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Regulation of Integrated Adenovirus Sequences During Adenovirus Infection of Transformed Cells

DAVID J. SPECTOR, DONALD N. HALBERT, AND HESCHEL J. RASKAS*

Department of Pathology and Department of Microbiology and Immunology, Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110

A human cell line (293) transformed by adenovirus type 5 encodes mRNA's and proteins from the early region 1 (E1) of the viral genome. These products correspond to those synthesized early after adenovirus infection of normal cells. This pattern of expression is different from that observed at later times in the lytic cycle. We have determined whether integrated sequences can undergo the early-late transition during infection of transformed cells. Cultures of 293 cells were infected with mutants of adenovirus type 5 that have deletions in E1 genes. In such infections, the integrated sequence complements the deletion mutants so that viral DNA replication, late mRNA and protein synthesis, and viral assembly occur. Because the infecting genomes lack E1 sequences, the products synthesized from the integrated DNA could be analyzed. In contrast to the early-late transition that occurs with E1 DNA in free viral genomes, the pattern of mRNAs and proteins made from the integrated sequences was restricted to the early pattern. Assuming that the viral sequences in 293 cells have not become altered during the history of the cells, our results suggest that regulation of integrated adenovirus genes may not be determined exclusively by nucleotide sequence recognition. Apparently, during infection certain factors prevent the integrated viral genes from responding to the regulatory signals which control late expression from free E1 DNA. The distinction between integrated and free viral sequences might reflect the different fates of viral and host transcripts during the lytic cycle of adenovirus.

In cells transformed by DNA tumor viruses, viral genetic information is integrated into the host genome (25, 44). The integrated sequences encode virus-specific RNAs and proteins that have a role in altering the growth properties of the cell (7, 10, 18, 19). Transformation by human adenoviruses requires expression of only a limited segment of the viral DNA; with adenovirus types 2 or 5, the necessary viral sequences represent no more than 11% of the 36-kilobase genome (17, 20, 22, 24, 43, 47). This transforming segment lies within early region 1 (E1) which covers map positions 0-11 (one map unit is 1% of the genome). E1 is one of four gene blocks expressed soon after productive infection (14, 15, 41, 46); it consists of two mRNA families, E1A and E1B. The DNA sequence of E1 is known (31a, 32, 48), as are many of the details concerning the mRNA's and proteins which are encoded (26). As a consequence, the expression of these sequences in transformed cells is amenable to detailed investigation.

It is possible that the host regulates production of mRNA from integrated adenovirus sequences (27). Investigation of the expression of integrated viral DNA might provide insight as to how great a role is exercised by the host cell. Such studies may pertain also to the question of how viral and host genes are discriminated during productive infection with adenovirus. Since it is possible that integrated and free E1 genes have the same nucleotide sequence, any differences in control might be due to host-imposed mechanisms that supplement sequence recognition. As a first step in examining the expression of integrated viral genes, we analyzed the products of adenovirus-specific sequences in a transformed human cell line, 293 (23). This line was transformed by viral DNA fragments and contains DNA from early regions 1 and 4 (1). Only region 1 is transcribed into mRNA (1). Our results demonstrate that the products of the integrated E1 sequences are similar to those found early in productive infection. Subsequently, we asked whether the integrated E1 sequences are capable of undergoing the normal early-late transition that occurs during productive infection. For these experiments we utilized adenovirus mutants having deletions in E1 genes (28). These mutants can replicate in 293 cells; one can then assay the expression of the integrated sequence without the background of the same products made from exogenous templates. The results suggest that the integrated sequence

is locked into its pattern of early expression and is not regulated as a free viral template.

MATERIALS AND METHODS

Cells and virus. KB cells were maintained in suspension and monolayer cultures; wild-type adenovirus types 2 and 5 were passaged in suspension cultures at 15 PFU per cell (15, 50).

Monolayers of 293 cells (23) were maintained on Joklik minimal essential medium containing 10% fetal or newborn calf serum. Mutant viruses dl 312 and dl 313 were obtained from T. Shenk and passaged at 3 to 5 PFU per cell. Infected cells were harvested when cytopathology was evident in all of the cells, usually 48 to 72 h after infection. Infected cells were washed two times with phosphate-buffered saline (PBS) and lysed by three cycles of freezing and thawing in PBS containing 0.9 mM magnesium chloride, 0.9 mM calcium chloride, and 0.1% albumin. Cellular debris was removed by centrifugation, and virus extracts were stored at −70°C.

Virus stocks were titrated on monolayers of 293 cells (F. Graham, personal communication).

Labeling and extraction of viral mRNA. Monolayers of 293 cells growing in 150-mm dishes were labeled when the cells were about 80% of confluence. A confluent monolayer contains 2×10^7 cells. Labeling was performed in 5 ml of medium containing 100 to 200 μCi of [^{3}H]uridine per ml (25 to 50 Ci/mmol, New England Nuclear Corp.). To stop incorporation, dishes were placed on ice and washed two times with ice cold PBS. Cells were scraped in ice cold isotonic buffer containing 0.05% Nonidet P-40 (50). Cytoplasmic extracts were clarified by centrifugation, and RNA was extracted with phenol-chloroform-isoamyl alcohol (34). Polyadenylic acid [poly(A)]-containing RNA was purified by chromatography on oligodeoxythymidylic acid [oligo(dT)]-cellulose (4).

Virus adsorption to 293 cells was performed in 2 to 5 ml of Joklik minimum essential medium without serum. Virus was allowed to absorb for 1 h, and then cells were fed with 20 ml of fresh medium containing serum.

Infections of KB monolayers and suspension cultures were performed as previously described (50). The preparation of labeled RNA from KB cells was performed as described above.

Analysis of viral mRNA. Poly(A)-containing RNA was displayed on 3.5% polyacrylamide gels containing 98% formamide (16). Electrophoresis of 10-cm gels was for 5.5 to 6 h at 2.5 mA/gel. Gels were sliced into 2-mm fractions, and RNA was eluted from each fraction by incubation of the gel slices in 6× SSC (1× SSC is 0.15 M NaCl–0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) for 40 to 48 h. Aliquots of eluted RNA were hybridized to nitrocellulose filters containing 0.5- to 1.0-μg equivalents of DNA fragments. Hybridization was at 66°C for 18 to 24 h. Labeled fragment-specific RNA was assayed after RNase treatment of hybrids (34). In experiments in which it was necessary to determine the actual quantity of labeled RNA, hybridization samples were checked for DNA excess by annealing supernatants with a second DNA filter. DNA excess was assumed when less than 25% of the original value was rehybridized.

Preparation of specific viral DNA fragments. For most of the experiments shown, analysis was performed with fragments of adenovirus type 2 DNA. Adenovirus type 2 DNA has over 98% homology with adenovirus type 5 DNA (33). Sequencing studies in region E1A suggest only minor base changes (38, 55). Viral DNA was prepared from purified virions (54). Primary restriction enzyme digests were performed with endo R·*Eco*RI and endo R·*Sma*I. Subcleavage of *Sma*I-E (2.9–10.7) was performed with endo R·*Hpa*I and endo R·*Bal*I. The E1 fragments used in this study were 0–2.9 (*Sma*I-J), 2.9–4.4 and 4.4–10.7 (*Sma*I-E subcleaved with endo R·*Hpa*I), and 8.7–10.7 (*Sma*I-E subcleaved with endo R·*Bal*I). Digestion conditions have been described elsewhere (50).

All restriction enzymes were prepared by published procedures (26, 50) except for endo R·*Hpa*I, which was purchased from Bethesda Research Laboratories.

In vitro translation of purified mRNA's. E1-specific mRNA's were purified by hybridization to nitrocellulose membranes containing 50- to 100-μg equivalents of DNA fragment followed by selective thermal elution (36). Purified RNA was chromatographed on oligo(dT)-cellulose and translated in reticulocyte lysates (26). ^{35}S-labeled peptides synthesized in vitro were analyzed by fluorography of SDS-polyacrylamide slab gels (26).

Analysis of proteins synthesized in vivo. To label proteins synthesized in vivo, monolayers (35-mm dishes) of 293 cells were infected with 50 PFU of wild-type virus or deletion mutants per cell as described above. At 24 h after infection, 20 μCi of [^{35}S]methionine (800 to 1,200 Ci/mmol, Amersham Corp., Chicago, Ill.) per ml was added to each plate in 1 ml of medium. Uninfected 293 cells were labeled under the same conditions. Three hours later, the medium was removed and the cells were washed three times with ice cold PBS. Cells were scraped off the plate with a rubber policeman, and washed two additional times in PBS. The cell pellet was then resuspended in 0.5 ml of sample buffer (2% SDS, 1% mercaptoethanol, 0.1 M Tris-hydrochloride [pH 6.8], 10% glycerol, and 0.1% bromophenol blue) and heated to 100°C with occasional mixing for 5 min. Aliquots were then analyzed in a 15% SDS-polyacrylamide gel (31). ^{35}S-labeled virion markers were prepared as described previously (26).

R-loop mapping. Cytoplasmic poly(A)-containing RNA was purified from 10^8 transformed cells infected with 50 PFU of dl 313 per ml and harvested 20 h after infection. RNA specific for E1B was selected by hybridization to 8.7–10.7 DNA (36). The RNA was used to form DNA-RNA hybridization loops in the presence of 70% formamide (53). Purified RNA (one-tenth of the sample) and whole adenovirus DNA (6.5 μg/ml) were incubated at 56°C for 15 h in 0.035 ml of 70% deionized formamide, 0.4 M CaCl, 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.8), and 0.01 M EDTA. Samples were spread and shadowed as previously described (11a).

R-loops were visualized in a Phillips 210 electron microscope at an accelerating voltage of 60 kV. Molecules were photographed on 35-mm film, negatives

were enlarged, and the lengths of structures were determined with a Numonics Graphics calculator. Coordinates are expressed as percentage of the total length of adenovirus DNA.

RESULTS

Expression of E1 in transformed cells. To understand the expression of E1 genes in 293 cells, it is necessary to recall the mRNA's transcribed from the region during lytic infection (Fig. 1). We reported previously that the accumulation of these mRNA's is regulated differently at early and late times (50). The RNAs shown in Fig. 1A are present before 6 h, whether the infection is performed in the presence or absence of inhibitors of DNA synthesis. At later times, one 9S RNA accumulates from E1A and another from E1B (Fig. 1B). Another characteristic of the late phase is the accelerated accumulation of the 13S RNA from E1B. These metabolic observations are summarized in Table 1.

To analyze the virus-specific RNAs made in 293 cells, cellular RNA was labeled with [^{3}H]-uridine. Cultures were labeled for 3 h and poly(A)-containing RNA was isolated from cytoplasmic fractions. After electrophoresis on polyacrylamide-formamide gels, RNA eluted from gel slices was hybridized to DNA probes for E1 RNA. As in early RNA samples from lytically infected cells (50, 56), 22S and 13S size classes were present. In infected cells (9, 26, 50, 56) or transformed cells, (8; see Fig. 4 below) the 13S size class includes a 13S species from E1B and 13S and 12S RNAs from E1A. The 13S size

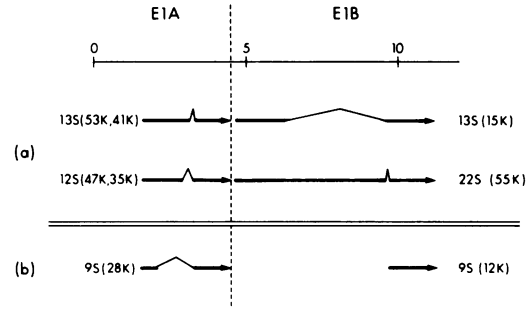

FIG. 1. *Map positions and in vitro translation products of adenovirus E1 mRNA's. The bold arrows indicate the map positions of mRNA's (8, 9, 12, 30, 50). Spliced out regions are denoted by thin lines connecting the arrow segments; mRNA species are identified by their relative migration rates in polyacrylamide gels containing 98% formamide (50). The values in parentheses indicate the molecular weights of the polypeptides encoded by each mRNA in vitro. (26, 49a). (a) mRNA's in 293 cells and from cells early after productive infection. (b) Additional mRNA's detected late after productive infection.*

TABLE 1. *Accumulation of E1 RNAs during productive infection*[a]

Characteristic	Region	mRNA species
Accumulate at constant rate during infection	E1A	12S, 13S
	E1B	22S
Accumulate at accelerated rates late times in infection	E1A	9S
	E1B	9S, 13S

[a] Based on studies of Spector et al. (50).

class from E1A was analyzed by hybridization to 0–2.9 DNA, and the 13S E1B species was analyzed by annealing to 4.4–10.7 DNA. The 9S E1A and E1B species present late in productive infection were not detected (for region 1 hybridization profiles in 293 cells, see Fig. 5 and 6, mock-infected samples). Berk et al. (8) have reported mapping results consistent with our data.

To confirm that E1 expression in 293 cells resembles early lytic patterns, we estimated the relative accumulation of the E1 RNAs in the two systems (Table 2). The number of ^{3}H counts per minute in each RNA size class was determined by hybridization in DNA excess. These values measure the amount of newly synthesized RNA. The amounts of incorporation into 22S E1B RNA as a proportion of total cytoplasmic poly(A)-containing RNA are quite similar. In both transformed cells and early infected cells the majority of radioactivity in 13S species is incorporated into E1A RNA. In addition, 13S RNAs in 293 cells are labeled to a lesser extent than in early infection. These observations are in contrast to late productive infection when the labeling of 13S RNA increases substantially and the E1B species predominates (50; see Fig. 6 below). Thus, the accumulation of cytoplasmic E1 RNAs in 293 cells resembles the pattern that is found early in lytic infection.

We proceeded to identify the in vitro products of the viral RNAs in 293 cells to confirm that all the RNA species shown in Fig. 1a were present and could function as mRNAs. Viral species were purified by preparative hybridization to DNA fragments specific for E1A or E1B (36). In vitro translation of E1A RNAs revealed four polypeptides with molecular weights of 35K to 53K (Fig. 2, lane 3). These correspond to the four early proteins made from the 13S and 12S RNAs isolated from lytically infected cells (26). In vitro translation of E1B RNAs produced 15K and 55K polypeptides (Fig. 2, lane 6). These also correspond to the products of early RNAs from lytically infected cells, in this instance the E1B 13S and 22S RNAs, respectively (49a). Peptides of 28K and 12K are synthesized from E1 RNA isolated at late times in productive infections

TABLE 2. *Labeling of E1-specific mRNA's*

Cell Line	Time of labeling (h)	Poly(A) RNA assayed (cpm × 10^{-6})	22S RNA		13S RNA		
			Hyb	% of Poly(A)$^{+a}$	Hyb	% of Poly(A)$^{+}$	% from E1A^{b}
293^{c}	3	1.2	1300	0.11	1400	0.12	ND
	3	2.5	2500	0.10	1200	0.05	63
KB Ad2 infected	3^{d}	0.42	520	0.12	2430	0.58	ND
	3^{e}	1.0	1560	0.16	3720	0.37	74

[a] Percentage of eluted poly(A)-containing RNA that is represented in this class of viral RNA.

[b] Aliquots of the RNA samples were hybridized to 0-2.9 DNA (E1A) or 4.4-10.7 DNA (E1B). The relative amounts of 13S counts per minute in E1A and E1B species were calculated from the data. The E1A data were corrected for the fact that 0-2.9 DNA detected only one-half of the label in the E1A species. ND, Not done.

[c] In two separate experiments, monolayers of 293 cells (4×10^{7}) were labeled with 100 μCi of [^{3}H]uridine per ml. Samples were harvested 3 h later. Poly(A)-containing RNA was purified from cytoplasmic extracts and displayed on formamide polyacrylamide gels. RNA eluted from gel slices was hybridized to nitrocellulose membranes containing the 0-58 adenovirus DNA fragment (RI-A). The counts per minute in the eluted samples used for hybridization were summed and are indicated as "Poly(A) RNA assayed (cpm × 10^{-6})". The counts per minute hybridized in the 22S and 13S peaks were summed and the values are indicated as "Hyb." Ad2, Adenovirus type 2.

[d] RNA prepared from monolayer cultures of KB cells labeled 3 to 6 h after infection with adenovirus type 2. All manipulations were as described above.

[e] RNA prepared from suspension cultures of KB cells labeled 3 to 6 h after infection with adenovirus type 2.

(26, 40, 49a, see Fig. 1b). These proteins were not detected after in vitro translation of 293 viral RNA. We conclude that the expression of integrated E1 sequences in 293 cells corresponds to that of genomic DNA early in productive infection (Fig. 1a).

Expression of viral mRNA in 293 cells infected with adenovirus deletion mutants. The adenovirus mutants dl 312 and dl 313 contain deletions in E1 (28; Fig. 3). In KB or HeLa cells, these mutants are blocked at an early stage in virus replication (29). The E1 products synthesized in 293 cells complement these mutants (28). Mutant dl 312 lacks the promoter region and most of the sequences coding for E1A mRNA's. Therefore, the 13S, 12S, and 9S RNAs specified by E1A cannot be synthesized from the dl 312 genome. Mutant dl 313 lacks most of the coding sequence for the 22S and 13S RNAs from E1B, part of the coding sequence for the E1B 9S RNA, and the likely promoter for this mRNA. Therefore, these RNAs cannot be produced from the dl 313 genome. In addition, dl 313 does not contain the coding sequences for the 3′ terminus of the E1A RNAs. Physical mapping of the RNAs produced in nonpermissive infections indicates that E1A RNAs made from the dl 313 genome have their 5′ end dictated by the E1A promoter and 3′ end at the E1B adenylation site (29; RNAs 1 and 2, Fig. 3). Although it has not been reported previously, this genome should contain the information for a third spliced RNA, using the splicing sites that produce the 9S RNA from E1A (compare Fig. 1b and RNA 3, Fig. 3). These three abnormal RNAs would all be slightly larger than the wild-type E1A RNAs.

As described above, E1 expression in 293 cells is similar to the early pattern in productive infection (Fig. 2, Table 2). Since the E1 deletion mutants replicate effectively in 293 cells, the mRNA's made from the integrated sequences can be studied in conditions analogous to late infection. By choosing the appropriate time, it should be possible to determine whether replicative events regulate an early-late transition of the integrated sequences. For example, late after dl 312 infection in 293 cells, any 9S RNA from E1A must be encoded by the integrated sequence. Mutant dl 313 infection of the same cells provides a system to examine E1B mRNA production; any 9S RNA from E1B, or the accelerated production of 13S RNA from this region, must be encoded by the integrated sequence.

Monolayer cultures of 293 cells were infected with dl 312 or dl 313 at 50 PFU per cell. At this multiplicity, no early (dl 312) or late (dl 313) mRNA was synthesized in nonpermissive infections of KB cells (data not shown). As a control, parallel cultures of 293 cells were infected with wild-type virus or mock-infected. RNA was labeled with [^{3}H]uridine added at 18 h after infection; the cells were harvested 2 h later, and poly(A)-containing RNA was analyzed as described above. To examine the late mRNA accumulating in cells infected by each virus, RNA samples were hybridized to the 0-58 fragment (R1-A, Fig. 4). This fragment contains sequences encoding at least three late families and two early blocks of viral mRNA (13, 21, 34, 35, 37). Most of the viral species migrate in the 18 to 28S size range (34, 51). All three virus infections resulted in a pattern of late mRNA characteris-

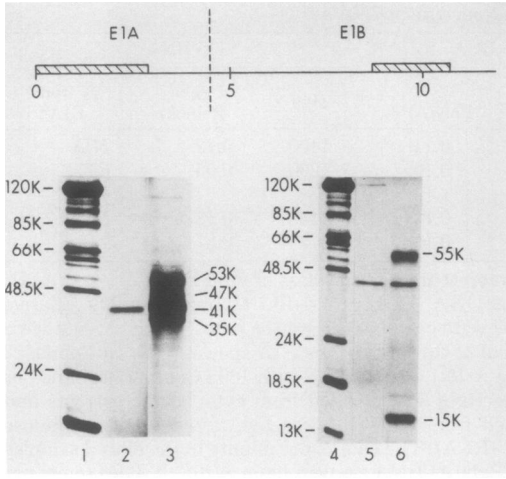

FIG. 2. *In vitro translation of purified E1-specific mRNA's from 293 cells. Cytoplasmic RNA was prepared from 1.8 × 10^8 cells labeled for 4 h with [^{3}H]-uridine. Poly(A)-containing RNA was hybridized preparatively to nitrocellulose membranes containing E1A DNA (0–2.9, SmaI-J) or E1B DNA (8.7–10.7, BalI-G/SmaI-E). Nonspecific RNA was eluted at 55°C, and specific RNA was eluted at 85°C. Samples were reselected for poly(A) content on oligo(dT)-cellulose and translated in reticulocyte lysates. The hatched bars represent the positions of the fragments used for the hybridization-selection. Lane 1, virion marker proteins; lane 2, no RNA added; lane 3, RNA selected by hybridization to 0–2.9 DNA. Lane 4, virion marker proteins; lane 5, no RNA added; lane 6, RNA selected by hybridization to 8.7–10.7 DNA.*

tic of wild-type-infected, non-virus-transformed cells. The mock-infected culture displayed the 22S and 13S E1 size classes typical of uninfected 293 cells.

E1A mRNA's synthesized in dl 312-infected 293 cells. Having shown that a late infection was established in 293 cells infected by deletion mutants, we proceeded to analyze the expression of the integrated sequences. The RNA in the gel samples of Fig. 4 was hybridized to DNA specific for E1A mRNA. To provide a reference, the fractions in the 13S region of the gel were annealed to the 0–2.9 fragment (Fig. 5, left panels), whereas the production of the induced 9S RNA was assayed by hybridization to 2.9–4.4 DNA (Fig. 5, right panels). The integrated sequences in mock-infected cells specify only the 13S RNA size class and not the 9S RNA. At 18 to 20 h after superinfection with wild-type virus, both size classes are detected. Since the wild-type-infected cells contain both integrated and free viral E1 sequences, this part of the experiment cannot distinguish which DNA serves as template for these E1A mRNA's. To achieve this distinction, RNA from the dl 312-infected sample was analyzed. In this instance, E1A mRNA's can only be transcribed from integrated sequences. The result was a profile similar to that of the mock-infected cell sample; 9S mRNA synthesis was not detected. We conclude that in 293 cells infected with dl 312, 9S mRNA cannot be made from the integrated E1A sequence.

Also shown in Fig. 5 is the result of the hybridizations to detect E1A mRNA's in dl 313-infected transformed cells. As discussed below, the data reveal hybrid RNAs made from the dl 313 genome which contain covalently linked E1A and E1B sequences (see Fig. 3). This finding will be analyzed in a subsequent section of Results.

E1B mRNA's synthesized in dl 313-infected cells. A cytoplasmic RNA analysis also was performed for region E1B (Fig. 6). In this case, RNA samples were hybridized to 4.4–10.7 DNA. The integrated E1B sequences in mock-infected cells specify only 22S RNA and a minor amount of 13S RNA. During infection of 293 cells with the wild-type or dl 312 virus, the accumulation of 13S RNA accelerates, and the 9S RNA species appears. As in infected normal human cells, this pattern can be detected only when viral DNA replication begins (data not shown). For both the wild type and dl 312 virus, it is impossible to distinguish which templates, free or integrated genomes, are responsible for the late regulatory E1B pattern in infected 293 cells. In the dl 313-infected cells, E1B mRNA can come only from the integrated sequence. The only differences from mock-infected cells

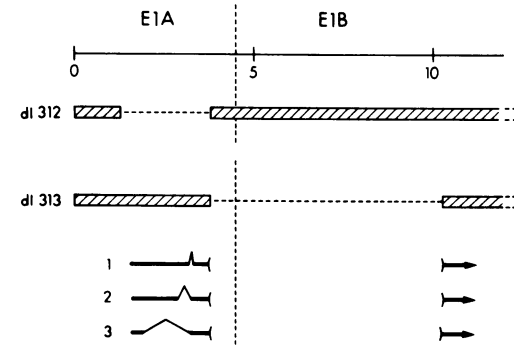

FIG. 3. *Molecular structure of two adenovirus type 5 deletion mutants. The location of the deleted sequences (horizontal dashed bars) in dl 312 and dl 313 genomes has been determined by Shenk and co-workers (48a). The E1 mRNA's synthesized in dl 313-infected cells are also illustrated. The parentheses indicate the deleted genome coding region. RNAs 1 and 2 have been reported previously (29). RNA 3 is the hypothetical product containing the splice normally present in 9S RNA from E1A.*

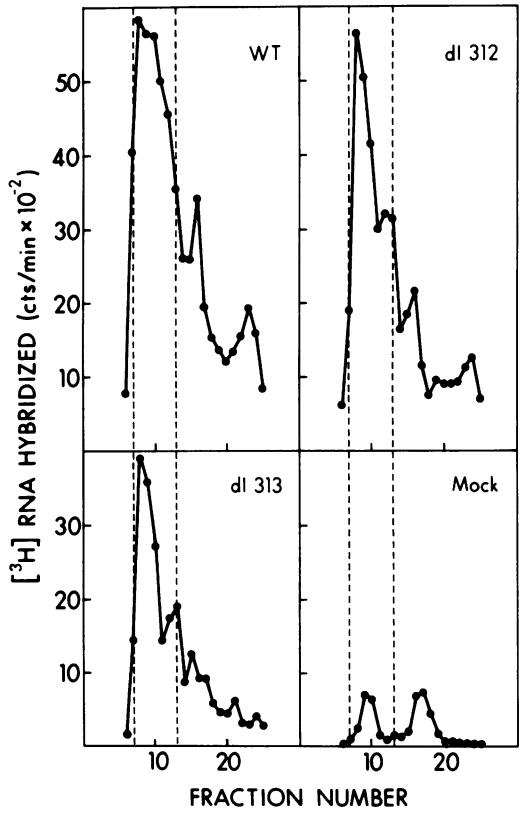

FIG. 4. *Viral mRNA synthesized late after infection of 293 cells with mutant and wild-type adenoviruses. Monolayers of 293 cells (4 × 10⁷ cells for each sample) were infected with 50 PFU per cell of the virus indicated or mock-infected (Mock). At 18 h after infection, RNA was labeled with 200 μCi of [³H]-uridine per ml. Cells were harvested 2 h later and poly(A)-containing RNA was prepared. After electrophoresis, RNA eluted from gel slices was hybridized to DNA filters containing the 0–58 fragment; 0.1 ml out of 0.5 ml fractions was assayed. The dashed lines indicate the migration of 28S and 18S rRNA markers. Counts per minute applied to gel: wild type, 9.3 × 10⁶; dl 312, 6.4 × 10⁶; dl 313, 7.1 × 10⁶; mock, 1.0 × 10⁷.*

were a slight increase in 13S RNA accumulation and the appearance of a small amount of 10S RNA. This result probably reflects the synthesis of E1A-E1B hybrid RNA species from the free dl 313 genomes (Fig. 3 and see below).

E1A-E1B hybrid RNAs in dl 313-infected 293 cells. After the abortive infection of HeLa cells with dl 313, E1 specific RNA is synthesized from the defective genome (29). The analysis of these transcripts by S1 nuclease mapping revealed colinear RNA molecules of the sizes predicted from the hypothetical structures of RNAs 1 and 2 shown in Fig. 3. These RNAs should contain covalently linked E1A and E1B sequences and are synthesized in the absence of viral DNA replication. When assayed by annealing of DNA fragments to radioactive RNA in gel fractions, they should hybridize to probes for E1A or E1B and migrate as slightly larger than normal E1A RNAs. These are probably included in the 13S RNA peak in dl 313-infected 293 cells whether an E1A (Fig. 5) or E1B (Fig. 6) probe is used. The accumulation of these RNAs does not vary substantially over the time course of infection (data not shown), a result consistent with the model that they are under the control of the E1A promoter.

Late in productive infection the dl 313 genomes should produce a third hybrid RNA (designated RNA 3 in Fig. 3) using the splicing site present in 9S RNA from E1A. Two small segments of this RNA would hybridize to E1A probes; the major portion of the molecule should hybridize to E1B. We detected an RNA species in fraction 21 of the gels shown in Fig. 4, 5, and 6 which fits these criteria and migrates as a 10S molecule. To confirm that RNA 3 molecules are

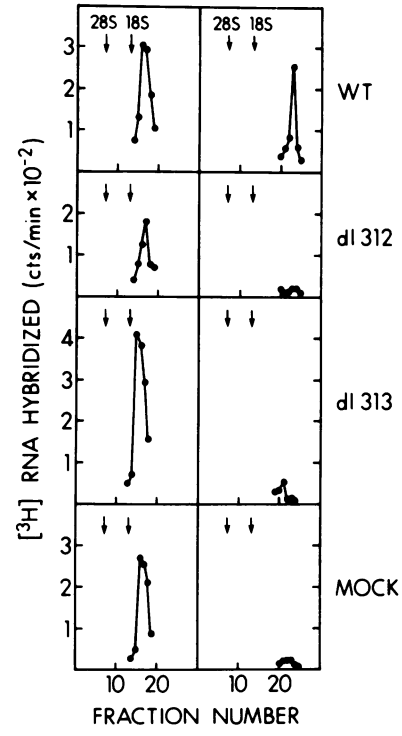

FIG. 5. *E1A mRNA synthesized in 293 cells infected with mutant and wild-type adenovirus. Left panels: 13S RNAs—0.1-ml aliquots of the gel samples from Fig. 4 were hybridized to 0–2.9 DNA in the 13S region of the gel. Right panels: 9S RNAs—0.15-ml aliquots were hybridized to 2.9–4.4 DNA in the 9S region of the gel.*

present in 293 cells infected by dl 313, E1B-specific RNA was purified by hybridization-selection (36) and rehybridized to whole adenovirus DNA under conditions favorable for the formation of R-loop structures. Hybrids were visualized in the electron microscope. The molecule shown in Fig. 7 conforms to the expected RNA 3. It contains two intervening sequences: a small sequence corresponds to the splice normally found in 9S RNA from E1A, and a second sequence corresponds to the deleted sequence in the dl 313 genome. Only one part of the DNA-RNA hybrid is long enough to give a visual R-loop. This corresponds to the E1B-specific portion and is about 400 nucleotides in length. We conclude that the 10S RNA observed in dl 313-infected cells is a good candidate for RNA 3 and is unrelated to the 9S RNA normally made from the E1B promoter active late in infection. We did not detect any R-loops corresponding to the E1B 9S RNA.

dl 313-infected cells do not make the product of the 9S RNA from E1B. The 9S RNA from E1B codes for a 12K virion polypeptide (IX) which is very abundant in extracts of late infected cells (40). As a further confirmation that no 9S RNA was made from integrated E1B genomes after dl 313 infection, we assayed the synthesis of peptide IX in mutant-infected 293 cells. Control cultures were infected with dl 312 or wild-type virus or mock infected. After 24 h, [^{35}S]methionine was added. Three hours later, cells were harvested, and labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 8). Wild-type (lane 1) and dl 312-infected (lane 4) cells synthesize all the recognizable viral structural proteins including a ^{35}S-labeled protein that comigrates with virion polypeptide IX (lane 5). Each of these viruses might synthesize the mRNA for peptide IX from either the integrated or free viral sequences. In dl 313-infected cells, where protein IX could only be made from the integrated sequence, no protein of this size was found even though the other viral proteins are synthesized (lane 3). A faint band of similar mobility was detected in mock-infected cells (lane 2). At the time this infection was performed, dl 313 inhibits the synthesis of at least some low-molecular-weight host proteins. This inhibition probably is responsible for the failure of the comigrating host band to label significantly in the dl 313-infected cells. The data confirm the inability of dl 313-infected cells to make functional 9S mRNA from the integrated E1B sequence. These results also explain the finding of Shenk and co-workers that dl 313 virions contain no polypeptide IX (28, 48a). Thus, from the transcriptional analyses (Fig. 6) and the protein labeling patterns (Fig. 8), we conclude that the integrated E1B sequences cannot express a late pattern in dl 313-infected 293 cells.

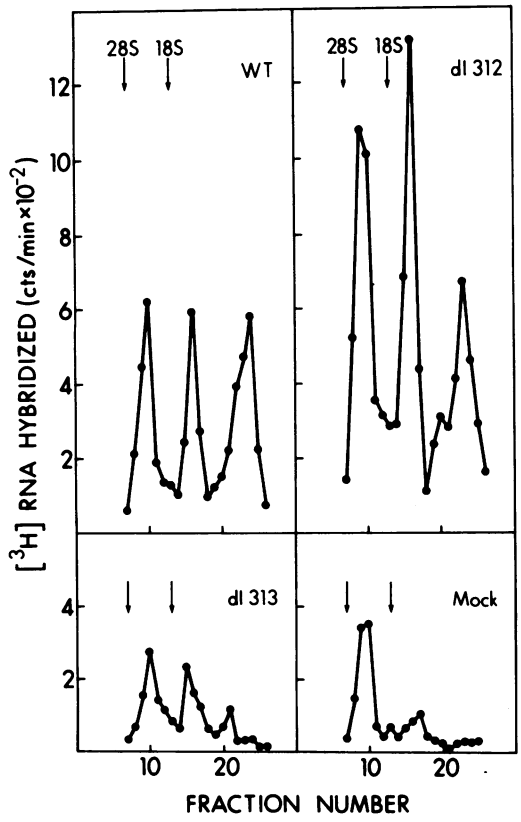

FIG. 6. *E1B mRNA synthesized in 293 cells infected with mutant and wild-type adenovirus. Aliquots of 0.05 ml of the samples in Fig. 4 were hybridized to 4.4–10.7 DNA.*

DISCUSSION

To study the regulation of integrated E1 viral sequences in an adenovirus-transformed human cell line, we have analyzed the accumulation of cytoplasmic mRNA. Our conclusion is that integrated E1 sequences maintain a constant pattern of expression even when the transformed cells are infected with adenovirus. The E1 sequences in viral genomes are regulated to change the amounts and kinds of mRNA's and proteins synthesized, but the integrated sequences do not display an altered pattern.

Regulation of the early-late transition of E1 genes. During normal productive infection, the accumulation of three different E1 RNAs is altered substantially at the time of the transition from early to late RNA synthesis (see Table 1). This transition occurs at about the same time that DNA replication begins in infected cells,

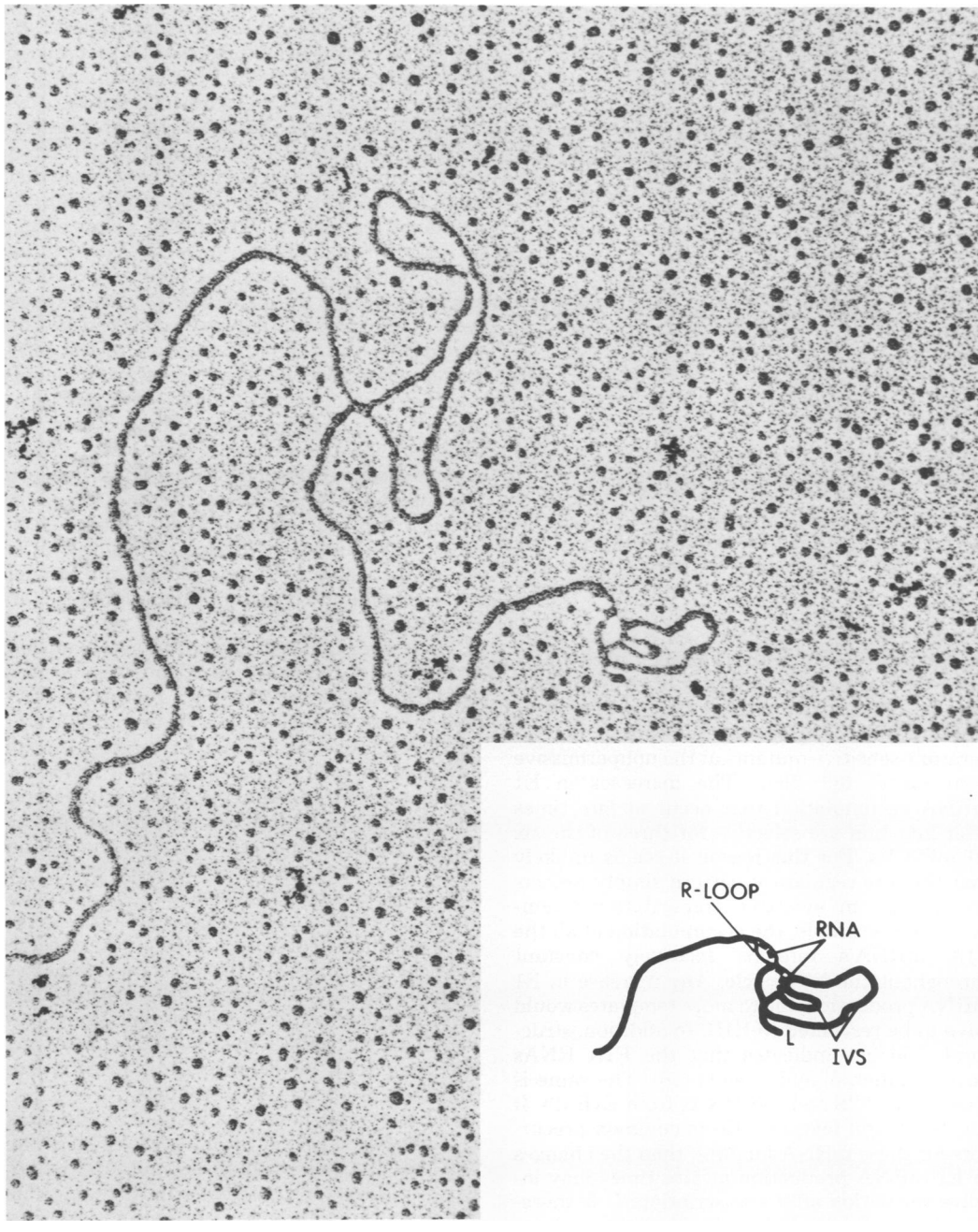

FIG. 7. *Electron microscopy of a dl 313 E1A-E1B hybrid RNA molecule. R-loops were prepared and examined as described in experimental procedures. Over 20 molecules containing R-loops in E1 were observed. Of these, eight unambiguous structures were identified. Five of these molecules corresponded to 22S RNA from E1B, and three E1A-E1B hybrid RNAs were detected. The insert indicates the various features of one of the two RNA 3 hybrid molecules. L, Left end of adenovirus DNA. IVS, Intervening sequences. The first intervening sequence maps from 1.5 to 3.0 map units (m.u.). The second maps from 3.2 to 9.7 m.u. R-loop maps from 9.7 to 10.7 m.u. RNA indicates location of the E1A-E1B hybrid RNA.*

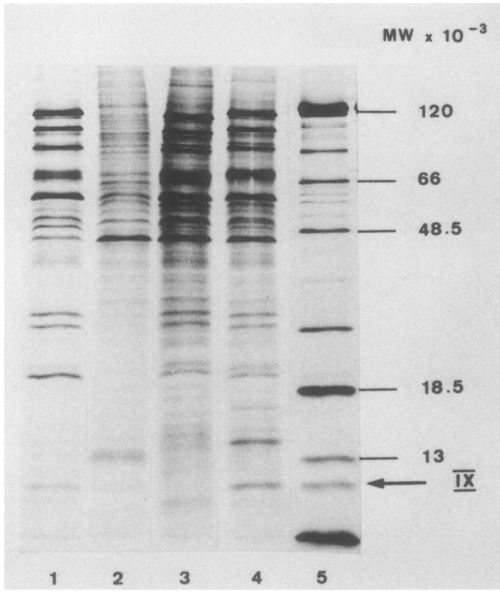

FIG. 8. *Synthesis of viral proteins in 293 cells infected with mutant and wild-type adenovirus. Lane 1, Wild-type virus; lane 2, mock infected; lane 3, dl 313 infected; lane 4, dl 312 infected; lane 5, virion proteins.*

although the act of replication may not be necessary. For example, the 9S mRNA from E1B can be made when viral DNA synthesis is blocked by drugs or by infection with early temperature-sensitive mutants at the nonpermissive temperature (39, 49a). The increases in E1 mRNA accumulation that occur at late times after infection are selective for three of the six E1 mRNA's. For this reason it seems unlikely that the late regulatory pattern simply reflects an increase in available transcriptional templates. For example, the accumulation of all the E1A mRNA's remains relatively constant throughout the lytic cycle. Any increase in E1 mRNA production due to more templates would have to be restricted to E1B. In addition, structural evidence indicates that the E1A RNAs have common 5′ and 3′ ends (38). The same is true for the 22S and 13S RNAs from E1B (2). If this structural feature reflects common precursors for these mRNA families, then the changes in E1 mRNA production at late times may involve regulation after transcription. It is therefore likely that additional elements, such as changes in template structure or soluble factors, are necessary for the altered pattern of E1 mRNA accumulation at late times.

Early-late transition from integrated E1 DNA is blocked. Analysis of the cytoplasmic viral RNAs made in 293 cells indicates that these RNA structures are the same as those made from viral E1 DNA early in productive infection. However, the E1 late pattern cannot be turned on even after the introduction into the cell of the viral factors that presumably accomplish this change. This restriction must involve a block in mRNA metabolism which prevents cytoplasmic accumulation and might be explained in three ways. First, the integrated E1 DNA might contain changes in primary sequence that alter recognition of late regulatory signals. A similar effect might result from the juxtaposition of host cell regulatory sequences. Such mutations could be the result of random events or may be selected for if the late E1 products are toxic. These possibilities can be distinguished by elucidating the primary structure of the integrated genes. Second, any regulatory phenomena that require increased numbers of transcriptional template due to replication would not apply to the integrated sequences. Finally, there may be secondary structural differences between the integrated templates and those free templates which produce the late E1 pattern. Such structural differences may prevent the integrated sequences or their products from becoming accessible to regulatory factors produced in late infection.

There is evidence of structural heterogeneity among DNA templates in adenovirus-infected cells. DNA structure has been probed by electron microscopy or analysis of the products of partial nuclease digestion. The results indicate different physical properties for host DNA, infecting parental templates at early or late times after infection, and progeny viral templates (45, 52). In addition, replicative intermediates and transcription complexes isolated at late times after infection appear to have different sedimentation and protein composition (11). These results are consistent with a model in which the physical structure of DNA-protein complexes confers some transcriptional specificity. Differences in the structure of RNA-protein complexes which are probably substrates for processing may also result in the absence of an early-late transition of integrated E1 sequences.

Our experiments rely on E1 deletion mutants to facilitate analysis of the integrated genes. The 293 cells infected with these mutants do not synthesize certain E1 mRNA's and proteins. The mutants also contain sequence alterations outside of region E1 (28). Therefore, the effect of missing products on E1 regulation must be considered. The data indicate that any missing functions do not prevent regulation of the E1 sequences in the viral templates as distinguished from chromosomal host DNA. In dl 312-infected

cells the accumulation of 13S mRNA from E1B can be accelerated and protein IX mRNA can be made. In dl 313-infected cells any deficiencies apparently do not block the splicing pathway for the 9S mRNA from E1A. We have detected dl 313 mRNA that probably is synthesized by this pathway (RNA 3 in Fig. 3). This RNA has been seen in the electron microscope (Fig. 7). It can be selected by hybridization to a DNA fragment (8.7–10.7) containing its 3′ sequences and rehybridized to E1A DNA (D. Spector, unpublished data). Thus, it is unlikely that the absence of encoded viral products is responsible, in itself, for the failure of integrated sequences to alter their pattern of expression after superinfection of 293 cells with deletion mutants.

Two other aspects of the 293 cell deletion mutant system warrant discussion. Because neither dl 312- nor dl 313-infected cells accumulate 28K protein mRNA, this protein may be nonessential for lytic growth. Also, some quantitative differences in expression of viral RNAs and proteins are suggested by the experiments shown in Fig. 4 and 8; however, these are difficult to evaluate. The particle-to-PFU ratios for stocks of mutant and wild-type viruses may be different. If noninfectious particles can function as templates for RNA transcription, then different particle-to-PFU ratios might result in different rates of mRNA production at the same multiplicity. Also, the time course of mutant virus replication might be altered, as is suggested by the failure of dl 313 to shut off completely host protein synthesis even after 27 h of infection of 293 cells.

Integrated viral sequences as representative of host genes. The failure of integrated adenovirus sequences to be regulated as free viral genes raises the possibility that they may be recognized by the virus as host genes. During productive infection of human cells with adenovirus, the synthesis of cellular proteins is shut off (3, 5, 42). One component of this restriction is the failure of newly synthesized cellular mRNA to accumulate in the cytoplasm at late times possibly due to a processing defect (6). However, mRNA formation from specific genes has not been studied after infection. If effects can be demonstrated on integrated viral sequences under the same conditions as when the metabolism of total cellular mRNA is altered, then our system may be useful in studying the details of host restriction at the molecular level. If the integrated sequence is treated as host and assuming it has not diverged from the viral sequence, then the regulatory distinction between host and viral genes after infection with adenovirus may not be contained in recognition of primary nucleotide sequences.

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