JOURNAL OF VIROLOGY, Oct. 1978, p. 227-239
0022-538X/78/0028-0227$02.00/0
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Vol. 28, No. 1
Printed in U.S.A.

Transcription Map for Adenovirus Type 12 DNA

J. R. SMILEY† AND S. MAK*
Department of Biology, McMaster University, Hamilton, Ontario, Canada L8S 4K1

Received for publication 23 March 1978

The regions of the adenovirus type 12 genome which encode l- and r-strand-specific cytoplasmic RNA were mapped by the following procedure. Radioactive, intact, separated complementary strands of the viral genome were hybridized to saturating amounts of unlabeled late cytoplasmic RNA. The segments of each DNA strand complementary to the RNA were then purified by S1 nuclease digestion of the hybrids. The arrangement of the coding regions of each strand was deduced from the pattern of hybridization of these probes to unlabeled viral DNA fragments produced by digestion with *Eco*RI, *Bam*HI, and *Hin*dIII. The resulting map is similar, if not identical, to that of adenovirus type 2. The subset of the late cytoplasmic RNA sequences which are expressed at early times were located on the map by hybridizing labeled, early cytoplasmic RNA to both unlabeled DNA fragments and unlabeled complementary strands of specific fragments. Early cytoplasmic RNA hybridized to the r-strand to *Eco*RI-C and *Bam*HI-B and to the l-strand of *Bam*HI-E. Hybridization to *Bam*HI-C was also observed. The relative rates of accumulation of cytoplasmic RNA complementary to individual restriction fragments was measured at both early and late times. Early during infection, most of the viral RNA appearing in the cytoplasm was derived from the molecular ends of the genome. Later (24 to 26 h postinfection) the majority of the newly labeled cytoplasmic RNA was transcribed from DNA sequences mapping between 25 and 60 map units on the genome.

The availability of bacterial restriction endonucleases which cleave a given DNA molecule to generate a defined set of fragments has allowed considerable progress to be made in the analysis of the transcription program of the group C human adenoviruses.

The DNA sequences encoding the mRNA's synthesized during the early and late phases of productive infection, as well as those expressed in transformed cell lines, have been mapped on the adenovirus type 2 (Ad2) (2, 4, 6, 28, 31, 39) and Ad5 (5, 7) genomes. The positions and polarities of the sites complementary to early and late mRNA are similar if not identical in the two genomes, a finding not expected in view of their extensive nucleotide sequence homology (10) and similar biological characteristics.

We are interested in the analysis of the transcription of the genome of Ad12, a member of the highly oncogenic group A human adenoviruses. These viruses share very few nucleotide sequences with the group C adenoviruses (17) and differ widely in a number of biological properties from both Ad2 and Ad5. We have previously shown that early in productive infection with Ad12, RNA complementary to about 30% of the viral genome, derived from both complementary DNA strands, is detectable in the cytoplasm of infected cells (33). The fraction of the genome expressed as cytoplasmic RNA increases to about 80% at late times. The majority of the RNA sequences which appear exclusively at late times are derived from the r-strand of the genome. Further experiments showed that the early RNA sequences are a subset of those present late in infection. We report here experiments which map the physical locations and strand derivation of the DNA sequences expressed as early and late cytoplasmic RNA, using a new method. In addition, the relative rates of accumulation of cytoplasmic RNA derived from different regions of the genome were monitored at different times after infection.

† Present address: Department of Therapeutic Radiology, Radiology Laboratories, Yale University School of Medicine, New Haven, CT 06510.

MATERIALS AND METHODS

Virus and cells. Purified Ad12 strain 1131 (24) and human KB cells grown in suspension were used throughout this study. Procedures for infection and virus purification have been described (12, 21). Viral DNA was extracted from purified virions as described by Green and Pina (13).

Separation of the intact complementary Ad12 DNA strands. The complementary strands of Ad12 DNA were separated by centrifuging denatured viral

DNA to equilibrium in neutral cesium chloride density gradients in the presence of polyuridylic acid-polyguanidylic acid [poly(U,G)], as previously described (18, 33).

Material pooled from the gradients was further purified by exhaustive self-annealing at 37°C in 0.14 M sodium phosphate buffer, pH 6.8, containing 50% formamide, followed by sedimentation in 5 to 20% (wt/wt) high-salt neutral sucrose gradients (35) at 38,000 rpm for 90 min in a Beckman SW40 rotor. Intact single strands (sedimenting at 70S) were pooled, exhaustively dialyzed against 0.01 M Tris (pH 7.3) containing 2% formamide, and used for hybridization.

RNA extraction. RNA was extracted from infected cells as previously described (33). Early RNA (labeled 5.5 to 7.5 h postinfection) was prepared by concentrating infected cells to 3×10^6/ml by centrifugation and adding [^{3}H]uridine (New England Nuclear, 20 Ci/mmol) to a final concentration of 10 μCi/ml.

Restriction endonucleases and electrophoresis. Endonuclease *Eco*RI was purified by the method of Pettersson and Philipson (26). Endonucleases *Bam*HI and *Hin*dIII were purchased from Bethesda Research Laboratories. Digestion buffers were 0.1 M Tris (pH 7.9)–0.01 M $MgCl_2$ for *Eco*RI, 0.02 M Tris (pH 7.4)–0.01 M $MgCl_2$–0.06 M NaCl for *Hin*dIII and 0.02 M Tris (pH 7.4)–0.01 M $MgCl_2$–0.002 M β-mercaptoethanol for *Bam*HI. Fragments were separated by electrophoresis through 0.7% (*Eco*RI) or 1% (*Hin*dIII and *Bam*HI) agarose gels for 11 h at 2 V/cm. The DNA bands were visualized under short-wave UV illumination after staining the gels with 0.5 μg of ethidium bromide per ml.

Separation of the complementary strands of DNA fragments by electrophoresis. DNA fragments in agarose gel slices were denatured in situ and subjected to re-electrophoresis in 1.0% agarose gels as described by Horowitz (16).

Restriction endonuclease cleavage map of Ad12 (1131) DNA. The cleavage patterns of Ad12 (1131) DNA by endonucleases *Eco*RI, *Bam*HI, and *Hin*dIII were examined. The *Eco*RI and *Bam*HI fragments of strain 1131 DNA were found to be identical to those produced from Ad12 (Huie) DNA (Smiley and Mak, unpublished data; C. Mulder, personal communication). Since identical sets of fragments were produced by digestion of the DNA of both strains of Ad12, we have assumed that the *Eco*RI and *Bam*HI cleavage maps of these strains are identical. The *Hin*dIII cleavage map of strain 1131 DNA was determined (Smiley and Mak, manuscript in preparation) and is shown with those for *Eco*RI and *Bam*HI in Fig. 2.

Purification of the segments of each DNA strand complementary to late cytoplasmic RNA. ^{3}H-labeled l (leftward transcription)- and r (rightward transcription)-strands (5×10^5 cpm/μg, 0.5 μg/ml) were separately hybridized to unlabeled late cytoplasmic RNA (2 mg/ml) for 24 h at 67°C in 0.3 M NaCl–0.01 M Tris (pH 7.3), conditions previously determined to be saturating. The material was treated with S1 nuclease as described previously (33) and then made 0.2 M sodium phosphate (pH 6.8)–1% sodium dodecyl sulfate (SDS), and yeast RNA (1 mg/ml) was added as carrier. The mixture was extracted with phenol saturated with 0.2 M sodium phosphate (pH 6.8), dialyzed against 0.01 M Tris (pH 8.1), and ethanol precipitated.

The pellets were taken up in 2 ml of 0.3 N NaOH, boiled for 15 min, neutralized, buffered at pH 7.3 with 0.1 M Tris, and dialyzed against 0.1× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium acetate) after the addition of 1 mg of yeast RNA per ml as carrier.

Hybridization of labeled nucleic acids to DNA fragments generated by restriction endonucleases. Unlabeled DNA fragments separated by agarose gel electrophoresis were denatured in situ and transferred to nitrocellulose strips by the method of Southern (34). Each strip was prepared from a gel containing 2 μg of Ad12 DNA. The labeled nucleic acid was hybridized to the strip in 2× SSC containing 0.1% SDS and 500 μg of yeast RNA per ml at 65°C for 20 h. The unhybridized nucleic acid was removed by one of two procedures. In [^{3}H]RNA-DNA hybridization, the strips were washed and treated with pancreatic RNase as described by Southern (34). In [^{3}H]DNA-DNA hybridization, the unhybridized [^{3}H]DNA was removed by washing for 1 h in 500 ml of 2× SSC plus 0.1% SDS at 65°C followed by 1 h in 500 ml of 4×10^{-3} M Tris plus 0.1× SSC (pH 9.4) at room temperature (21). The hybridized radioactivity was detected and quantitated by fluorography. Strips were oven dried, immersed in 20% (wt/vol) Omnifluor (New England Nuclear) in toluene, air dried, and exposed at −70°C to Kodak RP Royal X-omat (RP R14) film previously exposed to a hypersensitizing flash of white light (19). Preexposure was done to bring the background density of the film to 0.15 D above that of unexposed film. The resulting negatives were scanned with a Joyce-Loebl microdensitometer, and the relative amount of radioactivity hybridized to each band was estimated from the peak areas. In some cases, there was significant background radioactivity on the fluorographs. Estimation of the specifically hybridized radioactivity was more difficult, and the peaks were extrapolated to the base line of the microdensitometric trace. It is clear that this procedure results in an overestimation of the specifically hybridized radioactivity where there is a significant background.

Hybridization of labeled RNA to immobilized l- and r-strands. To prepare complementary strand-specific RNA, samples containing 5×10^6 cpm of ^{3}H-labeled late cytoplasmic RNA (about 150 μg of RNA) were hybridized to l- or r-strand Ad12 DNA immobilized on nitrocellulose filters. Hybridization was for 20 h in 2× SSC containing 0.1% SDS at 65°C. After hybridization, filters were washed with 1 liter of 2× SSC containing 0.1% SDS at 65°C for 20 min with stirring, followed by a further wash with 2× SSC under suction at room temperature. The hybridized RNA was eluted by boiling for 5 min in 0.1× SSC, and yeast RNA (500 μg/ml) was added as carrier. After dialysis against 0.01 M Tris (pH 7.3), any contaminating [^{14}C]DNA was degraded with 100 μg of pancreatic DNase per ml in the same buffer containing 0.01 M $MgCl_2$. The RNA was then extracted by the hot phenol-SDS method, ethanol precipitated, and dialyzed against 0.1× SSC.

RESULTS

Mapping the DNA sequences expressed as late cytoplasmic RNA. Most of the hybridization mapping studies of adenovirus published to date have utilized as probes labeled complementary strand-specific sequences of individual DNA fragments generated by restriction endonucleases. The complementary strands of individual fragments have been prepared by hybridization to excess, intact complementary strands (39) or by electrophoresis of denatured DNA fragments (7, 31). The complement excess hybridization method (39) required rather large amounts of intact complementary strands, quantities that are difficult to obtain with Ad12 DNA because of the relatively low yield of virus and the small density difference between the complements in poly(U,G)-cesium chloride density gradients (18, 33). Some of the Ad12 DNA fragments could not be separated directly by either electrophoresis or by binding to poly(U,G) followed by cesium chloride density centrifugation (23; Smiley, unpublished data). We have, therefore, adopted a mapping strategy which obviates the requirement for strand separating the individual DNA fragments. Radioactively labeled intact l- and r-strands were hybridized to saturating amounts of unlabeled cytoplasmic KB cell RNA extracted at 24 h after infection (late RNA). Late cytoplasmic RNA is complementary to 65% of the r-strand and 15% of the l-strand of Ad12 DNA (33). In this preparative hybridization the added RNA protected 63.8% of the r-strand and 16.2% of the l-strand from S1 nuclease, demonstrating that the hybridization reaction was saturated. The hybridized portions of each strand were purified by S1 nuclease digestion of the unhybridized nucleic acid, and the RNA was removed by alkaline hydrolysis as described in Materials and Methods. The DNA sequences recovered by this procedure correspond to the segments of l- and r-strands which are complementary to late cytoplasmic viral RNA and should be in equimolar amounts. We cannot, however, exclude the possibility that sequences from a small fraction of the genome which encodes extremely rare RNA species are underrepresented in the DNA selected in this manner.

These [^{3}H]DNA preparations were used as probes to determine the regions of the Ad12 genome complementary to these sequences. Each probe was hybridized to excess (2 μg) unlabeled DNA fragments generated by various restriction endonucleases. The pattern of hybridization of these selected sequences to the fragment sets generated by different restriction endonucleases provides information on the distribution of the coding regions of the l- and r-strands throughout the genome. In addition, within the limits discussed below, the relative amount of radioactivity hybridized to each fragment gives an estimate of the degree of complementarity of each fragment to the probe used. This in turn provides a measure of the fraction of the DNA fragment transcribed into stable cytoplasmic RNA in the infected cell (see below).

To determine how accurately the relative amount of radioactivity hybridized to each fragment reflects the degree of complementarity of the probe to that fragment, total ^{3}H-labeled Ad12 DNA, degraded to about 350 nucleotides in chain length by boiling in alkali, was hybridized to *Eco*RI fragments A to E, using the Southern technique (34). The amount of radioactivity bound to each fragment was quantitated as described in Materials and Methods. The results are shown in Table 1. It can be seen that the relative amount of ^{3}H-labeled Ad12 DNA hybridized to the unlabeled fragments is approximately equal to the expected values of complementarity between the labeled probe and the unlabeled DNA fragments. Several points concerning these data and their interpretation are noteworthy. First, although there is close agreement between experiments, the degree of complementarity of the probe to fragments A and C is overestimated, whereas that to D and E is underestimated. It is possible that this result reflects the relative guanine-plus-cytosine contents of the fragments, since the DNA probe was labeled with [^{3}H]thymidine, or some other source of systematic error. In some cases described below, the degree of complementarity between the r-specific probe and individual DNA fragments is also slightly overestimated, perhaps for similar reasons. Second, since the results are displayed as the fraction of the total hybridized radioactivity which has annealed to

TABLE 1. *Hybridization of ^{3}H-labeled Ad12 DNA to Ad12 DNA fragments generated by EcoRI*[a]

Ad12 DNA fragment	Fragment size fraction of the genome	Relative amt of ^{3}H-Ad12 hybridized	
		Expt I	Expt II
A	0.36	0.39	0.39
B	0.27	0.26	0.30
C	0.165	0.21	0.20
D	0.115	0.096	0.095
E	0.07	0.042	0.047

[a] Fragmented ^{3}H-labeled Ad12 DNA was hybridized to nitrocellulose strips bearing electrophoretically separated DNA fragments, and the hybridized radioactivity was quantitated by fluorography.

each fragment, the determinations within a given experiment are not independent. Systematic or spurious errors in the measurement of the fraction hybridized to one fragment necessarily introduce error into the other determinations. One of the factors contributing to spurious error is the background radioactivity on some of the fluorographs (e.g., Fig. 1).

To map the viral DNA sequences complementary to late RNA, ^{3}H-labeled l- and r-strand viral DNA sequences selected by hybridization to late cytoplasmic RNA were hybridized to DNA fragments generated by *Eco*RI, *Bam*HI, and *Hin*dIII, using the Southern technique. The results are shown in Fig. 1 and Table 2. Since the r-strand probe is derived from 0.65 of the total r-strand (33), the fraction of the probe hybridized to each unlabeled fragment must be multiplied by 0.65 to give the fraction of the total r-strand transcribed within this particular DNA fragment. Only 0.15 of the total l-strand is complementary to late cytoplasmic RNA (33). Therefore, the fraction of the total l-strand transcribed from each DNA fragment can be similarly estimated.

Data from Table 2 were used to construct a map of the sites expressed as late cytoplasmic RNA (Fig. 2). The map shown was assembled from the data as described below by assuming that cytoplasmic RNA is asymmetrically transcribed and by reducing the number of separate, contiguously transcribed regions to the minimum demanded by the data. Other, more complex arrangements for the coding regions are possible.

Consider first the transcription from the l-strand. A minimum of two separate regions of transcribed DNA were detected by hybridization to the *Eco*RI fragments; one within *Eco*RI-A, the other within *Eco*RI-C. the leftmost region, within *Eco*RI-C, may extend a small distance beyond the *Eco*RI-C–D junction, since in one experiment the probe hybridized to a small degree with *Eco*RI-D. This suggestion is supported by the weak hybridization to *Hin*dIII-C. The data obtained with the fragments generated by *Bam*HI demonstrate that *Eco*RI-A contains at least two separate regions of transcribed DNA, one within *Bam*HI-C and the other within *Bam*HI-E. (Although *Bam*HI-C and -D are not separable by gel electrophoresis, the failure of the l-strand-specific probe to hybridize with either *Eco*RI-B or *Hin*dIII-A, which both contain *Bam*HI-D, allows the assignment to *Bam*HI-C.) The fragments produced by *Hin*dIII allow a more precise localization of these three regions. The probe hybridized to only *Hin*dIII-C, -D, and -E (however, fragments J, K, and L were run out of the gels used in the analysis). Qualitative consideration of the data shows that the leftmost region is located to the right of *Hin*dIII-F (6.3 map units) and to the left of any of *Bam*HI-D, -H, or -I (25 map units). The region detected within *Bam*HI-C was not de-

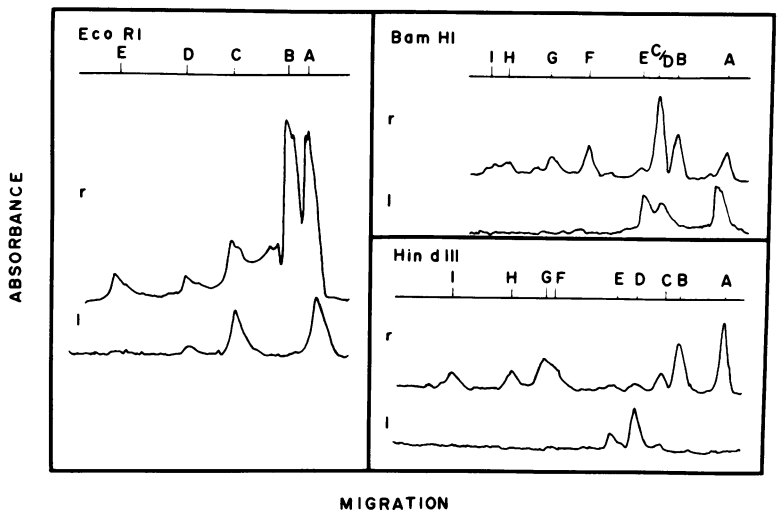


FIG. 1. *Hybridization of labeled l- and r-strand-specific probes complementary to late cytoplasmic RNA to unlabeled DNA fragments. Nitrocellulose strips bearing 2 μg of unlabeled DNA fragments produced by EcoRI, BamHI, and HindIII were incubated with 0.02 μg (10^4 cpm) of r-strand probe or 0.005 μg (2.5×10^3 cpm) of l-strand probe in 2× SSC plus 0.1% SDS at 65°C for 20 h. The hybridized radioactivity was detected by fluorography to flash-hypersensitized X-ray film. Microdensitometer scans of the resulting negatives are shown.*

TABLE 2. *Distribution of the l- and r-strand sequences complementary to late cytoplasmic RNA among specific DNA fragments*[a]

Probe	Fragment	Fragment size (fraction)	Fraction of probe hybridized	Fraction of genome represented in probe within fragment[b]
r	*Eco*RI-A	0.36	0.35	0.23
r	*Eco*RI-B	0.27	0.36	0.24
r	*Eco*RI-C	0.165	0.17	0.11
r	*Eco*RI-D	0.115	0.06	0.04
r	*Eco*RI-E	0.07	0.06	0.04
r	*Eco*RI-F	0.02	ND	ND
r	*Bam*HI-A	0.25	0.13	0.08
r	*Bam*HI-B	0.16	0.16	0.11
r	*Bam*HI-C	0.13	0.32[c]	0.08[c]
r	*Bam*HI-D	0.13		0.13[c]
r	*Bam*HI-E	0.115	0.04	0.03
r	*Bam*HI-F	0.075	0.12	0.08
r	*Bam*HI-G	0.063	0.10	0.07
r	*Bam*HI-H + I	0.065	0.12	0.08
r	*Hin*dIII-A	0.23	0.29	0.18
r	*Hin*dIII-B	0.155	0.19	0.12
r	*Hin*dIII-C	0.13	0.06	0.04
r	*Hin*dIII-D	0.115	0.02	0.01
r	*Hin*dIII-E	0.09	0.01	0.006
r	*Hin*dIII-F	0.063	0.13	0.08
r	*Hin*dIII-G	0.060	0.12	0.07
r	*Hin*dIII-H	0.049	0.10	0.06
r	*Hin*dIII-I	0.029	0.07	0.04
r	*Hin*dIII-J-L	0.071	ND	ND
l	*Eco*RI-A	0.36	0.56 (0.49)[d]	0.08 (0.07)
l	*Eco*RI-C	0.165	0.36 (0.51)	0.05 (0.08)
l	*Eco*RI-D	0.115	0.08 (<0.01)	0.01 (0.00)
l	*Bam*HI-A	0.25	0.39 (0.53)	0.06 (0.08)
l	*Bam*HI-C	0.13	0.31 (0.25)	0.05 (0.04)
l	*Bam*HI-E	0.115	0.30 (0.22)	0.05 (0.03)
l	*Hin*dIII-C	0.13	0.09 (0.07)	0.01 (0.01)
l	*Hin*dIII-D	0.115	0.67 (0.57)	0.07 (0.06)
l	*Hin*dIII-E	0.09	0.24 (0.36)	0.03 (0.04)

[a] ^{3}H-labeled l- and r-strand probes complementary to late cytoplasmic RNA were hybridized to nitrocellulose strips bearing electrophoretically separated DNA fragments, and the hybridized radioactivity was quantitated by fluorography (Fig. 1). DNA fragments which hybridize nondetectable amounts of radioactive probe are not presented in this table. ND, Not done.

[b] Calculated as the product of the effective sequence complexity of the probe used and the fraction of the total hybridized radioactivity annealed to each fragment. The effective complexity of the r-strand probe was 0.65 when hybridized to *Eco*RI and *Bam*HI fragments and 0.61 when annealed to the *Hin*dIII fragments A to I. Effective complexities of 0.15 and 0.106 were assumed for the l-strand probe (see text).

[c] Fragments *Bam*HI-C and -D comigrate. Together they contain sequences homologous to 32% of the r-strand probe (=21% of the total r-strand). The rationale for apportioning these sequences between the two fragments is given in the text.

[d] The two numbers represent results from two separate experiments.

tectable within *Hin*dIII-A to -I and, therefore, resides within one or more of the *Hin*dIII fragments excluded from the analysis (J-L). This region can be placed between *Eco*RI-E (62 map units) and *Hin*dIII-B (68 map units). The rightmost l-specific region maps to the right of 91 map units (entirely within *Hin*dIII-E). The relative sizes of these three regions were estimated from the fraction of the probe which hybridized to each DNA fragment (Table 2). From left to right these regions are 6 to 8, 4 to 5, and 3 to 5 map units in length. If it is assumed that the l-specific segment of *Hin*dIII-C is contiguous to that of *Hin*dIII-D, then the leftmost region can be placed from about 11.5 to 18.5 map units.

The r-strand probe hybridized, to varied degrees, with all of the DNA fragments assayed (Fig. 1). Given the above-mentioned constraints on the positions of the segments of the l-strand complementary to cytoplasmic RNA, this result demonstrates that at least three separate regions of the r-strand are transcribed into cytoplasmic RNA. One of these regions lies to the left of the leftmost l-specific block. The size of this segment, estimated from the extent of hybridization of the r-probe to *Eco*RI-C, *Bam*HI-A, and

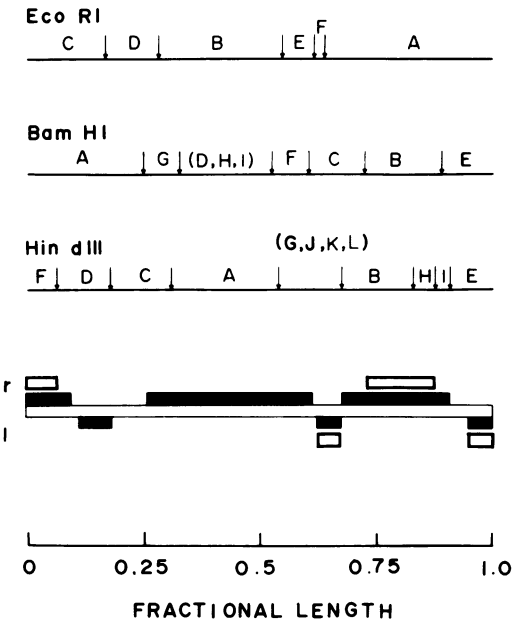


FIG. 2. *Map of sites of the Ad12 genome expressed as early and late cytoplasmic RNA. The map for late RNA was deduced from the data shown in Fig. 1 and Table 2. The map for early RNA was deduced from data shown in Table 3 and Fig. 5, together with information from Ortin et al. (24). Symbols:* (▬) *late RNA;* (▭) *early RNA.*

*Hin*dIII-F and -D, is 8 to 11 map units. The next region, which begins to the right of this l-strand block (i.e., some point beyond position 18.5) should encompass all or part of *Eco*RI-D, -B, and -E, *Bam*HI-G, -D, -H, -I, and -F, and *Hin*dIII-C, -A, and -G. (Although the relative order of *Hin*dIII-G, -J, -K, and -L is not known, *Hin*dIII-G is entirely contained within *Eco*RI-E [Smiley, unpublished data]). The relative extent of hybridization of the r-specific probe to these fragments suggests that most or all of *Eco*RI-B, *Bam*HI-G, -D, -H, -I, and -F, and *Hin*dIII-A and -G are complementary to the probe. However, it appears that only a portion of *Eco*RI-D and *Hin*dIII-C is complementary to the probe. Because of the uncertainties inherent in interpreting the quantitative data, as pointed out above, it is difficult to precisely locate the ends of this block. The available data suggest that it starts at or close to position 25 and ends with *Eco*RI-E or -F and is, therefore, about 35 map units in length. The third r-strand region maps to *Eco*RI-A, *Bam*HI-C, -B, and -E, and *Hin*dIII-B, -H, and -I. This segment begins to the right of the l-strand region, which maps to *Bam*HI-C, and, based on the low levels of hybridization to *Hin*dIII-E, appears to end around position 92.

Several difficulties were encountered in the interpretation of the data obtained with the r-strand probe. First, because *Bam*HI-C and -D were not separable electrophoretically, we obtained an estimate of the fraction of the r-strand transcribed from within both these regions. The data obtained with the fragments generated by *Eco*RI and *Hin*dIII indicate that virtually all of the r-strand within *Eco*RI-B and *Hin*dIII-A is transcribed as cytoplasmic RNA. The *Bam*HI-D fragment maps entirely within this region, and consequently we have assumed total transcription of the r-strand of this fragment and have assigned excess hybridization to *Bam*HI-C. Second, the method calculating the fraction of each strand transcribed within each region assumes that all of the DNA sequences complementary to the probe used are present in the analysis. Fragments *Hin*dIII-J to -L and *Eco*RI-F were in fact excluded because of their small size. The exclusion of *Eco*RI-F (2% of Ad12) has a negligible effect, but the exclusion of *Hin*dIII-J to -L (approximately 8% of Ad12) complicates the analysis. However, as discussed above, a region comprising 4 to 5 map units located entirely within fragments *Hin*dIII-J to -L is transcribed from the l-strand and can be excluded from the analysis of r-strand-specific RNA. Effectively, only about 3 to 4% of Ad12 is then excluded by not examining these fragments. Assuming that all of this DNA is represented as r-strand-specific cytoplasmic RNA, the effective complexity of the r-strand DNA probe becomes 0.61. The effects of this alteration in complexity are small. The values given in Table 2 were calculated using 0.61 as the complexity of the r-strand probe.

Analysis of the RNA sequences actively synthesized at late times. The results outlined above serve to map the positions and strand specificities of the DNA sequences represented in late cytoplasmic RNA but give no indication of which segments of the genome are actively transcribed at late times. To determine whether both strands are transcribed, Ad12-infected cells were labeled from 21 to 24 h postinfection with [^{3}H]uridine, and the cytoplasmic RNA was purified.

It was found that this [^{3}H]RNA could hybridize to both l- and r-strands of Ad12 DNA (data not shown). To determine which segments of each strand are actively transcribed at 24 h after infection, the ^{3}H-labeled viral RNA was selected by hybridization to l- and r-strand DNA, eluted, and rehybridized to unlabeled *Eco*RI fragments (Fig. 3). It is clear that most RNA species present in the cytoplasm at late times are actively synthesized and transported to the cytoplasm at

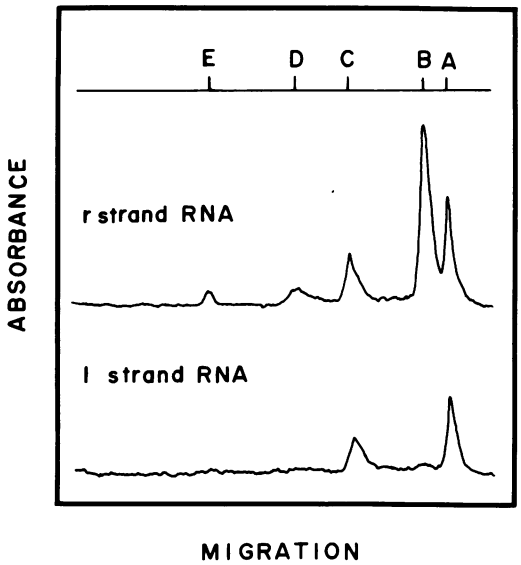


FIG. 3. *Hybridization of labeled late complementary strand-specific RNA to unlabeled EcoRI fragments. Cytoplasmic RNA labeled with [^{3}H]uridine from 21 to 24 h postinfection was strand selected as described in the text and hybridized to nitrocellulose strips bearing 1 μg of denatured, separated Ad12 EcoRI fragments. Hybridized radioactivity was detected as in Fig. 1.*

late times. This is in contrast to the result reported that at late times after infection with Ad2 the RNA is transcribed exclusively from the r-strand of the genome (26).

It is possible that the labeling times chosen are not comparable to the 16- to 18-h period used by Pettersson and Philipson (26) with Ad2 and that analysis of RNA synthesized later would reveal only r-strand transcription. It is worth noting, however, that the maximal rates of viral DNA and RNA synthesis occur at 24 h postinfection with Ad12 (21), whereas this occurs at 18 h postinfection with Ad2 (14). Since the strand-specific RNA used in the previous experiment was isolated by hybridizing near-saturating amounts of RNA to each strand, the results of the experiment displayed in Fig. 3 cannot be used to estimate the relative amounts of radioactivity incorporated in RNA derived from different regions of the genome.

To obtain estimates of RNA transcription rates from different regions of the genome, cytoplasmic RNA labeled 24 to 26 h postinfection with [^{3}H]uridine was degraded in alkali to about 500 nucleotides in chain length (1), and 2 μg of this RNA (specific activity, 3.4×10^4 cpm/μg) was hybridized directly to excess (2 μg) fragments generated by *Eco*RI and *Bam*HI. The relative amount of radioactivity hybridizing to each band was taken as a measure of the relative rates of accumulation of cytoplasmic RNA derived from each fragment. The data obtained (Table 3) indicate tht RNA species derived from different regions of the genome accumulate at widely differing rates. During the labeling period chosen, RNA derived from about 25 to 50 map units on the genome (*Eco*RI-B and -D) accumulates at a greater rate than any other class.

Mapping of the early regions. At early times, viral RNA forms an extremely small fraction of the total cytoplasmic RNA. Consequently, the method used to map the late RNA was difficult to apply to early RNA, since a large amount of viral RNA is required to saturate substantial quantities of radioactively labeled viral DNA.

Some information about the DNA sequences encoding early cytoplasmic RNA was obtained by hybridizing cytoplasmic RNA labeled with [^{3}H]uridine from 5.5 to 7.5 h postinfection to

TABLE 3. *Distribution of labeled late cytoplasmic RNA sequences among DNA fragments*[a]

Fragment	Fraction of total RNA hybridized	Specific accumulation rate[b]
*Eco*RI-A	0.07	0.19
*Eco*RI-B	0.60	2.22
*Eco*RI-C	0.07	0.42
*Eco*RI-D	0.20	1.74
*Eco*RI-E	0.06	0.86
*Bam*HI-A	0.06	0.24
*Bam*HI-B	0.03	0.19
*Bam*HI-C	0.05[d]	0.42[c]
*Bam*HI-D		3.93[c]
*Bam*HI-E	0.02	0.17
*Bam*HI-F	0.07	0.87
*Bam*HI-G	0.16	2.55
*Bam*HI-H+I	0.12	1.80

[a] Late cytoplasmic RNA labeled 24 to 26 h postinfection with [^{3}H]uridine degraded to about 500 nucleotides in chain length was hybridized to excess (2 μg) unlabeled DNA fragments, and the hybridized radioactivity was quantitated by fluorography.

[b] Defined as the fraction of the total hybridized radioactivity annealing to each fragment divided by the fractional length of that fragment.

[c] The relative distribution of hybridized radioactivity between *Bam*HI-C and -D was estimated by measuring hybridization to fragments produced by simultaneous cleavage with *Eco*RI and *Bam*HI. The ratio of hybridized radioactivity between *Bam*HI-C/*Eco*RIα (within *Bam*HI-C) and *Bam*HI-D was 0.07. *Bam*HI-C/*Eco*RIα represents 0.71 of *Bam*HI-C. Assuming that accumulation within *Bam*HI-C is uniform, this yields a specific accumulation rate of 0.42 for *Bam*HI-C.

[d] *Bam*HI-C and -D are not separable electrophoretically. This number represents the sum of the radioactivity hybridizing to each fragment.

DNA fragments produced by *Eco*RI, *Bam*HI, and *Hin*dIII. Relative amounts of [^{3}H]RNA hybridized to each fragment are shown in Table 4. Hybridization was detected with *Eco*RI-A, -B, and -C, *Bam*HI-A, -B, -C, and -E and *Hin*dIII-B, -C, -E, -F, and -H. Fragments *Eco*RI-F and *Hin*dIII-J, -K, and -L were not analyzed. These data demonstrate that early RNA is derived from at least three separate regions of the viral genome.

Positive hybridization to *Hin*dIII-E but not to *Hin*dIII-I indicated that one region is contained entirely within *Hin*dIII-E. Similarly, positive hybridization to *Hin*dIII-F but not -D places another region near the left end of the genome. Results from hybridization with *Eco*RI and *Bam*HI fragments are consistent with this conclusion. At least one region is located in *Bam*HI-B and -C. The [^{3}H]RNA hybridized to the *Bam*HI-C/D doublet was assigned to *Bam*HI-C by the following experiment. Ad12 DNA was cleaved simultaneously with *Eco*RI and *Bam*HI, and the fragments were separated by electrophoresis (Fig. 4A). *Bam*HI-D is not cleaved by *Eco*RI, but *Bam*HI-C is cleaved into three fragments (*Bam*HI-C/*Eco*RIα [fragment VI in Fig. 4A, lane 2] and two smaller ones). Results shown in Table 4 show that early [^{3}H]RNA hybridized only to *Bam*-C/*Eco*RIα (designated as *Bam*HI/*Eco*RI VI in Table 4) but not to *Bam*HI-D (designated as *Bam*HI/*Eco*RI III). The lack of hybridization to *Hin*dIII-A confirms this conclusion.

The observed hybridization of early RNA to *Eco*RI-B (6% of the total hybridized radioactivity; Table 4) does not agree with the data obtained with *Bam*HI, where no hybridization was seen with fragments D, H, I, and F and only very small amounts (<1% of the total) were found with G. Similarly, no hybridization was observed to *Hin*dIII-A and -G (<1%), and only small amounts of hybridization were observed to *Hin*dIII-C (1%). The rather high levels of hybridization of early RNA to *Eco*RI-B can perhaps be explained by postulating the existence of minority rearrangement genomes bearing sequences derived from the normal *Eco*RI-A and/or -C fragments on fragments of about the size of *Eco*RI-B. Such genomes have in fact been observed within Ad12 (Huie) populations (I. Mak, H. Ezoe, and S. Mak, manuscript in preparation). From data in Table 4, it can be seen that the different early RNA species accumulate at widely differing rates, with RNA encoded by the molecular ends of the genome accumulating most rapidly. These RNA species together comprise from 75 to 90% of the total viral RNA detected.

TABLE 4. *Distribution of labeled early RNA sequences among DNA fragments*[a]

Fragment	Fraction of total radioactivity hybridized annealed to fragment	Maximum size of region encoding the early RNA	Specific accumulation rate[b]
*Eco*RI-A	0.47		
*Eco*RI-B	0.06		
*Eco*RI-C	0.47		
*Bam*HI-A	0.60	0.067	8.96
*Bam*HI-B	0.11	0.15	0.73
*Bam*HI-C+D	0.09	0.05	1.80
*Bam*HI-E	0.21	0.05	4.20
*Bam*HI-G	<0.01		
*Hin*dIII-B	0.05		
*Hin*dIII-C	0.01		
*Hin*dIII-E	0.40		
*Hin*dIII-F	0.50		
*Hin*dIII-H	0.13		
*Bam*HI + *Eco*RI-I+II[c]	0.63		
*Bam*HI + *Eco*RI-IV	0.28		
*Bam*HI + *Eco*RI-VI	0.08		

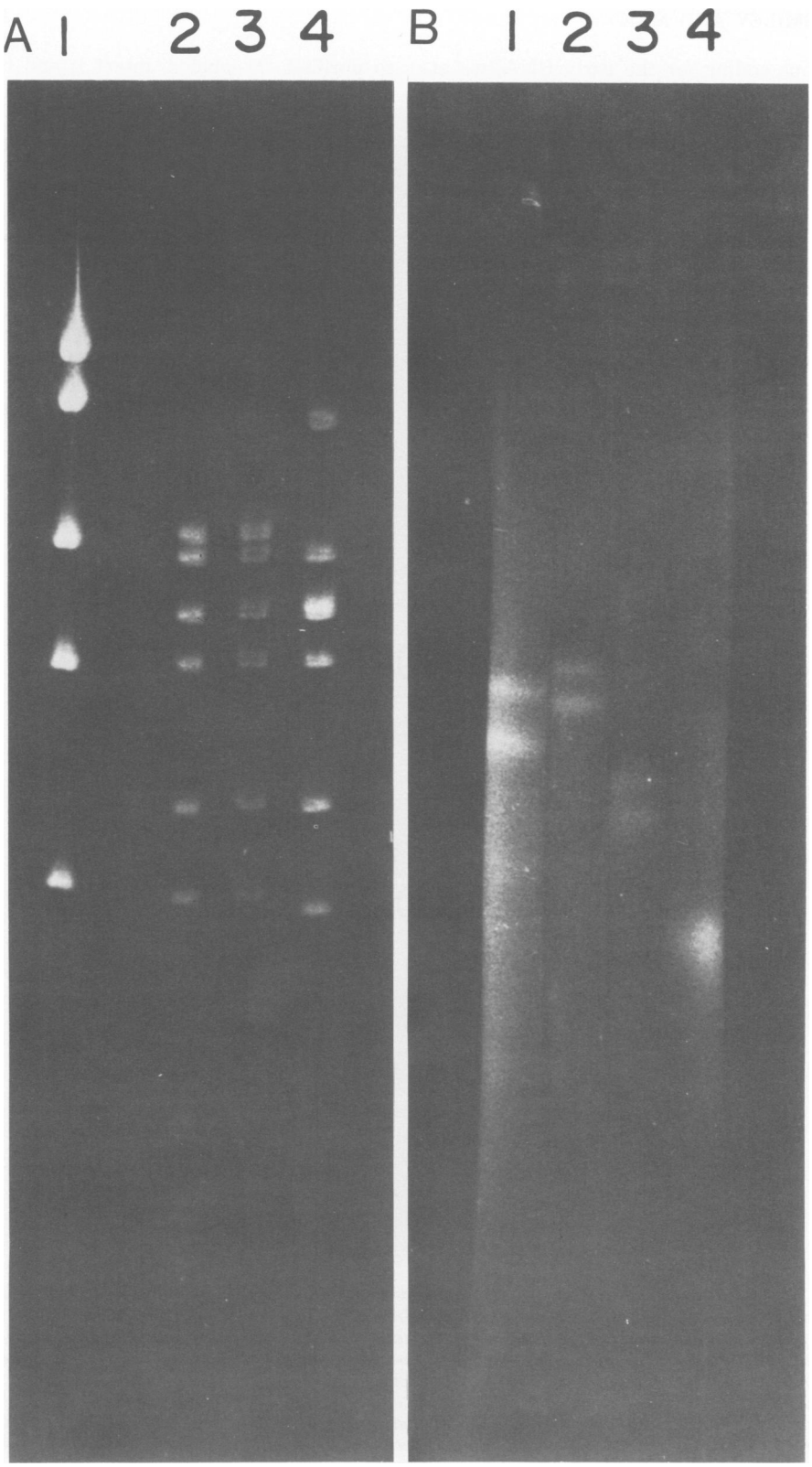
[a] Cytoplasmic RNA labeled 5.5 to 7.5 h postinfection was hybridized to 2 μg of unlabeled Ad12 DNA fragments. The analyses with *Eco*RI and *Bam*HI fragments were done with the same RNA preparation, whereas another lot of RNA was used for the experiments with the *Hin*dIII fragments. Fragments *Eco*RI-A–E, *Bam*HI-A–I, *Hin*dIII-A–I, and *Bam*HI + *Eco*RI-I–VII were assayed. Only those fragments which hybridized detectable radioactivity are tabulated. The limit of detection of the assay is about 1% of the total hybridized radioactivity.

[b] Defined as the fraction of the total hybridized radioactivity which annealed to a given fragment divided by the maximum size of the region encoding the early RNA.

[c] The identity of fragments produced by the double digestion are: I, *Eco*RI-C; II, *Bam*HI-B; III, *Bam*HI-D; IV, *Bam*HI-E; and VI, *Bam*-HI-C/*Eco*RIα.

Strand assignment of the early regions. To determine directly the strand derivation of

FIG. 4. *Electrophoretic strand separation of some Ad12 DNA fragments. Ad12 DNA was cleaved simultaneously with BamHI and EcoRI, and the resulting fragments were separated in 1% agarose gels. Fragments EcoRI-C, BamHI-B, BamHI-E, and BamHI-C/EcoRIα were isolated in gel slices, denatured in situ, and again subjected to electrophoresis. (A) Electrophoretic separation of fragments produced by EcoRI (1), EcoRI plus BamHI (2, 3), and BamHI (4) in 1% agarose gels, allowing the identification of the origin of some of the fragments produced by double digestion. (B) Electrophoresis of denatured DNA fragments in 1% agarose gels. 1, EcoRI-C DNA; 2, BamHI-B DNA; 3, BamHI-E DNA; 4, BamHI-C/EcoRIα DNA. Electrophoresis was for 8 h at 2 V/cm.*


A 1 2 3 4
B 1 2 3 4

the regions coding for the early RNA, we attempted to resolve the strands of the DNA fragments (i.e., *Eco*RI-C, *Bam*HI-B and -E, and the *Bam*HI-C/*Eco*RIα) by gel electrophoresis. The method used is that used by Horowitz (16). Figure 4B shows that fragments *Eco*RI-C and *Bam*HI-B and -E were resolved into doublets, whereas denatured *Bam*HI-C/*Eco*RIα migrated as a single band. To determine the strand derivation of the fast- and slow-migrating bands of *Eco*RI-C, *Bam*HI-B, and *Bam*HI-E, the relevant portion of each gel was excised and composites were made. The DNA from the composite gels was transferred to nitrocellulose strips by the Southern method (34) without further denaturation. These strips were then hybridized to purified, ^{3}H-labeled, intact l- and r-strands and fluorographed (Fig. 5). The labeled complementary strands hybridized primarily to one band of the doublet, allowing strand assignments to each band to be made. The fast-migrating band of *Eco*RI-C is derived from the intact l-strand, whereas the fast bands of *Bam*HI-B and -E are part of the intact r-strand. The strand derivations of early RNAs were then directly determined by hybridizing labeled RNA to such strips (Fig. 5). The results demonstrate that early cytoplasmic RNA is derived from the r-strand of *Eco*RI-C and *Bam*HI-B and the l-strand of *Bam*HI-E. The strand derivation of early RNA with *Bam*HI-C was not directly determined.

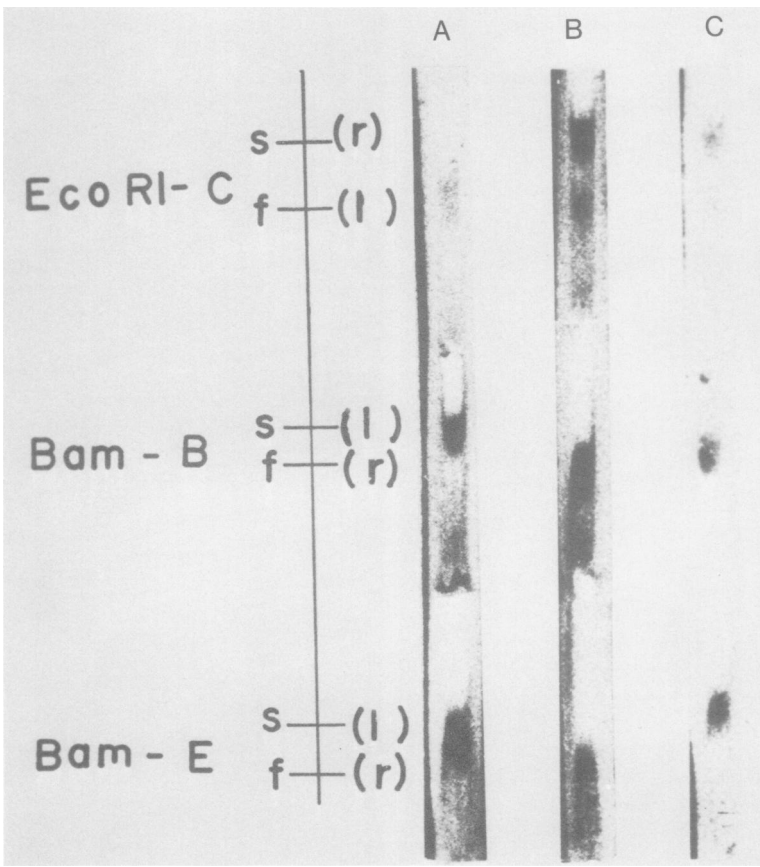


FIG. 5. *Hybridization of ^{3}H-labeled intact l- and r-strand DNA and early cytoplasmic RNA to complementary strand of EcoRI-C, BamHI-B, and BamHI-E DNA. Each DNA fragment was denatured and subjected to electrophoresis in agarose gels. The gels were examined under UV illumination after staining with 0.5 μg of ethidium bromide per ml. The region of each gel containing the double bands was excised, and composite gels were created by end-to-end alignment of the excised segments. The DNA in the composite was then transferred to a nitrocellulose membrane and hybridized to 10^4 cpm of ^{3}H-labeled r-strand Ad12 DNA (A) or l-strand Ad12 DNA (B) and to 5×10^6 cpm of ^{3}H-labeled early cytoplasmic RNA (C). Hybridized radioactivity was detected by fluorography. A schematic representation of the positions of each of the DNA bands in the composite gels is shown.*

DISCUSSION

We have developed a new method of mapping viral genes expressed late during infection. It involves the isolation of radioactively labeled l- and r-strand DNA sequences complementary to late cytoplasmic RNA and, subsequently, rehybridization of these sequences to DNA fragments generated by restriction endonucleases. The method has several advantages over the standard ones. First, the requirement for strand separating the individual DNA fragments is bypassed. Second, the transcribed segments of each strand can be hybridized to any set of ordered DNA fragments to refine the map with a minimum of additional effort. Drawbacks of the method include the lack of information on the abundance of different RNA species, the requirement for rather large quantities of viral RNA to prepare the DNA probes, and the reduced precision of the method as compared to saturation hybridization. The ends of blocks of transcribed DNA can be located only to within several map units by this procedure, and it is likely that small gaps within what we have assumed are regions of contiguous complementarity to cytoplasmic RNA would go unnoticed.

Using this new method and the DNA fragments generated by three restriction endonucleases, we have mapped the sites of the Ad12 (strain 1131) genome which are expressed as late cytoplasmic RNA. The map is similar to the Ad12 (Huie) map obtained by saturation hybridization of labeled, complementary strand-specific sequences generated by *Eco*RI and *Bam*HI (29). The close agreement of the two transcription maps indicates that our method is suitable for mapping late mRNA. The Ad12 transcriptional map for late cytoplasmic RNA is very similar to those previously obtained with Ad2 (27, 31) and Ad5, with the exception that a region of Ad12 DNA extending from approximately 17 to 25 map units is apparently not transcribed into stable cytoplasmic RNA. The corresponding region of Ad2 and Ad5 DNA is transcribed as stable RNA (27, 31).

Well before the onset of viral DNA synthesis, we have detected cytoplasmic RNA derived from a minimum of three separate regions of the Ad12 genome. Ortin et al. (23) have detected four separate blocks of DNA sequences encoding early Ad12 cytoplasmic RNA, mapping within the r-strand of *Eco*RI-C and *Bam*HI-B, and the l-strand of *Bam*HI-C and *Bam*HI-E. We were not able to separate the regions mapped in *Bam*HI-B and -C. The data of Ortin et al. (23) and those obtained in this study allow a quite precise mapping of the four major early regions. The leftmost block, transcribed from the r-strand, maps between 0 and 6.3 map units (within *Hin*dIII-F). Ortin et al. have estimated the size of this region as about 7.5% of Ad12. If it is assumed that the early RNA derived from *Bam*HI-C is transcribed entirely from the l-strand (23), this segment must lie between the *Eco*RI-E–F junction (62 map units) and the leftmost end of the *Hin*dIII-B (68 map units). The maximum size of this region is 4 to 5 map units. The total lack of hybridization of early cytoplasmic RNA to *Hin*dIII-I allows placing the right terminus of the early region within *Bam*HI-B to the left of 88.1 map units (the *Hin*dIII-H–I boundary). The maximum possible size of this block is then 15 map units, which is identical to the estimates obtained by saturation hybridization (23). The rightmost early region, transcribed from the l-strand, maps entirely to the right of 91 map units and is probably identical to the rightmost late l-specific region. The sites of these early regions are also similar to those of Ad2 (27, 31). It was of interest to note that the extreme left r-strand early region of Ad12 (at most 6.3 map units) is considerably smaller than the analogous region of Ad2 (11 map units) (27, 31).

At both early and late times postinfection, cytoplasmic RNAs complementary to different segments of the genome accumulate at widely different rates. Figure 6 shows the specific rates of accumulation in the cytoplasm of RNA-derived different regions of the genome at early and late times. The data obtained with early RNA have been normalized to the maximum possible size of each early coding region, whereas the late data have been normalized to fragment size. If not all DNA sequences are a template for the synthesis of late cytoplasmic RNA (for example, the region from 18 to 25 map units), this procedure will underestimate the specific accumulation rate of RNA derived from sequences flanking the nontranscribed segments. The major RNA species synthesized and transported to the cytoplasm during the interval between 5.5 and 7.5 h postinfection are derived from the molecular ends of the genome (*Hin*dIII-E and -F). Smaller amounts are detected from *Bam*HI-B and -C. By 24 to 26 h after infection the pattern has drastically changed, with RNA derived from between 25 to about 60 map units being by far the most rapidly accumulating class. This RNA constitutes the major class synthesized exclusively at late times. The corresponding region of Ad2 encodes the major virion proteins (20). If similar regions of the Ad2 and Ad12 genomes encode analogous proteins, this result is not surprising.

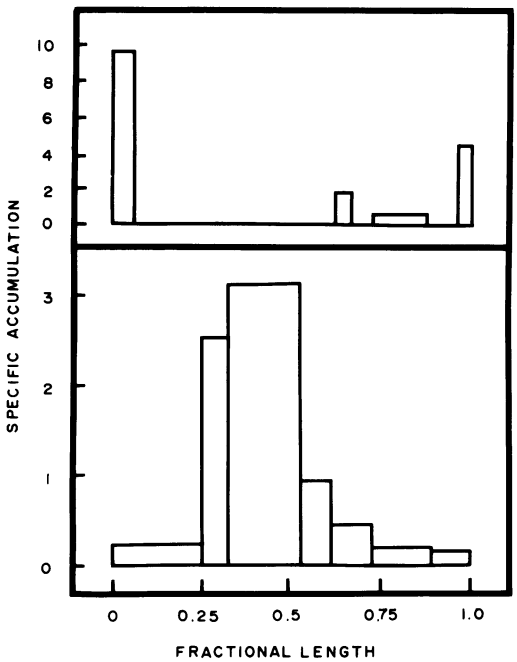


FIG. 6. *Correlation of the rates of accumulation of cytoplasmic RNA complementary to DNA fragments with map position. The data obtained with the BamHI fragments, shown in Tables 3 and 4, are displayed. Specific accumulation rates for early RNA species were calculated by dividing the fraction of the total early RNA present in each class by the maximum possible fractional size of the corresponding early coding region. (Upper) Cytoplasmic RNA labeled 5.5 to 7.5 h postinfection. (Lower) Cytoplasmic RNA labeled 24 to 26 h postinfection.*

These results clearly show that both the "early" and "late" cytoplasmic RNAs can be subdivided according to their rates of accumulation. By examining only two periods postinfection we are able to distinguish three classes of RNA on this basis: 1, RNA which accumulates rapidly early (derived from *Hin*dIII-E and -F); 2, those early RNA species which accumulate more slowly (within *Bam*HI-B and -C); 3, RNA not detectable early which accumulates rapidly late (derived from *Eco*RI-B, -D, and -E). It is possible that an examination of more time intervals will reveal further complexity. It is tempting to speculate that these RNA classes differ in the times of their maximal rate of accumulation.

Flint and Sharp (8) have quantitated the levels of Ad2 nuclear and cytoplasmic RNA derived from different segments of the viral genome at various times postinfection. At both 18 and 32 h postinfection, RNA derived from the region of the genome extending from approximately 65 to 90 map units constitutes the most abundant class in the nucleus and cytoplasm. At 32 h postinfection RNA mapping between 66 and 80 map units comprises over half of the total viral cytoplasmic RNA. Our data indicate that during the interval for 24 to 26 h postinfection with Ad12, RNA derived from 25 to 60 map units accumulates most rapidly in the cytoplasm. Since the labeling time chosen corresponds to the time at which the maximal rate of viral RNA synthesis occurs (14), this RNA will presumably eventually constitute the most abundant cytoplasmic class. This may indicate a difference in the fine control of Ad2 and Ad12 RNA accumulation.

The distribution of contiguous regions encoding early and late cytoplasmic RNA is virtually identical in Ad2, Ad5, Ad7 (37), and Ad12. The gross homology of genome organization of the group A, B, and C adenoviruses may indicate that genes with similar functions share common map positions. This supposition is strengthened by the finding that the gene(s) responsible for the maintenance of in vitro transformation in both groups of viruses map to the left (guanine plus cytosine rich) end of the genome (7, 9, 11; Mak et al., manuscript in preparation). The observed similarity further implies that during the evolutionary divergence of the group A, B, and C adenoviruses the overall layout of the genome has been preserved, whereas the DNA sequences of the individual genes have diverged to the point where very little sequence homology can be detected (17).

ACKNOWLEDGMENTS

This work was supported by grants from the National Cancer Institute of Canada and National Research Council of Canada. J.R.S. is a Research Student of the National Cancer Institute of Canada.

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