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Proteins Containing Only Half of the Coding Information of Early Region 1b of Adenovirus Are Functional in Human Cells Transformed with the Herpes Simplex Virus Type 1 Thymidine Kinase Gene and Adenovirus Type 2 DNA

DANIEL F. KLESSIG,[1,2]†* MARGARET P. QUINLAN,[2]‡ AND TERRI GRODZICKER[2]

Department of Cellular, Viral and Molecular Biology, University of Utah, Salt Lake City, Utah 84132,[1] and Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724[2]

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We introduced into tk^- human 143 cells adenovirus type 2 (Ad2) genes by transformation with a plasmid (p711) containing both Ad2 sequences and the herpes simplex virus type 1 (HSV-1) *tk* gene. p711 contained approximately the left 8% of the Ad2 genome inserted in the *Hin*dIII site of pBR322, whereas the fragment of HSV-1 containing the *tk* gene was inserted in the *Bam*HI site. Three tk^+ cell lines were isolated after selection in HAT medium. The arrangement of viral sequences in the three transformants was analyzed by restriction endonuclease digestion and filter hybridization. All three lines contained a single insertion of Ad2 DNA which was present at approximately one copy per cell. The arrangement of Ad2 sequences in these lines was identical to that found in the linear p711 DNA used in the transformation. S1 analysis of the Ad2-specified RNA from two of these lines indicated that the early region 1a mRNA's were synthesized, though in lower amounts than found in lytic infections. These cell lines contained only the left half of early region 1b (4.6 to 11.2), which encoded the 5′ portion of the 1b mRNA's. A complex pattern of 1b RNAs was made in these cell lines. Transcription of most of these RNAs began at or near the 1b promoter and proceeded through the 1b sequences into the flanking pBR322, HSV-1, or host sequences. Since many of the RNAs were terminated or spliced in the HSV-1 (anti-sense strand) or pBR322 sequences, new RNA processing sites must be used in the formation of these mRNA's. All three lines fully complemented the 1a deletion mutant Ad5 *dl*312. Surprisingly, these lines also permitted the growth of 1b deletion mutants (Ad5 *dl*313 and Ad5 *dl*434), although the complementation was not always complete. Presumably the new gene product(s) which contained only part of the 1b gene provided most of the essential function(s) required for viral multiplication. Alternatively, the 1b 19-kilodalton protein which was entirely encoded by the adenovirus sequences present in these cell lines was sufficient for viral growth even in the absence of the 1b 55-kilodalton protein.

Specific pieces of foreign DNA can be introduced into mammalian cells by a variety of methods, including cotransformation, viral vectors, and microinjection with glass capillaries, erythrocyte ghosts, or liposomes (34). When mouse L tk^- cells are transfected with the herpes simplex virus type 1 (HSV-1) thymidine kinase (*tk*) gene and a variety of other unlinked DNAs (cotransformation), many of the resulting tk^+ transformants also incorporate the unlinked DNA (49). Thus, φX174, pBR322, simian virus 40 (SV40), and rabbit or human β-globin sequence have been introduced into mouse tk^- cells by cotransformation (19, 35, 49, 50). We have shown that left-end segments of the human adenovirus type 2 (Ad2) genome can also be introduced into permissive tk^- human cells by this method (27). The unselected DNA can also be ligated to the selectable *tk* gene before transfection. By using this approach, rabbit β-globin, chick ovalbumin, and SV40 sequences have been introduced into mouse tk^- L cells (6, 19, 28, 32).

We have adopted this latter approach to introduce different adenovirus early genes into permissive human cells. Early region 1a and 1b genes encoded at the left end of the adenovirus genome are involved in DNA replication and

† Present address: Department of Cellular, Viral and Molecular Biology, University of Utah, Salt Lake City, UT 84132.
‡ Present address: Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115.

morphological transformation. In addition, one or more of the 1a gene products are required for efficient expression of the other early regions (2, 21, 30). As a source of the 1a gene which can be stably introduced into permissive cells, we have inserted the HSV-1 *Bam*HI fragment containing the *tk* gene into the plasmid F4/41 (40) to form plasmid p711. F4/41 contains approximately the left 8% of the Ad2 genome, which includes all of 1a as well as half of the 1b gene. To test the biological activity of p711, we have inserted it into human tk^- 143 cells and selected for expression of the HSV-1 *tk* gene.

MATERIALS AND METHODS

Cells and viruses. The 143 tk^- human cell line, derived after selection with bromodeoxyuridine (K. Huebner and C. Croce, personal communication) from the murine sarcoma virus-transformed line R970-5 (38), was a gift from C. Croce. The 293 cell line was derived by morphological transformation of human embryonic kidney cells with sheared Ad5 DNA (14). The 293 and HeLa cell lines were provided by J. F. Williams. All cell lines were cultivated in Dulbecco modified Eagle medium (Microbiological Associates) supplemented with 10% calf serum, 100 μg of streptomycin per ml, and 100 μg of penicillin per ml. tk^+ transformants were selected and grown in Dulbecco modified Eagle medium containing hypoxanthine (15 μg/ml), aminopterin (0.5 μg/ml), and thymidine (5 μg/ml) (HAT) and 10% calf serum, streptomycin, and penicillin.

Ad2 was propagated on monolayers of HeLa cells. Ad5 *dl*312, Ad5 *dl*313 (both provided by T. Shenk) and Ad5 *dl*434 (provided by D. Solnick) were grown on monolayers of 293 cells. Plaque titrations were performed as described previously (16), except that for 293 cells $MgCl_2$ was omitted from the overlay medium. For measurement of virus yields from various cell lines, confluent monolayers were infected at a multiplicity of infection of 10 PFU/cell except for *dl*313 (0.5 PFU/cell) in phosphate-buffered saline. After 75 min of incubation at 38.5°C, the inoculum was removed, and the monolayer was rinsed with phosphate-buffered saline and incubated in Dulbecco modified Eagle medium or HAT for 48 h. Progeny virus were released from infected cells by freeze-thawing and sonication.

Cell transformation. 143 cells were transformed by the procedure described by Wigler et al. (47), a modification of the DNA/calcium phosphate coprecipitation method (15). Dishes (90 mm) of 143 cells at 10% confluence were transfected with the following DNAs per plate: 30 ng of p711 linearized with *Sal*I and 15 μg of 143 tk^- cell carrier DNA. Transformed cells were selected for acquisition of the HSV-1 *tk* gene (1, 31, 33, 48) by HAT selection (44). Individual colonies were isolated by using cloning cylinders and grown into mass cultures. All experiments using recombinant DNA were performed in accordance with the National Institutes of Health guidelines.

DNA preparation. Adenovirus DNA was prepared according to the method of Pettersson and Sambrook (35). DNA from tissue culture cells was prepared as described by Wigler et al. (49). ptk2, a pBR322 plasmid carrying a 3.6-kilobase (kb) HSV-1 *Bam*HI fragment that contains the herpes thymidine kinase gene, was obtained from D. Hanahan. F4/41, provided by J. Sambrook (40), is a recombinant pBR322 plasmid that contains Ad2 sequences obtained from the morphologically transformed rat cell line F4. The viral sequences inserted at the pBR322 *Hin*dIII site in F4/41 include Ad2 sequences from 0 to 7.9% attached at their left end to a small inverted segment from the right end (97.1 to 96.0). Plasmid DNA was prepared according to the method of Tanaka and Weisblum (45). Restriction enzyme-generated fragments of viral DNA were fractionated on horizontal 1% agarose gels in Tris-borate buffer (90 mM Tris, 90 mM borate, and 2.5 mM EDTA, pH 8.3) by electrophoresis at a potential of 1 to 2 V/cm for 16 to 36 h. The separated fragments were eluted from the gel by electrophoresis (27).

Filter hybridization. Fifteen micrograms of high-molecular-weight DNA extracted from transformed cells was digested with 50 to 70 U of the different restriction endonucleases indicated (New England BioLabs or Bethesda Research Laboratories) in 100 to 150 μl for 3 to 7 h at 37°C. Forty picograms of Ad2 or 10 pg of p711 was digested with appropriate restriction endonucleases in the presence of 15 μg of calf thymus DNA to generate marker fragments. This mixture contains the equivalent of one-half copy of the viral or plasmid genome per diploid cell or about one copy per 143 cell, which contains ~80 chromosomes. The digestion products were fractionated by electrophoresis at 1.2 to 2 V/cm for 15 to 20 h on horizontal 0.8, 1, or 1.2% agarose gels (14 by 16 by 0.3 cm) in 36 mM Tris, 30 mM NaH_2PO_4, and 1 mM EDTA. The DNA fragments were transferred to a nitrocellulose filter by a modification of the Southern blotting technique (41). After staining in 0.5 μg of ethidium bromide per ml, the gel was treated for 90 to 120 min in 200 to 400 ml of 0.2 M NaOH and 0.6 M NaCl, rinsed in water, soaked for 60 to 90 min in 200 to 400 ml of 1 M Tris, pH 7.4, and 0.6 M NaCl, and washed for 5 to 15 min in 6× SSC (SSC = 0.15 M NaCl plus 0.015 sodium citrate) before transfer to nitrocellulose filters in 6× SSC for 36 to 72 h. Before hybridization, the filter was treated in 25 ml of 6× SSC, 10× Denhardt reagent (10), 1 mM EDTA, and 10 to 25 μg of denatured calf thymus DNA per ml for 6 to 16 h at 68 to 71°C, at which time sodium dodecyl sulfate (SDS) was added to a final concentration of 0.25 to 0.5%, and the incubation was continued for an additional 1 to 2 h at 68 to 71°C. The filter was hybridized to ~50 ng of ^{32}P-labeled probe DNA (specific activity, 1×10^8 to 5×10^8 cpm/μg) in 5 ml of 6× SSC, 5× Denhardt reagent, 100 μg of denatured calf thymus DNA per ml, 10 μg of denatured human 143 tk^- cell DNA per ml, 1 mM EDTA, and 0.5% SDS at 68 to 71°C for 14 to 24 h; it was then washed three times for 5 to 20 min each in ~250 ml of 2× SSC and 0.1% SDS at room temperature and three times for 45 to 60 min each in ~250 ml of 0.1× SSC and 0.1% SDS at 50 to 53°C. Filters were exposed to Kodak XR film, using du Pont Lightning Plus intensifying screens, for 1 to 4 days.

RNA analysis. Cytoplasmic RNA was prepared from the 143 cell line and its transformed derivatives as previously described (26). Cytoplasmic RNA extracted at 6 h postinfection from HeLa cells infected with 25 PFU of Ad2 per cell in the presence of 10 μM anisomycin was provided by J. Lewis. Polyadenylated RNA was selected on oligodeoxythymidine-cellulose

columns. The structure of the adenoviral RNA was determined by using a modification of the S1 nuclease mapping technique of Berk and Sharp (3). Polyadenylated RNA selected from 1 mg of total cytoplasmic RNA obtained from 143 tk^- or tk^+ transformants was hybridized to Ad2 fragments in 50 μl. The amount of the fragments was equivalent on a mole basis to 0.5 μg of the entire Ad2 genome, which is approximately 35.5 kb in length. In several experiments, an additional 100 μg of unselected cytoplasmic RNA was included. Cytoplasmic RNA (16 to 32 μg) isolated during the early phase of Ad2 infection was used as a positive control. The adenovirus sequence from the *Hin*dIII-cleaved p711 was not purified before hybridization to RNA. After S1 treatment, the sample was divided into two and fractionated on an alkaline 1.2% agarose gel and a neutral 1.4% agarose gel by electrophoresis. Neutral gels were electrophoresed in Tris-borate buffer. The fractionated DNA was transferred to a nitrocellulose filter and hybridized to ^{32}P-labeled Ad2 DNA (40 ng per filter; specific activity, 1×10^8 to 10×10^8 cpm/μg) as described above, except that the alkaline gel was treated in 1 M Tris (pH 7.4) and 0.6 M NaCl for 45 to 75 min while the neutral gel was exposed to 0.2 M NaOH and 0.6 M NaCl for 45 min and then to 1 M Tris (pH 7.4) and 0.6 M NaCl for 45 to 75 min. Filters were exposed to XR film for 2 to 10 days with intensifying screens.

RESULTS

The tk^- cell line 143 was transformed with the chimeric plasmid p711 to construct permissive human cell lines containing early region 1a of the adenovirus genome. Figure 1 shows the structure of p711. It was constructed by insertion of a 3.6-kb HSV-1 *Bam*HI fragment containing the *tk* gene into F4/41 in the orientation shown. The plasmid, F4/41, contains approximately the left 7.9% of the Ad2 genome on a *Hin*dIII fragment which was derived from the Ad2-transformed rat cell line F4 (40). p711 contains the entire 1a gene as well as half of the 1b gene which encodes the 5′ portion of the 1b mRNA's. After selection and growth in HAT medium, tk^+ colonies were obtained. Four colonies were picked and expanded into cell lines (the D lines).

Amount and arrangement of adenovirus sequences in the tk^+ transformants. The adenovirus sequences in the tk^+ cell lines were analyzed by restriction enzyme digestion and filter hybridization (41). Cell DNAs from the four lines were digested with *Bam*HI, an endonuclease which does not cut within the adenovirus segment contained in p711 (~0 to 7.9). Analysis of the *Bam*HI digestion products by electrophoresis, transfer, and filter hybridization using nick-translated Ad2 *Eco*RI-A (0 to 58.5%) as probe showed that three lines had a single insertion of adenovirus sequence (Fig. 2). The faint, diffuse, low-molecular-weight bands seen in Fig. 2 was observed only sometimes; we do not know if they represented true Ad2 sequences or were the result of spurious hybridization. The D7 cell line did not contain left-end sequences. In two of the three lines, it is clear that the fragment containing adenovirus sequences and adjoining host DNA was larger than the DNA used for transformation. We shall use the term "host DNA" to refer to the sequences connected to the chimeric plasmic insertions in the transformed lines. We also do not know whether these flanking sequences were other DNAs that were present in the transfection mixture or whether the chimeric plasmid sequences were integrated into a host chromosome. The intensity of the bands compared with the reconstruction indicates that the adenovirus sequences were present in approximately one copy per cell.

Extent of viral sequences present in tk^+ transformants. The adenovirus sequences in the three D cell lines were mapped by cleaving cell DNA with combinations of restriction endonucleases that cut several times within p711 and determining which restriction fragments comigrated with the corresponding plasmid (p711) fragments. Cell DNA was cleaved with various combinations of *Hin*dIII, *Bgl*II, *Sma*I, and *Kpn*I and analyzed by filter hybridization, using labeled Ad2 *Hin*dIII-G+F (0 to 7.9 and 89.5 to 97.1) DNA as probe (Fig. 3). The right-hand tracks in each panel of Fig. 3 are reconstructions that show the marker p711 fragments from each digestion that hybridized to the probe.

All three lines contained the same arrangement of Ad2 sequences present in the transfecting p711 plasmid (Fig. 3A, B). One cell line, D10, was analyzed by using four combinations of restriction enzymes. The results shown in panel C confirm that the Ad2 sequences in this line had a structure identical to that shown in p711 in Fig. 1.

To determine whether the Ad2 sequences were still attached to the HSV-1 *Bam*HI segment in the three D lines, the cellular DNAs were cleaved with *Bgl*II. *Bgl*II cleaved p711 near the distal end of both the Ad2 and HSV-1 inserts to produce two fragments (see Fig. 1); one contained primarily pBR322 sequences, and the other harbored almost the entire Ad2 and HSV-1 sequences joined by a short segment of pBR322. The Ad2 and HSV-1 sequences maintained this same arrangement in all three lines (Fig. 3D). In fact, further analysis indicated that the inserted sequences extended as far as the distal *Eco*RI sites in pBR322 and HSV-1 for all three lines, whereas in one line, D10, the chimeric plasmid DNA was intact through the distal *Bam*HI site (data not shown; see Fig. 1).

Viral RNAs in the transformed cell lines. Because these transformed lines have the entire 1a gene and only half of the 1b gene, we looked at the Ad2-specified RNAs. The amount and

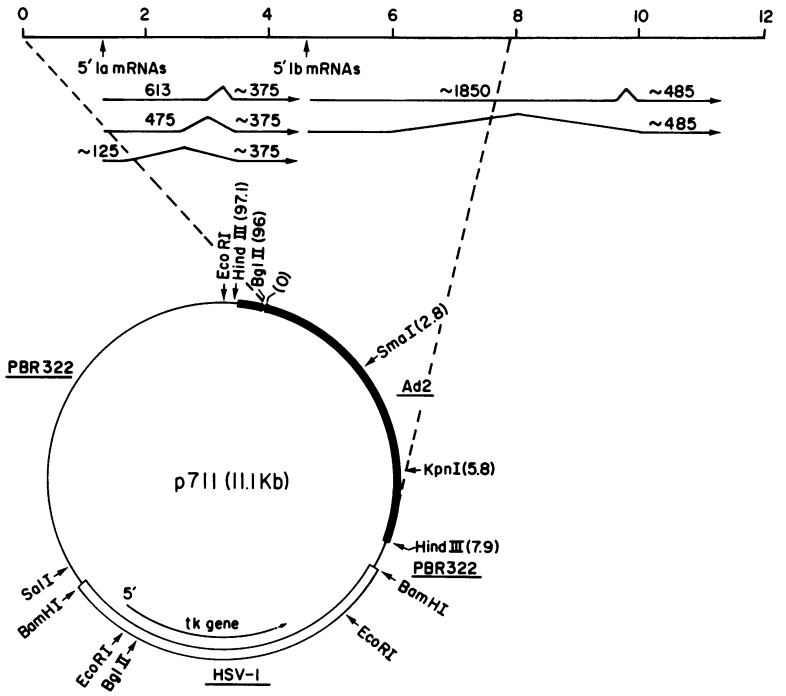


FIG. 1. Structure of the chimeric plasmid 711. The left 12% of the Ad2 genome is represented as a scale at the top with each segment corresponding to 1 map unit. The structure of the early region 1a and 1b mRNA's made in lytically infected cells are shown below the scale, with ⌒ denoting regions spliced out of the transcripts. The adenovirus sequences in the chimeric plasmid p711 are shown as a solid black segment; the HSV-1 3.6-kb fragment containing the *tk* gene is represented by the white segments in the circular diagram. pBR322 sequences are indicated by a single line. The position of several restriction endonuclease cleavage sites are indicated by arrows, with their map coordinates on the conventional Ad2 physical map given in parentheses. The lines inside the circles indicate the approximate position and direction of transcription of the *tk* mRNA (9, 11, 46).

structure of 1a and 1b mRNA's present in these cells was determined by using a modification of the S1 nuclease mapping technique of Berk and Sharp (3). To detect the low concentration of viral RNAs in these lines, RNA from the transformed cells was first annealed to unlabeled fragments of p711 or Ad2 DNA. The RNA-DNA duplexes were treated with S1, fractionated on either neutral or alkaline agarose gels, transferred to nitrocellulose filters, and hybridized to labeled Ad2 DNA (17).

Cytoplasmic, polyadenylated RNAs from the D10 and D27 cell lines were hybridized to several restriction fragments containing adenovirus DNA: *Hin*dIII-cleaved p711 (coordinates ~0 to 7.9); *Xba*I-*Xho*I-cleaved Ad2 (3.8 to 15.5); and *Sal*I-cut linear p711 DNA. Figure 4 shows the results of S1 mapping using these fragments as well as a graphic representation of the viral RNAs detected.

1a mRNA's. Two major 1a mRNA's have been observed in the early phase of adenovirus infection by the S1 mapping procedure (4). These two RNAs have common 5' (1.3) and 3' (4.4) termini but have different-size internal intervening sequences which are removed by splicing (see Fig. 1) (4, 8, 25, 42). The 625(630)-, 490(445)-, and 407-nucleotide (n) bands seen on alkaline gels (Fig. 4, panels I and IV) and the 950n(990) and 810n(820) bands observed on neutral gels (panels II and V) using p711 DNA as probe correspond to these two RNAs. Both of these 1a mRNA's, labeled RNA no. 1 and 2 in the graphic display at the bottom of Fig. 4, were present in the D10 and D27 cell lines; however, their concentration was considerably lower than the 700 to 1,200 copies per cell observed in early lytic infection (12). A comparison of the band intensities obtained by using ~16 (panels I and II) or ~32 (panels IV and V) times more RNA from the transformed cells than from early lytically infected cells suggested that the viral RNA concentrations were ~25-fold lower in D10 and D27 cells.

The 600n band seen with all three RNA preparations in panel V may correspond to a third 1a mRNA which has been mapped only by electron

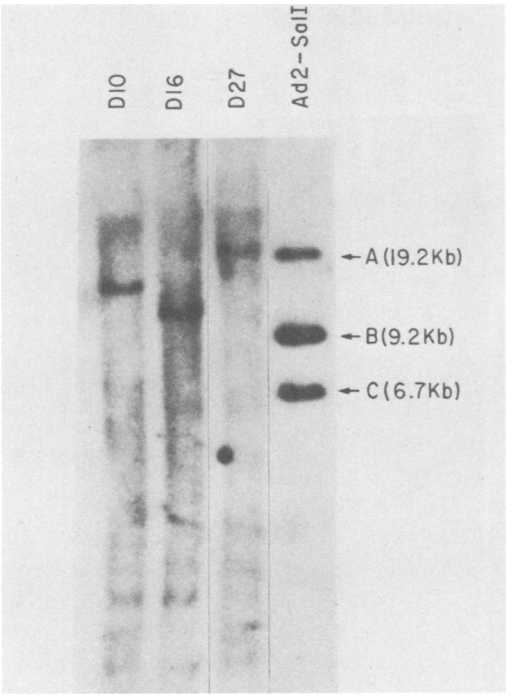


FIG. 2. Arrangement and number of adenovirus insertions in the tk^+-transformed cell lines. High-molecular-weight DNA from the cell lines indicated above each track was digested with *Bam*HI, fractionated on a 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized to a ^{32}P-labeled Ad2 *Eco*RI-A DNA fragment (0 to 58.5 map coordinates; see Materials and Methods). The right track is a reconstruction containing viral DNA equivalent to one copy per tetraploid cell. These cell lines as well as the parental 143 tk^- are subtetraploid. The sizes and notation of the Ad2 fragments generated by *Sal*I are indicated at the right.

microscopy (8). The probable structure of this RNA (no. 3) is shown in the lower portion of Fig. 4.

1b mRNA's. The early region 1b, like 1a, encodes two early mRNA's which share common 5′ (4.6) and 3′ (11.2) termini but have internal segments of different sizes which are removed by splicing (see Fig. 1) (4, 8, 25). Only the large 22S species produced by splicing out the small region corresponding to 9.7 to 9.9% has been observed by S1 mapping (2, 3). S1 digestion of D10 and D27 mRNA hybridized to *Hin*dIII-cut p711 (~0 to 7.9) or to a DNA fragment containing Ad2 sequences from positions 3.8 to 15.5 yielded a 1,055 to 1,130n band on both alkaline and neutral gels (panels I, II, and III in Fig. 4). This is the size expected (1,100n from DNA sequence analysis; R. Roberts and T. Gingeras, personal communication) for an RNA starting at the 1b promoter and continuing through the 1b sequences into flanking DNA. RNA from the lytically infected cells yielded the same 1,065n (1,130) band when hybridized to the *Hin*dIII fragment. As expected, RNA from lytically infected cells when annealed to a fragment that contained the entire 1b region (3.8 to 15.5) produced 1,800n and 480n bands on alkaline gels (panel III). These bands corresponded to the 22S 1b RNA. A comparison of the band intensities observed by using ~16-fold more RNA from the transformants suggested that the amount of 1b mRNA in these two lines is ~25-fold lower than in early lytic infections when 800 to 1,100 copies are present per cell (12).

Because the 1b segment in the D10 and D27 cell lines did not contain the splice sites or the polyadenylation site for the 22S 1b mRNA, we further analyzed the structure of the 1b-containing transcripts. D10 and D27 RNAs were hybridized to the same structure of p711 DNA (*Sal*I cleaved) used in the transformation. The duplexes were then digested with S1 and analyzed on neutral and alkaline gels. A complex pattern of 1b mRNA's was synthesized in these cells (Fig. 4, panels IV and V). A graphic representation of the RNAs is depicted in the lower portion of Fig. 4. Possible structures of these RNAs were deduced from the size and intensity of the bands on alkaline and neutral gels. The structures of most of these RNAs were compatible with the RNAs starting at the 1b promoter. Although there were other structures consistent with the data, we assume that one or more of the RNAs must be transcribed from the sense strand of the 1b gene because these cell lines complemented 1b deletion mutants (see below) and thus made partially functional proteins.

The D10 cell line produced one prominent mRNA that contained 1b sequences. A 1,300n band was seen on both alkaline and neutral gels. This RNA (no. 4) was probably initiated at the 1b promoter and continued ~1,300n through the 1b region into pBR322 sequences before being terminated or spliced into flanking host sequences. We suspect that the RNA was spliced rather than terminated at this position because the hexanucleotide sequence AATAAA which precedes the polyadenylation site by approximately 25n on most eucaryotic mRNA's (37) was not present in this region of pBR322. Although the sequence GT appeared several times in this region of pBR322 (43), no further similarities to the donor splice site consensus sequence were observed (5, 7).

Three prominent mRNA's containing 1b sequences were found in the D27 cell line. One of these RNAs produced a ~5,100n band on both neutral and alkaline gels. This finding is consistent with it (no. 5) being initiated at the 1b

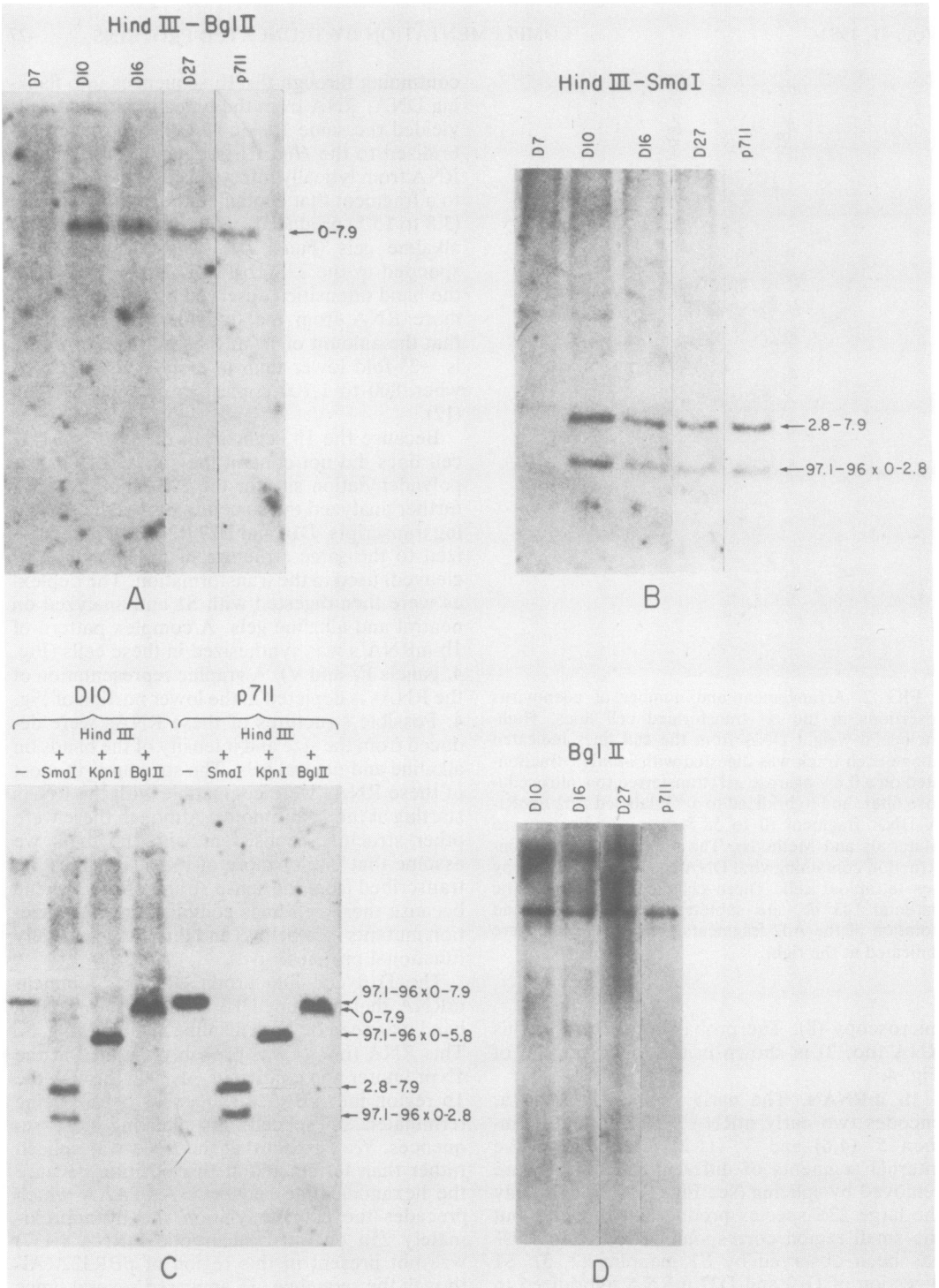


FIG. 3. Mapping of the viral sequences in the tk^+-transformed cell lines. In panels A, B, and D, high-molecular-weight DNA from each cell line indicated above each track was digested with the restriction endonucleases shown above the panel. In panel C, the D10 and p711 DNAs were digested with the *Hin*dIII plus the restriction endonuclease indicated above each track. The cleaved DNA was fractionated on 1 or 1.2% agarose gels, transferred to nitrocellulose filters, and hybridized to nick-translated *Ad*2 *Hin*dIII-G+F DNA (0 to 7.9 and 89.5 to 97.1 map coordinates, respectively; see Materials and Methods). The right track in panels A, B, and D is a reconstruction containing p711 DNA equivalent to one copy per tetraploid cell. The map coordinates on the Ad2 genome of each band are shown at the right. The positions of the cleavage sites on p711 are shown in Fig. 1.

promoter and extending through the remainder of the inserted DNA before terminating in flanking host sequences. The remaining two major RNAs (no. 6 and 7) were spliced within p711 sequences and yielded the ~2,750 and ~2,500 bands seen in panel IV of Fig. 4. These two RNAs on the neutral gel probably gave rise to the protected ~6,200n fragment. In both cases, it is likely that transcription initiated upstream from the 1b promoter. If the transcripts were initiated at the 1b promoter and transcribed through the entire linearized plasmid DNA, the longest RNA formed would be only ~5,300n. Assuming that transcription started upstream from the 1b promoter, a band larger than 1,100n should be observed when RNA is hybridized to *Hin*dIII-cleaved p711 or the 3.8 to 15.5% Ad2 fragment. Panel I shows a very faint band of 1,590n. If the left end of the Ad2-specified, alkaline-resistant segment of the 6,200n band (bands B and C in panel IV) were located 1,590 base pairs upstream from the 1b-pBR322 border, it would map near the acceptor splice site for 1a mRNA's. Thus, it is probable that RNA no. 6 and 7 shown in the bottom of Fig. 4 are initiated at the 1a promoter and use the 1a splice sites, but fail to utilize the 1a termination and polyadenylation sites.

Several other minor RNA species were also found in these two cell lines. Possible structures for some of these RNAs are illustrated at the bottom of Fig. 4. A comparison of the intensities of these minor bands with those of the bands generated from the 1a mRNA's suggests that these RNAs were present in only a few copies per cell.

Complementation of viral mutants in transformants. To see whether the left-end Ad2 DNA in the D cell lines produced functional proteins, the lines were tested for their ability to complement the growth of adenovirus mutants. The Ad5 deletion mutant *dl*312 is missing the sequences that mapped between 1.2 and 3.7 on the viral genome and thus lacks early region 1a (1.3 to 4.4). It was isolated as a host range mutant that failed to grow on HeLa cells but did replicate in the 293 cell line, which contains and expresses the left 11% of Ad5 (14). It also grows in tk^+ cotransformed human cell lines that contain the entire left end of the viral genome (17). Early region 1a was present in its entirety and was transcribed in the D cell lines. As expected, all three lines supported the efficient growth of this mutant. The level of complementation was similar to that observed with 293 cells, whereas HeLa, the parental 143 tk^- and D7 tk^+ cell lines (which did not contain Ad2 left end sequences) failed to support replication of the mutants (Table 1). The virus progeny obtained after infection of the three transformants retained their mutant phenotype, demonstrating that they arose by complementation and not recombination with viral sequences present in the cell (data not shown).

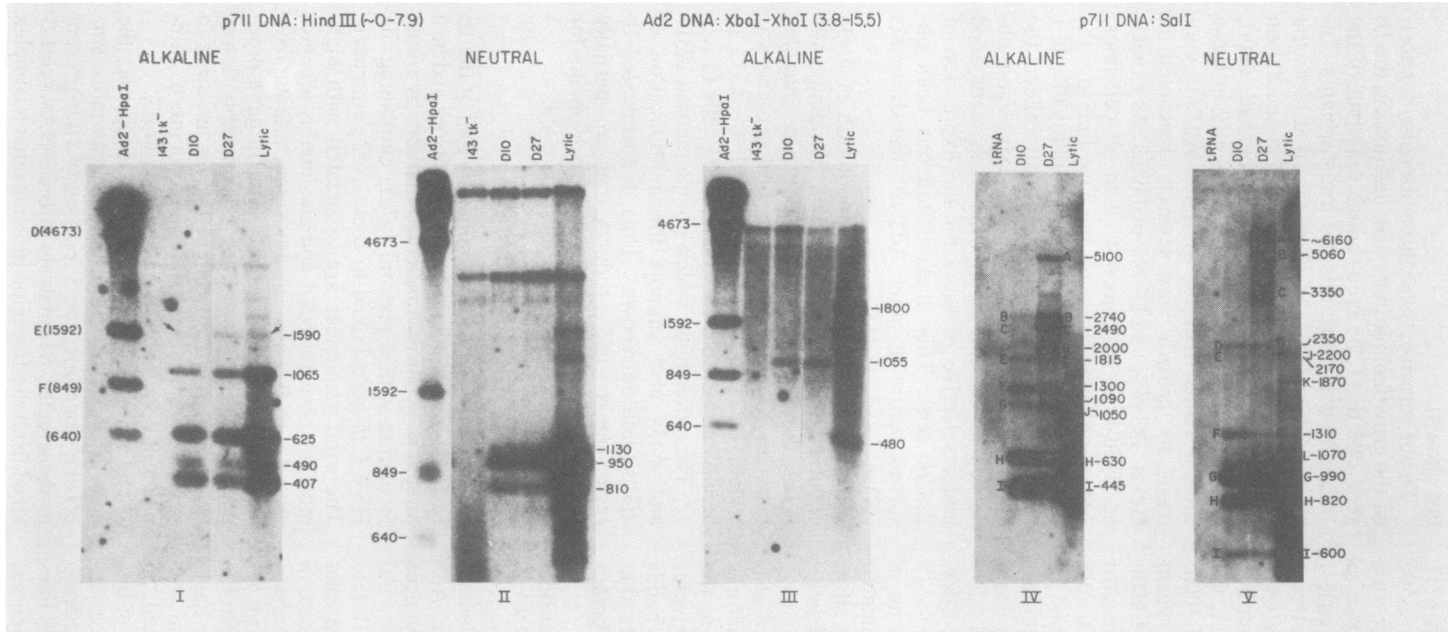
To our surprise, these three lines also supported the growth of mutants with deletions in the 1b gene (4.6 to 11.2), even though only half of the 1b region was present in the transformants (Table 1). Ad5 *dl*434, a 1a-1b mutant that lacks the viral sequences located between 2.6 and 8.7 on the genome (D. Solnick, unpublished data), grew only 8- to 10-fold less efficiently in these transformants than did wild-type Ad2. In contrast, its growth was severely restricted in HeLa, 143 tk^-, and D7 tk^+ cell lines. As expected, wild-type Ad2 grew well in all cell lines. After continued passage, the D16 line lost the ability to complement left-end mutants (Table 1 and data not shown). This probably reflects the instability of adenovirus gene expression observed in this line even when it is maintained under selective pressure for the expression of the *tk* gene. This phenomenon has been observed before in other Ad2 tk^+ transformants (17).

Since only partial complementation of *dl*434 occurred in these lines, a second 1b mutant, Ad5 *dl*313, that lacked the viral sequences mapping between 3.8 and 10.2 on the viral genome was tested. D27 supported the growth of *dl*313 to levels only 10-fold less than in 293 cells (Table 1). The other lines which complemented *dl*312 and *dl*434 also support the multiplication of *dl*313 (data not shown). Thus, the truncated 1b region must yield products that are partially functional and can supply the requirements for growth of the mutants in the lytic cycle.

DISCUSSION

The entire 1a and approximately half of the 1b early regions of adenovirus were introduced into human tk^- cells as unselected markers by transformation with a plasmid (p711) containing the HSV-1 *tk* gene linked to these Ad2 sequences. The viral sequences are associated with high-molecular-weight DNA and presumably are integrated into the host chromosome, although we have no direct proof of this. The 1a gene products, although present at considerably lower levels than in lytically infected cells, are made in sufficient quantities in the transformants to complement a 1a deletion mutant Ad5 *dl*312.

The ability of these cell lines to support the growth of 1b delection mutants was more surprising since only the 5′ portion of 1b is present in these cells. S1 analysis of the RNAs synthesized from the 1b region indicates that a complex pattern of cytoplasmic, polyadenylated RNAs is made in small quantities in the two cell lines (D10 and D27) examined. The majority of the


p7II DNA: HindIII (~0-7.9)
Ad2 DNA: XbaI-XhoI (3.8-15.5)
p7II DNA: SalI
ALKALINE
NEUTRAL
Ad2-HpaI
143 tk⁻
DIO
D27
Lytic
tRNA
D(4673)
E(1592)
F(849)
(640)
4673-
1592-
849-
640-
-1590
-1065
-625
-490
-407
-1130
-950
-810
-1800
-1055
-480
-5100
-2740
-2490
-2000
-1815
-1300
-1090
-1050
-630
-445
~6160
5060
-3350
2350
2200
2170
K-1870
-1310
L-1070
G-990
H-820
I-600
I
II
III
IV
V
Host
pBR322
97.1%
96%
0%
Ad2
2798
7.9%
0
28
375 bp
HSV-1
~3600 bp
5'Ia mRNAs
(498)
5'Ib mRNAs
(1699)
3'
5'
tk mRNA
Band designations
in panels
RNA No.
613
~375
475
~125
1310
(prominent only in DIO)
~5080
2740
~2500
2490
~2750
2000
1350
1815
530
1085
prominent
only in D27

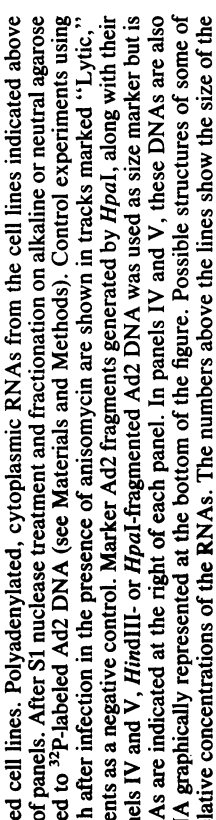


FIG. 4. S1 mapping of adenovirus RNA from the tk^+-transformed cell lines. Polyadenylated, cytoplasmic RNAs from the cell lines indicated above each track were hybridized to the DNA shown at the top of the sets of panels. After S1 nuclease treatment and fractionation on alkaline or neutral agarose gels, the DNA was transferred to nitrocellulose filters and hybridized to ^{32}P-labeled Ad2 DNA (see Materials and Methods). Control experiments using cytoplasmic early RNAs extracted from Ad2-infected HeLa cells 6 h after infection in the presence of anisomycin are shown in tracks marked "Lytic," and RNA from 143 tk^- cells or yeast tRNA was used in the experiments as a negative control. Marker Ad2 fragments generated by *Hpa*I, along with their sizes in base pairs, are indicated to the left of panels I to III; for panels IV and V, *Hin*dIII- or *Hpa*I-fragmented Ad2 DNA was used as size marker but is not shown. The sizes of DNA fragments complementary to the RNAs are indicated at the right of each panel. In panels IV and V, these DNAs are also marked with a letter which is used to denote the corresponding RNA graphically represented at the bottom of the figure. Possible structures of some of these RNAs are depicted with lines whose intensity indicates the relative concentrations of the RNAs. The numbers above the lines show the size of the RNA segment; ⌒ denotes regions spliced out of the RNAs. Arrows show the direction of transcription, and dotted lines denote that the existence or position of this segment of the RNA relative to the structure of the integrated p711 DNA in tk^+ cell lines (shown at the top of the illustration) is speculative. In the line illustration of the inserted plasmid DNA, the numbers with percentage signs for the Ad2 sequence denote the map coordinates on the conventional Ad2 physical map. The nucleotide number determined by DNA sequence analysis (Roberts and Gingeras, personal communication) is also given. Nucleotides 28 through 375 on the Sutcliffe (43) sequence of pBR322 are sandwiched between the Ad2 and HSV-1 DNA. The uncertainty concerning the site(s) on the plasmid where recombination into flanking host sequences has occurred is indicated by (⁓).

transcripts are initiated at the 1b promoter and continue through the Ad2 sequences into the flanking pBR322, HSV-1, or host sequences. Some of the mRNA's terminate or are spliced in pBR322 DNA or in the HSV-1 sequences which have an anti-sense orientation with respect to the *tk* gene. This implies that new processing (termination, cleavage, polyadenylation, or splicing) sites must be used. Examination of the nucleotide sequence near the right end of the adenovirus segment and flanking pBR322 DNA in these lines does not reveal the polyadenylation signal AATAAA, although the sequence GT appears several times and may act as a donor splice site. Use of quiescent splicing signals has previously been observed in other systems where the genome has been perturbed by genetic or bioengineering manipulation (24). Furthermore, in a polyoma *tsA*-transformed mouse cell line, the viral DNA containing the termination signal for early RNA has been lost. Transcription proceeds into and terminates in flanking mouse DNA (23).

Although nearly identical Ad2 and HSV-1 DNA sequences are present in D10 and D27, both quantitative and qualitative differences in the 1b RNAs are observed. D10 cells produce a subset of the complex pattern of partially Ad2-specified RNA seen in D27 cells. The most prominent 1b RNA in D10 contains ~1,300n encoded by p711 and is only a minor species in D27. Several major species are found in D27 cells which are either absent or present in minute quantities in D10. The segment of these transcripts encoded by p711 sequences is two to five times longer than that of the prominent D10 mRNA. These differences probably reflect different sites of integration which present alternative RNA processing sites to the 1b transcripts. The absence of a short segment of the distal part of the HSV-1 DNA in D27 may also affect mRNA synthesis.

Although the level of complementation of 1b deletion mutants of Ad5 (*dl*434 and *dl*313) in the transformants was slightly lower than that seen in 293 cells, it was considerably greater than expected for cells containing only half of the 1b region. Recent sequence determination of early region 1 of Ad5, Ad7, and Ad12 indicates that the 1b 19-kilodalton protein can be translated from the 5′ half of either of the two 1b mRNA's (Fig. 1). In contrast, the 55-kilodalton protein is encoded only on the large 22S mRNA and must utilize an internal AUG for initiation and a reading frame which differs from that of the 19-kilodalton protein (J. Bos and H. Van Ormandt, personal communication). Assuming that Ad2 has a similar arrangement, all of the information for the 19-kilodalton protein but only a portion for the 55-kilodalton protein is

TABLE 1. Complementation of adenovirus deletion mutants in *tk*-adenovirus transformed human cell lines[a]

Expt	Ad2	Virus yield (PFU/ml)		
		Ad5 *dl*312 (Δ1.3-3.7)[b]	Ad5 *dl*434 (Δ2.6-8.7)	Ad5 *dl*313 (Δ3.8-10.8)
1				
HeLa	3×10^8	3×10^6		
293	3×10^8	5×10^8		
143 *tk*$^-$	1×10^8	5×10^5		
D7	2×10^8	5×10^5		
D10	2×10^8	3×10^8		
D16	1×10^8	5×10^7		
D27	1×10^8	2×10^8		
2				
HeLa	1×10^8		5×10^5	
293	2×10^8		2×10^8	
143 *tk*$^-$	5×10^7		5×10^4	
D7	5×10^7		$<1 \times 10^3$	
D10	1.5×10^8		2×10^7	
D16	6×10^7		3×10^5[c]	
D27	6×10^7		8×10^6	
3				
HeLa	2×10^8			8×10^4
293	1×10^8			3×10^7
143 *tk*$^-$	6×10^7			8×10^4
D7	6×10^6			$<1 \times 10^4$
D27	6×10^7			4×10^6

[a] Cells were infected at a multiplicity of infection of 10 PFU/cell and incubated at 38.5°C for 48 h. However, with *dl*313 a multiplicity of infection of 0.5 PFU/cell was used. Ad5 *dl*313 shows multiplicity-dependent leakiness and grows to moderately high titers on HeLa cells at high multiplicity of infection (22).

[b] The mutants lack the DNA sequences which are given as map units on the conventional adenovirus physical map.

[c] When D16 had been carried for a short time, higher yields of *dl*434 on D16 were observed, suggesting that the expression of the Ad2 sequence was unstable in this line.

present in these transformed cell lines. Thus, either the 19-kilodalton protein provides most of the essential function(s) required for viral multiplication or the new RNA species observed in these cells must be translated into functional, novel proteins. Regardless, most if not all of the functions provided by the 1b protein(s) that is required for virus growth are encoded in the 5′ half of the mRNA(s). From preliminary analysis of the sequence of this region of the Ad2 DNA (Roberts and Gingeras, personal communication), it is likely that translation continues through the truncated 1b region present in p711 into the adjacent pBR322 sequences. We do not, however, have direct proof of this. Analysis of the DNA sequences of the short region of pBR322 adjacent to the 1b segment in p711 indicates that the presumptive open reading frame used for the synthesis of 1b-encoded proteins extends for seven sense codons beyond the Ad2-pBR322 border before reaching several closely spaced termination codons. Thus, it is likely that many of the novel, chimeric RNAs produced in these transformed lines encode a single, truncated, chimeric protein primarily specified by the 5′ half of the 1b gene.

Examples of truncated, chimeric proteins retaining some of the functions of the original gene product have been reported. There is a series of Ad2-SV40 hybrid proteins which maintain part or all of the SV40 large-T antigen functions (18, 20, 29). Furthermore, although both the 1a and 1b regions of adenovirus are required for morphological transformation of rodent cells (13, 39), Graham and colleagues (13) showed that cells could be transformed by the *Hin*dIII-G fragment of Ad5 which contains essentially the same sequence found in our *tk*$^+$ transformants, i.e., 1a and half of 1b early regions. In all of these cases, however, there was selection for the retention of particular functions of the original gene product(s). In the *tk*$^+$-transformed cells described here, no known selection was applied to maintain the 1b function(s), yet in all three lines most of the essential properties of the 1b gene products were retained.

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