Mapping of Adenovirus 12 mRNA's Transcribed from the Transforming Region

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Adenovirus 12 mRNA's transcribed from the transforming region were analyzed and mapped on viral DNA by the nuclease S1 gel and diazobenzyloxymethyl paper blot techniques in cells transformed by the *EcoRI*-C (leftmost 16.5%) and *HindIII*-G (leftmost 6.8%) fragments of adenovirus 12 DNA and in cells early and late after lytic infection with adenovirus 12. Two initiation sites of mRNA transcription and two kinds of splicing were found in each of early regions 1A and 1B. For early region 1A mRNA's, four species were found in lytically infected cells. Three of them were commonly found in cells transformed by either *HindIII*-G or *EcoRI*-C. Cells transformed by *HindIII*-G contained two additional 1A transcripts, which could be the 3' portions of chimeric mRNA's of cellular-viral or viral-viral sequences. Transcription in the 1B region diverged among the above cell lines. Early after lytic infection, no appreciable amount of 1B mRNA was detected, whereas two species of mRNA's, one corresponding to protein IX mRNA and another having splicing, were found at the late stage. In cells transformed by *EcoRI*-C, a distinct mRNA species with splicing was observed. Cells transformed by *HindIII*-G contained a transcript from the leftmost part of the 1B sequence at the 5' portion of chimeric mRNA species, suggesting the presence of tandem integration of viral DNA in the cells. Other mRNA species in the 1A and 1B regions were also detected in both transformed cell lines. The results are discussed in relation to the nucleotide sequence of the *HindIII*-G fragment of adenovirus 12 DNA.

The transforming ability of human adenovirus has been localized in the leftmost 7 to 8% of the viral genome by cell transformation studies with restriction DNA fragments of human adenovirus 2 (Ad2), Ad5, Ad7, and Ad12 (23, 25, 29). Cell transformation experiments with smaller DNA fragments have revealed a minimum size for the transforming fragments; the leftmost fragments *AccI*-H (*BpaI*-H; 4.7%, Ad12) (26), *BglIII*-H (4.5%, Ad7) (8), and *HpaI*-E (4.3%, Ad5) (A. J. Van der Eb, H. Van Ormondt, P. I. Schrier, J. H. Lupker, H. Jochemson, P. J. Van den Elsen, R. J. Deleys, J. Maat, C. P. Van Beveren, R. Dijkema, and A. De Waard, Cold Spring Harbor Symp. Quant. Biol., in press) still can transform cells, although the resultant cell lines show incomplete transformation phenotypes. In the early stage of lytic infection, before viral DNA replication, at least four regions of viral DNA are transcribed (18, 24). The leftmost region (11.5% of the viral DNA), which contains the entire transforming ability, is divided into two regions: 1A (0 to about 4.5 map units) and 1B (4.5 to 11.5 map units), with specific promoters (31). Thus, region 1A contains the information for the basic conversion of cells into a transformed cell line, but region 1B codes for proteins

which also play important roles in cell transformation (26; Van der Eb et al., in press). On the other hand, characterization of the host range deletion mutants of Ad5 has offered inferences into functions encoded in the transforming region; region 1A product(s) regulates expression of other early viral genes (2, 13).

Analysis of early mRNA's from early region 1 of Ad2 and Ad5 by the nuclease S1 gel technique (2-4) showed that at least two mRNA's were coded in region 1A and that one was coded in region 1B, all with spliced structures. Electron microscopic studies identified additional mRNA species with different splicings (6). Ad12 (17, 27) has essentially the same topography (9) as Ad2 (7, 18, 24), but no detailed mapping of mRNA species has been available. In this paper, we describe the mapping of region 1 mRNA's transcribed in Ad12-infected cells and in cell lines transformed by the Ad12 *EcoRI*-C (the leftmost 16.5%) and *HindIII*-G (the leftmost 6.8% of viral DNA) fragments. A notable difference from Ad2 (4), Ad5 (2), and Ad7 (33) was observed upon expression of 1B mRNA's in cells early after lytic infection. In transformed cell lines, several unique mRNA's which were not detected in productively infected cells were found. In the

nucleotide sequence of the *Hind*III-G fragment of Ad12 (K. Fujinaga, Y. Sawada, Y. Uemizu, T. Yamashita, H. Shimojo, K. Shiroki, H. Sugisaki, K. Sugimoto, and M. Takanami, Cold Spring Harbor Symp. Quant. Biol., in press; H. Sugisaki, K. Sugimoto, M. Takanami, K. Shiroki, I. Saito, H. Shimojo, Y. Sawada, Y. Uemizu, S. Uesugi, and K. Fujinaga, Cell, in press), specific sequences were found at the sites of initiation, termination, and splicing of RNAs mapped in this study.

MATERIALS AND METHODS

Cells and virus. Transformed cell lines CY1-1 and GY1-1 used in this study were established from cell line 3Y1 (established from a Fischer rat embryo) transfected with the *Eco*RI-C and *Hind*III-G fragments of Ad12 DNA, respectively, as described previously (25, 32). Cells were grown in monolayers in Eagle minimum essential medium containing 0.1 mM Ca^{2+} supplemented with 10% fetal bovine serum (GIBCO Laboratories). Ad12 (Huie) was propagated in KB cells, and viral DNA was isolated from purified virions as described (11, 12).

Enzymes. *Hind*III was purified in our laboratory. *Acc*I and *Hae*III were gifts from Mituru Takanami, Kyoto University, Kyoto, Japan. *Eco*RI, *Escherichia coli* DNA polymerase I, and nuclease S1 were purchased from Boehringer Mannheim Corp. Pancreatic DNase I was a product of Worthington Biochemicals Corp.

Preparation of ^{32}P -labeled restriction fragments of Ad12. ^{32}P -labeled virus was prepared from infected cells labeled with 100 μCi of $^{32}\text{P}_i$ per ml in phosphate-free minimum medium supplemented with 2% dialyzed calf serum for 48 to 72 h at 37°C. Viral DNA had a specific activity of 1×10^6 to 2×10^6 cpm/ μg and was digested with restriction endonuclease. DNA fragments were separated by 1.4% agarose gel electrophoresis and purified as described previously (23). Highly radioactive DNA fragments (5×10^7 to 2×10^8 cpm/ μg) were prepared with [α - ^{32}P]-dATP and [α - ^{32}P]dCTP by the nick translation procedure as previously described (21).

Isolation of cytoplasmic RNA. KB cells in monolayer culture were infected with Ad12 at a multiplicity of approximately 20 PFU per cell. Cells were harvested at 8 h (without cytosine arabinoside) or 12 h (with 20 μg of cytosine arabinoside per ml) after infection for preparation of Ad12 early RNA, and at 23 h after infection for preparation of Ad12 delayed early RNA. The cytoplasmic fractions of GY1-1, CY1-1, and infected KB cells were prepared by hypotonic Nonidet P-40 lysis in the presence of *N*-ethylmaleimide, spermine, and diethylpyrocarbonate by the method of Zieve and Penman (34). The cytoplasmic fraction was made 0.5% in sodium dodecyl sulfate, 10 mM in EDTA, and 0.1 M in NaCl and extracted by phenol and chloroform. RNA in the aqueous phase was precipitated by adding 2 volumes of ethanol and rinsing with 70% ethanol. Polyadenylic acid-containing RNA was selected by oligodeoxythymidylic acid-cellulose (Collaborative Research, Inc.) column chromatography.

Nuclease S1 gel analysis. Hybridizations for nu-

clease S1 gel analysis were carried out in hybridization buffer containing 80% formamide, 0.4 M NaCl, 0.04 M PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (pH 6.4), and 0.001 M EDTA (5), essentially by the method of Berk and Sharp (3) as previously described (33). The melting temperature (T_m) irreversible in this solvent was 46 to 47°C as determined by susceptibility of the Ad12 *Eco*RI-C fragment to nuclease S1 (Y. Sawada, unpublished data). Hybridizations were therefore carried out at 53°C, 6 to 7°C higher than the T_m of Ad12 *Eco*RI-C. RNA-DNA hybrids were digested by nuclease S1 (30) as described previously (3, 33) with slight modifications.

Alkaline gel electrophoresis (15) was carried out in 1.4% agarose slab gels (20 cm in length) at 40 V for 14 h. Gels were neutralized by soaking in 0.05 M Tris (pH 7.2) for 30 min, dried, and autoradiographed at -70°C with Kodak XR-1 or XRP-1 film and Du Pont tungstate intensifying screens. Lengths in nucleotides of cotranscripts were obtained in alkaline gels. In neutral gels, slightly larger values were observed for respective cotranscripts than were observed in alkaline gels (4). Two-dimensional electrophoresis (8a) was carried out as follows. After a first electrophoresis of a neutral agarose slab gel, the gel track containing respective RNA-DNA hybrids was cut out and equilibrated for alkaline electrophoresis buffer. The gel strip was set on top of a second alkaline agarose slab gel and electrophoresed.

Preparation of diazobenzoyloxymethyl paper. 1-[(*m*-Nitrobenzyloxy)methyl]pyridinium chloride was synthesized essentially by the procedure previously described (1). (*m*-Nitrobenzyloxy)methyl chloride (yellow liquid with a boiling point of 139 to 144°C at 2 mm of Hg) was made from paraformaldehyde, *m*-nitrobenzyl alcohol, and dry HCl in benzene and was added slowly to ice-cold pyridine by stirring. The crystalline pyridinium salt was collected on a sintered glass filter, washed with pyridine, washed with petroleum ether, and dried under reduced pressure. The 1-[(*m*-nitrobenzyloxy)methyl]pyridinium chloride was stored at -20°C. The aminobenzoyloxymethyl paper derived from a sheet of Toyo 514 paper (14 by 20 cm) was converted to diazobenzoyloxymethyl (DBM) paper by treatment with a freshly prepared solution containing 40 ml of water, 80 ml of 1.8 M HCl, and 3.2 ml of NaNO_2 (10 mg/ml) for 30 min at 0 to 4°C. The DBM paper was washed five times for 5 min each time with cold water and then twice for 10 min with ice-cold sodium phosphate buffer (25 mM, pH 6.8); it was used within 15 min after the final wash.

DBM paper blot analysis. Polyadenylic acid-containing RNAs were denatured with 1 M glyoxal in 50% dimethyl sulfate and 6.7 mM sodium phosphate (pH 6.8) for 60 min at 50°C as previously described (16). Restriction fragments of Ad12 DNA for size markers were also glyoxalated. After addition of tracking dye containing bromophenol blue and glycerol, denatured samples were electrophoresed on a 1.5% agarose slab gel in 10 mM sodium phosphate buffer (pH 6.8). After electrophoresis, the gel was immersed in 50 mM NaOH containing ethidium bromide (0.5 $\mu\text{g}/\text{ml}$) for 40 min to 1 h to dissociate the RNA-glyoxal compound and to stain the RNA bands. The gel was then neutralized by washing twice in 100 mM phosphate buffer (pH 6.8) for 20 min, followed by two washes of 25 mM phos-

phate buffer for 5 min each. The RNA was transferred by blotting to the DBM paper. The RNA blot was treated overnight at 42°C with hybridization buffer (50% formamide; 0.75 M sodium chloride; 75 mM sodium citrate; 0.02% [wt/vol] each of bovine serum albumin, Ficoll, and polyvinyl pyrrolidone; and 1.5 mg of sonicated denatured calf thymus DNA per ml) containing 1% (wt/vol) glycine (1). Hybridizations were carried out for 20 to 40 h at 42°C in plastic bags containing hybridization buffer (50 to 100 μ l/cm² of paper surface area) and the single-stranded ³²P-labeled probe (3 $\times$ 10⁴ to 5 $\times$ 10⁴ cpm/cm² of paper surface area). The paper was washed once in a plastic bag at 42°C and then again on a suction funnel at room temperature with a solution containing 50% formamide, 0.75 M sodium chloride, and 75 mM sodium citrate and finally with a solution containing 0.3 M sodium chloride and 30 mM sodium citrate. The paper strips were blotted to remove excess solution and air dried, and the RNAs were made visible by autoradiography as described above.

RESULTS

Mapping of mRNA's in the CY1-1 cell line by the nuclease S1 gel technique. The early region 1 sequences (0.9 to 11.2 map units [see below]) of Ad12 DNA are fully included in the *EcoRI*-C fragment (0 to 16.5 map units). CY1-1 cells contain the *EcoRI*-C fragment of Ad12 DNA at 5 to 6 copies per haploid cell (21). Four major and three minor virus-specific mRNA species were detected in CY1-1 cytoplasm, and all seven mRNA's had spliced structures (Fig. 1E). A very dense band at about 2,250 nucleotides in a neutral gel was observed after hybridization with the *EcoRI*-C fragment (Fig. 1A, lane 9). This apparently dissociated into two bands at 450 and 1,740 nucleotides in an alkaline gel (Fig. 1A, lane 2). Portions of this long mRNA were detected as bands by using the *HindIII*-G, *HindIII*-I, *AccI*-J, and *AccI*-F fragments as probes (Fig. 1A). These bands were assigned in region 1B as shown in Fig. 1D. Thus, the only 1B mRNA in CY1-1 cells has its 3' end at position 3,820 and 5' end at position 1,530, approximately. It has the spliced structure, having splicing points approximately at positions 3,270 and 3,370.

In region 1A, three mRNA's were found as major species (Fig. 1E). These were detected as bands in common with the fragments *EcoRI*-C, *HindIII*-G (0 to 6.8 map units), and *AccI*-H (0 to 4.7 map units) (Fig. 1A). Each of them had a spliced structure (760 plus 310, 620 plus 310, and 530 plus 310 nucleotides in length) as identified by two-dimensional analysis (see Fig. 4A). These 1A mRNA's were mapped in detail by using small DNA fragments produced by recleavage of *AccI*-H (positions 0 to 1,596) with *HphI*: *HphI*-A (positions 0 to 692), -BD (positions 692

to 1,214), and a mixture of -B (positions 692 to 1,105) plus -EC (positions 1,214 to 1,596) (Fig. 1D).

A band appearing at about 500 nucleotides with the B fragment in a neutral gel almost disappeared in an alkaline gel (Fig. 1B, lanes 3 and 9), whereas other bands did not alter. This indicates that all splicing of the three mRNA's in the region of 0 to 4.7 map units occurs around position 1,000. As two bands at 380 and 280 nucleotides were observed with the BD fragment as well as with the probe containing the B plus EC fragments in alkaline gel (Fig. 1B, lanes 9 and 10), there should be two donor sites for splicing approximately at positions 970 and 1,070. One band at 380 nucleotides is observed with the A fragment, indicating that at least one mRNA starts approximately at position 310. These assignments are schematized in Fig. 1D.

Hybridization with the *AccI*-H/*HaeIII*-A fragment (positions 442 to 1,596) produced only two bands at about 620 and 530 nucleotides (Fig. 1C, lane 3). This clearly showed that there are two promoter sites in region 1A of Ad12 DNA. The foregoing one should start transcription approximately at position 310 as mapped above. The relative density of the band at 620 nucleotides as compared with that at 530 nucleotides in lane 3 (with the *AccI*-H/*HaeIII*-A fragment) is higher than in lane 4 (with the *AccI*-H fragment). Thus, the 760-nucleotide-long cotranscript gives a band at about 620 nucleotides after hybridization with the A fragment, resulting in a doublet band with another 620-nucleotide-long cotranscript. As the donor sites for splicing are mapped approximately at positions 970 and 1,070 as above, the 530- and 620-nucleotide-long cotranscripts start approximately at position 450, very near the *HaeIII* site on the *AccI*-H fragment (Fig. 1D).

Three minor species were also found and identified as long mRNA's with the same 5' ends as 1A mRNA's and the same 3' end as 1B mRNA's (Fig. 1E). They contained the 2,130-nucleotide-long cotranscripts, whose left portions were detected as bands at 450 nucleotides with the *AccI*-H fragment (Fig. 1A1 and 1C, faint bands) and at 1,150 nucleotides with the *HindIII*-G fragment (Fig. 1A1) in alkaline gels. Two-dimensional analysis using the *AccI*-H fragment revealed, after extensive exposure, minor dots corresponding to 670-, 310-, 760-, 620-, 530-, and three 450-nucleotide-long cotranscripts. Each of the 760-, 620-, and 530-nucleotide-long cotranscripts was spliced together with a 450-nucleotide-long cotranscript, consistent with the above assignment of minor bands. The spliced mRNA of 670- and 310-nucleotide-long cotranscripts was mapped at its 5' and 3' ends at the common

FIG. 1. Autoradiograms of nuclease S1-digested RNA-DNA hybrids of CY1-1 cytoplasmic RNA and diagrams of the deduced RNA structures. Alkaline (A1) and neutral (A2) agarose gels of nuclease S1-digested CY1-1 RNA restriction fragment hybrids. Restriction fragments used are *EcoRI*-C (lanes 2 and 9), *HindIII*-G (lanes 3 and 10), *HindIII*-I (lanes 4 and 11), *HindIII*-F (lanes 5 and 12), *AccI*-H (lanes 6 and 13), and *AccI*-J (lanes 7 and 14). The reannealed fragment can be seen in each lane. Lanes 1 and 8 are the *HindIII* marker digests of Ad12 DNA. The lengths (in kilobases) of the Ad12 *HindIII* restriction fragments are: A, 5.40; B, 4.75; C, 3.70; D, 3.66 (C and D comigrate); E, 3.30; F, 2.51; G, 2.32; H, 1.80; I, 1.39; J, 1.10; K, 1.03; L, 0.98; M, 0.68; N, 0.54; O, 0.48; and P, 0.36. Since excess reaction of nuclease S1 in these experiments cut through RNA-DNA hybrids at splicing points, several bands corresponding to each cotranscript of spliced RNAs are also seen in the autoradiogram of the neutral gel (for example, the bands at about 1,200 nucleotides in lane 10 and at about 1,000 nucleotides in lane 11). Neutral (B1) and alkaline (B2) agarose gels of nuclease S1-digested

hybrids of *HphI* restriction fragments and cytoplasmic RNAs from CY1-1 (lanes 2, 3, and 4), GY1-1 (lane 6) and infected early cells (lane 5). Restriction fragments used are *AccI*-H/*HphI*-A (lanes 2, 5, 6, 8, 11, and 12), *AccI*-H/*HphI*-BD (lanes 3 and 9) and *AccI*-H/*HphI*-B plus EC (lanes 4 and 10) (see Fig. 1D and 1E for the physical map). Lanes 1 and 7 are the *HindIII* marker digests of Ad12 DNA. Alkaline (C1) and neutral (C2) agarose gels of nuclease S1-digested hybrids of restriction fragments and polyadenylic acid-containing cytoplasmic RNAs from CY1-1 and GY1-1 cells. Restriction fragments used are *HindIII*-G (lanes 2, 5, 6, and 9), *AccI*-H (lanes 4 and 7), and *AccI*-H/*HaeIII*-A (lanes 3 and 8) (see Fig. 1D for the physical map). Lanes 1 and 10 are the *HindIII* marker digests of Ad12 DNA. (D) Assignment of main bands detected in CY1-1 cells by the nuclease S1 gel procedure. Detected portions of each cotranscript are represented by open boxes with numbers in nucleotides of corresponding band lengths. Hatched boxes represent portions not identified by the relevant restriction fragments. Caret symbols represent splicing, and the 3' end of an RNA is indicated by an arrowhead. Numbers above vertical arrows represent the assigned positions of RNA initiation, termination and splicing sites. Restriction fragments relevant to the assignments are represented below the RNA structure. (E) Diagrams of the structure of Ad12-specific RNAs in CY1-1 cytoplasm deduced from the data in (A), (B), and (C). Viral sequences present in an RNA are represented by a line above the corresponding region of the Ad12 genome map. The genome map is marked off in kilobases and map units from the left end. Caret symbols represent splicing of colinear transcripts into one RNA molecule, and the 3' end of an RNA is indicated by an arrowhead. The more abundant cytoplasmic RNAs are represented by heavy lines, and less abundant cytoplasmic RNAs are represented by thin lines. Numbers above the lines represent the cotranscript lengths (in nucleotides). Restriction maps relevant to the analysis are represented below the genome map.

sites with the spliced one of 760 plus 310 nucleotides in length (see Fig. 3D).

Mapping of mRNA's in GY1-1 cell line by nuclease S1 gel technique. Three major 1A mRNA's detected in CY1-1 cells were also transcribed in GY1-1 cells. An additional 1A species was found as a band at 840 nucleotides with the *AccI*-H and *HindIII*-G fragments in alkaline gels

(Fig. 2A and 1C) and a band at 470 nucleotides with the *AccI*-H/*HphI*-A fragment (Fig. 1B, lane 12). This longer cotranscript was also spliced together with the 310-nucleotide-long cotranscript, as was resolved by two-dimensional, nuclease S1 gel analysis (see Fig. 4C). In the 1B region, one cotranscript specific to GY1-1 cells was mapped. After hybridization with the

FIG. 2. Autoradiograms of nuclease S1-digested RNA-DNA hybrids of GY1-1 cytoplasmic RNA and diagrams of the deduced RNA structures. (A) Alkaline (left) and neutral (right) agarose gels of nuclease S1-digested GY1-1 RNA restriction fragment hybrids. Restriction fragments used are *HindIII*-G (lanes 4 and 5), *AccI*-H (lanes 3 and 6), and *AccI*-J (lanes 2 and 7). Lanes 1 and 8 are the *HindIII* marker digests of Ad12 DNA. (B) Diagrams of the structure of Ad12-specific RNAs in GY1-1 cytoplasm deduced from the data in (A) and Fig. 1 (B and C). Representation is as in Fig. 1.

*Hind*III-G fragment, a band at 560 nucleotides was found in an alkaline gel (Fig. 2A, lane 4). Its right portion was detected with the *Acc*I-J fragment as a band at 490 nucleotides. This 560-nucleotide-long cotranscript was not found in either CY1-1 or KB cells after infection with Ad12, and is considered a GY1-1-specific cotranscript.

Three minor mRNA species were detected in GY1-1 cytoplasmic RNA after hybridization with the *Hind*III-G fragment. The band at 950 nucleotides in alkaline gels (Fig. 2A, lane 4, and Fig. 1C, lane 2) is attributed to the 3' portion of these minor species. These were mapped through the 1A and 1B regions (positions 230 to 2,090, 310 to 2,090 and 450 to 2,090) and had the same spliced structure (positions 1,070 to 1,140) as was determined for the major 1A mRNA's (Fig. 2B). There was another minor species whose 5' portion was detected as a band at about 560 nucleotides with the *Acc*I-H/*Hph*I-A fragment in an alkaline gel (Fig. 1B, lane 12). It is also considered to be a GY1-1-specific species, starting approximately at position 120 with the same spliced structure as other minor species (not shown in Fig. 2B).

Mapping of mRNA's in lytically infected cells by nuclease S1 gel technique. In cells early after lytic infection with Ad12, four major mRNA species were found for the 1A region whether 1- β -D-arabinofuranosylcytosine was present or not (Fig. 3A and B). These were mapped in detail with the *Acc*I-H/*Hph*I fragments (shown in part in Fig. 1B) and the *Acc*I-H/*Hae*III fragment (Fig. 3B). Three of the four mRNA's of infected early cells corresponded to the three 1A mRNA's transcribed in CY1-1 and GY1-1 cells. The fourth started at the same site as the longest one of the three and spliced as the shortest (Fig. 3D). Their spliced structure was resolved by two-dimensional analysis (Fig. 4B). In contrast with CY1-1 or GY1-1 cells, no appreciable band was observed on autoradiograms of nuclease S1 gel analysis for 1B mRNA's from infected early cells. Long species through the 1A and 1B regions were not found, either.

In cells late after lytic infection with Ad12, all of the four 1A mRNA's found in the early stage were also detected (Fig. 3C). In addition, two mRNA's were found in the 1B region at the late stage. One was detected at 1,150 nucleotides with *Eco*RI-C in a neutral gel (Fig. 3C2, lane 2) and at 530 nucleotides with *Hind*III-G and 340 nucleotides with *Hind*III-I in an alkaline gel (Fig. 3C1, lanes 3 and 4). This mapped at positions 1,530 to 3,820 and spliced at positions 2,060 and 3,370. A second was detected at 450 nucleotides with *Eco*RI-C and at 340 nucleotides with *Hind*III-I in an alkaline gel (Fig. 3C1, lanes 2

and 4) and mapped at positions 3,370 to 3,820.

Data presented in Fig. 3C1 and C2 also show at least four mRNA species comprising four cotranscripts of 1,400, 720, 150, and 80 nucleotides with two different splicings. These can be mapped in the region which is apparently outside early region 1 and corresponds to the region on the *l* strand assigned for protein IVa2 in the case of Ad2 (14). A prominent band at 1,400 nucleotides was commonly found with the *Eco*RI-C, *Hind*III-F, and *Acc*I-F fragments in alkaline gels (Fig. 3C1). In neutral gels, two bands at 1,620 and 1,550 nucleotides were detected with the *Hind*III-F fragment, one was detected at 1,550 nucleotides with the *Eco*RI-C fragment, and one was detected at 1,500 nucleotides with the *Acc*I-F fragment (Fig. 3C2). Thus, there are two major species having the 5' ends at positions approximately 5,600 and 5,670, near the *Acc*I (position 5,530) and the *Eco*RI (position 5,600) sites. They presumably have a common 3' end near position 3,850 and a splice at positions 5,250 and 5,520. Two minor species were composed of the 720-nucleotide-long cotranscripts (presumably having the common 3' end as the 1,400-nucleotide-long cotranscripts) (data not shown). A band at 430 nucleotides found with the *Hind*III-F fragment in an alkaline gel (Fig. 3C, lane 5, corresponding to a band at 460 nucleotides in a neutral gel) can be mapped at the right-hand end of the fragment. It could be the 3' portion of mRNA species corresponding to the early region 5 species of Ad5, where the *ts36* gene has been mapped (10).

Analysis of mRNA's by DBM paper blot hybridization. The procedure of Alwine et al. (1) was applied to characterize mRNA's. DBM papers carrying polyadenylic acid-containing RNAs from CY1-1, GY1-1, and infected early cells were hybridized with ³²P-labeled *Acc*I-H and *Acc*I-J fragments specific to the 1A and 1B regions, respectively (Fig. 5). In CY1-1 cells, mRNA species of about 3,200 and 1,000 to 1,200 nucleotides in length were found in region 1A. For the 1B region, mRNA's with lengths of about 3,200 and 2,300 nucleotides were detected. Long species of about 3,200 nucleotides in length apparently seem to be transcribed from the 1A and 1B regions contiguously. This corresponds well with the three minor species detected by nuclease S1 gel analysis. 1A-specific species of 1,000 to 1,200 nucleotides and 1B-specific species of 2,300 nucleotides in length compare well with those identified by nuclease S1 gel analysis.

In GY1-1 cells, however, mRNA species were found with sizes unexpected from the results of nuclease S1 gel analysis. Species with sizes of about 3,600, 2,500, 1,900, 1,700, and 1,000 to 1,200 nucleotides were detected with the ³²P-labeled

FIG. 3. Autoradiograms of nuclease S1-digested RNA-DNA hybrids of early region 1 cytoplasmic RNA in cells lytically infected with Ad12, and diagrams of the deduced RNA structures. (A) Alkaline agarose gel of nuclease S1-digested early RNA restriction fragment hybrids. Ad12 early RNA was prepared from lytically infected KB cells incubated in the presence of 20 μ g of 1- β -D-arabinofuranosylcytosine per ml of culture fluid. Restriction fragments used are EcoRI-C (lane 2), HindIII-G (lane 3), HindIII-I (lane 4), HindIII-F (lane 5), AccI-H (lane 6), AccI-J (lane 7), and AccI-F (lane 8). Lane 1 is the HindIII marker digest of Ad12 DNA. (B) Alkaline agarose gel of nuclease S1-digested early RNA restriction fragment hybrids. 1- β -D-Arabinofuranosylcytosine was not present in the course of Ad12 infection. Restriction fragments used are HindIII-G (lane 1), HindIII-I (lane 2), AccI-H (lane 3), AccI-H/HaeIII-A (lane 4), and AccI-J (lane 5). (C1 and C2) Alkaline and neutral agarose gels, respectively, of nuclease S1-digested delayed early RNA restriction fragment hybrids. Cytoplasmic RNA was prepared from cells 23 h after lytic infection. Restriction fragments used are EcoRI-C (lane 2), HindIII-G (lane 3), HindIII-I (lane 4), HindIII-F (lane 5), AccI-H (lane 6), AccI-J (lane 7), and AccI-F (lane 8) for both C1 and C2. Lane 1 in C1 and C2 is the HindIII marker digest of Ad12 DNA. (D) Diagrams of the deduced structures of early region 1 mRNA's in cells lytically infected with Ad12. The mRNA's detected at the early stage are represented by bold lines, and those detected only at the late stage are represented by open boxes; representation is otherwise the same as in Fig. 1.

AccI-H fragment, and one of about 1,700 nucleotides was detected with the AccI-J fragment (Fig. 5). Very large species (more than 8,000 nucleotides) were also found with both probes. The 1,000- to 1,200-nucleotide-long species correspond to the three major 1A mRNA's mapped in GY1-1 cells by nuclease S1 gel analysis. There are three other mRNA's (3,600, 2,500, and 1,900 nucleotides in length) specific to the 1A region of the viral genome, but these have larger sizes than expected for any 1A mRNA species analyzed by nuclease S1 gel analysis, suggesting that they are chimeric mRNA's of cellular and viral sequences. These should contain the GY1-1-specific species (840 plus 310 nucleotides in length) as viral components. The 1,700-nucleotide-long species detected with both probes could comprise at least two mRNA's. One could start at position 450 by one of the 1A promoters, read contiguously through the 1B region, and terminate at a cellular signal, which is assumed to be close after the viral-cellular DNA junction. A second could start at position 1,530 by the 1B promoter and terminate at the 1A termination signal, if the viral DNA fragments are integrated in a tandem array. In the nuclease S1 gel analysis with the *Hind*III-G fragment (Fig. 2A, lane 5), a 1,480-nucleotide-long band was observed in the neutral gel, which cannot be mapped on the Ad12 DNA in the previous section. This species could be explained by the second possibility assumed above, comprising the 560- and 840-nucleotide-long cotranscripts. It could then splice with the 310-nucleotide-long cotranscript to be the 1,710-nucleotide-long mRNA. Such chimeric molecules of viral sequences have been identified in cells transformed by a DNA fragment of Ad7 (33).

DISCUSSION

We have defined the structure of Ad12-spe-

cific mRNA's encoded in the transforming region by the methods of Berk and Sharp (3, 4) and Alwine et al. (1). The structures of these mRNA's as detected in transformed cell lines carrying only transforming DNA segments (CY1-1 and GY1-1) and in cells early and late after lytic infection with Ad12 are summarized in Fig. 1E (CY1-1), 2B (GY1-1), and 3D (early and late). In these figures, the genome sequences represented in an mRNA are depicted by a line above the genome map of Ad12. Intervening sequences between continuously transcribed sequences (cotranscripts), which are spliced out in the mature mRNA, are shown by a caret symbol. Abundant mRNA's are represented by bold lines, and minor species present at lower concentrations are represented by thin lines. We can find specific sequences in the close vicinity of initiation, splicing, and termination sites of these mRNA species on the nucleotide sequences of the *Hind*III-G fragment (2,320-nucleotide-long) of Ad12 DNA (Fujinaga et al., in press; Sugisaki et al., in press), as summarized in Fig. 6.

In cells lytically infected with Ad12, four 1A mRNA's (RNAs I to IV) were found to a similar extent. These are generated by combinations of two initiation sites and two splicings as summarized in Fig. 3D. The presence of two promoter sites was supported by our preliminary experiments using exonuclease VII (kindly provided by J. W. Chase, Albert Einstein College of Medicine). In CY1-1 and GY1-1 cell lines, three of the four 1A mRNA's were found (RNAs I, III, and IV). There are sequences corresponding to the Hogness box (19) for these mRNA's: TAT-TATA at position 276 and TATTTAA at position 414 (numbers in nucleotides from the left terminus of the viral genome). Assumed cap sites are found at positions 306 and 445, 30 and 31 nucleotides downstream from the signal sequences, respectively (Fig. 6). The RNA map-

FIG. 3. D

FIG. 4. Diagrams and autoradiograms of two-dimensional analyses of nuclease S1-digested RNA-DNA hybrids. The AccI-H fragment was hybridized with polyadenylic acid-containing cytoplasmic RNAs from CY1-1 cells (A), infected early cells (B), and GY1-1 cells (C). The first electrophoresis was from left to right in a neutral gel, and the second was from top to bottom in an alkaline gel. The numbers to the left of the dots represent the lengths of corresponding cotranscripts in nucleotides. Cotranscripts derived from a given mRNA species fall on the respective vertical line.

ping data on the initiation sites described in this study are in good agreement with these two signals, which are separated by 138 nucleotides. There is another Hogness box-like sequence TATTTA at position 292, but this is not assumed here to be the signal because it is only 122 nucleotides upstream from the expected Hogness box at position 414. There are possible donor site sequences (22) TT/GTAAGT at position 975 and CA/GTAAGT at position 1,068 and an acceptor site sequence (22) TTTTAG/GT at position 1,138. In the vicinity of an expected termination site for 1A mRNA's, a sequence AATAAA (20) is also present at position 1,436 (Fig. 6).

In cells lytically infected by Ad2 (4), Ad5 (2), and Ad7 (33), only two major species are detected for 1A mRNA's. The third 1A species is detected in an appreciable amount in Ad2-transformed cells (J. Sambrook, R. Green, J. Stringer, T. Mitchison, S. L. Hu, and M. Botchan, Cold Spring Harbor Symp. Quant. Biol., in press). Ad12 seems to be in a somewhat different situation from the above three serotypes. We typically detected four major 1A mRNA's, but in some cases two species initiated by the preceding Hogness box (RNAs I and II) decreased their relative abundance in cytoplasmic RNA preparations (Y. Sawada, unpublished data). Only the remaining two species (RNAs III and IV) were detected by Segawa et al. (K. Segawa, I. Saito, K. Shiroki, and H. Shimojo, Virology, in press). It is possible that there are unknown controls on the synthesis or processing of the Ad12 1A transcripts in lytically infected cells. There should also exist different controls on the expression of 1A mRNA's among lytically infected cells and transformed cell lines described in this study.

There are distinct features to the expression of 1B mRNA among lytically infected cells and transformed cell lines. In CY1-1 cells, one long species of about 2,200 nucleotides with a small splice (RNA V) was detected in prominent abundance, and mapped approximately at position 1,530 to position 3,820. The relevant Hogness box TATAAA is seen at position 1,498 and the

FIG. 5. DBM paper blot hybridization of mRNA's from CY1-1, GY1-1, and infected early cells. Polyadenylic acid-containing cytoplasmic RNAs were purified from CY1-1, GY1-1, and KB cells early after lytic infection with Ad12 (in the presence of 20 μ g of 1- β -D-arabinofuranosylcytosine per ml of culture medium). RNA blots prepared as described in the text were hybridized with 32 P-labeled DNA probes specific to early regions 1A (AccI-H) and 1B (AccI-J) of Ad12 and autoradiographed.

FIG. 6. Diagrams of the transcription map and predicted signal sequences in the *HindIII*-G fragment. All of the main mRNA species, except chimeric ones, detected in CY1-1, GY1-1, and infected KB cells are presented in the upper diagram. Numbers above the lines represent RNA lengths in nucleotides. Caret symbols represent the splicing of primary transcripts. The 3' ends of RNAs are indicated by arrowheads. In the lower diagram, predicted regions composing mRNA's are indicated by hatched and filled lines. Numbers above the lines represent nucleotide positions of assigned initiation and termination codons, splice sites, and signal sequences for transcription start and stop. Their sequences are also shown below the lines. Numbers within parentheses show the assumed cap sites.

presumed cap site is seen at position 1,527 on the nucleotide sequences of the Ad12 *HindIII*-G fragment (Fig. 6). This mRNA corresponds to 1B mRNA's detected in cells lytically infected with Ad2 (4), Ad5 (2), and Ad7 (33) and in a cell line transformed by Ad2 (Sambrook et al., in press). However, no appreciable amounts of mRNA was found in cells early after lytic infection with Ad12, by either nuclease S1 gel analysis or DBM paper blot hybridization (this study; Segawa et al., in press). The long 1B mRNA was not clearly found in this study even at the late stage, although it was detected as a minor species in some experiments (data not shown). Instead, two 1B mRNA species, one of about 980 nucleotides in length (530 plus 450) with splicing (RNA VI) and another of about 450 nucleotides in length, were found at the late stage, corresponding to the results of previous work on Ad2 mRNA's (6, 7, 28, 31). A presumed donor site for splicing of the former is AT/GTACTT at position 2,059 (Fig. 6). The latter species corresponds to protein IX mRNA identified for Ad2 (7), and could have its own promoter sequences preceding its 5' end. These species were not found in

the CY1-1 cell line, as is the case with the Ad2-transformed cell line (Sambrook et al., in press). Clearly, the expression of 1B mRNA species of Ad12 is controlled by distinct mechanisms between lytically infected cells and transformed cell lines. In addition, Ad12 is distinguished from Ad2, Ad5, or Ad7 by the 1B mRNA expression in lytically infected cells. The DNA sequences of the 1B region beyond the *HindIII*-G fragment of Ad12 are now under current investigation.

In GY1-1 cells, only a 560-nucleotide-long cotranscript was detected in the 1B region, and it mapped at about position 1,530 to position 2,090. This species is apparently initiated by the promoter at position 1,498, as is the case in CY1-1 cells and in lytically infected cells. However, no signal sequences for termination or splicing are found around position 2,090. This cotranscript could be the 5' portion of chimeric RNA species containing viral-cellular and viral-viral sequences as suggested by DBM paper blot analysis. In the former case, mRNA transcription starts by the 1B promoter, continues beyond the viral-cellular DNA junction, and terminates at an appropriate cellular signal. In the latter case,

mRNA transcription starts by the 1B promoter and terminates at the 1A signal in the adjacent viral DNA, which is integrated in a tandem array (33). The band at 1,480 nucleotides detected with the *Hind*III-G fragment in the neutral gel by the nuclease S1 gel procedure (Fig. 2A, lane 5) needs the presence of tandem integration of viral DNA in GY1-1 cells as described above. The 840-nucleotide-long cotranscript specific to GY1-1 cells should comprise the 3' portion of the chimeric RNA species, since no promoter-like sequences were found in the vicinity of its 5' end on the DNA sequence of Ad12. The DBM paper blot analysis offered evidence for the presence of at least three species of cellular-viral chimeric mRNA's. Such species were also detected in cells transformed by Ad2 (Sambrook et al., in press). In GY1-1 cells, another chimeric mRNA species containing the 840- and 950-nucleotide-long cotranscripts is suggested by nuclease S1 gel mapping (Fig. 2B). These data reflect the complicated integration of multiple copies of viral DNA in GY1-1 cells (21).

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