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Relationship of mRNA from Productively Infected Cells to the Complementary Strands of Adenovirus Type 2 DNA

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The complementary strands of adenovirus type 2 (Ad2) DNA were separated by buoyant density gradient centrifugation with poly (U, G). The complementary strand DNA was shown to remain intact through the course of strand separation. The l-strand of Ad2 DNA, appearing in the less dense complex with poly (U, G) in neutral CsCl density gradients, was shown to have a buoyant density in alkaline (pH 12.5) CsCl density gradients which is 2 to 3 mg per ml greater than that of its complement (h-strand). Renaturation of purified complementary strand DNA was observed only in mixtures of h- and l-strand DNA, and then with the second-order reaction rate expected for Ad2 DNA. Hybridization of the complementary strands of Ad2 DNA with cytoplasmic mRNA isolated from infected HeLa cells was performed in liquid phase and analyzed by hydroxylapatite chromatography. Before viral DNA synthesis (6 h after infection), 13 to 18% of the h-strand and 30 to 35% of the l-strand were represented in viral mRNA. Late (18 h) after infection the mRNA represented 20 to 25% and 63 to 68% of the h- and l-strands, respectively. Most, if not all sequences present in viral mRNA before viral DNA synthesis were also present in the cytoplasm late in infection.

During productive infection with adenovirus type 2 (Ad2), two classes of virus-specific RNA have been distinguished, appearing before (early- or E-RNA) and after (late- or L-RNA) the onset of viral DNA synthesis (26). A two-step DNA-RNA and DNA-DNA hybridization technique with filter-bound viral DNA was used to assess the fraction of the Ad2 genome represented in L-RNA. The results suggested that 80 to 100% of the genome (conventionally assumed to be the equivalent of one complete strand of Ad2 DNA, 1.1×10^7 daltons; reference 7) was transcribed late after infection (5). Hybridization competition techniques were pursued to investigate the fraction of the genome represented in E-RNA (8 to 20%; references 4, 26) as well as the persistence and continued transcription of early genes late in infection (4, 12, 26). Green and colleagues (4, 7, 26) maintain that all early sequences are also present late in infection and that about 20% of these early sequences continue to be transcribed late after infection. Lucas and Ginsberg (13) observed that about 30% of the early sequences were synthesized late after infection; however, their results also suggested that the remaining E-RNA (70%) was absent in L-RNA preparations, degraded as soon as 9 to 14 h after infection. These disagreements may in part have been due to technical pitfalls inherent in the filter assay of RNA hybridization competition (13).

The separation of the complementary strands of Ad2 DNA was important for a more detailed investigation of Ad2 genome transcription. Landgraf-Leurs and Green (11) reported the separation of the complementary strands of adenovirus DNA using the synthetic ribocopolymer binding technique. Green and colleagues (7) further studied Ad2 transcription by hybridization of RNA to separated strands of Ad2 DNA immobilized on filters. From these studies it was concluded that both E-RNA and L-RNA contain transcripts from both complementary strands of Ad2 DNA. Although h-strand transcripts were detected both early and late after infection, the predominant fraction of virus-specific RNA was found to be complementary to l-strand DNA. The h- and l-strand DNA refer to the complementary strands of Ad2 DNA having, respectively, higher and lower buoyant density, as DNA-poly (U, G) complexes in CsCl density gradients.

We have investigated the relationship of virus-specific mRNA, from productively infected cells, to the complementary strands of Ad2 DNA by employing liquid phase hybridization

of nucleic acids and analysis by hydroxylapatite chromatography (10, 23). This method allows direct determination of the fraction of each complementary strand of Ad2 DNA represented in mRNA appearing at different times after infection. A greater representation of l-strand DNA sequences than h-strand sequences was observed with both E- and L-RNA. The fractions of the h- and l-strands of Ad2 DNA represented in cytoplasmic E-RNA were found to be 13 to 18% and 25 to 30%, respectively. The cytoplasmic mRNA appearing late in productive infection accounts for about 20 to 25% of the h-strand and 63 to 68% of the l-strand of Ad2 DNA. Our results further indicate that at least 90%, if not all E-RNA sequences, are present late after infection.

MATERIALS AND METHODS

Cells and infection. HeLa-S3 cells obtained originally from J. Maizel (Albert Einstein College of Medicine, New York) were maintained in suspension in the Eagle Spinner medium (3) with 7% calf serum. At the time of infection with Ad2, the cells were concentrated to 2×10^7 cells/ml and infected with 2,000 particles per cell. After 30 min, the infected cells were diluted to 3×10^5 to 4×10^5 cells/ml. In some cases, for the preparation of E-RNA, cytosine arabinoside was added to 20 μg/ml to block DNA synthesis. E- and L-RNA were prepared from cells harvested 6 and 18 h after infection, respectively.

Extraction of RNA. RNA from productively infected cells in suspension culture was prepared essentially as described by Penman (20). The cells were collected by centrifugation, washed once with phosphate-buffered saline, and resuspended in isotonic buffer (12) with 0.6% Nonidet P-40. The cells were disrupted at a density of 10^8 cells/ml using a Dounce homogenizer with a tightly fitting pestle. The cytoplasmic fraction was collected as the supernatant after centrifugation (1,000 $\times$ g for 3 min). Cytoplasmic RNA was prepared by chloroform-isoamyl alcohol-phenol (2.0/0.02/10) extraction at room temperature, pH 9.0, according to Brawerman et al. (2). After addition of NaCl to 0.15 M; the aqueous phase was precipitated with 2 volumes of ethanol. The precipitated RNA was dissolved in 10 mM Tris-hydrochloride, 1 mM EDTA, pH 7.5, and subsequently precipitated with 2 M LiCl at 4 C overnight (1). The precipitate was then dissolved in 10 mM Tris-hydrochloride, pH 7.5, with 2 mM $MgCl_2$ and incubated with 40 μg of pancreatic DNase per ml (Worthington, electrophoretically purified) for 20 min at 37 C. After phenol extraction and ethanol precipitation the RNA was fractionated by affinity chromatography on poly (U)-Sepharose as described elsewhere (12). The poly (A)-containing mRNA fraction was precipitated with ethanol and finally dissolved in 10 mM Tris-hydrochloride, pH 7.5, and stored at −70 C.

Preparation of viral DNA. [^{3}H]Ad2 virus was prepared and purified by three equilibrium density gradient centrifugations in CsCl as previously described (27). ^{14}C-labeled virus was prepared by including 5 mCi of [^{14}C]formate per liter of medium during the course of infection. Viral DNA was obtained after disruption of the virus with Sarkosyl and incubation with Pronase B (Calbiochem) as previously described (27).

Separation of the complementary strands of Ad2 DNA. In general, the methods described by Szybalski et al. (25) and applied for adenovirus DNA by Landgraf-Leurs and Green (11) were followed. Poly (U,G) was purchased from Schwarz/Mann (lot no. 7001, U/G ratio 3.3). This was the only lot of several tested which was found applicable to the separation of Ad2 complementary DNA strands. Freshly purified [^{14}C]formate-labeled Ad2 virus (0.3 ml, $A_{260} = 9$) in buoyant CsCl solution was diluted with 5 volumes of 10 mM Tris-hydrochloride, 1 mM EDTA, pH 8.5. Sarkosyl and Pronase B (preincubated for 15 min at 37 C) were added to 5 mg/ml and 1 mg/ml, respectively. After 30 min of incubation at 37 C, NaOH was added to give a concentration of 0.17 M. After 10 min at room temperature, 0.5 mg of poly (U,G) (1 mg/ml in distilled water) was added, and the mixture was immediately neutralized with HCl and buffered with 0.10 M Tris-hydrochloride, 10 mM EDTA, pH 8.5. Solid CsCl was added to give a final density of 1.78 g/ml. The volume was adjusted subsequently by adding CsCl solution of the same density and buffer concentration. The mixture was placed in polyallomer tubes and centrifuged 48 to 60 h at 35,000 rpm, 20 C, in a Spinco Ty50 rotor. Fractions (0.1 ml) were collected from the bottom of the gradients and 5-μliter portions were removed to determine the distribution of ^{14}C-labeled DNA (see Fig. 1A).

Alkaline CsCl density gradients. Samples were centrifuged in 7.0-ml CsCl density gradients buffered with 50 mM K_3PO_4, pH 12.5, for 40 h, 20 C, in a Ty65 rotor. The initial density was 1.74 g/ml. Fractions (52) were collected dropwise, from the bottom of each gradient, onto glass filters and dried.

RNA-DNA hybridization. Fragmented, separated strands of ^{14}C-labeled Ad2 DNA were incubated with different amounts of RNA in mixtures which contained 1 M NaCl, 0.14 M phosphate (equimolar NaH_2PO_4 and Na_2HPO_4), 4 mg of sodium dodecyl sulfate (SDS) per ml. Each mixture contained approximately 1,500 counts/min or 0.03 μg of Ad2 DNA in a total volume of 0.1 ml. The samples were incubated at 65 C for at least 36 h and were then diluted 10-fold with 0.14 M phosphate, 4 mg of SDS per ml, and were analyzed by chromatography on hydroxylapatite.

Hydroxylapatite chromatography. Analysis of nucleic acid hybridization was performed using hydroxylapatite columns (BioRad HTP) at 60 C as described by Khoury et al. (10) and Sambrook et al. (23). Hydroxylapatite (Clarkson Hypatite) chromatography at room temperature and in the presence of formamide (27) was used in the purification of the separated complementary strands of Ad2 DNA. With sufficiently degraded Ad2 DNA (300 to 500 nucleotide pieces) each method affords quantitative separation

of single-stranded DNA from duplex or partially duplex molecules (22, 27).

Alkaline sucrose gradients. Samples (about 5 μliters) were diluted in 0.2 ml of 0.20 M NaOH and layered onto 5 to 20% linear alkaline sucrose gradients as described by Morrow and Berg (14). Centrifugation followed for 3 h at 55,000 rpm in an SW56 rotor, 20 C. Thirty equal fractions were collected dropwise from the bottom of each gradient. Intact Ad2 DNA (alkaline sedimentation coefficient 34*S*; reference 7) was recovered as a sharp band with a peak in fraction 6 (see Fig. 2).

Liquid scintillation counting. Liquid samples were counted in toluene-methanol-based scintillation fluid as previously described (27) and filters in a toluene-based scintillation fluid.

RESULTS

Strand separation and purification of complementary strand Ad2 DNA. ^{14}C-labeled Ad2 virus was disrupted with Sarkosyl and Pronase and the DNA was denatured with sodium hydroxide. After addition of poly (U,G) and neutralization, the mixture was banded in CsCl density gradients as described in Materials and Methods. The resulting distribution (Fig. 1A) showed two nearly equal bands (h and l) and a broader, lesser band at lower density (u). The h- and l-bands had buoyant densities of 1.800 and 1.785 g/ml, respectively, in reasonable agreement with the results of Landgraf-Leurs and Green (11) for the density shifts of the complementary strand-poly (U,G) complexes under similar centrifugation conditions. Similar distributions have been obtained in experiments covering a wide range of poly (U,G) and DNA concentrations and also when purified DNA (27) rather than virus was used as starting material. The amount of DNA appearing in the u-band was variable with respect to that in the h- and l-bands. [^{14}C]formate and labeled viral protein fragments from the Pronase digestion account for the background of radioactivity in the gradient. Fractions comprising the h- and l-bands were pooled as indicated in Fig. 1A and rebanded in CsCl density gradients under the same centrifugation conditions as before, but without additional poly (U,G). Fig. 1B and C show the distribution of the rebanded h- and l-strand-poly (U,G) complexes. Fractions were pooled as indicated and stored in polyallomer tubes at 4C for several months without evidence of degradation of the DNA. Analysis of samples of h- and l-bands by sedimentation in alkaline sucrose gradients revealed that they contained DNA only of unit length (34*S*) (Fig. 2). Electron microscopy also revealed unit length DNA including single-stranded circular species resulting from the inverted terminal repetition of Ad2 DNA (6, 29).

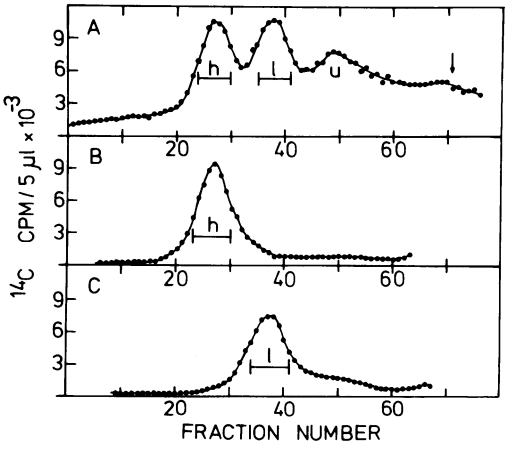

FIG. 1. *Separation of the complementary strands of [^{14}C]Ad2 DNA as complexes with poly (U,G). Samples were prepared and banded in CsCl density gradients as described in Materials and Methods. Material in the h- and l-bands (two gradients of which only one is shown in A) was pooled as indicated and rebanded in second CsCl density gradients (B and C). Material in the rebanded h- and l-band distributions was pooled as indicated and stored at 4 C. The arrow in A shows the position (density 1.730 g/ml) for denatured Ad2 DNA in gradients lacking poly (U,G). The u-band in panel A is described in the text (Results).*

Complementary strands of Ad2 DNA, prepared as described above, were characterized with respect to buoyant density in alkaline CsCl density gradients (Fig. 3). The h-strand DNA was found to be 2 to 3 mg/ml less dense than the l-strand DNA in alkaline CsCl.

The h- and l-strand DNA-poly (U,G) complexes were degraded by incubation in 0.24 M NaOH at room temperature for 30 min. The samples were then dialyzed for 4 days at 37 C against 50% formamide, 0.12 M phosphate, and 4 mg of SDS per ml at a DNA concentration of about 5 μg/ml. This would allow all contaminating complementary strand DNA to renature with the complement in excess, since the time corresponds to between 250 and 500 times that required for 50% reassociation of denatured Ad2 DNA under these conditions (27). The resulting h- and l-strand samples were sonically treated and passed over hydroxylapatite columns equilibrated with 50% formamide, 0.12 M phosphate, and 4 mg of SDS per ml at room temperature (27). In each case 94% of the total self-annealed DNA applied to the columns was recovered in the single-stranded DNA fraction. The resulting h- and l-strand DNA were stored in the same formamide-phosphate-SDS buffer at room temperature.

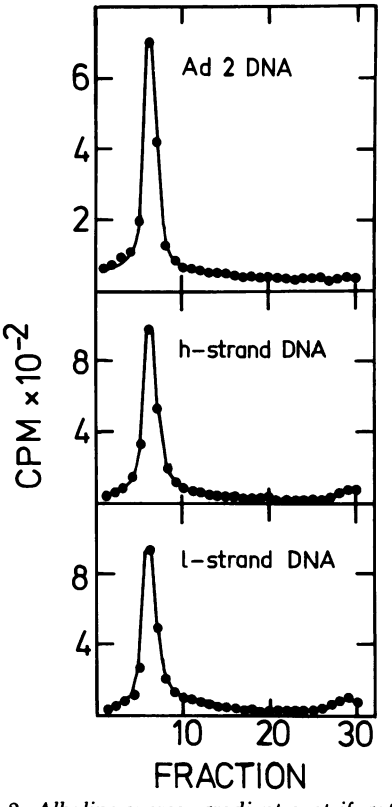

FIG. 2. *Alkaline sucrose gradient centrifugation of intact [³H]TdR-labeled Ad2 DNA (27) and rebanded [¹⁴C]formate-labeled h- and l-strand DNA. Sedimentation is shown from right to left and was performed as described in Materials and Methods. The small fraction of radioactivity at the top of the gradients with h- and l-strand DNA is background [¹⁴C]formate remaining after rebanding in CsCl (Fig. 1B and C). This was removed by dialysis during the subsequent self-annealing procedure (see text).*

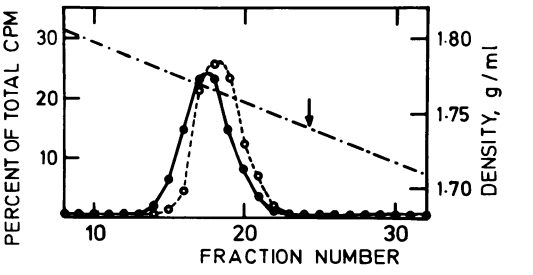

FIG. 3. *Distributions of [³H]h-strand DNA (○), 1,900 counts/min and [¹⁴C]l-strand DNA (●), 1,500 counts/min in a bouyant alkaline (pH 12.5) CsCl density gradient. The density gradient (-·-·-·-) was calculated from initial density (1.740 g/ml), dimensions of the Ty65 rotor, centrifugation conditions (see Materials and Methods) and data from Vinograd and Hearst (28). The h-strand DNA was found to be about 2.5 ± 0.5 mg/ml less dense than l-strand DNA in alkaline CsCl solution. The same result was obtained in gradients containing [¹⁴C]h-strand DNA and [³H]l-strand DNA.*

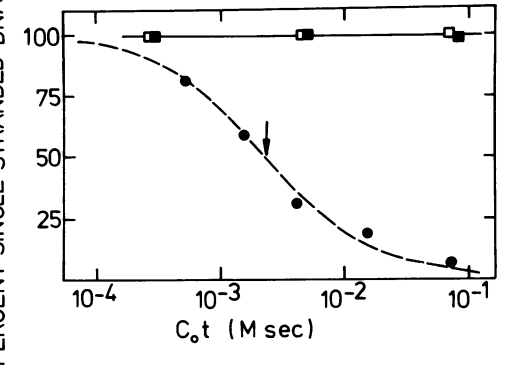

FIG. 4. *Reassociation kinetics of mixed, fragmented h- and l-strand DNA. Samples were prepared, incubated at 65 C, and analyzed on hydroxyapatite columns as indicated in Materials and Methods. Each reaction contained 0.3 μg of DNA per ml. Reactions containing only h-strand (□) or l-strand (■) DNA showed no signs of duplex formation. A reaction containing 0.15 μg/ml of each complementary strand (●) showed duplex formation with 50% reassociation after 45 min. The dashed line corresponds to the contour expected for ideal second-order reassociation kinetics.*

To ascertain that DNA from the h- and l-bands contained separated complementary strands of Ad2 DNA, h- and l-strand DNA samples were incubated separately or mixed at 65 C under conditions described in Materials and Methods. When separately incubated the h- and l-strand DNA remained single-stranded, whereas the mixture of equal amounts of h- and l-DNA renatured with expected second-order kinetics (Fig. 4). Self-annealing experiments with purified u-band DNA showed that this material contained nearly equal amounts of h- and l-strand DNA. The nature of the u-band material has not been further investigated.

Hybridization of cytoplasmic mRNA to separated strands of Ad2 DNA. Prior to hybridization with RNA, the h- and l-strand DNA was further fragmented by limited alkaline hydrolysis at 100 C in 0.3 M NaOH for 20 min. This gave a rather narrow distribution of fragment size with an average sedimentation coefficient of 5.5*S* in alkaline sucrose gradients (not shown). The three smallest specific R_1 endonuclease cleavage fragments of Ad2 DNA (D, E, and F; reference 21) were used as markers in parallel gradients. This sedimentation coefficient corresponds to a single-stranded DNA

molecular weight of about 1.1×10^5 (24), which suggests that the average size of the resulting fragmented h- and l-strand DNA is 330 nucleotides, about 1% of a full-length strand of Ad2 DNA (8).

Increasing amounts of cytoplasmic RNA, extracted from infected cells at different times after infection, were incubated with ^{14}C-labeled, separated strands of Ad2 DNA in solution as described in Materials and Methods. After 36 to 48 h at 65 C the fraction of each strand of DNA which had hybridized with RNA was determined by chromatography on hydroxylapatite. The hybridization mixtures contained 0.3 μg of h- or l-strand DNA per ml. No reassociation of h- or l-strand DNA was observed when incubated separately. When equal amounts of h- and l-strand DNA were mixed and incubated under identical conditions (Fig. 4) the observed value of $Cot_{\frac{1}{2}}$ (the product of DNA concentration and time required for 50% reassociation under our conditions, 1.21 M Na^+) was 2.4×10^{-3} M (nucleotide) s. This value is consistent with previous determinations from Ad2 DNA renaturation kinetics (22, 27). The time of incubation of the RNA-DNA hybridization mixtures was then estimated to be 100 to 130 times the time required for 50% hybridization of 0.3 μg of h- or l-strand DNA per ml with complementary RNA sequences present at a number equivalent concentrations. Thus, at saturation the fraction of DNA in duplex or partially duplex DNA-RNA hybrid structure should correspond to the fraction of DNA complementary to the RNA used in the hybridization mixture.

No hybridization was obtained with h- or l-strand DNA and cytoplasmic RNA isolated from uninfected cells (Fig. 5A).

Saturation curves for cytoplasmic E-RNA are presented in Fig. 5B-D. This RNA contained sequences representing 13 to 18% of h-strand DNA and 30 to 35% of l-strand DNA. This result was independent of the presence of cytosine arabinoside during the infection (Fig. 5C and D). The same values were also obtained with cytoplasmic RNA that was not fractionated by affinity chromatography on poly (U)-Sepharose (Fig. 5B). This result confirmed that this step of our purification of mRNA did not lead to selective loss of any sequences of virus-specific RNA.

Saturation curves for L-RNA, isolated 10 and 18 h after infection, are presented in Fig. 6. L-RNA was found to contain sequences representing 20 to 25% of h-strand DNA and 63 to 68% of l-strand DNA.

When saturating amounts of E- or L-RNA were first incubated in 0.5 M NaOH, 37 C, for 18 h and then neutralized, less than 2% hybridization was observed after standard incubations with h- or l-strand DNA at 65 C. This control

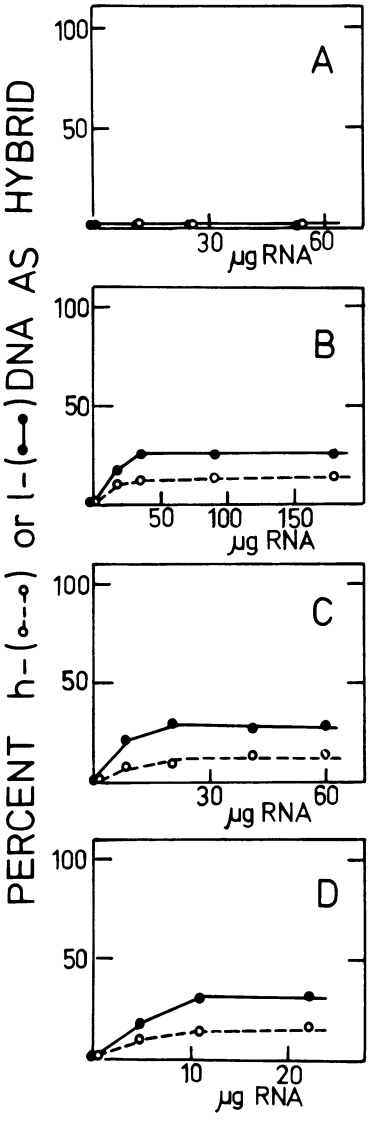

FIG. 5. *Saturation of h-strand* (O) *and l-strand* (●) *Ad2 DNA sequences by incubation with increasing amounts of RNA. The results presented are for cytoplasmic RNA from uninfected cells (A) or infected cells early (6 h) after infection. Panel B describes E-RNA which was not fractionated by poly (U)-Sepharose chromatography. Panels C and D describe E-RNA preparations from cells grown without and with added cytosine arabinoside, respectively, as described in Materials and Methods, as are conditions of RNA-DNA hybridization and analysis. The reactions each contained 0.03 μg of h- or l-strand DNA (1,600 counts/min of ^{14}C).*

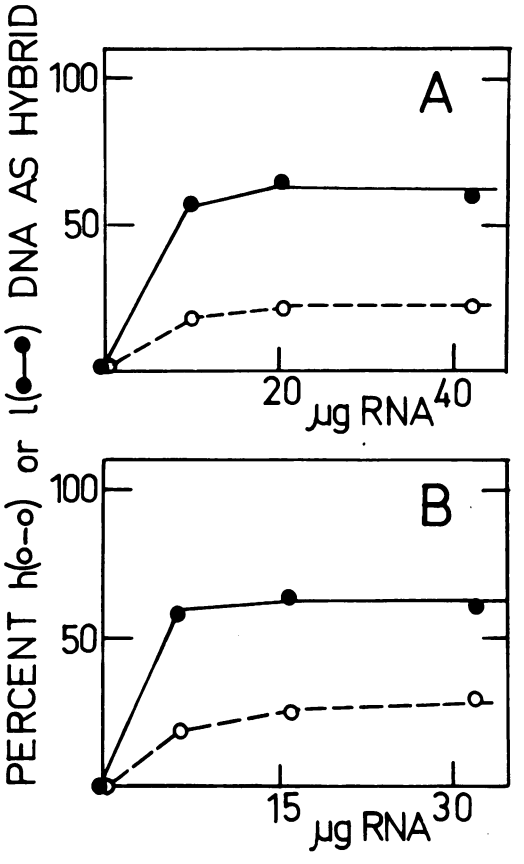

FIG. 6. *Saturation of h-strand* (○) *and l-strand* (●) *Ad2 DNA with increasing amounts of L-RNA. Panels A and B describe results with L-RNA isolated 10 and 18 h after infection, respectively. Hybridization conditions and analysis are described in Materials and Methods. Each reaction contained 0.03 µg of DNA (1,600 counts/min of ^{14}C).*

establishes that the RNA preparations were free of Ad2 DNA. The h- or l-strand DNA in hybridization mixtures which was retained as duplex, or partially duplex nucleic acid thus reflected RNA-DNA hybrid structure.

Mixtures of E- and L-RNA (in amounts previously shown to saturate complementary sequences in h- and l-DNA) were incubated with the separated strand DNA to determine if E-RNA sequences are still present late after infection. The h- and l-strand DNA did not hybridize to significantly greater extents with mixtures of E- and L-RNA as compared to L-RNA alone (Table 1).

The complementary strands of Ad2 DNA were prepared by separation of denatured DNA-poly (U, G) complexes, as described by Landgraf-Leurs and Green (11). The single strands of Ad2 DNA remained intact through the course of strand separation (Fig. 2). Renaturation of DNA occurred only when the separated complementary strands were remixed (Fig. 4). The complementary strands are referred to as h- and l-strand DNA, having, respectively, higher and lower buoyant density, as DNA-poly (U, G) complexes, in neutral CsCl density gradients.

When mixtures of differently labeled h- and l-strand DNA were banded in CsCl density gradients at pH 12.5 h-strand DNA was found to be 2 to 3 mg/ml less dense than l-strand DNA (Fig. 3). This density difference is too small to afford practical separation of the complementary strands of Ad2 DNA. However, it is analytically useful as a means of confirming the identification of strands prepared by using different ribopolymers or eventually by different procedures.

Results of previous studies of Ad2 transcription during the course of productive infection were briefly reviewed in the introduction. The complexities of the two-phase (liquid-filter) nucleic acid hybridization techniques employed in those studies suggest that quantitative interpretations of the resultant data be made carefully. The results presented in this paper were obtained using single (liquid) phase hybridization conditions and chromatographic analysis as described previously (10, 22, 23). The distinct advantage of the method lies in the direct determination of the amount of DNA present in hybrid structure, and there is no ambiguity with regard to possible sequence-selective losses or seclusion of filter-bound DNA. The major sources of experimental error are the analysis of radioactivity and the operation of the hydroxylapatite separations of single-stranded from duplex and partially duplex nucleic acids. In

TABLE 1. *Fractions of each complementary strand of Ad2 DNA represented in E- and L-RNA and in mixtures of E- and L-RNA*

RNA	Ad2 DNA (%) in hybrid[a]	
	h-Strand	l-Strand
Early	13	31
Late	22	66
Early and late	24	67

[a] Average values from two experiments employing different preparations of E- and L-RNA. Values are given with an associated experimental error of ±3%. The amounts of E- and L-RNA used in each experiment were sufficient to saturate sequences of h- and l-strand DNA as determined prior to the incubations in experiments similar to those shown in Fig. 4 and Fig. 5.

the experiments we have presented the experimental error associated with values of percent DNA in hybrid (or duplex) structure is about ±3% on a scale from 0 to 100%. DNA fragments were derived from a random cleavage process and single-stranded DNA tails could occur in RNA molecules with terminal hybrid structures. The method of hybrid analysis is thus prone to an overestimation of the fraction of the DNA sequences represented in an RNA sample. The overestimate is at most equivalent to the ratio of the average size of the DNA probe (330 nucleotides) to the length of the RNA sequence in question. If several RNA sequences are consecutive with respect to the genome, or separated on the genome by fewer nucleotides than present in the average DNA probe molecule, then the actual overestimate will be less than suggested above. The average size of viral-specific mRNA is about 3,200 nucleotides, estimated from distributions in sucrose gradients and polyacrylamide gels (12, 17, 18). The fraction of each complementary strand represented in E- and L-RNA could thus be overestimated by a factor of about 1.1. Sambrook et al. (23) performed experiments which suggested that in practice the analytical overestimate is much less than suggested above. They hybridized fragmented, separated strands of simian virus 40 (SV40) DNA (average length 360 nucleotides) with RNA extracted from KB cells infected with the hybrid virus Ad2+ND_1. This virus contains 16% tof the SV40 genome and Sambrook et al. observed 19% hybridization of E-strand SV40 DNA with the RNA and no hybridization of SV40 L-strand DNA. Assuming that the entire SV40-specific region of the Ad2+ND_1 genome was transcribed in KB cells, their result represents overestimation by a factor of 1.19. This is 2.6 times less than that suggested by the ratio of DNA fragment size to the fraction of SV40 genome present in Ad2+ND_1 DNA (360/776 = 0.46). In the same study of SV40 transcription, the DNA probe length for the DNA-RNA hybridizations represented 7.5% of a complete SV40 DNA strand. In our experiments the length of DNA fragments in hybridization reactions represented only 1% of the Ad2 genome. We can therefore consider that the magnitude of the analytical overestimation was less than 5% of the fractions of h- or l-strand DNA retained on hydroxylapatite as DNA-RNA hybrids, since the size of the DNA probe is small with respect to both the average size of Ad2 mRNA and of the Ad2 genome.

From our results we have concluded that, early after infection, before DNA synthesis and independent of the presence of cytosine arabinoside, 13 to 18% of h-strand DNA and 30 to 35% of l-strand DNA sequences are represented in the cytoplasm. Taken together, half of the Ad2 genome is present as mRNA. The extent of transcription early after infection must therefore be at least 45% of the genome. This value is significantly higher than 8 to 20% of the genome as estimated by Thomas and Green (26) and Fujinaga and Green (4) using total cell RNA early after infection. Their estimate was derived from the combined results of the two-step hybridization experiments with late total cell RNA (5) and the subsequent saturation hybridization competition experiments with early and late RNA.

Late after infection the combined fractions of the h- and l-strands represented in cytoplasmic RNA, 0.88 ± 0.05, is in accord with 80 to 100% transcription of the genome late in infection, estimated by Fujinaga et al. (5).

Results of hybridization competition experiments on several DNA-containing animal virus systems have suggested that, in general, early RNA sequences are present in the population of late RNA species, although they are not necessarily all transcribed late in infection (9, 15, 16, 23). The results pertaining to Ad2 transcription have been contradictory. Green et al. (4, 7, 26) maintained that all or nearly all early sequences are present late after infection, a view supported by our own results (Table 1).

The results of a preliminary experiment with sequential hybridization of late and early RNA are also in accord with this conclusion. Single-stranded h- and l-strand DNA was isolated after saturation with late RNA, treated with alkali to hydrolyze any remaining late RNA, and then hybridized with early RNA sufficient to saturate number equivalent concentrations of complete h- and l-strands. The experimental error associated with such a multi-step procedure is higher than for the simple mixing experiment (Table 1), yet it was clear that more than 80 and 90% of h- and l-strand sequences, respectively, represented in early RNA are also represented in the cytoplasm late after infection.

Lucas and Ginsberg have reported, however, that 70% of early RNA sequences are no longer transcribed late in infection, and these sequences were degraded as early as 9 to 14 h after infection (13). In addition to the technical pitfalls associated with hybridization competition experiments reviewed by these workers (13) differences in the kinetics of RNA turnover in different cell lines may also play a part in their clearly differing results. All these studies have been made with human cell lines in culture (HeLa and KB), but differences similar

to those described of HeLa and mouse L-cells (16) may eventually have to be considered.

The predominant transcription of the l-strand of Ad2 DNA relative to the h-strand was reported by Green et al. (7) and later presented among the results of Patch et al. (19). Their experiments were based on determination of the relative fraction of total hybridizable radioactive RNA, labeled throughout the course of infection, which bound to h- and l-strand DNA immobilized on filters. To interpret their results quantitatively requires critical assumptions concerning the abundance and the specific activities of radioactively labeled viral-specific RNA species. The results we have obtained, under saturation conditions and independent of RNA labeling, confirm the predominance of l-strand transcripts both early and late after infection. The relation of mRNA to the complementary strands of Ad2 DNA in the course of productive infection is summarized in Table 1. The pattern of late gene expression appears to be established by 10 h (Fig. 5A).

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