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Functions of the Two Adenovirus Early E1A Proteins and Their Conserved Domains in Cell Cycle Alteration, Actin Reorganization, and Gene Activation in Rat Cells

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Rat embryo cells were infected with adenovirus type 5 mutants that code for only one of the two early E1A proteins, mutants with defects in one of the two conserved regions common to the two proteins, or mutants with defects in the 46-amino-acid region unique to the 289-amino-acid E1A protein. Cells were scored for altered cell cycle progression, disruption of actin stress fibers, and activation of E2A expression. Mutants lacking either E1A protein were able to cause all of these effects; but mutants lacking a 243-amino-acid protein had less effect, and mutants lacking a 289-amino-acid protein much less effect, than wild-type virus. A mutation in any of the three conserved regions caused a defect in each E1A effect. To investigate the reported function of conserved domain 2 in mitosis, we monitored by fluorescence-activated cell sorter the reduction in Hoechst 33342 fluorescence that occurs when cells divide after undergoing a round of DNA replication in 5-bromodeoxyuridine. A smaller percentage of adenovirus-infected cells than mock-infected cells divided within a given period after completing a round of DNA replication. Viruses with mutations in conserved domain 2 were defective for initiation of cellular DNA replication, as were all other E1A mutants we have examined, but had no specific defect in cell division compared with wild-type virus. Thus, although there may be some specialization of function between the two E1A proteins and between their conserved domains, it was not apparent in the aspects of E1A function and the mutants that we examined.

The adenovirus E1A gene collaborates with the E1B gene, or heterologous genes such as *ras* or polyomavirus middle T antigen, to cause complete transformation (35). Alone, E1A induces morphologically altered cell lines that grow continuously in culture but lack other transformed properties (17, 45). Consistent with these effects, adenovirus infection of rat cells results in changes to the cytoskeleton and control of the growth cycle, both of which are direct effects of E1A (2, 7, 19). E1A activates transcription of early adenovirus genes (3, 20) and some cellular genes (6, 9, 38, 46), but it also inhibits expression of other genes controlled by some viral and cellular enhancers (6, 16).

E1A produces 12S and 13S early mRNA species that code for proteins of 243 amino acids (aa) and 289 aa, respectively (4, 10, 34). These differ only by 46 aa unique to the 289-aa protein. Both E1A proteins are required for full transformation (18, 26). Each can to some extent immortalize cells (28, 29) and induce DNA replication in quiescent cells (21). The unique region of the 289-aa protein is essential for gene activation but apparently not for some transformation-related functions (30, 45). Nevertheless, many mutants with mutations in the unique region have transformation defects (1, 8, 12, 13), and all that we have tested have a reduced ability to alter the cell growth cycle (2, 7).

To clarify the roles of the two early E1A proteins and their conserved domains in different effects of E1A cells, we infected rat embryo cells with genetically engineered viruses that code for only one of the two E1A products, similar viruses with defects in conserved regions common to the two proteins, or mutants with defects in the region unique to the 289-aa protein. The infected cells were examined for altered cell cycle progression, disruption of actin stress fibers, and activation of viral gene expression. The results suggested that both E1A proteins and all three conserved domains are involved in all three functions.

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MATERIALS AND METHODS

Viruses and cells. The origins of engineered and mutant viruses were as follows.

(i) Mutants coding only for the 289-aa protein. *pm*975 (27) was from A. J. Berk, University of California, Los Angeles; *dl*348 (44) was from J. R. Cutt, State University of New York at Stony Brook; and the 13S cDNA virus (27) was from E. Moran, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

(ii) Mutants coding only for the 243-aa protein. *dl*347 (44) was from J. R. Cutt, and the 12S cDNA virus (27) was from E. Moran.

(iii) Mutants with defects in common conserved regions. Mutants with defects in common conserved regions were generous gifts from E. Moran. Construction of 12S *pm*705 (conserved region 1), 12S *pm*961, E1A *pm*928, and 12S *pm*928 (conserved region 2) have been described (28, 46).

(iv) Mutants with defects in the 289-aa unique region. The origins of mutants *hr*1, *hr*3, and *in*500 have been described previously (2, 7, 8, 15, 18).

All mutants were grown in 293 cells, and titers were determined on 293 and HeLa cells. The preparation and growth of rat embryo fibroblasts have been described previously (7). Baby rat kidney (BRK) cells were prepared as described by Ruley (35).

Detection of viral gene activation and actin stress fiber reorganization. Viral gene activation was detected by indirect immunofluorescence with an antiserum that reacts with the E2A gene product, and reorganization of stress fibers

was observed by staining with 7-nitro-2-oxa-1,3-diazole phallacidin as described previously (19).

Cell cycle analysis. To 5×10^5 cells in 1 ml of medium was added 50 μl of propidium iodide (5 mg/ml) in 5% Triton X-100, followed by 10 μl of RNase (8,000 U/ml) (41). After standing for 30 min on ice, 25,000 cells were analyzed in a Becton-Dickinson FACS IV fluorescence-activated cell sorter (FACS) with excitation at 388 nm with a 600-nm long-pass filter. The G_1 peak in mock-infected cells was set to channel 50. The number of cells with DNA contents corresponding to the G_1, S, and G_2-M phases of the cell cycle were determined (41) as well as those with a DNA content of >4n. As an overall quantitative measure of the effect of a virus on the cell cycle, the percentages of cycling and resting cells were calculated.

FACS analysis of cell division and completed rounds of DNA replication. When cells replicate DNA in the presence of 5-bromodeoxyuridine (BudR) and divide, their fluorescence is reduced to half of that of G_1 cells (G_1') when stained with a thymidine-specific fluor such as Hoechst 33342 (5, 37). This phenomenon was used to monitor cell division in mutant adenovirus-infected cells. Rat embryo fibroblasts (REF; 1.5×10^6 per 5-cm dish) were infected with the viruses to be tested. Sixteen hours later, the medium on one dish was replaced by 5 ml of fresh medium, that on another was replaced by medium containing 10 μg of BudR per ml and 8 μg of deoxycytidine per ml, and that on a third dish was replaced by medium containing 1 μg of colchicine per ml. After incubation for 24 h, cells were trypsinized, suspended at 5×10^5/ml, and stained with 100 μl of Hoechst 33342 (200 μg/ml) per ml in medium containing 1% Nonidet P-40. Fluorescence analysis was done with an argon ion laser adjusted to 30 mW at 351 and 363 nm and a 400 nm long-pass filter. The G_1 peak was set at channel 70, and the G_1' peak at about channel 35 in BudR-treated cells was integrated and halved to determine the number of cells that had divided. Cells that had completed DNA replication during the same period were determined by staining colchicine-treated cells and integrating the G_2-M peak.

RESULTS

A mutation in either E1A protein affects control of the rat cell cycle. The adenovirus E1A gene is able to induce quiescent rat cells to enter the growth cycle and to alter cycle progression in growing cells (2, 7, 9, 31, 32). In a previous paper in which mutants with defects in the 289-aa unique region were used, we concluded that the 289-aa E1A protein was essential for cell cycle effects, and that the 243-aa protein contributed to the full effect but either was unable to act alone or was much less effective than the 289-aa protein (2). To further explore the roles of the two E1A proteins in cell cycle effects, we infected rat embryo cells with adenoviruses in which the wild-type (wt) E1A region was replaced by cDNA for the 13S mRNA (13S virus and *dl*348, which produce only the 289-aa protein), or by cDNA for the 12S mRNA (12S virus and *dl*347, which produce only the 243-aa protein) (28, 44). Cell cycle progression was then monitored under conditions in which most control cells were arrested in G_1 by confluence. FACS profiles from one experiment are shown in Fig. 1, and data from several experiments are combined as plots of percent cycling cells versus input multiplicity in Fig. 2a and b. Based on the mutants that we tested, each E1A protein was able to alter control of the cell cycle acting alone; but the 13S cDNA viruses had less effect, and the 12S cDNA viruses much less effect, than wt virus.

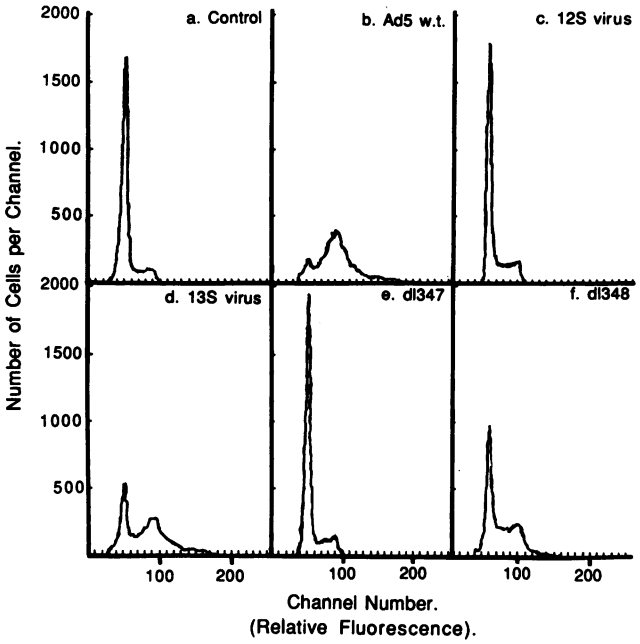

FIG. 1. Cell cycle FACS profiles of cDNA virus-infected cells. Rat embryo cells were mock infected (a) or infected with 50 IU of wt adenovirus (b), 12S cDNA virus (c), 13S cDNA virus (d), *dl*347 (e), or *dl*348 (f) per cell. At 64 h after infection, cells were harvested, stained with propidium iodide, and analyzed in the FACS as described in Materials and Methods.

Spindler et al. (39) reported that a mutant (*dl*1500) (26) that produces only the 243-aa E1A protein was able to induce cellular DNA synthesis in serum-starved cells of a continuous rat cell line (CREF) to the same level as wild-type virus, whereas the splicing mutant *pm*975, which makes only the 289-aa protein (27), was partly defective. This result with *dl*1500 contrasts with out results in confluence-arrested primary cells, in which both 12S cDNA (243-aa protein) viruses were more defective for increases in cellular DNA content than were their 13S cDNA (290-aa protein) counterparts (Fig. 1 and 2). We therefore repeated our experiments in rat cells that were incubated in medium containing 0.5% serum for 2 days before infection and 3 days after. Both 12S cDNA (243-aa protein) viruses induced cycling in the serum-starved cells but were quantitatively defective compared with wt virus (Table 1). Likewise, a 13S cDNA virus (*dl*348) and a splicing mutant (*pm*975) that produce only the 289-aa E1A protein both induced cell cycling at a lower level than did the wt virus (Table 1). Thus in serum-starved rat cells, as in confluence-arrested cells, results with those mutants suggest that each E1A protein can induce cell cycle effects but at a lower level than in the wt virus; the maximum quantitative effect requires both proteins.

Another factor known to affect E1A function is cell type. It has been reported that 12S cDNA constructs have high ability to induce cell DNA replication and proliferation in BRK cells (28; E. Moran, personal communication). We compared the ability of 13S cDNA and 12S cDNA constructs to induce cell cycling in REF and BRK cells. Analysis in the FACS showed that 12S cDNA viruses were indeed more active in BRK cells, and their 13S counterparts also showed a slight increase in activity (Table 2), whereas wt adenovirus showed no such increase. BRK cells therefore contain a factor that augments suboptimal E1A function; nevertheless

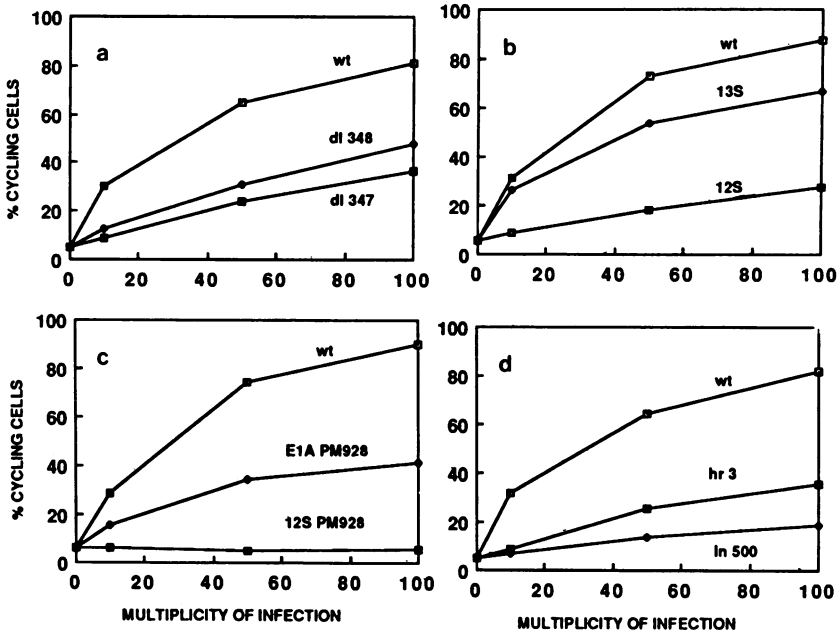

FIG. 2. Cell cycle effects of cDNA viruses and mutants with defects in conserved regions 2 and 3. Cells were mock infected or infected at the indicated multiplicities with wt virus (□) or the following: (a) *dl*348 (◆) or *dl*347 (■); (b) 13S cDNA virus (◆) or 12S cDNA virus (□); (c) E1A *pm*928 (◆) or 12S *pm*928 (■) in region 2; or (d) *hr*3 (■) or *in*500 (◆) in region 3. Cells were harvested 64 h later, stained with propidium iodide, and analyzed in the FACS as described in Materials and Methods. The percentage of cycling cells was then calculated (40).

it appears that, at least for *dl*347 and *dl*348, the augmented function is still less than that of wt E1A.

Both E1A proteins are involved in actin reorganization and gene activation. As in human cells (3, 20), the adenovirus E1A gene is responsible for activating other viral early genes such as E2A in rat cells (2, 19). E1A also causes disruption of actin microfilaments and stress fibers early in infection of rat cells (19). In earlier work with the constructs *pm*975, *dl*347, and *dl*348, we obtained evidence that the 289-aa protein is essential for both of these functions but that the 243-aa protein might also be required for a full effect (19). We used the 13S cDNA and 12S cDNA viruses (28) to verify and extend these observations. Rat cells were infected with wt adenovirus or 13S cDNA or 12S cDNA virus at 20 IU/cell and after 48 h examined for E2A expression by immunofluorescence and stress fiber organization by staining with 7-nitro-2-oxa-1,3-diazole phallacidin. Each E1A protein was able to induce both E2A expression and reorganization of the actin stress fibers when acting alone (Fig. 3); but again the 289-aa protein was less effective, and the 243-aa protein was much less effective, than the wt adenovirus. The maximum quantitative effect required both E1A proteins.

Requirement for all three conserved regions. E1A has three regions that are conserved between serotypes and important in function. Regions 1 (aa 40 to 80) and 2 (aa 121 to 139) are common to both proteins, whereas region 3 corresponds to the 46 aa unique to the 289-aa protein (22, 30, 42, 45). These regions may vary in their relative importance for different E1A functions (28, 30, 45, 46). To explore this further, we infected rat cells with a number of viruses, each of which was mutated in one of the three regions, and scored them for altered cell cycle control, disruption of stress fibers, and gene activation. Mutant 12S *pm*705, a 12S cDNA virus with a mutation in conserved region 1, was severely defective for cell cycle effects (Fig. 4), gene activation, and stress fiber disruption (Fig. 3). The same was true of the region 2 mutant 12S 961 (Fig. 3 and 4). A point mutation at nucleotide 928 in region 2 caused a severe defect in a 12S cDNA virus but only

TABLE 1. Each E1A protein can induce some cycling in serum-starved cells, but the wt effect requires both[a]

Virus	E1A proteins		% Cycling cells		
	289 aa	243 aa	Expt 1	Expt 2	Expt 3
Mock infection	−	−	6.4	12.6	14.2
Adenovirus type 5 wt	+	+	48.1	70.0	37.8
*pm*975	+	−	23.2	57.5	
*dl*348	+	−		37.8	19.9
12S virus	−	+	27.1	28.4	
*dl*347	−	+	20.5	52.5	

[a] REF cultures were incubated in medium containing 0.5% fetal calf serum for 2 days and then infected with the viruses shown in medium without serum. After 1.5 h at 37°C, fresh medium containing 0.5% serum was added. Cells were stained with propidium iodide 64 h after infection and analyzed in the FACS.

TABLE 2. Cell cycle effects of E1A cDNA viruses in REF and BRK cells[a]

Virus	E1A proteins		% Cycling cells	
	289 aa	243 aa	REF	BRK
Mock infection	−	−	18.7	19.0
Adenovirus type 5 wt	+	+	85.3	77.7
13S virus	+	−	66.1	72.5
*dl*348	+	−	49.6	61.6
12S virus	−	+	28.9	62.0
*dl*347	−	+	20.3	34.9

[a] BRK cells or REF cultures were infected with the viruses shown. After 64 h of incubation, the cells were stained with propidium iodide and analyzed in the FACS.

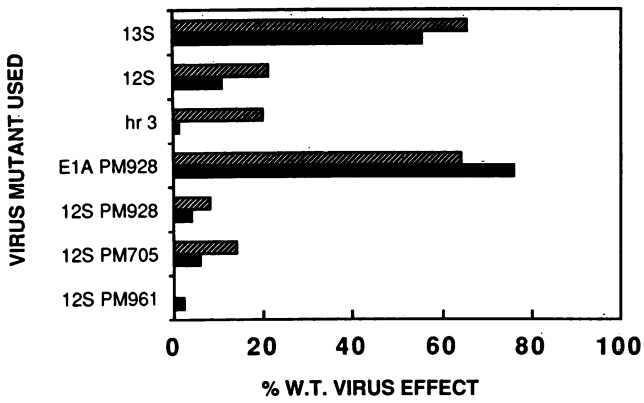

FIG. 3. Gene activation and reorganization of the cytoskeleton in mutant-infected cells. Rat embryo cells were infected with 20 IU of wt adenovirus or the mutants indicated per cell, fixed after 48 h, and stained for expression of E2A (■) or disruption of stress fibers (▨) as described previously (19). Results are expressed as percentages of the wt virus effect after subtracting mock-infected control values.

a mild defect in the equivalent virus with both E1A proteins (Fig. 2 and 3). Mutants *hr*1, *hr*3, and *in*500, with defects in conserved region 3, also had reduced ability to induce cell cycle effects (Fig. 2 and 4), activate expression of E2A, and disrupt the cytoskeleton (Fig. 3), consistent with our previous observations (2, 19). Thus all of the conserved regions appear to be required for each of the three E1A functions we have examined.

Is conserved domain 2 required to drive mitosis or DNA replication? It has been reported that mutant *pm*928, which is mutated in conserved domain 2, and Cxdl, which has a deletion of conserved domain 2 and 10 amino acids of

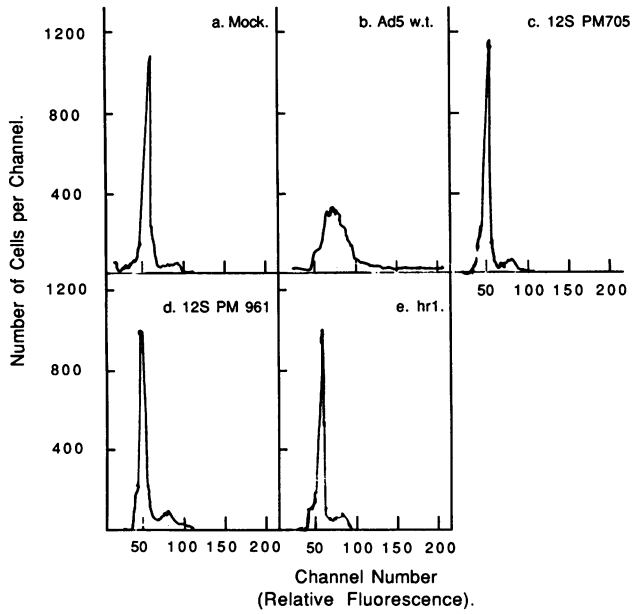

FIG. 4. Mutants in all conserved domains are defective for cell cycle effects. Rat embryo cells were mock infected (a) or infected with 50 IU of the following per cell: wt adenovirus type 5 (b), 12S *pm*705 (conserved domain 1; c), 12S *pm*961 (conserved domain 2; d), or *hr*1 (conserved domain 3; e). Cells were harvested 64 h later, stained with propidium iodide, and analyzed in the FACS.

conserved domain 3, are able to stimulate cellular DNA synthesis but are defective for induction of mitosis and cell proliferation in BRK cells (29, 46). We found this surprising, because in our experiments conserved domain 2 mutants had defects in induction of DNA replication (Fig. 2 and 4), and we had previously found no evidence for a specific mitotic induction function of wt E1A (31, 32). We therefore investigated the cell cycle effects of domain 2 mutants in more detail.

To follow cell division in the FACS, we incubated cells in BudR for 24 h and stained with the thymidine-specific fluor Hoechst 33342 (37). To test the method, we first incubated growing REF in BudR and stained with Hoechst 33342. Compared with cells not exposed to the drug (Fig. 5a), BudR-treated cells lacked the S and G_2+M parts of the normal fluorescence profile (due to failure of the BudR-labeled daughter strands to fluoresce) and showed a peak at half the G_1 peak fluorescence (G_1' in Fig. 5b), corresponding to cells that had divided after incorporating BudR. That the G_1' peak indeed corresponds to cells that had divided was shown by its absence in BudR-treated cells in which cell division was blocked by colchicine (Fig. 5d). The number of cells that had completed DNA replication in the same time period (irrespective of division) was estimated by integrating the G_2+M peak of cells treated with colchicine without BudR (Fig. 5c).

The method was then applied to confluence-inhibited REF infected by adenovirus type 5 and mutants with defects in conserved domain 2. Hoechst 33342 profiles of infected cells not treated with drugs are shown in Fig. 6. Mutants Cxdl and E1A *pm*928 were clearly defective for initiation of S phase compared with wt adenovirus type 5, whereas 12S *pm*928 and 12S *pm*961 were defective compared with 12S virus. Note also that if the mutants initiated S phase but mitosis did not occur, the G_1 peak should be reduced to the same extent as in wt-infected cells, and cells with G_2+M DNA content should accumulate; this was not observed. The numbers of cells that completed DNA replication and that divided were calculated from profiles of cells treated with colchicine or BudR. Conserved domain 2 mutants were less able than their wt counterparts to induce and complete a round of cellular DNA replication in the 24-h period studied (Table 3). Differences in the numbers of cells that divided in the same period were small (Table 3). When the ratio of cell division to completed DNA replication was calculated, it was clear that fewer adenovirus type 5-infected cells than uninfected cells divided after completing a round of DNA replication within the 24-h period. E1A *pm*928 had a marginally lower ratio of cell division to completed DNA replication than did wt adenovirus type 5, but the other three conserved domain 2 mutants had higher ratios than appropriate controls (Table 3). Thus there was evidence that conserved domain 2 mutants had reduced ability to induce and complete rounds of DNA replication but not that they had a specific defect in the ability to cause cell division.

DISCUSSION

The existence of two separate, related E1A mRNAs and proteins has led to the hypothesis that they have qualitatively different functions. This is favored by reports that the 289-aa protein is solely or mainly responsible for activation of early gene expression (14, 25, 27, 28); however, other reports suggest that the 243-aa protein is able to activate viral gene expression, albeit less efficiently (11, 23, 24, 43). When E1A functions other than gene activation are consid-

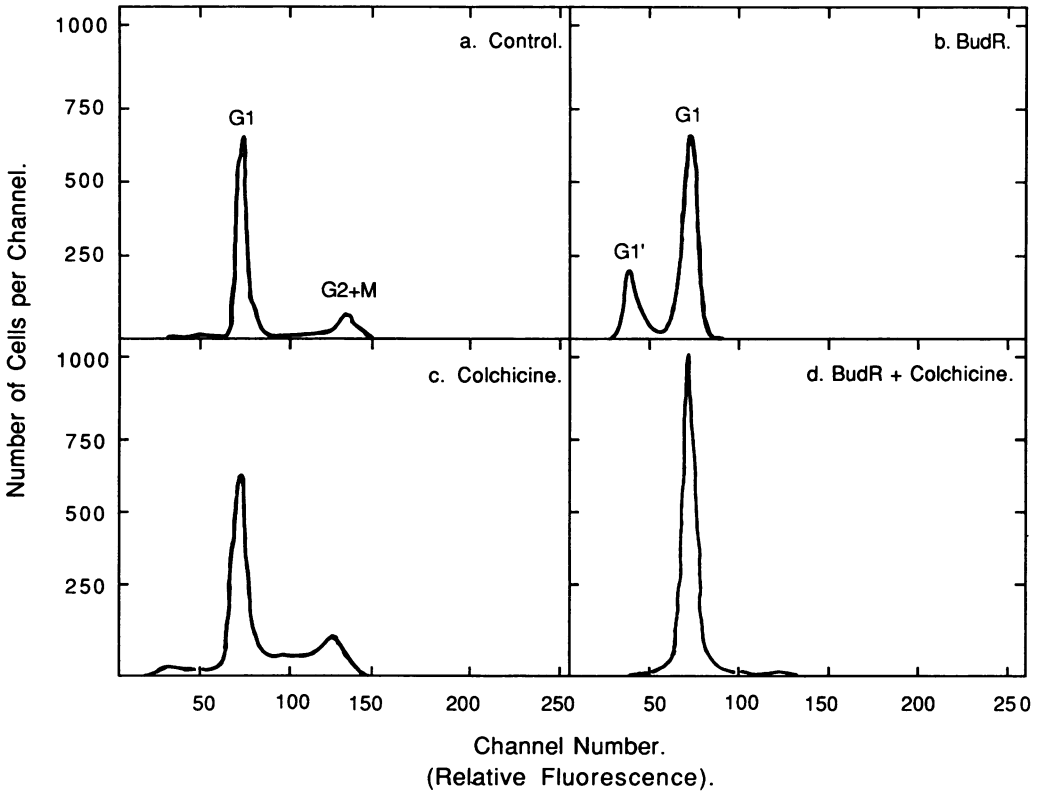

FIG. 5. Hoechst 33342–BudR analysis of cell division in REF. Growing REF were treated for 24 h with normal medium (a); medium containing BudR and deoxycytidine (b); colchicine (c); or BudR, deoxycytidine, and colchicine (d) as described in Materials and Methods. Cells were stained with Hoechst 33342 and analyzed in the FACS.

ered, there is considerable evidence that either E1A protein can cause each effect. The 289-aa and 243-aa proteins are each capable of transformation (18, 26), induction of cellular DNA replication (21, 40), immortalization of cells (30, 45), cooperation with other oncogenes in transformation (45), induction of cyclin (46) and E1A-induced heat shock proteins (38), and repression of enhancers (6, 16, 43). Both 13S and 12S cDNA viruses induce cellular thymidylate synthetase, although it is delayed in cells infected by 12S virus (46). Our own results, with the mutants we tested, indicate that each E1A protein is able to induce cell cycle progression in quiescent rat embryo fibroblasts; this is consistent with a recent report that plasmids carrying either the 13S or the 12S E1A cDNA can induce cycling in quiescent rat 3Y1 cells (33). We found that, for the mutants we tested, stress fiber disruption and early viral gene expression could also be induced by either E1A protein, and each of the three E1A effects we investigated requires both E1A proteins to achieve wt levels of response. In each case the 243-aa protein was less efficient than the 289-aa protein when acting alone, but there was no unequivocal evidence of specialization of function between the two proteins; rather it seemed that they act together to produce each E1A effect.

The discovery of domains in the E1A proteins that are conserved in evolution has likewise led to the hypothesis that they may correspond to regions of different specialized function; evidence in favor of this view has been reported (23, 25, 29, 46). We found that a mutation in any of the three conserved domains reduced the ability of adenovirus to alter cell cycle control, disrupt the actin cytoskeleton, and activate viral early gene expression, with no clear evidence of specialization of function in the parameters we investigated. We previously reported that mutants *sub*315 and *sub*316, with defects in the 3′ exon of E1A, are severely defective for cell cycle effects and less efficient than wt virus in early gene activation (7). We have investigated altogether 18 different E1A mutants with locations that range over 86% of the 289-aa protein; each had a demonstrable defect in both cell cycle progression and viral gene activation, although these varied quantitatively.

It has been reported that mutants with defects in conserved domain 2 are able to induce cellular DNA replication but have a specific defect in inducing mitosis and cell proliferation (29, 46). This has led to the suggestion that wt E1A has a specific function in cell division, separable from its ability to induce a cell cycle and DNA replication in quiescent cells, and that this mitotic function but not the DNA replication function is located in conserved domain 2 (29). Our previous results, however, suggested that mitosis in adenovirus-infected cells occurs only as part of the program to which cells are normally committed once they traverse the G_1-S boundary; indeed, when E1A is overproduced, many infected cells go through two or more rounds of DNA replication before dividing (32). Our experiments with the BudR–Hoechst 33342 method in the FACS confirmed that wt adenovirus-infected cells are, if anything, less likely than their mock-infected counterparts to divide within a given time of completing DNA replication. We could find no detectable defect in cell division in cells infected by domain 2 mutants; in fact, cells infected by three of the four mutants had a higher ratio of cell division to completed DNA replication than did cells infected by wt virus. We did, however, find conserved domain 2 mutants to be defective for initiation and completion of rounds of cellular DNA

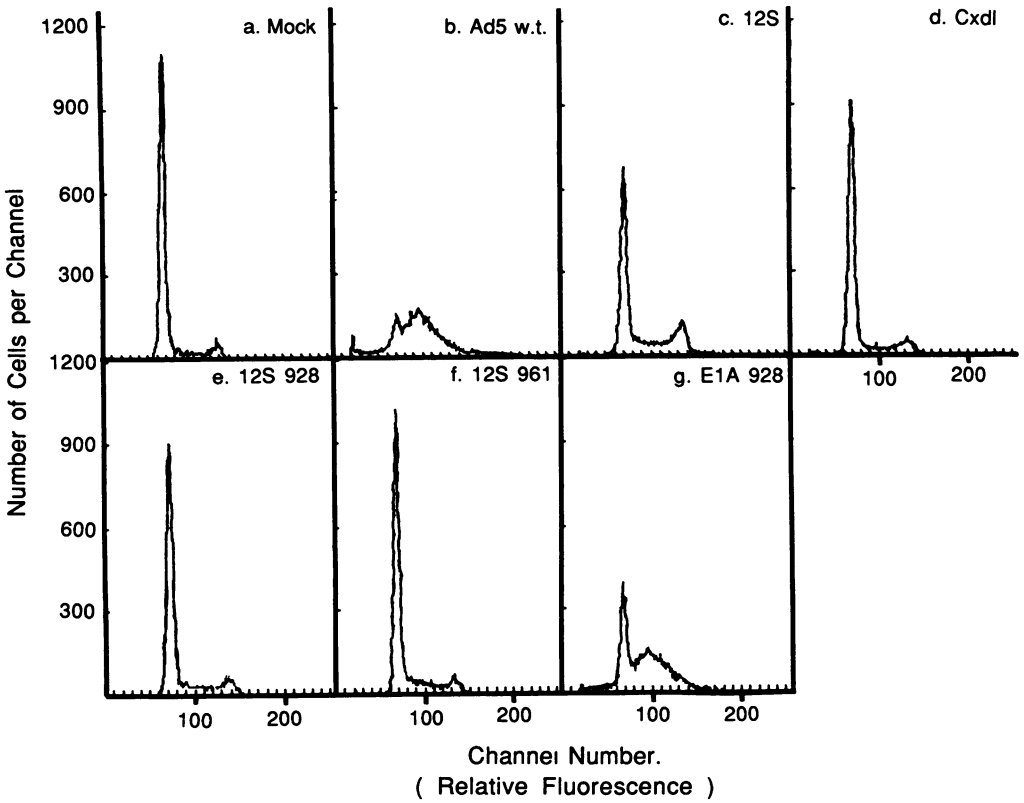

FIG. 6. Hoechst 33342 profiles of cells infected by conserved domain 2 mutants. REF were mock infected (a) or infected by 50 IU of adenovirus type 5 (b), 12S virus (c), Cxdl (d), 12S *pm*928 (e), 12S *pm*961 (f), or E1A *pm*928 (g) per cell. Sixteen hours later the medium was changed to normal medium, BudR plus deoxycytidine, or colchicine. At 40 h after infection, cells were stained with Hoechst 33342 and analyzed in the FACS. The profiles shown are of cells without drug. Quantitative data from these profiles and those of coldricine- and BudR-treated cells are shown in Table 3.

replication compared with appropriate controls and no different in this respect from all other E1A mutants we have tested. The reasons for the discrepancy between our results and those of Zerler et al. (46) are not known, although they could include the different cell types and experimental methods used. We suggest that the case for a specific mitotic function of domain 2 needs confirmation before it can be accepted.

Other functions have also been reported to be mainly or exclusively located in conserved domain 2. Lillie et al. (25) reported that the ability of E1A to repress enhancers resides in conserved domain 2 of the 243-aa protein and that the 289-aa protein has no repressor activity. However, other authors reported that both E1A proteins have repressor activity (6, 43), and Schneider et al. (36) reported that conserved domain 1 is more important than conserved domain 2 in repressor function. Conserved domain 2 has been reported to be essential for establishment of primary cells and cooperation with *ras* in transformation (45), but conserved domain 1 is at least as important for both functions (36). It is clear that conserved domain 2 is important for establishment, cooperation with *ras*, and perhaps repression, but it is less certain that these properties of E1A reside exclusively, or even mainly, in conserved domain 2. Our results suggest that conserved domain 2 is also involved in induction of cell cycle progression, disruption of the actin cytoskeleton, and activation of early viral gene expression, although it is less important for these effects than is conserved domain 3. Microinjection of mutant E1A proteins also suggests that conserved domain 2 is part of a large

TABLE 3. DNA replication and cell division in REF infected by adenovirus type 5 mutants defective in E1A conserved domain 2[a]

Treatment cycle parameter (virus)	% Untreated cycling cells ($S+G_2+M$)	% Completed DNA replication (colchicine-treated cells) (G_2+M)	% Completed division (BudR-treated cells) ($G_1'/_2$)	Division/DNA replication (%)
Mock infection	18.3	17.6	4.9	27.8
Adenovirus type 5 wt	73.3	36.5	3.8	10.4
E1A 928	62.9	27.2	2.5	9.2
E1A Cxdl	22.0	13.0	4.1	31.5
12S	44.5	40.3	5.4	13.4
12S 928	34.5	19.2	4.0	21.1
12S 961	24.8	18.9	4.7	24.9

[a] Experimental details are shown in the legend to Fig. 6.

central region of the E1A proteins important for viral early gene activation (23).

From our results, it appears that a mutation anywhere in either E1A protein affects all of the E1A functions we have examined to a greater or lesser extent. The evidence for functional specialization of domains has generally been based on observations that a given mutation affects one E1A function more than another. We believe that these results should be interpreted with caution. Different mutants with defects in the same domain may have different phenotypes. For instance, mutant *hr*1, with a mutation in conserved domain 3 (the 289-aa protein unique region), has 50% or more of wt effect on the actin cytoskeleton but is severely defective for cell cycle effects and gene activation (19). However, this does not mean that domain 3 is required for cell cycle effects and gene activation but not for disruption of the actin cytoskeleton; mutants *in*500 and *hr*A in the same domain are severely defective for cytoskeletal effects, whereas *hr*3 has an intermediate phenotype (19). The clearest domain effect is the requirement for a 289-aa protein with a functional domain 3 for high-level viral gene activation; but many other E1A functions are affected by mutations in domain 3, and viruses entirely lacking domain 3 are able to activate gene expression to some extent. Results with recombinant mutant E1A proteins suggest that domain 3 is only part of a central region of the E1A proteins important for gene activation that also includes domain 2 and part of the 3′ exon (23). Outside this central region, mutations have a smaller but still detectable effect on gene activation. This is consistent with our results.

Microinjection of mutated E1A proteins has identified a domain at the C termini of the E1A proteins responsible for their nuclear localization and separate from the central region important in gene activation (23). However, the more extensive the C-terminal deletion of these proteins, the longer nuclear localization was delayed, and the more severe was their defect in gene activation. Although these experiments clearly identified the C termini of the proteins as responsible for nuclear localization, the evidence for separation of localization from effector functions such as gene activation was thus not as clear.

E1A has pleiotropic effects on cells, and it is not known whether these result from a single or from several primary functional activities. Some of the effects of E1A are probably indirect and result from its disturbance of cellular regulatory networks. When the tertiary structures and direct mechanism(s) of action of the E1A proteins have been determined, it may be possible to interpret some of the effects of different mutations on events in infected cells with confidence. Meanwhile, we suggest that it may be premature to equate the locations on the linear sequence of mutations that differentially influence the various effects of E1A on cells with domains of qualitatively different effector function in the protein.

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