested by scraping. Cell pellets were lysed in NET2$^+$ buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris [pH 7.4], 0.5% Na deoxycholate, 0.5% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS]), and the lysates were precleared twice with 0.1 volume of a 10% suspension of fixed *Staphylococcus aureus* (IgG Sorb; The Enzyme Center, Inc.) in NET2$^+$ containing 2% bovine serum albumin (BSA). Precleared lysates were incubated for 1 h on ice with 100 μl of M73 hybridoma supernatant containing monoclonal antibody to E1A (courtesy of E. Harlow); then, 30 μl of a 10% suspension of *S. aureus* in NET2$^+$ BSA was added, and incubation was continued for 30 min on ice. Precipitates were washed with NET2$^+$, and bound proteins were resolved in 12.5% polyacrylamide–SDS gels (38), which were subjected to fluorography (9) and exposed for 2 to 3 days at −70°C.

RESULTS

Experimental design. Previous work showed that the E1A 12S gene product activates the PCNA promoter upon cotransfection into HeLa cells (49). The goal of the present study is to define the regions of E1A that are required for this transactivation function, with a view to correlating the PCNA transactivation function with other known activities of E1A 12S and with its associated proteins (Fig. 1A).

Most of the experiments described here employed a construct that contains PCNA sequences from −87 to +62, relative to the PCNA transcription initiation site at +1, that are fused upstream of the CAT reporter gene. The assay consists of transfecting HeLa cells with the PCNA-CAT construct and an E1A 12S expression plasmid that contains the CMV early promoter directing the expression of E1A 12S or mutants thereof (Fig. 1B). To determine basal levels of PCNA promoter activity in the absence of E1A 12S expression, we transfected a construct (E1A 12S.FS) that encodes the first 22 amino acids of the 243R protein followed by four out-of-frame amino acids and a termination codon (Fig. 1B). A third plasmid that expresses the E1B 19-kDa protein from the CMV promoter was included in the transfections to improve sensitivity. The E1B 19-kDa protein stimulates expression from the PCNA promoter without any apparent sequence specificity (26, 46). To normalize for transfection efficiency, we also included pON260, a plasmid encoding β-galactosidase under the control of the CMV promoter (66).

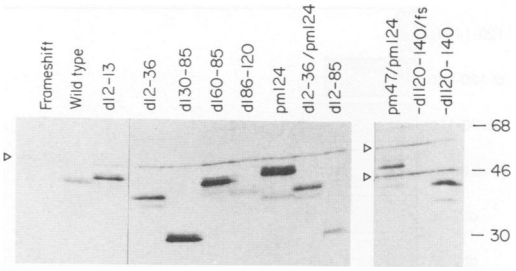


FIG. 2. Immunoprecipitation of E1A proteins from transfected cells. Cells were cotransfected with the indicated plasmids and were labeled with [^{35}S]methionine. Cell lysates were reacted with monoclonal antibody M73, and the immunoprecipitates were displayed in an SDS-polyacrylamide gel. Numbers on the right indicate the positions of molecular weight markers (in thousands). Arrowheads, nonspecific bands.

To ensure that E1A 12S mutants defective for PCNA-CAT activation were expressed at levels comparable to that of the wild-type protein, cells were labeled with [^{35}S]methionine from 24 to 28 h posttransfection. The E1A protein levels were examined by immunoprecipitation with M73, a monoclonal antibody that reacts with an epitope near the C terminus of E1A (70). Although the precise levels for the individual mutants showed experimental variation, all the mutant proteins were synthesized at levels comparable to that of the wild type, indicating that their stabilities are similar to that of the wild-type protein (Fig. 2). Moreover, several of the constructs that displayed low transactivation function gave the same results when tested at higher concentrations, confirming that their defects are not attributable to reduced expression of the mutant E1A proteins. The 12S.FS and *dl*120-140/fs products did not give rise to immunoprecipitable protein, since they lack sequences in exon 2 and, therefore, could not be detected in this assay.

Induction of PCNA depends on multiple domains in the 12S product. As a first step toward identifying the E1A regions that are involved in PCNA induction in HeLa cells, we tested large deletions that span entire domains of the 243R protein. This approach was designed to determine whether PCNA induction can be broadly correlated with any of the regions of the 12S product to which various functions have been assigned (Fig. 1A) (for a review, see references 43 and 61). Figure 3 displays the effects of large deletions that individually encompass one or more domains and, in combination, delete all of the regions of the 12S product (Fig. 1B).

Mutant *dl*2-85 eliminates the first 85 amino acids including the nonconserved N-terminal region and CR1. Figure 3 shows that this mutant is essentially inactive at inducing PCNA-CAT expression. We then tested two mutants that separately delete the two regions deleted in *dl*2-85. Mutants *dl*2-36 and *dl*30-85, which lack the nonconserved N-terminal region and CR1, respectively, were also severely impaired for activation of PCNA-CAT expression (Fig. 3). Extending the analysis farther downstream, we tested mutant *dl*86-120, which lacks the nonconserved segment between CR1 and CR2. This construct exhibited about 30% of the activity of

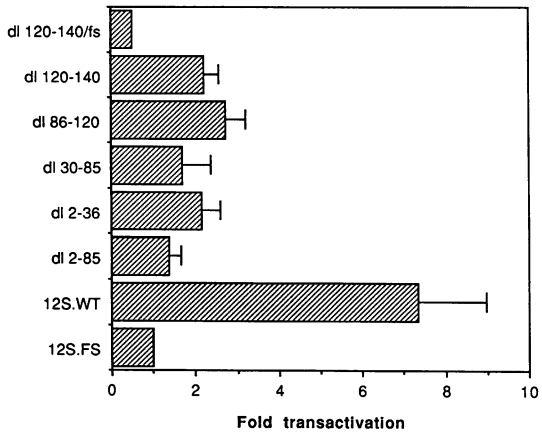


FIG. 3. Regions of 243R required for transactivation of the PCNA promoter. Wild-type 243R (12S.WT) and the indicated mutants were assayed for transactivation of the PCNA promoter by cotransfection with the −87 to +62 PCNA-CAT reporter. The fold increase in CAT activity indicated on the abscissa (about sevenfold for 12S.WT) is normalized to the negative control (12S.FS; set at 1.0). The results are expressed as CAT/β-galactosidase activity ratios and represent the means of three independent transfections done in duplicate.

the wild-type protein. Deletion of CR2 (*dl*120-140) also resulted in about 30% of the activity of the wild-type protein. In combination with removal of exon 2 sequences, in *dl*120-140/fs, this mutation resulted in complete loss of activity (Fig. 3). However, we cannot exclude the possibility that this is due to instability of the *dl*120-140/fs protein, since the antibody reacts with sequences that are deleted in this construct.

From these results, we conclude that induction of PCNA-CAT expression is severely reduced by deletions throughout the 243R protein, indicating that multiple regions of the 12S product are needed to activate the promoter fully. On the basis of known functions of the 12S product and their domain associations, this suggests that more than one function is required for full induction of PCNA-CAT. Alternatively, it is possible that PCNA induction activity might depend on the overall structure of the native protein.

Relationship with cellular E1A-binding proteins. To assess the role of specific E1A functions in PCNA induction, we examined more subtle alterations in the protein that selectively impaired the binding of one or more cellular proteins (Table 1). Through these experiments, we hoped to distinguish effects of overall protein structure from a multifactorial induction mechanism.

The N-terminal region binds to the cellular protein p300 and mediates functions such as transformation (32, 69, 71, 78), transcriptional repression (32, 69), and induction of DNA synthesis (69). As discussed above, the N-terminal mutant, *dl*2-36, was significantly defective for PCNA transactivation (Fig. 3). This protein is defective for p300 binding (69) but not for the binding of any of the other known proteins that interact with E1A, suggesting that PCNA induction may involve p300. The requirement for p300 association was tested with two mutants, *dl*2-13 and *dl*60-85 (Fig. 1B), that are also specifically impaired with respect to p300 binding (78). Mutant *dl*2-13 was partially impaired for transactivation of PCNA-CAT, exhibiting about 50 to 60% of the activity of the wild-type protein. On the other hand, the activity of mutant *dl*60-85, which lacks residues in the

TABLE 1. Properties of E1A proteins

12S construct	Binding of cellular proteins[a]					PCNA activation[b]
	p300	Cyclin A	p105-RB	p130	p107	
12S.FS	−	−	−	−	−	−
12S.WT	+	+	+	+	+	+++
*dl*2-13	−	+	+	+	+	+
*dl*2-36	−	+	+	+	+	+/−
*dl*30-85	−	−	−	−	+	+/−
*dl*60-85	−	+	+	+	+	++
*dl*2-85	−	−	−	−	+	−
*dl*86-120	+	+/−	+	+	+	+
*dl*120-140	+	−	−	−	−	+/−
*pm*47	+	+[c]	−	−	+	++
*pm*124	+	+	−	+/−	+	+++
*dl*2-36/*pm*124	−	+[c]	−	−	+	+/−
*pm*47/*pm*124	+	−[c]	−	−	−	+/−

[a] Relative to binding by 12S.WT. +, binding observed at wild-type levels; +/−, weak binding; −, no binding.
[b] Relative to activation by 12S.WT. +++, 75 to 100%; ++, 50 to 75%; +, 25 to 50%; +/−, 10 to 25%; −, <10%.
[c] Binding characteristic as expected on the basis of interaction with p107.

C-terminal half of CR1, was only slightly impaired (Fig. 4A). Unless there are hitherto unrecognized differences among these mutants in their p300-binding capacities, the effects of these three mutants are not entirely consistent with a requirement for p300 in mediating the 12S response of the PCNA promoter (see Discussion). These results, therefore, suggest a requirement for the N-terminal region, particularly

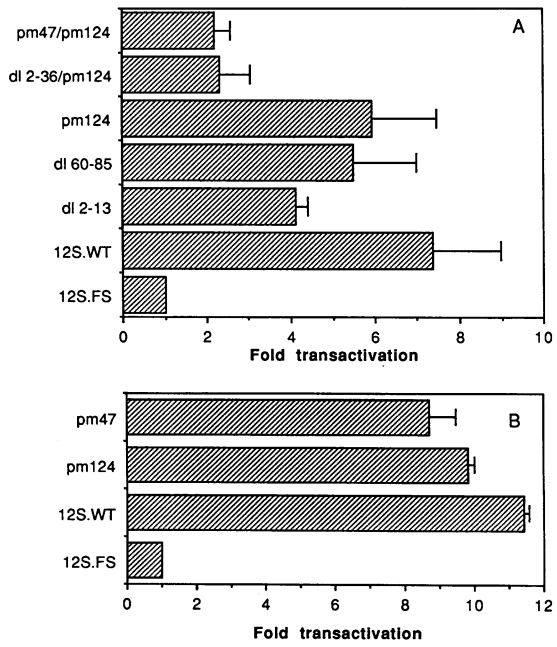


FIG. 4. The effects of small deletions and point mutations in 243R on transactivation of the PCNA promoter. (A) The designated 12S construct (indicated on the ordinate) was assayed for transactivation of the PCNA promoter as described in the legend to Fig. 3. The results are expressed as CAT/β-galactosidase activity ratios and represent the means of three independent transfections done in duplicate. (B) The same procedures were used, except that transfections contained 10 μg of the −87 to +62 PCNA-CAT reporter together with the indicated 12S construct. The results are means of single transfections performed in triplicate.

for sequences between amino acids 2 and 60, but are less definitive about the nature of the protein association responsible.

Recent work from a number of laboratories has indicated that the cellular anti-oncogene product, p105-RB, exerts its antiproliferative effects by binding to and presumably sequestering a variety of growth-responsive transcription factors such as E2F and c-*myc* (3, 4, 62, 63). The oncogene product from a number of DNA tumor viruses, including E1A of adenovirus, can bind p105-RB, releasing and thereby activating these growth-responsive transcription factors (14, 17, 29, 53). To test the possibility that p105-RB is involved in the transactivation of the PCNA promoter by the 12S product, we exploited a mutant with a point mutation in CR2, *pm*124 (Fig. 1B), which is defective for binding p105-RB and for transformation (45, 69). Mutant *pm*124 retained a PCNA induction activity that was similar to that of the wild-type construct (Fig. 4A). Furthermore, when combined with *dl*2-36 in *dl*2-36/*pm*124, the *pm*124 mutation did not abolish the residual activity of the *dl*2-36 mutant (Fig. 4A). These results indicate that p105-RB binding by E1A 12S does not play a role in the response of the PCNA promoter in this assay.

In addition to p105-RB, CR2 binds several other proteins that may play a transcriptional role (63) and serve other cell cycle regulatory functions (55). The reduced transactivation ability of the *dl*120-140 mutant (Fig. 3) indicated a requirement for CR2 in the induction of PCNA-CAT. We, therefore, investigated the effects of mutants with point mutations in CR1 and CR2 that are defective in binding the CR2-associated proteins. The double point mutant *pm*47/*pm*124, is defective for binding p105-RB, p107, p130, and p60-cyclin A (Table 1). Figure 4A shows the effect of this mutant on PCNA-CAT transactivation. It is only about 30% as active as the wild type, suggesting that one or more of the associations that are lost by this mutant protein are involved in PCNA transactivation. In order to define the defect more precisely, we compared the effect of the double point mutant with the effects of the mutants carrying one of the single mutations that constitute it. As described above, *pm*124, which is defective only for p105-RB binding, is similar to wild-type 12S in activation of the PCNA promoter. The effects of *pm*47, a mutant with a single point mutation in CR1, and *pm*124 are shown in Fig. 4B. The *pm*47 mutant is defective for binding p105-RB and p130 (76), an uncharacterized protein that interacts with E1A (22). Surprisingly, the ability of this mutant to induce PCNA was similar to that of the wild type and comparable to that of *pm*124 (Fig. 4B). Thus, the single point mutants have little or no effect on PCNA induction on their own but display reduced activity in combination, and the binding of p107 and that of cyclin A respond in a similar fashion (Table 1). These results indicate that, of the known E1A-binding proteins, cyclin A and p107 are potential candidates for mediating the response of the PCNA promoter to the 12S product.

From the results described above, it appears that full induction of PCNA-CAT expression in HeLa cells requires two regions of E1A: (i) amino acids 2 to 60, as indicated by the effects of mutants *dl*2-36, *dl*30-85, and *dl*60-85, and (ii) CR2, as shown by *dl*120-140 and *pm*47/*pm*124. The N-terminal region of E1A 12S represents a distinct activity and can cooperate with CR2 but is functionally independent of it (44, 69), suggesting that these two regions may represent sites for two independent binding events that are both required for PCNA induction. To test whether they might be able to complement one another in *trans*, the N-terminal mutant

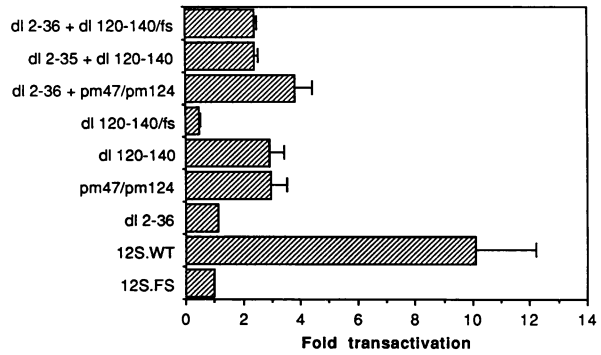


FIG. 5. Complementation assay for PCNA-CAT induction. The −87 to +62 PCNA-CAT reporter was cotransfected with one or more of the 12S constructs as indicated. The fold increase in CAT expression was normalized for β-galactosidase activity and represents the mean of two independent transfections done in duplicate.

*dl*2-36 was cotransfected with CR2 mutant *pm*47/*pm*124, *dl*120-140, or *dl*120-140/fs. As shown in Fig. 5, cotransfecting the N-terminal mutant with CR2 mutants did not substantially increase PCNA-CAT activity above the levels obtained with either of the mutants alone. This result lends no support to the complementation idea and is consistent with the existence of a single functional domain with regard to PCNA induction but does not prove it.

Response of the full-length PCNA promoter. The experiments described to this point were conducted with a truncated form of the PCNA promoter, −87 to +62 PCNA-CAT, because this construct produces a greater response to the activating properties of E1A 12S than constructs with longer upstream sequences (49). PCNA-CAT reporter constructs with longer upstream promoter sequences than that of the −87 to +62 PCNA-CAT display higher basal expression but respond less well to the 243R activation function (49). The reduced responses of the longer constructs might stem from upstream-site activities opposing the effects of 243R. To address this possibility, the −1265 to +62 PCNA-CAT construct was compared with the −87 to +62 PCNA-CAT construct for its response to the E1A 12S wild type and mutants of E1A 12S defective in transcriptional repression function (69). Figure 6 shows the responses of both PCNA promoters to the pCMV12S wild type and to mutants *dl*2-36, *pm*124, and *dl*2-36/*pm*124. Point mutant *pm*124 was similar to the wild type in activity, while the *dl*2-36 mutant and double mutant *dl*2-36/*pm*124 displayed little or no ability to induce PCNA-CAT expression from either promoter. Although the construct with a longer upstream genomic sequence was less responsive to E1A as expected, its relative response to mutant 12S constructs was similar to that of the −87 to +62 PCNA-CAT. Therefore, sequences upstream of −87 do not appear to confer a differential response to E1A in HeLa cells.

DISCUSSION

Recent studies of the transcriptional properties of the 12S product suggest that it modulates transcription via its interactions with a number of cellular proteins (4, 12, 15, 63) that regulate cell cycle and growth (18, 55) and are linked to the E1A 12S transforming function (78). It is likely that transcriptional regulation of cellular genes by E1A 12S is related to its role as a transforming protein. To ask whether the

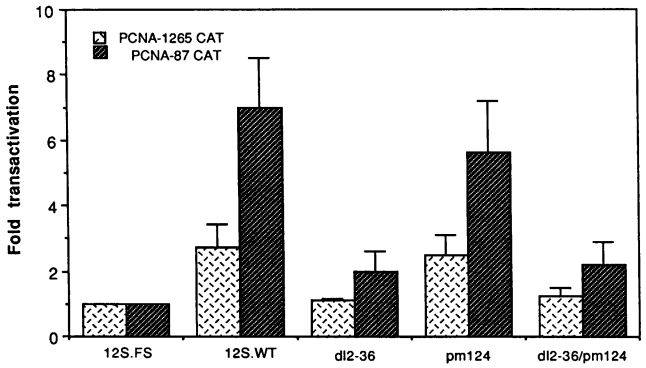


FIG. 6. Transactivation of the PCNA promoters by E1A 12S products. The −1265 to +62 PCNA-CAT and −87 to +62 PCNA-CAT constructs were assayed by cotransfection with pCMV12S or with the designated mutants thereof as indicated. Results are expressed as CAT/β-galactosidase activity ratios relative to the activity obtained in the presence of 12S.FS. The basal activities obtained for the −1265 to +62 and for the −87 to +62 PCNA-CAT constructs were about 120 and about 10 CAT units, respectively. Values are means of three independent transfections done in duplicate.

induction of the PCNA promoter by 12S can be attributed to any of the known properties of the 243R protein, we examined the domain requirements for transcriptional activation of the cellular PCNA gene by E1A 12S in HeLa cells. The results indicate that the determinants are distributed throughout the protein and that sequences in the nonconserved N terminus as well as in CR1 and CR2 and the second exon are involved. Table 1 summarizes the binding properties of the various E1A mutants used here as detected by coimmunoprecipitation (22, 76, 78). The efficiency of activation of the PCNA promoter by these mutants relative to that of the wild type is indicated to facilitate correlations of the functions of E1A with PCNA activation.

The N-terminal region of E1A is one of the domains required for transformation. While it is well recognized that the N terminus mediates the repression of enhancers and the growth-inducing activities of E1A (69), stimulation of gene expression via this region has not been carefully studied. The only known cellular protein that interacts with the N-terminal region is p300 (Fig. 1A and Table 1), a partially characterized protein whose biological function is not yet fully understood. This observation suggests that p300 may participate, directly or indirectly, in the induction of PCNA. An obstacle to this conclusion is the observation that mutants with deletions of 243R that are impaired in p300 binding showed different degrees of activity with respect to PCNA-CAT induction (Table 1). However, it is possible that the PCNA-CAT stimulation assay is sensitive to differences in p300 affinity that are not detectable in vitro. This interpretation is supported by the results of a study of the effects of E1A 12S mutants on the PCNA promoter in rodent cells. In this system, the full-length PCNA promoter is repressed by 243R (34, 46). Repression correlates with p300 binding, and the loss of functions other than p300 binding has no effect. Furthermore, for the mutants that are defective for p300 binding, the relative order of activity is the same as for PCNA activation in HeLa cells, i.e., *dl*60-85 > *dl*2-13 > *dl*2-36, consistent with a partial loss of function in vivo. Together, these findings argue that p300, or some other unidentified factor that binds to the N-terminal region of 243R, is involved in transactivation of the PCNA promoter. Recent studies have indicated that p300 has a sequence-specific DNA binding activity (59). Although this observation suggests a mechanism of action for p300 in mediating E1A effects, there is no homology in the −87 to +62 construct to the consensus DNA sequence for p300 binding (59). Prior work linked p300 binding by E1A to transcriptional repression, but the requirement of the N terminus in activation of PCNA raises the possibility that the same protein might mediate two opposite effects. PCNA is an essential replication factor, and the stimulation of DNA synthesis by E1A correlates with p300 binding (28, 69), so its involvement in PCNA induction would not be out of place.

Transactivation of the PCNA promoter by E1A 12S is mediated through an ATF site (49) and differs from that of E2F-responsive promoters in that it occurs in HeLa cells (2). The constitutive expression of the E7 protein of human papillomavirus in HeLa cells masks some of the effects of the 12S product, because the E7 protein contains regions of homology to CR1 and CR2 of E1A (54). Thus, activation of PCNA expression by E1A in HeLa cells may reflect the involvement of functions that are not shared with the E7 protein. Whether this is the case or not, cotransfection of pCMV12S transactivates the truncated (−87 to +62) PCNA-CAT construct in a variety of cell lines other than HeLa cells. These include mouse 3T3 cells, rat CREF cells, and human WI-38 fibroblasts (34). We chose HeLa cells for the present study, despite the overlapping functions of the endogenous human papillomavirus E7 protein, because the low basal expression and reduced response to transactivation by E1A 12S make induction difficult to quantify in other cells. Most importantly, this study benefits from the understanding of cellular E1A-binding proteins that has been developed with HeLa cells (22, 76, 78).

The requirement for CR2 and the second exon in PCNA-CAT transactivation suggests that additional interactions are involved in the 12S response. Since the *pm*124 and *pm*47 mutants of E1A 12S transactivate the PCNA promoter nearly as well as wild-type 12S (Table 1), we conclude that p105-RB binding is not required for PCNA-CAT activation in HeLa cells. Mutant *dl*86-120, which lacks residues within the nonconserved region between domains 1 and 2, was defective in transactivating the PCNA promoter. This deletion mutant retains all the binding properties of E1A, except that it appears to have reduced binding to cyclin A (22). As evidenced by the mutants that are defective in interactions mediated by CR2 (Table 1), the most likely intermediary in the response is p107-cyclin A. The involvement of exon 2 sequences in PCNA induction is most likely due to an inability to localize in the nucleus by *dl*120-140/fs, since exon 2 is required for nuclear localization of E1A (43). Production of a cellular growth factor has also been ascribed to exon 2 sequences (58), which also may account for the different activities of *dl*120-140 and *dl*120-140/fs. We have not further investigated a possible role of exon 2 sequences in PCNA transactivation, since these sequences are dispensable for transformation and our goal is to relate PCNA transactivation with E1A transforming functions.

The observation that mutants defective in either N-terminal or domain 2 function are impaired for induction of PCNA-CAT indicates that interactions involving both of these regions are required for full activity. The absence of complementation between the N terminus and CR2 in our assay does not appear to be consistent with two independent binding events. It is possible that two or more proteins involved in transactivation of the PCNA promoter by E1A

must be bound to the same E1A molecule, but the existence of an unidentified protein that binds to multiple sites on E1A 12S could also explain these data. A precise determination of the mechanisms involved must await further characterization of cellular complexes targeted by the 12S product.

Our findings agree well with analyses of PCNA induction in BRK cells infected with E1A mutants bearing adenovirus. Zerler et al. (81) showed that E1A 12S containing a deletion of CR2 or a mutant with a point mutation in CR2, *pm*124, can induce PCNA synthesis. In our experimental system, we observed the same effect for the *pm*124 mutant, although CR2 was required for full activation. Jelsma and colleagues (32) found that small deletions throughout the first exon did not eliminate PCNA induction by adenovirus and concluded that multiple regions of the protein are involved. The results of the present study, which specifically examines the effect of the 12S product on the PCNA promoter in the absence of other viral genes, also lead to the conclusion that there is functional overlap in regard to PCNA induction by E1A 12S. Some mutants of E1A 12S that were partially defective in the cotransfection assay were scored as positive in the viral infection assay, but this discrepancy probably merely reflects the more quantitative nature of the present data.

The pattern of regulation of PCNA by growth and oncogenic stimuli is representative of a large number of cellular proteins that respond in a similar fashion (20, 21). As an essential DNA replication factor (30), induction of PCNA expression is a prerequisite for cell growth. The existence of redundant mechanisms for the induction of PCNA by E1A bespeaks the importance of the task and probably serves to increase the variety of cell types in which E1A 12S can exert its mitogenic potential. The activation effect described here involves both the transformation domains of the 12S product. An understanding of the mechanisms underlying this response could provide valuable insights into the control of cellular proliferation and into the regulation of growth-responsive genes in particular.

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