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Complementation of Adenovirus Type 5 Host Range Mutants by Adenovirus Type 12 in Coinfected HeLa and BHK-21 Cells

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We have studied the ability of adenovirus type 12 (Ad12) to complement the Ad5 transformation-defective host range (*hr*) mutants during infection of human cells (HeLa) or hamster cells (BHK-21). The group I mutant *hr*3 (mapped within 1.3 to 3.7 map units), which is incapable of synthesizing viral DNA, was complemented for both DNA synthesis and infectious virus production in nonpermissive HeLa cells during coinfection with Ad12. Similarly, the group II mutant *hr*6 (6.1 to 9.4 map units), which does synthesize DNA, was also shown to be complemented for virus production. When the host cells were BHK-21, an established hamster cell line that is permissive for Ad5 but nonpermissive for Ad12 DNA synthesis and virus production, coinfection with Ad5 and Ad12 did not overcome the block to Ad12 DNA synthesis. Coinfection of BHK-21 cells with Ad12 and either *hr*3 or *hr*6 leads to the complementation of only the group I mutant (*hr*3). The inability of Ad12 to complement *hr*6 in BHK-21 cells may be due to the failure of Ad12 to express an early gene product from the region corresponding to early region 1B (4.5 to 11 map units) of Ad5 where *hr*6 and the other group II mutations are located.

Expression of the adenovirus genome in productively infected cells is temporally divided into two distinct phases, early and late, corresponding to events occurring before and after the onset of DNA replication. During the early phase only a portion of the genome, corresponding to five discrete regions and involving both strands of the viral DNA, is expressed (6). Early cytoplasmic RNA is transcribed from the *r* strand of early region I (1.5 to 11.2 map units), from the *l* strand of early region II (61.6 to 74.9 map units), from the *r* strand of early region III (76.8 to 86.0 map units), and from the *l* strand of early region IV (91.4 to 99.1 map units) (3). Recent evidence indicates that sequences complementary to map positions 11.2 to 14.5 and 19.8 to 23.5 of the *l* strand are also present as polyadenylated cytoplasmic RNA early in infection but at a lower copy number than mRNA from the other early regions mentioned above (9).

Early region I contains the viral genes necessary and sufficient for transformation of cultured rodent cells (8, 11, 14, 21, 30). The mRNA's complementary to this region define two distinct, nonoverlapping blocks (3): EI A, between 1.5 and 4.4 map units, and EI B, between 4.5 and 11.2 map units. A number of Ad5 host range (*hr*) mutants defective in early region I functions have been isolated (16). These mutants are unable to replicate efficiently in some established human cell lines, such as HeLa or KB, but can grow on the 293 line of Ad5-transformed human embryonic kidney cells which contain the extreme left 12.5% of the Ad5 genome and transcribe viral mRNA's from early region I (1, 13). The *hr* mutants isolated on these cells fall into two complementation groups (16), the group I mutants (within 0 to 4.5 map units), which are incapable of synthesizing DNA but will induce a semiabortive or abnormal transformation, and the group II mutants (within 6.1 to 9.4 map units), which do synthesize DNA but are incapable of transforming cells (7, 12, 17). These complementation groups are both distinct from the groups defined by the temperature-sensitive (*ts*) early mutants of Ad5 (7, 16).

The positions and polarities of the sites complementary to early and late mRNA are similar if not identical in Ad5 and the distantly related (in terms of DNA sequence homology) Ad12 (18, 23, 27). This conservation of physical organization between the genomes of highly oncogenic Ad12 and nononcogenic Ad5 suggests that genes with similar functions may share common map positions. In particular, the DNA sequences responsible for oncogenic transformation by both Ad5 and Ad12 are at the left ends of both genomes (11, 14, 30). In studies by Williams et al. (29) Ad5 *ts* mutants were tested for complementation by Ad12 to determine the degree of functional relatedness between the two viruses. Of

the 15 mutants tested, 8 produced enhanced yields of infectious virus upon coinfection with Ad12, whereas the remainder were complemented poorly or not at all. No complementation was detected between Ad12 and any of the Ad5 mutants temperature sensitive for early genes. Because Ad5 and Ad12 differ in the ability to induce tumors after injection into newborn animals, it is of some importance to understand the relatedness of the functions coded by the transforming genes of the two viruses. We were therefore interested in determining whether the defects of the Ad5 *hr* mutants could be overcome by coinfecting cells with wild-type (wt) Ad12. This communication reports the results of such studies for one mutant from each of the two *hr* complementation groups. It is shown that Ad12 complements both *hr* groups during coinfection of HeLa cells, but only the group I *hr* mutants in BHK-21 cells, an established hamster line that is nonpermissive for Ad12 virus production (4).

MATERIALS AND METHODS

Cells. 293 cells used for propagation and titration of wt virus and *hr* mutants have been described previously (13). These cells were grown in Joklik modified medium supplemented with 10% horse serum. BHK-21 and HeLa cells were grown in αMEM plus 10% fetal bovine serum.

Virus. The wt strain of Ad5 and the *hr* mutants used in this study have been described previously (12, 16). Mutants *hr*1 and *hr*3 belong to complementation group I, and *hr*6 belongs to complementation group II. Ad12 strain 1131 was obtained from S. Mak, McMaster University, Hamilton, Ontario. Purification procedures for the virus have been described previously (15). Concentrations of Ad12 virus were determined by optical density at 260 nm, assuming that 1 optical density unit was equivalent to 2×10^{11} particles, whereas titers of wt Ad5 and *hr* mutants were determined by plaque assay on monolayers of 293 cells.

The particle/PFU ratio for both wt Ad5 and *hr* mutants was in the range of 50 to 100, whereas for Ad12 the range was 1,000 to 2,000.

Infection of cells. Confluent monolayers of HeLa or BHK cells in 60-mm petri dishes (Corning Glass Works) were infected with 5 (unless otherwise indicated) PFU of Ad5 virus or *hr* mutant per cell in 0.2 ml of phosphate-buffered saline. Inocula for coinfection studies also contained appropriately diluted Ad12. After 30 min of adsorption at room temperature, the cultures were incubated at 37°C in MEM-F11 supplemented with 5% horse serum. At 40 h postinfection, the infected cells were rinsed with phosphate-buffered saline lacking divalent cations and processed for DNA analysis or harvested for assay of infectious virus yields.

Complementation analysis. Analysis of viral DNA production was carried out as follows. Infected cells were rinsed once with phosphate-buffered saline without cations, lysed with 1 ml of 0.4% sodium dodecyl sulfate–0.01 M EDTA plus 500 μg of pronase per ml in 0.01 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.3), and digested for 2 h at 37°C. Samples (usually 10 to 20 μl) were centrifuged to equilibrium in a CsCl density gradient at 20°C in a Beckman model E analytical ultracentrifuge equipped with UV optics and a photoelectric scanner using a six-place AnG rotor. In some experiments infected cells were scraped into 2 ml of phosphate-buffered saline and lysed by sonication for 15 s in a Biosonic sonicator, and virus yields were determined by plaque assay.

RESULTS

Viral DNA synthesis in coinfected HeLa cells. The buoyant densities of Ad5 DNA, Ad12 DNA, and the host cell DNA are all sufficiently different from one another to permit a clear separation of bands and a reasonably accurate measure of the amounts of viral DNA relative to the total DNA extracted from coinfected cells. A typical series of UV scanner traces from the analytical ultracentrifuge is shown in Fig. 1. No

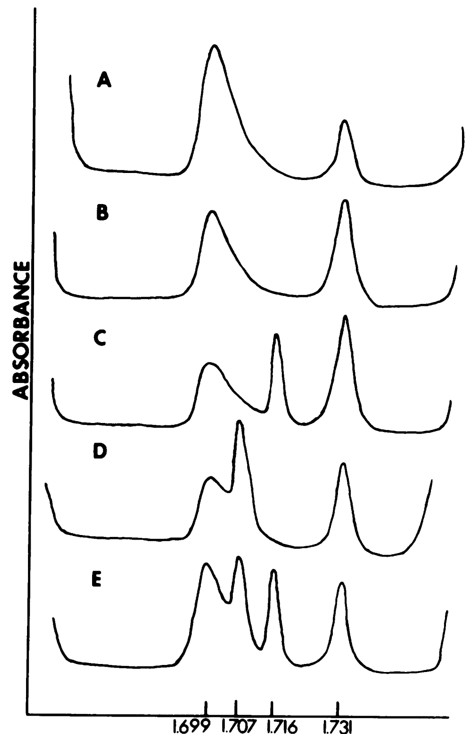

FIG. 1. *Equilibrium buoyant density analysis of DNA extracted from HeLa cells. Samples were prepared and centrifuged as described in the text. In each case, 1.0 μg of Micrococcus luteus DNA (1.731 g/cm³) was added as a marker. The traces are for DNA extracted from (A) mock-infected cells or cells infected with (B) hr3, 5 PFU/cell; (C) wt Ad5, 5 PFU/cell; (D) Ad12, 1,000 particles per cell; or (E) hr3, 5 PFU/cell and Ad12, 1,000 particles per cell.*

significant difference was observed in the patterns obtained from uninfected cells (Fig. 1A) and from cells infected with *hr*3 at a multiplicity of 5 PFU/cell (Fig. 1B). On the other hand, DNA profiles for cells infected with either wt Ad5 (Fig. 1C) at 5 PFU/cell or Ad12 at 1,000 particles per cell (Fig. 1D) displayed prominent bands of viral DNA with distinctly different buoyant densities. When HeLa cells were coinfected with *hr*3 at 5 PFU/cell and Ad12 at 1,000 particles per cell (Fig. 1E), two bands of viral DNA were readily distinguishable, corresponding to Ad5 and Ad12 DNA.

The scan in Fig. 1E indicated that coinfection with Ad12 could overcome the *hr* block and allow *hr*3 to replicate its DNA in HeLa cells. Thus, DNA extraction and model E analysis provided a convenient assay with which to examine some of the parameters affecting complementation of the *hr* defect by Ad12.

HeLa cells were infected at various multiplicities ranging from 0 to 25 PFU/cell with either wt Ad5 or the group I mutant *hr*3 and superinfected with 1,000 particles of Ad12 strain 1131 per cell. Alternatively, the multiplicity of wt Ad5 or *hr*3 was held constant, and the Ad12 multiplicity was varied from 0 to 1,000 particles per cell. The relative yields of viral DNA were calculated from the areas under the peaks of scans such as those shown in Fig. 1. Figures 2A and B demonstrate the effects on viral DNA synthesis of increasing *hr*3 and wt Ad5 input multiplicity, respectively. Yields of viral DNA from singly infected cells were maximal or near maximal for wt Ad5 at 5 PFU/cell, but essentially nondetectable for *hr*3 at this multiplicity. At multiplicities higher than about 20 to 30 PFU/cell the group I *hr* mutants showed a multiplicity-dependent leakiness (17) which resulted in a small amount of *hr*3 DNA synthesis detected in singly infected cells. Figures 2C and D illustrate the effects of increasing Ad12 input multiplicity on *hr*3 and wt Ad5 DNA synthesis, respectively. Ad12 input multiplicities of less than 250 particles per cell resulted in less than wt Ad5 levels of mutant DNA synthesis, demonstrating the role of multiplicity in determining whether complementation will be observed. It should be noted that in cells singly infected with Ad12, levels of viral DNA synthesis reached a plateau at this multiplicity.

Virus yield from coinfected HeLa cells. To compare DNA yields with yields of infectious virus, a parallel series of HeLa cells infected with 5 PFU of wt Ad5 or *hr*3 per cell was superinfected with Ad12 at increasing multiplicities. Titers of the Ad5 mutants could be scored without interference from Ad12 since within the 6-day period of the Ad5 plaque assay, Ad12 plaques did not develop on 293 cell monolayers. Small, pinpoint plaques of Ad12 were only observable after 12 to 14 days of incubation; in

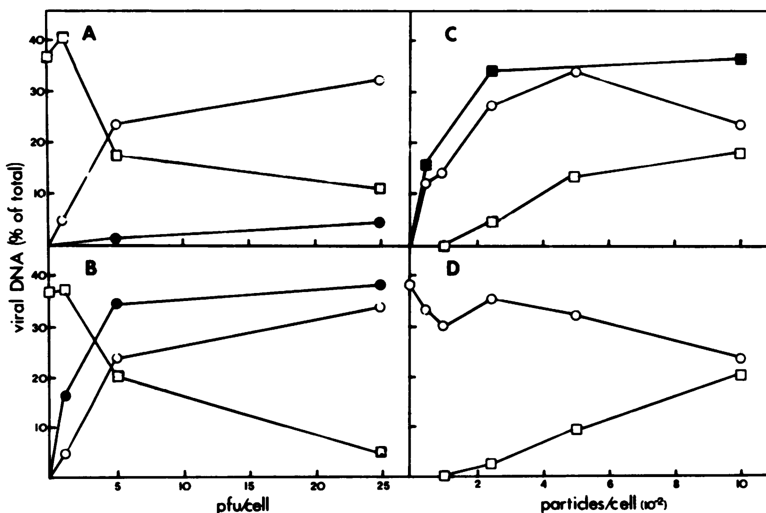

FIG. 2. *Viral DNA synthesis in singly infected and coinfected HeLa cells as a function of multiplicity of infection. The relative yields of viral DNA were calculated from the areas under the peaks in the UV scans of buoyant density gradients. (A and B) HeLa cells infected with an increasing multiplicity of hr3 and wt Ad5, respectively; hr3 or wt Ad5 DNA in singly infected (●) and in coinfected cells (○); Ad12 DNA in cells coinfected with 1,000 particles of Ad12 per cell (□). (C and D) HeLa cells infected with 5 PFU of hr3 or wt Ad5 per cell, respectively, and an increasing multiplicity of Ad12; hr3 or wt Ad5 in coinfected cells (○) and Ad12 DNA in singly infected (■) and in coinfected cells (□).*

addition, yields of virus from Ad12-infected cells were always 2 to 3 log units lower than yields of Ad5 from cells singly infected with *hr* mutants. The data in Fig. 3A show that coinfection of cells with Ad12 virus at multiplicities as low as 250 particles per cell raised the yields of *hr*3 up to wt levels, consistent with the data obtained from DNA analysis. At high multiplicities of coinfecting Ad12 virus (greater than 500 particles per cell) yields of both wt Ad5 and *hr*3 were reduced.

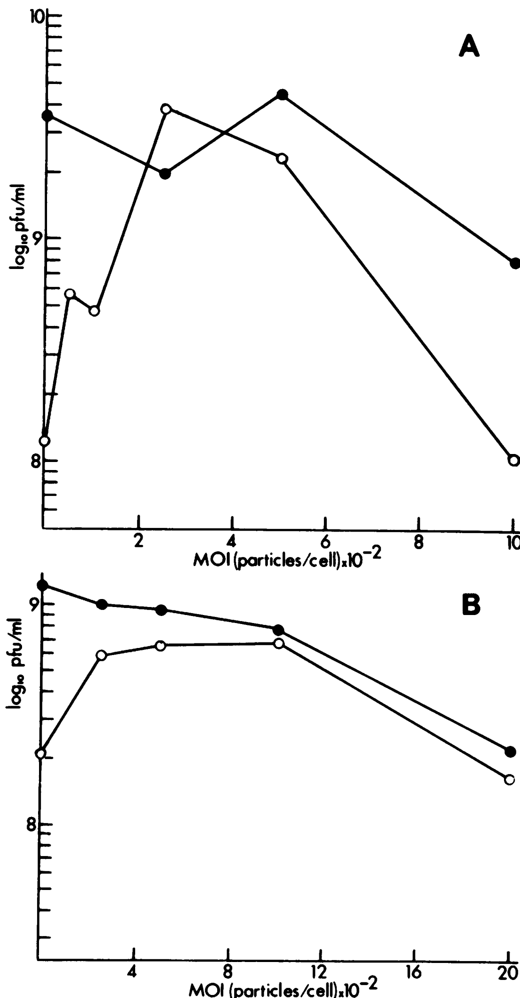

FIG. 3. *Yields of infectious virus from HeLa cells coinfected with hr mutants and Ad12. At 40 h postinfection infected cells were scraped into 2 ml of phosphate-buffered saline with Ca^{2+} and Mg^{2+}, sonicated, and titrated on 293 cell monolayers. Yields are plotted as a function of increasing multiplicity of infection (MOI) of coinfecting Ad12 with: (A) (○) hr3 and (●) wt Ad5; and (B) (○) hr6 and (●) wt Ad5.*

It was reported previously (17) and confirmed in the present study (data not shown) that group II mutants synthesize wt levels of viral DNA in singly infected HeLa cells. Consequently, it was not possible to assay complementation by analyzing Ad5 DNA yields in mixed infections of Ad12 and *hr* group II mutants. Therefore, to determine whether Ad12 was capable of complementing the group II defect, a series of cells coinfected with Ad12 and *hr*6, a group II mutant, were assayed for virus yields on monolayers of 293 cells. Yields of *hr*6 were enhanced by coinfection with Ad12 up to a multiplicity of 500 to 1,000 particles per cell (Fig. 3B). Again, increasing the Ad12 multiplicity beyond this point lowered the yields of both *hr*6 and wt Ad5 significantly.

Viral DNA synthesis in coinfected BHK-21 cells. Ad5 infects BHK-21 cells productively (26), whereas Ad12 undergoes an abortive cycle during which early genes are transcribed (22), but no viral DNA synthesis is detectable (4). Because the *hr* mutants map in early region I of Ad5 it was of interest to determine whether the Ad12 functions expressed in BHK cells were capable of complementing the *hr* defects.

Figure 4 shows the results of model E analysis of DNA extracted from BHK cells 40 h after infection with wt Ad5 (trace A), *hr*3 (trace B), Ad12 (trace C), or from cells coinfected with Ad12 and *hr*3 (trace D) or Ad12 and wt Ad5 (trace E). A number of observations were made. First, wt Ad5 could replicate its DNA in BHK cells, whereas *hr*3 could not, as was found for infected HeLa cells. Second, Ad12 did not synthesize viral DNA either in singly infected BHK or in BHK cells coinfected with Ad5 (compare Fig. 4 with Fig. 1). Third, the *hr*3 defect could be overcome in mixed infections (Fig. 4, trace D) in the absence of any Ad12 DNA synthesis.

A comparison of DNA yields, measured as a percentage of the total DNA extracted from infected cells, is presented in Fig. 5. As was found with infected HeLa cells, the yield of Ad5 DNA from BHK cells coinfected with *hr*3 and Ad12 increased with increasing multiplicity of the coinfecting Ad12 virus and reached maximal levels of DNA synthesis at 250 to 500 particles of Ad12 per cell, resulting in *hr*3 yields approaching that of wt Ad5.

Yields of infectious virus from coinfected BHK-21 cells. To determine whether the viral DNA yields were reflected in increased production of infectious virus, BHK-21 cells coinfected with *hr*3 and Ad12 were harvested at 40 h postinfection and titrated on 293 cell monolayers. A parallel series of cells was coinfected with *hr*6 and Ad12 and assayed in the same manner. As

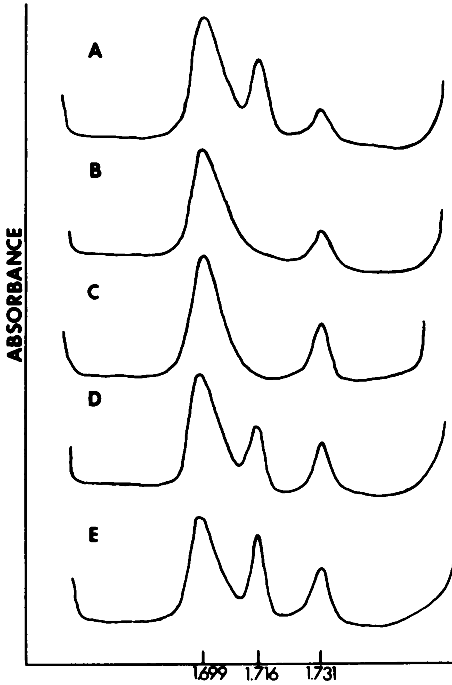

FIG. 4. *Equilibrium buoyant density analysis of DNA extracted from BHK-21 cells. Samples were prepared as described in the text, and 1.0 μg of M. luteus DNA (1.731 g/cm³) was added to each sample as a marker. The traces are for DNA extracted from cells infected with (A) wt Ad5, 10 PFU/cell; (B) hr3, 10 PFU/cell; (C) Ad12, 1,000 particles per cell; (D) hr3, 10 PFU/cell and Ad12, 1,000 particles per cell; and (E) wt Ad5, 10 PFU/cell and Ad12, 1,000 particles per cell.*

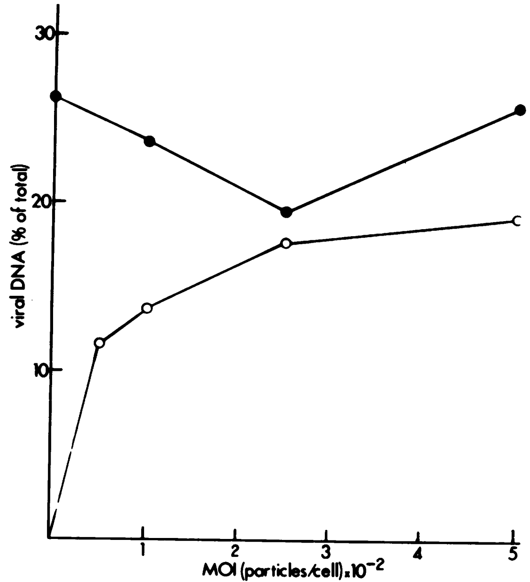

FIG. 5. *Viral DNA synthesis in BHK-21 cells coinfected with hr3 or wt Ad5 and Ad12. Relative yields were determined as described in the legend to Fig. 2 for (○) hr3 and (●) wt Ad5 at 10 PFU/cell at increasing multiplicities of infection (MOI) of Ad12.*

was found in HeLa cells, *hr*6 was capable of synthesizing viral DNA in BHK cells with or without coinfecting Ad12 (data not shown). The yield of *hr*3 increased to wt Ad5 levels with increasing input of coinfecting Ad12 (Fig. 6); however, the yield of *hr*6 virus was unaffected by the multiplicity of coinfecting Ad12 and remained at the same level as in singly infected cells. Thus, in BHK-21 cells, under conditions of Ad12 coinfection which fully complemented the group I *hr* defect, the group II *hr* mutant was not complemented.

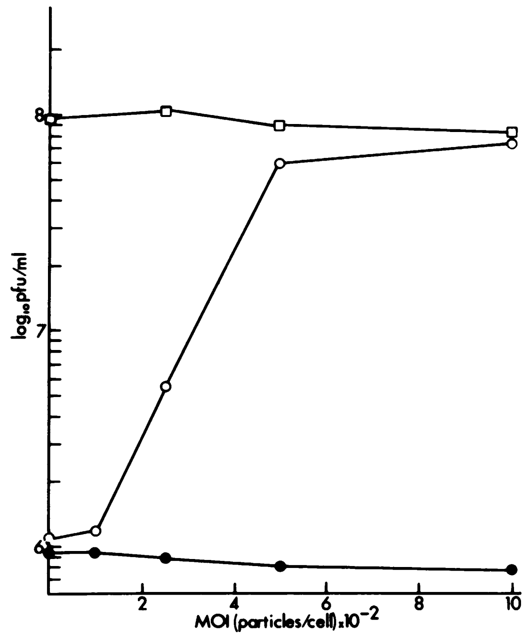

FIG. 6. *Yields of infectious virus from BHK-21 cells coinfected with hr mutants and Ad12. Samples were prepared as described in the legend to Fig. 3. Yields are plotted as a function of increasing multiplicity of infection (MOI) of coinfecting Ad12 and (○) hr3, (●) hr6, and (□) wt Ad5.*

DISCUSSION

The *hr* group I mutants of Ad5 do not synthesize viral DNA during infection of nonpermissive cells. This provides a convenient method for examining complementation between this group of mutants and coinfecting Ad12 virus. The defect caused by the *hr*3 lesion can be fully complemented by the coinfecting Ad12, a result

which has been confirmed for another group I mutant, *hr*1 (data not shown). During coinfection *hr*3 acts in the same manner as wt Ad5 to reduce DNA yields of Ad12 (Fig. 2C). Coinfection of HeLa cells at low multiplicities with wt Ad5 and Ad12 had little effect on Ad5 DNA synthesis (Fig. 2B) or virus production (Fig. 3A), a finding which differs from results previously reported (19) showing that at low multiplicities Ad12 interferes with Ad2 replication. It is not clear at present whether this difference is due to a difference in the serotypes (Ad2 versus Ad5) or a strain difference in the Ad12 used, since only Ad5 and Ad12 (strain 1131) have been used in this investigation. It should also be noted that coinfection of BHK-21 cells with Ad12 and Ad2 has been reported to have the effect of greatly reducing Ad2 virus yields (28), although the multiplicity of the Ad12 virus used in those experiments greatly exceeds the range of multiplicities used in the present study. During coinfection of HeLa cells at very high multiplicities (greater than 5,000 particles per cell) of Ad12, yields of *hr*3 and Ad5 were greatly reduced (data not shown). It was therefore necessary to carefully select the proper coinfecting multiplicities for both viruses to observe the complementation of *hr* mutant DNA synthesis.

It was also possible to observe complementation of virus yield during coinfection of HeLa cells. Yields from HeLa cells singly infected with *hr* mutants at 5 PFU/cell were reduced relative to wt yields by approximately 2 orders of magnitude for group I mutants and by about 1 order of magnitude for group II mutants (Fig. 3). The ratio of the yields of coinfected cells to singly infected cells for *hr*3, therefore, showed a considerable enhancement in type 5 output, whereas similar experiments with *hr*6 did not demonstrate as great an increase in output. However, when the yields of *hr*6 were examined as a function of the multiplicity of the coinfecting Ad12 virus, a clear pattern of complementation could be observed when compared with wt Ad5 (Fig. 3B).

Ad5 is capable of productively infecting BHK-21 cells, though growth in this cell line results in reduced yields compared with growth in established human cell lines and yields of the *hr* mutants are reduced even further. Since infection of hamster cells with Ad12 results in an abortive cycle of infection with no detectable production of infectious virus (4), viral DNA (5), or capsid antigens (24), it was of interest to determine whether coinfections of *hr* mutants and Ad12 would result in complementation of the *hr* defect in this cell type. Three observations were made from the complementation studies in BHK cells. First, the block in Ad12 DNA synthesis (and presumably expression of late mRNA and production of virus) was not overcome by coinfecting wt Ad5 (Fig. 4E) or *hr* mutants (Fig. 4D). Second, the group I mutant *hr*3 was efficiently complemented by Ad12 for both viral DNA synthesis and production of infectious virus in the absence of any Ad12 DNA synthesis. Third, there was no increase in yield of infectious virus for the group II mutant, *hr*6, a result quite different from that obtained in HeLa cells.

Since no late Ad12 RNA is synthesized in singly infected BHK cells it has been suggested that the lack of Ad12 viral DNA synthesis may be a consequence of a failure to transcribe some of the early viral genes or of improper processing of the RNA (20, 25). Ortin and colleagues (22), studying stable nuclear and polysomal mRNA isolated early after infection by hybridization with specific restriction fragments of the Ad12 genome, found that for virus-specific nuclear RNA there was no significant difference between Ad12-infected BHK 21 cells and early viral RNA synthesized in infected KB cells. However, the amount of polysome-associated mRNA hybridizing to the *Eco*RI fragment C (left 16% of genome) in abortively infected BHK-21 cells was one-third less than that in productively infected KB cells (22). Thus, it is possible that a gene product of the early region in Ad12, corresponding to early region 1B (4.5 to 11 map units for Ad5) where *hr*6 and the other group II mutants map (10), may not be expressed in BHK-21 cells. Other explanations, such as improper post-translational modification of the Ad12 gene product or failure of the product to interact with hamster cell functions, are also possible. It does not seem likely that the lack of an Ad12 function capable of complementing the Ad5 *hr* group II defect is alone responsible for the abortive cycle of Ad12 in hamster cells, for it is known that the Ad5 group II defect does not affect viral DNA synthesis or severely impair late polypeptide synthesis (17). An understanding of the failure of Ad12 to complement *hr*6 in BHK-21 cells might clarify the molecular basis for the differences in oncogenicity among the adenovirus groups as well as the processes involved in transformation.

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