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Transcription of Adenovirus 5 Early Region 1b Is Elevated in Permissive Cells Infected by a Mutant with an Upstream Deletion

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Early region 1b (E1b) of adenovirus 5 consists of a single transcription unit that lies from 1,702 to 4,070 nucleotides from the conventional left end of the genome. The effect of mutations that map upstream of E1b on the production of E1b mRNA was examined in vivo with mutants defective in gene functions from the neighboring early region 1a (E1a) transcription unit (499 to 1,632 nucleotides from the left end). These host range mutants replicate in the adenovirus 5-transformed human cell line 293. E1b mRNA accumulation was assayed by DNA-RNA hybridization late after productive infection when the E1b transcripts are abundant in the cytoplasm. Cells infected with deletion mutant *dl*312 accumulated two- to fourfold more E1b mRNA than cells infected by wild-type virus, mutant *dl*311, or mutant *hr*1. The elevated levels of E1b mRNA were also detected in steady-state nuclear RNA, pulse-labeled polyadenylated nuclear RNA, and pulse-labeled total nuclear RNA. These data indicate that E1b transcription was elevated in human 293 cells infected with *dl*312. There was no evidence of increases in genomic DNA in *dl*312-infected cells, suggesting that the rate of transcription may be elevated. When mixed infections with a 10-fold excess of either *dl*312 or wild-type virus were performed, the phenotype was that of the more abundant genome. This result suggests that the respective phenotypes were *cis* dominant. The increased rate of transcription can be attributed to *cis*-active regulatory effects of the deletion of nucleotides 448 to 1,349 in mutant *dl*312 DNA.

Regulation at the level of gene transcription is a common mechanism for control of gene expression in procaryotic and eucaryotic cells. In cells infected by adenovirus 5, the transcription of viral DNA depends on both cellular and viral components. As an example, gene products from viral early region 1a (E1a) are required in *trans* for efficient transcription of other early viral genes by cellular RNA polymerase II (8, 32, 44, 51, 65). The proper initiation of transcription of adenovirus genes (30, 38, 66, 67, 69), as of other viral (7, 41) and cellular genes (15, 24, 66; M. Goldberg, Ph.D. thesis, Stanford University, Stanford, Calif., 1978), is often dependent upon the presence of specific nucleotide sequences in *cis*.

Transcription of adenovirus early region 1b (E1b; nucleotides 1,702 to 4,070 from the conventional left end) (11, 12, 34, 49) appears to require the contribution of viral components in both *cis* and *trans*. The initiation site of transcription is most likely determined by an A-T-rich heptanucleotide sequence (Goldberg, thesis) 30 bases upstream from nucleotide 1,702 (5, 20, 37, 63). E1b mRNA also may be initiated at the E1a transcription start at nucleotide 499 (9, 28, 35). This site also contains an A-T-rich sequence upstream (5, 63). Like other early viral genes, E1b transcription is regulated positively in *trans* by E1a gene products (8, 32, 44, 46).

This communication describes altered transcriptional regulation of E1b genes in permissive cells infected with a host range deletion mutant of adenovirus 5, *dl*312 (31). Data suggest that increased transcription of E1b from *dl*312 genomes was a *cis*-dominant property of this viral DNA.

MATERIALS AND METHODS

Cells and virus. KB cells were obtained from H. J. Raskas, Washington University School of Medicine, St. Louis, Mo.; 293 cells (human embryonic kidney cells transformed by adenovirus 5) (22) also were obtained from H. J. Raskas and from the American Type Culture Collection. The maintenance of both cell lines as monolayer cultures, and of KB cells as suspension cultures, has been described previously (16, 60, 61).

Adenovirus 5 was obtained from H. J. Raskas. Mutants *dl*311 and *dl*312 (31) were generously provided by T. Shenk (SUNY at Stony Brook, Stony Brook, N.Y.), and mutant *hr*1 (27) was a gift from J. Williams (University of Pittsburgh, Pittsburgh, Pa.). The virus strains were plaque purified, and stocks were prepared as described previously (16, 60, 61). All stocks were titrated on monolayers of 293 cells.

Labeling and extraction of RNA from infected cells. Infections of monolayer cultures of 293 cells were performed as described (60). For transcription assays, cells were removed from dishes and centrifuged at 800 × *g* at room temperature. Pellets of cells were suspended at 10^7 cells per ml in fresh medium containing 500 μCi of [^{3}H]uridine per ml (25 to 50 Ci/mmol; New England Nuclear Corp.). Cultures were incubated at 37°C for 5 min; incorporation was halted by pouring the cell suspension over crushed, frozen phosphate-buffered saline.

To analyze radioactive viral RNA by polyacrylamide gel electrophoresis in 98% formamide, labeling was performed as described previously (60) by using 200 to 250 μCi of [^{3}H]uridine per ml. For tracer labeling of samples to be analyzed by blotting, cells were exposed to 10 μCi per ml of [^{3}H]uridine in 10 ml of medium for 2 h.

To prepare nuclear RNA, labeled cells were washed twice with ice-cold phosphate-buffered saline and once with ice-cold isotonic buffer. Cells were suspended in ice-cold isotonic buffer containing 0.1% Nonidet P-40. After vigorous pipetting, the free nuclei were pelleted by centrifugation at 800 × *g*. Nuclei were washed once with isotonic buffer and suspended in a volume of isotonic buffer equal to the packed pellet. Labeled RNA to be analyzed by polyacrylamide gel electrophoresis in 98% formamide was extracted from nuclei by a modification (21) of the procedure of Holmes and Bonner (29). Complete separation of nuclear RNA from DNA was required for analyses of total nuclear RNA. In these cases, the extraction procedure of Zimmer et al. (70) was used. Cytoplasmic fractions were prepared, and the RNA was extracted as described previously (42, 61).

Preparation of DNA probes. DNA probes were prepared from adenovirus 2 or adenovirus 5 DNA. These serotypes are greater than 98% homologous in DNA sequence (23, 40). For some experiments, DNA fragments generated by restriction endonuclease cleavage of purified viral DNA were prepared as described previously (25, 60, 61). Viral DNA fragments were also available as cloned recombinant molecules in plasmid pBR322. Expressed in map units (M.U.; one M.U., 365 nucleotides) from the left end, these included the 0 to 4.5 fragment (obtained from D. Lee, Washington University School of Medicine), the 4.5 to 7.8 fragment (obtained from J. Nevins, Rockefeller University, New York, N.Y.), and the 59.5 to 70.7 DNA fragment (obtained from S. Bhaduri, Washington University School of Medicine). The purified L strand of the 58.5 to 70.7 viral DNA fragment cloned in bacteriophage M13 was a gift of C. Goldenberg (Washington University School of Medicine) and S. Bhaduri. Plasmid DNA was extracted by the alkaline method of Birnboim and Doly (10), and the partially purified nucleic acid was digested with RNase A (100 U/ml) and RNase T_1 (8 U/ml). *n*-Lauroyl sarcosine was added to a concentration of 0.2%, and residual protein was removed by proteinase K treatment (100 μg/ml). The nucleic acid was purified by extraction with phenol-chloroform-isoamyl alcohol. After ethanol precipitation, nucleic acid was dissolved in water, and oligoribonucleotides were removed by precipitation of the DNA by polyethylene glycol in the presence of 0.5 M NaCl (43). Nitrocellulose membranes containing bound single-stranded DNA were prepared as described previously (42), except that plasmid DNA was boiled in 0.1 N NaOH for 5 min to denature the strands. Nick translation (33, 53) of DNA to a specific activity of 3 × 10^8 cpm/μg was performed with a kit from Amersham Corp.

Analysis of virus-specific RNA. (i) Transcription assays. Filter hybridization assays to detect viral RNA in pulse-labeled samples were performed according to J. Nevins (personal communication). RNA samples were treated with 0.1 N NaOH at 0°C for 15 min to reduce the size of the molecules. The solution was neutralized with 2 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and adjusted to 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS). Hybridization to DNA bound to nitrocellulose membranes (BA85; Schleicher & Schuell Co.) was carried out at 66°C for 24 h. Membranes were washed five times with 2× SSC, treated with 20 μg/ml of RNase A and 8 U/ml of RNase T_1 for 60 min at room temperature, and washed again with 2× SSC five times. Membranes were dried, and radioactivity was determined in organic-based scintillant.

(ii) Assays of radiolabeled size-separated viral RNA. Polyadenylated RNA was purified by oligodeoxythymidylate-cellulose chromatography (4). The detection of viral RNA eluted from slices of polyacrylamide gels containing 98% formamide (18) has been described previously (42).

(iii) Assays of steady-state viral RNA by blot hybridization. Modifications of the original RNA blotting procedure of Alwine et al. (3) were employed. RNA samples were run on 1.0% agarose gels cast in 3.7% formaldehyde solution (36, 45). Horizontal slab gels were prepared in 0.02 M morpholinopropanesulfonic acid (pH 7.0), 0.005 M sodium acetate, and 0.001 M EDTA. Running buffer contained the same constituents and formaldehyde concentration. Samples were dissolved in running buffer containing 50% formamide and heated to 60°C for 5 min before electrophoresis. Gels were run at 4 V/cm for 6 h or 1.5 V/cm for 16 to 20 h. Gels were blotted onto nitrocellulose paper in 10× SSC (62). After transfer, the paper was washed for 15 min in 5× SSC to remove residual formaldehyde, dried, and baked at 80°C in vacuo for 2 to 3 h.

Paper was prehybridized for 24 h at 42°C in buffer containing 50% formamide, 6× SSC, 1× Denhardt solution (17), and 0.1% SDS. ^{32}P-labeled viral DNA was mixed with 250 μg of salmon sperm DNA that had been sheared by sonic treatment and denatured. The DNA was boiled for 10 min and diluted into 50% formamide, 0.01 M EDTA, 2× SSC, 0.1% SDS, and 10% dextran sulfate (64). Hybridization was carried out at 42°C for 24 h.

After hybridization, the nitrocellulose was washed once with 2× SSC containing 0.05% SDS at room temperature, three times with 0.1× SSC containing 0.05% SDS at 52°C, and two times with the latter solution at room temperature. The paper was covered

with plastic wrap and exposed to Kodak XAR-5 or Du Pont Cronex 4 film at −70°C with one or two Lightning Plus intensifying screens (E. I. du Pont de Nemours & Co., Inc.).

Analysis of virus-specific DNA by blot hybridization. DNA was extracted from infected cells harvested at the same time as cultures harvested for RNA analysis by blot hybridization. The procedure used was a modification of the procedure of Cohen et al. (14). Cells were suspended in 0.05 M Tris-hydrochloride (pH 8.1), 0.01 M EDTA, and 0.15 M NaCl. SDS and pronase were added to 1% and 1 mg/ml, respectively. Digestion was allowed to proceed for 4 h at 37°C and overnight at room temperature. Nucleic acid was extracted by treatment with phenol and chloroform-isoamyl alcohol (24:1). RNA was removed from precipitated nucleic acids by suspending in 0.02 M Tris-hydrochloride (pH 7.5), 0.001 M EDTA, 0.02 M NaCl and digesting with RNase A (200 μg/ml) and RNase T_1 (8 U/ml), followed by additional phenol extraction.

DNA was digested by endo R · *Hin*dIII and separated on a 0.8% agarose gel. The DNA was transferred to nitrocellulose paper by the procedure of Southern (58) as modified by Wahl et al. (64) and hybridized to ^{32}P-labeled DNA as described before for nitrocellulose-bound RNA.

RESULTS

Structure of regions E1a and E1b. The pattern of RNA expressed from region E1b of the adenovirus genome is described in Fig. 1. Region E1b specifies two predominant mRNA species, 22S and 13S (12, 13, 34, 49, 61), which may be processed from a common precursor extending from nucleotides 1,702 to 4,070 (5, 28, 35). Some E1b mRNA may be produced from precursor transcripts extending from nucleotide 499, the upstream E1a initiation site, to nucleotide 4,070 (9, 28, 35). The location of E1a is also shown in the figure.

Overproduction of E1b mRNA in permissive cells infected with host range deletion mutant *dl*312. Adenovirus 5 mutant *dl*312 (31) lacks sequences which include the initiation site for transcription of the E1a gene (see Fig. 1) (5, 20, 28, 35). Replication of the virus can occur in human embryonic kidney cells transformed by adenovirus 5 (line 293). The 293 cells contain integrated E1 DNA, which expresses early E1a and E1b gene products (1, 8, 60). The E1a gene products complement the E1a expression deficiency in mutant *dl*312 (32, 45).

When wild-type virus or *dl*312-infected 293 cells are allowed to proceed to the late phase (after viral DNA replication commences), abundant quantities of E1b mRNA accumulate (61, 68). This RNA consists of two size classes (22S and 13S) and is presumably comprised of transcripts from the integrated E1 sequences as well as from the infecting viral genomes (61). In the experiment shown in Fig. 2, infected cells were labeled for 2 h before harvesting. Polyadenylated cytoplasmic RNA was purified and displayed on a polyacrylamide gel containing 98% formamide. RNA eluted from each fraction was hybridized to nitrocellulose filters containing excess E1b DNA probe. The profile shows the hybridization of the E1b mRNAs. The data indicate that appreciably more 22S and 13S RNA were labeled in *dl*312-infected 293 cells than in wild-type-infected 293 cells. Also shown is the profile obtained when RNA was extracted

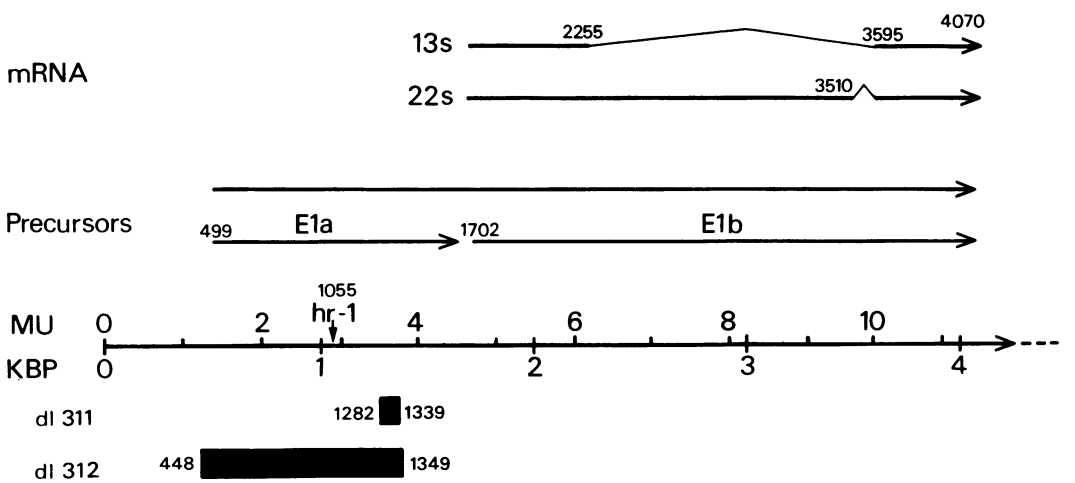

FIG. 1. Genetic organization of adenovirus 5 region E1b. mRNA, structures of the abundant E1b mRNAs; thin lines indicate the position of intervening sequences. Precursors, the structures of possible precursors to E1b mRNA are indicated along with the location of the E1a transcription unit. All numbers shown are nucleotides as numbered from the left end of the genome (11, 48, 49, 63). The location of these structures is shown relative to the adenovirus map in M.U. and kilobase pairs (KBP); 365 bp = 1 M.U. (11). Also illustrated are the locations of the point mutation in *hr*1 DNA (52) and the sequences deleted in *dl*311 and *dl*312 DNA (57).

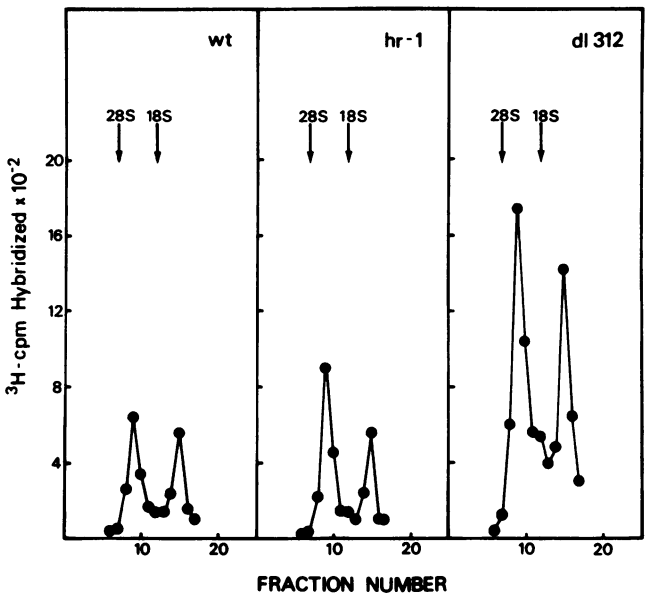

FIG. 2. Accumulation of E1b mRNA late after infection of 293 cells by wild-type (wt) or mutant adenovirus 5. Monolayers of 293 cells were infected with 50 PFU of the virus indicated (wt, *hr*1, or *dl*312) per cell. At 16.5 h after infection, cells were labeled with 250 μCi of [^{3}H]uridine per ml. Two hours later, cells were harvested, and polyadenylated cytoplasmic RNA was prepared from each sample. Radiolabeled size-separated viral RNA was assayed as previously described (42). The probe was 4.5 to 11.0 viral DNA generated by endo R · *Sma*I and endo R · *Hpa*I digest of viral DNA. The total counts per minute (cpm) in the samples used for hybridization were: wt, 5.0×10^5; *hr*1, 4.8×10^5; and *dl*312, 4.4×10^5. The positions of 28S and 18S rRNA markers run in parallel gels are indicated.

from 293 cells infected with the host range mutant *hr*1 (see Fig. 1). Like *dl*312, this mutant is defective in E1a expression (8). However, unlike *dl*312, *hr*1 does not have a deletion in viral DNA sequences but, rather, is a point mutation expressed as a premature polypeptide chain terminator (52). The accumulation of E1b mRNA in *hr*1-infected cells does not differ from that accumulation with wild type.

The quantitative data from this and other similar experiments are presented in Table 1. The percentages of ^{3}H-labeled RNA shown were based upon the total counts per minute in the portions of eluates of gel fractions hybridized to E1b-specific DNA. In each case, infection with *dl*312 resulted in at least a twofold increase, and in some cases up to fourfold increase in the accumulation of 22S and 13S RNA. The E1a deletion mutant *dl*311, which lacks nucleotides 1,282 to 1,339 (see Fig. 1) (57) and is also host range, although to a lesser extent than *dl*312 (31), exhibited the same phenotype as wild-type virus in this assay. When mRNA specific for another early region (E2) was quantitated, no significant difference was seen between *dl*312-infected cells and cells infected with the other mutants or wild-type virus.

Overproduction of nuclear RNA in *dl*312-infected 293 cells. The increased accumulation of

TABLE 1. ^{3}H-labeled viral mRNA accumulated in adenovirus-infected 293 cells[a]

Expt no.	Postinfection labeling from (h):	Virus	% of ^{3}H-labeled mRNA hybridized[b]		
			E1b		E2
			22S	13S	
1[c]	16.5 to 18.5	wt Ad5[d]	0.21	0.14	0.26
		*dl*312	0.87	0.60	0.29
		*hr*1	0.25	0.13	0.21
2	18 to 20	wt Ad5	0.23	0.16	ND[e]
		*dl*312	0.55	0.60	ND
3	20 to 22	wt Ad5	0.10	0.06	0.17
		*dl*311	0.12	0.10	0.27
		*dl*312	0.30	0.18	0.22

[a] Experiments were performed as described in the legend to Fig. 1.

[b] The counts per minute hybridizing to 4.5 to 11.0 DNA (E1b, generated by endo R · *Sma*I and endo R · *Hpa*I digestion of adenovirus 5 DNA) or the L strand of 58.5 to 70.7 DNA (E2, cloned in bacteriophage M13) were summed from gel profiles similar to that shown in Fig. 1. The percent hybridization was calculated by comparing the total counts per minute in viral RNA to the total counts per minute in the samples of eluted RNA used for hybridization.

[c] Shown in Fig. 1.

[d] Wild-type adenovirus 5.

[e] ND, Not determined.

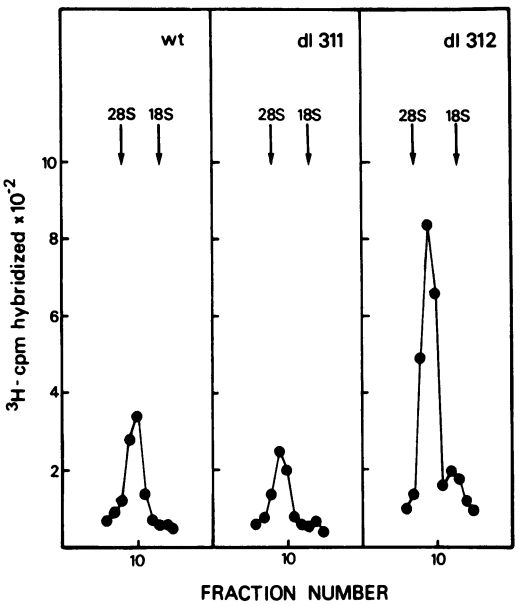

FIG. 3. Accumulation of newly synthesized, polyadenylated E1b RNA in the nucleus of 293 cells late after infection by wild-type (wt) or mutant adenovirus 5. Monolayers of 293 cells were infected with 50 PFU of the virus indicated (wt, *dl*311, or *dl*312) per cell. At 16 h after infection, cells were labeled with 200 μCi of [^{3}H]uridine per ml, and 15 min later, cells were harvested, and nuclear RNA was prepared by a modification (21) of the method of Holmes and Bonner (29). Polyadenylated nuclear RNA was analyzed as described in the legend to Fig. 2. Total counts per minute (cpm) in the samples hybridized were: wt, 3.7×10^5; *dl*311, 4.2×10^5; and *dl*312, 2.6×10^5.

E1b mRNA in *dl*312-infected 293 cells might derive from an increase in the synthesis of precursor RNA in the nucleus. To examine this question directly, cells were labeled for 15 min with [^{3}H]uridine, and polyadenylated nuclear RNA was analyzed as described before. Under these labeling conditions, most of the E1b-specific counts per minute appeared in 22S RNA. This size class presumably includes both spliced and unspliced E1b precursor RNAs (see Fig. 4 below). There was also a reproducible small peak of E1b RNA at 13S, which probably represented spliced 13S RNA before its transport to the cytoplasm. The labeling of these species in *dl*312-infected cells was clearly much greater than in wild-type- or *dl*311-infected cells (Fig. 3). Therefore, the increase in labeling of cytoplasmic mRNA probably originated from increased labeling of nuclear RNA precursors. This result suggests that the increase in E1b RNA accumulation in *dl*312-infected 293 cells occurred at a step close to transcription.

To characterize further the E1b RNA species present in the nucleus, polyadenylated nuclear RNA was analyzed by blot hybridization (Fig. 4). Two bands migrating as 22S could be distinguished. These bands probably correspond to unspliced and spliced 22S RNA which were resolved despite the fact that they differ by only 80 nucleotides. Nuclear RNA showed a predominant amount of the slower migrating unspliced RNA. The cytoplasmic mRNA marker (M) showed a predominance of the faster migrating spliced form, as expected. Some unspliced RNA was present as well, suggesting that the cytoplasmic RNA sample may be contaminated with nuclear RNA. Also present in the marker lane were 13S mRNA from E1b and a 9S mRNA,

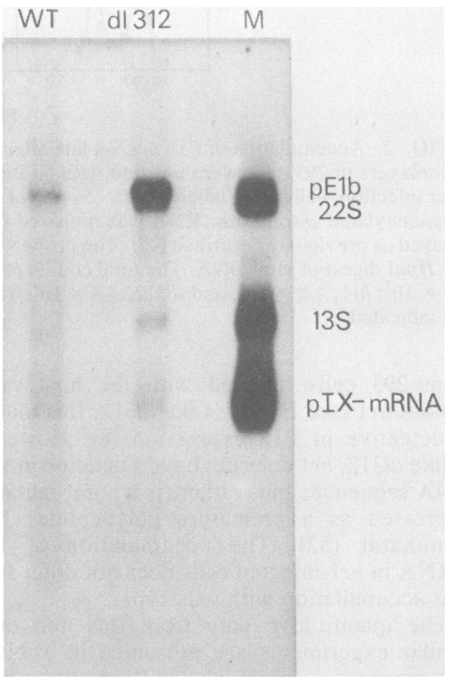

FIG. 4. Steady-state levels of polyadenylated E1b RNA in the nucleus of infected 293 cells at late times. Cells infected (as described in the legend to Fig. 3) with wild-type (WT) or *dl*312 virus were harvested at 20 h after infection. Polyadenylated nuclear RNA was prepared as described in the legend to Fig. 3, and steady-state viral RNA was analyzed by blot hybridization (see the text). The probe used was 2.8 to 11.0 viral DNA (*Sma*I-F fragment). This probe also detects RNA from the adjacent E1a region, but the quantity of E1a RNA in these samples is small compared with E1b (data not shown). Also shown as a marker (M) is late polyadenylated E1b cytoplasmic RNA from adenovirus 5-infected KB cells. The nomenclature of the bands is: pE1b, putative precursor to E1b mRNA, unspliced; 22S, spliced 22S E1b RNA; 13S, spliced 13S E1b RNA; and pIX mRNA, nuclear RNA precursor to the mRNA for protein IX.

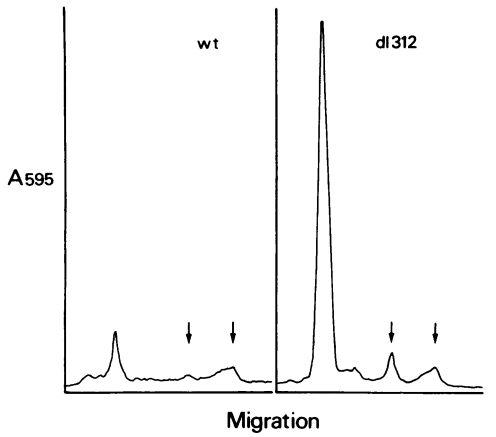

FIG. 5. Densitometric scanning of steady-state polyadenylated nuclear E1b RNA. Lanes from the autoradiogram shown in Fig. 4 were scanned at 595 nm with a Beckman Du-8 spectrophotometer with a gel scanning microprocessor accessory. The major peaks correspond to 22S (both spliced and unspliced forms). The left and right arrows indicate the positions of 13S and 9S RNA, respectively.

which was detected by the E1b probe used in these experiments. This mRNA was probably the message for protein IX (2, 50, 61). The amount of 9S RNA served as a convenient internal control for quantities of RNA loaded in a gel lane, since the amount of 9S RNA did not vary with infection by the different viruses (data not shown). In the two lanes containing adenylated nuclear RNA (*dl*312 and wild type), the unspliced 22S species was predominant, although there were lesser amounts of spliced 22S and 13S RNA and 9S RNA in the nucleus.

Densitometric scanning of the autoradiogram shown in Fig. 4 produced the patterns shown in Fig. 5. As expected, roughly equivalent amounts of 9S RNA (indicated by the arrow at the far right) were present in both samples. The *dl*312-infected cells clearly contained more 22S and 13S RNAs than wild-type-infected cells. This assay was not strictly quantitative due to the possibility of nonquantitative transfer of RNA to the nitrocellulose and because the hybridization was not performed with excess probe. Furthermore, the intensity of the autoradiographic signals was not a linear function of the radioactivity present. Nevertheless, this assay reproducibly showed clear qualitative differences between *dl*312- and wild-type-infected cells in steady-state adenylated nuclear E1b RNA, and the data supported the results obtained with pulse-labeled nuclear RNA.

Increased transcription of E1b RNA in *dl*312-infected 293 cells. The results with polyadenylated nuclear RNA indicated that the overproduction of E1b mRNA in *dl*312-infected 293 cells might occur at a step very close to transcription. To test directly whether more E1b RNA was transcribed in *dl*312-infected 293 cells, samples were pulse-labeled for 5 min. In such a short pulse, only newly transcribed nuclear RNA is labeled. Total nuclear RNA was extracted and hybridized to nitrocellulose membranes containing a DNA fragment (4.5 to 7.8 M.U.) that detects transcription only from the E1b promoter. The data in Table 2 indicate that in *dl*312-infected cells more RNA was transcribed from E1b than in wild-type-infected cells. As a control, transcription of a second region of the genome was also measured in this assay. This region, defined by the DNA fragment from 59.5 to 70.7 M.U., specifies nuclear RNA from both strands at late times after infection; predominant transcription is rightward. Transcription of this region was slightly more efficient in wild-type-infected cells. If this small difference is significant, the increased transcription of E1b relative to total viral transcriptional activity may actually be underestimated by normalizing to total counts per minute, as in this experiment.

Dominance of the *dl*312 phenotype in *cis*. The increased transcription of E1b in *dl*312-infected 293 cells could arise due to the lack of a diffusible factor that normally regulates E1b transcription in *trans* in cells infected by wild-type virus. For example, *dl*312-infected 293 cells fail to accumulate the mRNA for the smallest E1a protein late in infection (60). Alternatively, the source of increased transcription could be that the *dl*312 genomes themselves were defective in *cis* for E1b transcriptional regulation.

TABLE 2. Transcription of viral genes in adenovirus-infected 293 cells[a]

Time postinfection (h)	Virus	% of ^{3}H-labeled mRNA hybridized	
		4.5–7.8 (E1b)	59.5–70.7
20	wt Ad5[b]	0.07	3.0
	*dl*312	0.13	2.5
19	wt Ad5	0.07	5.4
	*dl*312	0.16	3.5

[a] Cells infected with 50 PFU of the virus indicated per cell were labeled for 5 min at the times shown, as described in the text. RNA was extracted from nuclei by the method of Zimmer et al. (70), and hybridizations were performed as described in the text. Cloned viral DNA probes were used for hybridization. All reactions were checked for DNA excess by using higher input levels of RNA. Excess was assumed if the same percent hybridization was obtained under these conditions. The background hybridizing to pBR322 DNA (less than 50 cpm) was subtracted. The results of duplicate assays are represented in each value.

[b] Wild-type adenovirus 5.

To distinguish these possibilities, human 293 cells were infected with *dl*312 or wild-type virus alone (50 PFU/cell) or in combination. The multiplicity of each virus in the coinfections was adjusted as follows. Sufficient amounts of each virus were employed (at least 5 PFU/cell) so that every cell would be infected by both viruses. In one sample, a 10-fold excess of *dl*312 virus (50 PFU/cell) was used. The reasoning was that the mutant phenotype should be observed if the *dl*312 templates were dominant in *cis*, whereas the wild-type phenotype would be observed if the wild-type phenotype were dominant in *trans*. The latter result would depend upon the gene dosage of the *trans*-acting wild-type virus factor being sufficient to regulate the transcription of the *dl*312 genomes under these conditions. A second coinfection sample was prepared by using a 10-fold excess of wild-type virus in case the *dl*312 phenotype were dominant in *trans* due to the production of an undetected product of the mutant genome.

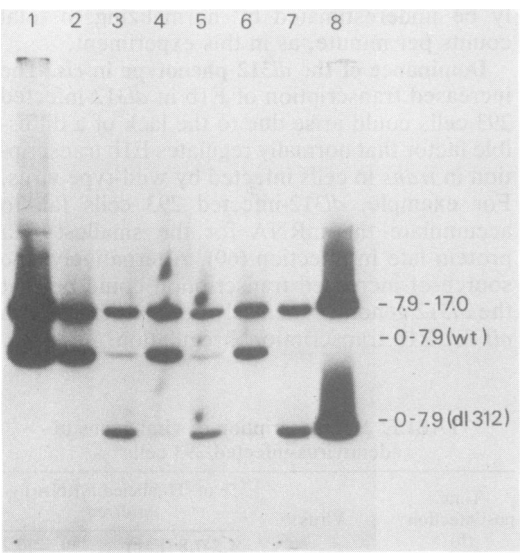

FIG. 6. Viral DNA accumulated in 293 cells coinfected with *dl*312 and wild-type adenovirus 5. DNA was extracted from infected cells at 22 h (experiment 1, lanes 2 and 3), 17.5 h (experiment 2, lanes 4 and 5), or 18 h (experiment 3, lanes 6 and 7). DNA was digested with endo R · *Hin*dIII and analyzed by blot hybridization as described in the text. The probe used in hybridization was ^{32}P-labeled *Sma*I-F fragment (2.8 to 11.0). Each gel lane contains 1 μg of digested DNA from infected cells; 0 to 7.9 and 7.9 to 17.0 refer to the *Hin*dIII DNA fragments generated from cleavage of viral DNA, as expressed in M.U. Lane 1, *Hin*dIII-digested adenovirus 5 DNA; lanes 2, 4, and 6, DNA from cells infected with 50 PFU of wild-type virus and 5 PFU of *dl*312 per cell; lanes 3, 5, and 7, DNA from cells infected with 50 PFU of *dl*312 and 5 PFU of wild-type virus per cell; lane 8, *Hin*dIII-digested *dl*312 DNA.

For this experiment to provide the necessary information, the ratio of templates from which late transcription occurred must reflect the input multiplicities in coinfected cells. Since no direct assay for transcription templates was available, the accumulation of viral DNA was measured. The accumulation of viral DNA in coinfected cells late after infection actually reflected the ratio of input multiplicities (Fig. 6). In this figure, the patterns of *Hin*dIII-generated DNA fragments obtained from cells coinfected with *dl*312 and wild-type virus were compared with fragments produced from viral DNA of either type. The hybridization probe used (*Sma*I-F fragment, 2.8 to 11.0 M.U.) detected the *Hin*dIII-E fragment (7.9 to 17.0 M.U.) in both DNAs. The probe also detected the wild-type *Hin*dIII-G fragment (0 to 7.9 M.U.) and the equivalent *dl*312 fragment, which is missing 900 base pairs (57). The altered migration of this fragment allowed comparison of the amount of *dl*312 or wild-type DNA. Blot hybridization of DNA extracted from coinfected cells clearly showed that cells infected with a 10-fold excess of *dl*312 virus produced substantially more *dl*312 left-end fragment; cells infected with a 10-fold excess of wild-type virus accumulated substantially more wild-type left-end fragment. In this experiment, cold *Sma*I-F DNA was added to the ^{32}P-labeled probe so that hybridization conditions approaching DNA excess would be achieved. Therefore, the band intensities were roughly proportional to the amount of DNA in the samples. Assuming that these intensities reflect the ratio of transcriptional templates available in the cell, the desired conditions appear to have been obtained.

These data also suggest that more viral DNA accumulated in coinfected cells when wild-type virus was in excess (compare 7.9 to 17.0 M.U. bands in each lane). This result may reflect more efficient replication of wild-type DNA or a higher effective multiplicity of wild-type virus infection. More DNA could lead to more transcriptional template and explain why transcription from the 59.5 to 70.7 region was somewhat higher in wild-type-infected cells than in *dl*312-infected cells (Table 2).

The amount of E1b nuclear RNA accumulated in coinfections was compared with that accumulated in infections with either virus alone by blot hybridization as described in the experiment shown in Fig. 4. Again, this assay provided a qualitative rather than strictly a quantitative analysis of RNA accumulation. Densitometric scanning of autoradiograms obtained (described in legends to Fig. 4 and 5) is shown in Fig. 7. The profiles indicated, first of all, that roughly the

same amount of 9S RNA (indicated by the arrow at the far right) accumulated in all infections. Furthermore, adding a small amount of the heterologous virus did not alter either the wild-type or *dl*312 phenotype. The conclusion is that the phenotype was dependent upon the dominant template in *cis*. Once again, this conclusion assumes that gene dosage of a possible *trans*-acting factor was not limiting in coinfection. Furthermore, the number of templates for transcription in cells infected by an excess of wild-type virus was at least as large as and probably larger than the number of templates in cells infected with an excess of *dl*312. Thus, the increased transcription of E1b in *dl*312-infected cells (Table 2) probably reflected an increased rate of transcription under these conditions.

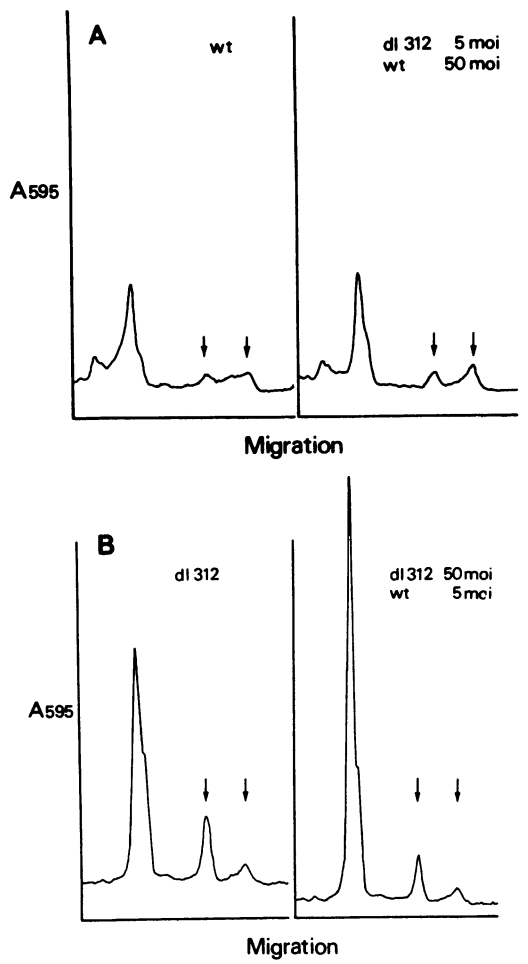

FIG. 7. Steady-state levels of total E1b RNA in the nucleus of 293 cells infected with wild-type virus, *dl*312, or both. The 293 cells, infected with 50 PFU/cell of wild-type (wt) virus (A) or 50 PFU/cell of *dl*312 (B), or coinfected with multiplicities (moi) of 5 for *dl*312 and 50 for wild type (A) or 50 for *dl*312 and 5 for wild type (B), were harvested at 17 h after infection. Total nuclear RNA was extracted by the method of Zimmer et al. (70), and steady-state E1b RNA was detected as described in the legend to Fig. 4. Densitometric scanning was performed as described in the legend to Fig. 5. The major peak is 22S RNA; the left and right arrows indicate the positions of 13S and 9S RNA, respectively.

DISCUSSION

The accumulation of E1b mRNA, especially the 13S size class, increases substantially at late times after infection of KB cells with wild-type virus, compared with accumulation at early times (61, 68). At least part of this increased accumulation is due to altered mRNA stability (68); increased transcription of E1b at late times does not occur during very high-multiplicity infections (68). It was this normal high level of E1b mRNA production late in infection that served as the control for the experiments described here.

These data indicate that the accumulation of E1b mRNA was abnormally high late after infection of adenovirus-transformed human 293 cells with adenovirus 5 deletion mutant *dl*312 and that the altered phenotype was due to an increase in the rate of transcription of the E1b gene. Previous studies of RNA synthesis from the integrated E1 sequences in 293 cells after infection with wild-type adenovirus 5 or deletion mutants indicated that the expected increase in E1b mRNA accumulation at late times probably occurs from infecting genomes rather than from the integrated sequences (60). This conclusion was based on the failure of E1b mRNA to accumulate in productive infections with mutant *dl*313, which does not encode its own genomic E1b mRNA (32). If this conclusion is correct, then most likely the abnormally high rate of E1b transcription in *dl*312-infected 293 cells occurred from viral templates and not from the integrated sequences.

It is not clear whether the altered E1b transcriptional activity of *dl*312 templates was unique to late infection. No significant differences in E1b mRNA accumulation have been observed between wild-type- and *dl*312-infected cells before the onset of viral DNA replication (data not shown). Under these conditions, however, the amount of total E1b mRNA made was only slightly greater than that made in uninfected 293 cells.

Substantial synthesis of E1b mRNA from integrated cellular sequences continued at late times after infection. In fact, mock-infected cells accumulated about half as much 22S RNA as wild-type virus-infected cells at late times (60). The amount of 13S RNA in mock-infected cells was negligible compared to that in wild type (data not shown). The contribution by integrated E1b

DNA accounts for the relatively high level of 22S RNA seen in wild-type virus-infected 293 cells. In the *dl*312-infected cells, substantial increases in both 22S and 13S RNA were seen (Fig. 2, Table 1). Thus, the increased rate of transcription of E1b is accompanied by the accumulation of an unusually large proportion of 22S mRNA, compared to that normally seen in wild-type virus-infected KB cells (61). If the increase in E1b mRNA was due to transcription of *dl*312 templates, then the proportional increase, compared to wild-type infections, has probably been underestimated in these experiments. In this regard, a host cell permissive for *dl*312 which does not contain E1b DNA would be useful for further investigation of this phenomenon.

The results of the coinfection experiment suggest that the absence of a *trans*-acting factor from *dl*312-infected 293 cells was not responsible for the mutant phenotype. However, the possibility remains that the gene dosage of such a factor was insufficient for activity in cells receiving 10 copies of mutant DNA for every copy of wild-type DNA. Along these lines, it is instructive to consider which viral products are absent from *dl*312-infected 293 cells and could be responsible for such an effect. Aside from defects in region E3 (31) (also a characteristic of mutant *dl*311, which is wild type for the E1b phenotype), the only known viral product missing in *dl*312-infected 293 cells is the 9S mRNA for a late E1a protein (60). If this RNA or its protein product (28,000 daltons on polyacrylamide gels) (19, 59) were involved in E1b regulation in this system, it would have to be a negative regulator of transcription. The E1a region appears to encode products early in infection that are positive regulators of viral transcription (44).

There are three possible explanations for the mutant phenotype. First, the deleted sequence of *dl*312 but not *dl*311 may contain a recognition site for a negative regulatory factor. Repression of early simian virus 40 transcription by simian virus 40 T antigen requires a binding site near the promoter (26, 54). In the *dl*312 genome, the deleted sequence is 350 nucleotides upstream from the initiation site at 1,702. If there is a deleted recognition site, then sequences substantially upstream must participate in regulation of this unit. One intriguing possibility is that such a site is contained in the sequences common to the two introns found in E1a mRNA. These sequences are between nucleotides 1,112 and 1,229. This region is unusual in that it is over 70% A-T base pairs (63).

A second possibility is that the region to the left of nucleotide 448 contains a positive control element that, in *dl*312 DNA, can influence the E1b promoter due to the removal of sequences between 448 and 1,349. Such an element might be analogous to the papovavirus enhancer sequences that appear to enhance transcription of nearby genes (6). A similar sequence may be present in retrovirus genomes (47). In *dl*312 genomes, this element may affect E1b because of the removal of the E1a promoter, or it may simply influence any promoter within a certain distance. The *dl*312 deleted sequences may bring the 1,702 initiation site close enough to this element to bring about increased transcriptional activity at this site. The magnitude of enhancement of E1b transcription by the adenovirus sequence is much less than that observed when the simian virus 40 enhancer sequences are placed in proximity to the cellular β-globin gene (6). This would not be an unexpected finding if E1b is already controlled by a promoter which is relatively efficient.

Finally, the deletion in *dl*312 might eliminate competition for RNA polymerase II by the E1a promoter and initiation site at 499 in some undefined way. Analysis of viral DNA molecules with different sequence compositions may help resolve these possibilities.

There is one further point to be made about *dl*312-infected 293 cells. The overproduction of E1b mRNA in these cells may be manifested as an increase in E1b protein synthesis. Analysis of ^{35}S-labeled proteins from infected cells indicated that a band migrating at 15,000 daltons was labeled more substantially in *dl*312-infected cells than in wild-type-infected cells (60). This band is probably the E1b-encoded 15,000-dalton protein(s) (25, 39). If so, then there is the possibility that *dl*312-infected cells may make substantial amounts of the large E1b protein as well, a 55,000-dalton protein (19, 25, 55) implicated directly in cellular transformation by adenovirus (56). *dl*312-infected permissive cells may prove a useful source of E1b proteins for further study.

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