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Reduced Microfilament Organization in Adenovirus Type 5-Infected Rat Embryo Cells: a Function of Early Region 1a

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The actin microfilament organization in rat embryo cells was examined by fluorescence microscopy with 7-nitrobenz-2-oxa-1,3-diazole-phallacidin and by electron microscopy, after mock infection or infection with adenovirus type 5 (Ad5). Infected cells showed severely reduced numbers of actin microfilaments and stress fibers, detectable early after infection. Mutants defective in Ad5 early genes were used to show that reduced microfilament organization was a function of the Ad5 transformation early gene 1a (E1a) and did not require expression of any other viral gene. The product of the E1a 13s mRNA was essential for the effect, although the 12s mRNA product appeared to contribute. Ad5 infection of the cells had no observable effect on total cell actin levels or on the ratio of monomeric to polymeric actin. E1a, therefore, affected only the higher-order organization of actin.

All adenoviruses are able to transform semipermissive cultured rodent cells (18, 34, 35). The genes responsible for transformation are located in the left-hand 12 to 14% of the viral genome, thus indicating that viral early genes 1a (E1a) and 1b (E1b) are needed for transformation (17, 45, 51). In fact, these two genes are both necessary and sufficient for full transformation (22, 29, 47).

E1a encodes three coterminal mRNAs during infection (5, 14, 40). A 9s mRNA is synthesized late in infection, that is, after viral DNA replication (49), but this mRNA has no role in transformation (23). Two mRNAs are synthesized early during infection, a 13s and a 12s mRNA, encoding products of 289 and 243 amino acids (aa), respectively. These two mRNAs have common 5′ and 3′ termini, differing only in the extent of splicing and a region encoding the 46 aa difference between their products (19, 40, 41).

The use of mutants defective in E1a has shown that both the 289- and the 243-aa products are required for full transformation (36, 57). E1a is already known to have several functions which may be relevant to the transformation process. These include the induction of two cellular genes: the 70,000-dalton heat-shock protein (39) and thymidine kinase (13). E1a is also responsible for the induction of cell cycle abnormalities and chromosome aberrations (2, 9, 10). Thymidine kinase induction, the cell cycle abnormalities, and chromosome aberrations have been shown to be functions of the 289-aa product (2, 2a, 9). The 243-aa product has recently been shown to have a role in efficient viral replication in growth-arrested cells (37, 50), and the presence of this protein also seems to be essential for anchorage-independent growth in transformed cells (37). In the transformation process, E1a is known to immortalize primary cultured cells (27, 44).

Transformation by a number of agents has been shown to result in severely reduced numbers of actin microfilaments and stress fibers. Viruses known to have this effect include adenovirus type 5 (Ad5) (20), simian virus 40 (43, 52), and Rous sarcoma virus (8, 53). Little is known of the mechanism of the microfilament reduction. In Rous sarcoma virus transformation, strong evidence suggests that the Rous sarcoma virus transforming protein $pp60^{v\text{-}src}$ phosphorylates tyrosine residues on vinculin (28, 46). Vinculin is located in the cell membrane close to where actin microfilaments attach and may in fact be the link between actin and the membrane (54). Levels of an actin-binding protein, tropomyosin, have also been shown to be reduced in transformed cells (26, 32, 33). The significance of these observations to reduction in microfilament numbers is not understood.

We are presently investigating early events in Ad5 infection of semipermissive rat cells that might be relevant to the transformation process. As part of this study we have examined the microfilament and stress fiber organization in normal and Ad5-infected rat cells.

MATERIALS AND METHODS

Cell growth and culture. Primary cultures of Wistar rat embryo fibroblasts were prepared as previously described (3). Cultures of cells were grown in Autopow medium (Flow Laboratories Inc., McClean, Va.), supplemented with 10% fetal calf serum in 175-cm^2 plastic tissue culture flasks.

Viruses and virus growth. Ad5 and mutant strains *dl*808, *in*351, *dl*350, *dl*356, *dl*327, *ts*37, and *ts*125 were grown and titrated in HeLa cells. Mutants *dl*312, *hr*1, *hr*A, *in*500, *PM*975, *dl*347, *dl*348, *dl*313, and *hr*7 were all grown and titrated in Ad5-transformed human 293 cells. Titrations were performed by the fluorescent cell-counting method (42). Virus inocula were stored at −70°C as crude cell lysates.

Ad2/5 PM975 (37) was a generous gift from A. J. Berk, University of California, Los Angeles; Ad5 *hr*A (48) was obtained from D. Solnick, Yale University; strain *in*500 (11) was from N. C. Jones, Purdue University; strain *hr*1 (24) was from B. W. Stillman, Cold Spring Harbor; strains *dl*312 (29), *dl*347 (57), and *dl*348 (57) were from T. Shenk, Princeton University.

The locations of the mutations in E1a are shown in Fig. 1. Mutants defective in E1b, *hr*7 (24) and *dl*313 (29), were obtained from B. W. Stillman, Cold Spring Harbor, and T. Shenk, Princeton University, respectively. Mutants *ts*37 (55) and *ts*125 (16) were gifts from J. Williams, Carnegie-Mellon University, Pittsburgh, Pa.

Mutant *dl*808, with a deletion from between 91.4 and 92.0

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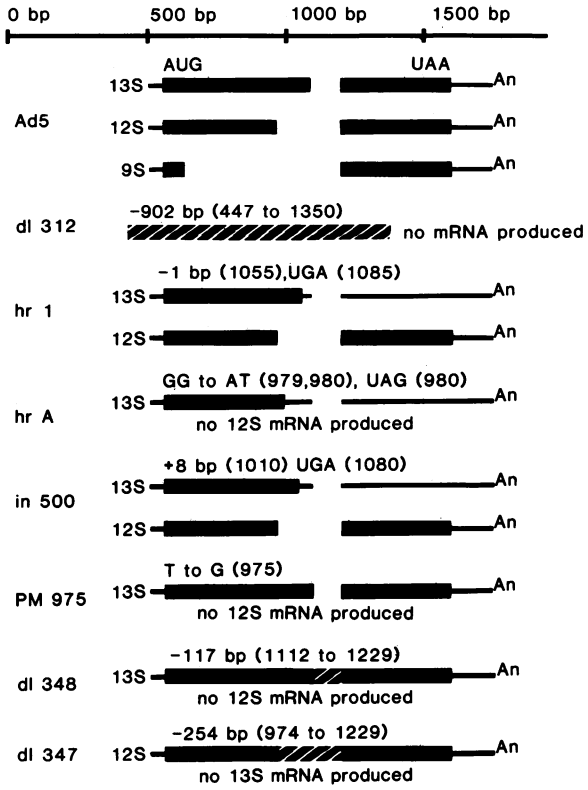

FIG. 1. Physical map of E1a mutants used for this study, modified from a previous map by Logan and Shenk (31). Symbols: ▬, protein coding region of the mRNA (—); ▰▰▰, sequence deletion. The numbers above mRNA symbols indicate the nature of the mutation and the mutation position in base pairs from the left end of the DNA. References for individual mutants are given in the text.

to between 97.2 and 98.4 map units (mu) (12) and thus defective in subregions 1 or 2 through 7 of early region E4, was kindly supplied by G. Ketner, The Johns Hopkins University. Other mutants defective in E3 and E4 were isolated and supplied by J. R. Cutt (D. N. Halbert, J. R. Cutt, and T. Shenk, submitted for publication). Briefly, strain *dl*327 has a deletion of 78.5 to 84.3 mu which causes extensive deletion of E3. The remaining mutants have a deletion from 75.9 to 84.3 mu, which removes the promoter and most of the encoding regions of E3, and additional E4 defects as follows: *dl*350, −3 base pairs at 98.4 mu in subregion 1; *in*351, +5 base pairs at 98 mu in subregion 1; and *dl*356, −2 base pairs at 91.9 mu in subregion 7.

Growth of cells. Primary rat cells were seeded at a density of 2.5×10^4 cells into 50-mm glass petri dishes containing three glass cover slips, covered with 5.0 ml of Autopow medium–10% fetal calf serum, and cultured at 37°C in a 5% CO_2, humidified atmosphere, until the cell density doubled.

Infection. After removal of the medium, cells were infected with Ad5 in 1.0 ml of Autopow medium–1% fetal calf serum for 1.5 h or with only 1.0 ml of Autopow medium–1% fetal calf serum for mock infection. The original medium was then replaced, and the cells were incubated at 37°C in a 5% CO_2, humidified atmosphere until examined. Experiments with strains *ts*37 and *ts*125 were done at both the permissive (32.5°C) and nonpermissive (39.5°C) temperatures.

Examination of microfilaments. Cover slip cultures were examined for microfilament organization by staining with 7-nitrobenz-2-oxa-1,3-diazole-phallacidin (NBD-Phallacidin) (1). The cultures were washed with phosphate-buffered saline (PBS), fixed in 3.7% paraformaldehyde in PBS for 10 min at room temperature, rinsed twice with PBS, extracted with acetone for 3 to 5 min at −18°C, and air dried. Each cover slip was then covered with 50 μl of PBS containing 8 ng of NBD-Phallacidin for 20 min at room temperature. Controls were performed by omitting the NBD-Phallacidin. Cover slips were then rinsed twice with PBS, mounted cell surface down in 50% glycerol–PBS, and sealed with nail varnish. Slides were examined at ×400 magnification by using an Olympus BH2 microscope with a BH-RFL-W fluorescence vertical illuminator, excitation filter blue (490 nm), and 0530 Barrier filter. For photography an Olympus PM-2 camera was used with Kodak Tri-X-Pan film.

Test for viral antigens. Cover slip cultures were examined for viral antigen by the indirect-fluorescent-antibody technique with P-antiserum (3, 25). Slides were viewed as described above.

Scoring of microfilament disruption or P-antigen-positive cells. Coded cover slip cultures were scored for the presence of P-antigen or the absence of microfilaments and stress fibers. In each case three cover slip cultures per experiment were examined, and 10 counts were made per cover slip. Counts were expressed as the percentage of the total cells counted that showed P-antigen or absence of microfilaments and stress fibers. The significance of the results was assessed by Student's *t* test.

Transmission electron microscopy. Cells were grown on cover slips as described above, fixed in 2% glutaraldehyde for 1 h, washed twice in 0.1 M cacodylate buffer, and refixed in 2% osmium for 1 to 2.5 h. After being washed with distilled water, cells were labeled with 1% uranyl acetate for 1.5 h, dehydrated serially in 50 to 95% ethanol twice,

TABLE 1. Effect of time and MOI on the expression of viral P-antigen or on microfilament disruption in Ad5-infected rat cells

MOI (iu of Ad5 per cell)	% Cells (±SE), at hours postinfection, that:							
	Expressed P-antigen[a]				Lacked microfilaments[b]			
	6	24	48	72	6	24	48	72
Mock infected	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	8.8 (±3.4)[c]	11.3 (±2.4)	21.3 (±8.6)	17.4 (±3.1)
1	2.9 (±1.5)[c]	5.4 (±2.0)[c]	4.8 (±1.5)[c]	9.0 (±1.5)	11.8 (±2.6)[c]	24.1 (±5.2)	56.5 (±12.2)	51.0 (±6.4)
20	1.8 (±1.0)[c]	7.2 (±2.3)[c]	22.3 (±2.0)	28.2 (±4.5)	12.6 (±3.5)[c]	35.6 (±3.9)	55.9 (±9.5)	48.1 (±5.2)
50	7.3 (±1.9)[c]	14.4 (±2.5)	39.1 (±3.4)	32.3 (±5.2)	13.2 (±4.4)[c]	53.6 (±8.0)	71.7 (±6.5)	69.6 (±9.0)
100	4.4 (±3.0)[c]	19.3 (±5.6)	37.1 (±7.0)	44.7 (±7.1)	39.6 (±5.6)	49.3 (±7.7)	78.1 (±9.2)	62.7 (±2.7)

[a] Determined by indirect-immunofluorescence staining with P-antiserum as described in the text.
[b] Determined by NBD-Phallacidin staining as described in the text.
[c] Results not significant at the 1.0% level. All other results were significant at the 1.0% level.

embedded in 50% ethanol–resin for 3 h, and left overnight in absolute resin. Cover slips were removed from the resin with liquid nitrogen. Thin sections of resin were prepared and examined with a Philips 201 transmission electron microscope.

Measurement of actin content. Cells were infected as described above, except that cells were grown in 75-cm^2 plastic tissue culture flasks. Lysed cell suspensions were prepared as described by Gowing et al. (21), except that cells were detached from culture flasks by gentle scraping with a rubber policeman and that cells in suspension were counted with a hemocytometer.

The total actin and monomeric actin of cell suspensions were measured by using the DNase 1 inhibition assay (6, 7). Under the conditions used, polymeric actin has no significant effect on the activity of DNase 1. The assay of DNase 1 activity is based on an increase in the A_{260} of DNA when it is hydrolyzed by DNase 1. There is a linear relationship between the degree of inhibition of enzyme activity and the actin monomer content between 20 and 70% inhibition; therefore, an appropriate standard curve enables calculation of actin monomer contents of lysed cell suspensions. The total actin content can be similarly determined by treatment of a sample of lysed cell suspension with 0.75 M guanidinium hydrochloride which depolymerizes polymeric actin to native monomer actin. Standard curves were prepared by using freshly purified monomeric actin from rabbit skeletal muscle, a generous gift from R. L. Tellam.

RESULTS

Microfilament organization in mock-infected and Ad5-infected cells. We examined the microfilament organization in normal and Ad5-infected rat cells. In all cases culture conditions were chosen in which low cell density and 10% serum assured that density-dependent and serum-dependent inhibition of growth were not in effect. We initially decided to compare the microfilament organization in mock-infected cells and in cells infected with 20 infectious units (iu) of Ad5 per cell, at 48 h after mock or virus infection. The 48-h incubation period was chosen to ensure that all viral early genes and most late genes had been expressed including the Ad5 delayed early gene E2a (38) whose product is the main target of P-antiserum. That viral E2a protein was present in virus-infected cells was confirmed by indirect immunofluorescence staining with P-antiserum, as described above (Table 1; Fig. 2A and B).

In mock-infected cells, NBD-Phallacidin staining of microfilaments revealed numerous bundles of microfilaments, oriented parallel to each other. These microfilament bundles often extended the whole length of the cells (Fig. 2C), which were fibroblastic in morphology. In contrast, Ad5-infected cells were more rounded, and microfilament bundles were rare or absent (Fig. 2D).

To determine whether microfilaments were indeed absent from the Ad5-infected cells, we examined thin sections of cells by transmission electron microscopy. In mock-infected cells, we observed bundles of microfilaments, together with individual filaments which were 6 to 7 nm in diameter (Fig. 2E). In serial sections of Ad5-infected cells, no microfilament bundles or individual filaments were found even at double the magnification used for mock-infected cells (Fig. 2F).

Kinetics of microfilament disappearance. Having observed that Ad5 infection caused a severe disruption of microfilament organization, we then determined when this effect first appeared during infection. Rat cells infected with 1, 20, 50, or 100 iu of Ad5 per cell, together with mock-infected cells, were examined at 6, 24, 48, or 72 h after infection. Cells were stained for viral P-antigen or microfilaments. The results are summarized in Table 1.

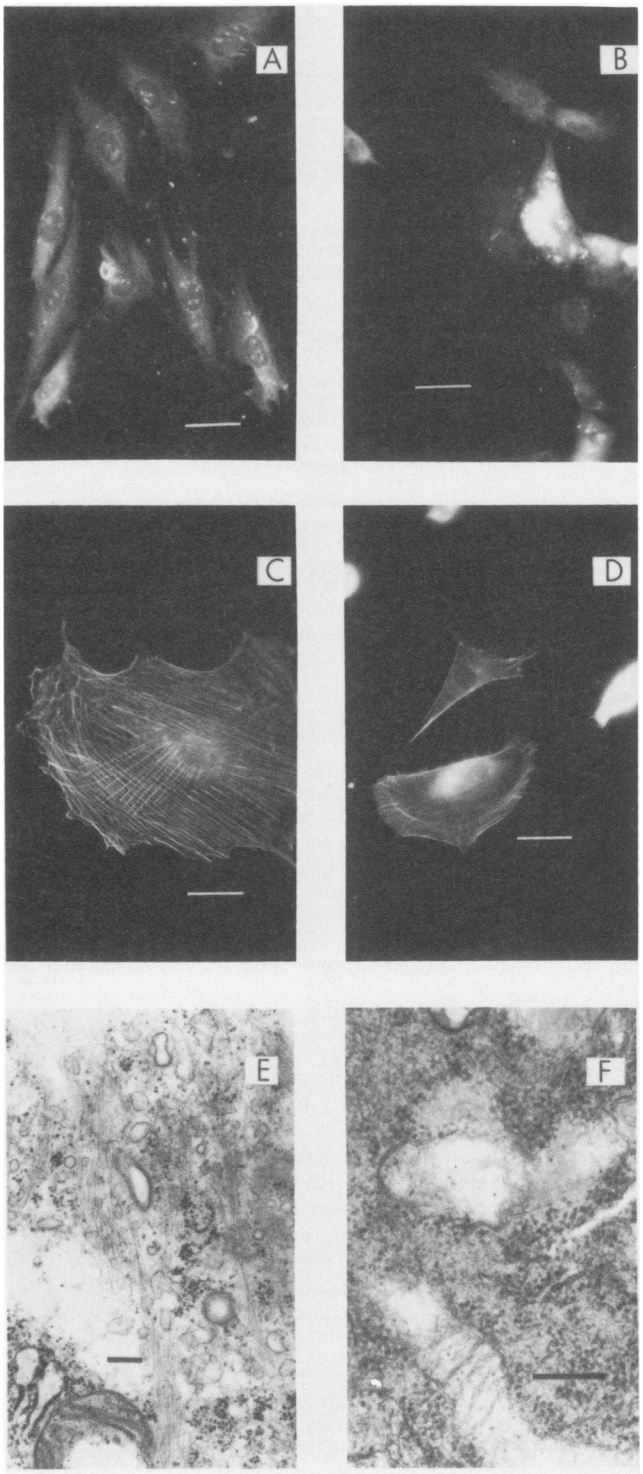

FIG. 2. P-antigen expression and microfilament organization in mock-infected (A, C, E) and Adenovirus-infected (B, D, F) rat embryo cells. Cells were stained with P-antiserum (A, B) or NBD-Phallacidin (C, D) or thin sectioned for electron microscopy (E, F) as described in the text. Bars, 10 μm (A–D) and 100 nm (E, F).

In infected cells the results for P-antigen production confirmed previous findings (38), which showed that infected rat cells positively staining for P-antigen first appeared about 24 h after infection and reached a peak 48 to 72 h after infection. Our results also showed that a small but nonsignificant proportion of cells positively stained for P-antigen at 6 h after infection. NBD-Phallacidin staining of microfilaments in mock-infected cells showed that about 20% of cells (see Table 1) did not contain microfilaments. In subsequent experiments we found that this high value was a function of the time allowed for cells to adhere to the cover slips. By increasing this time from 1 to 2 days before performing an experiment, the level was reduced to 3%. Infected cells showed that a significant reduction in microfilament organization had occurred by 24 h after infection, increasing to a peak by 48 h after infection. Cells infected with 100 iu of Ad5 per cell also showed a significant reduction in microfilaments as early as 6 h after infection in this experiment. In subsequent experiments, the effect was not significant until 12 to 14 h after infection. These results indicated that microfilament disruption was an early effect of Ad5 infection and occurred before the expression of viral delayed early genes.

E1a gene is responsible for microfilament disruption. The early appearance of infected cells lacking microfilaments indicated that a viral early gene might be responsible for this disruption. We used a series of mutants defective in Ad5 early genes to infect cells and determine whether or not these mutants could produce viral P-antigen or microfilament disruption. The mutants used are described above (E2, E3, and E4 mutants) and in Fig. 1 (E1a mutant *dl*312). Cells were mock infected or infected with 20 iu of Ad5 per cell and incubated for 48 h before being examined.

The E2a gene whose product is labeled by P-antiserum is known to be under positive control of the 13s mRNA product of E1a (4, 30, 31, 57). For all of the mutants except *dl*312, P-antigen expression was unaffected by the mutations present (Fig. 3). *dl*312, an E1a mutant, produced no significant P-antigen staining. The results were very similar for microfilament disruption. Mutant *dl*312 caused no significant microfilament disruption, whereas mutants defective in E1b, E2, E3, and E4 gave wild-type levels of microfilament disruption. These results indicated that the Ad5 E1a gene was the only gene essential for this effect.

Responsibility of the 13s mRNA product of E1a for microfilament disruption. With the knowledge that the E1a gene was essential for microfilament disruption, we used a series of mutants with E1a defects to examine the roles of the E1a products in microfilament disruption. As in the previous study with viral early-gene mutants, we also examined the ability of the E1a mutants to produce P-antigen as an indication that the mutants were functioning as expected. The mutants are described in Fig. 1. The cells were mock infected or infected with 20 iu of virus per cell and incubated for 48 h before examination; the results for these mutants are illustrated in Fig. 4. The absence of P-antigen staining or microfilament disruption in cells infected with virus strain *hr*A (severely truncated 13s mRNA product and no 12s mRNA produced), results also seen with mutant *dl*312 (see above), confirmed a requirement for E1a in these effects. The absence of P-antigen-staining cells and microfilament disruption were also seen for cells infected with strains *in*500 (truncated 13s mRNA product and normal 12s mRNA produced) and *dl*347 (no 13s mRNA and normal 12s mRNA produced). These results confirmed the known requirement for the 13s mRNA product in early-gene expression but also indicated a requirement for the 13s mRNA product in

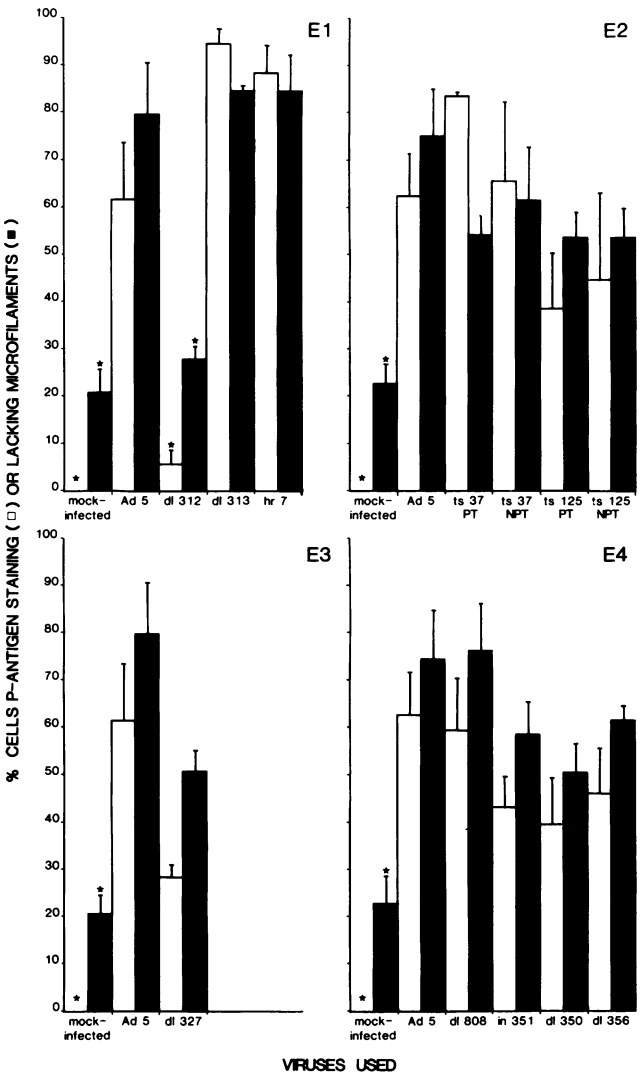

FIG. 3. Effect of adenovirus early-gene mutations on viral P-antigen expression and microfilament organization in infected rat embryo cells. Cells were infected and examined as described in the text. NPT and PT, for *ts*37 and *ts*125, indicate the nonpermissive (39.5°C) and permissive (32.5°C) temperatures, respectively. Results labeled with a star (★) are significantly less than Ad5 results at the 1.0% level. References for individual mutants are given in the text.

microfilament disruption. Infected cells staining positively for P-antigen and showing a significant microfilament disruption were seen with both strains *PM*975 and *dl*348 (normal 13s mRNA and no 12s mRNA produced), which indicated that the 12s mRNA product was not essential for either function. However, the levels of microfilament disruption were slightly less than levels caused by wild-type Ad5, which indicated a possible requirement for the 12s mRNA product for a full viral effect.

Strain *hr*1 (truncated 13s mRNA product and normal 12s mRNA produced) gave small but significant levels of P-antigen-staining cells and levels of microfilament disruption much greater than those in mock-infected cells, although significantly less than levels caused by the wild-type Ad5. We suggest that these two features indicated a residual 13s mRNA function in strain *hr*1 (see below).

Actin content in Ad5-infected cells. To determine whether

the disruption of microfilaments by Ad5 was due to decreased levels of total cell actin or polymerized actin, we investigated these levels in mock-infected and Ad5-infected rat cells. The results of one such assay are summarized in Table 2. Repeat experiments gave similar results (data not shown).

The total actin contents of mock-infected and Ad5-infected cells were found to be very similar. Similarly, the ratios of actin monomer (G-actin) to total actin were almost identical for the two cell lysates. We conclude from these results that Ad5 had no observable effect on the total actin content or ratio of G-actin to total actin in infected cells.

DISCUSSION

Our studies have shown that Ad5 infection of semipermissive rat embryo cells caused a disruption in the microfilament organization of such cells. This disruption could be detected early after infection, before detection of the viral delayed early gene E2a in these cells. Since Ad5-transformed rodent cells have been shown to have few microfilaments (20), we believe that this effect of Ad5 infection may be important in leading to production of the transformed phenotype. Transformed cells often exhibit reduced substrate adhesion and altered morphology. Indeed other workers have suggested a relationship between cell shape and adhesion and microfilament disruption (15, 56). In our studies microfilament disruption occurred with cells that were still attached to the cover slips but with a more rounded morphology; therefore, we suggest that microfilament disruption could be related to altered cell morphology leading to reduced adhesion of the cells.

By using a series of mutants in viral early genes, we were able to show that the microfilament disruption was a function of Ad5 transformation gene E1a and did not involve the other transformation gene E1b. Furthermore, by the use of mutants defective in E1a, we established an essential role for the 13s mRNA product.

Our result for mutant *hr*1 did not seem to fit into this interpretation (see Fig. 4). *hr*1 and *in*500 were similar E1a mutants (see Fig. 1) with a truncated 13s mRNA product and a normal 12s mRNA produced. Mutant *in*500 gave no significant P-antigen expression or microfilament disruption, but *hr*1 gave low levels of P-antigen expression and levels of microfilament disruption that were much greater than those of mock-infected cells, although still significantly less than those caused by wild-type virus. The difference between the mutants is a small region of 39 nucleotides in the 5′ portion of the E1a mRNA (see Fig. 1). This region is normal in *hr*1 but has been altered by a frameshift mutation in *in*500. We suggest that this region of 39 nucleotides confers a residual 13s mRNA function in *hr*1, enabling microfilament disruption to proceed, but is not sufficient for the P-antigen expression seen with wild-type Ad5.

We also found that in mutants lacking a 12s mRNA E1a product, the levels of microfilament disruption were always slightly less than those seen for wild-type Ad5. We suggest that the E1a 12s mRNA product might be required for a full virus effect, perhaps by a cooperative interaction between the products of the 13s and 12s mRNAs of E1a, but that the 243-aa product of the 12s E1a mRNA is unable to act alone. A similar requirement for both E1a products has been shown for full transformation by Ad2 (36).

We found that Ad5-infected cells had similar total actin contents to those of mock-infected cells. Thus, the microfilament disruption could not be caused by an E1a reduction of actin biosynthesis to levels below the critical actin concentration required for actin monomers to become polymerized or by degradation of actin. The ratio of actin monomer to total actin content was also very similar in infected and mock-infected cells. By subtraction, the levels of polymer-

TABLE 2. Actin content of cell lysates[a]

Cell sample	Total actin per cell (pg)	Actin monomer per cell (pg)	Actin polymer per cell (pg)
Mock infected	16.5	4.4	12.1
Ad5 infected	15.7	4.1	11.6

[a] See text for a description of the DNase 1 inhibition assay of actin content.

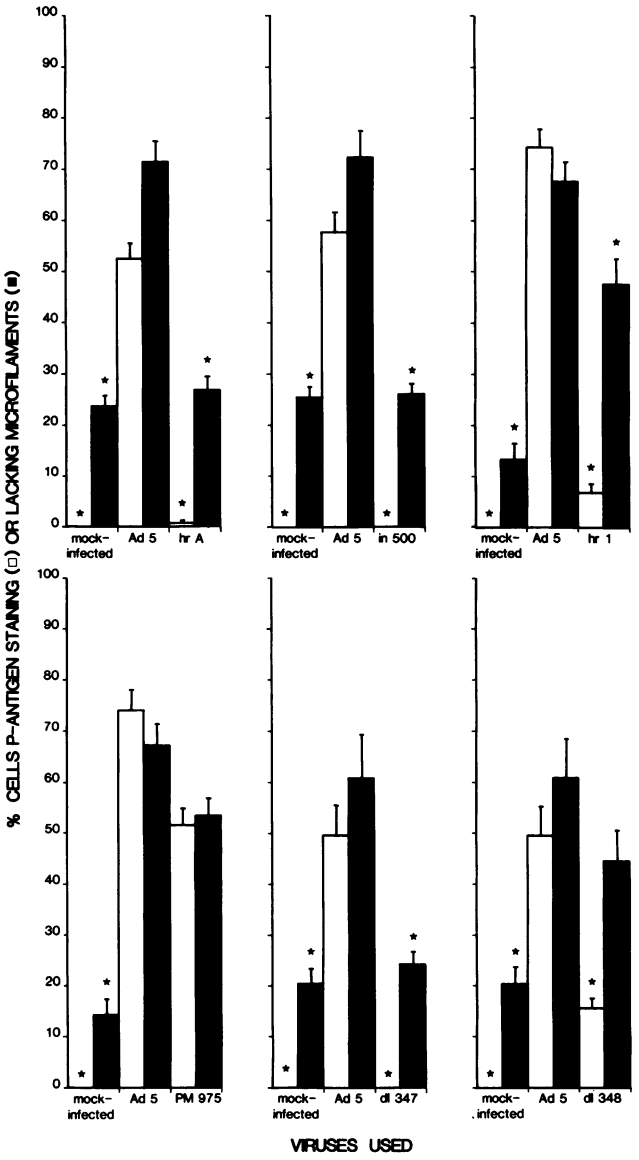

FIG. 4. Effect of adenovirus E1a mutations on viral P-antigen expression and microfilament organization in infected rat embryo cells. Cells were infected and examined as described in the text. Results labeled with a star (★) are significantly less than Ad5 results at the 1% level. References for individual mutants are given in the text.

ized actin were, therefore, very similar in the two cell samples. The assay, though, does not distinguish between different higher-order structures of polymeric-actin assembly. We believe the loss of microfilaments and stress fibers in infected cells could be caused by alterations in the regulation of assembly of such higher-order actin structures. This regulation is normally under the control of various actin-binding proteins (for a review, see reference 54). Increased levels of phosphorylation of vinculin, an actin-regulatory protein, have already been shown for Rous sarcoma virus-transformed cells (46). Levels of tropomyosin, another actin-regulatory protein, have also been shown to be reduced in cells transformed by a number of agents including adenovirus (26, 32, 33). We are therefore investigating possible effects of E1a on the actin-regulatory proteins.

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