Role of Adenovirus Type 2 Early Region 4 in the Early-to-Late Switch during Productive Infection

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Deletion of 98% of the adenovirus type 2 E4 transcription unit resulted in a delay of several events characteristic of the intermediate stage of infection, including viral DNA synthesis, induction of the cellular gene coding for dihydrofolate reductase, and the onset of the switch from viral early to late gene expression. Although delayed, both viral DNA replication and dihydrofolate reductase induction eventually approached wild-type levels. Events characteristic of the late stage of infection were both delayed and diminished in mutant-infected cells. Therefore, the viral E4 gene family is involved in the transition from the early to late stages of infection, and events whose timing is coincident or dependent upon the early-to-late switch are delayed.

Productive infection of human cells by adenovirus type 2 can be subdivided into early, intermediate, and late stages as characterized by stage-specific events and temporal expression of viral gene products (2, 13). The onset of viral DNA replication marks the beginning of the intermediate phase of infection, in which the infected cell switches from the production of host and viral early gene products to the production of viral late gene products. During the intermediate phase, several viral early gene products accumulate to levels above those observed at early times (6, 13), and cellular dihydrofolate reductase (DHFR) is induced (11, 12). The late stage of infection is characterized by maximal and almost exclusive expression of viral late genes and concomitant shutoff of most host genes, including the DHFR gene (1, 2, 13).

We have previously used human cell lines containing amplified DHFR genes to monitor the effects of virus infection on the expression of host genes (11, 12). The induction of DHFR and other DNA synthetic enzymes is likely to be controlled by a viral early gene product. However, the maximal induction of expression of the DHFR gene occurs at a time when the expression of E1a gene products is minimal (4, 7) and does not result from increased transcription as does the induction of other viral and host genes subject to E1a control (5, 7, 8). It is unlikely, therefore, that DHFR induction is directly controlled by E1a.

To investigate the role of early gene products in the modulation of expression of the DHFR gene by adenovirus, infection of DHFR-amplified cells was done with the E4 deletion mutant isolated by Weinberg and Ketner (9), *dI808*, which lacks 98% of the E4 transcription unit. The high level of expression of the DHFR gene in the amplified cells (500-fold) permits direct visualization of DHFR on protein gels and easy quantitation of DHFR RNA sequences via hybridization to available human DHFR probes (12). Comparison of the relative synthesis of DHFR (as monitored by [³⁵S]methionine incorporation) at different times after *dI808* infection indicated that maximal induction occurred with delayed kinetics, compared with a wild-type infection (Fig. 1). Virus-mediated shutoff of DHFR synthesis was also delayed (and diminished) in *dI808*-infected cells. Induction and shutoff were delayed by 11 and 16 h, respectively,

indicating that the delay in intermediate events translated to a similar delay in late events. The delayed induction and shutoff of DHFR protein correlated with a delayed induction and shutoff of DHFR mRNA levels (data not shown). Therefore, the level of DHFR protein synthesis at any given time after infection was directly proportional to the amount of DHFR-specific cytoplasmic RNA available for translation.

Synthesis of viral late proteins also began later than normal in *dI808* infections. Hexon synthesis, for example, began at 13 to 18 h postinfection with wild-type adenovirus type 2 but was not observed until 30 h postinfection with *dI808*. The extent of hexon synthesis was greatly diminished during *dI808* infection as well, amounting to roughly 10 to 20% of maximal production in a wild-type infection.

The delayed and diminished production of viral late proteins in *dI808*-infected cells correlated with a decreased and late production of viral late RNA (Fig. 2). Relative levels of viral late cytoplasmic RNA were depressed to a level 10 to 20% of that observed in wild-type infections and first appeared 8 to 12 h later than in a wild-type infection.

The delayed phenotype observed in *dI808* infections was not the result of incomplete infection. Examination of viral early gene products by Western blot analysis of nuclear proteins with anti-72-kilodalton serum indicated that the 72-kilodalton polypeptide appeared with wild-type kinetics in mutant-infected cells (data not shown). Other laboratories have also reported that E4 deletions do not depress the production of early gene products (3, 10).

To determine whether the delayed phenotype of *dI808* can be correlated with alterations in synthesis of viral DNA, synthesis of viral DNA was monitored during infection (Fig. 2). Replication of *dI808* DNA began 6 to 12 h later than did replication of wild-type DNA. An 8-h lag in the replication of *dI808* has also been observed by Weinberg and Ketner in unamplified HeLa cells (10). The delay in the onset of replication caused a 50% reduction in the amount of viral DNA in *dI808*-infected cells from 10 to 28 h postinfection. By 46 h postinfection, however, incorporation of [³H]thymidine into viral DNA occurred at the same rate in mutant- and in wild-type-infected cells, indicating that the blockage in mutant-infected cells alters the timing of the onset of replication of viral DNA rather than the mechanism of replication itself.

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FIG. 1. Protein synthesis during *dl/808* infection. HeLa S3-1000 cells were infected with 20 PFU of *dl/808* or wild-type adenovirus type 2 per ml (11, 12). At various times postinfection (shown at top in hours), cells were labeled with [^{35}S]methionine for 1 h. Total cytoplasmic protein was prepared, and equal amounts of acid-precipitable radioactivity were applied onto each lane of a gradient 7.5 to 19.5% polyacrylamide gel (12). The positions of DHFR and hexon are indicated.

Examination of the rate of transcription by using runoff assays in *in vitro* nuclei isolated at various times after infection with mutant or wild-type virus indicated that transcription of viral late RNA also began later in *dl/808*-infected cells than in wild-type-infected cells. Identical viral DNA probes were used to quantitate the replication and transcription data shown in Fig. 2 to permit comparisons of extents of replication, transcription, and accumulation of steady-state cytoplasmic RNA. The initial appearance of transcriptional activity correlated with the beginning of viral DNA replication. Furthermore, during the initial stages of replication, the extent of viral transcription from the major late transcription unit correlated with the level of viral DNA replication. Both were depressed to a level approximately 30% of that of wild type. At later times, however, the extent of transcription in mutant-infected cells was depressed relative to the amount of replication, suggesting that the observed low levels of viral late mRNA and protein in *dl/808*-infected cells are caused by blocks in both replication of viral DNA and viral late transcription.

Comparison of the kinetics of viral DNA replication and DHFR induction in *dl/808*-infected cells indicated that both processes were delayed in mutant-infected cells. Induction of DHFR occurred just as synthesis of viral DNA was initiated, 24 to 30 h postinfection in *dl/808*-infected cells. Induction of DHFR during wild-type infection also occurred during the onset of replication of viral DNA, 13 to 18 h postinfection. Induction of DHFR, therefore, occurred during the intermediate period of infection when the cell was leaving the early phase of infection and entering the late stage. The simultaneous delay in DHFR induction, synthesis of viral DNA, viral late transcription, and the appearance of late cytoplasmic RNA and proteins suggests that the most comprehensive phenotype produced by deletion of the E4 family of early viral gene products is the prolongation of the early phase of infection and a defective switch into the late stage of infection; this delay causes depressed synthesis of late gene products and continued synthesis of host gene products.

The complex phenotype of E4 mutations has been re-

FIG. 2. Production of viral DNA and RNA during *dl/808* infection. (A) Cells infected with *dl/808* (○) or wild-type (●) adenovirus type 2 were labeled with [^3H]thymidine for 30 min at various times postinfection. DNA was prepared and hybridized to viral DNA immobilized on filters (12). Each point represents the average from two experiments (values plotted represent 500 to 10,000 cpm). (B) Nuclei were prepared at various times postinfection and used for *in vitro* transcription with [^{32}P]UTP for 20 min (11). RNA was prepared and hybridized to viral DNA immobilized on filters (values plotted represent 1,000 to 20,000 cpm). (C) Cytoplasmic RNA was prepared at various times postinfection, spotted onto nitrocellulose filters, and hybridized with ^{32}P -labeled, nick-translated, virus-specific probes (12). The viral *Bam*HI D fragment was used as probe for all three experiments.

ported to be different in different cell lines (3, 9, 10). Thus, many cells, including HeLa suspension cells, are quite permissive for E4 deletions. The results presented here derive from a line of monolayer HeLa cells containing amplified DHFR genes and are in substantial agreement with those obtained by Weinberg and Ketner (10) with *dl808* in an unamplified human line and by Halbert et al. (3) with a different deletion mutant (*dl366*) of the E4 region in a different line of HeLa cells. Several differences, however, exist between the behavior of *dl808* and *dl366*. Weinberg and Ketner (10) observed an effect of *dl808* on DNA replication. They observed both a delay and a depression in the amount of viral DNA synthesis. They did not observe the late recovery of replication that we did. Halbert et al. (3) also reported a delay and a reduction in viral DNA replication in *dl366*-infected cells. Because Halbert et al. did not examine replication at very late times, it is not clear whether they would have observed competent late replication as we did. Halbert et al. also examined late transcription in *dl366*-infected cells and found it to be depressed. Although they reported that the observed level of transcription correlated with the level of replication at 24 h postinfection, they did not examine levels at later time points. Thus, it is not clear whether they would have observed the late replication-independent depression of transcription which we observed. These differences point out the necessity of obtaining data at multiple times after infection when working with mutants that have pleiotropic phenotypes, which are characteristic of early adenovirus mutants.

Comparison of the effects of mutation in the viral genome on both cellular and viral expression of genes is necessary to understand the complicated interplay between viral and host regulatory mechanisms operating during productive adenovirus infection. Some of the differences in phenotypes observed in different laboratories working with E4 deletion mutants are probably caused by differences in the cell lines employed. The availability of an amplified human cell line enabled us to examine the effects of an E4 deletion on a single host gene and permitted a better estimate of the requirement for E4 gene products during the intermediate stage of infection than has been previously possible. Thus, many of the reported effects of E4 deletions on the late stage of infection may reflect the response of a given cell line to E4-mediated intermediate events rather than a direct requirement of E4 gene products for late events.

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