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Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis

(adenovirus type 5/E1A mutants)

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ABSTRACT **Previous work has suggested that oncogenic transformation by the E1A gene products of adenovirus type 5 may be mediated through interactions with at least two cellular proteins, the 105-kDa product of the retinoblastoma growth suppressor gene (p105-Rb) and a 300-kDa protein (p300). By using viral mutants, we now show that the induction of cellular DNA synthesis in quiescent cells by E1A differs from transformation in that E1A products induce synthesis if they are able to bind to either p105-Rb or p300, and only mutant products that bind to neither are extremely defective. These results suggest that p105-Rb and p300 (or cellular proteins with similar E1A-binding properties) provide parallel means by which DNA synthesis can be regulated.**

The familiar explanation that cancer is due to uncontrolled cell growth has been given sharper focus recently by developments in the concept of cellular anti-oncogenes or growth suppressor genes, that is, genes whose products normally arrest cell growth. Unlimited cell growth is thought to occur if these genes are deleted or altered or if their products are inactivated. The main experimental basis for this concept has been recent findings on the retinoblastoma (*Rb*) gene, indicating that retinoblastomas arise when both alleles of this gene are lost or inactivated. This work, which essentially began with a study by Knudson (1), has been reviewed by Weinberg (2).

Further insights into the mechanisms of action of the *Rb* gene have been obtained through studies with the early region 1A (E1A) oncogene of human adenovirus type 5 (Ad5). Expression of this gene can immortalize cells, cause cells to proliferate, and, in collaboration with a second oncogene such as Ad5 E1B or activated *ras*, induce oncogenic transformation (3). Transformation seems to require specific interactions between E1A polypeptides and key cellular proteins. Several cellular proteins to which E1A proteins bind have been observed (4, 5). Two of these have molecular masses of 105 kDa and 300 kDa (p105 and p300, respectively). p105 has been identified as the product of the *Rb* gene (p105-Rb) by Whyte *et al.* (6), and this was subsequently confirmed by our group (7). It has further been found that E1A mutants whose proteins fail to bind to p105-Rb also fail to transform cells (7–10). It is widely believed that the formation of a complex with E1A products inactivates p105-Rb, thereby permitting the uninhibited growth associated with oncogenically transformed cells. However, this association is clearly not the only event required for E1A-mediated transformation as mutant E1A products that bind p105-Rb normally but fail to bind p300 are also defective for transformation (8–10). This suggests that p300 is involved in growth control as well.

The major products of the E1A region of Ad5 are proteins of 289 and 243 residues. They are produced from 13S and 12S mRNAs, respectively, and are identical except for an extra internal sequence of 46 amino acids in the larger protein. To learn more about the way these proteins function in transformation, we have constructed and studied the properties of a series of small in-frame deletion mutants spanning the whole of the coding sequence of the E1A gene (9, 11). We have also used these mutants to map the sites on E1A proteins involved in binding to p105-Rb, p300, and another cellular protein of 107 kDa (p107) (8).

It has been known for some time that E1A induces cellular DNA synthesis in a variety of growth-arrested cells (12–14). Studies on the phosphorylation of p105-Rb during the cell cycle (15–18) led to the suggestion that this protein may help to regulate the progress of cells from G_0/G_1 phase into S phase (16, 17). In an attempt to understand more clearly the role of both p105-Rb and p300 in this regulation, we have used our series of E1A mutants to examine the relationship between the induction of cellular DNA synthesis by E1A products and their ability to bind to p105-Rb and p300. The results suggest that binding of E1A proteins to either p105-Rb or p300 (or to other cellular proteins with similar binding properties) is sufficient to induce synthesis. These data provide further suggestive evidence that p105-Rb regulates entry into S phase but that p300 is an alternative control element for DNA synthesis.

MATERIALS AND METHODS

Cells and Viruses. The 293 cell line (19) was maintained in Eagle's modified minimal essential medium (MEM) supplemented with 10% (vol/vol) newborn calf serum. KB cells were maintained in α-modified MEM supplemented with 10% (vol/vol) fetal bovine serum. Primary baby rat kidney (BRK) cells were prepared from 4- to 6-day-old Wistar rats as described (9) and were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum.

The construction of E1A mutations dl1101–1109 and pm1131 and their transfer into an E1A plasmid pLE2/520, which expresses only the 12S E1A mRNA, have been described (8, 9, 11). pLE2/520 contains a deletion from Ad5 dl520 that removes the 5′ splice site for the 13S E1A mRNA (9, 20). E1A mutations dl1141–1143 were constructed and transferred into pLE2/520 in a similar manner. Mutant pLE2/520 plasmids containing two deletions in exon 1 were created by combining appropriate restriction fragments from plasmids containing single exon 1 deletions. Mutated E1A regions were transferred from pLE2/520 into the phenotypically wild-type (wt) virus Ad5 dl309, by using either the

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Abbreviations: Ad5, adenovirus type 5; BRK, baby rat kidney; p105-Rb, 105-kDa retinoblastoma protein; p300 and p107, cellular proteins of 300 kDa and 107 kDa, respectively; wt, wild type; pfu, plaque-forming unit(s); moi, multiplicity of infection.
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method of McGrory et al. (21) or the method of Stow (22), as described (9). After screening, each mutant virus was plaque-purified twice, amplified, and titrated on 293 cells.

Induction and Measurement of DNA Synthesis. The procedure for measuring DNA synthesis was that of Zerler et al. (14). BRK cells were plated on 60-mm dishes and infected 3 days later at a multiplicity of 10 plaque-forming units (pfu) per cell. At the time of infection or 24 or 48 hr after infection, 50 μCi of [^{3}H]thymidine (Amersham) in 5 ml of fresh medium was added. After labeling for 24 hr, the cells were harvested and resuspended in 0.3 M NaOH. DNA was precipitated with trichloroacetic acid and collected on Whatman glass-fiber filters, and radioactivity was measured in a scintillation counter.

To estimate the extent of viral DNA synthesis in BRK cells infected with 12S virus, duplicate sets of quiescent cells were infected either with the phenotypically wt Ad5 dl309 or with the 12S virus Ad5 dl520 and labeled for three 24-hr periods. Controls were provided by mock-infected cells or by cells infected with Ad5 dl312, which lacks essentially the whole E1A region. With one set of cells, incorporation of [^{3}H]-thymidine into total DNA was measured as before. From a second set of cells, viral DNA was extracted by the Hirt method (32), the samples were electrophoresed on 0.6% agarose gels, and the radioactivity in each of the viral DNA bands was measured by scintillation counting. Because of lysis, measurements on cells infected with dl309 could only be made over the first 24-hr labeling period.

Analysis of E1A and Associated Cellular Proteins. Proteins from virally infected KB cells were analyzed as described (8). KB cells were infected at a multiplicity of 70 pfu per cell and labeled with [^{35}S]methionine. To lysates of these cells was added either the E1A-specific monoclonal antibody M73 (Oncogene Science, Manhasset, NY) to precipitate E1A and associated cellular proteins or the Ad5 E1B-specific antibody 9C10 (Oncogene Science) as a control. Precipitates were analyzed by SDS/polyacrylamide gel electrophoresis, followed by fluorography.

RESULTS

Several previous studies have shown that E1A products are able to induce cellular DNA synthesis in quiescent primary BRK cells infected with adenovirus (14, 23–25). This is also the system we have chosen for examining the role of E1A protein binding in this process, as it enables us to relate results directly to our earlier work on the transformation of BRK cells by E1A mutants (9). BRK cells are semipermissive for Ad5, and cell killing was prevented by infecting with virus containing mutated E1A regions transferred into a dl520 background. dl520 expresses only the E1A 12S mRNA and its product, the smaller 243-residue protein (20), and, in the absence of the 289-residue protein, which is required to activate transcription of other early viral genes, viral replication is limited (14, 23). Throughout our work, DNA synthesis was measured by the incorporation of [^{3}H]thymidine over three 24-hr intervals, namely, 0–24, 24–48, and 48–72 hr after viral infection. Three measurements were made to discover whether the incorporation induced by each of our E1A mutants was rapid, delayed, or transient (cf., refs. 23 and 25). In no case did we detect any significant trend, and so we report below the incorporation over the total 72 hr after infection for each mutant as a percentage of that by dl520.

Estimation of Viral and Cellular DNA Synthesis in BRK Cells Infected with 12S Virus. To establish the extent to which viral DNA synthesis affected our results, quiescent BRK cells were infected with the phenotypically wt Ad5 dl309 or with dl520 and labeled with [^{3}H]thymidine. Uptake of radioactivity was then measured into total DNA and into viral DNA. Ad5 dl309 produced much more viral DNA than did dl520 (see also ref. 14). By comparing incorporation into total DNA and viral DNA for these two viruses (data not shown), it was possible to arrive at an upper limit for the fraction of the incorporation with dl520 due to viral DNA synthesis. Results from two experiments estimated this at 10% or less of total DNA synthesis and, therefore, of minor importance compared to the synthesis of cellular DNA.

Induction of DNA Synthesis by E1A Mutants Containing Single Deletions. A number of published studies with E1A mutants have attempted to define the regions of the 243-residue protein essential for the induction of DNA synthesis (14, 23, 24, 26, 27). The results suggested that the N-terminal 85 residues are required, although other evidence (25, 26) indicated that sequences at the C-terminal end of exon 1 (Fig. 1) are also necessary.

To investigate this question further, we examined a series of Ad5 mutant viruses, dl1101/520, dl1102/520, etc., in which single deletions in the coding region of E1A (Fig. 1) were combined with dl520. Initially, we tested dl1101/520 through dl1109/520, dl1141/520, dl1142/520, and pm1131/520 for their ability to stimulate incorporation of [^{3}H]thymidine into DNA in quiescent BRK cells. Except for pm1131/520, these mutants all contain a small deletion in exon 1 of E1A and include dl1101/520, dl1103/520, and dl1104/520, which should lack the principal binding sites for p300, and dl1107/520 and dl1108/520, which should lack those for p105-Rb and p107 (Fig. 1). pm1131/520 contains a termination mutation in exon 2 that deletes the C-terminal 71 residues of the E1A protein. Collectively, these mutants delete almost the entire exon 1 and a major portion of exon 2 (Fig. 1). Fig. 2 shows

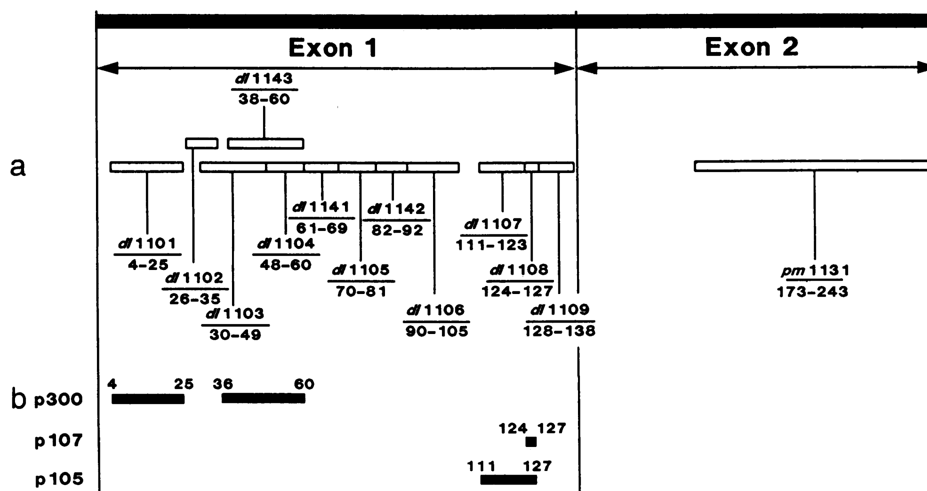

FIG. 1. Map of the Ad5 243-residue E1A protein, showing the deletions used in the present study, with the residues deleted (a) and the principal sites affecting binding to p105-Rb, p107, and p300 (b) from Egan et al. (8).

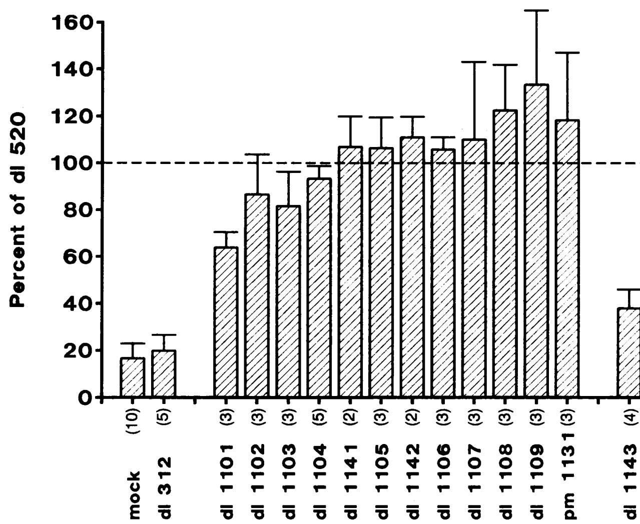

FIG. 2. Incorporation of [^{3}H]thymidine into DNA over a 72-hr period by BRK cells infected with 12S viruses containing single E1A deletions. For each mutant, the average incorporation for the number of experiments indicated in parentheses is shown as a percentage of the incorporation by the wt 12S control virus, dl520, in the same experiments. Error bars show SD.

the incorporation of [^{3}H]thymidine for each mutant over a 72-hr period after infection expressed as a percentage of that with dl520. All the mutants were able to induce DNA synthesis, although those with deletions in the N-terminal 60 residues were slightly less efficient than dl520. The most significant result, however, was that with none of these mutants was induction of DNA synthesis seriously impaired. Thus individually, none of the deletions in the 243-residue protein impeded the ability of this protein to induce DNA synthesis.

Smith and Ziff (24) found that small deletion-substitution mutants in the N-terminal region of the 243-residue protein also had no effect on induction of DNA synthesis in BRK cells. However, these authors (24) and Moran and Zerler (23) showed that, in these cells, viruses with deletions beyond residue 20 that were larger than 25 residues were defective. To confirm the adverse effect of a larger deletion on induction of DNA synthesis, we constructed dl1143/520 to delete residues 38–60 (Fig. 1). Induction with this mutant was markedly reduced, although not to the level in mock-infected cells or in cells infected with Ad5 dl312, a mutant that lacks essentially the whole E1A sequence (Fig. 2).

As part of a routine study of E1A mutants, we tested dl1143/520 for the ability of its E1A protein to bind to cellular proteins. This was done by infecting human KB cells, immunoprecipitating the E1A proteins, and analyzing the precipitates on denaturing gels (8). Fig. 3 shows that binding of p300 to the E1A protein from dl1143/520 occurred at greatly reduced levels. This was expected as we had found (8) that another mutant, dl1104, which contains a deletion in the same region, also gave reduced binding to p300 (see Fig. 1). However, dl1143/520 showed reduced binding to p105-Rb as well, but binding to another cellular protein, p107, was not noticeably affected (Fig. 3). [Whyte *et al.* (10) observed similar changes in binding with their mutant dl646N, which deleted residues 30–85.]

Induction of DNA Synthesis by E1A Mutants Containing Double Deletions in Exon 1. The results just described suggested that the reduced induction of DNA synthesis by dl1143/520 may have been related to a failure of its altered E1A protein to bind to both p105-Rb and p300. To test this hypothesis, we made a series of mutants containing two deletions in exon 1. For brevity, a double mutant combining, for example, dl1101 and dl1106 within the dl520 background was designated dl01/06/520. Among these double mutants, we combined a deletion such as dl1101, which destroys the ability of E1A proteins to bind to p300, with a deletion such as dl1107, which eliminates the p105-Rb binding site (see Fig. 1). As controls we constructed other double mutants in which the two exon 1 deletions should yield E1A products still capable of binding to p105-Rb, to p300, or to both. These mutants were tested for their ability to stimulate incorporation of [^{3}H]thymidine into DNA as before, and results obtained over a 72-hr period after infection for all the mutants are presented in Table 1. Each of these mutants was tested for its ability to bind to p105-Rb, p107, and p300 in studies such as those shown in Fig. 3. The results from the binding studies are summarized in Table 1.

It is clear that double mutants that were defective at inducing DNA synthesis—namely, dl01/07/520, dl01/08/520, and dl43/08/520—were those that failed to bind to both

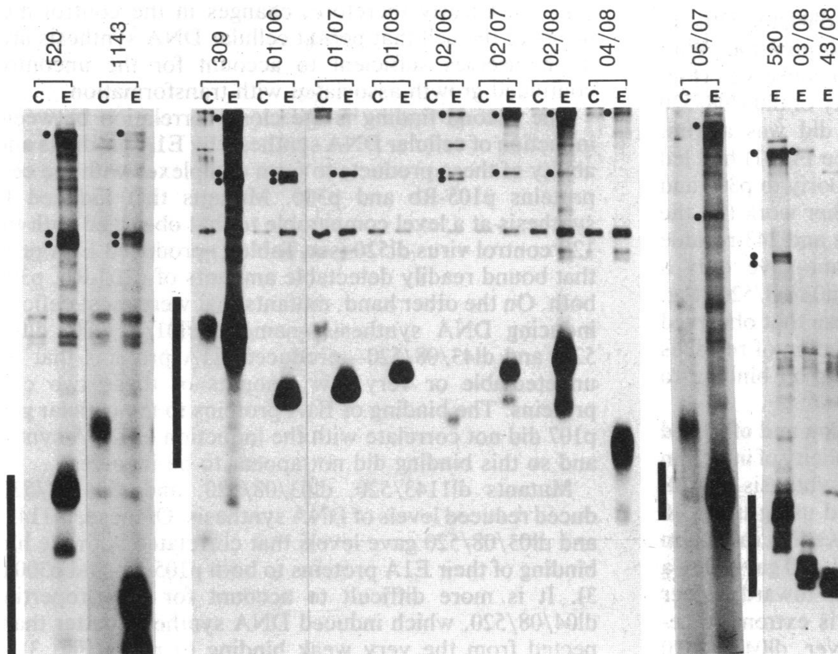

FIG. 3. Fluorographs of denaturing gels of extracts of KB cells infected with Ad5 viruses containing E1A deletions in a dl520 background, compared to infections with dl520 or with the phenotypically wt dl309. In many cases, extracts were immunoprecipitated with either an antiserum to Ad5 E1B proteins as control (lanes C) or an E1A-specific monoclonal antibody (lanes E). Bands due to p300, p107, and p105-Rb are indicated by dots, and those due to E1A proteins are indicated by bars. For some unknown reason, dl02/08/520 showed a faint band of p107. This does not normally occur when dl1108 is present: see dl01/08/520, dl04/08/520, and dl43/08/520 in neighboring lanes and dl1108 in Egan *et al.* (8).

Table 1. Ability of E1A 243-residue-related proteins from mutant Ad5 viruses to induce DNA synthesis in BRK cells compared to their ability to bind to p105-Rb, p107, and p300 in KB cells

Mutant	Binding			DNA synthesis, % of control	Experiments, no.
	p105-Rb	p107	p300		
Mock	−	−	−	15 ± 4	11
dl520	+	+	+	100	11
dl312	−	−	−	18 ± 5	4
dl01/07/520	−	+	−	20 ± 4	7
dl01/08/520	−	−	−	29 ± 6	3
dl43/08/520	−	−	−	14 ± 4	2
dl03/08/520	−	−	↓	48 ± 9	3
dl04/08/520	−	−	↓	41 ± 10	4
dl1143/520	↓	+	−	38 ± 8	4
dl01/06/520	+	+	−	69 ± 6	3
dl02/06/520	+	+	+	121 ± 15	3
dl02/07/520	−	+	+	80 ± 12	3
dl02/08/520	−	↓	+	89 ± 18	3
dl05/07/520	−	+	+	92 ± 17	3

DNA synthesis for each mutant is the incorporation of [^{3}H]-thymidine as a percentage of that for dl520 (control) (mean ± SEM). Mutants are grouped according to their ability to induce cellular DNA synthesis poorly (lines 4–6), at a reduced level (lines 7–9), or at a level comparable to that of dl520 (lines 10–14). Binding to p105-Rb, p107, and p300 is shown as similar to (+), or much reduced compared to that with dl520 (↓), or not detectable (−).

p105-Rb and p300. In contrast, the ability to bind to p107 did not correlate with induction of DNA synthesis. In no case was loss of induction due to failure to produce E1A proteins, as the defective mutants produced at least as much E1A protein as other mutants (Fig. 3). Lack of induction was also not due simply to the presence of two separate deletions in exon 1, as double mutants dl02/06/520, dl02/07/520, dl02/08/520, and dl05/07/520 stimulated DNA synthesis efficiently (Table 1) and dl01/06/520 stimulated DNA synthesis to the same level as dl1101/520 alone (Fig. 2).

From these results, it appeared, therefore, that the ability of a mutant to induce DNA synthesis correlated with its ability to bind to p105-Rb, to p300, or to both and that it was only mutants that bound to neither of these proteins that were markedly defective at inducing DNA synthesis.

Two double mutants, dl03/08/520 and dl04/08/520, induced intermediate levels of thymidine incorporation. With dl04/08/520, only weak binding to p300 was observed (Fig. 3); we discuss this mutant below. The ability of dl03/08/520 to induce DNA synthesis at the level it did was at first surprising, as results with dl1103 (ref. 8; see Fig. 1) had led us to believe that this mutant would bind poorly to p300 and not at all to p105-Rb. However, in this earlier work (8), the mutant used produced both the 289-residue and 243-residue E1A proteins, whereas the present mutant gave only a 243-residue-related product. In fact, with dl03/08/520 (Fig. 3), binding to p300 was somewhat better than that observed with dl1103 (8). These data suggest that deletion of residues 30–49 may have somewhat different effects on binding to p300 by the 289- and 243-residue proteins.

Effects of Different Multiplicities of Infection and of Mixed Infections. To investigate the effect of multiplicity of infection (moi) on the level of induction of DNA synthesis, dl520, dl01/07/520, and dl04/08/520 were assayed using up to 40 pfu per cell (Fig. 4). The plot for dl520 shows that induction was maximal at moi values above 5. dl01/07/520 gave only a slight increase in thymidine incorporation toward higher values of moi, indicating that this mutant is extremely defective at inducing DNA synthesis. However, dl04/08/520

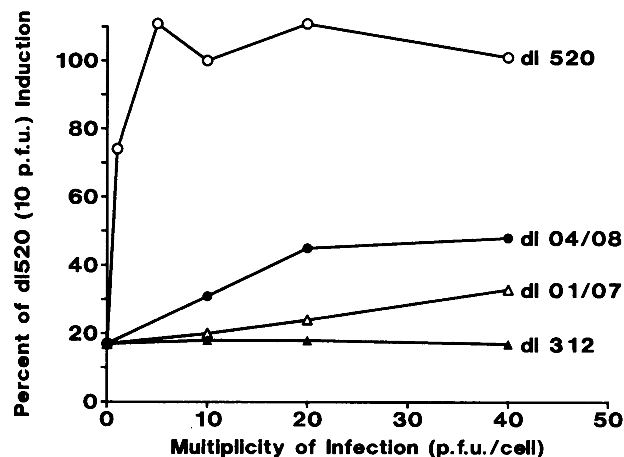

FIG. 4. Incorporation of [^{3}H]thymidine into DNA by E1A mutants in a dl520 background as a function of moi.

showed a moderate increase in induction up to 20 pfu per cell. This mutant is discussed in the next section.

If the induction of cellular DNA synthesis is a consequence of the E1A protein binding to cellular proteins such as p105-Rb and p300, then, in a mixed infection, the E1A product of a mutant such as dl02/08/520, which induces near wt levels of incorporation and binds p300 well, should predominate over the product of a mutant such as dl01/07/520, which is defective for induction and binds undetectable amounts of p105-Rb and p300. This was tested in an experiment in which BRK cells were infected with these two mutants. The incorporations obtained, as percentages of the level with dl520, were as follows: dl312, 25%; dl01/07/520 alone, 30%; dl02/08/520 alone, 110%; dl01/07/520 plus dl02/08/520, 120%. Thus the effect of dl02/08/520 predominated over that of dl01/07/520.

DISCUSSION

There are two significant findings in the results presented here. First, as others have found (14, 24), there exist E1A mutants such as dl1101, dl1103, dl1107, and dl1108 that in BRK cells are defective for transformation (9) but, nevertheless, induce significant levels of cellular DNA synthesis (Fig. 2). Clearly therefore, changes in the control mechanisms of the cell that permit cellular DNA synthesis are not in themselves sufficient to account for the uncontrolled continued growth associated with transformation.

The second finding is the close correlation between the induction of cellular DNA synthesis by E1A products and the ability of these products to form complexes with the cellular proteins p105-Rb and p300. Mutants that induced DNA synthesis at a level comparable to that observed with the wt 12S control virus dl520 (see Table 1) produced E1A proteins that bound readily detectable amounts of p105-Rb, p300, or both. On the other hand, mutants that were most deficient at inducing DNA synthesis—namely, dl01/07/520, dl01/08/520, and dl43/08/520—produced E1A proteins that bound undetectable or very low amounts of these two cellular proteins. The binding of E1A proteins to the cellular protein p107 did not correlate with the induction of DNA synthesis, and so this binding did not appear to be involved.

Mutants dl1143/520, dl03/08/520, and dl04/08/520 induced reduced levels of DNA synthesis. Of these, dl1143/520 and dl03/08/520 gave levels that correlated with the limited binding of their E1A proteins to both p105-Rb and p300 (Fig. 3). It is more difficult to account for the properties of dl04/08/520, which induced DNA synthesis better than expected from the very weak binding to p300 (Fig. 3). One

possibility is that the BRK equivalent to p300 binds more efficiently to this E1A product than does human p300. Alternatively, induction of DNA synthesis may be due to another, unidentified cellular protein with binding properties similar but not identical to those of p300 (see below).

A connection between the induction of DNA synthesis and binding to p105-Rb and p300 readily explains our results with double infections and with different values of moi. If binding of the cellular proteins to E1A products is responsible for inducing DNA synthesis, then, in an infection with two mutants, we would expect a mutant whose protein binds to predominate over one whose protein does not bind. This is what we found. In experiments on the induction of DNA synthesis at increasing values of moi (Fig. 4), induction by dl520 was not critically dependent on moi values above 5 pfu per cell. This suggests that if binding to p105-Rb and p300 is required for induction, the amount of E1A protein produced at these moi values is not limiting. However, if a mutant E1A protein were sufficiently weak at binding, then the amount of it produced in the cell could become limiting, and induction might then increase as the value of moi increased. This appeared to be the case with dl04/08/520, the E1A protein of which binds extremely weakly to p300.

Thus our results are consistent with the hypothesis that the binding of the E1A 243-residue product to p105-Rb and p300 is responsible for the induction of cellular DNA synthesis and that binding to either of these proteins is sufficient for this induction.

However, the evidence for this hypothesis is not conclusive. The present results suggest that binding of at least two cellular proteins to different sites on the 243-residue polypeptide, toward the N- and C-terminal ends of exon 1, respectively, is involved in the induction, but it is not certain that p105-Rb and p300 are these proteins. A variety of other cellular proteins besides p105-Rb, p107, and p300 can bind to the 243-residue E1A product (4, 5, 28), and any that bind to the same sites as p105-Rb and p300 could be important for induction. So far, we have not identified other cellular proteins with such binding properties.

How might E1A products affect regulatory processes in the cell? In the induction of DNA synthesis, one or both of the two cellular proteins could be inducers that are activated when the E1A product binds. Alternatively, one or both could be suppressors that are inactivated by binding the E1A product. If both are inducers, our data indicate that it is sufficient for the E1A protein to interact with either to induce synthesis. If p105-Rb is one of the proteins, it is likely to act as a suppressor. From studies on the phosphorylation of p105-Rb during the cell cycle (15–18), it has been proposed that this protein may help to block passage of cells from G_0/G_1 into S phase (16, 17) and that a viral protein like E1A may remove this block by binding to p105-Rb and inactivating it as a suppressor (16). Should one of the proteins be a suppressor and the other an inducer, induction by mutants, such as dl1101/520 and dl1107/520, requires that activation of the inducer and inactivation of the suppressor can each on their own lead to DNA synthesis. Finally, if both cellular proteins are suppressors, they must act cooperatively rather than in parallel, as these same mutants show that inactivation of either suppressor by binding to the E1A product is sufficient for DNA synthesis to occur.

Were p105-Rb and p300 to be the cellular proteins affected by E1A in the induction of cellular DNA synthesis and in cell transformation, several other questions follow. For example, it has been reported that the *Rb* gene is expressed in a wide variety of tissues (29). Is p300 equally ubiquitous? We would expect the induction of cellular DNA synthesis to be intimately associated with cell transformation. What is the relationship? Earlier work (8, 9) suggested that transformation required E1A proteins to bind to p105-Rb and p300. The evidence for this was that mutants such as dl1101, which failed to bind to p300, and dl1107, which failed to bind to p105-Rb, were defective for transformation in combination with *ras*. Thus to permit uncontrolled growth associated with transformation, the E1A protein must bind to p105-Rb and p300 whereas, to induce DNA synthesis, it must bind to either one. This would suggest that the pathways by which p105-Rb and p300 participate in the control of cell growth are more complex than those that control the onset of DNA synthesis and that in the latter case, the two proteins provide alternative means by which DNA synthesis can be induced. It is already known that sequences in exon 1 of E1A also affect mitosis and passage of cells through the cell cycle (23, 30, 31). Our E1A mutants can be used to examine the relationship of these processes to the binding of p105-Rb and p300 and to explore some of the other questions raised here.

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