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Interactions between Cell Growth-Regulating Domains in the Products of the Adenovirus E1A Oncogene

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Among the various biological activities expressed by the products of the adenovirus E1A gene are the abilities to induce cellular DNA synthesis and proliferation in quiescent primary baby rat kidney cells. The functional sites for these activities lie principally within two regions of the E1A proteins: an N-terminal region and a small second region of approximately 20 amino acids further downstream. To study the biological functions of the first domain, we constructed an in-frame deletion of amino acid positions 23 through 107 of the E1A products. This deletion did not impede the ability of the E1A products to transactivate the adenovirus early region 3 promoter in a transient-expression assay in HeLa cells. The ability to induce DNA synthesis in quiescent baby rat kidney cells was, however, lost in the absence of these sequences. Deletion of the small second region induced a form of S phase in which DNA synthesis occurred in the apparent absence of controls required for the cessation of DNA synthesis and progression through the remainder of the cell cycle. These cells did not appear to accumulate in or before G2, and many appeared to have a DNA content greater than that in G2. The functions of both domains are required for production of transformed foci in a *ras* cooperation assay. Focus formation occurred, however, even when the two domains were introduced on two separate plasmids. This complementation effect appeared to require expression of both of the mutant proteins and did not appear to result merely from recombination at the DNA level.

Several biological activities have been attributed to the adenovirus type 2 (Ad2) early region 1A (E1A) gene. This gene is required for transcriptional transactivation of the remainder of the adenovirus early genes during productive infection in permissive human cells (2, 25, 33, 41, 57). Expression of the E1A gene can also repress the effect of certain transcriptional enhancers (4, 22, 23, 55, 56), and introduction of the E1A gene into undifferentiated F9 cells has been shown recently to induce morphological differentiation (36). In addition, the products of the E1A region are sufficient to induce cellular DNA synthesis in quiescent cells (27, 49) and to establish stable cell lines from primary rodent cells (24, 44). The E1A products can also act in concert with the adenovirus E1B region (6, 13, 17, 26, 48, 54) or with certain other oncogenes, such as the *ras* gene product (12, 44), to transform various rodent cells fully. We are exploring the relationship between these functions and the structure of the E1A proteins to probe the mechanisms underlying control of gene expression during cell growth.

Comparison of the predicted amino acid sequences of the E1A regions of several adenovirus subgroups shows that the E1A regions are composed of three highly conserved domains alternating with sequences of relatively low homology (28, 52). Two major spliced products, the 12S and 13S mRNAs, are formed from the E1A region early in infection (3, 7, 30, 42). These products are predicted to encode proteins 243 and 289 amino acids long, respectively, distinguished only by the presence of 46 amino acids at positions 140 through 185, which are unique to the 13S product. Two of the three highly conserved domains occur in both the 12S and 13S products, while the third consists essentially of the 13S unique region (see Fig. 1).

Mutational analysis has begun to correlate some of the biological activities of the E1A products with specific conserved domains. The presence of a functional conserved domain 3 correlates in most assays with the ability of the E1A gene products to accelerate transactivation of the other virus early genes (5, 14, 35, 37, 38, 51) and some cellular genes (59, 61), although this domain does not appear to be essential for efficient transactivation in a protein microinjection assay (11). This domain is dispensable for the induction of DNA synthesis (27, 49) and immortalization of primary cells (19, 37, 61). The nonconserved regions appear to be dispensable for both of these functions, suggesting that the active sites for immortalization localize to domain 1 and 2 (reviewed in reference 39). Single point mutations within conserved domain 2 abolish the activity which induces proliferation of primary epithelial baby rat kidney (BRK) cells (34, 38, 47), but various deletions, together encompassing all of the sequences after amino acid position 86 (including all of conserved domain 2) remain competent to induce a substantial level of DNA synthesis in these cells, suggesting that this activity is a function of the first conserved domain (62). No direct requirement for this region of E1A in the induction of DNA synthesis was demonstrated in these experiments, however.

To study the biological functions of domain 1, we constructed an in-frame deletion of amino acid positions 23 through 107 of the E1A region and report here that this mutation abolished the ability of adenovirus to induce DNA synthesis, as well as cell proliferation in infected quiescent cells. Furthermore, the suggestion that the E1A gene encodes more than one function required for the induction of proliferation in BRK cells is supported by the demonstration that separate constructs that express only domain 1 or 2 could complement one another in a *ras* cooperation assay for formation of transformed foci. The relative independence of the several gene-regulating domains in the E1A products is also supported by the demonstration that the domain 1

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deletion mutant retained substantial ability to activate an adenovirus early promoter in a transient-expression assay.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of 293 and HeLa cell lines were maintained as described previously (38). All viruses were propagated on 293 monolayers, except for Ad5dl309, which was propagated on HeLa cell monolayers. Ad5dl309 and Ad5dl312 (26) were obtained from T. Shenk. The construction of the cDNA viruses and mutant viruses other than E1A-PSdl has been described (40). Primary BRK cells were prepared by collagenase-dispase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) treatment of kidneys from 6-day-old Fisher rats. Cells were infected 2 to 3 days after plating as described previously (38).

Construction of the PSdl deletion. To obtain an E1A construct with unique *Pvu*II and *Sty*I sites, the *Hin*dIII-to-*Bam*HI fragment of pMT-E1A (61) was transferred to a variant of pAT153 in which the *Sty*I site had been destroyed. After digestion of this plasmid with *Pvu*II and *Sty*I, the single-stranded overhang of the *Sty*I end was cut back with mung bean nuclease as recommended by the supplier (New England BioLabs, Inc., Beverly, Mass.). The resulting blunt ends were ligated, and constructs which had lost the *Pvu*II-to-*Sty*I sequences of E1A were isolated and identified by restriction digest analysis. The mutant sequence was verified by dideoxy sequencing (46). Base pairs 625 through 879 were removed to delete 85 amino acids from positions 23 to 107. The *Bst*XI fragment of the deletion mutant plasmid was transferred to an otherwise complete E1A plasmid to make pE1A-PSdl-pUC118, which contains adenovirus sequences from approximately base pair 1 to base pair 1830 (the *Pst*I site). The 12S-PSdl variant was made in a similar way, by transfer of the *Bst*XI fragment into a 12S-WT plasmid in pUC18. Viruses were constructed from the mutant plasmids as described previously (38).

Transfection assays. Transfections were carried out by a modified calcium phosphate coprecipitation technique (18, 58), as described previously (40).

For analysis of transient expression of chloramphenicol acetyltransferase (CAT) activity, 6-cm-diameter HeLa cell monolayer cultures were transfected with 5 μg of E3-*cat* plasmid DNA (pKCAT23; 57) plus 5 μg of appropriate E1A plasmid DNA per plate. Cells were harvested 36 to 48 h posttransfection and assayed for CAT activity (16). The extent of conversion of chloramphenicol to its acetylated forms, separated by thin-layer chromatography, was quantitated by analysis in an AMBIS beta-scanning system (Automated Microbiology Systems, Inc., San Diego, Calif.).

For the focus formation assay, 6-cm-diameter monolayer cultures of primary BRK cells were transfected with 1 μg of T24 Ha-*ras* (10, 15) plasmid DNA plus 2 μg of total E1A plasmid DNA per plate. The numbers reported (see Table 2) represent the total number of foci on 10 to 20 plates. The number of plates counted for each mutant sample was constant in a single experiment.

Immunoprecipitation. [^{35}S]methionine-labeled cell lysates were immunoprecipitated with anti-E1A-*trp*E fusion protein mouse monoclonal antibody, series 73, as described previously (62).

Virus growth functions. Monolayer cultures of HeLa cells were infected at a multiplicity of 1 and incubated at 37°C for the times indicated. Unlabeled lysates were titrated on 293 cells as described previously (38).

Analysis of DNA synthesis. Infected BRK cells were labeled with [^{3}H]thymidine (ICN Pharmaceuticals, Inc., Irvine, Calif.) and assayed as described previously (62). The standard deviation in the experiments described (see Fig. 4) was 23%.

Emulsion autoradiography. Monolayer cultures of primary BRK cells prepared as described above were infected at a multiplicity of 10 PFU per cell. Cells were labeled at the time of infection with [^{3}H]thymidine (ICN) (10 μCi/2 ml of medium on a 35-mm-diameter plate). At 72 h postinfection, cells were fixed in 3.7% Formalin in phosphate-buffered saline, covered with liquid autoradiographic emulsion (Kodak NTB-2), and exposed for 4 days, after which the emulsion was developed and the cells were photographed.

Flow cytometry. BRK cells were infected at a multiplicity of 10 PFU per cell, harvested, and fixed overnight in ethanol at the postinfection times indicated. The cells were rehydrated in phosphate-buffered saline, treated with RNase, and stained with propidium iodide. Immediately before flow cytometry, each cell sample was sonicated lightly to break up possible cell clumps and filtered through a 0.36 μm (aperture size) mesh. Fluorescence intensities were determined by quantitative flow cytometry with an Epics C system (Coulter Electronics, Inc., Hialeah, Fla.). A total of 10,000 cells was analyzed from each sample. The first peak of fluorescence intensity indicates the number of cells in G1. Cells showing double this amount of fluorescence were considered to have reached G2 or mitosis (M), while an intermediate level of fluorescence between the G1 and G2-M peaks indicates cells in the S phase. Cells showing S or G2-M levels were considered to be cycling. Fluorescence greater than that at the G2-M level suggests the occurrence of polyploidy. Fluorescence that occurred before the G1 peak was presumed to be cellular debris and was omitted from Table 1; this component became more significant with increasing time postinfection.

RESULTS

Construction of the PSdl deletion mutation. Our previous experiments analyzing mutants which in combination remove all sequences after amino acid position 86 (see Fig. 1) have provided indirect evidence that the N-terminal 85 amino acids of the E1A region are sufficient for induction of DNA synthesis in quiescent primary BRK cells (62). The N-terminal 85 amino acids encode only the first of the three conserved amino acid domains, which extends approximately from position 40 to position 80. To learn whether sequences in this region are required for induction of DNA synthesis in quiescent cells, a restriction fragment extending from the *Pvu*II site at base pair 622 in the E1A gene to the *Sty*I site at base pair 875 was removed to create the plasmid pE1A-PSdl. Removal of these nucleotide sequences is predicted to result in an in-frame deletion of 85 amino acids from position 23 to position 107 (Fig. 1). This mutation was rebuilt into genomic and 12S cDNA viruses which were propagated on 293 cells, an Ad5-transformed human cell line that expresses the early region 1 proteins constitutively and can therefore complement E1A defects (17).

Virus early gene transactivation and growth functions. The ability of pE1A-PSdl to activate expression from a virus early promoter was tested in HeLa cells in a transient-expression assay in which expression of the bacterial *cat* gene is dependent on E1A-induced activation of the adenovirus E3 promoter (57). E3-*cat* was activated as well by pE1A-PSdl as by the wild-type E1A plasmid (Fig. 2), indicating that the entire conserved domain 1 is dispensable for this activity. An E1A 12S cDNA plasmid which lacks

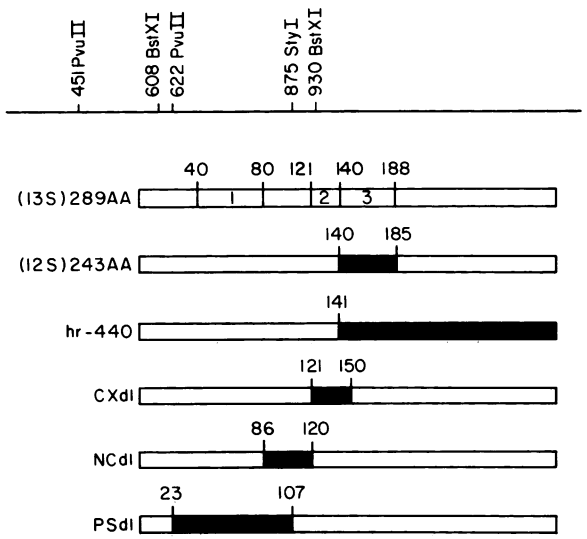

FIG. 1. Schematic representation of the amino acid structure of E1A wild-type and mutant variants. The nucleotide positions of selected restriction sites in exon 1 of the E1A gene are represented on the top line. The E1A proteins (open bars) contain three domains of highly conserved amino acid sequences, alternating with nonconserved regions. Conserved domains 1 and 2 occur in both of the major E1A splice products, whereas domain 3 is unique to the 13S product. Deleted sequences are indicated by blackened regions. The amino acid position numbers above the bars refer to the positions as they would occur in the 13S product.

domain 3 as a result of differential splicing showed no activity in this assay.

The ability of E1A mutants to activate virus functions required for productive growth can be assayed with greater sensitivity during virus infection when the multiplicity of infection can be kept low. HeLa cells, infected at a multiplicity of 1 PFU per cell, were harvested at 24-h intervals for up to 4 days after infection, and the relative virus yields (Fig. 3) were determined by titration on 293 cells. (Virus yield did not increase in any of the infections after 4 days). The rate of production of E1A-PSdl virus particles was slower than that of wild-type Ad5dl309 virus but greater than that of the 12S cDNA virus. These results indicate that amino acids between positions 23 and 107 are less important than domain 3 but are not completely dispensable for the E1A function required for productive growth in proliferating HeLa cells.

Induction of DNA synthesis. Infection of primary BRK cells with the E1A-PSdl or 12S-PSdl virus failed to induce observable cell proliferation when proliferating cells were visualized by vital-cell staining (data not shown). To determine whether this proliferation defect includes a defect in the ability to induce DNA synthesis, BRK cells were infected with a series of nonlytic viruses that carry mutations in the E1A region and labeled for 24-h periods with [^{3}H]thymidine. The structures of the mutant E1A regions are shown in Fig. 1. DNA synthesis was measured as the incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable counts. The results (Fig. 4) indicated that [^{3}H]thymidine incorporation was low in uninfected cells or in cells infected with the near-total E1A region deletion mutant control virus Ad5dl312 (25). As reported previously (62), a high level of DNA synthesis is induced in cells infected with the 12S-WT cDNA virus or with each of a series of deletion mutant viruses, E1A-hr440, E1A-CXdl,

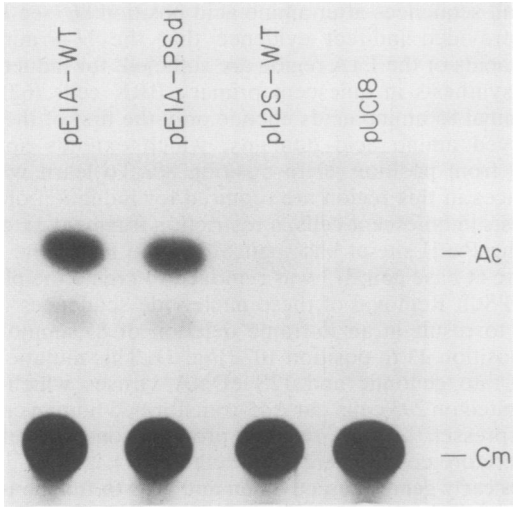

FIG. 2. Transactivation of an adenovirus E3-promoted *cat* gene. HeLa cells were cotransfected with pKCAT23 (57) and the E1A mutant variants indicated. Chloramphenicol acetyltransferase activity, measured after cotransfection with the E1A mutant plasmids relative to activity after cotransfection with pE1A-WT and averaged for three independent experiments, was 1.1 for pE1A-PSdl and <0.1 for p12S-WT. The positions of chloramphenicol (Cm) and its major acetylated form (Ac) are indicated.

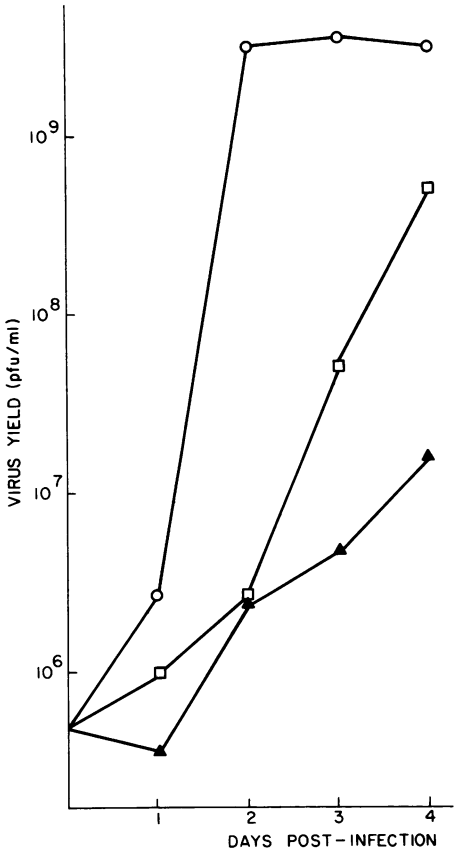

FIG. 3. Growth curves of E1A mutant viruses. HeLa cells were infected with dl309 (○), E1A-PSdl (□), or 12S-WT (▲) virus at a multiplicity of 1 PFU per cell and harvested at the indicated times postinfection. Virus yields were titrated on 293 cells.

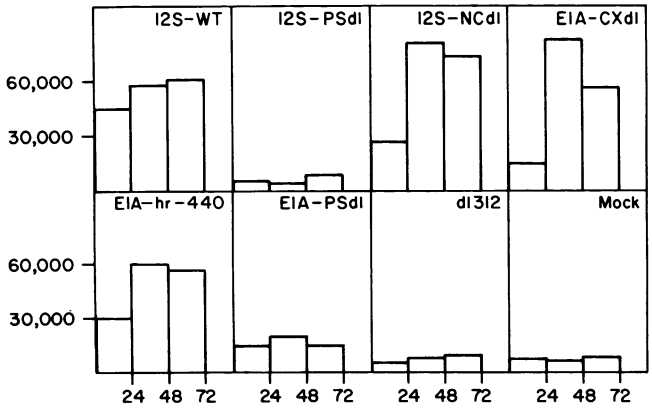

FIG. 4. DNA synthesis in infected primary BRK cells. Incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable material was measured in primary BRK cells infected at a multiplicity of 10 PFU per cell and labeled for 24-h intervals ending at 24, 48, or 72 h postinfection. The numbers of the left indicate trichloroacetic acid-precipitable counts.

and 12S-NCdl, which in combination lack all but the N-terminal 85 amino acids. Although the second conserved domain is required for induction of proliferation, total deletion of this domain in the E1A-CXdl virus delays, but does not abolish, induction of DNA synthesis. Infection with the E1A-PSdl virus, however, barely induces DNA synthesis above the level that occurs in uninfected cells.

Although the E1A-PSdl mutant supported some productive virus growth in proliferating HeLa cells, this virus had a limited cytopathic effect within 72 h after infection of quiescent BRK cells. Nevertheless, to rule out any possible cytopathic effect as the reason for the failure to induce DNA synthesis, the experiment was also performed with a 12S-PSdl virus. Like the E1A-PSdl virus, the 12S-PSdl virus induced little DNA synthesis above the level found in uninfected cells. The defect in induction of DNA synthesis is not likely to be due to instability of the PSdl mutant proteins, since these proteins, like previously constructed in-frame deletion mutations of the E1A gene, were translated in BRK cells with an efficiency similar to that of their wild-type counterparts (see Fig. 7). Western immunoblots and indirect immunofluorescence assays in HeLa cells showed that the protein levels of the E1A-PSdl products were similar to wild-type protein levels and that the E1A-PSdl products, like their wild-type counterparts, are localized in the nucleus (data not shown).

Autoradiography. The ability of domain 1 or 2 deletion mutants to induce DNA synthesis in primary BRK cells was also assayed by autoradiography after exposure of infected cells to [^{3}H]thymidine. In this type of assay, the number of cells responding is determined, rather than the counts incorporated into the culture as a whole. The results (Fig. 5) indicated that few uninfected primary BRK cells incorporated detectable levels of [^{3}H]thymidine. Approximately 70% of the cells infected with the 12S-WT cDNA virus incorporated [^{3}H]thymidine, detected as black cell nuclei in the autoradiogram. The domain 1 deletion mutant virus E1A-PSdl failed to induce [^{3}H]thymidine incorporation above the level seen in mock-infected cells. The domain 2 deletion virus E1A-CXdl induced [^{3}H]thymidine incorporation into approximately half as many cell nuclei as did the 12S-WT virus, consistent with the results shown in Fig. 4 and Table 1 (see below).

The ability of the E1A-CXdl virus to induce [^{3}H]thymidine incorporation into half as many cells as those stimulated by the 12S-WT virus contrasts with the 10-fold lower mitotic index induced by the E1A-CXdl virus than that induced by infection with the 12S-WT virus (62). These effects were examined further in the following experiments.

Cell cycle effects. Expression of the first conserved E1A domain appears to be sufficient to induce DNA synthesis but not cell division (62). To determine the effect of this activity on the cell cycle distribution of BRK cells, infected cells were fixed at 24-h intervals after infection, stained with propidium iodide, and analyzed for DNA content in a flow cytometer. The results (Table 1 and Fig. 6) showed that, by 48 h after infection, most of the cells infected with the 12S-WT virus were cycling, while most of the uninfected cells or cells infected with the domain 1 deletion mutant E1A-PSdl remained in G1. The effect of infection with the E1A-CXdl mutant, which expressed domain 1 and induced DNA synthesis but not proliferation, was unusual. Whereas infection with this mutant reduced the number of cells in G1 in a manner consistent with the results shown in Fig. 4 and 5, the stimulated cells did not appear to accumulate in or before G2-M. Rather, an aberrantly large number of cells appeared to accumulate a DNA content greater than that in G2-M, suggestive of polyploidy. The fluorescence intensity of cells infected with the E1A-CXdl virus reached as high as four to five times the G1 level and was essentially evenly distributed after the G1 peak, indicating no tendency for the cells to come to rest at any particular point after the stimulation of DNA synthesis. These results suggest that a domain 2 function is involved in controlling the normal cessation of DNA synthesis before progression through the remainder of the cell cycle.

Cooperation with the *ras* oncogene. Conserved domain 2 of the E1A gene is not required for induction of DNA synthesis in infected primary BRK cells but is required for the ability of the E1A gene to cooperate with the *ras* oncogene to transform primary BRK cells (31, 34, 40, 47). To determine whether the E1A function lost with the PS deletion is also required for *ras* complementation, focus formation was assayed after cotransfection of primary BRK cells with the Ha-*ras* gene and the mutant plasmid pE1A-PSdl. The results

TABLE 1. Cell cycle effects of E1A mutants

Time (h) and virus	% of cells in each phase[a]		
	G1	S + G2-M	>G2-M
24			
12S-WT	60.2	36.9	1.1
E1A-PSdl	74.5	20.9	1.4
E1A-CXdl	78.4	15.4	1.6
Mock	76.1	18.5	1.4
48			
12S-WT	30.0	64.4	5.6
E1A-PSdl	69.8	20.5	3.6
E1A-CXdl	51.8	18.3	28.7
Mock	74.6	17.3	1.3
72			
12S-WT	36.4	57.6	4.2
E1A-PSdl	62.8	19.2	2.7
E1A-CXdl	53.7	19.1	10.4
Mock	76.2	13.0	1.2

[a] Experimental details are described in Materials and Methods.

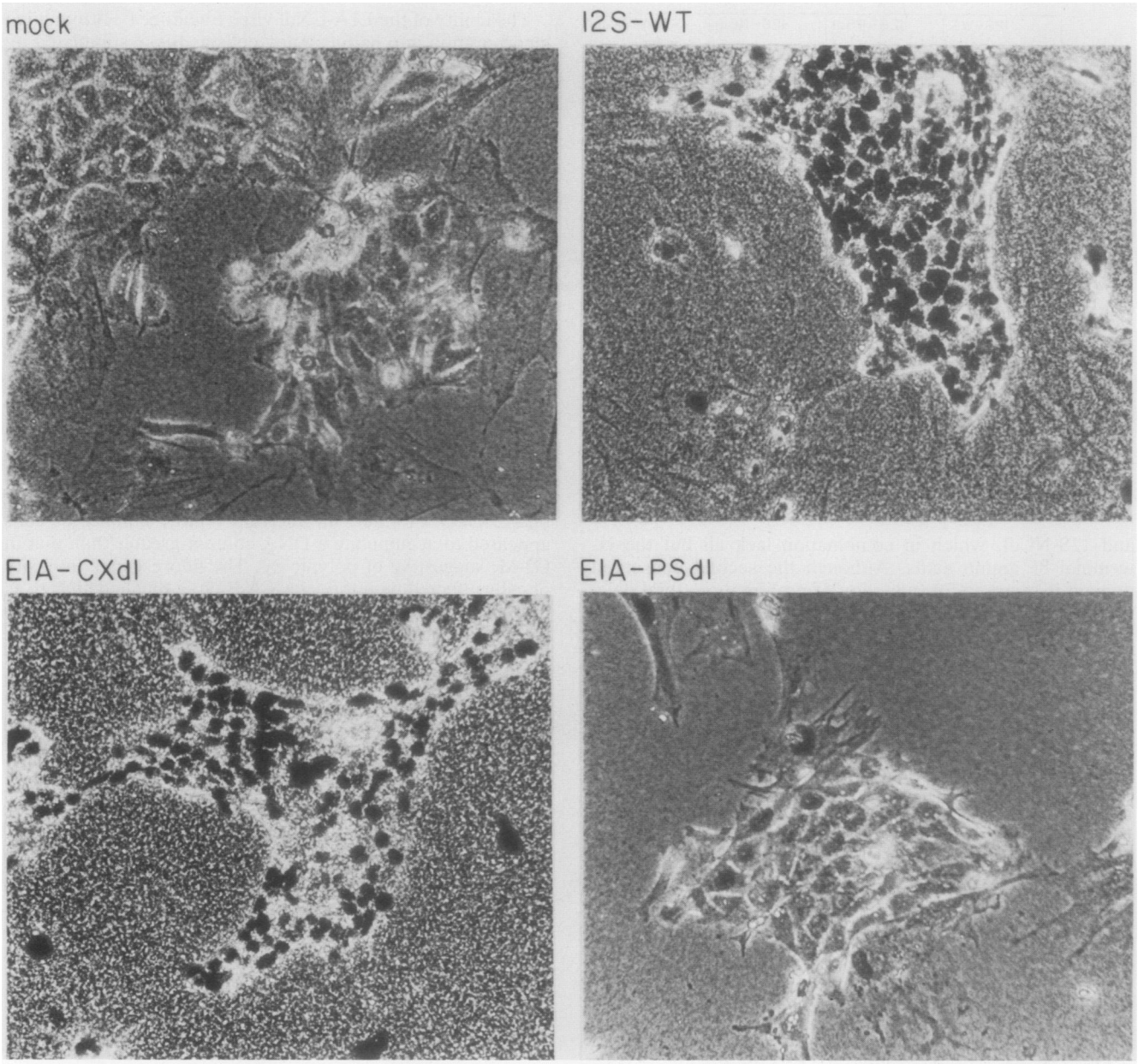

FIG. 5. Autoradiography of infected primary BRK cells. Monolayer cultures of primary BRK cells in 5% fetal bovine serum were infected at a multiplicity of 10 PFU per cell with the E1A deletion mutant viruses indicated. The cells were labeled at the time of infection with [^{3}H]thymidine, fixed at 72 h postinfection, and prepared for autoradiography. Black nuclei indicate active DNA synthesis.

(Table 2) indicated that pE1A-PSdl, like the domain 2 deletion plasmid pE1A-CXdl, had no detectable ability to cooperate with *ras*. Each of these plasmids is as much as 100-fold less active than the wild-type E1A plasmid in this assay. The E1A-PSdl *ras* cooperation defect is consistent with the recent demonstrations that single amino acid substitutions, as well as smaller deletions within conserved domain 1, can also impair *ras* cooperation in BRK cells (35, 47, 50, 53).

The previous demonstration that E1A mutants that carry a deletion of most of the sequences between domains 1 and 2 remain able to cooperate with *ras* (40, 47) or induce proliferation of BRK cells (47, 62) suggested that any interaction between domains 1 and 2 required for their function is essentially independent of the spacing between them. To explore further the relationship between domains 1 and 2, they were introduced on two separate plasmids, pE1A-PSdl and pE1A-CXdl, together with *ras*, in a triple plasmid cotransfection experiment (Table 2). The total amount of E1A DNA was kept constant in each transfection, so transfections with two mutant plasmids did not contain more E1A DNA than did a transfection with the wild-type E1A plasmid. Transfection of both of these E1A mutant plasmids together with the Ha-*ras* gene did result in focus formation, although at a lower efficiency than that found with the full-length E1A gene. The individual foci produced in the triple plasmid transfection experiments were readily grown out as rapidly proliferating stable cell lines.

One explanation of these results is that the activities of conserved domains 1 and 2, though both required for the *ras*

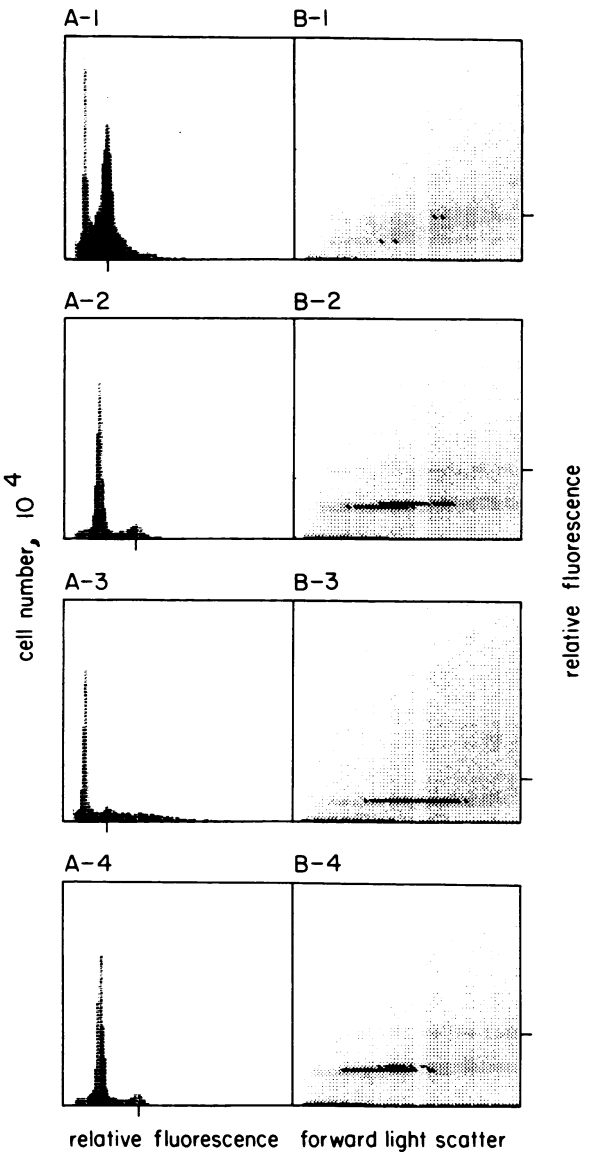

FIG. 6. Cell cycle effects of E1A mutants. Infected BRK cells were prepared as described in Materials and Methods and analyzed in a flow cytometer. The plots presented in this figure were derived from samples fixed at 48 h after infection. The numerical analysis of these data, as well as data from samples fixed at 24 and 72 h after infection, is presented in Table 1. (A) Relative fluorescence is plotted against the number of cells analyzed. (B) Relative fluorescence is plotted against forward light scatter, an indicator of cell size. Increasing cell size is indicated by a shift to the right in panel B. The tick marks on the bottom of panel A and the right side of panel B indicate the G2 peak of relative fluorescence. Fluorescence to the right of this mark in panel A or above this mark in panel B is indicative of DNA content greater than that in G2-M. The mutants analyzed in this figure were as follows (samples): 1, 12S-WT; 2, E1A-PSdl; 3, E1A-CXdl; 4, mock-infected cells.

cooperation effect, are independent enough to function effectively even when expressed in different polypeptides. Another possibility is that DNA recombination occurred by means of the 39 base pairs of common sequence corresponding to amino acid positions 108 through 120, remaining between the deleted regions of pE1A-PSdl and pE1A-CXdl and that the establishment of each of the permanent cell lines

TABLE 2. Focus formation on primary cultured BRK cells

Plasmid(s) cotransfected with Ha-*ras*	No. of foci in expt no.[a]:			
	1	2	3	4
pE1A-WT	21	103	64	
pE1A-PSdl		0	0	
pE1A-CXdl		0	0	
pE1A-PSdl + pE1A-CXdl	5	14	8	27
pE1A-PSfs + pE1A-CXdl			0	2
pE1A-WT + pE1A-PSfs				27
pUC18	0	0	0	0

[a] Experimental details are described in Materials and Methods.

resulted from integration of a reconstituted single gene encoding both domains. (The amino acid endpoints of the deletions are shown in Fig. 1.) The second possibility was tested in the two ways described below.

Expression of the E1A products in each of five independently isolated cell lines was assayed by immunoprecipitation with E1A-specific mouse monoclonal antibodies (20). The results with a representative cell line, 101.4H, are shown in Fig. 7. Each cell line analyzed expressed, like 101.4H (lane 4), a total set of E1A proteins equivalent to the sum of those produced separately during infection of primary BRK cells with either deletion mutant virus E1A-PSdl (lane 3) or E1A-CXdl (lane 2). (Each E1A mutant, like the wild-type gene, produced several different protein bands because of differential splicing and posttranslational modifications [8, 9]). No proteins of wild-type appearance could be detected in these cell lines (compare lane 4 with lane 5), even after extensive overexposure of the autoradiograms.

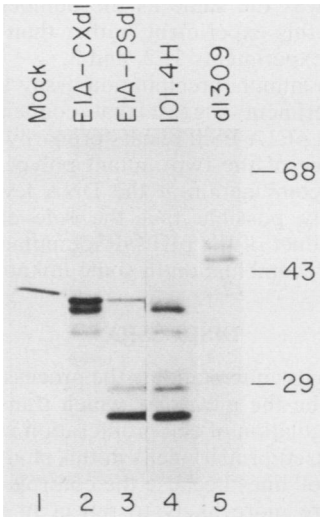

FIG. 7. Immunoprecipitation of E1A mutant products in BRK cells. [^{35}S]methionine-labeled protein extracts from primary BRK cells uninfected (lane 1) or virus infected at a multiplicity of 20 PFU per cell (lanes 2, 3, and 5) or from a cloned cell line derived from primary BRK cells cotransfected with E1A mutant plasmids and the T24 Ha-*ras* plasmid (lane 4) were absorbed with anti-*trp*E-E1A fusion protein mouse monoclonal antibodies, series 73 (20), precipitated on protein A-Sepharose, and fractionated in 15% sodium dodecyl sulfate-polyacrylamide gels. Ad5dl309 (lane 5) is the parental virus for all of the E1A mutants discussed in this report and contains a wild-type E1A gene. Numbers on the right indicate kilodaltons. Various amounts of a non-E1A-specific protein band of approximately 40 kilodaltons coprecipitated in all samples.

Because this type of experiment cannot exclude the possibility that an undetectable level of recombinant E1A products is responsible for the observed complementation, the possibility that the results observed are due to DNA recombination was tested in another type of experiment. An E1A variant, pE1A-PSfs, was constructed in a way similar to that used for the E1A-PSdl mutant, but by filling in instead of cutting back the *Sty*I site, four additional nucleotide base pairs were retained in addition to the 39 base pairs remaining between the deleted regions of pE1A-PSdl and pE1A-CXdl. In the pE1A-PSfs construct, therefore, the translation frame is altered after the *Sty*I site, resulting in a peptide consisting of 23 amino acids of authentic E1A sequence followed by 26 nonsense residues. Since recombination would correct the frameshift at the DNA level, the pE1A-PSfs mutant plasmid would be expected to show the same recombination frequency as the in-frame mutant plasmid pE1A-PSdl. The difference in complementing ability between these two plasmids is likely, therefore, to reflect a requirement for actual expression of a functional polypeptide from the pE1A-PSdl construct. The frameshift mutant plasmid pE1A-PSfs was at least 10-fold less active than the pE1A-PSdl variant in its ability to cooperate with pE1A-CXdl and Ha-*ras* in the triple plasmid assay (Table 2), suggesting that most of the cooperation observed between pE1A-CXdl and pE1A-PSdl requires expression of the pE1A-PSdl polypeptide and not just the presence of the DNA sequence from this construct. The E1A-WT plasmid was also cotransfected with pE1A-PSfs in experiment 4 (Table 2) to ensure that expression of the E1A-PSfs nonsense peptide does not abolish the effect of the wild-type plasmid. In this case, the amount of wild-type E1A plasmid was half that in the samples in which pE1A-WT was the only E1A plasmid in the assay, which may in part explain why the number of foci in the pE1A-PSdl–pE1A-CXdl cotransfection was the same as the number of wild-type-induced foci in this experiment, rather than four- to eight-fold less, as in experiments 1, 2, and 3.

Together, the immunoprecipitation assay and the genetic frameshift experiment suggest that cooperation between pE1A-CXdl and pE1A-PSdl results primarily from the independent presence of the two mutant polypeptides and not merely from recombination at the DNA level, although it remains formally possible that the role enacted by the polypeptide product of the pE1A-PSdl mutant plasmid is to promote DNA recombination in some unknown way.

DISCUSSION

Our approach to understanding the process of transformation was to probe the means by which transforming genes influence the regulation of cell proliferation in normal quiescent cells. We used primary cells in this study in preference to established cell lines because the controls that operate in primary cells are more likely to reflect in vivo cell cycle controls than are the controls that operate in established cell lines that have been immortalized by some means, such as passage through cell crisis, so that normal regulation is disrupted even before the effects of specific transforming genes are studied.

Primary BRK cell cultures consist mostly of epithelial cells which become quiescent within 2 to 3 days after plating, even in the presence of serum and at subconfluent densities (43). If untreated, these cells die off in about a week. Products of the adenovirus E1A gene have the ability to induce DNA synthesis and rapid proliferation in quiescent primary BRK cells independently of the presence of serum (43) and can cooperate with the *ras* oncogene to transform these cells fully (44). These activities appear to lie principally within two amino acid regions of the E1A products: the N-terminal 85 amino acids and a second region extending approximately from amino acid position 120 to position 139. Previous results suggested that the E1A products supply at least two functions required to induce extended proliferation of primary BRK cells: one in the N-terminal region sufficient for induction of DNA synthesis and one in domain 2 required for the cells to progress further through the cell cycle (62).

Here, we examined the effect of total deletion of conserved domain 1. Loss of these sequences did not impair activation of the virus E3 early promoter in a transient-expression assay in HeLa cells. Virus growth in HeLa cells was not wholly unaffected by this deletion, however. Virus yield during infection with the PSdl variant virus was about 10-fold less than the wild-type yield. Similar observations have been reported for an 11S Ad5 strain that does not contain domain 1 (50, 53) and E1A mutants that contain deletions within domain 1 (47).

Deletion of the sequences from positions 23 to 107 abolishes the ability of the E1A products to induce either proliferation or DNA synthesis in quiescent primary BRK cells. These results are consistent with the suggestion that during the induction of proliferation in quiescent primary BRK cells, conserved domain 1 of the E1A products is primarily important for induction of DNA synthesis, whereas conserved domain 2 is required for further cell cycle progression. The function required for induction of DNA synthesis in quiescent BRK cells does not appear to be obligatory for virus growth in actively proliferating HeLa cells. In BRK cells, expression of the N-terminal region containing conserved domain 1 in the functional absence of conserved domains 2 and 3 (for example, in the E1A-CXdl construct) induced a form of S phase in which DNA synthesis occurred but in the absence of controls required for progression through the remainder of the cell cycle. These cells did not appear to accumulate in or before G2-M, and many appeared to have a DNA content greater than that in G2-M. These results raise the possibility that the domain 2 function is involved in controlling the cessation of DNA synthesis before further progression through the cell cycle.

The results presented here are consistent with the suggestion, made previously (62), that each of the three conserved domains in the E1A gene has an independent gene-regulating function. This does not exclude the possibility that these activities interact, are additive, or are even competitive in some cases. But each of several biological effects of E1A seems to be distinctly more sensitive to alterations in one domain than in others. The possibility that domains 1 and 2 evolved independently and were linked later in the E1A gene is suggested by the recent realization that domain 1, like domain 3, is contained within a separate intron in the E1A gene which can be removed by alternative splicing (50, 53).

The functions of both domains 1 and 2 are required for production of transformed foci in a *ras* cotransfection assay. Focus formation occurred, however, even when the two domains were introduced on two separate plasmids. This complementation effect did not appear to result merely from recombination at the DNA level, since a frameshift variant of the PSdl mutation had at least 10-fold less complementing activity than the variant which produces an authentic E1A polypeptide. In addition, no evidence of expression of wild-type E1A protein products could be detected in the cell lines established by the complementing E1A mutants.

The means by which regions 1 and 2 cooperate to induce

BRK cell proliferation or *ras* cooperation may or may not include physical interaction between these two polypeptides or between each of these polypeptides and a cellular protein(s), a variety of which can coprecipitate with the E1A proteins (21, 60). We are currently testing the possibility that such complexes occur.

The demonstration that certain pairs of oncogenes, such as E1A and *ras*, can act cooperatively but not singly to induce transformation of primary cells (32, 44) suggested that transformation is at least a two-step process. The results presented here suggest, however, that the E1A gene, at least, actually contributes two functions to the cooperation effect, with initial activation of the proliferative state requiring control both at the beginning of the S phase and also at some point before cell division, suggesting that induction of DNA synthesis and onset of cell division can sometimes be uncoupled in mammalian cells and that at least three controlling steps can be distinguished during induction of transformation.

Separate points of cell cycle control, operating at the G1-to-S phase and the G2-to-M borders, have been demonstrated in yeast cells (reviewed in reference 45). Premitotic controls have also been demonstrated in developing embryos (reviewed in reference 29), and a factor which promotes mitosis in *Xenopus laevis* oocytes has been partially purified from HeLa cells (1). Identification of the roles played by mammalian cell cycle control genes has been difficult, however, possibly because mammalian genes in situ are under the regulation of extracellular and cytoplasmic signals. Since the E1A gene products seem to exert their effects independently of these signals, they appear to be excellent tools for probing the biochemical mechanisms of cell cycle control.

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