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Regulation of Adenovirus Gene Expression in Human WI38 Cells by an E1B-Encoded Tumor Antigen

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Adenovirus mutants carrying alterations in the gene encoding the E1B 19-kilodalton tumor antigen (19K protein) cause enhanced cytopathic effect (*cyt* phenotype) and the degradation of host-cell chromosomal DNA (*deg* phenotype) upon infection of human HeLa or KB cells. Furthermore, E1B 19K gene mutant viruses are defective for cellular transformation. We report that these mutant viruses possess a host-range phenotype for growth in human cells. In human HeLa cells the mutant viruses grew to the same levels as the wild-type virus, but they were severely defective for growth in KB cells. In human WI38 cells, the E1B 19K gene mutant viruses had a substantial growth advantage over the wild-type virus, yielding 500-fold-higher titers. Viral DNA synthesis was reduced 10- to 20-fold in WI38 cells infected with the wild-type virus relative to that synthesized by the E1B mutant viruses. Viral early and late protein synthesis was similarly reduced in wild type- relative to mutant-infected cells. These reduced levels of early gene expression in wild-type virus-infected cells were paralleled by comparably reduced levels of early cytoplasmic mRNA. The primary cause of this host-range phenotype appeared at the level of early gene transcription, since transcription of viral early genes in the mutant-infected cells was substantially greater than levels found in cells infected with the wild-type virus. These results implicate the E1B 19K tumor antigen in the regulation of adenovirus early gene expression. Specifically, the E1B 19K protein directly or indirectly exerts a negative effect on early gene transcription accounting for efficient gene expression from the E1B mutant viruses in WI38 cells. Based on these findings it is probable that the *cyt* and *deg* phenotypes observed in mutant-infected HeLa and KB cells are the result of the pleiotropic effect of this altered gene regulation.

The left end of the adenovirus genome, as it is conventionally represented, contains two nonoverlapping transcription units, the early regions E1A and E1B. Both E1A and E1B are required for productive infection and oncogenic transformation by adenovirus. The E1A region encodes polypeptides which are required for the regulation of transcription in adenovirus-infected cells (reviewed in reference 47). The E1A gene products *trans*-active transcription of all the early adenovirus gene regions, including transcription of the E1A gene itself (2, 7, 13, 15, 23, 28, 32, 35, 37, 44, 55). The primary role of E1A in transformation appears to be in the immortalization of cells, but expression of the E1B gene, or other oncogenes, is required for complete cell transformation by adenovirus (25, 46).

In contrast with E1A, the role of the E1B region in either productive infection or cell transformation has not been established. The E1B region produces two major mRNAs, the 22S and 13S species that encode the major E1B polypeptides, the 19- and 55-kilodalton tumor antigens (19K and 55K proteins) (11, 17, 34). The larger 22S mRNA can encode both E1B proteins, whereas the smaller 13S mRNA only encodes the 19K polypeptide (6, 11). These two mRNA species are differentially spliced from a common precursor mRNA, and their relative abundance is regulated throughout infection (36, 49). The E1B 55K protein is required for productive infection in most human cells. Recent evidence from the characterization of viral mutants which contain lesions within the 55K protein-coding region has implicated a role for this protein in the shutoff of host-cell protein synthesis and and the transport or processing of late viral mRNA (4, 43; J. Williams, B. D. Karger, Y. S. Ho, C. L. Castiglia, T. Mann, and S. J. Flint, Cancer Cells, in press), although additional functions in viral DNA replication have been demonstrated (48, 52).

Viral mutants containing either point mutations or a deletion within the coding region for the 19K protein produce several interesting phenotypes in infected cells. The first is the induction of enhanced or unusual cytopathic effect, designated the cytocidal or *cyt* phenotype (42, 54, 56, 57, 60). The *cyt* phenotype is also manifest by the formation of large plaques (54, 56, 57, 60), with some E1B 19K gene mutant viruses being designated *lp* or large-plaque mutants (8, 54). Second is the induction of host-cell chromosomal DNA degradation, or the *deg* phenotype (9, 12, 31, 42, 53, 59, 60). The *deg* phenotype occurs in the absence of the 19K gene product and requires only adenovirus early gene expression (42, 59, 60). On the basis of these data, we have proposed that another adenovirus early gene product is responsible for inducing the DNA degradation in the absence of the 19K protein or its function (60). Third, most E1B 19K mutants are defective for transformation of rat cells (tra^-) (3, 8, 14, 33, 42, 54, 56, 57, 60), implicating the 19K protein in a proviral role in the process of oncogenic transformation by adenovirus.

The E1B 19K protein is localized transiently in cytoplasmic membranes early in infection and accumulates in the nuclear envelope at late times after infection (41, 59). In transformed cells it is found in cytoplasmic membranes, the cell surface, and the nuclear envelope (59; E. White and B. Stillman, unpublished data). The functional significance of this association of the 19K protein with different cellular membranes is not known. Studies with 19K mutant viruses, however, have indicated that the presence of the 19K protein in the nuclear envelope may be required to prevent the degradation of host-cell chromosomal DNA during productive infection (59).

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In addition to the *cyt*, *deg*, and *tra* phenotypes of the E1B 19K viral mutants, there was some indication that these mutant viruses might also possess a host-range phenotype. Takemori et al. (58) originally observed that the *cyt* mutants of adenovirus type 12 (Ad12) grew in one line of human KB cells yet failed to propagate in another KB cell line. E1B 19K mutant viruses of the Ad2 serotype grow poorly in KB cells, with yields reduced 100-fold compared with the wild-type virus (54), whereas no differences, or at most 10-fold reductions in virus yields, have been reported in HeLa cells (33, 42, 60). Viral DNA replication is reduced in mutant-infected KB cells (54) but not in HeLa cells (42, 60). These reported differences in lytic functions in E1B 19K mutant virus-infected cells varied with the type of host cell and were suggestive of a host-range phenotype. Mutant viruses with a host-range phenotype are potentially useful tools, as they present permissive and restrictive conditions for examining gene function. We therefore investigated this possibility by screening human cell lines for growth of the E1B 19K gene mutant viruses.

A comparison of virus yields, viral DNA synthesis, and early and late viral protein synthesis in wild type- and E1B 19K gene mutant-infected cells was performed on a variety of human cell lines. From these studies we determined that there are at least three classes of human cells which respond differently to E1B 19K mutant virus infections. Infection of HeLa cells with the E1B 19K mutant viruses induces the *cyt* and *deg* phenotypes, but virus yields are not substantially affected. Infection of KB cells with the mutant viruses results in severe *cyt* and *deg* phenotypes, and consequently viral DNA replication and virus production are greatly reduced. In mutant-infected WI38 and HEK cells there is no *deg* phenotype (E. White and B. Stillman, submitted), and in WI38 cells mutant viruses grow to higher yields than the wild-type virus owing to higher levels of early gene transcription. This raises the possibility that the *cyt* and *deg* phenotypes displayed in infected HeLa and KB cells may be a pleiotropic effect of the underlying function of E1B 19K gene in the regulation of adenovirus gene expression.

MATERIALS AND METHODS

Cells and viruses. Human diploid embryonic lung cells (WI38) and secondary human embryonic kidney (HEK) cells were purchased from Whittaker M.A. Bioproducts (Walkersville, Md.). KB cells were obtained from G. Chinnadurai (St. Louis University Medical Center, St. Louis, Mo.). All cells were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum.

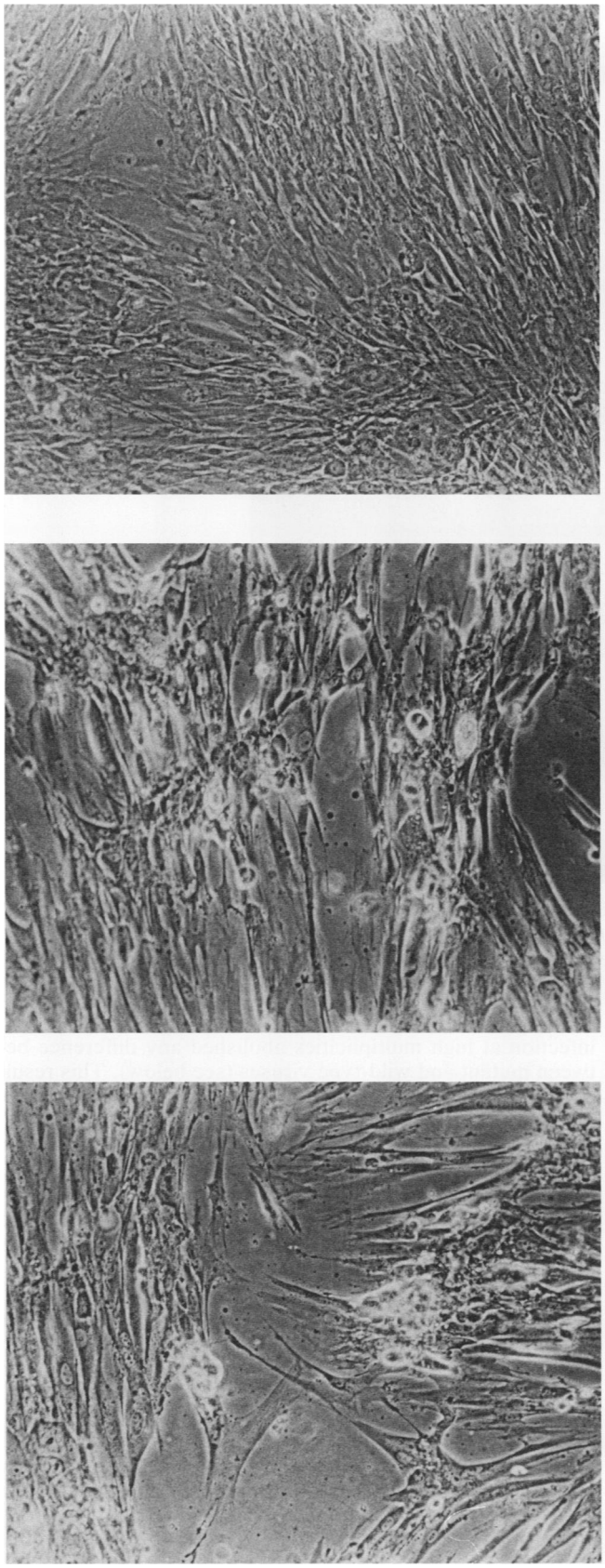
The adenovirus E1B mutant viruses used in these studies have been described elsewhere. Briefly, Ad2*cyt*106 contains a single point mutation at nucleotide 1769 within the E1B 19K-coding region which results in an amino acid substitution (Ser to Asn at amino acid 20) (60). Ad2*lp*3 and Ad2*lp*5 were obtained from G. Chinnadurai (St. Louis University Medical School) and also contain point mutations within the E1B 19K gene (8). The Ad2*lp*3 mutation results in an Ala to Val substitution at amino acid 3, whereas Ad2*lp*5 contains two mutations; the first results in an Asp to Tyr substitution at amino acid 82, and the second alters the termination codon causing the addition of 20 extra amino acids to the carboxy terminus of the 19K protein. Ad5*dl*337 was obtained from T. Shenk (Princeton University, Princeton, N.J.). This virus is derived from Ad5*dl*309 and contains a large out-of-frame deletion between nucleotides 1770 and 1916 within the E1B 19K-coding region (42). E1B mutant viruses were grown on monolayers of 293 cells, which are adenovirus-transformed human embryo kidney cells that contain and express E1 of Ad5 (16), and virus stocks were maintained as crude lysates. The E1B 19K gene mutant viruses are complemented by, and are not defective for growth in, 293 cells, and virus yields of the mutant viruses are comparable to yields of the wild-type virus. Titers of all viruses were determined by plaque assays on 293 cells.

WI38 and HEK cells were grown in monolayer culture and infected 2 to 3 days after reaching confluence and growth arrest. Cultured cells were infected with a multiplicity of infection based on the cell number per dish of a confluent monolayer. The number of WI38 cells per dish varied somewhat from batch to batch, so when the multiplicity of infection was critical for the experiment, a plate of cells was trypsinized, and the exact cell number was determined by counting the cells with a hemacytometer. Whenever possible, the wild-type Ad2 was used as a control for the Ad2-derived mutant viruses, and the parental Ad5*dl*309 virus was used in experiments with Ad5*dl*337. Slight differences were observed between Ad2 and Ad5*dl*309 (see below) which may be due to the Ad5 serotype origin of Ad5*dl*309 (28) and the fact that this virus contains a deletion of early region 3.

DNA isolation and analysis. Low-molecular-weight DNA was isolated by the method of Hirt (24) with minor modifications (60). DNA was analyzed by electrophoresis through 1% agarose gels and in Southern blots by standard procedures. For DNA quantitation experiments, Hirt supernatant DNAs were immobilized on nitrocellulose filters with a dot-blot manifold. Hybridization of nitrocellulose filters with nick-translated adenovirus DNA probe (45) was at 42°C in the presence of 50% formamide followed by washing at 55°C (59). The quantitation of adenovirus DNA was performed by scintillation counting (Cerenkov) an comparing the results with filters containing known amounts of adenovirus DNA standards.

Early and late protein synthesis. Monolayers of infected HeLa, WI38, HEK, and KB cells were labeled with 10 μCi of [^{35}S]methinonine per ml for 5 h at varying times postinfection and washed with phosphate-buffered saline, and the cells were lysed in Laemmli (30) sample buffer. Samples were subjected to electrophoresis through 10 or 15% sodium dodecyl sulfate (SDS)-polyacrylamide gels (30). Unlabeled cell extracts were prepared in essentially the same way and analyzed by immunoblotting to nitrocellulose filters as previously described (59). Polyclonal antibodies were prepared against purified 72K DNA-binding protein (DBP) (B. Stillman, unpublished data). Antibodies against the E1B 19K-*trpE* fusion protein were made by using the expression vector constructed by Spindler et al. (50). The anti-hexon serum was obtained from C. Anderson (Brookhaven National Laboratory, Upton, N.Y.). Polyclonal antisera directed against the E1A proteins were obtained from K. Spindler (50). The antibodies were used in immunoblot analysis to detect these antigens in infected cells.

RNA preparation and analysis. Cytoplasmic mRNA was isolated from infected cells by the method of Anderson et al. (1). For cell-free translation, total cytoplasmic RNA was used to program a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine (10, 40). The labeled translation products were analyzed by SDS-polyacrylamide gel electrophoresis. Poly(A)$^+$ mRNA was isolated from infected cells by chromatography of equal amounts of total cytoplasmic RNA on oligo(dT)-cellulose (2) and used for Northern blot analysis. Selected mRNA was subject to electrophoresis on



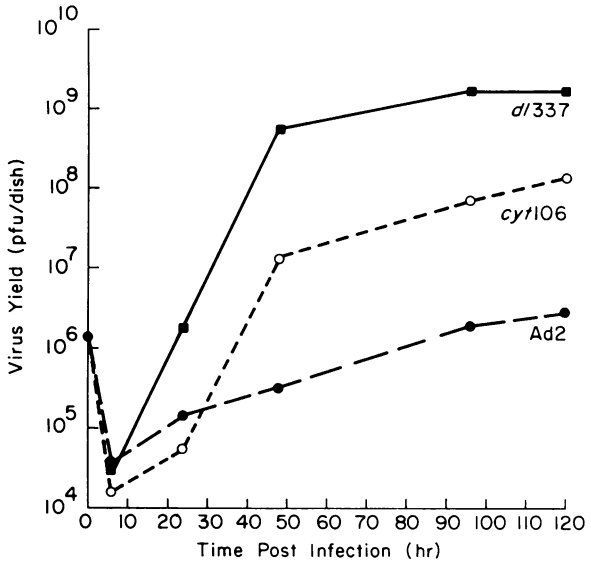


FIG. 2. Virus yield in wild-type- and mutant virus-infected WI38 cells. Cells were infected at a multiplicity of 5 PFU per cell, and the yield of an entire plate was determined by plaque assay on 293 cells at the indicated times postinfection.

formaldehyde-agarose gels and transferred to nitrocellulose by standard techniques. SP6 RNA polymerase was used to generate high-specific-activity ^{32}P-labeled RNA robes for the E1A, E1B, and E2A regions of the adenovirus genome. Hybridization of RNA probes to mRNA immobilized on filters was at 65°C in the presence of 50% formamide (50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 8× Denhardt, 50 mM $NaPO_4$ [pH 6.5], 0.1% SDS, 250 μg of salmon sperm DNA per ml, 500 μg of yeast tRNA per ml) with washes at 68°C.

Transcription analysis. Nuclei from mock-infected and infected WI38 cells were used in nuclear run-on transcription assays (18, 19). Equal amounts of labeled mRNA transcripts generated *in vitro* were hybridized to denatured adenovirus DNA probes for each of the early region genes that were immobilized on nitrocellulose filters. Hybridization was in 50% formamide at 42°C with washes at 55°C (59).

RESULTS

Induction of cytopathic effect. One of the most obvious indications of an adenovirus infection is the cytopathic effect observed in permissive host cells. Confluent monolayers of WI38 cells were infected at a multiplicity of 20 PFU per cell with the wild-type Ad2 or Ad5*dl*309 and the E1B 19K gene mutant viruses. The cultures were then observed for the appearance of cytopathic effect. By 48 h postinfection cytopathic effect was clearly evident in cells infected with the E1B mutant viruses Ad2*cyt*106 and Ad2*lp*5, but cultures infected with the wild-type Ad2 virus resembled mock-infected cultures (Fig. 1). Similar induction of cytopathic effect was obtained with the E1B 19K gene mutants Ad2*lp*3 and Ad5*dl*337, whereas the cytopathic effect induced by Ad5*dl*309 wild-type strain was less marked (data not shown).

FIG. 1. Cytopathic effect in wild type- and mutant-infected WI38 cells. Confluent monolayers of WI38 cells were infected with the wild-type Ad2 (A), Ad2*cyt*106 (B), and Ad2*lp*5 (C), at a multiplicity of 20 PFU per cell. Photographs were taken at 48 h postinfection.

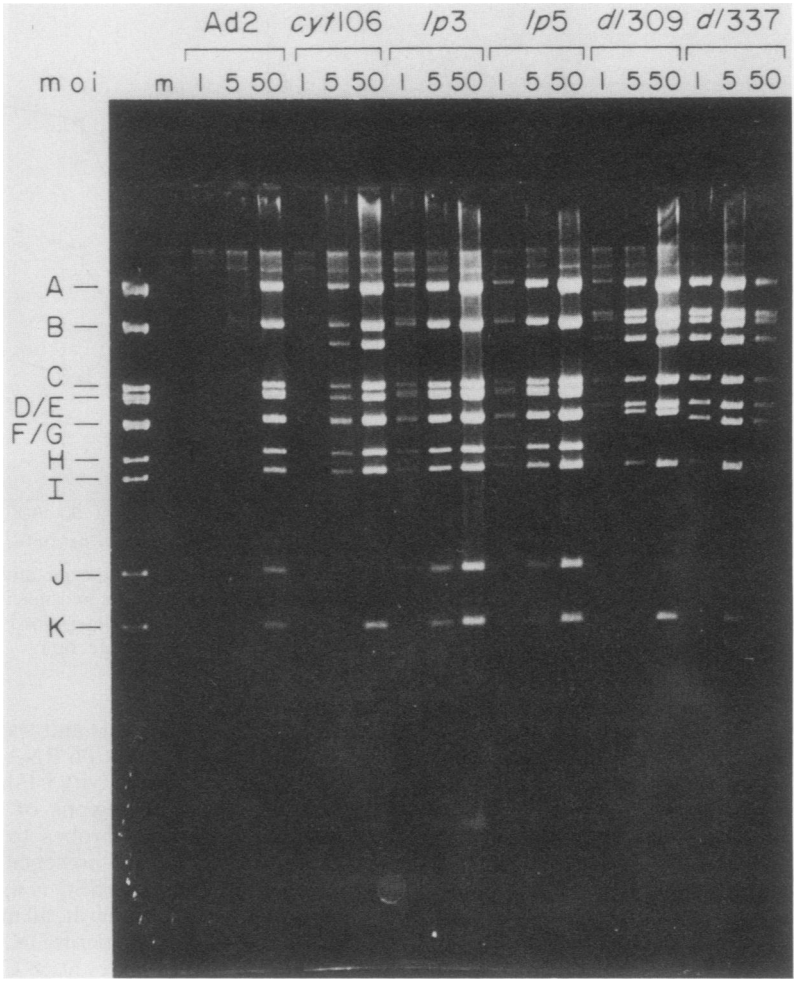


FIG. 3. Viral DNA replication in wild type- and mutant-infected WI38 cells. Ethidium bromide-stained agarose gel of *Hin*dIII-digested Hirt DNAs isolated from wild-type (Ad2 and Ad5*dl*309)- and mutant (Ad2*cyt*106, Ad2*lp*3, Ad2*lp*5, Ad5*dl*337)-infected cells at 48 h postinfection. Multiplicities of infection (moi) of 1, 5, and 50 PFU per cell are shown. Lane m, Mock-infected cells; markers are *Hin*dIII-digested Ad2 virion DNA. Quantitation of the viral DNA is described in the text.

Increased virus yield in mutant-infected cells. The occurrence of cytopathic effect in the mutant- but not wild type-infected WI38 cells indicated that the E1B mutant viruses may have a selective growth advantage over the wild-type virus. Alternatively, the cytopathic effect induced by the E1B mutant viruses may be a manifestation of the *cyt* phenotype observed in other cell lines. To distinguish between these two possibilities, we measured directly the ability of the different viruses to produce infectious virus in WI38 cells. WI38 cells were infected at a multiplicity of 5 PFU per cell (as determined by plaque assay on 293 cells), and at increasing times postinfection, crude lysates were made, and the titer of the virus produced was determined by plaque assay on 293 cells. In comparison with mutant-infected WI38 cells, the wild-type Ad2-infected cells produced a reduced virus yield in WI38 cells. Infection with the E1B mutant viruses resulted in 100- to 1,000-fold more infectious virus per dish by 40 to 50 hours postinfection than Ad2-infected cells (Fig. 2). The yields of virus produced in mutant-infected WI38 cells were slightly less than the yields of both wild-type and mutant viruses produced in HeLa cells. This poor growth of the wild-type virus in WI38 cells, however, was found to be multiplicity dependent since infection at high multiplicities abolished any difference between mutant and wild-type viruses (see below). This result demonstrates that the lack of cytopathic effect in Ad2-infected WI38 cells was due to a less productive infection.

In contrast to the results with the WI38 cells, infection of KB cells with the E1B 19K mutants Ad2*cyt*106 and Ad5*dl*337 resulted in 100- to 1,000-fold-lower titers than the corresponding wild-type virus infections (data not shown). The *cyt* and *deg* phenotypes are also present in KB cells. This is in good agreement with the previously published data (53, 54). Infection of HeLa cells with Ad2*cyt*106 or Ad5*dl*337 also induced the *cyt* and *deg* phenotypes, although virus yields were reduced at most 10-fold (42, 60).

Viral DNA replication. Having established that the E1B 19K gene mutant viruses have a selective growth advantage over the wild-type virus in WI38 cells, we sought to determine which viral replicative functions were deficient in the wild type-infected WI38 cells. This was accomplished by investigating the individual viral processes responsible for producing a lytic infection; viral DNA replication, early and late protein synthesis, viral mRNA synthesis and transcription.

The amount of viral DNA synthesized in mutant- and wild

type-infected WI38 cells was examined. At low multiplicities (1 and 5 PFU per cell) only scant amounts of viral DNA were detected at 46 h postinfection with the wild-type Ad2, whereas considerable viral DNA synthesis had occurred in the mutant (Ad2*cyt*106, Ad2*lp*3, Ad2*lp*5)-infected cells (Fig. 3). The wild-type strain Ad5*dl*309 synthesized more viral DNA than Ad2; however, Ad5*dl*337, which is an E1B 19K gene deletion mutant in an Ad5*dl*309 background, still produced more viral DNA than its parent (Fig. 3). The levels of viral DNA in this experiment were quantitated by hybridization to labeled adenovirus DNA probe. At a multiplicity of 5, Ad2*cyt*106, Ad2*lp*3, and Ad2*lp*5 synthesized 7.2-, 21-, and 22-fold more viral DNA, respectively, than Ad2 wild type. At a multiplicity of 1, Ad5*dl*337 synthesized 20-fold more viral DNA than Ad5*dl*309. These results are in contrast to similar experiments performed in HeLa cells in which E1B gene mutant viruses synthesize the same amount of viral DNA as the wild-type virus (42, 60). It is evident from Fig. 3, however, that at a multiplicity of 50, less of a difference in viral DNA synthesis existed between mutant- and wild type-infected cells. This indicates that deficient viral DNA replication was a multiplicity-dependent phenomenon, as was the virus yield (Fig. 2). The effect of the multiplicity of infection on the levels of viral DNA synthesized in wild type- versus mutant-infected WI38 cells is shown in Fig. 4. In this more detailed analysis, it can be seen that although greater than 10-fold more viral DNA was synthesized in E1B mutant-infected WI38 cells than was synthesized by the wild-type virus at low multiplicities, any difference between mutant and wild-type viruses was abolished at high multiplicities of infection (50 to 100 PFU per cell). The reason for the multiplicity dependence of the wild-type virus for growth and DNA replication in WI38 cells is not known; however, a similar phenotype has been reported for E1A mutant viruses in HeLa cells (26, 28).

We also observed during the course of examining viral DNA replication in WI38 and HEK cells that the E1B 19K mutant viruses did not induce the degradation of host-cell chromosomal DNA as they do in HeLa and KB cells (White and Stillman, submitted). The absence of the *deg* phenotype in WI38 and HEK cells may be related to the effect of the 19K protein on early gene expression (see below).

Early and late protein synthesis. We investigated whether the positive effect of the E1B mutations on viral DNA synthesis was the primary result of the mutations or the indirect result of a perturbation of viral gene expression. Expression of early and late viral genes in the wild-type- and E1B 19K mutant virus-infected human cells was examined by labeling infected cells in vivo with [^{35}S]methionine at different times postinfection and analyzing the products on SDS-polyacrylamide gels. Infection of HeLa cells (Fig. 5) or KB cells (data not shown) with the E1B 19K mutant viruses was not dramatically different from a wild-type infection with respect to the amount of viral protein synthesis. Early and late viral gene expression was evident in HeLa cells by 17 h postinfection in both wild type- and mutant-infected cells, and viral polypeptides represented the bulk of proteins synthesized at 25 and 41 h postinfection. Levels of viral polypeptides synthesized in Ad2*cyt*106-infected cells appeared slightly reduced in comparison with levels in Ad2-infected cells and may be an indirect effect of the *cyt* and *deg* phenotypes. A different situation emerged in infected WI38 cells. First, the overall infection progressed more slowly in comparison with HeLa cells (Fig. 5). Second, shutoff of host protein synthesis, which normally occurs during a productive adenovirus infection, was not evident (Fig. 5). Third,

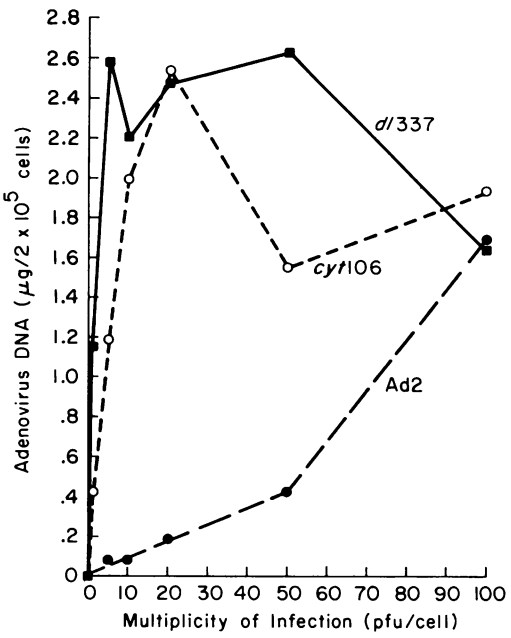


FIG. 4. Quantitation of viral DNA synthesized at 48 h postinfection in wild type- and mutant-infected WI38 cells at increasing multiplicities of infection.

synthesis of viral polypeptides was barely detectable in Ad2-infected WI38 cells by 41 h postinfection, but in the Ad2*cyt*106-infected cells viral proteins were readily observed by 25 h and were more pronounced by 41 h (Fig. 5). As with the WI38 cells, when HEK cells were infected with the E1B mutant viruses, viral gene expression was accelerated with respect to wild type-infected cells (Fig. 5). By 17 h synthesis of the 72K DBP and hexon occurred in Ad2*cyt*106-infected cells, but these same proteins were not synthesized until 25 h postinfection in Ad2-infected HEK cells.

It appears that the overall effect of the E1B 19K gene mutations on gene expression is a more rapid and productive infection in WI38 and HEK cells. In WI38 cells, this effect is more pronounced, with a considerable increase in the levels of synthesis of specific viral polypeptides. To further investigate this possibility, we performed an examination of the accumulated levels of specific early and late viral polypeptides during a time course of infection of WI38 cells. In gels containing wild type-infected cell extracts, the presence of the major structural protein hexon was virtually undetectable by Coomassie blue staining or by immunoblotting with anti-hexon antiserum, even at 120 h postinfection (Fig. 6). There were, however, abundant amounts of hexon present in Coomassie blue-stained gels or immunoblots of Ad2*cyt*106- and Ad5*dl*337-infected cells (Fig. 6). A similar situation emerged with an early protein, the 72K DBP, in immunoblots. Large amounts of the DBP were present in the E1B mutant- but not wild type-infected WI38 cells (Fig. 6).

Synthesis of the DBP and the other early viral gene products is dependent on *trans*-activation of the early viral transcription units by E1A gene products (5, 28). Immunoblots probed with E1A-specific antiserum detected E1A protein in the mutant-infected WI38 cells, but the amount of E1A protein in the Ad2-infected cells was below the level of detection (data not shown). Therefore, the reduction in viral early gene expression in Ad2-infected

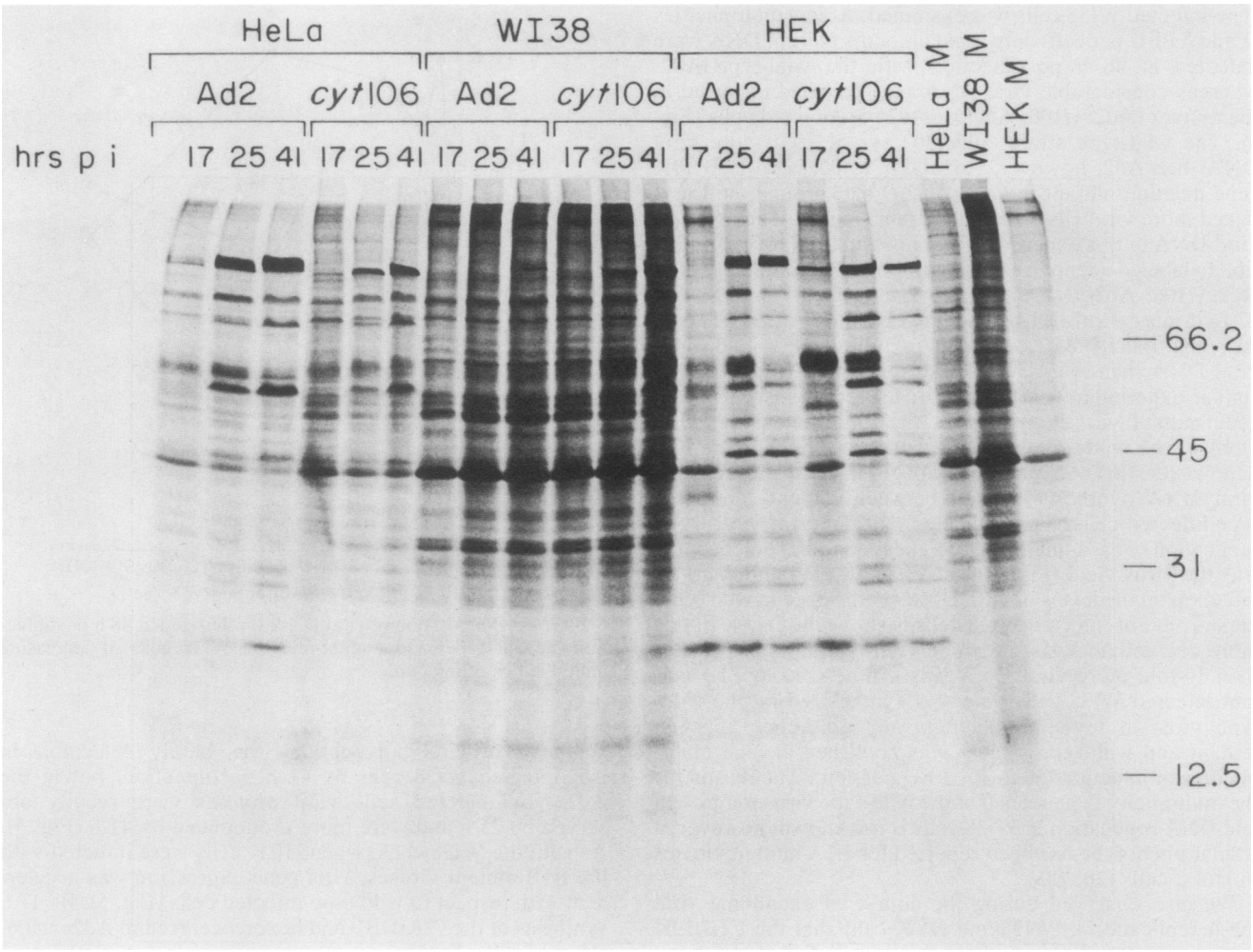


FIG. 5. Protein synthesis in wild type- and mutant-infected HeLa, WI38, and HEK cells labeled in vivo with [^{35}S]methionine. Cells were infected at a multiplicity of 10 PFU per cell, labeled at the indicated times, and analyzed by SDS-polyacrylamide gel electrophoresis. Mock (M)-infected cells are on the right, and the molecular size markers in kilodaltons. pi, Postinfection.

WI38 cells may be directly or indirectly a consequence of limiting amounts of E1A protein necessary to *trans*-activate early gene transcription.

In contrast to the results with hexon, the 72K DBP, and E1A, the presence of the E1B 19K protein was detectable in Ad2-infected WI38 cells (Fig. 6). This may be due to the fact that the E1B transcription unit is the least-dependent of all the early viral transcription units on E1A for the *trans*-activation of transcription (39). Limiting amounts of E1A protein in Ad2-infected WI38 cells may be sufficient for activating transcription of E1B but not for activating E2A transcription and subsequent synthesis of the DBP. Synthesis of the E1B 19K protein in wild-type virus-infected WI38 cells also indicates that the Ad2 virus is infecting the WI38 cells and that it is the regulation of early gene expression which is perturbed after infection. The 19K protein synthesized in Ad2-infected WI38 cells is found in the nuclear envelope, indicating that despite the absence of other viral early proteins, the localization of the 19K protein is unaffected (data not shown). No E1B 19K protein was detected in Ad2*cyt*106- and Ad5*dl*337-infected WI38 cells (Fig. 6). This was expected since, as a result of the E1B 19K gene mutations, neither virus synthesizes a significant amount of the 19K gene product in HeLa cells (42, 60).

In summary, viral early and late gene expression is severely limited in WI38 cells infected with the wild-type virus. This is not the case for the E1B 19K gene mutant viruses, in which early and late gene expression, viral DNA replication, and virus production appear normal. Since early gene transcription is the first step in this cascade of events, we examined adenovirus early gene expression at the RNA level in WI38 cells.

Cytoplasmic mRNA. Cytoplasmic RNA isolated from Ad2-, Ad2*cyt*106-, and Ad5*dl*337-infected WI38 cells was translated in vitro, and the translation products were analyzed by SDS-polyacrylamide gel electrophoresis. The products of translatable cytoplasmic mRNAs for adenovirus polypeptides are abundant in mutant- but not in wild type-infected cells (Fig. 7). Northern analysis of poly(A)$^+$ mRNA revealed that the reduction in translatable cytoplasmic message in the Ad2 infection is due to reduced amounts of adenovirus early message in the cytoplasm (Fig. 8). Similarly reduced levels of early message were found in the nuclei of Ad2- compared with E1B mutant-infected WI38 cells (data

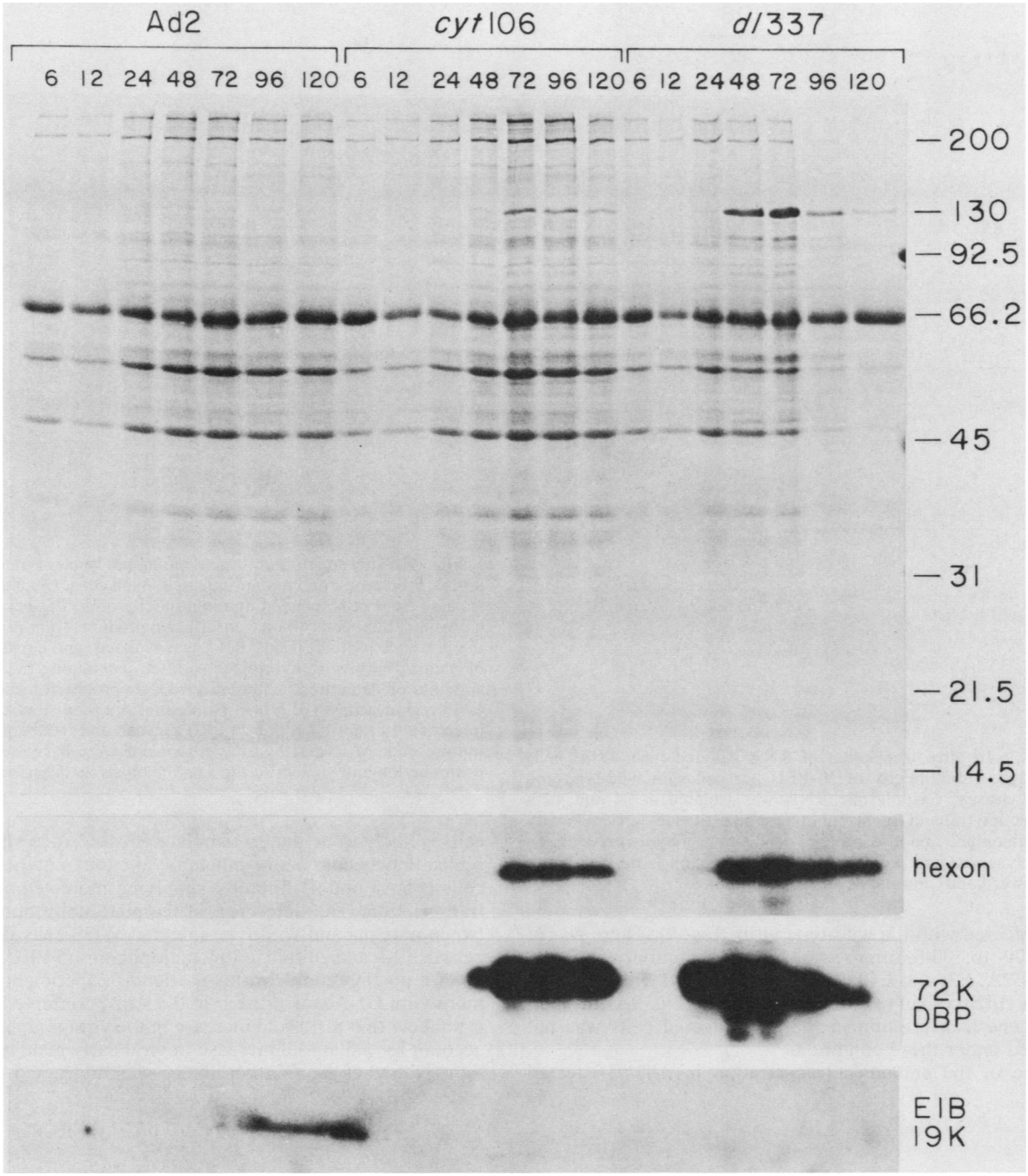


FIG. 6. Immunoblot analysis of early and late protein synthesis in wild type- and mutant-infected WI38 cells. (Top) Coomassie blue-stained SDS-polyacrylamide gel of total proteins synthesized in WI38 cells infected at a multiplicity of 20 PFU per cell with wild-type and mutant viruses at the indicated times postinfection (6 to 120 h). (Bottom) Immunoblots of gel as in top panel stained with anti-hexon, anti-72K DBP, and anti-E1B 19K antisera. Only the regions of the immunoblots containing relevant antigens are shown. Molecular size markers are in kilodaltons.

not shown). The observations that the adenovirus early message detected in the cytoplasm is of the correct size and can be translated in vitro suggest that it is processed normally.

In vitro transcription analysis. The general overproduction of nuclear and cytoplasmic adenovirus early mRNA in E1B 19K gene mutant virus-infected WI38 cells could be the result of enhanced early gene transcription, increased mRNA stability, transport, or processing. To distinguish between some of these possibilities, we measured transcription in wild type- and mutant-infected WI38 cell nuclei directly by run-on transcription assays in vitro (18, 19). Labeled transcripts generated in vitro were hybridized to denatured plasmids containing sequences from each of the early region genes. Transcription of all the viral early genes was dramatically greater in E1B mutant-infected cells than in

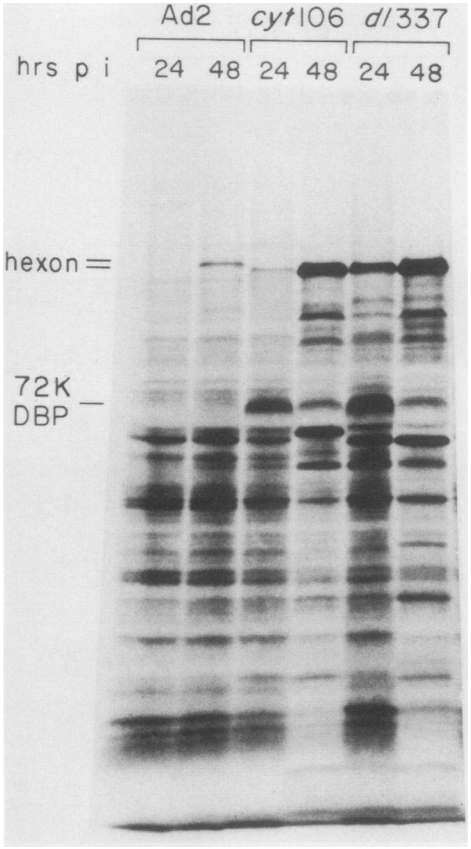


FIG. 7. In vitro translation of RNA isolated from WI38 cells infected at a multiplicity of 20 PFU per cell with wild-type and mutant viruses. Cytoplasmic RNA was isolated at 24 and 48 h postinfection (pi) and translated in a rabbit reticulocyte system with [^{35}S]methionine, and the protein products were analyzed on an SDS-polyacrylamide gel. The positions of hexon (Ad2, top band; Ad5, lower band) and the 72K DBP are marked.

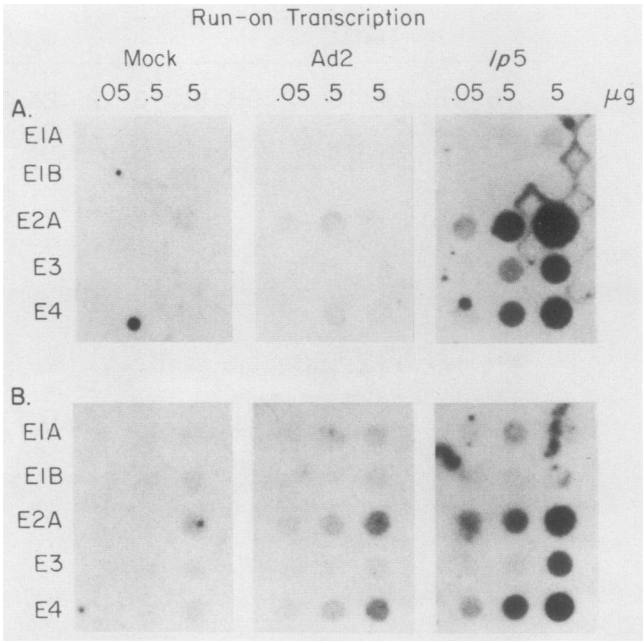


FIG. 9. Nuclear run-on transcription analysis of nuclei from mock-, wild type-, and mutant-infected WI38 cells. (A) Nuclei were isolated from cells infected at a multiplicity of 5 PFU per cell at 43 h postinfection, and run-on transcription products were produced in the presence of [α-^{32}P]UTP. RNA was isolated, and equal amounts of radioactivity were hybridized to filters containing the indicated amounts of denatured, immobilized DNA probes for each of the early region genes. (B) Same as in panel A except that cells were infected at a multiplicity of 20 PFU per cell and incubation was in the presence of 10 mM hydroxyurea. Cells were harvested at 40 h postinfection and subject to the same analysis as described above.

cells infected with the wild-type virus (Fig. 9). There was at least 100- to 500-fold more transcriptional activity for early genes E2A, E3, and E4 in WI38 cells infected with E1B 19K mutant Ad2*lp5* than in Ad2-infected cells (Fig. 9A). In fact, early gene transcription in the Ad2-infected cells was not detected under these conditions.

Some of the enhanced transcription in Ad2*lp5*-infected cells is likely to be due to template effects, since viral DNA replication occurs more efficiently in the mutant-infected cells (Fig. 3 and 4), thereby supplying more templates for transcription. The difference in template copy number between mutant- and wild type-infected WI38 cells is usually between 10- and 20-fold at low multiplicities (5 PFU per cell) at 48 h postinfection. In this particular experiment, 10-fold more viral DNA was present in the Ad2*lp5*-infected cells. It is unlikely that at 10-fold increase in the viral template could account for a 500-fold increase in viral early gene transcription. Nonetheless, this possibility was addressed directly.

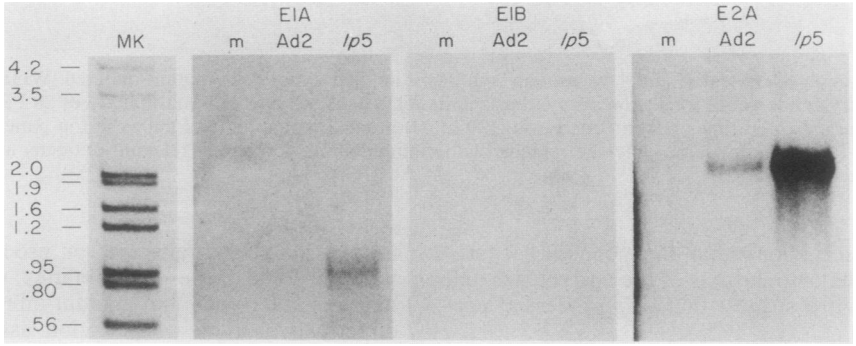


FIG. 8. Northern analysis of poly(A)$^+$ cytoplasmic mRNA from WI38 cells infected at a multiplicity of 5 PFU per cell. Equal amounts of cytoplasmic RNA, isolated at 43 h postinfection, were chromatographed on oligo(dT)-cellulose and checked by in vitro translation before electrophoresis on formaldehyde-agarose gels. After transfer to nitrocellulose, blots were hybridized to SP6-generated RNA probes specific for the E1A, E1B, and E2A regions of the adenovirus genome. Markers (MK) are in kilobases.

Mock-, Ad2-, and Ad2*lp5*-infected WI38 cells were incubated in the presence of hydroxyurea for the duration of the infection. Hydroxyurea effectively blocks viral DNA replication and late gene expression, keeping the amount of viral DNA at input levels. Nuclei were isolated from hydroxyurea-treated cells, and in vitro transcription products were produced and analyzed. Again, there was considerably more adenovirus early gene transcriptional activity, approximately a 20-fold increase, in nuclei from Ad2*lp5*-infected WI38 cells compared with nuclei from Ad2-infected WI38 cells (Fig. 9B). When the copy number of template DNA in wild type- and mutant-infected cells was compared in this experiment, the mutant showed a three- to fivefold-higher amount of DNA. This small difference could be due to the error in determining multiplicity of infection by plaque assays between different virus stocks. Nevertheless, the results suggest that transcription of early virus genes is elevated four- to sevenfold in mutant-infected WI38 cells compared with the level in wild type-infected cells. We conclude that the E1B 19K protein present in wild type-infected WI38 cells negatively regulates transcription of early viral genes.

DISCUSSION

Infection of human WI38 cells with adenovirus mutants carrying lesions within the coding region for the E1B 19K tumor antigen causes greater viral yields and more rapid and productive viral DNA replication than does infection with the wild-type virus. The basis for this growth advantage of the mutant viruses was increased early gene expression occurring at the level of transcription. All the E1B 19K mutants we tested (Ad2*cyt*106, Ad2*lp3*, Ad2*lp5*, and Ad5*dl*337) have this host range phenotype irrespective of whether they express an altered 19K protein or none at all. Therefore, the absence of either the E1B 19K protein or its function is responsible for the increase in early gene transcription. Evidently, the E1B 19K tumor antigen is acting as a negative regulator of early gene expression in wild type-infected WI38 cells, thereby accounting for increased viral gene expression in mutant-infected cells in which the 19K protein is absent or nonfunctional.

The negative effect of the 19K protein on early gene transcription could be direct or indirect. First, the 19K protein could be acting as a repressor in the classical sense, via a direct binding to DNA. We feel that this is unlikely, given the intracellular localization of the 19K protein in cytoplasmic membranes and the nuclear envelope and not in the interior of the nucleus (41, 59). Second, the nuclear envelope localization of the 19K protein could afford it the ability to alter nuclear structure. Changes in the structure of the nucleus might render alterations in chromatin structure and subsequently gene expression. Evidence for this mode of regulation of gene expression is indirect and, for the most part, speculative (reviewed in references 20 and 27). Third, the 19K protein may repress the activity of a transcription factor or interfere with the assembly of transcription complexes, thereby having an indirect but negative effect on transcription. Fourth, and we feel the most probable, the E1B 19K tumor antigen may interact with the E1A proteins or mRNA. The E1A proteins are known to *trans*-activate transcription of the adenovirus early genes (5, 28, 37) as well as some endogenous cellular genes (26, 29, 51). Inhibition of the E1A *trans*-activation process by the 19K protein would result in an indirect negative regulation of adenovirus early gene expression. Modulation of E1A activity could be achieved by a variety of mechanisms, both direct and indirect. For instance, modification of the E1A proteins or altered levels of E1A proteins or RNA in infected cells could affect *trans*-activation. We observed elevated levels of E1A proteins in mutant-infected WI38 cells, which alone might account for the observed enhancement of transcription. The E1A proteins are also known to autoregulate their own transcription (5, 23, 37, 38), and a small increase in the amount of E1A protein (or activity) in infected cells could feed back and exaggerate the initial, more subtle effect. This effect would also be compounded by an increase in the template copy number as more viral DNA is synthesized as a result of the increase in viral gene expression.

Why is the effect of the E1B 19K protein on adenovirus early gene expression so clearly demonstrated in WI38 cells, but not in HeLa or KB cells? One striking difference is in the time course of adenovirus infection in the various cell lines: adenovirus replicates within 24 to 30 h in HeLa, KB, and HEK cells, but takes 4 to 5 days in WI38 cells. This may be due to differences in growth physiology between the cell types. WI38 cells are a nontransformed human diploid secondary lung fibroblast cell line which are easily growth arrested (21, 22). In contrast, HeLa and KB cells are rapidly growing transformed cells that serve as extremely productive hosts for adenovirus lytic infection. A tightly controlled growth physiology of the WI38 cells may result in limited amounts of host-cell factors that are required for adenovirus gene expression and DNA replication. This is suggested by the observation that HeLa cells, but not WI38 or HEK cells, are capable of supporting the growth of E1A$^-$ viruses at high multiplicities of infection (26, 28, 39). The difference in growth physiology between HeLa and KB cells on the one hand, and WI38 and HEK cells on the other hand, may also account for the absence of the *deg* phenotype in WI38 and HEK cells.

We initiated studies on the role of the E1B 19K protein in the regulation of gene expression during adenovirus infection. Current experiments indicate that the 19K protein is indeed regulating early gene expression in HeLa cells, since we demonstrated that 19K$^-$ viruses overproduce early virus-encoded proteins relative to the levels found in wild type-infected HeLa cells (unpublished observations). This uncontrolled expression of early genes during E1B 19K mutant adenovirus infection of HeLa cells leads to overproduction of early and late proteins and may cause enhanced cytopathic effect and premature cellular destruction. The E1B 19K protein may therefore contribute to the temporal regulation of adenovirus gene expression during lytic infection. Further experimentation will determine whether this hypothesis is correct.

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