Adenovirus Type 5 Early Region 1b Gene Product Is Required for Efficient Shutoff of Host Protein Synthesis

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To determine the role adenovirus 5 early region 1b-encoded 21- and 55-kilodalton proteins play in adenovirus productive infection, mutants have been isolated which were engineered to contain small deletions or insertions at 5.8, 7.9, or 9.6 map units. By using an overlap recombination procedure involving H5dl314 (Δ 3.7 to 4.6 map units) DNA cleaved at 2.6 map units with *Cla*I and the adenovirus 5 *Xho*I-C (0 to 15.5 map units) fragment containing the desired mutation, viral mutants were isolated by their ability to produce plaques on KB cell line 18, which constitutively expresses only viral early region 1b functions (Babiss et al., J. Virol. 46:454-465, 1983). DNA sequence analysis of the viral mutants isolated (H5dl118, H5dl110, H5in127, and H5dl163) indicates that all of the viruses contain mutations which affect the 55-kilodalton protein, whereas dl118 should also produce a truncated form of the 21-kilodalton protein. When analyzed for their replication characteristics in HeLa cells, all of the mutant viruses exhibited extended eclipse periods and effected yields that were reduced to 10% or less of that produced by H5sub309 (parent virus of the mutants which is phenotypically identical to wild-type adenovirus 5). When compared with characteristics of sub309, the early and late transcription and DNA replication of the mutants were similar, but synthesis of late polypeptides and late cytoplasmic mRNAs was greatly reduced. Quantitation of mutant virus-specific late mRNAs associated with polysomes revealed a threefold reduction when compared with that of sub309. Analysis of infected cell extracts further revealed that these mutants were incapable of efficiently shutting off host cell protein synthesis, suggesting that the 55-kilodalton protein plays a role in this process. These data suggest that early region 1b products may function by interacting with additional viral or host cell macromolecules to modulate host cell shutoff or that some late viral mRNA or polypeptide may potentiate this reaction.

The early region 1a (E1a) and E1b genes of adenovirus 5 (Ad5) (0 to 11 map units [m.v.]) have been shown to encode proteins which are capable of initiating and maintaining a transformed cell phenotype when the genes are stably maintained and expressed in susceptible rodent cells (19, 20, 22, 23). In addition to these functions, the 51-kilodalton (kd) protein encoded by the 13S mRNA from E1a has been shown to enhance the transcription of additional early gene regions before the onset of viral DNA replication (4, 28, 43). The E1b gene codes for 55- and 21-kd polypeptides (15, 24), whose functions during the viral replicative cycle remain unclear. The 55-kd protein has been shown to be a product of spliced 23S mRNA (15, 38), whereas the 21-kd protein can be translated from either 23S or 13S mRNAs (5). Tryptic peptide analysis of both proteins revealed that they are unrelated (6), which Bos et al. (5) confirmed by DNA sequence analysis.

A number of suspected point mutations (9, 25) and deletion and insertion mutations (29) have been introduced into the E1b gene of various adenovirus serotypes, and they have resulted in a variety of phenotypes. These include: the generation of large clear plaques by both Ad2 and Ad12 (58) mutants on KB cell lines; degradation of cellular and viral DNAs during nonpermissive infections by Ad2 (13), Ad5 (33), and Ad12 (16) mutants; overproduction of E2a- and E3-encoded proteins during infections of HeLa cells with Ad5 mutants (34, 52); and the need for an E1b gene product(s) for the efficient replication of adeno-associated virus DNA (35,

47). Although most of these mutants have been phenotypically well characterized, the nature of the specific mutational lesion present in the E1b gene usually has been undefined, and the E1a region also has been usually encumbered. Thus, these mutations do not permit relating the phenotypes of the mutants to the altered proteins produced.

In an effort to define more clearly the function(s) of the 21- and 55-kd proteins encoded in the E1b regions, deletions and insertions were introduced into the E1b gene of Ad5, and the resultant mutants were isolated by their ability to replicate on a complementing KB cell line that constitutively expresses only viral E1b gene functions (2). DNA sequence analyses of the viral mutants isolated (dl118, dl110, in127, and dl163) indicate that they should produce truncated forms of the 55-kd protein, whereas only dl118 contains a mutation which also affects the 21-kd protein. As a consequence of the mutations, all of the viral mutants showed a reduced ability to shut off host protein synthesis efficiently upon infection of HeLa or KB cells, suggesting that the 55-kd protein may play some role in this process. In addition, all of the mutants produced reduced amounts of late polypeptides, and evidence is presented which suggests that some stage in the processing or transport of late viral transcripts is perturbed.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of human KB, 293, (21), HeLa, and KB cell line 18 (a KB cell line that constitutively expresses the bacterial XGPRT gene and the Ad2 E1b gene; 2) cells were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum. Suspension cultures of human KB cells were grown in modified

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minimal essential medium supplemented with 10% human serum.

H5*sub309* and H5*dl314* (Δ 3.7 to 4.6 m.u.) were provided by Thomas Shenk (29), and were propagated in KB cell suspension cultures or 293 cell monolayers, respectively.

Construction of recombinant viruses. The Ad5 *XhoI*-C (0 to 15.5 m.u.) DNA fragment was cloned into the plasmid vector pMK2004 (30) as previously described (2). This cloned viral DNA was cleaved at the unique *KpnI* (5.8 m.u.), *HindIII* (7.9 m.u.), or *BglII* (9.6 m.u.) site, and deletions were introduced by digestion with 0.001 U of nuclease *Bal31* (Bethesda Research Laboratories) for 10 to 30 min at 37°C. To construct an insertion mutation, plasmid DNA was linearized by digestion with *HindIII*, and the single-stranded tails generated were filled in by treating the DNA with 2 U of DNA polymerase (large fragment; Boehringer Mannheim Biochemicals). These altered molecules were recircularized with T4 ligase, cleaved with the appropriate restriction enzyme to remove molecules that escaped the initial exonuclease digestion, and introduced into *Escherichia coli* HB101 cells. The transformed bacterial cells were selected by their acquired ampicillin resistance. All plasmids were isolated and purified by the Sarkosyl-lysate procedure of Clewell and Helinski (11).

The overlap recombination method, as previously described (1a, 9), was used to introduce the mutated viral sequences contained within the hybrid plasmids into a complete viral genome (Fig. 1). Viral plaques were isolated 11 days after transfection of KB cell line 18 cells (2) with a mixture of 1 μ g of *dl314* (29) DNA-protein complex (10) cleaved with *ClaI* (2.6 m.u.) and a 1- μ g equivalent of plasmid DNA cleaved with *XhoI* and *BamHI*.

FIG. 1. Diagram of the steps used to construct mutants in E1b genes of Ad5. The diagram represents the method for constructing *dl118*, *dl110*, *in127*, and *dl163* by overlap recombination between the mutated 0- to 15.5-m.u. fragment (equivalent to 1 μ g) and the 2.6- to 100-m.u. fragment of H5*dl314* (1 μ g), which contains a deletion extending from 3.7 to 4.6 m.u. Wild-type and mutated 0- to 15.5-m.u. DNA fragments of Ad5 were cloned into the unique *BamHI* and *XhoI* sites in pMK2004 (30), and before transfection, the viral DNA was released from the intact plasmid by cleavage with *BamHI* and *XhoI*. Filled-in boxes denote areas of deleted or inserted DNA sequences. The dotted line represents a recombination event which would regenerate the parent virus *dl314*, but its presence was not detected since KB cell line 18 cells (2) only complement viral E1b gene defects. Curved bold lines 1 and 2 denote recombination events which generate wild-type or E1b mutant viruses, respectively.

All viruses were plaque purified three times before producing stocks for characterization and study. Mutant viruses were identified by their *KpnI* (H5*dl118*), *HindIII* (H5*dl110* and H5*in127*), or *BglII* (H5*dl163*) digestion patterns, which revealed the loss of one enzyme site (data not shown). The size of the deletion(s) or insertion(s) generated was determined by DNA sequence analysis by the Maxam and Gilbert technique (41).

Virological methods. Replicative cycles of each virus in HeLa or KB cell line 18 cells were performed as previously described (2). Viral yields were titrated on 293 cells by indirect immunofluorescence, using rabbit antiserum directed against Ad5 late antigens (59), or by plaque assay (65).

Viral DNA replication was monitored and quantitated by labeling *dl118*- and *sub309*-infected HeLa cells for 20 min with [3 H]thymidine (10 μ Ci/ml) at different times after infection and hybridizing total cell DNA to nitrocellulose filters containing 5 μ g of *sub309*-denatured DNA (39, 64). Viral DNA replication was additionally assayed by equilibrium density centrifugation (14). The size and integrity of the DNA molecules synthesized during mutant virus and *sub309* virus infection of HeLa cells were assayed by alkaline sucrose gradient centrifugation (16).

RNA analysis. *dl118*- and *sub309*-infected KB cell RNAs were labeled for 10 min with [3 H]uridine (200 μ Ci/ml) at the times indicated in the figure legends and on the table. Total cytoplasmic and nuclear RNAs were isolated from ca. 2×10^7 cells (2) and hybridized to nitrocellulose filters containing 5 μ g of Ad5 DNA fragments extending from 50 to 60 m.u. (region L3 specific) or 83 to 90 m.u. (region L5 specific), as described by Nevins (44).

Pulse-labeled RNAs associated on polysomes in KB cells infected with 5 PFU of *dl118* of *sub309* per cell were isolated by the method of Palmiter (48). Virus-specific RNAs were identified and quantitated by DNA-RNA filter hybridization as described above.

Polyadenylated [poly(A)⁺] cytoplasmic RNA was isolated from *dl118*-, *dl63*-, or *sub309*-infected KB cells at 20 and 40 h postinfection by chromatography on oligodeoxythymidylic acid-cellulose (type 3; Collaborative Research, Inc.; 2). Virus-specific L3 and L5 mRNA species were identified by electrophoretically fractionating denatured RNAs on agarose-formaldehyde gels (12), transferring the RNAs to nitrocellulose filters (62), and hybridizing with nick-translated, [32 P]-labeled (50) adenovirus L3- and L5-specific DNA probes.

VA RNA analysis was performed by labeling virus-infected KB and 293 cells with [32 P]_i (200 μ Ci/ml) from 2 to 16 h postinfection and fractionating the total cytoplasmic RNA on 6% polyacrylamide gels containing 8 M urea in Tris-borate buffer for 12 h at 500 V (61).

Protein analysis. The proteins of mutant- or *sub309*-infected HeLa cells, cultured in Dulbecco modified Eagle medium containing 5% of the usual concentration of methionine, were labeled with [35 S]methionine (10 μ Ci/ml) at various times after infection. At the end of the labeling period, cells were harvested, and samples containing equal numbers of trichloroacetic acid-precipitable counts either were processed for electrophoretic fractionation on sodium dodecyl sulfate (SDS)-polyacrylamide gels containing 17.5% polyacrylamide and 0.75% bisacrylamide (32) or were processed further by immunoprecipitation to quantitate late viral polypeptides (46). After electrophoresis, gels were fixed (50% [vol/vol] methanol, 10% acetic acid), enhanced (En³Hance; New England Nuclear Corp.), dried, and exposed to X-ray film.

For polypeptide transfer-immunoblot studies, infected HeLa cells were harvested 10 h after infection and resuspended in 1 ml of reticulocyte standard buffer (0.01 M NaCl, 0.01 M Tris-hydrochloride [pH 7.4], 0.0015 M MgCl₂ containing 10% Nonidet P-40) at a density of 6×10^6 cells per ml. Nuclei were pelleted at $3,000 \times g$, resuspended in 0.5 ml of TE buffer (0.1 M Tris-hydrochloride [pH 8.0], 0.001 M EDTA), and sonicated for 10 s on ice. Samples (10 and 20 μ l) were mixed with $3 \times$ sample buffer (6% SDS, 0.03% bromophenol blue, 30% glycerol, 15% β -mercaptoethanol) and boiled for 3 min. The samples were fractionated on SDS-polyacrylamide gels as described above. For the transfer of proteins to nitrocellulose filters (17), gels were soaked in transfer buffer (Tris base, 3 g/liter; glycine, 14.4 g/liter; 20% [vol.vol] methanol) for 30 min and then electrophoresed for 3 h at 170 mA. The filters were then incubated in RIPA buffer (0.05 M Tris-hydrochloride (pH 7.4), (0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS; 31) containing 0.5% bovine serum albumin for 30 min and then in 5 ml of RIPA buffer containing the desired dilution of rabbit antiserum to the 72-kd DNA-binding protein or the 21-kd E1b polypeptide (kindly provided by Maurice Green, St. Louis University School of Medicine) for 90 min. After several washes with RIPA buffer, the filters were incubated in RIPA buffer containing [¹²⁵I]-labeled staphylococcal protein A (1 μ Ci; Amersham Corp.) for 90 min, washed with

RIPA buffer and 0.5 M Tris-hydrochloride (pH 7.4), dried, and exposed to X-ray film.

RESULTS

Construction of viral mutants. The steps involved in the construction of deletion and insertion mutations in the E1b gene of Ad5 are illustrated in Fig. 1. The initial goal was to introduce mutations that would alter the reading frames of the E1b-encoded 55- and 21-kd proteins, to determine the differential effects of their expression. To produce the desired mutations, the Ad5 *Xho*I-C DNA fragment (0 to 15.5 m.u.) was cloned into a plasmid vector, and the defects were generated at unique restriction endonuclease sites: *Kpn*I (5.8 m.u.), *Hind*III (7.9 m.u.), or *Bgl*II (9.6 m.u.), using exonuclease Bal31 to produce deletions; insertions of DNA sequences were made at the *Hind*III enzyme site, using the *E. coli* large fragment DNA polymerase. The altered E1b gene sequences were introduced into a complete viral genome by overlap recombination (9), with the terminal DNA-protein complex fragment (2.6 to 100 m.u.) from the deletion mutant *dl*314 (29) cleaved with *Cl*aI. By using KB cell line 18, which constitutively expresses only viral E1b gene products (2), no background due to incomplete cleavage of the *dl*314 or progeny resulting from a recombination event 5' to the E1a deletion (Fig. 1, dotted line) were observed, since no E1a gene products were present to allow their replication. Fur-

FIG. 2. Nucleotide sequences of deletions and insertions constructed into the E1b region of Ad5. A DNA fragment extending from 5.0 to 7.9 m.u. derived from *dl*118 was cloned into a plasmid vector containing the herpes simplex virus TK gene (42) and was propagated in dam strain GM33 of *E. coli* (40). The DNA sequence from a *Bst* E11 site (1,915 bp from the terminus) was determined by the chemical degradation method of Maxam and Gilbert (41). The size of the deletion present in *dl*163 was determined by cloning a DNA fragment extending from 7.9 to 10.1 m.u. into the same plasmic vector and then sequencing toward the deleted *Bgl*II site (9.6 m.u.) from a 5'-end-labeled *Av*cI site. For both mutants *dl*110 and *in*127, a DNA fragment extending from 5.6 to 9.6 m.u. was cloned into the plasmid vector pMK2004. The size of the deletion present in *dl*110 was determined by sequencing toward the *Hind*III site (7.9 m.u.) from a 5'-end-labeled *N*coI DNA site (2,207 bp from the terminus). The integrity of the insertion present at 7.9 m.u. for *in*127 was confirmed by sequencing from a 5'-end-labeled *R*saI site (2,762 bp from the terminus). Hatched boxes show the deleted (Δ) DNA sequence in each mutant, and the filled box indicates an insertion (∇) of DNA. Dashed lines represent sequences removed during the processing of the 23S and 13S mRNAs. The nucleotide positions of the splice junctions and 5' and 3' termini are from Bos et al. (5).

thermore, the frequency of recombination that yielded progeny virus containing the altered E1b gene was inversely proportional to the distance between the E1a deletion and E1b mutation. Therefore, the number of progeny viral plaques produced by mutants containing the desired E1b phenotype could be predicted (63).

All of the mutant viruses isolated were plaque purified three times on either 293 cells or cell line 18 cells. Since the plating ratios of all mutant viruses isolated were smaller on 293 and cells line 18 cells, it could be assumed that, as a result of the recombination event, no other viral gene sequences were altered. This was further confirmed by analyzing restriction enzyme digest patterns of mutant viral DNAs and by marker rescue of *dl118*, using a DNA fragment extending from 4.5 to 9.6 m.u. (data not shown).

DNA sequence analysis of E1b mutants. DNA sequence analysis was done to determine how the mutations present in the four viral mutants isolated affected the coding capacity of the E1b 23S and 13S mRNAs. The positions of the deletions or insertions were mapped by sequencing (41) different restriction endonuclease pieces of cloned DNA fragments derived from each of the isolated mutant viral genomes. The data indicate that *dl118* contained a 543-base-pair (bp) deletion extending from 2,049 to 2,591 bp (Fig. 2). As a result, the reading frame for the 55-kd protein was not changed; therefore, it would be predicted that a ca. 35-kd deleted form of the protein should be produced. The 21-kd protein encoded by the 23S mRNA should be reduced to 12-kd, although its presence could not be detected in *dl118*-infected HeLa cells (see Fig. 4). The 13S mRNA should also be altered by this mutation since the 5' donor splice site at nucleotide 2,255 was deleted.

Mutant virus *dl110* was determined to contain a 472-bp deletion extending from 2,333 to 2,804 bp (Fig. 2). As a result of this mutation, a frameshift was introduced into the reading frame of the 23S mRNA, producing a stop codon (TAA) at 2,851 to 2,853 bp. The 23S mRNA would now be predicted to produce a truncated polypeptide of only 14 kd. This mutation should not alter the production of the 21-kd protein from either viral mRNA (Fig. 2).

Like the mutations in *dl110*, the mutations present in both *in127* and *dl163* should not alter the expression of the 21-kd protein in HeLa cells. However, by virtue of the 4-bp insertion at the *HindIII* site at 7.9 m.u. in *in127*, a frameshift mutation was introduced into the 23S mRNA, resulting in premature termination of translation at newly inserted nucleotides 2,808 to 2,810 (TAG). *dl163* contained a frameshift mutation as a result of an 8-bp deletion extending from 3,326 to 3,333 bp and should produce a premature stop codon (TGA) at positions 3,354 to 3,356. All of the sequence data generated by analyses of these viral mutants were in complete accord with the Ad5 sequence obtained by Bos et al. (5).

Replication characteristics of *sub309* and E1b deletion and insertion mutants. Plaquing ratio analysis, using HeLa cells and KB cell line 18 cells, suggested that the four E1b mutants were not completely defective for replication in noncomplementing HeLa cells (data not shown). However, to determine what aspect of the viral replicative cycle was altered by the E1b mutations, a careful examination of the replicative cycle of each virus in HeLa cells and cell line 18 cells was necessary.

Replicate monolayers of HeLa cells and cell line 18 cells were infected with the appropriate viral mutant or *sub309* at 50 PFU per cell; individual samples were obtained at various intervals, and viral yields were determined, using 293 cells or

cell line 18 cells. *H5sub309* was used for comparison in all of our studies, since it has been determined that its replicative characteristics are indistinguishable from wild-type Ad5, and with the exception of the *XbaI* site of 3.8 m.u., the genome structures of *sub309*, *dl314*, and all of our mutants are identical.

Sub309 replication on both cell lines was indistinguishable, as was the ability of *sub309* and all of the mutant viruses to replicate in the complementing KB cell line 18 (data not shown). In contrast, all of the viral mutants replicated poorly in HeLa cells (Fig. 3), producing slower replication cycles and yields of less than 10% in comparison with cycles and yields of *sub309*. For all of the mutants, the replicative process was still exponential as late as 72 h postinfection, whereas the *sub309* infection had attained maximum yield by at least 48 h. It should be noted that replication of the viral mutants was unaffected by temperatures ranging from 32 to 39.5°C. It is of interest that *dl118* was much more defective for replication in HeLa cells than were the other viral mutants tested. Since *dl118* does not encode normal 21- or 55-kd proteins and the remaining viral mutants do express functional 21-kd polypeptide, it is possible that both proteins play a role in the viral replication cycle in HeLa cells.

FIG. 3. Viral replicative cycles in HeLa cells. Monolayers of HeLa cells were infected with 50 PFU of *dl118* (■), *dl110* (▲), *in127* (△), *dl163* (●), or *sub309* (○) per cell and incubated at 37°C. Samples were taken at various times postinfection (P.I.), frozen and thawed six times to release intracellular virus, and titrated for infectivity by the fluorescent focus assay, using KB cell line 18 or 293 cells cultured at 37°C. FFU, Focus-forming units.

Additional experiments were done, using HeLa cells and *dl118* at multiplicities of infection of 5 and 100 PFU per cell (data not shown), since it has been previously shown that the degree of defectiveness of these types of viruses is inversely proportional to the multiplicity of infection (55). These studies illustrated that the final viral yield at 72 h was affected by the amount of input virus, but the overall kinetics of the viral replicative cycle were unaltered.

To determine what stage of the viral replicative cycle was altered to reduce the yield of progeny virus in infected HeLa cells, the major biosynthetic events of the replicative cycle were monitored.

Early mRNA and protein synthesis. In HeLa cells infected with *sub309* and the four E1b mutants, synthesis of early cytoplasmic poly(A)⁺ containing mRNAs and early proteins was analyzed by RNA filter transfer-DNA hybridization (12, 62) and immunoblot assays (17), respectively (Fig. 4). The steady-state levels of early mRNAs from regions E1a and E4 (data not shown) and E2a and E1b were similar for all of the viral mutants and *sub309* at 10 h after infection. In addition, due to the mutations present, *dl118* produced a deleted form of both the 23S and 13S E1b mRNAs; the 13S mRNA appears to be of normal size, which implies that it employed an alternative splice site. As expected, *dl110* transcribed a shortened RNA affecting only the 23S message. The amounts of both the 72-kd protein encoded by the E2a gene and the 21-kd protein encoded by the E1b gene were approximately the same in *dl110*-, *in127*-, *dl163*-, and *sub309*-infected HeLa cells at 10 h after infection. *dl118* did not

translate a truncated form of the 21-kd protein that could be detected by using a polyclonal antiserum raised against the wild-type protein.

Viral DNA synthesis. To study viral DNA synthesis, HeLa cells were infected with 5 PFU of *dl118* or *sub309* per cell, the cells were labeled with [³H]thymidine at various intervals, and total infected-cell DNA was hybridized to filters containing excess *sub309* DNA. It is striking that the overall kinetics of viral DNA replication for both viruses were similar (Fig. 5). In addition, when DNA replication products were analyzed by equilibrium density centrifugation, all of the viral mutants generated profiles that were indistinguishable from that of *sub309*, and all were capable of shutting off host cell DNA replication (data not shown).

These findings suggest that E1b gene products do not play a role in the processes required for synthesis of progeny viral DNA; this was also shown when *H5hr6* was used (34). However, studies in which an Ad5 host range, deletion mutant, *dl313* (29), was used suggested that one E1b product may function as an antinuclease (33, 35). To determine whether any of the newly constructed E1b mutants produced DNA degradation, infected cells were pulse-labeled with [³H]thymidine from 12 to 13 h postinfection and chased for an additional 12 h, after which time total cellular DNA was isolated and analyzed on alkaline-sucrose gradients. None of the four E1b mutants studied showed any evidence of viral or cellular DNA degradation, whereas *dl313* degraded viral and host cell DNAs as previously described (data not shown).

FIG. 4. Expression of adenovirus E1b- and E2a-specific mRNAs and polypeptides in HeLa cells. HeLa cell monolayers were infected with 5 PFU of each virus per cell at 37°C, and the RNAs were isolated at 10 h postinfection. Five micrograms each of cytoplasmic poly(A)⁺ RNAs from *dl118*-, *dl110*-, *in127*-, *dl163*-, and *sub309*-infected HeLa cells was denatured and electrophoresed in 0.8% agarose gels containing 2.2 M formaldehyde as described in the text. After transfer to nitrocellulose filters, RNAs were hybridized with nick-translated E1b (5 to 10.1 m.u., panel A) or E2a (60.2 to 63.6 m.u., panel B) DNA. The two E1b-specific mRNAs synthesized during a *sub309* infection of HeLa cells were 2.3- and 1.03-kb in size. The E2a-specific mRNA produced for all of the viruses was 2.3-kb in size. Early polypeptides were detected by infecting HeLa cells at a multiplicity of 5 PFU of each virus per cell, harvesting the cells at 10 h postinfection, and preparing nuclear extracts. After electrophoresis in SDS-polyacrylamide gels, the proteins were electrophoretically transferred to nitrocellulose filters, and the viral E1b (panel C) and E2a (panel D) polypeptides were localized by using polyclonal serum to the 21- or 72-kd species followed by [¹²⁵I]-labeled protein A as described in the text. Lanes 1 and 2 represent 1.2×10^6 and 2.4×10^6 cells, respectively; unif, uninfected.

FIG. 5. DNA replication of *sub309* and *dl118* in HeLa cells. Monolayers of HeLa cells were infected with *dl118* (●) or *sub309* (○) at a multiplicity of 5 PFU per cell, and total cell DNA was labeled using [³H]thymidine (10 μ Ci/ml) for 20 min at the times (P.I., postinfection) indicated. Infected cell DNA was isolated and hybridized to nitrocellulose filters containing 5 μ g of denatured *sub309* DNA. All counts were corrected for background and nonspecific hybridization and normalized for the efficiency of hybridization, as determined by using [³²P]-labeled *sub309* DNA.

Synthesis of late viral transcripts and mRNAs. To determine whether the transcription rates of late viral RNAs differed in *dl118*- and *sub309*-infected KB cells, RNAs were pulse-labeled with [³H]uridine, and the cytoplasmic and nuclear RNAs were quantitated by DNA-RNA filter hybridization analysis (44) at various times after infection. These studies indicate that the overall kinetics and quantities of transcripts arising from two late transcription units, L3 (encodes the hexon polypeptide) and L5 (encodes the fiber polypeptide), were almost identical for both *dl118* and *sub309* (Fig. 6). Therefore, the poor yield of *dl118* progeny virus in KB cells could not be attributed to a decrease in late transcription.

The steady-state levels of poly(A)⁺-containing cytoplasmic RNAs isolated at 20 and 40 h after *dl118*, *dl163*, and *sub309* infection of KB cells were analyzed by RNA-transfer, DNA-filter hybridization, using [³²P]-labeled L3- and L5-specific DNA probes (Fig. 7). The data indicate that both E1b mutants and *sub309* produced spliced mRNA species of the appropriate sizes. However, at 20 h after infection, the quantities of the cytoplasmic mRNA species present in both *dl118*- and *dl163*-infected cells were reduced when compared with those present in *sub309*. At 40 h postinfection, when the yield of *sub309* virions had attained maximum levels and when viral replication was continuing to increase in *dl118*- and *dl163*-infected cells (Fig. 3), the levels of L3 and L5 mRNAs were not as disparate for all three viruses. Since both mutants exhibited these same characteristics of late mRNA production, it is most likely that the apparent defect in processing or transport of late mRNAs is related to the absence of the completely functional 55-kd protein, since *dl163* produced the 21-kd polypeptide.

To examine whether the apparent reduction in late mRNAs further resulted in a decrease in the amount of viral mRNAs associated with polysomes, *dl118*- and *sub309*-infected KB cells were analyzed. RNA-transfer DNA hybridization analysis of fractions isolated from sucrose gradi-

FIG. 6. RNA transcribed from L3 and L5 regions in KB cells infected with *dl118* or *sub309*. KB cell spinner cultures infected with 5 PFU of *dl118* (□, nuclear; ■, cytoplasmic) or *sub309* (○, nuclear; ●, cytoplasmic) per cell were labeled with [³H]uridine (200 μ Ci/ml) for 10 min at the times (P.I., postinfection) indicated. Nuclear and cytoplasmic RNAs were isolated and hybridized to nitrocellulose filters containing 5 μ g of denatured L3 (50 to 60.1 m.u.) or L5 (83 to 90 m.u.) DNA, as described in the text. All counts shown were corrected for background, non-specific hybridization, and the efficiency of hybridization.

ent polysome profiles (27) of *dl118*- and *sub309*-infected KB cell extracts revealed that both L3 and L5 mRNAs were present predominantly in the polysome peak at 20 h postinfection (data not shown). To quantitate the amount of viral mRNAs associated on polysomes at 20 and 40 h after infection of KB cells with *dl118* or *sub309*, polysomes were isolated (48), and the viral mRNAs associated were quantitated by DNA-RNA filter hybridization (44). At 20 h postinfection, *dl118*-infected KB cells contained about one-third the quantity of total late mRNAs associated on polysomes as did *sub309*-infected cells (Table 1). At 40 h after infection, the levels of virus-specific mRNAs for both viruses were similar, but at this time the production of virions in *sub309*-infected cells had begun to decline, whereas viral replication in *dl118*-infected cells was still in the incremental phase.

Late protein synthesis. To determine whether decreased production of late viral proteins was responsible for the reduced yield of infectious virus in cells infected with the E1b mutants under study, *dl118*- and *sub309*-infected HeLa cells were labeled for the indicated period of time (Fig. 8) with [³⁵S]methionine, and the polypeptides were analyzed by electrophoresis. Mutant virus-infected HeLa cells revealed very little virus-specific late polypeptides as late as 42 h after infection, in contrast with *sub309*-infected cell extracts. It is also striking that *dl118*-infected HeLa cell extracts did not exhibit the same level of shutoff of host cell protein synthesis as compared with *sub309*-infected cells extracts. This inability to shut off host protein synthesis did not appear to be multiplicity dependent since HeLa cells infected with 100 PFU of *dl118* per cell still displayed the continued synthesis of large amounts of host proteins (data not shown). However, as the multiplicity of *dl118* increased, the relative level of virus-specific polypeptides among the total protein population of infected cells did increase.

This phenomenon was also observed for *dl110*, *in127*, and *dl163* (data not shown), suggesting that the ability to shut off host cell protein synthesis effectively may require function of the E1b 55-kD protein. However, the 55-kD protein probably does not act independently to block host protein synthesis, since cell line 18 and 293 cells continue to synthesize host proteins and undergo cell divisions in the presence of the constitutively expressed 55-kD protein.

To quantitate the amounts of late polypeptides produced

FIG. 7. Analysis of viral mRNA species present at late times after infection of KB cells with *dl118*, *dl163*, or *sub309*. Poly (A)⁺ cytoplasmic RNAs were isolated from KB cells 20 and 40 h after infection with 5 PFU of *dl118*, *dl163*, or *sub309* per cell. One-tenth-microgram (lane 1) and one-microgram (lane 2) quantities of denatured RNA were fractionated on 0.8% agarose gels containing 2.2 M formaldehyde as described in the text. After transfer to nitrocellulose filters, RNAs were hybridized with nick-translated L3 (50 to 60.1 m.u., panel A) or L5 (83 to 90 m.u., panel B) DNA. The L3-specific mRNAs produced by *sub309* were 3.96 and 3.24 kb in size, and the L5-specific mRNAs were 1.93 and 1.8 kb in size.

TABLE 1. Quantitation of virus-specific polysome-associated late mRNAs

Virus	Time of ³ H pulse (h) ^a	Total counts hybridized ^b	
		Expt. 1	Expt. 2
<i>sub309</i>	20	373,746	151,557
	40	148,962	67,893
<i>dl118</i>	20	120,575	50,208
	40	110,552	52,107

^a KB cell monolayers were infected with 5 PFU per cell and labeled at the indicated time with 200 μ Ci of [³H]uridine per ml for 10 min.

^b All counts determined were corrected for background and efficiency of hybridization.

in E1b mutant virus- and *sub309*-infected HeLa cells, immunoprecipitations of [³⁵S]-labeled cell extracts were done. For *dl118* at a multiplicity of infection of 5 PFU per cell, the amount of late virus-specific polypeptides synthesized between 18 and 22 h or between 38 and 42 h was reduced when compared with the amount synthesized for *sub309* (Fig. 9). To quantitate this reduction, trichloroacetic acid-precipitable [³⁵S]methionine counts were determined for each immunoprecipitated sample, and they revealed that late poly-

FIG. 8. SDS-polyacrylamide gel autoradiogram of [³⁵S]-labeled cell extracts from *dl118*- and *sub309*-infected HeLa cells. HeLa cell monolayers were infected with 5 or 50 PFU of *dl118* or *sub309* per cell and labeled at the times indicated with [³⁵S]methionine (10 μ Ci/ml) in Dulbecco modified Eagle medium containing one-twentieth the usual concentration of methionine. After a 4-h labeling period, cells were harvested, disrupted in sample buffer (1% SDS, 1% β -mercaptoethanol, 0.05 M Tris-hydrochloride [pH 0.8], 10% glycerol), and boiled for 3 min. Electrophoresis was done on SDS-polyacrylamide gels as described in the text. Each lane represents an extract from ca. 5×10^4 cells; Unif., uninfected.

FIG. 9. Immunoprecipitation of late viral polypeptides in virus-infected HeLa cells. HeLa cells were infected with 5 or 50 PFU of *dl118* per cell or 5 PFU of *dl110*, *in127*, *dl163*, or *sub309* per cell and labeled at the indicated times with [35 S]methionine (10 μ Ci/ml). Whole-cell extracts were prepared, and virus-specific late polypeptides were detected by immunoprecipitation by using (+) polyclonal serum to Ad5-infected cell lysates or (-) normal rabbit serum. The samples were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Unif., Uninfected.

peptide production in *dl118*-infected HeLa cells was reduced by 12-fold between 18 and 22 h and by ca. 6- to 8-fold between 38 and 42 h in comparison with production in *sub309*-infected HeLa cells (data not shown). At 50 PFU per cell, the synthesis of late polypeptides was increased somewhat, but the synthesis of cell-specific proteins (e.g., actin) appeared to remain constant (Fig. 8). Similar observations were made when *dl110*, *in127*, and *dl163* were used, but these observations were in agreement with the kinetics of the viral replicative cycle (Fig. 3), the other mutants synthesizing slightly higher quantities of late polypeptides than did *dl118*.

VAI RNA synthesis. Thimmappaya et al. (61) have shown that VAI RNA is required for efficient translation of viral mRNAs at late times after infection. However, when that synthesis of VAI RNA was determined in *dl118*-, *dl163*-, and *sub309*-infected KB cells and 293 cells by labeling with 32 P, between 2 and 14 h after infection, it was found that the levels of VAI (G) RNAs all appeared similar (Fig. 10). Therefore, the decreased translation of viral late mRNAs cannot be explained by failure to produce adequate amounts of VAI RNA. In addition, all of the viruses used in the present studies are unable to synthesize VAI (A) RNAs, and they synthesized greatly reduced amounts of the VAII RNA species. Thimmappaya et al. (60, 61) have shown that these RNAs are not essential for viral replication in the HeLa cells.

DISCUSSION

Transcriptional studies involving Ad5-infected mammalian cells have revealed that at least six regions on the viral genome are expressed before viral DNA replication (for a review, see reference 37). Although mutations have been introduced into most of these regions, still very little is known about the functions of the early polypeptides encoded therein. The adenovirus E1b has been extensively studied since it has been shown to play a role in the process of cellular transformation (19, 20, 22, 23). Studies with viruses containing mutations introduced into the E1b region to determine the exact role the 55- and 21-kd proteins may serve in transforming cells suggest that the 21-kd protein may act in conjunction with an E1a gene product to initiate and maintain the transformed state (8; L. E. Babiss, P. B. Fisher, and H. S. Ginsberg, manuscript in preparation), whereas the 55-kd protein may function to express fully the characteristics of the completely transformed cell (53, 56; Babiss et al., manuscript in preparation). Transfection studies in which an Ad5 DNA fragment extending from 0 to 7.9 m.u. was used revealed that E1a and partial E1b gene products (21-kd and truncated 55-kd proteins) can effect complete transformation of rodent cells at a frequency similar to a DNA fragment containing both the E1a and intact E1b genes (22). However, in studies in which a cloned rat embryo cell line cells (18) and Ad5 viruses constructed to

contain deletions or insertions at 7.9 or 9.6 m.u. were used, it was found that completely transformed foci were generated, but that mutations at either site reduced the frequency of transformation and that foci appeared at a progressively reduced frequency as the mutations approached the N-terminal end of the 55-kd protein (Babiss et al., manuscript in preparation).

The role of the 55- and 21-kd proteins during the viral replicative cycle remains unclear. Previous studies, using Ad5 host range mutants containing either supposed point mutations (34) or deletion and insertion mutations, which also encompass the E1a region (29), have suggested that E1b gene products do not function to replicate viral DNA but do affect the synthesis of late viral polypeptides. However, the exact nature of the mutations present in some of these viruses or what effect the mutation had on either viral polypeptide is unknown.

As a means of relating a viral function to a specific E1b polypeptide, a series of mutations was engineered into the E1b region of Ad5, and the exact nature of the lesion was

determined by DNA sequence analysis (Fig. 2). The viral mutant *dl118* was shown to be defective for the production of both the 55- and 21-kd polypeptides (Fig. 4; unpublished data), and consequently, its replication in HeLa cells was reduced ca. 50-fold (Fig. 3). Viral mutants *dl110*, *in127*, and *dl163* all synthesized the 21-kd protein (Fig. 4) and a predictably truncated 55-kd polypeptide, and all exhibited a ca. 10-fold reduction in viral yields in HeLa cells. From these observations, it appeared possible that both intact proteins were necessary for optimum viral replication but that they were not absolutely required for production of infectious virus.

All of the viral mutants appeared to synthesize wild-type quantities of viral DNA during infections of KB cells (Fig. 5; unpublished data), in agreement with previous studies in which H5hr6 was used (34). However, none of the mutants studied produced degradation of either viral or cellular DNA, as had been described for the Ad5 deletion mutant *dl313* (33) and several Ad12 *cyt* mutants (16). These findings suggest that if in fact an E1b gene product does function as an antinuclease, a possible candidate would be the 21-kd protein, whose truncated form in *dl118*-infected KB cells may retain partial functionality. We are presently investigating this possibility by constructing additional mutations in the E1b gene, which would alter only 21-kd polypeptide expression. It is also possible that a mutation at the 3' terminus of E1a, e.g., H5*dl313* which also must affect the 12S and 13S mRNAs, may abrogate an antinuclease activity.

Analysis of the kinetics of transcription from late regions L3 and L5 revealed no differences between *dl118*- and *sub309*-infected KB cells (Fig. 6). However, the steady-state levels of spliced, poly(A)⁺, cytoplasmic mRNAs from these two regions were reduced in *dl118*-infected KB cells and, to a lesser extent, in *dl163*-infected KB cells (Fig. 7). Since both mutants shared this phenotype, it is most likely that the 55-kd protein is involved in some process affecting mRNA maturation, stability, or transport.

Concomitant with this decrease in virus-specific late mRNAs and the reductions in late polypeptides (Fig. 9) was the fact that the ability of all of the mutants to shut off host protein synthesis efficiently was perturbed (Fig. 8). Again, the 55-kd protein was implicated in this process since all of the mutants shared this characteristic.

Sarnow et al. (54) showed that during a productive infection of HeLa cells with Ad5, monoclonal antibodies against the 55-kd protein coprecipitated a protein of 25 kd. Further analysis revealed that this protein was a product of the E4 gene and that a physical complex between both proteins existed. Two mutations which directly affect the expression of the 25-kd protein have been engineered into the E4 gene of Ad5 (7; J. R. Cutt, D. N. Halbert, and T. Shenk, personal communication). Both of these mutants resemble the E1b mutants described in this communication in that host cell protein synthesis is not appreciably abated during infections of HeLa cells. It is of particular interest to note that additional E4 mutations, which did not alter the expression of the 25-kd protein, were phenotypically indistinguishable from wild-type virus. In addition, since 293 and KB cell line 18 cells are viable and replicate, it is clear that expression of the 55-kd protein alone is not sufficient to turn off host protein synthesis. It is therefore likely that the 55-kd-25-kd protein complex formed during a productive viral infection may mediate this intracellular modification directly, or it may alter the efficient expression of a late polypeptide which potentiates this function.

This latter notion was approached by using an Ad5 mutant

FIG. 10. Autoradiogram of a polyacrylamide-urea gel containing [³²P]-labeled cytoplasmic VA RNAs from *dl118*-, *dl163*-, and *sub309*-infected KB and 293 cells. Monolayers of KB and 293 cells were infected with 5 PFU of *dl118*, *dl163*, or *sub309* per cell and labeled between 2 to 14 h after infection with ³²P_i (200 μCi/ml) in phosphate-free Dulbecco modified Eagle medium containing 2% dialyzed calf serum. Total cytoplasmic RNA was isolated, and 5-μg samples of each was applied to a 6% polyacrylamide gel containing 8 M urea in Tris-borate buffer. Electrophoresis was carried out for 12 h at 500 V. Unif., Uninfected.

which fails to produce VAI RNAs (61). Babich et al. (1) showed that HeLa cells infected with this VAI-defective virus normally reduced host cell protein synthesis, despite the marked decrease in the production of late viral polypeptides. These findings support the hypothesis that early viral gene products, such as the 55-kd–25-kd protein complex, are responsible for mediating the shutoff of host protein synthesis.

During the course of a viral infection of mammalian cells, the turnoff of host cell protein synthesis is a common phenomenon. Numerous studies have been performed to determine the mechanism(s) by which the virus induces this event and have suggested the following: alteration of the host cell translation apparatus occurs (51, 57); competition between viral and host cell mRNAs for ribosomes exists (27, 36); or degradation of preexisting host cell mRNAs during herpes simplex virus or fowl plague virus infection occurs (26, 45, 49).

Our present studies do not reveal the mechanism by which adenovirus early gene products may induce alterations in cellular gene expression. Beltz and Flint (3) have shown that during the late (20 h post infection) stage of an Ad5 productive infection of HeLa cells, the transcription of cellular genes is not greatly altered, but the appearance of newly synthesized cellular mRNAs in the cytoplasm is reduced. Babich et al. (1) extended these studies to show that the steady-state level of several cellular cytoplasmic mRNAs remained constant as late as 18 h after infection, but due to the synthesis of a discriminating factor, viral mRNA translation was favored. From additional experiments it was concluded that adenovirus capture of the host cell translational machinery was not due to competition between viral and host cell mRNAs for ribosomes (1). However, it is plausible that cellular analogs to the viral VAI RNAs may exist in the uninfected cell (U RNAs) and that an early stage in the turnoff of host cell gene expression may involve viral early gene products down-regulating their expression or transport to the cytoplasm. This would explain why viral mRNAs are translated in preference to cellular mRNAs and would further support the contention that it is an early viral gene function(s) that initiates the events leading to the modification of host cell mRNA translation.

The data described in which viruses with mutations in the E1b genes affecting both the 55- and 21-kd proteins were used suggest that both proteins are important for optimum virion production. Additional studies show that the 55-kd protein may also function to initiate the events leading to the shutoff of host cell gene expression. Indirect evidence suggests that this protein may interact with another viral early gene product, the 25-kd protein encoded by E4, to mediate this cellular modification. With the use of additional viruses containing mutations in both early regions and with the use of cell lines expressing these early viral functions, these questions are presently being approached.

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