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Adenovirus Type 12-Specific RNA Sequences During Productive Infection of KB Cells

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The complementary strands of adenovirus type 12 DNA were separated, and virus-specific RNA was analyzed by saturation hybridization in solution. Late during infection whole cell RNA hybridized to 75% of the light (l) strand and 15% of the heavy (h) strand, whereas cytoplasmic RNA hybridized to 65% of the l strand and 15% of the h strand. Late nuclear RNA hybridized to about 90% of the l strand and at least 36% of the h strand. Double-stranded RNA was isolated from infected cells late after infection, which annealed to greater than 30% of each of the two complementary DNA strands. Early whole cell RNA hybridized to 45 to 50% of the l strand and 15% of the h strand, whereas early cytoplasmic RNA hybridized to about 15% of each of the complementary strands. All early cytoplasmic sequences were present in the cytoplasm at late times.

The human adenoviruses are divided into four subgroups based on their oncogenicity for newborn hamsters (9, 13). Group A adenoviruses, which include adenovirus type 12 (Ad12), are highly oncogenic in newborn hamsters, whereas group C adenoviruses (e.g., Ad2) are nononcogenic. Ad12 and Ad2 differ widely in a number of other properties. They share few or no DNA sequences in common (10; J. R. Smiley and S. Mak, unpublished data), and consequently their cleavage patterns by endo *Eco*RI are entirely unrelated (16). Ad2 is capable of replicating in hamster cells, whereas Ad12 is not (2). Purified preparations of Ad12 have a higher particle to PFU ratio than Ad2 (18).

The transcription of Ad2 DNA has been intensively studied in infected human cells and in transformed rat cells. Early work by Green and his collaborators using the technique of membrane filter hybridization indicated that whole cell RNA isolated late after infection hybridized to 80 to 100% of the viral genome (4) and that most viral RNA sequences were derived from the light (l) strand of the viral DNA molecule (5). By competition hybridization it was determined that early whole cell RNA was complementary to 8 to 20% of the viral genome (5).

Recent studies using the technique of liquid-phase saturation hybridization of radioactive viral DNA strands and of complementary strand-specific sequences of defined fragments of the viral genome generated by bacterial restriction endonucleases have resulted in a map of the sites of the Ad2 DNA molecule expressed as messenger RNA (17, 20, 23, 24). Some authors have detected early cytoplasmic RNA sequences that are not detectable late (1, 12), whereas others have not (20, 24). RNA sequences that are not detectable in the cytoplasm have been found in Ad2-infected cell nuclei both at early and late times (5, 17, 20, 25). Late nuclear RNA contains sequences derived from 100% of both DNA strands (20).

Because of the lack of nucleotide sequence homology between Ad2 and Ad12 DNA and their differing biological characteristics, it is of considerable interest to determine how similar the transcription patterns of the two viruses are. Membrane filter hybridization experiments have been reported indicating that late Ad12-specific whole cell RNA is complementary to 80 to 100% of the viral genome (15) and that early whole cell RNA is complementary to about 50% of the viral genome (5). We have studied AD12-specific RNA by liquid-phase saturation hybridization of radioactive purified complementary viral DNA strands by unlabeled infected cell RNA.

MATERIALS AND METHODS

Virus and cells. Human Ad12 and KB cells grown in suspension were used throughout this study. Cells were maintained in minimal essential medium (Joklik modified) supplemented with 5% horse serum. Virus infection and purification procedures have been described (6, 14). Cells were infected with purified Ad12 at 2×10^3 virions/cell to obtain infected cell RNA.

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Separation of the Ad12 DNA strands. Viral DNA from purified virions having a specific activity of 5×10^5 counts/min per μg was obtained by adding [^{3}H]thymidine (20 Ci/mmol; New England Nuclear, Boston, Mass.) to infected cultures at a final concentration of 5 μCi/ml at 19 h postinfection. To separate the DNA strands, the ribopolymer binding technique (22) as modified by Landgraf-Leurs and Green (11) and Tibbets et al. (24) was used. Purified virions were dialyzed against 0.01 M Tris (pH 8.1). To each milliliter of virus suspension (containing 50 to 100 μg of DNA) 0.15 ml of 0.05 M EDTA, 0.15 ml of 1.0 M sodium phosphate (pH 6.0), 0.05 ml of Pronase (20 mg/ml, B grade, Calbiochem, Los Angeles, Calif.), and 0.1 ml of 10% sodium dodecyl sulfate was added. The mixture was incubated at 37 C for 30 min and cooled to room temperature. Viral DNA was denatured by the addition of NaOH to 0.17 N. After 10 min the mixture was neutralized with HCl and buffered with 0.1 M Tris, pH 8.1. Polyuridylic-guanylic acid [poly(U,G)] (U-G ratio, 3.03:1; Biogenics lot 111) in 0.01 M Tris (pH 8.1), 0.001 M EDTA was added to give a poly(U,G)-DNA ratio of 5 to 10:1. Most of the sodium dodecyl sulfate was removed by centrifugation at 25,000 $\times$ *g* at 0 C for 20 min. The solution was made to 4.1 ml with 0.1 M Tris (pH 8.1), and solid cesium chloride was added to give a final density of 1.76 g/ml. The solution was overlayed with mineral oil and centrifuged at 33,000 rpm at 15 C in siliconized polyallomer tubes in the Beckman 50 titanium rotor for 72 h. Six-drop fractions were collected from the bottom, and 5-μl aliquots were removed for radioactivity determination. Fractions corresponding to the heavy (h) and light (l) strands were separately pooled, denatured and rebanded in CsCl gradients after adding fresh poly(U,G). Fractions were pooled, dialyzed against 0.01 M Tris (pH 8.1), and centrifuged through 16.5 ml of 5 to 20% alkaline sucrose gradients at 24,000 rpm for 13 h at 20 C in the SW27.1 rotor. Fifty-drop fractions were collected, and 10-μl aliquots were analyzed for radioactivity. Fractions containing full-length molecules were pooled, neutralized, extracted with phenol at pH 8.1, and exhaustively dialyzed against 0.01 M Tris, pH 7.3.

Separation of nuclear and cytoplasmic fractions. A composite detergent lysis-mechanical shear method was used. Infected cells were washed with isotonic buffer (0.15 M NaCl; 0.01 M Tris, pH 7.9; 0.01 M EDTA) (9) and resuspended to a cell concentration of 5×10^7/ml in isotonic buffer containing 0.7% Nonidet P-40 (Shell Chemical Corp., New York, N.Y.) at 0 C. The mixture was shaken vigorously for 10 min, and nuclei and unlysed cells were pelleted by centrifugation. The supernatant was withdrawn and used as the cytoplasmic fraction. The pellet was resuspended in isotonic buffer lacking Nonidet P-40 to the original volume and homogenized to minimize cytoplasmic contamination of the nuclei. The nuclei were pelleted, washed twice with buffer, and used for RNA extraction.

RNA extraction. RNA was extracted by the hot phenol-sodium dodecyl sulfate method (26). After ethanol precipitation the nucleic acid was dissolved in 0.01 M Tris (pH 7.3) and 0.01 M $MgCl_2$ and incubated with 100 μg of pancreatic deoxyribonuclease (RNase free, Worthington Biochemical Corp., Freehold, N.J.) per ml for 1 h at 37 C. RNA was reextracted by the hot phenol method, ethanol-precipitated, and dialyzed against 0.01 M Tris, pH 7.3. An aliquot of each preparation was assayed for contaminating viral DNA by hybridization to a ^{3}H-labeled l strand after degrading the RNA with NaOH (0.5 N, 24 h, 37 C). All RNA preparations used were DNA free. RNA concentration was determined by absorption at 260 nm, assuming that an optical density of 1 at 260 nm corresponds to 42 μg of RNA per ml.

To isolate double-stranded RNA, the RNA was annealed 24 h in 2$\times$ SSC (0.15 M NaCl plus 0.015 M sodium citrate) at 67 C and then digested with 100 μg of pancreatic ribonuclease per ml for 1 h at 37 C in 2$\times$ SSC. The residual RNA was extracted with phenol and was ethanol precipitated.

Hybridization. Liquid-phase hybridization of [^{3}H]DNA with unlabeled RNA was done in a total volume of 0.4 ml in siliconized glass tubes. [^{3}H]DNA (about 4×10^{-3} μg) was incubated alone or with RNA from infected cells in 0.3 M NaCl and 0.01 M Tris (pH 7.3) at 67 C for the indicated times. In each experiment, the RNA concentration of all samples was made identical by adding yeast RNA to give a total RNA concentration of 1.25 mg/ml. This step is necessary to eliminate DNA binding to the hybridization vessel. The fraction of the DNA in hybrid form was determined by digestion with S_1 nuclease (21).

All complementary strand preparations were exhaustively self-annealed before use in hybridization experiments. The background S_1-resistant radioactivity observed in the absence of infected cell RNA (5% for pure strand preparations) was subtracted from both the input radioactivity and the S_1-resistant radioactivity observed after hybridization. Therefore the results of hybridization experiments are expressed as the fraction of the initially S_1-sensitive radioactivity that has been protected by RNA.

S_1 nuclease digestion. S_1 nuclease used in early experiments was a generous gift from Kaken Chemical Co., Ltd., Tokyo, Japan. Subsequent lots were purchased from Seikagaku Kogyo Co. Ltd., Tokyo, Japan. Enzyme was stored in S_1 buffer (0.3 M NaCl, 0.0018 M $ZnCl_2$, 0.05 M sodium acetate, pH 4.5) plus 20% glycerol at -20 C. Digestion was done in S_1 buffer, plus 10 μg of denatured calf thymus DNA per ml, and 10 μg of S_1 nuclease per ml for 1 h at 37 C. To remove residual RNA the solution was made 0.5 N NaOH and incubated for an additional 2 h at 37 C. The solution was then neutralized with HCl, precipitated with cold 10% trichloroacetic acid in the presence of 100 μg of calf thymus DNA as carrier and slowly filtered onto Millipore filters (0.2-μm pore size). Radioactivity was determined in a Beckman LS230 or LS233 scintillation system. This procedure renders sonicated denatured Ad12 DNA 97% acid-soluble, native Ad12 DNA less than 5% acid-soluble, and eliminates the otherwise severe quenching due to unhydrolyzed RNA.

RESULTS

Purification of the Ad12 complementary DNA strands. Tritium-labeled denatured Ad12 DNA was banded in a cesium chloride density gradient in the presence of poly(U,G) as detailed in Materials and Methods. Two major peaks were observed (Fig. 1), in agreement with the data of Landgraf-Leurs and Green (11). The light band consistently contained more radioactivity than the heavy band, although the ratio of the radioactivity between the two peaks varied from experiment to experiment. The light band was poorly resolved from a less dense component, which varies widely in quantity between experiments. This band is probably analogous to the U band observed by Tibbets et al. in similar gradients of Ad2 DNA (24). Its nature has not been investigated.

The indicated fractions were pooled, denatured, and rebanded in the presence of fresh poly(U,G). The starting densities used for rebanding the h and l strands were 1.77 and 1.75 g/ml, respectively. The h band was virtually homogeneous, whereas the l band was resolved into two minor peaks and one major peak (Fig. 2). The unequal efficiency of recovery of h and l DNA from the second gradient is not consistently observed. Fractions were pooled and further purified in alkaline sucrose gradients (Fig. 3). Most of the DNA sedimented as one size class, which cosedimented with intact Ad12 DNA (data not shown). Only full-length molecules were pooled and used for hybridization experiments.

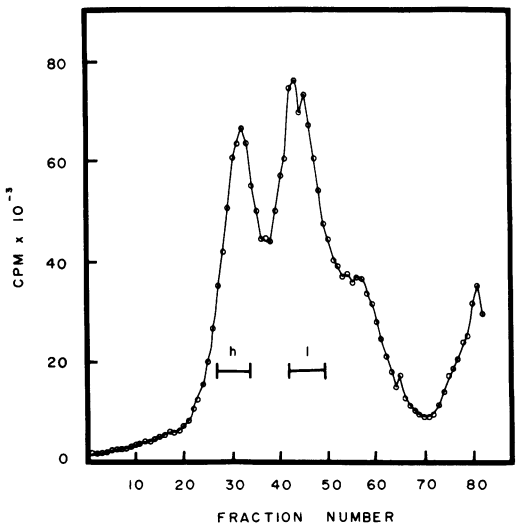

FIG. 1. *Separation of the Ad12 DNA strands in a poly(U,G)-cesium chloride density gradient. Denatured [³H]Ad12 DNA was banded as described in Materials and Methods. Six-drop fractions were collected from the bottom (left), and 5-μl aliquots were analyzed for radioactivity. The indicated fractions were pooled for rebanding.*

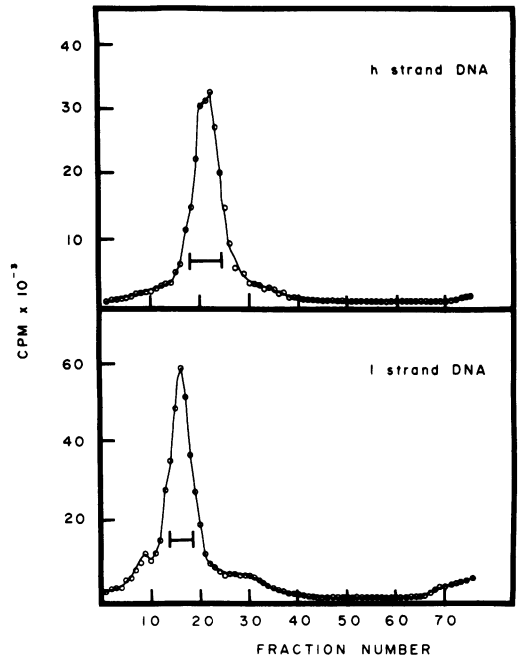

FIG. 2. *Rebanding pattern of partially purified h- and l-strand DNA. Material pooled from the gradient displayed in Fig. 1 was rebanded in a CsCl gradient after addition of fresh poly(U,G). Fractions were processed as in Fig. 1. Starting densities were 1.77 and 1.75 g/ml for h and l DNA, respectively. The indicated fractions were pooled for size analysis.*

Recovery of [³H]DNA after hybridization. Single-stranded DNA tends to adhere to even siliconized glassware. Early experiments (data not shown) demonstrated that [³H]DNA was lost during DNA-RNA hybridization and that the loss was not observed in samples containing large amounts of RNA. It seemed possible that RNA prevented the loss by competing with the DNA for sites on the surface of the tube. Therefore, we attempted to prevent the loss of DNA by adding unlabeled yeast RNA to the solution. ³H-labeled l-strand DNA was incubated alone or with 1.25 mg of yeast RNA per ml under the standard hybridization conditions for 48 h, and samples were removed and counted directly in an aqueous scintillation system using Aquasol (New England Nuclear). The loss of [³H]DNA in the absence of RNA was both severe (60 to 80%) and variable from sample to sample, whereas virtually no loss was observed in the presence of RNA. Since the yeast RNA did not affect the assay for single-stranded DNA with S_1 nuclease (Table 1), we

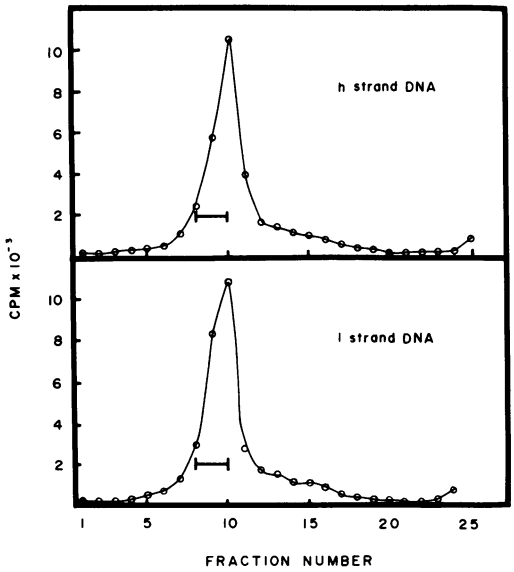

FIG. 3. *Size distribution of the rebanded h and l DNA. Fractions pooled from the gradients displayed in Fig. 2 were centrifuged through 16.5 ml of 5 to 20% alkaline sucrose gradients as detailed in Materials and Methods. Fifty-drop fractions were collected from the bottom (left), and 10-μl aliquots were analyzed for radioactivity.*

decided to include it in all incubations to bring the total RNA concentration of each sample to 1.25 mg/ml.

Purity of h and l DNA. To test the purity of the isolated strands the h and l preparations were incubated separately and together under annealing conditions at a DNA concentration of 0.1 μg/ml for 100 h in the presence of yeast RNA. Renaturation was assayed by digestion with S_1 nuclease (Table 1). Both strand preparations were initially 5 to 6% resistant to S_1 nuclease, and this value did not change after annealing and was not reduced by heat denaturation followed by immediate digestion. The background resistance was reduced to 3% by sonication followed by denaturation. Table 1 also shows that an equimolar mixture of h and l DNA became 84% resistant to S_1 nuclease after annealing. To exclude the possibility that the incomplete renaturation observed was a consequence of some of the DNA being degraded to a nonhybridizable form, we also performed complement excess hybridization experiments. ^{3}H-labeled h strand (0.015 μg/ml) was hybridized to 1.2 μg of ^{14}C-labeled h- or l-strand DNA per ml for 24 h. Excess l strand protected over 95% of the initially S_1-sensitive ^{3}H label from the nuclease, whereas incubation with excess h strand protected only 5% of the ^{3}H label. This data indicate that the h and l preparations were essentially free of contaminating sister strands. It is possible that some of the background resistance to S_1 nuclease is due to specific intrastrand base pairing as a consequence of the inverted terminal intrastrand complementary sequences in adenovirus DNA (6, 25).

Viral RNA sequences in whole cell RNA. To examine the strand specificity of Ad12 RNA synthesized during productive infection we performed saturation hybridization experiments between unlabeled RNA extracted from infected KB cells early (7.5 h) and late (24 h) during the infective cycle and from the purified complementary viral DNA strands. Under the conditions of infection used, Ad12 DNA replication is first detectable 11 to 12 h postinfection (15).

The first experiments were done with whole cell RNA (Fig. 4). Late whole cell RNA hybridized 75% of the l strand and 15% of the h strand at apparent saturation, whereas early RNA hybridized 45 to 50% of the l strand and 15% of the h strand. No hybridization was observed with uninfected KB cell RNA (Table 2). A mixture of apparently saturating amounts of early and late whole cell RNA hybridized the same fraction as late RNA alone, indicating that the early sequences detected in this experiment are a subset of the late sequences (Table 2).

Nuclear and cytoplasmic RNA sequences. Since numerous reports have shown that nuclear RNA in cells infected with Ad2 contains sequences not found in the cytoplasm (5, 17, 20, 25) we examined early and late Ad12 cytoplasmic RNA and late nuclear RNA. Late cytoplasmic RNA hybridized 65% of the l strand and 15% of the h strand, and early cytoplasmic RNA hybridized 17% of the l strand and 14% of the h strand at saturation (Fig. 5). A mixture of saturating amounts of early and late cytoplasmic RNA hybridized the same fraction of both

TABLE 1. *Purity of h and l DNA as assayed by self-annealing and digestion with S_1 nuclease*[a]

DNA	Fraction remaining acid precipitable after S_1 digestion
h, Denatured	0.056
l, Denatured	0.056
h, Annealed[b]	0.058
l, Annealed[b]	0.065
h + l, Annealed[b]	0.842
h, Sonicated, denatured	0.032
Ad12, intact, native	0.957

[a] Ad12 DNA (4×10^{-3} μg) was digested with S_1 nuclease as detailed in Materials and Methods.

[b] Annealing was for 100 h at a DNA concentration of 0.1 μg/ml.

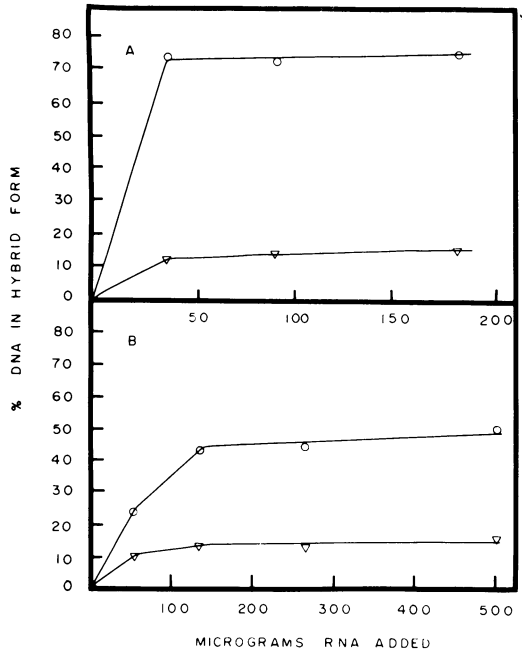

FIG. 4. *Hybridization of early and late whole cell RNA to the complementary DNA strands. h (▽) or l (○) [^{3}H]DNA at 2 × 10^3 counts/min was incubated for 48 (late) or 72 h (early) with the indicated amounts of unlabeled RNA from infected cells, plus enough yeast RNA to give a total RNA concentration of 1.25 mg/ml in each sample. Hybridization was assayed as in Materials and Methods. (A) Late RNA; (B) early RNA.*

TABLE 2. *Sequence relationship of early and late whole cell and cytoplasmic RNA*[a]

RNA	Fraction of DNA hybridized	
	l strand	h strand
Late whole cell (17 μg)	0.736	0.113
Early whole cell (265 μg)	0.463	0.183
Early whole cell (265 μg) + late whole cell (17 μg)	0.715	0.124
Late cytoplasmic (200 μg)	0.663	0.149
Early cytoplasmic (1,000 μg)	0.174	0.131
Late cytoplasmic (200 μg) + early cytoplasmic (1,000 μg)	0.641	0.152
Uninfected KB cell (900 μg)	0.000	0.000

[a] The indicated amounts of RNA, previously determined to be saturating amounts, were hybridized to h and l DNA for 72 h (whole cell and uninfected cell RNA) or 48 h (cytoplasmic RNA).

strands as late cytoplasmic RNA alone (Table 2), demonstrating that all early cytoplasmic sequences are present in the cytoplasm at late times. Since late RNA hybridizes the same fraction of the h strand as early RNA, it appears that all Ad12 late specific RNA is derived from the l strand.

Late nuclear RNA hybridizes more than 90% of the l strand and at least 36% of the h strand (Fig. 6A). Therefore, sequences complementary to at least 25% of the l strand and 21% of the h strand are detectable in the nucleus but not in the cytoplasm (cf. Fig. 5A and 6A).

Detection of symmetrically transcribed RNA. Since a greater than one-strand equivalent of DNA is represented as nuclear RNA, at least part of the viral genome is transcribed symmetrically. To demonstrate directly the presence of symmetrically transcribed RNA we have isolated double-stranded RNA molecules from late whole cell RNA by digestion of self-annealed RNA with pancreatic ribonuclease. The resistant RNA was denatured and hybridized to the complementary strands (Fig. 6B). No hybridization was observed without denaturing the RNA or after hydrolysis with 0.5 N NaOH for 24 h at 37 C. The double-stranded RNA hybridized 37% of the l strand and 30% of the h strand. The reactions are probably not saturated. Ideally the two curves should be congruent, and the difference observed is not yet understood. Possibly the regions of the l strand hybridized have a higher thymidine content than do the corresponding regions of the h strand.

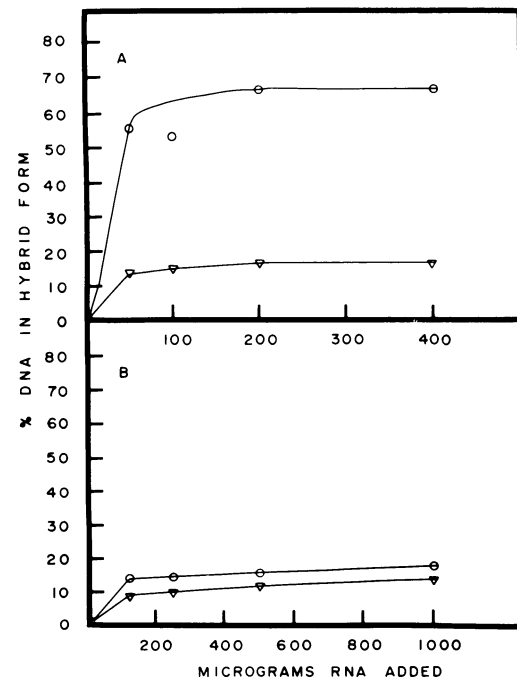

FIG. 5. *Hybridization of early and late cytoplasmic RNA to the complementary DNA strands. h (▽) or l (○) [^{3}H]DNA at 2 × 10^3 counts/min was incubated with the indicated amounts of unlabeled infected cell RNA for 48 h. (A) Late cytoplasmic RNA; (B) early cytoplasmic RNA.*

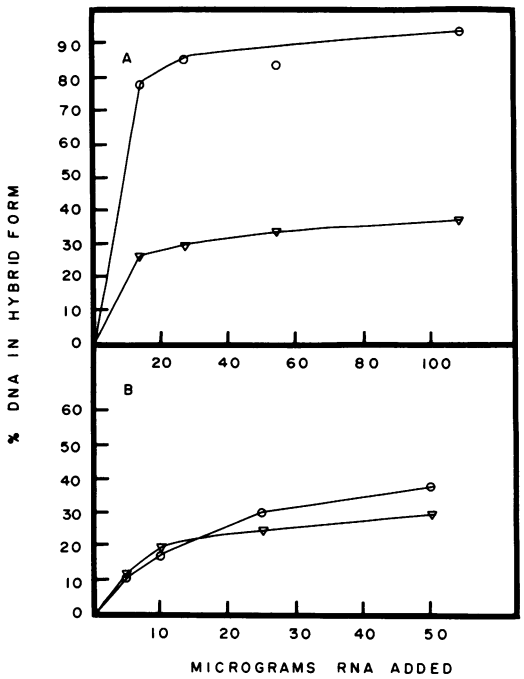

FIG. 6. *Hybridization of late nuclear RNA and denatured RNase-resistant RNA isolated from late whole cell RNA. h (▽) or l (○) [³H]DNA was incubated with the indicated amounts of unlabeled infected cell RNA for 48 h. (A) Late nuclear RNA; (B) denatured RNase-resistant RNA.*

DISCUSSION

Whole cell RNA from Ad12-infected cells hybridizes 75% of the Ad12 l strand and 15% of the h strand at late times and 45 to 50% of the l strand and 15% of the h strand at early times. These data agree with previous reports using the method of membrane filter hybridization, which suggested that Ad12 whole cell RNA is derived from 80 to 100% of the viral genome at late times (14) and about 50% at early times (5).

Late cytoplasmic Ad12 RNA is complementary to 65% of the l strand and 15% of the h strand, whereas early cytoplasmic RNA is derived from 17% of the l strand and 15% of the h strand. All early cytoplasmic RNA sequences are present in late cytoplasmic RNA. This data is similar to that obtained with Ad2 (20, 24), except that all Ad12 late specific cytoplasmic RNA sequences seem to arise from the l strand. We cannot, however, rule out the possibility of an extremely small late specific region on the h strand. Some caution is necessary in the interpretation of the saturation levels because of the possibility that small amounts of RNA leak from the nuclei during the preparation of the cytoplasmic fraction. This could inflate the saturation levels.

Our results for early RNA are difficult to correlate with those of Scheidtmann, et al. (19), who examined the size distribution of Ad12-specific polysome-associated RNA. These authors calculate that early mRNA is derived from approximately 45% of the genome, assuming that each size class observed corresponds to a unique RNA species. It is not clear whether our estimate of about 30% is significantly different from theirs.

Late nuclear RNA is derived from at least 90% of the l strand and 36% of the h strand and is almost certainly the source of the double-stranded RNA isolated from whole cells. The fraction of the genome symmetrically transcribed is unknown, since saturation was not reached with the double-stranded RNA, but is at least 30%. RNA complementary to 100% of both DNA strands has been detected in Ad2-infected nuclei (20). It is clear that there is sequence specificity during intranuclear processing and/or transport of RNA out of the nucleus. Saturation has not been reached with early nuclear RNA. However, early whole cell RNA consistently hybridizes more of the l strand than early cytoplasmic RNA, suggesting that early nuclei contain sequences not found in the early cytoplasm. Since there appears to be more RNA sequences present in both early and late nuclei as compared to the corresponding cytoplasm, it is possible that processing and transport of viral RNA is important in the switch from early to late patterns of protein synthesis. Whether or not there is also transcriptional control is unknown.

An interesting point brought out by these studies is that more viral RNA sequences are detectable in nuclear late RNA than in late whole cell RNA. We feel that this indicates that a large fraction of the nuclear-specific RNA sequences are complementary to the cytoplasmic RNA sequences. These complementary sequences would form RNA-RNA hybrids rapidly during the hybridization reaction due to the excess amount of cytoplasmic sequences. Consequently, the nuclear-specific sequences would be unavailable for DNA-RNA hybridization.

The saturation values given here are almost certainly slight underestimates of the true values, because S_1 nuclease hydrolyzes double-stranded nucleic acid at a slow but measurable rate. Under the digestion conditions used, 4.3% of native Ad12 DNA is hydrolyzed, and the rate of hydrolysis of RNA-DNA hybrids may differ from that of duplex DNA. However, less than 7% of the DNA component of the hybrids is degraded during the assay, since late nuclear RNA protects up to 93% of the l strand from S_1 nuclease. Another possible source of error is the

use of thymidine as a DNA label. The results are biased to the extent that thymidine is incorporated nonuniformly throughout the genome. There is evidence of nonrandom distribution of adenine plus thymine and guanine plus cytosine pairs in Ad12 DNA from denaturation mapping (3).

The results are remarkably similar to those obtained by Tibbets et al. (24) and Sharp et al. (20) with Ad2. The similarity is interesting in view of the almost complete lack of nucleotide sequence homology between the two viral DNA molecules (10; Smiley and Mak, unpublished data).

ACKNOWLEDGMENTS

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