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An adenovirus type 5 early gene function regulates expression of other early viral genes

(host range deletion mutants/early viral RNAs)

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ABSTRACT **We have identified an adenovirus type 5 (Ad5) early gene function located in early region 1 which is required for the production of early cytoplasmic mRNAs corresponding to early regions 2, 3, and 4. Mutant *dl312* (lacks the segment between 1.5 and 4.5 map units) grows as well as wild-type virus in 293 cells (Ad5-transformed human embryonic kidney cells), but its growth is severely restricted in HeLa cells. We detect no viral RNAs in the cytoplasm of *dl312*-infected HeLa cells. Viral RNA sequences are present, however, in *dl312*-infected HeLa cell nuclei.**

At least four segments of the adenovirus type 5 (Ad5) genome are represented in cytoplasmic transcripts synthesized at early times after infection. Two of these segments [region 1 (1.5–11.5 map units) and region 3 (76–86 map units)] are located on the rightward-reading viral DNA strand, and two are on the leftward-reading DNA strand [region 2 (62–76 map units) and region 4 (91–99 map units)] (1–4). Each region contains at least one promoter (5, 6).

Recently, we isolated a group of deletion mutants that lack portions of early region 1 (unpublished data). These mutants were isolated by selecting viral DNAs that lack the *Xba* I restriction endonuclease cleavage site at 4 map units by the procedure of Jones and Shenk (7). These mutants are propagated in 293 cells (an Ad5-transformed human embryonic kidney cell line; ref. 8); they do not replicate in HeLa cells.

In this report we show that the deletions in these mutants make predictable alterations in the cytoplasmic mRNAs encoded by early region 1. Further, we find that one of these mutants, *dl312*, defines a region 1 gene function that is required for the production of early cytoplasmic mRNAs corresponding to early regions 2, 3, and 4.

MATERIALS AND METHODS

Cells and Viruses. The 293 cells were provided by F. Graham and have been described (8). The cells were maintained in Dulbecco's modified minimal essential medium containing 10% fetal calf serum. HeLa cells were obtained from J. Williams. They were grown in Dulbecco's modified medium containing 5% fetal calf serum. Wild-type Ad5 (H5*wt300*) is a plaque-purified derivative of a virus stock originally obtained from H. Ginsberg. Mutant H5*ts36* was from J. Williams (9) and H5*ts125* was from H. Ginsberg (10). Mutants H5*dl311*, H5*dl312*, and H5*dl313* were selected for the loss of the *Xba* I endonuclease cleavage site at 4 map units (unpublished data).

Enzymes, DNAs, and RNAs. Restriction endonucleases were purchased from Bethesda Research (Rockville, MD), and S1 endonuclease and electrophoretically purified deoxyribonuclease I were from Sigma. Viral DNA was prepared from virions as described (7). ^{32}P-Labeled Ad5 DNAs, labeled *in vivo*, were prepared by the method of Tibbetts and Pettersson (11) except that 293 cells were substituted for HeLa cells. The specific activity of these DNAs ranged from 1 to 5 × 10^6 cpm/μg. Ad5 DNA fragments were labeled with ^{32}P *in vitro* by the nick translation method of Rigby *et al.* (12). The specific activity of these probe DNAs ranged from 8 × 10^7 to 2 × 10^8 cpm/μg.

RNA was isolated from cells (treated with 20 μg of cytosine arabinoside per ml) 8 hr after infection at a multiplicity of 50 plaque-forming units (PFU)/cell. Infected cells were divided into nuclear and cytoplasmic fractions. Cells were suspended in isotonic buffer (10 mM Tris·HCl, pH 7.8/1.5 mM $MgCl_2$/150 mM NaCl), and Nonidet P-40 was added to 0.6% after the mixture was cooled to 4°C. The mixture was held on ice for 10 min, then mixed on a Vortex for 10 sec; the nuclei were pelleted by centrifugation. The supernatant was the cytoplasmic fraction and the pellet was the nuclear fraction after two additional washes in isotonic buffer.

We prepared nuclear RNA by dissolving the nuclear pellet in a low pH sodium dodecyl sulfate buffer (50 mM NaOAc, pH 5.2/100 mM NaCl/10 mM EDTA/0.5% sodium dodecyl sulfate), extracting the RNA twice at room temperature with an equal volume of phenol (equilibrated with 50 mM NaOAc, pH 5.2), extracting it once with chloroform/isoamyl alcohol, 49:1 (vol/vol), and precipitating the RNA by addition of 2 vol of ethanol. The RNA was resuspended in 10 mM Tris·HCl, pH 7.4/10 mM $MgCl_2$, electrophoretically purified DNase I was added to 50 μg/ml, and the solution was incubated at 37°C for 30 min. Finally, the RNA was reextracted with phenol and with chloroform/isoamyl alcohol, and precipitated with ethanol. Cytoplasmic RNA was prepared by mixing the cytoplasmic cellular fraction with 3 vol of 100 mM Tris·HCl (pH 9), extracting twice at room temperature with phenol (pH 9)/chloroform/isoamyl alcohol, 500:100:1 (vol/vol) and once with chloroform/isoamyl alcohol, 49:1 (vol/vol), and precipitating with 2 vol of ethanol.

S1 Mapping of Cytoplasmic mRNAs. The protocol of Berk and Sharp (1) was used. Hybridizations in 80% formamide (13) were in 20 μl with cytoplasmic RNA (5 mg/ml) and a restriction endonuclease-generated, ^{32}P-labeled DNA fragment (3 μg equivalents per ml). Hybridization was for 3 hr at 59°C. The RNA·DNA hybrids were treated with S1 endonuclease [100 units (1) in 200 μl of 30 mM NaOAc/250 mM NaCl/1 mM $ZnCl_2$/5% (wt/vol) glycerol for 30 min at room temperature], and the digestion products were analyzed by electrophoresis in 3.8% polyacrylamide slab gels (1.5 mm thick, 20 cm long) containing 8 M urea and Tris borate buffer (50 mM Tris base/50 mM boric acid/1 mM EDTA).

Liquid Hybridizations. A ^{32}P-labeled DNA probe (approximately 400 nucleotides in size) and either cytoplasmic or

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Abbreviations: Ad5, adenovirus type 5; PFU, plaque-forming units.

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nuclear RNA (1 mg/ml) were mixed in distilled water and the DNA was denatured by boiling for 3 min. Hybridization was in 10 mM Tris·HCl, pH 7.5/1 M NaCl at 68°C. Aliquots were withdrawn and treated with S1 endonuclease (same buffer and enzyme concentration as above) and acid-precipitable radioactivity was determined.

RESULTS

The physical and phenotypic characteristics of the early region 1 deletion mutants studied in this report are summarized in Table 1. The deletions range in size between 150 and 2350 base pairs and they are located between 1.5 and 10.5 map units on the Ad5 chromosome. They all lack the *Xba* I endonuclease cleavage site at 4 map units. The deletion mutants all grow as well as wild-type virus in 293 cells, but their growth is severely restricted in HeLa cells. Their defect in HeLa cells occurs at an early stage of the viral growth cycle since none of the mutants synthesize viral DNA in these cells. Finally, although *dl311* is able to transform rat embryo cells as efficiently as the wild-type, mutants *dl312* and *dl313* are defective for transformation.

Deletion Mutations Alter Early Region 1 Viral mRNAs. Berk and Sharp (14) have defined the structure of the major Ad2 cytoplasmic RNAs produced during the early phase of infection. To map mRNAs, they digested hybrids formed between cytoplasmic RNA and ^{32}P-labeled restriction endonuclease-generated DNA fragments of the viral genome with S1 endonuclease or exonuclease VII and analyzed the products by gel electrophoresis. S1 endonuclease digests both single-stranded ends and loops (corresponding to intervening sequences) in RNA·DNA hybrids to generate ^{32}P-labeled DNA fragments the size of coding sequences. Exonuclease VII digests only the single-stranded ends, producing ^{32}P-labeled DNA fragments the size of coding plus intervening sequences. This procedure identified three major mRNAs encoded by Ad2 early region 1. The two left-most mRNAs (region 1a, Fig. 1) contain information from the same segment of the viral chromosome (1.5–4.5 map units) and differ only in the size of their intervening sequences. The third mRNA is coded by the region between 4.5 and 11.5 map units (region 1b, Fig. 1), and it lacks a small intervening sequence at 10 map units.

Because our region 1 deletion mutants were not in an Ad2 background, but derived from the closely related Ad5, it was necessary to confirm that wild-type Ad5 synthesized the same region 1 RNAs as Ad2. Accordingly, cytoplasmic RNA from *wt300* Ad5-infected HeLa cells was hybridized to a ^{32}P-labeled Ad5 DNA fragment (*Xho* I-C fragment, 0–15 map units) and the hybrids were digested with S1 endonuclease. Then the digestion products were analyzed by polyacrylamide gel electrophoresis (denaturing gel containing 8 M urea) (Fig. 2, region 1). Fragments were identified that corresponded in size to each coding sequence previously described for Ad2: 660-, 485-, and 375-nucleotide fragments composing the region 1a mRNAs; and 1850- and 485-nucleotide fragments corresponding to the region 1b mRNA. The locations of the Ad5 coding sequences

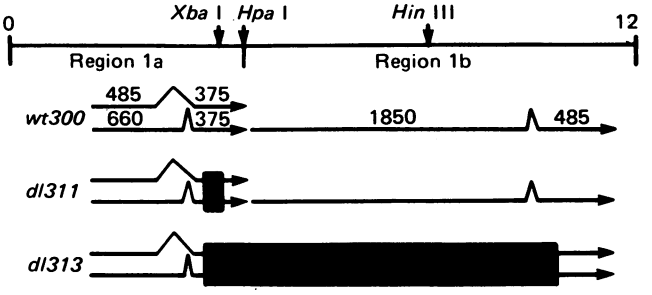

FIG. 1. Portion of the Ad5 physical map (0–12 map units) showing the early region 1 cytoplasmic RNAs synthesized by wild-type Ad5 and mutants *dl311* and *dl313*. The structure and map locations of the mRNAs were determined by Berk and Sharp (14) for Ad2 and have been confirmed for Ad5. The lines represent coding sequences, caret symbols denote intervening sequences, and arrow heads mark the 3′ ends of mRNAs. Segments deleted in the mutants are indicated by heavy bars.

were confirmed by repeating the experiment with several additional ^{32}P-labeled probe DNAs. The *Hpa* I-E fragment (0–4.5 map units) produced 660-, 485-, and 350-nucleotide fragments (data not shown), indicating that the *Hpa* I endonuclease cleavage site at 4.5 map units is near the 3′ end of the 375-nucleotide coding sequence (as was the case for Ad2, ref. 14). The *Hin*dIII-G fragment (0–8 map units) generated 1250-, 660-, 485-, and 375-nucleotide fragments (data not shown), indicating that the *Hin*dIII endonuclease cleavage site at 8 map units is within the 1850-nucleotide coding sequence (as was the case for Ad2, ref. 14). Apparently, the size and location of the major early region 1 mRNAs of Ad2 and Ad5 are identical.

Next, the early region 1 mRNAs synthesized in mutant-infected HeLa cells were examined. S1 endonuclease analysis of *dl311* RNA produced 1850-, 660-, and 485-nucleotide fragments, but no 375-nucleotide fragment (Fig. 2, region 1). Instead, a 225-nucleotide fragment was observed, exactly the expected result because *dl311* lacks a 150-base-pair segment at the map position of the 375-nucleotide coding sequence of region 1a (Fig. 1). A similar analysis of *dl313* region 1 RNAs produced 660- and (faint) 485-nucleotide fragments, but no 1850- or 375-nucleotide species (Fig. 2, region 1). Instead, a 440-nucleotide fragment was observed. Apparently, the 5′ portion of the 375-nucleotide coding sequences from region 1a have been fused to the 3′ portion of the 485-nucleotide coding sequence from region 1b (Fig. 1). Finally, no viral RNAs corresponding to region 1 were detected in *dl312*-infected HeLa cells (Fig. 2, region 1). Apparently, the *dl312* mutation either greatly reduces or prevents cytoplasmic expression of these RNAs.

Mutant *dl312*-Infected HeLa Cells Do Not Contain Cytoplasmic mRNAs Corresponding to Early Regions 2, 3, or 4. Cytoplasmic mRNAs synthesized in HeLa cells infected with wild-type or mutant virus were examined with ^{32}P-labeled DNA probes specific for early regions 2, 3, and 4. Fragments corresponding in size to coding sequences identified by Berk

Table 1. Characteristics of host-range deletion mutants

Virus	Deletion: Size, base pairs	Deletion: Location, map units*	Host range, PFU HeLa/PFU 293	Viral DNA synthesis (HeLa cells)	Transformation†
wt300	—	—	1	+	+
dl311	150	3.5–4.5	10^{-3}	−	+
dl312	1030	1.5–4.5	$>10^{-7}$	−	−
dl313	2350	3.5–10.5	$>10^{-7}$	−	−

* Locations are ±0.5 map unit.
† Foci formation assay on rat embryo or rat embryo brain cells.

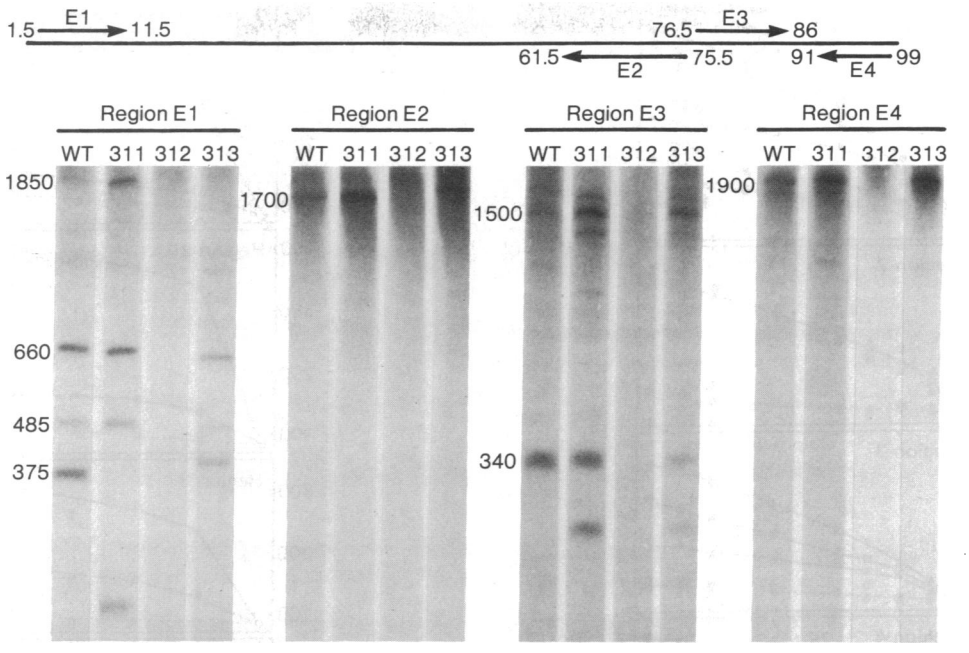

FIG. 2. Autoradiograms of S1 endonuclease-digested RNA·DNA hybrids formed between ^{32}P-labeled viral DNA probes and cytoplasmic RNA from HeLa cells infected with wild-type and mutant viruses. The ^{32}P-labeled DNA probes were derived as follows: early region 1, wild-type or mutant *Xho* I-C fragment (0–15 map units); early region 2, wild-type *Bam* I/*Eco*RI fragment (59–76 map units); early region 3, wild-type *Eco*RI-C fragment (76–83.5 map units); and early region 4, wild-type *Eco*RI-B fragment (83.5–100 map units). Numbers of nucleotides are shown.

and Sharp (14) were evident in wild-type, *dl311* and *dl313* cytoplasmic mRNA preparations (Fig. 2, regions 2, 3, and 4). A 1700-nucleotide species was found by using a region 2 probe, and a 1900-nucleotide species was present in region 4. In region 3, the 1500- and 340-nucleotide species identified by Berk and Sharp (14) were present, and, in addition, a prominent 280-nucleotide fragment was protected by *dl311* and *dl313* cytoplasmic RNA preparations. We believe this additional species is due to the presence of a substitution mutation that these mutants carry in this region (they were derived from *sub304*, ref. 7).

Unexpectedly, when the same set of ^{32}P-labeled probes were hybridized to a cytoplasmic RNA preparation derived from *dl312*-infected HeLa cells, no RNA species corresponding to early regions 2, 3, or 4 were detected (Fig. 2, regions 2, 3, and 4).

To confirm this result, we prepared ^{32}P-labeled DNA probes corresponding to early regions 1, 2, 3, and 4. They were denatured and then reassociated in the presence of RNA from the cytoplasm of wild-type and mutant-infected HeLa cells. The reassociation of the region 1 probe was accelerated by all RNA preparations tested (Fig. 3, region 1). The increase was the same in the presence of RNA from cells infected with either wild-type or *dl311* virus. Cytoplasmic RNA from mutant *dl313*-infected HeLa cells accelerated the reassociation of the probe to a lesser extent. This is reasonable because *dl313* lacks a large DNA segment in this region of the genome and, as a result, cannot produce RNAs corresponding to a portion of the probe DNA. Some region 1 RNA is present in the cytoplasm of *dl312*-infected HeLa cells since this RNA also accelerated the reassociation of the probe to a small extent. This RNA was not detected in the S1 endonuclease mapping experiment shown in Fig. 1. Viral RNAs corresponding to regions 2, 3, and 4 were evident in cytoplasmic RNA preparations from HeLa cells infected with wild-type, *dl311*, and *dl313* viruses; in each case the RNA accelerated the reassociation of the region-specific DNA probe (Fig. 3). In contrast, cytoplasmic RNA from *dl312*-infected HeLa cells failed to accelerate the reassociation of these DNA probes. We conclude that *dl312*-infected HeLa cells do not contain detectable amounts of cytoplasmic RNAs corresponding to regions 2, 3, and 4.

Mutant *dl312*-Infected HeLa Cells Contain Nuclear RNAs Corresponding to Early Regions 1, 2, 3, and 4. The same group of ^{32}P-labeled DNA probes were used to search for *dl312*-specific nuclear RNAs in infected HeLa cells. The reassociation rates of all probe DNAs were increased in the presence of nuclear RNA from cells infected with either wild-type or *dl312* virus (Fig. 4). The increase was abolished if the nuclear RNA was treated with ribonuclease before the reassociation (Fig. 4, region 2), ruling out the possibility that the increased reassociation rate was due to contaminating DNA. We conclude that viral RNAs are present in the nuclei of *dl312*-infected HeLa cells.

In each case, wild-type nuclear RNA accelerated the reassociation of the probe DNA to a much greater extent than did *dl312* nuclear RNA. The significance of this observation is unclear. Possibly, more viral RNA is present in wild-type-infected nuclei than in *dl312*-infected nuclei. Alternatively, this difference may simply reflect contamination of the wild-type nuclear RNA preparation with cytoplasmic RNAs.

Mutant *dl312*- and *dl313*-Infected HeLa Cells Do Not Contain VAI RNAs. The VAI RNAs are small (156- and 160-nucleotide) adenovirus-coded RNAs synthesized by RNA polymerase III (15). They are the most abundant viral RNAs present in adenovirus-infected cells at late times after infection (16). HeLa cells, infected with wild-type virus, labeled from 2 to 18 hr after infection in the presence of cytosine arabinoside (20 μg/ml, to block viral DNA synthesis and the onset of late functions) contained VAI RNAs (Fig. 5). In contrast no VAI RNAs were detected in *dl312*- or *dl313*-infected HeLa cells (no cytosine arabinoside present) labeled during the same period (Fig. 5). However, VAI RNA synthesis is not specifically dependent on the *dl312* and *dl313* gene functions because *ts36*-infected HeLa cells (20 μg of cytosine arabinoside per ml, 39°C) also did not contain VAI RNAs (Fig. 5). The same was true for *ts125* (data not shown). These experiments suggest that

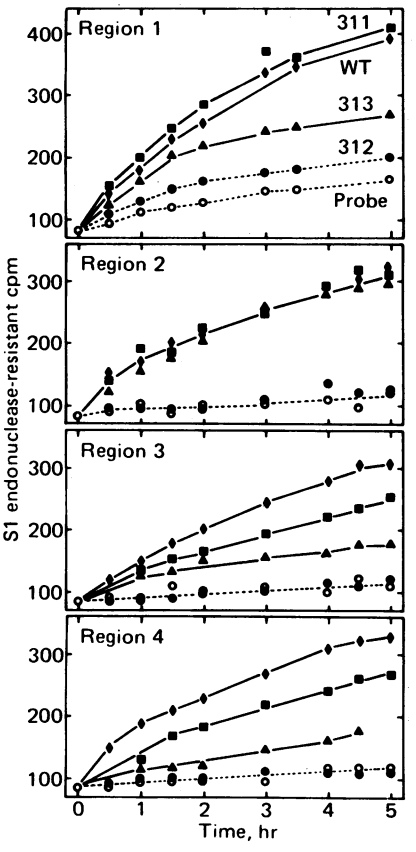

FIG. 3. Reassociation of ^{32}P-labeled viral DNA probes in the presence of cytoplasmic RNA from HeLa cells infected with wild-type and mutant viruses. The same restriction endonuclease-generated fragments described in Fig. 2 served as probes after being ^{32}P labeled by nick translation. Each time point sample contained 1500–2000 ^{32}P cpm. ○, Probe DNA plus uninfected HeLa cell RNA (1 mg/ml); ♦, probe DNA plus wild-type-infected HeLa cell RNA (1 mg/ml); ■, probe DNA plus *dl311*-infected HeLa cell RNA (1 mg/ml); ●, probe DNA plus *dl312*-infected HeLa cell RNA (1 mg/ml); ▲, probe DNA plus *dl313*-infected HeLa cell RNA (1 mg/ml).

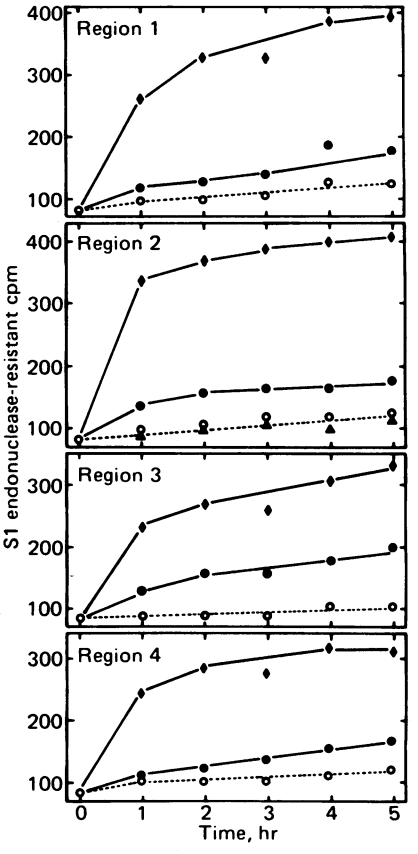

FIG. 4. Reassociation of ^{32}P-labeled viral DNA probes in the presence of nuclear RNA from HeLa cells infected with wild-type and *dl312* viruses. The probes were the same as in Fig. 3. ○, Probe DNA plus uninfected HeLa cell RNA (1 mg/ml); ♦, probe DNA plus wild-type-infected HeLa cell RNA (1 mg/ml); ●, probe DNA plus *dl312*-infected HeLa cell RNA (1 mg/ml); ▲, probe DNA plus *dl312*-infected HeLa cell RNA (1 mg/ml) treated for 10 min at 37°C with pancreatic ribonuclease (10 μg/ml) in 10 mM Tris·HCl/1 mM EDTA before annealing.

VAI RNA is not synthesized at a detectable level until late times after infection (after the onset of viral DNA synthesis). We suspect that cytosine arabinoside is not completely effective in blocking this late function in wild-type cells since four different "early" mutants do block its synthesis.

DISCUSSION

Mutant *dl312* lacks an early viral function required for the expression of cytoplasmic RNAs encoded by regions of the viral chromosome far removed from the site of the mutation. The early viral function that *dl312* lacks must map at least partially between 1.5 and 4.5 map units, the location of the *312* deletion. Two major early transcripts are encoded by this region of the adenovirus genome (ref. 14; Fig. 1, region 1a), and one (or both) of these is probably responsible for the function that *dl312* lacks. Because *dl312* is unable to transform rat embryo or rat embryo brain cells (unpublished data), this gene function probably also plays a critical role in transformation.

A trivial alternative to the above interpretation of the *dl312* gene function could be that *dl312* virions are not transported to the nucleus or uncoated subsequent to infection. As a result, the viral chromosome is not made accessible for transcription. Precedent for this argument is found in simian virus 40 temperature-sensitive mutants of the D complementation group (17–19). These mutants contain alterations in minor structural polypeptides. At the nonpermissive temperature, they are not uncoated and they fail to complement other temperature-sensitive mutants. This alternative is quite unlikely in *dl312*. Mutant *dl312* complements *dl313* for lytic growth in coinfected HeLa cells (unpublished data), and virus-specific nuclear RNAs are present in *dl312*-infected HeLa cells. Both of these observations suggest that the *dl312* chromosome reaches the nucleus and is capable of being transcribed.

Our finding that an early Ad5 gene product is required for the expression of other early viral transcripts predicts that inhibitors of protein synthesis added at or before infection should prevent the expression of early regions 2, 3, and 4. However, when cycloheximide is added at 1 hr after infection, the levels of region 2, 3, and 4 mRNAs increase rather than decrease (20, 21). We have performed similar experiments, adding cycloheximide (25 μg/ml) 1 hr prior to infection of HeLa cells with wild-type virus. In this case, the early mRNAs assayed (regions 1 and 2) were still synthesized, but they were present in somewhat lesser amounts than in untreated cultures. Low levels of protein synthesis could be detected in the cycloheximide-treated HeLa cells. In the presence of the drug, about 5% the amount of ^{35}S-labeled methionine was incorporated into acid-precipitable products as in untreated cells. As a result, it is likely that a small amount of the *dl312* gene product is made in the presence of cycloheximide. This, in turn, facilitates the synthesis of transcripts corresponding to other early regions. Experiments with other inhibitors of protein synthesis or a combination of inhibitors may help to resolve this issue.

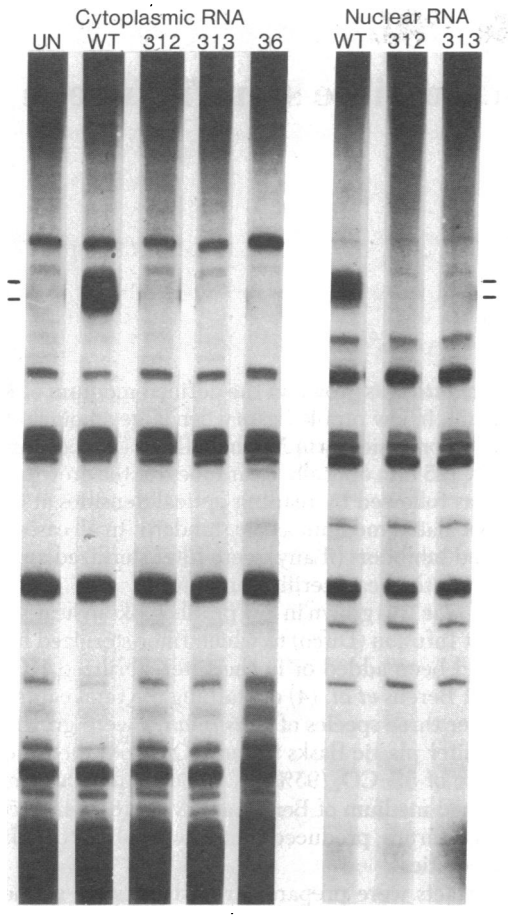

FIG. 5. Autoradiogram of ^{32}P-labeled cytoplasmic and nuclear RNAs from uninfected HeLa cells and cells infected with wild-type and mutant viruses. Infected HeLa cell cultures were labeled with $^{32}PO_4^{2-}$ (200 μCi/ml) from 2 to 18 hr after infection. Cells infected with wild-type and *ts36* viruses received cytosine arabinoside (20 μg/ml) at 1 hr after infection; the rest did not. Cultures infected with *ts36* were maintained at 39°C. After cytoplasmic and nuclear RNAs were extracted, low molecular weight $^{32}PO_4^{2-}$ was removed on Sephadex G-50, and 3×10^5 cpm aliquots of each sample were applied to an 8% polyacrylamide slab gel (0.6 mm thick, 40 cm long, containing 8 M urea in Tris borate buffer). Electrophoresis was for 8 hr at 1000 V.

We cannot yet exclude the possibility that the gene function required for the production of mature cytoplasmic mRNAs is not a protein but rather the RNA molecule itself, which is transcribed from the region that *dl312* lacks. This alternative is compatible with both the cycloheximide data (mRNA levels do not decrease in the presence of the inhibitor; refs. 20 and 21) and the results reported here for *dl312*-infected cells.

There are two promoters in region 1: one controls the synthesis of region 1a transcripts and the other controls region 1b transcripts (22–24). Are the region 1b transcripts under the control of the *dl312* gene product? Our data suggest that they probably are. We know that *dl312* and *dl313* complement for lytic growth in HeLa cells (unpublished data). Because *dl313* lacks most of the segment coding for the region 1b mRNAs, these functions must be supplied by *dl312* in the coinfected cells. The region 1b mRNAs are not synthesized in HeLa cells infected by *dl312* virus (Fig. 2). Therefore, in the complementing situation, the *dl312* genome must synthesize these mRNAs in response to a gene function supplied by *dl313*. This argues that the *dl312* function is required for the synthesis of the region 1b mRNAs.

Mutant *dl312* lacks the segment between 1.5 and 4.5 map units. Its deletion either includes or comes very close to the 5′ ends of both region 1a and 1b mRNAs. Because *dl312* can produce the region 1b gene products in the presence of *dl313*, a region 1 promoter must have remained intact in this mutant. Presumably this is the region 1b promoter because (as argued in the previous paragraph) region 1b in *dl312* appears to require the *dl312* gene product for expression. Possibly, the region 1a promoter is also present in *dl312*, since a small amount of cytoplasmic region 1 RNA was detected in HeLa cells by liquid hybridization kinetics (Fig. 3). If this is the case, the transcriptional termination or processing signals for region 1a mRNAs must also be intact. Otherwise *dl312*-infected HeLa cell cytoplasm would likely contain a region 1b RNA fused to the extreme 5′ ends of the region 1a RNA.

The precise nature of the *dl312* defect is unknown. Because viral RNAs can be detected in the nuclei of *dl312*-infected HeLa cells (Fig. 4), it is possible that primary transcripts are made that correspond to all regions of early transcription. This would place the block at a step subsequent to the synthesis of primary transcripts, but prior to the appearance of a mature mRNA in the cytoplasm. However, we have no evidence that these nuclear RNAs represent bona fide early transcripts. Possibly, this RNA is the result of random, nonspecific transcription of the viral chromosome in the nucleus.

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