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Second-Strand Synthesis Is a Rate-Limiting Step for Efficient Transduction by Recombinant Adeno-Associated Virus Vectors

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The ability of recombinant adeno-associated virus (AAV) to transduce cells with a marker gene in vitro was found to be substantially increased by the presence of adenovirus. Transfection experiments with adenovirus genomic DNA suggest that this increase is not facilitated by adenovirus-mediated viral uptake but is instead dependent on adenovirus gene expression. Using various adenovirus mutants, we were able to map this function to early-region E4 open reading frame 6. Plasmid expression of open reading frame 6 protein in cells infected with recombinant AAV increased transduction between 100- and 1,000-fold. The increase in transduction was not dependent on the recombinant AAV gene cassette but instead appeared to involve an immediate early step of the AAV life cycle. Chemical and physical agents that have been shown to induce helper-free replication of wild-type AAV were also able to stimulate recombinant AAV transduction, suggesting that the phenomenon might affect AAV DNA replication. Further experiments showed that viral uncoating was not affected and that the rate-limiting step involved synthesis of a second strand on the single-stranded genomic AAV DNA. These data suggest that the adenovirus E4 region, as well as chemical and physical agents, can play an essential role in an immediate-early step of the AAV life cycle, specifically in second-strand synthesis, and have important implications for the use of AAV vectors in gene therapy protocols.

Adeno-associated virus (AAV) is a nonenveloped virus with a single-stranded linear DNA genome of 4,680 bp (6, 28). AAV relies on the presence of a helper virus, e.g., adenovirus (Ad) or herpesvirus, for efficient lytic growth. When AAV infects a cell in the absence of a helper virus, it generally does not undergo lytic replication. Rather, it remains latent (46), integrating into the host cell genome (11). This integration of the AAV genome is preferentially targeted to the q terminus of chromosome 19 (19, 20, 36). Ad superinfection of cells latently infected with AAV results in the rescue and replication of the AAV proviral genome, bridging the two components of the biphasic life cycle of AAV (5).

The role of Ad in the early steps of an AAV infection is complex, with at least five Ad genes contributing to the helper effect: E1A, E1B, E2A, VA, and E4 open reading frame 6 (ORF6) (4, 7, 8, 16, 34). These effects of the Ad genes on AAV include transcriptional activation (E1A), mRNA maturation (E1B and E4 ORF6), and translation enhancement (VA and E2A). Although these Ad genes are necessary for efficient replication in a coinfection, limited replication of AAV can occur in the absence of Ad helper virus if the cells are treated with toxic agents such as UV light (45), hydroxyurea (HU) (46), or heat shock (47). Taken together, these results suggest that the specific cellular milieu, which is significantly modulated by Ad infection, may provide all the rate-limiting factors required for both the replicative and latent components of the AAV life cycle.

The generation of infectious AAV plasmids has made it possible to dissect the AAV life cycle and to test AAV as a vector for gene therapy (21, 31, 32). Studies with such plasmids have shown that the AAV 145-bp inverted terminal repeats are the only *cis*-acting sequences necessary for rescue from the infectious plasmid, replication, and integration of the AAV genome (22, 31, 33, 35).

In this study, we have used an AAV recombinant, consisting of the viral *cis*-acting termini and a marker gene, to further explore the role of Ad in the early steps of an AAV infection. By using an AAV β-galactosidase (*lacZ*) recombinant (rAAV), we were able to determine that an immediate-early step, namely, second-strand synthesis, was rate limiting in the absence of Ad. In coinfected cells, the E4 ORF6 region of Ad facilitated this reaction. It appears that this function is independent of previous AAV helper roles assigned to E4 ORF6 (16, 34). The demonstration that E4 ORF6 is required for an immediate-early step in the AAV life cycle also suggests that it may play an as yet unknown role in Ad infections. The Ad E4 ORF6 function can be supplied to various degrees in rAAV-transduced cells by exposure to heat shock or genotoxic reagents. Our results suggest that all these factors are acting through a similar pathway that results in the efficient production of a duplex AAV molecule. These results have important bearing on the AAV life cycle, as well as on the use of AAV vectors for gene therapy. Second-strand synthesis is apparently a rate-limiting step for transduction of therapeutic genes by current AAV vectors.

MATERIALS AND METHODS

Cells, viruses, and DNA. Human 293 (15) and HeLa (13) cells were grown in Dulbecco's modified Eagle's medium (Gibco, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum at 37°C under 5% CO_2. The Ad mutants *dl*309 (18), *dl*312 (12, 18), *dl*324 (39), *dl*327 (29, 38), and *dl*331 (39) have been described previously. The Ad mutants *dl*366*, E4*dl*ORF1-4, E4*in*ORF6, E4*in*ORF3, E4*in*ORF3/inORF6, *dl*366*+ORF1–2, *dl*366*+ORF3, *dl*366*+ORF4, and *dl*366*+ORF6/7 were generous gifts from Pat Hearing, State University of New York, Stony Brook, and are described elsewhere (16). When applicable, Ad virus titers were determined by a plaque assay (43). Ad particle numbers were determined by diluting 15 μl of the virus preparation in 285 μl of buffer containing 0.1% (wt/vol) sodium dodecyl sulfate, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA; agitating the mixture for 5 min; pelleting debris by centrifugation

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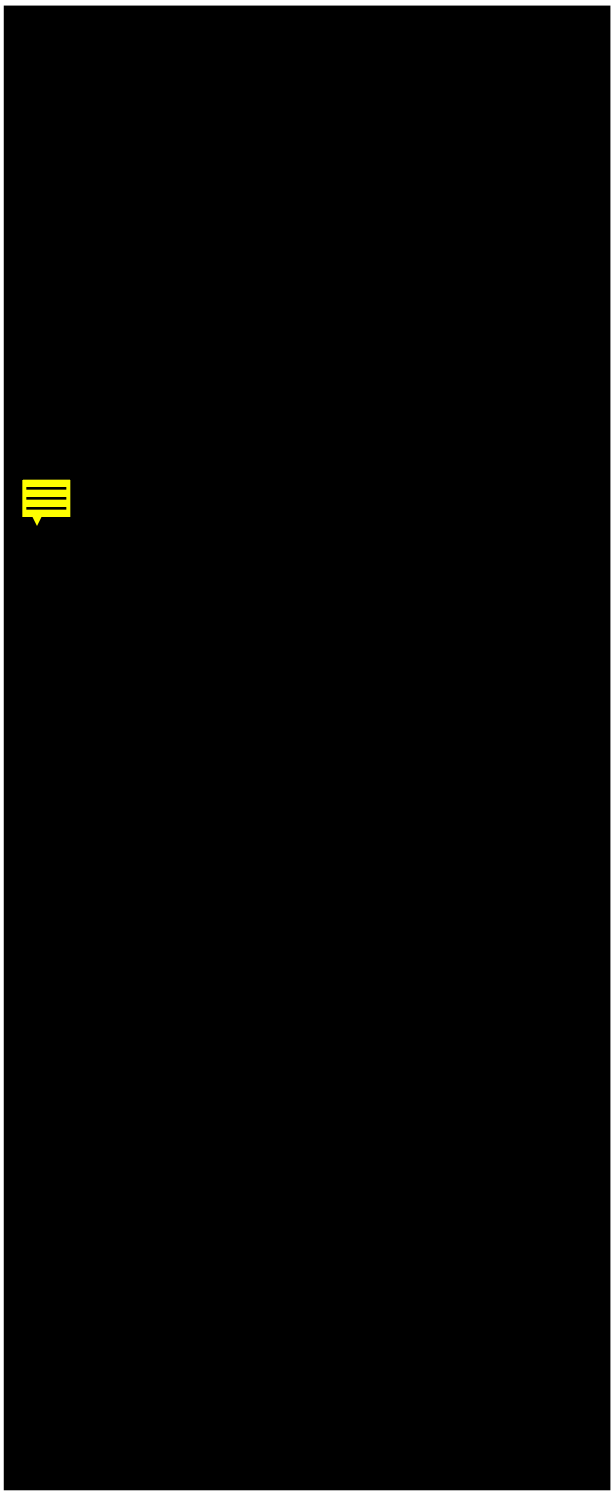


FIG. 1. Effect of Ad on transduction by rAAV. (A and B) 293 cells infected with either rAAV alone (A) or rAAV plus Ad (B). (C) Effect of increasing amounts of Ad on rAAV transduction. Cells positive for transduction have dark blue nuclei. All cells were fixed and stained 24 h p.i. as described in Materials and Methods.

(13,000 × *g*) for 5 min; and measuring the A_{260} of the supernatant (1 A_{260} unit = 10^{12} particles per ml).

Plasmids pAB-11 and pAAV/Ad were described previously (14, 33). Briefly, pAB-11 contains the β-galactosidase gene under the control of the cytomegalovirus (CMV) immediate-early promoter and flanked by the AAV inverted terminal repeats. pAAV/Ad contains the AAV replication and capsid protein-coding regions flanked by the Ad terminal repeats. Plasmid pCMV/E4ORF6 was created by amplifying the Ad5 E4 ORF6 region by PCR and subsequently cloning it into the pBK/CMV (Stratagene, La Jolla, Calif.) eukaryotic expression plasmid. The sequence was confirmed by sequencing (27).

TABLE 1. Effect of Ad mutants on rAAV expression[a]

Ad mutant	Region affected	Enhancement
*dl*309	E3	+
*dl*312	E1a	+[b]
*dl*324	E1a, E1b, IX, E3	+[b]
*dl*327	E3	+
*dl*331	VAI	+
*dl*366*	E4	−

[a] HeLa cells were coinfected with rAAV and the indicated Ad mutants. The cells were infected and stained for β-galactosidase activity as described in Materials and Methods.

[b] A high multiplicity of infection (>1,000 particles per cell) is needed to overcome the dependence of other genes on the E1 region (3, 17).

Viral infections and transient transfections. At 24 h before infection, 293 and HeLa cells were seeded at 8.5×10^4 and 4.3×10^4 cells per cm^2, respectively. The cells were infected for 1 h at 37°C with the indicated virus diluted in DMEM–2% heat-inactivated fetal bovine serum. For all infections involving rAAV, a multiplicity of infection of 1 was used. To stop the adsorption process, the virus-containing medium was removed and replaced with DMEM–10% heat-inactivated fetal bovine serum. The infections were allowed to continue for the indicated time.

Calcium phosphate (GIBCO-BRL, Grand Island, N.Y.)-mediated transfection was used for all transfections as specified by the manufacturer directions. All transfections were allowed to proceed for 12 h.

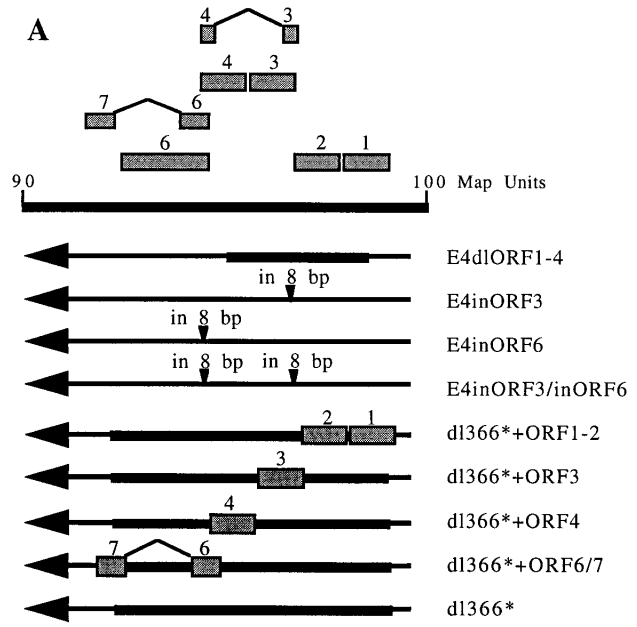
Generation of rAAV. rAAV was generated as described previously with minor modifications (14). Dishes (diameter, 10 cm) of subconfluent 293 cells were transfected with pAB-11 (1 μg), pAAV/Ad (9 μg), and *dl*309 DNA digested with *Xba*I (10 μg). The cells were collected 48 h after transfection, and the virus was released from the cells by sonication. The rAAV particles were purified by isopycnic centrifugation in CsCl (ρ = 1.4 g/ml) in an SW41 rotor (Beckman, Palo Alto, Calif.) at 40,000 rpm for 48 h at 10°C. Fractions from the gradient were collected and assayed for *lacZ* transduction ability. Peak fractions were pooled, dialyzed against 15% glycerol–1 M NaCl–10 mM Tris-HCl (pH 7.5)–1 mM EDTA, and stored at −20°C.

β-Galactosidase staining. Cells were fixed and stained for β-galactosidase activity by published procedures (37). We determined the number of blue nuclei 24 to 48 h after staining. Each plate or well was examined with an inverted microscope, and an optimum magnification that showed between 1 and 50 blue nuclei per field was chosen. The blue nuclei were counted in 10 randomly selected fields, the results were averaged, and the standard error of the mean was determined.

Treatment of cells. Cells were seeded as described above and subjected to X-ray or UV treatment the next day. For X-ray treatment, the cells were subjected to an X-ray beam from a linear accelerator, and for UV treatment, a Stratalinker (Stratagene, La Jolla, Calif.) was used. Immediately following the treatment, the cells were infected. For heat shock experiments, the medium was exchanged with medium heated to 42.5°C and the cells were incubated at 42.5°C for the indicated times. Immediately following the heat shock, cells were infected as described above. For HU treatment, the cells were seeded as described above and were treated 24 h later by supplementing the medium with HU to a final concentration of 10 mM.

Isolation of DNA from cells. Virion-protected DNA was isolated by resuspending 5×10^5 cells in 500 μl of 0.2% deoxycholate–10% ethanol–50 mM Tris (pH 8) for 1 h. The lysate was then centrifuged (13,000 × *g*) for 10 min, the supernatant was collected, RNase was added to 20 μg/ml, $CaCl_2$ and $MgCl_2$ were added to 2 mM, and DNase was added to 200 μg/ml. The mixture was incubated at 37°C for 90 min. EDTA and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were added to a final concentration of 10 mM, 1/20 volume of 10% sarcosine was added, and the mixture was heated to 70°C for 10 min. The mixture was then cooled to 37°C, proteinase K was added to a final concentration of 1 mg/ml, and the mixture was incubated for 2 h at 37°C. The mixture was then subjected to phenol extraction followed by chloroform-isoamyl alcohol (24:1) extraction, and DNA was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol. The DNA was resuspended in Tris-EDTA (TE) and allowed to reanneal for 2 h at 37°C before being subjected to fractionation on an 0.8% agarose gel.

Low-molecular-weight genomic DNA was isolated by the method of Hirt as described by McMaster et al. (24). Agarose gel electrophoresis, alkaline agarose gel electrophoresis, and Southern blots were performed as described previously (23).



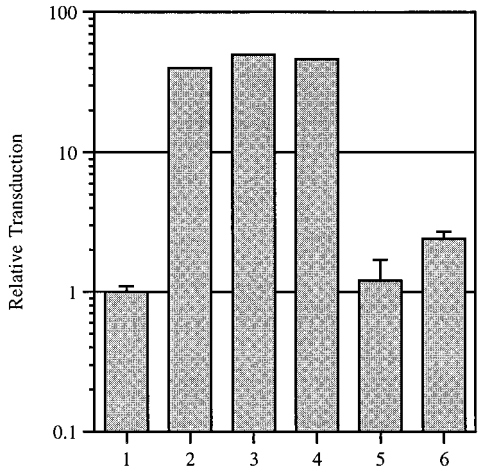


FIG. 3. Expression of E4 ORF6 from a plasmid is able to increase transduction by rAAV. 293 cells were transfected with plasmid DNA as indicated. At 24 h later, the cells were infected with the appropriate virus. The cells were fixed and stained for β-galactosidase activity as described in Materials and Methods 24 h p.i. Lanes: 1, rAAV alone; 2, rAAV plus *dl*309; 3, rAAV plus pCMV/E4ORF6; 4, rAAV plus pCMV/E4ORF6 plus E4inORF6; 5, rAAV plus pUC119; 6, rAAV plus pUC119 plus E4inORF6.

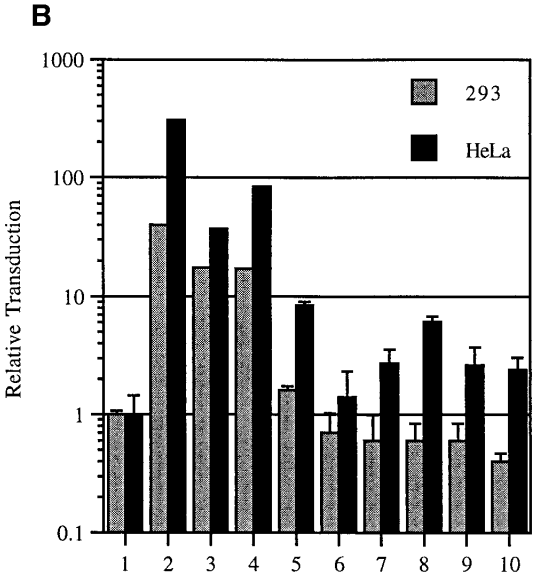


FIG. 2. Effect of various Ad E4 mutants on rAAV transduction. 293 or HeLa cells were infected and stained for β-galactosidase activity as described in Materials and Methods. (A) Maps of various mutations (thick black lines indicate deletions; black arrows indicate insertions; gray boxes indicate ORFs). (B) Ability of various mutants to induce rAAV transduction. Lanes: 1, no virus; 2, *dl*309; 3, E4*dl*ORF 1-4; 4, E4*in*ORF3; 5, E4*in*ORF6; 6, E4*in*ORF3/*in*ORF6; 7, *dl*366*+ORF1–2; 8, *dl*366*+ORF3; 9, *dl*366*+ORF4; 10, *dl*366*+ORF6/7.

RESULTS

rAAV vector transduction is enhanced by Ad coinfection. We studied the effect of Ad on the efficiency of transduction by an AAV vector by using an rAAV that carries the *lacZ* gene controlled by the human CMV major immediate-early promoter (CMV/*lacZ*). The number of cells expressing a detectable amount of β-galactosidase from the rAAV virus increased by as much as a factor of 1,000 in the presence of Ad (Fig. 1A and B). The maximal increase in rAAV transduction was achieved at an input of about 100 Ad particles per cell (Fig. 1C). Since 100 Ad particles is equivalent to 1 to 2 PFU, this result indicates that only about one functional Ad unit is required per cell. This effect is not unique to 293 cells; it is equally dramatic in other cell lines such as HeLa (see Fig. 2B).

Ad could affect the efficiency of rAAV transduction by directly modulating the expression of the CMV/*lacZ* gene carried on the rAAV genome. However, this mode of action seems unlikely, since Ad did not influence expression of this marker gene when it was introduced to cells on a plasmid by transfection or when it was present integrated in a stable cell line (data not shown). These results favor the hypothesis that the enhancing effect of Ad is due to a unique aspect of AAV virion-mediated gene delivery.

Ad induction of rAAV transduction maps to the Ad E4 transcription unit. To determine which Ad gene product(s) is necessary for the induction of rAAV gene expression, a panel of Ad mutants was used in a coinfection assay. These viruses carry specific mutations in the E1 (*dl*312 and *dl*324), VAI (*dl*331), E3 (*dl*309, *dl*324, and *dl*327), or E4 (*dl*366) regions of Ad (Table 1). Earlier studies demonstrated that the E1, VAI, and E4 regions are necessary for efficient replication of wild-type AAV (7, 8, 26, 34, 39). It is also possible that these regions are involved in an immediate-early step of the AAV life cycle. The above Ad mutants were individually coinfected into HeLa cells with rAAV and scored for their ability to enhance transduction by assaying *lacZ* expression 24 h postinfection (p.i.). Analysis of these coinfections demonstrated that only *dl*366*, an early-region E4$^-$ mutant, was unable to enhance *lacZ* transduction by rAAV (Table 1). This result focused the search for the Ad gene responsible for the increased transduction to the seven known ORFs of the E4 region.

Ad E4 ORF6 enhances rAAV transduction. The Ad E4 region has the potential to code for seven proteins, at least one of which, ORF6, is necessary for a productive wild-type AAV

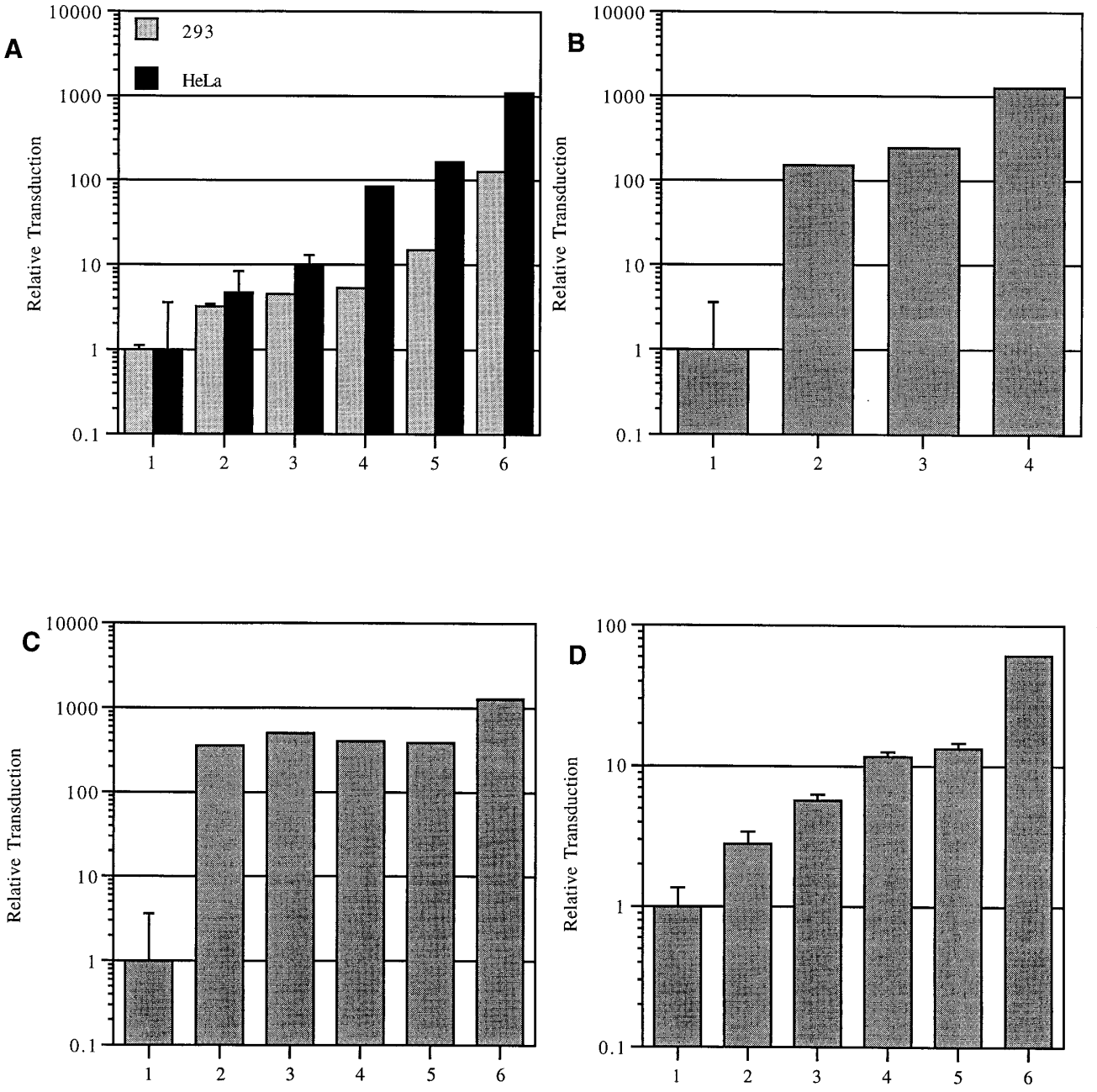


FIG. 4. Effect of various treatments on transduction by rAAV. 293 or HeLa cells were infected with rAAV, treated with the indicated agents, and stained for β-galactosidase activity as described in Materials and Methods. (A) Heat shock treatment at 42.5°C. Lanes: 1, rAAV alone; 2, 1 h at 42.5°C; 3, 2 h at 42.5°C; 4, 3 h at 42.5°C; 5, 4 h at 42.5°C; 6, rAAV plus *dl*309. (B) HU treatment. Lanes: 1, rAAV alone; 2, rAAV plus *dl*309 plus HU; 3, rAAV plus HU until infection; 4, rAAV plus *dl*309. (C) UV treatment. Lanes: 1, rAAV alone; 2, 15 J/m^2; 3, 20 J/m^2; 4, 25 J/m^2; 5, 30 J/m^2; 6, rAAV plus *dl*309. (D) X-ray treatment. Lanes: 1, rAAV alone; 2, 200 rads; 3, 800 rads; 4, 1,400 rads; 5, 2,000 rads; 6, rAAV plus *dl*309.

infection (9, 16, 26, 34). To determine which E4 gene(s) is necessary for induction, a number of Ad mutants that express one or more of the E4 proteins (Fig. 2A) were individually coinfected into HeLa or 293 cells with rAAV and assayed for transduction (Fig. 2B). In 293 cells, *dl*309 (wild-type Ad) increased the transduction by rAAV approximately 40-fold compared with rAAV alone (Fig. 2B, compare lanes 1 and 2). Ad mutants that did not express ORF6 were unable to increase the transduction by rAAV compared with rAAV alone (compare lane 1 with lanes 5 to 10). Finally, Ad mutants that expressed ORF6 were able to increase transduction to levels only twofold lower than those achieved by use of *dl*309 (compare lane 2 with lanes 3 and 4). Similar results were obtained with HeLa cells (Fig. 2B). The identification of E4 ORF6 as a critical player in the life cycle of rAAV is consistent with previous studies indicating a role for this gene in the wild-type AAV life cycle (16, 34). While these data strongly suggest that the E4 ORF6 gene is necessary for increased transduction by rAAV, the use of mutant viruses precludes identification of this gene as being sufficient. Since the Ad genome will provide a number of other

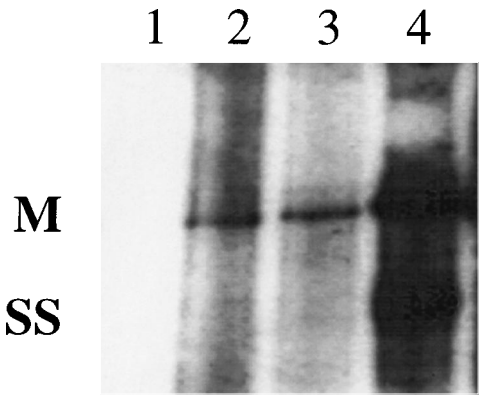


FIG. 5. Effect of E4 ORF6 on rAAV uncoating. 293 cells were transfected and then infected with rAAV as described in Materials and Methods. At 24 h p.i., nuclease-resistant virion DNA was isolated and electrophoresed through 0.8% agarose, transferred to a nylon membrane, and probed with a ^{32}P-labeled β-galactosidase sequence. Lanes: 1, 293 cells plus 1.6 ng of rAAV DNA; 2, 293 cells plus pCMV/E4ORF6 plus rAAV; 3, 293 cells plus rAAV plus pUC119; 4, 10^6 rAAV virions. M, monomer size; SS, single stranded.

gene products that could be affecting rAAV transduction, we attempted to determine if the E4 ORF6 product is active in the absence of a viral background.

To determine if the expression of E4 ORF6 is necessary to induce rAAV transduction, a plasmid that expresses only E4 ORF6, pCMV-E4ORF6, was transiently transfected into 293 cells and rAAV transduction was measured as described above (Fig. 3). Both *dl*309 virus and the E4 ORF6 plasmid were able to increase the transduction by rAAV approximately 40-fold compared with that achieved by rAAV alone (Fig. 3, compare lanes 1 through 3). When a nonspecific DNA (pUC119) was used, no increase in transduction was observed (lane 5). We assayed for the effect of other Ad genes on the E4 plasmid induction by transfecting pCMV-E4ORF6 and infecting 293 cells with a viral mutant that does not express E4 ORF6 (Fig. 3, E4 in ORF6). This combination did not augment the amount of rAAV transduction provided by E4 ORF6 alone (compare lanes 3 and 4), suggesting that the E4 ORF6 gene product is necessary and sufficient to enhance rAAV transduction in cells.

Induction of rAAV gene expression in the absence of Ad genes. The results thus far have suggested that transduction of cells by rAAV follows an infectious path similar to that used by wild-type AAV and that E4 ORF6 plays a critical role in an immediate-early step of the AAV life cycle. Previous studies have shown that cells subjected to genotoxic agents or stress can support limited helper-independent replication of wild-type AAV (29, 38, 44–47). These observations would suggest that at least for wild-type AAV, the dependence on Ad helper functions can be substituted by treatment of cells with these agents. If the increase in transduction seen with rAAV and E4 ORF6 is mimicking the wild-type AAV life cycle, we would predict that agents which stimulate productive wild-type AAV infections should also affect transduction of cells by rAAV. To test this hypothesis, HeLa and/or 293 cells were subjected to conditions that induce helper-independent replication of wild-type AAV, such as heat shock, HU, and UV irradiation. These cells were then infected with rAAV and assayed for *lacZ* transduction 24 h p.i. (Fig. 4A to C). All these treatments increased the number of *lacZ*-transduced cells by at least 2 orders of magnitude. In addition, X-ray treatment, which has not been reported to induce helper-independent replication of wild-type AAV, was able to increase transduction by rAAV by slightly more than 1 order of magnitude (Fig. 4D). Similar effects of DNA-damaging agents on AAV transduction have recently been reported by others (1, 30). These data, as measured by rAAV transduction, suggest that the E4 ORF6, genotoxic, and physical stresses all may be acting through a common pathway which is essential for the AAV life cycle.

Ad E4 ORF6 and UV treatment enhance second-strand DNA synthesis by rAAV. To identify the rate-limiting step in rAAV transduction, we initially assayed the effect of E4 ORF6 on rAAV uncoating. 293 cells were transfected with the E4 ORF6 plasmid or control DNA and then with rAAV. At 24 h p.i., virion-protected DNA was isolated by treating the cell lysate with DNase to remove all uncoated rAAV genomes. This was followed by inactivation of the DNase, protease treatment of the residual virions, and detection of the protected virion DNA by Southern hybridization. As illustrated in Fig. 5 (lane 1), rAAV DNA added to control samples was completely degraded after the addition of DNase. No difference was observed between the number of protected genomes in E4 ORF6-transfected cells and a plasmid control (lanes 2 and 3). Comparison of the initial input rAAV dose (lane 4) with the remaining uncoated virions (lanes 2 and 3) suggests that most of the rAAV genomes are uncoated efficiently regardless of the presence of E4 ORF6.

The previous experiment suggests that an immediate-early step in rAAV transduction, which occurs after uncoating, is being facilitated by E4 ORF6. A unique aspect of the AAV life cycle is that the viral template is packaged as a single-stranded DNA molecule and that second-strand synthesis is required before gene expression can take place. Second-strand synthesis is facilitated by a self-priming mechanism involving the AAV terminal repeat and results in the specific production of a double-stranded AAV molecule nicked at one end (see Fig. 8). To test the possibility that second-strand synthesis from rAAV was rate limiting for transduction, UV-irradiated or E4 ORF6-transfected cells were infected with rAAV. Low-molecular-weight DNA was isolated by Hirt extraction 24 h p.i. and fractionated by electrophoresis on alkaline agarose gels. Southern hybridization (Fig. 6) detected input rAAV DNA migrating as single-stranded DNA in all samples. However, rAAV molecules migrating at the expected molecular weight for the duplex replicative intermediate were detected at significantly greater levels only in the UV-irradiated cells (Fig. 6A, lane 2), and E4 ORF6-transfected cells (Fig. 6B, lane 1). Figure 6B, lane 2, shows the mobility of the AAV genome in the absence of transfected ORF6. This result indicates that the conversion of

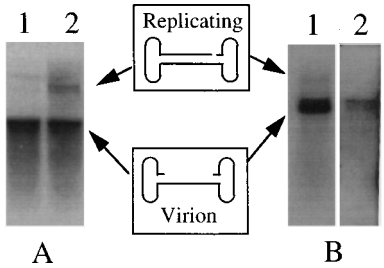


FIG. 6. Effect of E4 ORF6 and UV treatment on rAAV second-strand synthesis. 293 cells transfected with a plasmid expressing E4 ORF6 and 293 cells treated with UV (20 J/m^2) were infected as described in Materials and Methods. At 24 h p.i., the low-molecular-weight DNA was isolated by Hirt extraction, subjected to electrophoresis in a 0.8% agarose gel, transferred to a nylon membrane, and probed with a [^{32}P]DNA corresponding to the β-galactosidase coding region. (A) Lanes: 1, 293 cells plus rAAV; 2, 293 cells plus rAAV plus UV. (B) Lanes: 1, 293 cells plus rAAV plus E4 plasmid; 2, 293 cells plus rAAV.

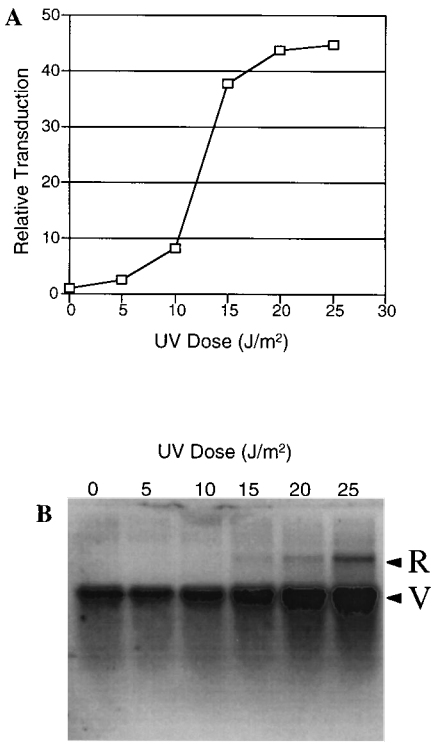


FIG. 7. Increased transduction corresponds to increased second-strand synthesis. Duplicate plates of 293 cells were irradiated with the indicated amounts of UV as described in Materials and Methods. The cells were then infected with rAAV. At 24 h p.i., the cells were either stained and the number of transduced cells was calculated (A) or subjected to the Hirt procedure to isolate low-molecular-weight DNA (B). The DNA samples were electrophoresed through a 0.8% alkaline agarose gel, transferred to a nylon membrane, and probed with a ^{32}P-labeled β-galactosidase sequence. R, replicating form; V, virion form.

rAAV single-stranded DNA to a double-stranded state is enhanced by UV light and E4 ORF6.

While the indication was that second-strand synthesis correlated with increased transduction from rAAV in the presence of E4 ORF6 and UV irradiation, we used our ability to dose escalate transduction by UV as a means of determining the direct relationship between second-strand synthesis and rAAV transduction. 293 cells were irradiated with various doses of UV and then infected with rAAV. The cells were assayed for *lacZ* staining at 24 h p.i., and a duplicate plate was characterized for second-strand synthesis as described above. Increased *lacZ* transduction by rAAV at different doses of UV (Fig. 7A) directly correlated with increased accumulation of duplex rAAV replicative intermediates (Fig. 7B), supporting the view that the rate-limiting step of rAAV transduction is the conversion of the single-stranded rAAV genome into a double-stranded intermediate.

DISCUSSION

By using AAV β-galactosidase (*lacZ*) recombinants (rAAV), we demonstrated up to a 1,000-fold increase in transduction frequency when cells were coinfected with Ad (Fig. 1 and 2) or exposed to genotoxic and physical stress (Fig. 4). These analyses may help resolve some of the discrepancies observed when characterizing rAAV generated with crude lysate compared with gradient-purified virus. For Ad coinfection, enhanced transduction occurred when the Ad genome was introduced into rAAV-infected cells via infection or transfection (data not shown). These data suggest that the enhanced transduction is not mediated by cointernalization of AAV with Ad virions but support the premise that expression of an Ad gene product is necessary for this effect. By using various Ad mutants, we have mapped this function to the E4 ORF6 region of Ad (Table 1; Fig. 2). Transfection experiments with plasmid DNA (Fig. 3) suggest that ORF6 of the E4 region is necessary to supply the Ad helper function needed for an early step in the AAV life cycle, specifically, second-strand synthesis (Fig. 6 and 7).

Effect of Ad E4 on AAV. The E4 region of Ad has been implicated in AAV helper function in various assays (9, 16, 26, 34). Early studies demonstrated the need for this region by assaying AAV replication or yield. Extended characterization of E4 mutants in Ad infection revealed the role of E4 ORF6 in the efficient accumulation of Ad late mRNAs (16, 42), a function also used by wild-type AAV for accumulation of its mRNA (34). All these previous studies were complicated by the fact that measurements of wild-type AAV DNA replication or gene expression were influenced by the AAV *rep* gene. AAV *rep* expression, in turn, is dependent on numerous Ad early-gene functions (E1A [transcriptional activation], E1B and E4 [mRNA maturation], and VA and E2a [translation enhancement]) (7, 9). As a result, it has been difficult to distinguish effects of *rep* from direct effects of Ad helper functions on other aspects of the AAV growth cycle. By using rAAV vectors to assay the immediate-early steps in AAV infection, we have avoided this complication in interpretation. This assay is functionally different from previous studies because reporter gene expression is independent of *rep* and its impact on the AAV life cycle. Our data show that AAV uncoating was not altered in the presence of E4 ORF6. However, it should be noted that although we saw similar amounts of DNase-protected DNA from E4 ORF6-positive and -negative cells, this assay could not distinguish between potential uncoating intermediates.

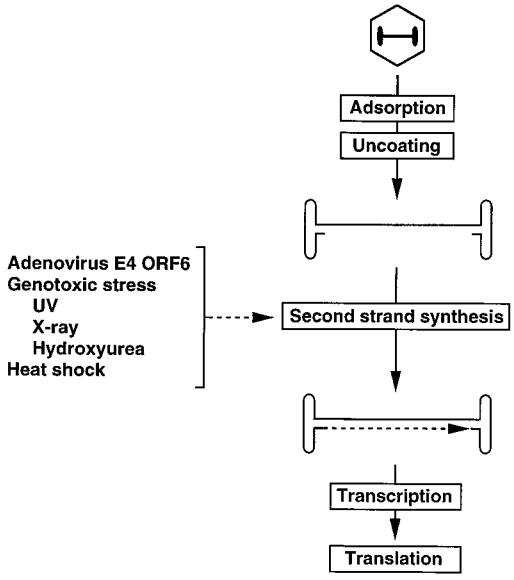


FIG. 8. Early events in the AAV life cycle cascade. When AAV encounters a host cell, it must first adsorb to the cell, uncoat its viral genome to release the single-stranded genome (solid line), and finally convert the genome to a double-stranded form (dashed line) before transcription and translation can take place. Second-strand synthesis appears to be the rate-limiting step of this cascade and can be facilitated by a number of agents including expression of the Ad E4 ORF6.

Our experiments clearly demonstrate that Ad E4 ORF6 facilitates second-strand synthesis of AAV DNA delivered to cells as a single-stranded molecule in a virus particle (Fig. 6 and 8). The mechanism by which the ORF6 protein enhances this reaction is unclear. It is interesting that E4 ORF6 prevents the generation of Ad DNA concatemers (41). We might speculate that the roles of E4 ORF6 in Ad DNA concatemer formation and AAV second-strand synthesis are directly related. Future studies are required to elucidate this relationship.

The phenomenon elicited by the Ad E4 ORF6 can be reproduced to different degrees in rAAV-infected cells by exposure of cells to heat shock or genotoxic reagents (Fig. 4, 6, and 7). Our studies support the hypothesis that increased rAAV transduction is due to enhanced AAV second-strand synthesis and that E4 ORF6, genotoxic, and physical stresses may all be acting through a common, as yet undefined, mechanism.

Impact on AAV vectors. AAV is being tested as a vector for gene delivery in vivo (2, 25, 40). Recently, a clinical protocol involving AAV-mediated gene delivery for cystic fibrosis was approved by both the Recombinant DNA Advisory Committee and the Food and Drug Administration. It is clear that rAAV vector transduction can be improved dramatically in cultured cells by the physical and chemical manipulations described above (Fig. 4, 6, and 7) and elsewhere (1, 30), suggesting that such reagents, which manipulate the intracellular milieu, could be coupled with current rAAV vector strategies to enhance the delivery of therapeutic genes. For example, rAAV transduction of bone marrow stem cells may be enhanced through the use of HU, a reagent which is currently being used in the treatment of sickle cell anemia (10). On the basis of our studies, current AAV vectors may be best suited for cancer gene delivery when the target cell is usually treated with agents such as X rays or chemotherapy that also enhance transduction by AAV. The use of AAV vectors aimed at transducing the bone marrow stem cells of patients with Fanconi's anemia (an autosomal recessive disorder characterized by eventual susceptibility to malignancy) in the presence of mitomycin has achieved promising results (40). Alternatively, systematic identification of cells in vivo which are competent for efficient AAV transduction may simultaneously obviate the need for additional steps required for enhanced transduction and direct the use of vector to these appropriate targets. The direct role of E4 ORF6 in increasing rAAV vector transduction could also be exploited by dual vector systems involving both rAd and rAAV carrying the therapeutic gene. Such a combination could combine high-efficiency gene transduction (rAd and rAAV) along with long-term persistence (rAAV). Alternatively, understanding the mechanism by which E4 functions to enhance rAAV transduction may suggest the possibility of identifying small molecules which could replace the role of Ad and enhance the efficiency of gene delivery by AAV vectors.

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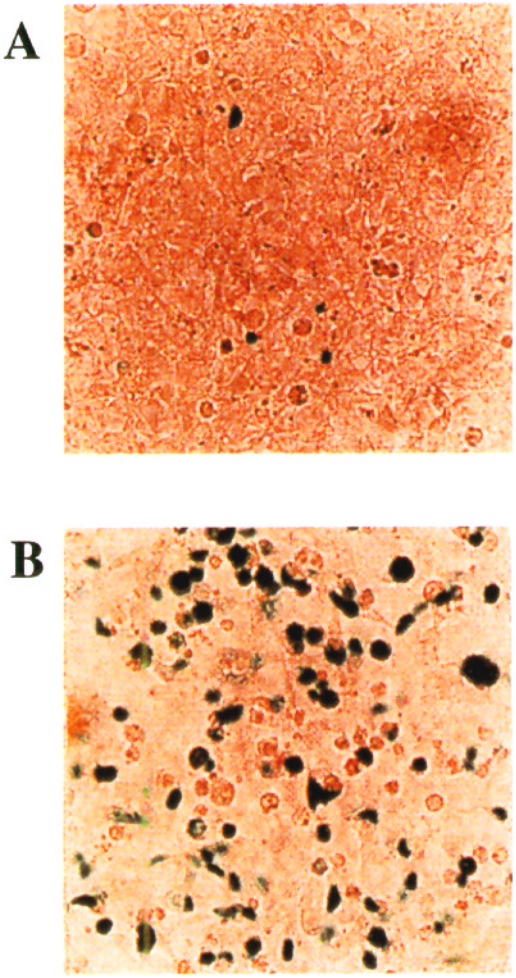
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