JOURNAL OF VIROLOGY, Nov. 1976, p. 355-372
Vol. 20, No. 2

Printed in U.S.A.

Transcription of the Genome of Adenovirus Type 12

III. Maps of Stable RNA from Productively Infected Human Cells and Abortively Infected and Transformed Hamster Cells

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Received for publication 25 March 1976

The adenovirus type 12-specific mRNA and the stable nuclear RNA from productively infected KB cells, early postinfection, from abortively infected BHK-21 cells, and from the adenovirus type 12-transformed hamster lines T637 and HA12/7 have been mapped on the genome of adenovirus type 12. The intact separated heavy (H) and light (L) strands of adenovirus type 12 DNA have been used to determine the extent of complementarity of the mRNA or nuclear RNA from different cell lines to each of the strands. More precise map positions have been obtained by the use of the H and L complements of the fragments of adenovirus type 12 DNA which were produced with the *Eco*RI and *Bam*HI restriction endonucleases. The results of the mapping experiments demonstrate that the mRNA's isolated early from productively and abortively infected and from two lines of transformed cells are derived from the same or similar regions of the adenovirus type 12 genome. The map positions on the adenovirus type 12 genome for the mRNA from the cell lines as indicated correspond to regions located approximately between 0 and 0.1 and 0.74 and 0.88 fractional length units on the L strand and to regions between 0.63 and 0.74 and 0.89 and 1.0 fractional length units on the H strand. The HA12/7 line lacks mRNA complementary to the region between 0.74 and 0.88 fractional length units on the L strand. Similar data are found for the nuclear RNA, except that the regions transcribed are more extensive than those observed in mRNA. The polarity of the H and L strands of adenovirus type 12 DNA has been determined. The H strand has its 3'-end on the right terminus in the *Eco*RI A fragment, and the L strand has its 3'-end on the left terminus in the *Eco*RI C fragment. Thus, the H strand is transcribed from right to left (l = leftward strand); and the L strand is transcribed from left to right (r = rightward strand). The designations H and L refer to the relative heavy and light densities of the two strands in polyuridylic-polyguanylic acid-CsCl density gradients. The *Eco*RI C-H and D-H complements have been shown to be part of the intact L strand; thus, there is a "reversal in heaviness" on the left terminus of the viral DNA.

Adenovirus type 12 (Ad12) infects human KB cells productively, and during the transcription of the Ad12 genome an early and late phase can be distinguished. The late phase starts with the onset of viral DNA replication, i.e., 12 to 14 h postinfection (40). In contrast, Ad12 undergoes an abortive cycle in BHK-21 cells (8, 9, 45), and viral DNA replication cannot be detected in this system (8, 9, 13, 17, 35). In BHK-21 cells, only that portion of the Ad12 genome that is expressed early after productive infection is transcribed (35).

In previous publications from this laboratory, the size classes and the kinetics of appearance of Ad12 mRNA in polysomes have been described for abortively infected and transformed hamster cells (30), as well as for productively infected human cells (40). It was shown that the Ad12-specific mRNA synthesized in abortively infected and in transformed hamster cells corresponded in size to some of the viral mRNA's observed early in productive infection. However, a precise correlation between the different size classes of Ad12 mRNA synthesized in different cell systems was not possible without the establishment of a detailed transcriptional map of the Ad12 genome.

For several virus systems transcriptional

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maps of the viral genome have been presented, e.g., for simian virus 40 (24, 25, 39), for polyoma virus (23), and for Ad2 (33,34, 42, 48, 49). Functional maps of Ad2 were obtained by in vitro translation of viral mRNA isolated from productively infected cells (1, 16, 26, 29). The Ad2-specific mRNA's were preselected on specific restriction endonuclease fragments of Ad2 DNA, and the translation products were characterized by polyacrylamide gel electrophoresis (1, 16, 26) or immunoprecipitation (29).

There is evidence that Ad12 DNA becomes integrated into the host genome in abortively infected BHK-21 cells (7, 9). In Ad12-transformed hamster cells, viral DNA has been shown to persist (18, 19), and different fractions of the viral genome are represented in unequal amounts (17). The viral DNA sequences persisting in 10 lines of Ad2-transformed and in two lines of Ad5-transformed cells have been well characterized (38). However, there is as yet scarce information about the actual physical state of the viral genome in transformed cells. For an avian adenovirus evidence has been presented for the integrated state of the viral genome in transformed hamster cells (3), and similarly for Ad12 DNA in several lines of transformed hamster cells (20; J. Groneberg, Y. Chardonnet, and W. Doerfler, submitted for publication). It is conceivable that viral DNA persisting in an integrated state in the cellular genome is subject to cellular transcriptional controls. This possibility exists also in productively infected cells, as evidence has been adduced for an integrated state of Ad2 DNA in productively infected cells as well (5, 10, 41).

In this paper we report on the transcriptional maps of Ad12-specific mRNA and stable nuclear RNA isolated from productively infected KB cells, from abortively infected BHK-21 cells, and from two Ad12-transformed cell lines, T637 and HA12/7. The T637 (46) and HA12/7 (55) cell lines were derived by in vitro transformation of BHK-21 cells and primary Syrian hamster cells, respectively, with Ad12. Moreover, the 3'-5' polarity of the two strands of Ad12 DNA was determined relative to the restriction map of Ad12 DNA.

MATERIALS AND METHODS

Cell lines and virus. The HA12/7 cell line was derived from primary Syrian hamster kidney cells by transformation with Ad12 (55) and was obtained from Harald zur Hausen, Erlangen. The HA12/7 cells were grown either in monolayers in reinforced Eagle medium supplemented with 5% fetal calf serum (Flow Laboratories) or in suspension cultures in Eagle medium modified for suspension culture containing 10% newborn calf serum (Flow Laboratories). The origin of the BHK-21 (44), T637 (46), previously termed HB line (30), KB cells (15) and of human Ad12, as well as the media and methods used in their propagation, have been described elsewhere (30). Some of the properties of the T637 and HA12/7 cell lines are summarized in Table 1.

Radioisotopes and chemicals. [6-^{3}H]thymidine (specific activity, 24 to 26 Ci/mmol), [5-^{3}H]uridine (specific activity, 25 to 30 Ci/mmol), and the four deoxyribonucleoside triphosphates (specific activity, 20 to 40 Ci/mmol) were purchased from Amersham-Buchler, Braunschweig, Germany.

Agarose (electrophoretic grade) was bought from Sigma Chemical Co., St. Louis, Mo., or from l'Industrie Biologique, Gennevilliers, France (indubiose A 37). Hydroxyapatite (Hypatite C) was obtained from Clarkson Co., Williamsport, Pa., and α-amylase was from the Sigma Chemical Co. Polyuridylic-polyguanylic acid [poly(U,G)] (Schwarz/Mann, lot no. 7001) was a gift from L. Philipson, Uppsala, Sweden. The sources of all other chemicals and reagents have been described previously (30).

Enzymes. The single-strand-specific nuclease S1 was purified from α-amylase as described elsewhere (50) and was a gift from J. Schick and E. Fanning of our laboratory. Restriction endonuclease *Eco*RI (21) was isolated according to the procedure of R. N. Yoshimori (Ph.D. thesis, Univ. of California, San Francisco, 1971), as described previously (10).

Restriction endonuclease *Bam*HI from *Bacillus amyloliquefaciens* H-I (53) was purified as follows. Strain HM of *B. amyloliquefaciens*, a gift from E. Viñuela, Madrid, was grown in L broth (6), and the cells were harvested in mid-logarithmic phase. The cells (approximately 12 g) were suspended in 12 ml of 0.01 M Tris-hydrochloride, pH 7.5, 0.01 M β-mercaptoethanol and disrupted by ultrasonic treatment at 0°C for five 1-min periods, and the crude extract was clarified by centrifugation at 105,000 × *g* for 1 h at 0°C in an L2-65B Spinco ultracentrifuge. The supernatant was adjusted to 1.0 M NaCl and applied to a Bio-Gel A-0.5m column (4 by 50 cm), which was equilibrated and eluted with 1.0 M NaCl, 0.02 M Tris-hydrochloride, pH 7.5, 0.01 M β-mercaptoethanol at 40 ml/h. The fractions were assayed for enzymatic activity by incubating Ad2 DNA (1 μg) in

TABLE 1. *Properties of Ad12-transformed hamster cell lines*

Cell line	Cells used for transformation	Multiplicity of Ad12 used in transformation	T antigen	Oncogenicity	Reference
T637	BHK-21	350	+	+	46
HA12/7	Primary Syrian hamster	10	−/+[a]	+	55

[a] These cells are T antigen negative by immunofluorescence (H. zur Hausen, personal communication), but T antigen positive by complement fixation (K. Raška, Jr., personal communication). The content of viral DNA in the two cell lines has been determined (17).

0.01 M Tris-hydrochloride, pH 7.5, 0.01 M $MgCl_2$, 0.01 M β-mercaptoethanol (*Bam* buffer) with 3- to 5-μl portions of each fraction at 37°C for 2 h and by subjecting the reaction mixture to electrophoresis on 1% agarose slab gels as described elsewhere (43). The fractions that contained restriction endonuclease activity were pooled and dialyzed against two changes of 0.01 M KPO_4, pH 7.5, 0.01 M β-mercaptoethanol, 0.0001 M EDTA, and 10% glycerol at 4°C for 24 h. Subsequently, the material was applied to a phosphocellulose (P-11, Whatman) column that had been equilibrated with the same buffer. The column was washed with the same buffer and eluted with a linear gradient from 0 to 0.7 M KCl in the same buffer at 12 ml/h. The restriction endonuclease eluted at 0.55 M KCl. Generally, 1 μl of purified enzyme was sufficient to digest completely 1 μg of Ad2 DNA, as assayed by slab gel electrophoresis in 1% agarose gels.

Exonuclease III was prepared as described by Richardson et al. (36) or was a gift of Heinz Schaller, Heidelberg. Reverse transcriptase from avian myeloblastosis virus (AMV) was isolated according to the procedure described by Kacian et al. (22) and was a gift of Karin Moelling, Berlin.

DNase I, electrophoretically purified, was obtained from Worthington Biochemicals Corp. (Freehold, N.J.) and was inactivated with iodoacetate as described elsewhere (54).

Pronase (B grade), free of endonuclease, was purchased from Calbiochem and preincubated at 37°C for 2 h before use.

Preparation of ^{3}H-labeled Ad12 DNA of high specific activity. Confluent monolayers of KB cells were inoculated with Ad12 at a multiplicity of infection of 1 to 10 PFU/cell. After a 2-h adsorption period, the inoculum was removed and fresh Eagle medium with 10% dialyzed calf serum was added. At 8 h postinfection, [6-^{3}H]thymidine (20 μCi/ml) was added, and the infected cells were harvested 50 to 70 h postinfection. The labeled virus was purified by three cycles of equilibrium centrifugation as described previously (8). The viral DNA was isolated from the purified virions, as detailed elsewhere (14), and had specific activities of 0.5×10^6 to 10^6 cpm/μg).

Isolation of Ad12 DNA fragments generated by the *Eco*RI and *Bam*HI restriction endonucleases. The ^{3}H-labeled Ad12 DNA was prepared as described in the preceding section. A total of 20 to 40 μg of Ad12 DNA was incubated with 20 to 40 μl of *Eco*RI endonuclease (Bio-Gel fraction) in 0.05 M Tris-hydrochloride, pH 7.5, 0.01 M $MgCl_2$ for 2 h at 37°C. Subsequently, the reaction mixture was extracted once with Tris-equilibrated phenol. The fragments of viral DNA were separated by electrophoresis in 1.5% polyacrylamide-0.8% agarose gels for 12 h (31) or in 1.0% agarose gels for 3 to 4 h (43). Gels were sliced, and the DNA was eluted from each slice by incubation at 37°C for 18 h in 0.1% sodium dodecyl sulfate (SDS), 0.01 M Tris-hydrochloride, pH 7.5, 0.001 M EDTA.

Alternatively, Ad12 DNA was fragmented by incubation with the *Bam*HI restriction endonuclease as outlined above, and the fragments were isolated by electrophoresis in 1.5% polyacrylamide-0.8% agarose gels as described for the *Eco*RI fragments.

In some experiments, the *Bam*HI C fragment was subsequently digested with *Eco*RI endonuclease to yield the C* fragment (see Fig. 1c; 9). For this purpose, the *Bam*HI C + D fragments were purified after elution from mixed polyacrylamide-agarose gels by centrifugation for 16 h at 40,000 rpm and 20°C in a discontinuous CsCl density gradient (SW56 rotor) consisting of 1.5-ml layers of densities 1.8 and 1.4 g/cm^3. Fractions containing the DNA fragments were pooled, dialyzed against *Eco*RI buffer, and incubated with *Eco*RI endonuclease as described. Again, the fragments generated were separated by electrophoresis on polyacrylamide-agarose gels.

Reassociation kinetics. The genetic complexities of the Ad12 DNA fragments were determined by reassociation kinetics (4, 52). The details of this procedure were described elsewhere (10). Alkali-denatured DNA was reannealed in 1.0 M NaCl, 0.01 M Tris-hydrochloride, pH 7.5, at 68°C. The amount of ^{3}H label in double-stranded DNA was measured after digestion with S1 nuclease, as detailed below.

Separation of the complementary strands of intact Ad12 DNA and of Ad12 DNA fragments. Intact Ad12 DNA or restriction endonuclease fragments of Ad12 DNA were denatured in 0.2 N NaOH for 10 min at ambient temperature. The samples were neutralized with NaH_2PO_4, and poly(U,G) was added in an amount equivalent to three times the weight of Ad12 DNA used in the experiment. The poly(U,G)-DNA complexes were separated by equilibrium sedimentation in CsCl density gradients (initial density, ρ = 1.775 g/cm^3) for 60 h at 35,000 rpm and 20°C in the 50-Ti fixed-angle rotor. The fractions containing the "heavy" (H) and "light" (L) strands were pooled, and the poly(U,G) was degraded by incubating the fraction in 0.2 N NaOH for 60 min at 37°C. Subsequently, the fractions were neutralized with HCl and subjected to ultrasonic treatment for 3 min at 0°C. The DNA was then dialyzed at 4°C against 1.0 M NaCl, 0.02 M sodium phosphate, pH 6.8, 0.05% Sarkosyl and reassociated at 68°C to a C_0t value of 500 times the $C_0t_{1/2}$ of Ad12 DNA. For all strand separation experiments reported in this paper one batch of poly(U,G) was used (lot no. 7001, Schwarz/Mann).

The single-stranded DNA in excess was purified by hydroxyapatite chromatography as follows. Upon reassociation, the DNA was diluted 10× in 0.02 M sodium phosphate, pH 6.8, and applied to hydroxyapatite columns of 0.5-ml volume at 60°C. The single-stranded DNA was eluted with 5 column volumes of 0.14 M sodium phosphate, pH 6.8, 0.1% SDS, and double-stranded DNA was eluted with 5 column volumes of 0.4 M sodium phosphate, pH 6.8, 0.1% SDS at 60°C. The isolated strands separated by this method self-annealed to less than 5%, as determined by digestion with S1 nuclease under conditions that allowed 40 to 100% reassociation of the mixed H and L complements (Table 2). The isolated strands of intact Ad12 DNA were also assayed for their ability

to reassociate by themselves or in an equimolar mixture (Table 2). Contamination of the H and L strands with the complement was ≤5%.

Each of the complementary strands from restriction endonuclease fragments of Ad12 DNA was assigned as being derived from the H or L strand of Ad12 DNA by hybridization to each of the intact complementary strands (Table 4). The method of nucleic acid hybridization is described below.

For those experiments in which the assignment of the two complements of each of the restriction enzyme fragments of Ad12 DNA was tested, the separated strands of intact Ad12 DNA were prepared as follows. ^{14}C-labeled Ad12 DNA was alkali denatured, and the two strands were separated by equilibrium sedimentation in poly(U,G)-CsCl density gradients. The fractions pooled from the heavy- and light-density strata were dialyzed against 0.01 M Tris, pH 7.5, 0.001 M EDTA and subsequently adjusted to 0.3 N NaOH. The H and L DNA fractions were then sedimented into alkaline 5 to 20% sucrose density gradients in an SW41 rotor at 35,000 rpm and 4°C for 7 h. Fractions containing full-length strands were selected and further purified by reassociation and chromatography on hydroxyapatite as described above.

TABLE 2. *Test for purity of the isolated strands of individual EcoRI and BamHI fragments as well as of intact Ad12 DNA* [a]

Fragment	% of ^3H cpm resistant to S1 nuclease after incubation under reannealing conditions of:		
	H strand	L strand	H + L strands
*Eco*RI			
A	2	1	81
B	3	2	34
C	2	1	80
D	3	4	100
E	2	3	39
F	5	5	50
*Bam*HI			
A	2	1	88
B	2	1	85
C*	7	3	50
E	1	1	68
G	2	2	64
Intact Ad12 DNA	3	5	70

[a] The ^3H-labeled *Eco*RI and *BAM*HI fragments of Ad12 DNA were isolated, and the complementary strands of each of the fragments were separated by equilibrium centrifugation in poly(U,G)-CsCl density gradients. After reannealing, the strands were purified by chromatography on hydroxyapatite as described in the text. The isolated H and L strands of each fragment were either self-annealed or annealed in an equimolar mixture. At the end of the annealing reaction, the amount of double-stranded DNA was determined after digestion with S1 nuclease. As a control, the separated strands of intact Ad12 DNA were also tested by self-annealing. The fact that the H + L strands of some of the fragments hybridize relatively inefficiently is probably due to low DNA concentrations.

Isolation of mRNA. The mRNA from mock-infected or Ad12-infected KB or BHK-21 cells, as well as from the transformed T637 and HA12/7 lines, was isolated from polysomal RNA by affinity chromatography on poly(U)-Sepharose as previously reported (30).

Isolation of nuclear RNA. Nuclei were isolated as described previously (30), resuspended in 0.15 M NaCl, 0.01 M Tris-hydrochloride, pH 8.0, 0.001 M EDTA, and lysed at 4°C by the addition of SDS and Sarkosyl to a final concentration of 1% each. The lysate was extracted with phenol-chloroform-isoamyl alcohol according to the procedure of Weinberg et al. (51). The nucleic acids were precipitated overnight at −20°C by adding 2 volumes of ethanol. The precipitate was resuspended in 0.1 M NaCl, 0.05 M Tris-hydrochloride, pH 7.5, 0.01 M $MgCl_2$ and incubated at 37°C with DNase I (20 μg/ml) for 60 min. The reaction was stopped by the addition of EDTA and SDS to final concentrations of 0.02 M and 1%, respectively, and the RNA was extracted with phenol-chloroform-isoamyl alcohol. The DNase treatment was repeated, and the RNA was precipitated again with ethanol. The RNA was resuspended in 0.1× SSC and stored frozen at −20°C. The procedures used were identical irrespective of whether KB or hamster cells were used.

DNA-RNA hybridization conditions. Hybridization reactions were carried out in mixtures containing various amounts of mRNA or nuclear RNA and 1 to 3 ng of Ad12 DNA in 50 to 100 μl of 1.0 M NaCl, 0.1% SDS, 0.01 to 0.05 M sodium phosphate, pH 6.8, and 1 μg of carrier RNA per μl. The H or L strand of Ad12 DNA (specific radioactivity, 0.5×10^6 to 10^6 cpm/μg) or corresponding molar amounts of the H or L strand of the *Eco*RI or *Bam*HI fragments were used in the hybridization reactions and incubated at 68°C for 100 h. Subsequently, the samples were quickly cooled to 0°C, diluted to 1.0 ml with 0.3 M NaCl, 0.03 M sodium acetate, pH 4.6, 0.005 M $ZnSO_4$, and incubated with S1 nuclease (2 to 10 μl) for 2 h at 45°C. The amount of S1 nuclease used was sufficient to degrade all single-strand DNA present, as determined in reconstruction experiments. The fraction of DNA remaining resistant to S1 nuclease was determined by precipitation with 10% trichloroacetic acid for 10 min at 0°C. The precipitates were collected on glass-fiber filters (Whatman GF/C).

DNA-DNA reannealing experiments to determine the purity of isolated intact DNA or fragment complements or the assignment of fragment complements to the intact strands of Ad12 DNA were performed under the same conditions. A mixture of equimolar amounts of each of the complementary strands reassociated to 40 to 100%, whereas each of the complements by itself annealed to ≤5% (Table 2).

Saturation hybridization. Saturation hybridization experiments were performed using the isolated strands of the ^3H-labeled *Eco*RI and *Bam*-HI fragments of Ad12 DNA and increasing amounts of un-

labeled RNA, as indicated in Fig. 8. Saturation levels were calculated as described by Lucas and Ginsberg (27).

Addition hybridization. To determine the extent to which the regions on the Ad12 genome expressed early in productive infection coincide with those transcribed in Ad12-infected BHK-21 cells, T637 cells, and HA12/7 cells, addition hybridization experiments were performed. The H or L strands of the ^{3}H-labeled *Eco*RI and *Bam*HI fragments of Ad12 DNA were incubated simultaneously with 35 μg of unlabeled RNA isolated from Ad12-infected KB cells 8 h postinfection (p.i.) and with saturating amounts of unlabeled RNA from Ad12-infected BHK-21, T637, and HA12/7 cells. The degree of hybridization obtained under these conditions was compared with that observed in experiments in which RNA from KB cells was omitted.

Determination of the polarity of the H and L strands of Ad12 DNA relative to the restriction map. The method of Arrand et al. (2) was followed, with minor modifications. Ad12 DNA (2 to 4 μg) was treated with *Escherichia coli* exonuclease III to digest approximately 10% of the nucleotides on the 3'-terminus of each strand. Conditions for the exonuclease III reaction were described elsewhere (2, 36). After treatment with exonuclease III, the single-stranded ends were filled in with ^{3}H-labeled deoxythymidine triphosphate (20 to 40 Ci/mmol) using reverse transcriptase from AMV under conditions similar to those reported by Kacian et al. (22). The reaction mixture consisted of 60 μl of exonuclease III-treated DNA in 0.01 M Tris-hydrochloride, pH 7.9, 0.002 M $MgCl_2$, 7 μl of 0.1 M $MgCl_2$, 5 μl of 1 M KCl, 1 μl of 0.1 M dithiothreitol, 2 μl each of 0.01 M dATP, dGTP, and dCTP, 1 μl of 0.005 M dTTP, 250 μCi of [^{3}H]dTTP (corresponding to approximately 5 nmol), 10 μl of 50% glycerol, and 10 μl of AMV reverse transcriptase. At the end of the incubation, Pronase B and SDS were added at 250 μg/ml and 0.5%, respectively, and the mixture was extracted with Tris-saturated phenol. The DNA was efficiently separated from the deoxyribonucleoside triphosphates on a minicolumn (4 by 0.5 cm in diameter) of P-30 (Bio-Rad), which was eluted with 0.01 M Tris-hydrochloride, pH 7.5, 0.002 M $MgCl_2$. The DNA was then cleaved with the *Bam*HI restriction endonuclease as described above, and the fragments were separated on polyacrylamide-agarose gels. Lastly, the strands of the ^{3}H-labeled terminal fragments (E and A; Fig. 1c) were separated by equilibrium centrifugation in CsCl density gradients in the presence of poly(U,G) (see above). The position of the H and L strands was identified by the buoyant density and comparison to the banding pattern of uniformly ^{3}H-labeled fragment strands in a control tube.

The strand assignment of the terminal fragment strands (*Bam*HI A and E fragments), which were labeled in vitro after exonuclease III digestion with the AMV reverse transcriptase, was determined by DNA-DNA hybridization (for experimental details, see above) to the isolated intact strands of Ad12 DNA and to the isolated strands of the terminal *Bam*HI or *Eco*RI fragments (Table 5).

Determination of radioactivity. The radioactivity on dried filters was measured in a toluene-based scintillator. The radioactivity in liquid samples was determined in the toluene-methanol-based scintillator as described elsewhere (30). Tri-Carb liquid scintillation spectrometers, Packard models 3380 or 3330, were used in all studies.

RESULTS

Isolation and characterization of the *Eco*RI and *Bam*HI fragments of the DNA of Ad12. Restriction endonucleases *Eco*RI and *Bam*HI were used to fractionate the genome of Ad12, to facilitate mapping by DNA-RNA hybridization of the Ad12-specific mRNA and nuclear RNA from cells infected or transformed by Ad12. The resolution of the transcriptional maps obtained in this manner is obviously dependent on the average size of the DNA fragments. For the establishment of a complete transcriptional map, it would also be necessary to have available the isolated complementary strands of each of the fragments, since the approach of using the isolated complementary strands avoids high backgrounds in the DNA-RNA hybridization reaction due to reannealing of the complementary DNA strands.

The Ad12 DNA fragments generated by the *Eco*RI and *Bam*HI restriction endonucleases can be separated by gel electrophoresis as demonstrated in Fig. 1a and b, respectively. The scheme in Fig. 1c illustrates the location of each of the fragments on the Ad12 DNA molecule (28; H. Delius and C. Mulder, personal communication). In keeping with the convention that the more deoxyadenosine-deoxythymidine-rich terminus of a DNA molecule be positioned to the right (11, 12), the *Eco*RI A fragment has been placed on the right end (Delius and Mulder, personal communication). The molecular weights of the *Bam*HI restriction endonuclease fragments of Ad12 DNA were estimated from their relative mobilities as determined by gel electrophoresis, using the *Eco*RI and *Bam*HI fragments of Ad2 DNA as references (cf. inset to Fig. 1b), and are presented in Table 3.

Separation of complementary strands. The separation of the complementary strands of either ^{3}H-labeled intact Ad12 DNA or the ^{3}H-labeled restriction endonuclease fragments of Ad12 DNA was accomplished by differential poly(U,G) binding and equilibrium centrifugation of the resulting complexes in CsCl density gradients (Fig. 2), as described by Tibbetts et al. (49). To avoid single-strand breaks in the high-specific-activity DNA, strand separation was performed within 3 days of the preparation of labeled virus, when intact Ad12 DNA was

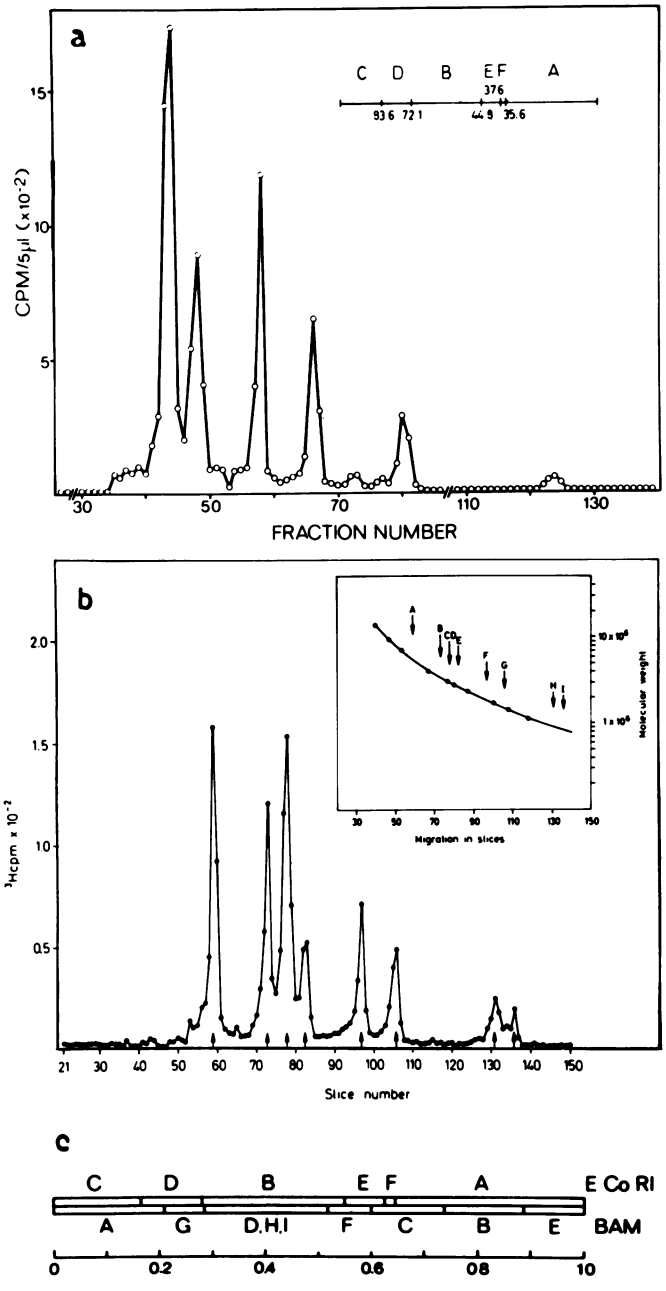

FIG. 1. *Separation by gel electrophoresis of the EcoRI (a) and BamHI (b) restriction endonuclease fragments of Ad12 DNA.* ^{3}*H-labeled Ad12 DNA was digested with either EcoRI (a) or BamHI (b) restriction endonucleases as described in the text, and the digestion products were analyzed by electrophoresis on cylindrical (0.6 by 12 cm for EcoRI digests or 0.6 by 15 cm for BamHI fragments) gels consisting of 1.5% polyacrylamide and 0.8% agarose. The samples were subjected to electrophoresis for 12 or 15 h at 40 V and ambient temperature. Subsequently, the gels were sliced into 0.9-mm disks, the DNA was eluted, and 5-μl portions of each fraction were counted. The inset to (a) presents the map locations and relative sizes (percentage of total length) of the EcoRI fragments of Ad12 DNA (28). From the inset to (b) the molecular weights of the BamHI fragments of Ad12 DNA (vertical arrows) can be calculated (cf. Table 3). The curve is drawn through a plot of the molecular weights of the EcoRI (32) and BamHI fragments (R. J. Roberts, personal communication) of Ad2 DNA. In (c) are shown the maps of the EcoRI and BamHI restriction nuclease fragments of Ad12 DNA (Delius and Mulder, personal communication).*

TABLE 3. *Molecular weights of the BamHI restriction endonuclease fragments of Ad12 DNA*[a]

Fragment	Mol wt (× 10^{-6})[b]
A	4.9
B	3.3
C	2.8
D	2.8
E	2.6
F	1.9
G	1.7
H	1.0
I	0.9

[a] The DNA of Ad12 was cleaved with the *Bam*HI restriction enzyme, and the fragments were separated on composite polyacrylamide-agarose gels as described. The ^{14}C-labeled *Eco*RI and *Bam*HI fragments of Ad2 DNA were used as molecular weight markers. Compare experiment presented in the inset to Fig. 1b.

[b] Average of five determinations.

used, or within 1 week, when Ad12 DNA fragments were used. The reassociation of the separated H and L strands of Ad12 DNA followed second-order kinetics (Fig. 3). Similar results were obtained upon reannealing of denatured Ad12 DNA without prior separation of the complements. The intactness of the isolated strands was ascertained by zonal sedimentation in alkaline sucrose density gradients.

The efficiency of separation of the complementary strands of the restriction fragments was dependent on the fragment used. Some of the fragments gave very good separation, like *Eco*RI A and C or the *Bam*HI fragments A, B, and E; other fragments, like *Eco*RI B, separated very poorly (Fig. 2). For each restriction endonuclease fragment the H and L fractions were purified further by self-annealing and chromatography on hydroxyapatite as described above. The fraction of DNA remaining single stranded upon self-annealing could then be used in DNA-RNA mapping experiments.

Assignment of fragment complements to the intact strands of Ad12 DNA. The assignment of the separated strands of each restriction enzyme fragment was determined by annealing the H and L strands of each restriction fragment to the intact H and L strands of Ad12 DNA (Table 4).

The complementarities of the H and L strands of *Eco*RI fragments A, B, E, and F to the L and H strands, respectively, of the intact Ad12 DNA molecule allow the conclusion that the H and L complements of these fragments are parts of the intact H and L strands, respectively, of Ad12 DNA (Table 4, part 1). The situation on the left-hand end of the molecule (cf. Fig. 1c) is complicated in that the H and L strands of the *Eco*RI fragments C and D hybridize to the intact H and L strands of Ad12 DNA (Table 4, part 1). A similar apparent anomaly is observed when the extent of annealing of the H

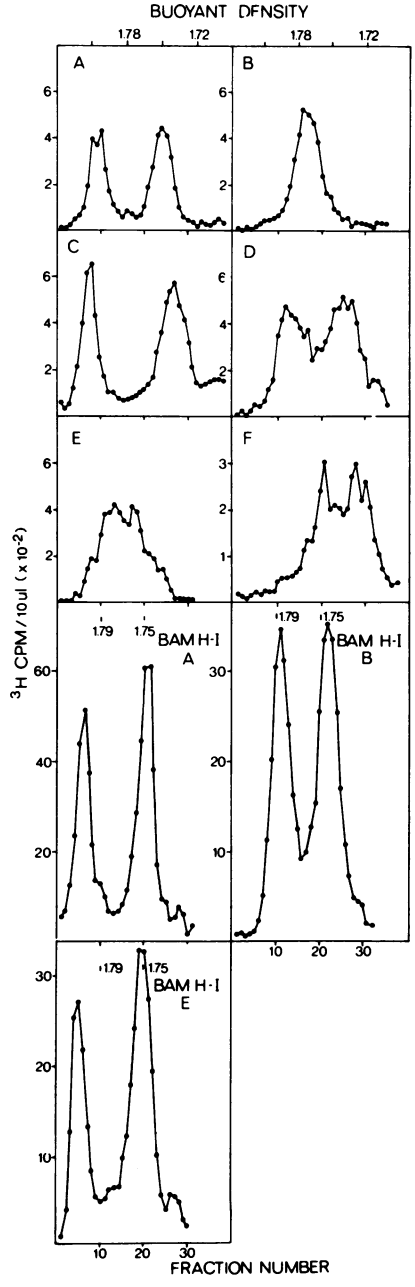

FIG. 2. *Separation of the complementary strands of EcoRI fragments A, B, C, D, E, and F and BamHI fragments A, B, and E of Ad12 DNA by poly(U,G) binding and equilibrium sedimentation in CsCl density gradients. Experimental details of the separation technique are described in the text.*

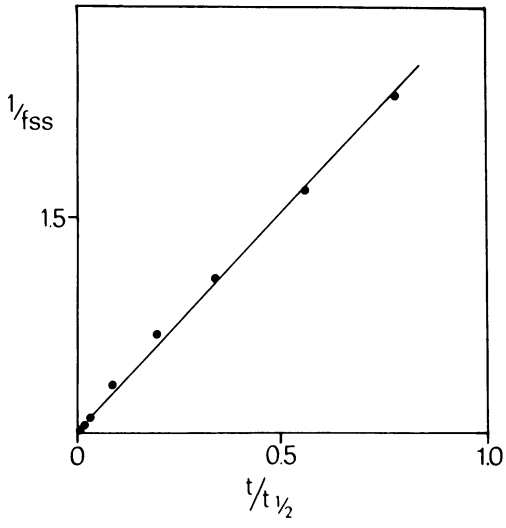

FIG. 3. *Reassociation of the separated H and L strands of Ad12 DNA. Equal amounts of ^3H counts per minute of the H and the L complements of Ad12 DNA were mixed and reassociated under conditions as described. At various times after starting the reaction, samples were withdrawn and processed as described in the text. The data were plotted as described by Sharp et al. (42).*

and L strands of the *Bam*HI fragments A and G (left end of molecule, cf. Fig. 1c) to the intact H and L strands of Ad12 DNA is measured (Table 4, part 2). On the other hand, the H and L complements of the *Bam*HI fragments C*, B, and E (located on the right end of the molecule, cf. Fig. 1c) hybridize to the intact L and H strands, respectively, as one would have expected (Table 4, part 2). The simplest explanation for these results is that on the left-hand end of the Ad12 DNA molecule comprising about 0.25 to 0.30 fractional length unit (cf. Fig. 1c, 11), the H complement, which binds more poly(U,G) in CsCl density gradients, is part of the intact L strand and vice versa. The limit of this "strand reversal" must lie to the left of the right terminus of the *Bam*HI G fragment, since the H strand of the *Bam*HI G fragment shows significant hybridization to the intact L strand (16%) and the fragment L strand to the intact H strand (23%) (Table 4, part 2). This result indicates that the strand assignment in the rightmost part of the *Bam*HI G fragment might be "normal." Hybridization of the H and L strands of the *Eco*RI fragments C and D to the L and H strands of the *Bam*HI A fragment is as expected (Table 4, part 3), since one scores for complementarities of strands from the same region of the Ad12 DNA (cf. Fig. 1c).

Further support for the conclusions advanced here comes from the results of hybridization experiments using the in vitro labeled termini of Ad12 DNA (see below).

Polarity of the H and L strands of Ad12 DNA. The polarity of the H and L strands of AD12 DNA was determined as outlined previously (2) and as described in detail in Materials and Methods. The results of this series of experiments are summarized in Fig. 4. Upon digestion of intact, unlabeled Ad12 DNA with *E. coli* exonuclease III and resynthesis of the single-stranded 3'-termini with ^3H-labeled nucleoside triphosphates using the AMV reverse transcriptase, the terminal fragments of the mole-

TABLE 4. *Identification of the complementary strands of the EcoRI fragments A through F and the BamHI fragments A, B, C*, E, and G by annealing to the intact H and L strands of Ad12 DNA*[a]*

Part	Restriction enzyme fragment (^3H labeled)		Strand	% of ^3H cpm hybridized to unlabeled or ^{14}C-labeled, intact Ad12 DNA	
				H strand	L strand
1	*Eco*RI	A[b]	H	4	88
			L	70	3
		B[b]	H	16	58
			L	45	16
		C[c]	H	61	7
			L	12	65
		D[c]	H	80	2
			L	6	54
		E[b]	H	11	77
			L	47	7
		F[b]	H	12	91
			L	45	4
2	*Bam*HI	A[c]	H	78	8
			L	8	47
		G[c]	H	66	16
			L	23	59
		C*[b]	H	8	46
			L	79	11
		B[b]	H	7	73
			L	74	3
		E[b]	H	7	83
			L	96	1
3[d]	*Eco*RI	C[b]	H	16	96
			L	99	3
		D[b]	H	0	83
			L	100	12

[a] The preparation of intact strands of Ad12 DNA and of reannealing conditions have been described in the text.

[b] Mean of five experiments. In some of the experiments independently made preparations of intact and fragment strands were used.

[c] These values represent the mean of eight determinations from three independent experiments.

[d] Results for part 3 are given as percentage of ^3H counts per minute hybridized to unlabeled *Bam*HI fragment A.

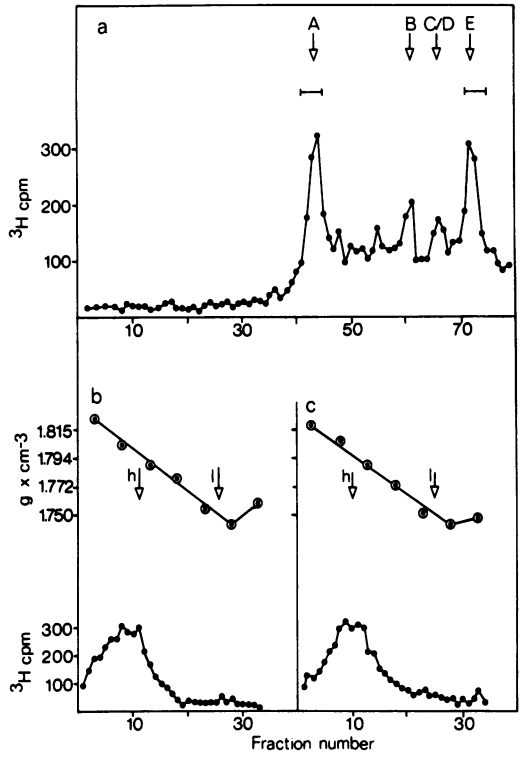

FIG. 4. *Determination of the polarity of the H and L strands of Ad12 DNA. Ad12 DNA was degraded with E. coli exonuclease III, and the single-stranded ends of the DNA were resynthesized with AMV reverse transcriptase as described in the text using [^{3}H]dTTP. (a) The labeled Ad12 DNA molecules were subsequently cleaved with the BamHI restriction endonuclease, and the fragments were separated on composite agarose-polyacrylamide gels. The arrows indicate the positions of the BamHI fragments of Ad12 marker DNA subjected to electrophoresis in a parallel gel. A and E refer to the terminal BamHI fragments of Ad12 DNA. The bars indicate the fractions pooled from which the DNA was extracted for poly(U,G)-CsCl equilibrium centrifugation. (b) BamHI fragment A was alkali denatured, poly(U,G) was added, and the mixture was subsequently neutralized and centrifuged to equilibrium in a CsCl density gradient as described in the text. The arrows indicate the positions of the H and L strands of homogeneously ^{3}H-labeled fragment A in a parallel poly(U,G)-CsCl density gradient (⊙——⊙). (c) BamHI fragment E was analyzed as described for fragment A in (b). The arrows indicate the positions of the H and L strands of homogeneously ^{3}H-labeled fragment E analyzed in a parallel gradient (⊙——⊙).*

cule, *Bam*HI fragments A (left terminus) and E (right terminus), became preferentially labeled. This was shown by cleavage with the restriction endonuclease *Bam*HI, fractionation of the fragments on gels (Fig. 4a), and separation of the strands of the terminal *Bam*HI fragments A (left end) and E (right end) by equilibrium sedimentation in poly(U,G)-CsCl density gradients. The ^{3}H-label was found in the H complement in both the A and E fragments (Fig. 4b and c).

On the left terminus of the Ad12 DNA molecule, the H complement of an 0.25- to 0.30-fractional-length-unit fragment is part of the intact L strand (Table 4). Thus, the data presented in Fig. 4 demonstrate that the intact H strand carries the 3'-end on the right terminus and the L strand has its 3'-end on the left terminus of the molecule (Fig. 9–11). The left 3'-terminal fragment strand, however, is the H strand in poly(U,G)-CsCl density gradients (Fig. 4).

To substantiate this interpretation, the H complements of the terminal *Bam*HI A and E fragments which were labeled in vitro on their 3'-termini were hybridized to the H and L strands of intact Ad12 DNA. The *Bam*HI A–H complement, part of the intact L strand, hybridizes to the intact H strand, the *Bam*HI E–H complement, part of the intact H strand, hybridizes to the intact L strand (Table 5). As one would expect, the in vitro labeled *Bam*HI A–H strand hybridizes to the *Bam*HI A–L and *Eco*RI C–L complements, as well as to the *Eco*RI D–L strand. In the latter case, the overlap with the *Bam*HI A–H strand is only partial, and hence the extent of hybridization is only 21% (Table 5; cf. Fig. 1c). The *Bam*HI E–H complement, labeled in vitro, anneals to the *Bam*HI E–L strand. Thus, the strand assignment of the terminal fragments and the polarity of the strands is unequivocal.

Strand homologies of Ad12-specific mRNA and stable nuclear RNA. Both the mRNA and stable nuclear RNA from Ad12-infected and -transformed cells were analyzed for homology with the separated H and L strands of Ad12 DNA by saturation hybridization. Polysomal or nuclear RNA was isolated from KB cells early, at 8 h p.i., from BHK-21 cells at 12 h p.i., and from the Ad12-transformed hamster cell lines T637 and HA12/7.

The data presented in Fig. 5 demonstrate that, as expected, uninfected KB cells do not contain Ad12-specific RNA (Fig. 5a) and that, 8 h p.i., the Ad12-specific mRNA corresponds to about 20% of each strand (Fig. 5b). Thus, early after infection, about 40% of the transcriptional capacity of the Ad12 genome is expressed. From the amount of mRNA required to saturate 1 ng of ^{3}H-labeled Ad12 DNA, it can be calculated that, 8 h p.i., approximately 0.05% of the total mRNA in the polysome fraction is Ad12 specific.

TABLE 5. *Strand assignment of the heavy complements of the terminal BamHI A and E fragments labeled in vitro by digestion with E. coli exonuclease III and resynthesis by AMV reverse transcriptase*[a]

Fragment complement labeled in vitro	%[b] ^{3}H cpm hybridized to:									
	Intact Ad12 DNA		*Bam*HI A fragment		*Eco*RI C fragment		*Eco*RI D fragment		*Bam*HI E fragment	
	H	L	H	L	H	L	H	L	H	L
*Bam*HI A-H	82	10	4	70	2	44	1	21		
*Bam*HI E-H	3	94							0	75

[a] The 3'-termini of either strand of intact Ad12 DNA were ^{3}H labeled by digestion with exonuclease III from *E. coli* and subsequent resynthesis with AMV reverse transcriptase using [^{3}H]TTP as described in the text. The labeled molecules were then cleaved with the *Bam*HI restriction endonuclease, the terminal fragments *Bam*HI A and E were isolated, and the complementary strands of either fragment were separated as described in Fig. 4. For both fragments A and E, the ^{3}H label was found in that complement that had the higher buoyant density in poly(U,G)-CsCl density gradients. Each of the two labeled heavy fragment complements was identified as to strand assignment by hybridization in a liquid system to the H and L strands of intact Ad12 DNA. In control experiments, the heavy strand of the *Bam*HI A fragment was hybridized to the isolated strands of the *Bam*HI A and the *Eco*RI C and D fragments. Similarly, the heavy strand of the *Bam*HI E fragment was annealed with the complementary strands of the *Bam*HI E fragment. The method of hybridization was outlined in the text.

[b] The values represent the mean of five determinations in two experiments.

The same type of analysis was carried out with the mRNA from mock-infected (Fig. 6a) and Ad12-infected BHK-21 cells (12 h p.i.) (Fig. 6b), as well as with mRNA from the Ad12-transformed hamster cell lines T637 (Fig. 6c) and HA12/7 (Fig. 6d). The results of these experiments demonstrate that BHK-21 cells are devoid of Ad12-specific mRNA and that, for the Ad12-infected or transformed cells, the mRNA isolated from BHK-21 cells 12 h p.i. with Ad12 contains sequences complementary to approximately 10% of the H and about 8% of the L strands. In T637 cells the Ad12-specific mRNA corresponds to approximately 25% of the H and about 18% of the L strands. In HA12/7 cells, however, mRNA complementary to the L strand amounts to only approximately 3 to 4%, whereas the H strand is transcribed up to a saturation value of about 15%. From these results, it has been calculated that in Ad12-infected and -transformed hamster cells 0.01 to 0.02% of the mRNA isolated from polysomes represents virus-specific sequences.

Similar experiments were performed with nuclear RNA isolated from uninfected KB cells and from KB cells at 8 h p.i. with Ad12 (Fig. 5c) or from uninfected BHK-21 cells (Fig. 7a), BHK-21 cells at 12 h p.i. with Ad12 (Fig. 7b), T637 cells (Fig. 7c), and HA12/7 cells (Fig. 7d). In general, the results are similar to those obtained with mRNA, except that the level of transcription appears to be increased, especially for the H strand transcribed early in productively infected KB cells. In HA12/7 cells the amount of nuclear RNA complementary to the L strand seems very low.

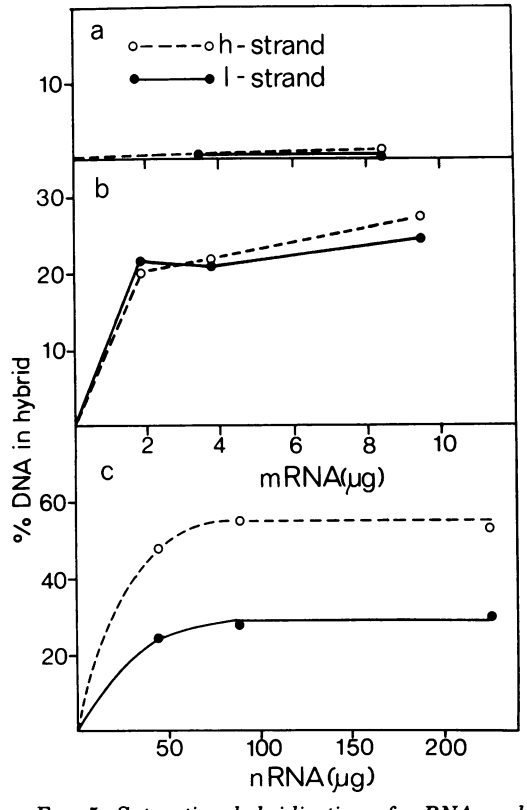

FIG. 5. *Saturation hybridization of mRNA and nuclear RNA from KB cells to the separated intact H and L strands of ^{3}H-labeled Ad12 DNA. The H or L strand of Ad12 DNA (1 ng/reaction mixture) was hybridized with increasing amounts of mRNA which was isolated from polysomes of mock-infected (a) or Ad12-infected KB cells 8 h p.i. (b) by affinity chromatography on poly(U)-Sepharose. In (c) the nuclear RNA isolated from KB cells 8 h p.i. was hybridized to the H and L strands of Ad12 DNA. The procedures for the isolation of mRNA and nuclear RNA and for DNA-RNA hybridization were described in the text. The purity of the isolated strands of Ad12 DNA was tested as described in Table 2.*

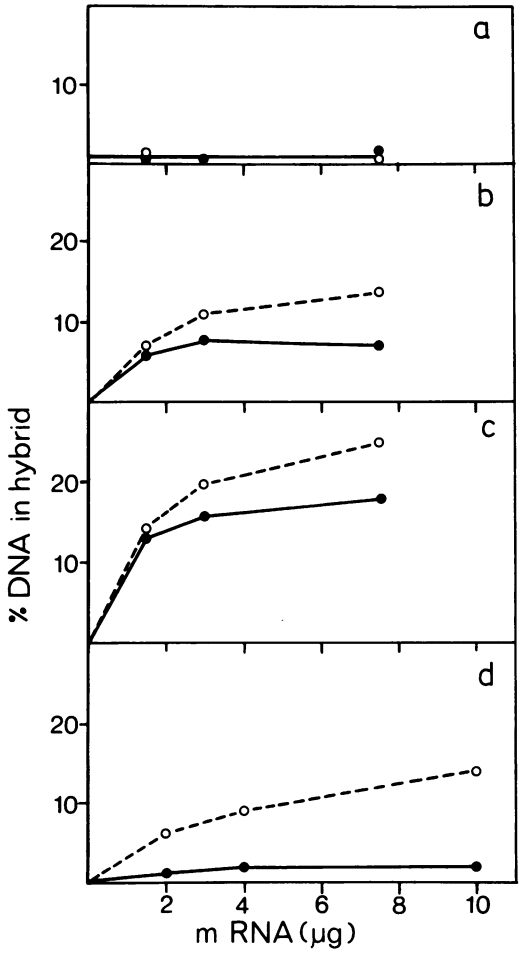

FIG. 6. *Saturation hybridization of mRNA from hamster cells to the separated intact H (○) and L (●) strands of ^{3}H-labeled Ad12 DNA. Experimental conditions were similar to those described in the legend to Fig. 5, except that mRNA was isolated from uninfected BHK-21 cells (a), BHK-21 cells 12 h p.i. with Ad12 (b), T637 cells (c), and HA12/7 cells (d).*

In all saturation hybridization experiments the DNA-RNA hybrid nature of the material resistent to S1 nuclease was ascertained by alkali hydrolysis (0.4 N NaOH, 90°C for 30 min) of the RNA preparation prior to hybridization. The level of hybridization was reduced to background values by this procedure. The levels of self-annealing of each of the ^{3}H-labeled probes used is demonstrated in Table 2, and these values have been subtracted from the data presented in Fig. 5 through 7.

Mapping of Ad12-specific mRNA and stable nuclear RNA on Ad12 DNA. (i) mRNA. The Ad12-specific sequences both in the mRNA and stable nuclear RNA fractions isolated from Ad12-infected or -transformed cells were localized on the Ad12 genome by hybridization of saturating amounts of unlabeled mRNA (cf. Fig. 8) or nuclear RNA with the separated strands of each of the ^{3}H-labeled *Eco*RI restriction endonuclease fragments and of the *Bam*HI fragments C*, B, and E. The latter set of fragments was used to obtain a more detailed map within *Eco*RI fragment A on the right molecular end (cf. Fig. 1c), which comprises 35.6% of the length of the molecule.

The amounts of unlabeled mRNA or nuclear RNA required to saturate a certain quantity of viral DNA were experimentally determined by using the isolated strands of restriction endonuclease fragments of Ad12 DNA. This type of experiment is exemplified by the data presented in Fig. 8, in which mRNA was used from Ad12-infected KB cells (Fig. 8a–e) and T637 cells (Fig. 8 f–j). Similar experiments were per-

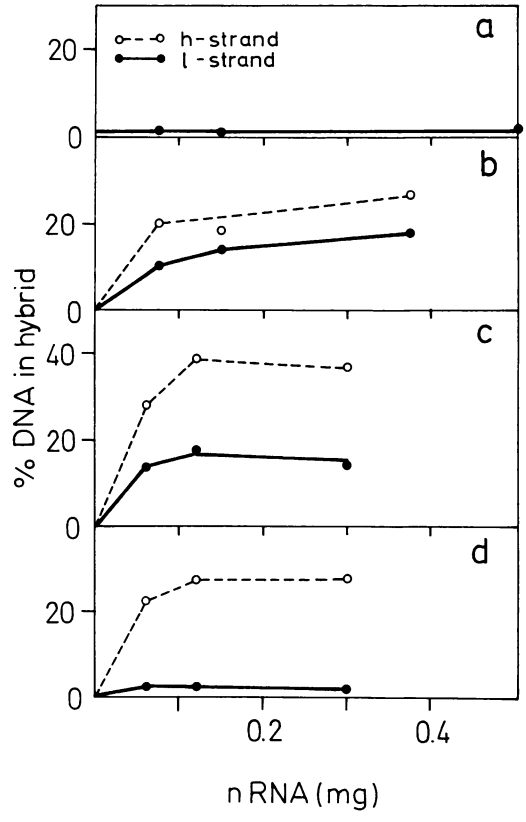

FIG. 7. *Saturation hybridization of nuclear RNA from hamster cells to the separated intact H and L strands of ^{3}H-labeled Ad12 DNA. Experimental conditions were similar to those described in the legend to Fig. 6, except that nuclear RNA from uninfected BHK-21 cells (a), BHK-21 cells 12 h p.i. with Ad12 (b), T637 cells (c), and HA12/7 cells (d) was used.*

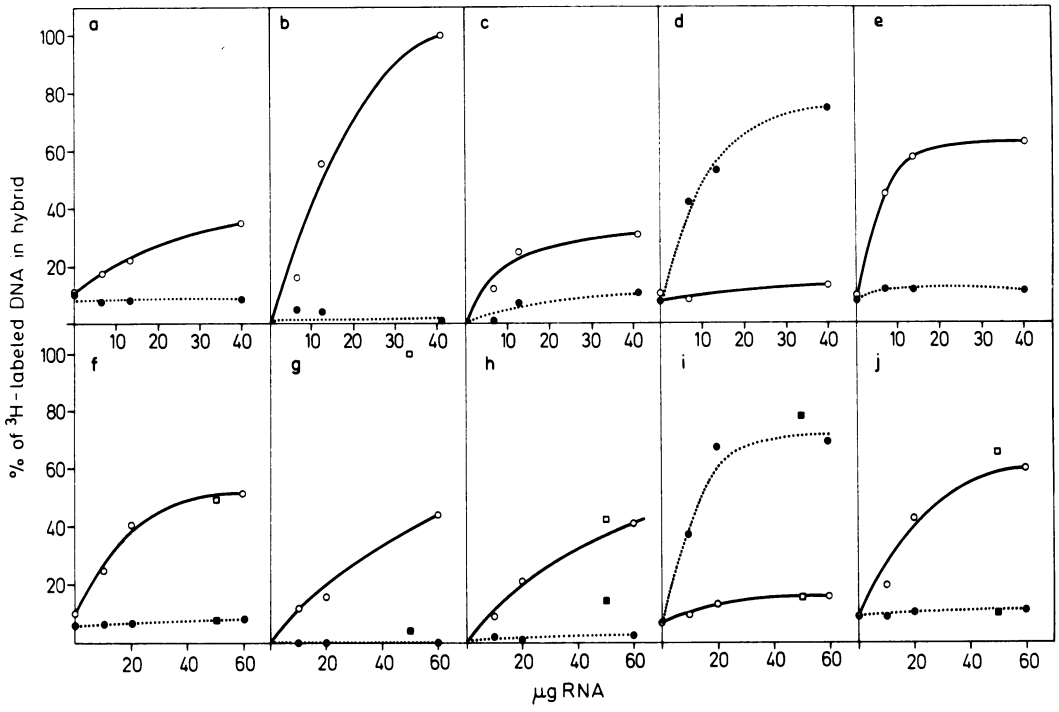

FIG. 8. *Saturation hybridization of mRNA to the isolated strands of EcoRI and BamHI fragments of Ad12 DNA. Increasing amounts of mRNA isolated from Ad12-infected KB cells 8 h p.i. (a–e) or from T637 cells (f–j) were hybridized to the H (○) or L (●) strand of the EcoRI C fragment (a and f), EcoRI F fragment (b and g), BamHI C* fragment (c and h), BamHI B fragment (d and i), and BamHI E fragment (e and j). In addition hybridization experiments, 35 µg of unlabeled mRNA isolated from Ad12-infected KB cells at 8 h p.i. was annealed together with mRNA from T637 cells, in amounts as indicated, to the H (□) or L (■) strand of the EcoRI or BamHI fragments described above. In all hybridization experiments the ^{3}H-labeled H or L strands of individual fragments of Ad12 DNA and unlabeled RNA were used. Conditions of hybridization and the methods of analysis are described in the text.*

formed with mRNA from Ad12-infected BHK-21 cells and HA12/7 cells (data not shown). From the results of saturation hybridization experiments, the saturating amounts of mRNA were calculated for each fragment strand and for the different cell lines investigated according to the method of Lucas and Ginsberg (27). These data are listed in Table 6 and indicate that the early mRNA from Ad12-infected KB cells is complementary to the *Eco*RI fragments C-H, A-H, A-L, and F-H. Hybridization experiments with the complementary strands of *Bam*HI fragments C*, B, and E reveal that the mRNA in Ad12-infected KB cells 8 h p.i. complementary to the *Eco*RI fragment A is predominantly derived from the C*-H, B-L, and E-H complements (Fig. 9). In this notation the designations A-H and A-L, for example, refer, respectively, to the H and L complements of *Eco*RI fragment A, etc. Sequences homologous to the *Eco*RI fragments B-L, D-H, D-L, E-H, and E-L appear to be represented in the mRNA population to a less significant extent (Table 6) and have not been included in the scheme presented in Fig. 9.

The mapping pattern on Ad12 DNA of Ad12-specific mRNA from abortively infected BHK-21 cells and the transformed hamster cell lines T637 and HA12/7 demonstrates that the Ad12-specific mRNA from these cells is derived, neglecting minor differences, from sections of the viral genome similar to those expressed early in productive infection (Table 6; Fig. 9). Similar conclusions based on hybridization competition experiments have been reached previously (35).

This interpretation was verified independently by addition hybridization experiments in which mRNA isolated 8 h after infection of KB cells with Ad12 was added to the annealing experiment simultaneously with mRNA from T637 cells (Fig. 8 f-j). Addition of mRNA from KB cells early after infection with Ad12 did not lead to increased levels of hybridization, indicating that the mRNA synthesized early in productive infection was derived from the same or from similar regions of the Ad12 genome as

was mRNA from T637 cells. Similar observations were made with mRNA from Ad12-infected BHK-21 and HA12/7 cells when mRNA from Ad12-infected KB cells was added to the hybridization experiments. It is concluded that in BHK-21 cells abortively infected with AD12, and in T637 and HA12/7 cells, those genes in Ad12 DNA that are expressed early in productive infection are transcribed.

There are distinct patterns of hybridization for each of the cell lines investigated. In comparison to the mRNA from Ad12-infected KB cells isolated at 8 h p.i., there is one major difference observed for the mRNA from the Ad12-transformed HA12/7 line. This mRNA lacks sequences complementary to the *Bam*HI B-L segment of Ad12 DNA (Table 6; Fig. 9).

TABLE 6. *Hybridization of saturating amounts of mRNA isolated from KB cells infected with Ad12 and from hamster cells abortively infected or transformed by AD12 with the ^{3}H-labeled complementary strands of the EcoRI fragments and BamHI fragments C*, B, and E of Ad12 DNA*[a]

Fragment strand (^{3}H labeled)	% of ^{3}H-labeled DNA in hybrid with unlabeled mRNA isolated from:[b]			
	KB cells · Ad12 at 8 h p.i.	T637 cells	HA12/7 cells	BHK-21 · Ad12 at 12 h p.i.
*Eco*RI				
A-H	29	37	21	31
A-L	33	25		14
B-H	5	3	7	1
B-L	8	2		
C-H	46	63	68	29
C-L	8	7	7	7
D-H	6	2	1	3
D-L	8	5	2	
E-H	8	7	3	2
E-L	6	4	1	
F-H	100	100	9	57
F-L	5	6		
*Bam*HI				
C*-H[c]	41	100	16	19
C*-L	6	2	3	5
B-H	11	13	7	9
B-L	93	81	7	56
E-H	69	94	28	63
E-L	12	11	9	10

[a] Experimental conditions have been described in the text. Levels of saturation hybridization were determined by annealing experiments similar to the ones described in Fig. 8. The amounts of mRNA used were: 7, 14, and 42 μg for mRNA from Ad12-infected KB cells; 4, 8, and 24 μg for mRNA from Ad12-infected BHK-21 cells; 10, 20, and 60 μg for mRNA from T637 cells; and 4, 8, and 23 μg for mRNA from HA12/7 cells.

[b] Mean of two to three independent determinations.

[c] The fragment C* was obtained by digesting *Bam*HI fragment C with *Eco*RI endonuclease (see Materials and Methods and Fig. 1c).

It may be possible to further improve the map of mRNA's from various Ad12-infected and -transformed cell lines on the Ad12 DNA molecule by using even smaller specific fragments of viral DNA. It must be mentioned that the maps in Fig. 9 and 10 have been arranged to allow for a minimal number of strand switches. More complicated maps are conceivable, but perhaps less likely.

(ii) Stable nuclear RNA. When the stable nuclear RNA fractions isolated from the Ad12-infected and -transformed cell systems described in the preceding section are analyzed by saturation hybridization, the results presented in Table 7 and Fig. 10 are obtained. At 8 h after the productive infection of KB cells with Ad12, the nuclear RNA contains sequences homologous to the *Eco*RI fragments A-H, A-L, B-H, B-L, C-H, D-H, D-L, E-H, E-L, and F-H (Table 7; Fig. 10). Some of these sequences, B-H, B-L, D-H, D-L, and E-L, are not represented in the mRNA fraction, but rather correspond to some of the late and anti-late sequences of mRNA isolated 53 h after productive infection of KB cells with Ad12 (Scheidtmann and Doerfler, manuscript in preparation).

Similar results are observed with the stable nuclear RNA from BHK-21 cells 12 h after abortive infection with Ad12 and from the Ad12-transformed hamster cell lines T637 and HA12/7. As already described for mRNA, the nuclear RNA from the HA12/7 line lacks sequences complementary to most of the L strand, except for a minor degree of homology to the D-L complement, which would not have been detectable in the experiment described in Fig. 7d.

Again, a more precise map of the *Eco*RI fragment A was obtained by hybridizing the ^{3}H-labeled H and L strands of the *Bam*HI fragments C*, B, and E (cf. Fig. 1c; Fig. 10; Table 7) to unlabeled nuclear RNA. The data show that the general pattern observed for mRNA (Fig. 9) is also reflected in the nuclear RNA (Fig. 10). The nuclear RNA sequences are, however, more extensive, and the general transcriptional pattern found suggests that at least a portion of the right half of the Ad12 genome may be transcribed symmetrically in the KB · Ad12 (8 h p.i.), BHK · Ad12 (12 h p.i.), and T637 systems. Hence, complementary sequences would be expected to be present in the nuclear RNA from these cells. The problem of symmetric transcription in Ad12-infected and -transformed cells requires further investigations.

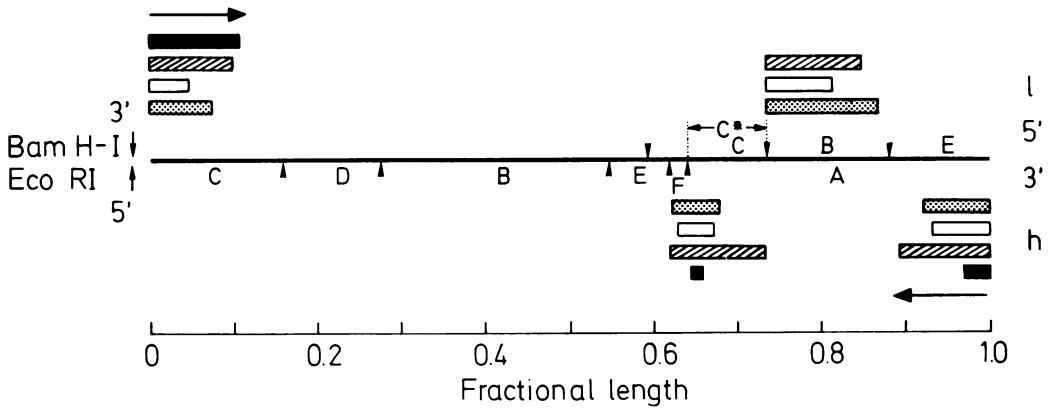

FIG. 9. *Summary of mapping experiments using polysome-associated mRNA from cells infected with or transformed by Ad12. The Ad12 genome is represented by the horizontal line in the center, and the cleavage sites by the EcoRI (▲) and BamHI (▼) restriction endonucleases are designated by arrows and the resulting fragments by capital letters. The C* fragment produced by digestion of the BamHI C fragment with the EcoRI enzyme is also indicated. The blocks represent mRNA sequences complementary to the H and L strands of sections of the Ad12 genome, as apparent from the scheme. The lengths of these blocks correspond to the percent values expressing the degree of homology between the respective Ad12 DNA fragments and mRNA from cell lines as indicated (cf. Table 6). The polarity of the H and the L strands is also included (cf. Fig. 4). The horizontal arrows indicate the polarity of transcription on the L (rightward) and H (leftward) strands. Symbols: dotted bar, KB·Ad12, 8 h p.i.; open bar, BHK-21·Ad12, 12 h p.i.; striped bar, T637; closed bar, HA12/7.*

DISCUSSION

Anatomy of the Ad12 DNA molecule. In accordance with the recommendation that the deoxyadenosine-deoxythymidine-richer terminus of the adenovirus DNA molecule should be designated the right end (11, 12), the *Eco*RI A fragment of the Ad12 DNA molecule must be positioned to the right, since partial denaturation experiments have shown that the *Eco*RI A fragment is richer in deoxyadenosine-deoxythymidine base pairs than the opposite end of the molecule (Delius and Mulder, personal communication). The restriction map presented in Fig. 1c is oriented in this way.

The polarity of the two strands of Ad12 DNA has been determined according to the method of Arrand et al. (2), relative to the restriction map. The scheme presented in Fig. 11 summarizes our findings. The intact L strand carries the 3'-end on the left terminus, and the intact H strand carries the 3'-end on the right terminus. The designations H (heavy) and L (light) strands refer to the properties of these strands to bind larger and lesser amounts of poly(U,G), respectively, and hence assume heavier or lighter buoyant densities in CsCl density gradients. This operational distinction between the two strands of Ad12 DNA should be complemented by a functional one. On the basis of the known polarity of the two strands, it is apparent that the L strand is transcribed from left to right and can be termed the "rightward" (r) strand. Similarly, the H strand is transcribed from right to left and can be designated the leftward (l) strand (Fig. 9–11). This nomenclature has been adopted from the transcriptional map for bacteriophage lambda (47) and has been recommended also for the map of Ad2 (42).

The scheme in Fig. 11 also accounts for the finding that the strand assignment of the H and L complements of the *Eco*RI fragments C and D and the *Bam*HI fragments A and G, which comprise 0.25 to 0.30 fractional length units on the left terminus of the moelcule, is apparently reversed (Table 4). Thus, the heavier complements of these fragments are part of the L strand and vice versa. This observation is paralleled in Ad2 DNA, where a similar strand switch in "heaviness" has been described for the left terminus of the molecule (42). The following results document the strand switch model as presented in Fig. 11.

(i) The H complements of *Eco*RI fragments C and D and *Bam*HI fragments A and G hybridize with the intact H strand, and the L complements of the same fragments hybridize with the intact L strand. The complements of all the other fragments, however, hybridize only with the opposite intact strand (Table 4).

(ii) On either terminus of the Ad12 DNA molecule, in vitro labeling of the 3'-terminus leads to incorporation of [^{3}H]dTTP into the H complements of the terminally located *Bam*HI

A and E fragments (Fig. 4). The label in the *Bam*HI A-H complement anneals with the intact H strand; that in the *Bam*HI E-H complement anneals with the intact L strand (Table 5).

The observed strand switch in "heaviness" comprising between 25 and 30% of the Ad12 molecule may explain the finding that the separation of the intact strands of Ad12 DNA is marginal both by equilibrium centrifugation in poly(U,G)-CsCl gradients and in alkaline CsCl density gradients.

A comparison of the strand polarity and the transcription maps between Ad12 (Fig. 9; 10) and Ad2 (34, 42) reveals striking similarities.

Comparison of the maps of Ad12-specific mRNA and stable nuclear RNA. With regard to the localization of the Ad12-specific mRNA, the conclusion can be drawn that those regions of the viral genome expressed early in productive infection are also represented in BHK-21 cells abortively infected with Ad12 and in two Ad12-transformed hamster cell lines, except that in the HA12/7 line there is a lower level of transcription of the L strand into polysome-associated mRNA (Fig. 9). Minor apparent differences between the patterns of different cell lines may be resolved by using even smaller fragments of Ad12 DNA in future mapping experiments. The RNA sequences complementary to the H and L strands on either molecular end have been placed terminally (Fig. 9; 10), although it is not likely that transcription extends to the terminal repeats in adenovirus DNA. It is, however, conceivable, though not proven, that circularized viral genomes (10, 37) may be involved in the transcription process.

TABLE 7. *Hybridization of saturating amounts of nuclear RNA isolated from KB cells 8 h after productive infection with Ad12, from BHK-21 cells 12 h after abortive infection with Ad12, and from the transformed lines T637 and HA12/7 with the ^{3}H-labeled complementary strands of the EcoRI fragments and Bam-HI fragments C*, B, and E of Ad12 DNA*[a]

Fragment strand (^{3}H labeled)	% of ^{3}H-labeled DNA in hybrid with unlabeled nuclear RNA isolated from:[b]			
	KB cells · Ad12 at 8 h p.i.	T637 cells	HA12/7 cells	BHK-21 cells · Ad12 at 12 h p.i.
*Eco*RI				
A-H	66	51	41	31
A-L	30	26	1	13
B-H	11	5	2	
B-L	16	2	3	6
C-H	83	83	77	77
C-L	11	6	5	2
D-H	28	7	7	7
D-L	15	5	15	2
E-H	44	2	5	13
E-L	10		2	7
F-H	100	25	10	63
F-L	3	1	2	
*Bam*HI				
C*-H[c]	100	100	16	100
C*-L	53	40	5	4
B-H	48	30	13	21
B-L	76	48	3	42
E-H	89	29	64	72
E-L	12	2	2	5

[a] Experimental details have been described in the text. Saturation hybridization experiments were performed as outlined in Table 6, footnote *a*.

[b] Mean of three independent determinations.

[c] See footnote *c*, Table 6.

Evidence has been presented that Ad2-specific nuclear RNA has to be processed into mRNA (33, 34, 42). The mechanism by which processing occurs is unknown as yet. A comparison of the transcriptional maps of Ad12 DNA for mRNA (Fig. 9) with that for stable nuclear RNA (Fig. 10) provides similar evidence for the Ad12 system. The same or very similar sequences of the viral genome represented in the polysome-associated mRNA fractions in KB cells 8 h p.i., in BHK-21 cells 12 h p.i. with Ad12, and in the Ad12-transformed T637 and HA12/7 lines are also detected in the stable nuclear RNA population. Due to the design of the experiments with nuclear RNA reported here, only the stable nuclear RNA is included in the analysis. It must also be emphasized that the results presented in Fig. 10 may be dependent upon the RNA concentration used. The regions of the Ad12 genome complementary to nuclear RNA are more extensive than the corresponding sections homologous to mRNA, particularly in the *Eco*RI fragments D-H and E-H and the *Bam*HI fragments C*-H, C*-L, B-H, and E-L. Some of the regions represented in the nuclear RNA from the different cell lines investigated, as derived from the *Eco*RI fragments C-L, D-L, D-H, B-H, and B-L are absent from mRNA. Considering the mapping pattern of nuclear RNA, particularly in the right half of Ad12 DNA, it will be interesting to search for complementary RNA sequences. Extensive symmetric transcription is also observed in Ad12-infected KB cells 48 h p.i. (Scheidtmann and Doerfler, manuscript in preparation).

Persistence and expression of Ad12 DNA in transformed hamster lines. In Ad12-transformed BHK-21 cells, the T637 line, all segments of the Ad12 DNA, i.e., all the *Eco*RI restriction enzyme fragments of Ad12 DNA, have been shown to persist in multiple copies,

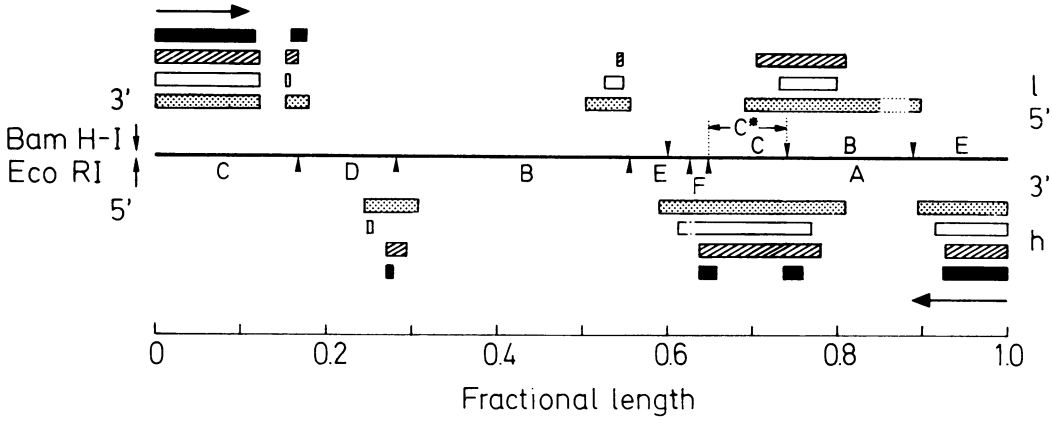

FIG. 10. *Summary of mapping experiments using nuclear RNA from cells infected with or transformed by Ad12. The scheme is similar to the one reproduced in Fig. 9. The dotted lines connecting blocks indicate that transcription might be continuous in these regions. All other elements of the scheme are as explained in the legend to Fig. 9.*

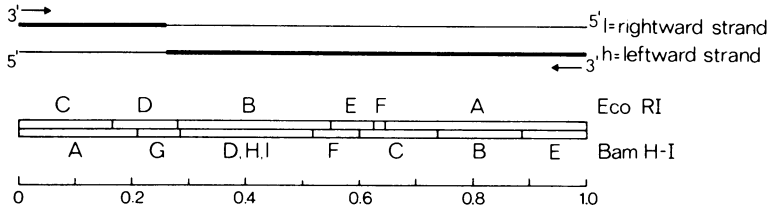

FIG. 11. *Anatomy of the Ad12 DNA molecule. The scheme illustrates the polarity of the H (leftward) and L (rightward) strands relative to the restriction map of Ad12 DNA. The "reversal in heaviness" on the left terminus of the Ad12 DNA molecule is also indicated. The exact limit of this reversal towards the right has not been determined; it is estimated to lie between 0.25 and 0.30 fractional length units. Symbols: thin line, L strand; heavy line, H strand. The scale on the bottom gives fractional length units, and the EcoRI and BamHI restriction maps of the Ad12 DNA molecule are also indicated.*

although different regions of the viral genome are represented non-stoichiometrically (17). Nevertheless, RNA sequences homologous to only a few specific regions of the Ad12 genome have been found to occur in the nuclear and mRNA populations of T637 cells (Fig. 9; 10). Similar findings have been made for Ad12 DNA in the HA12/7 cells (17; Fig. 9; 10). The facts that different sections of the Ad12 genome are represented in unequal amounts in the T637 and the HA12/7 lines (17) and that only a limited subset of the persisting segments is transcribed into polysome-associated mRNA suggest a complex mechanism which controls the expression of viral genes in transformed, and perhaps also in abortively infected, cells. These control mechanisms are unknown. It is, however, conceivable that these complex controls are somehow related to the specific site(s) of integration in the host genome of those segments of the viral DNA which are expressed in transformed cells. There is evidence that Ad12 DNA persists in T637 and HA12/7 cells in an integrated state (Groneberg et al., submitted for publication; Groneberg and Doerfler, Hoppe Seyler's Z. Physiol. Chem. 357:317, 1976).

A comparison of the transcriptional patterns of the Ad2 genome in Ad2-transformed rat cells (42) reveals that in those lines in which the entire viral genome, e.g., line T_2C_4, or large parts of the viral genome, e.g., line 8617, have been found to persist (38, 42), only early viral RNA sequences are expressed (42). Some of the Ad2-transformed rat cell lines, such as F17 or F18, contain DNA sequences homologous to only 14% of the left end of the Ad2 DNA (38), and these sequences are partly expressed (42). Thus, in the case of Ad2-transformed rat cells, similarly complex regulatory processes as in Ad12-transformed hamster cells must be operative in the regulation of the expression of persisting viral genes.

Further work will be required to locate each of the known size classes of Ad12-specific mRNA from productively infected (40) and from abortively infected and transformed cells (30) on the transcriptional map and to refine the transcriptional maps presented in this report.

Moreover, it will be interesting to determine precisely the relative quantities of individual mRNA populations synthesized in different cell systems.

ACKNOWLEDGMENTS

We thank Karin Moelling, Berlin, for a sample of avian myeloblastosis virus reverse transcriptase, Lennart Philipson, Uppsala, for a gift of poly(U,G), Heinz Schaller, Heidelberg, for exonuclease III, W. A. Strohl, Piscataway, N.J., for the T637 line, E. Viñuela, Madrid, for the *B. amyloliquefaciens* strain, and H. zur Hausen, Erlangen, for the HA12/7 line. We are also greatly indebted to C. Mulder, Worcester, Mass., for providing unpublished information on the localization of the *Bam*HI restriction fragments on the Ad12 genome.

This work was initiated while J. O. was a fellow of the European Molecular Biology Organization. This research was supported by the Deutsche Forschungsgemeinschaft (SFB 74).

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