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Synthesis of Complementary RNA Sequences During Productive Adenovirus Infection

(nuclear RNA/separated strands/specific DNA fragments)

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ABSTRACT **Liquid RNA·DNA hybridization with separated strands of adenovirus type 2 DNA revealed that late nuclear RNA can hybridize to about 85% of the l-strand and 10–15% of the h-strand, whereas late cytoplasmic RNA hybridizes to 65–70% and 25% of the l- and h-strand, respectively. With separated strands from the six *Eco*RI fragments of adenovirus type 2 DNA as probes, it was shown that late nuclear RNA hydridizes to 85–90% of the l-strand from all six *Eco*RI fragments. Since late cytoplasmic RNA hybridizes to 40–50% of the h-strand from both fragments *Eco*RI-B and *Eco*RI-C, complementary viral RNA sequences are synthesized during adenovirus infection. Complementarity between nuclear and cytoplasmic RNA could also be demonstrated by showing that late cytoplasmic RNA which had been preincubated with late nuclear RNA hybridized to a smaller fraction of the h-strand of fragment *Eco*RI-C than without preincubation. Double-stranded RNA which contains sequences that correspond to at least 60% of the viral genome was isolated from infected cells. However, less than 2% of the newly synthesized late RNA became double-stranded after incubation under annealing conditions, which suggests that RNA derived from one of the strands is present at a low concentration. Accordingly, it was shown that nearly all viral cytoplasmic RNA which is synthesized late after infection is derived from the l-strand.**

In recent years our knowledge about transcription of bacterial and phage DNA has expanded rapidly. Messenger RNA is synthesized by RNA polymerase which recognizes specific initiation and termination signals*. In some cases a large initial transcript appears to be cleaved into separate messenger RNA molecules by an enzyme which is specific for double-stranded RNA (2). Studies on transcription in animal cells have been hampered by the complexity of the eukaryotic genome. One way to evade this problem is to study transcription in cells which have been infected with animal DNA viruses. RNA synthesis in uninfected and adenovirus infected cells appears to proceed in a similar manner. In both cases, large RNA molecules can be detected in the nucleus which are polyadenylated and cleaved before entering the cytoplasm (1, 3–6). Messenger RNA both early and late after infection with adenovirus type 2 (ad2) hybridizes to both strands of the viral DNA (7, 8). The shift from early to late phase takes place at the onset of viral DNA replication (6-8 hr after infection) and leads to qualitative as well as quantitative changes of viral RNA synthesis. Most early messenger RNA can be detected also late after infection (8), but studies by presaturation hybridization on filters have indicated that some early RNA sequences are not synthesized late (9). The presence of small amounts of complementary viral RNA in adenovirus infected cells has also been described (10).

In the present report the relationship between nuclear and cytoplasmic RNA late after adenovirus infection has been studied by hybridization of RNA with separated strands of ad2 DNA and with separated strands of specific fragments which are obtained by cleavage of viral DNA with endonuclease *Eco*RI (11). This enzyme cleaves ad2 DNA (23 $\times 10^6$ daltons) into six fragments, designated A–F according to decreasing size. Their order on the genome has been determined to be A–B–F–D–E–C (12). In this communication we report that late after infection the nucleus contains viral RNA which is not exported to the cytoplasm and that some nuclear RNA sequences are complementary to cytoplasmic RNA sequences.

MATERIALS AND METHODS

Isolation of Cytoplasmic and Nuclear RNA from Infected Cells. Adenovirus type 2 was propagated in HeLa cells as described previously (8).

Poly(A) containing cytoplasmic RNA was extracted and fractionated by oligo(dT) cellulose chromatography as described previously (13). After lysis of the cells with 0.65% Nonidet P40 (Shell Oil Co.) in isotonic buffer (3), the nuclei were washed with isotonic buffer and extracted with phenol by the method of Holmes and Bonner (14), DNA was eliminated by digestion with 100 μg/ml of DNase for 60 min at 37° two or three times (Worthington Inc., New Jersey, "electrophoretically purified"). After each digestion the RNA was extracted with phenol:chloroform:isoamylalcohol at pH 9.0 as described by Brawerman *et al.* (15). All RNA preparations were analyzed for viral DNA contamination by hybridizing separated DNA strands to RNA samples which had been hydrolyzed in 0.3 M NaOH at 100° for 15 min.

Specific Fragmentation of Adenovirus 2 DNA. Endonuclease endo R. *Eco*RI was extracted from *Escherichia coli* strain Ry 13 (kindly made available by the Cold Spring Harbor Laboratory). The enzyme, referred to as *Eco*RI, was purified by phosphocellulose and DEAE chromatography. Ad2 DNA was digested and fragments separated as previously described (11).

Separation of Complementary Strands of Adenovirus 2 DNA. Separated strands of unlabeled adenovirus 2 DNA were ob-

Abbreviation: ad2, adenovirus type 2.

* The symposium, ref. 1, reviews the mechanisms for transcription both in prokaryotic and eukaryotic cells.

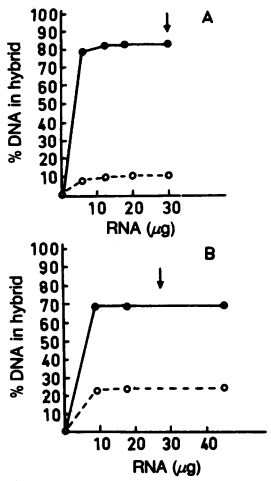

FIG. 1. Hybridization of late nuclear (A) and late cytoplasmic (B) RNA with ^{32}P-labeled separated strands of ad2 DNA. Each reaction mixture contained about 0.002 μg of probe DNA. The fraction of l-(●——●) and h-strand (○- - -○) DNA in hybrid was determined by chromatography on hydroxylapatite. The arrows indicate the molar RNA:DNA ratio which was used to hybridize late nuclear and cytoplasmic RNA with *Eco*RI fragments of ad2 DNA as given in Table 1.

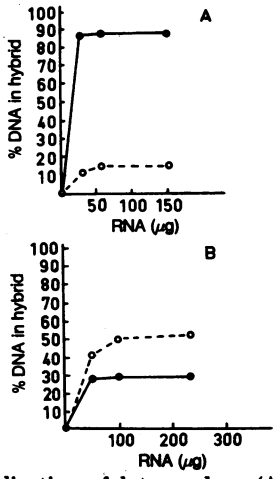

FIG. 2. Hybridization of late nuclear (A) and cytoplasmic (B) RNA with ^{32}P-labeled separated strands of fragment *Eco*RI-C. Hybridization mixtures with l-strand (●——●) and h-strand (○- - -○) DNA were assayed as described in the legend to Fig. 1.

tained by CsCl gradient centrifugation of denatured DNA in the presence of poly(U,G) (8). ^{32}P-Labeled strands of adenovirus 2 DNA or *Eco*RI fragments were prepared by allowing labeled denatured DNA to renature in the presence of an excess of one of the complementary strands as outlined in detail in a separate communication (13). Before use in hybridization experiments, the strands were degraded to an approximate size of 350 nucleotides by partial alkaline hydrolysis (11).

Nucleic Acid Hybridization. Hybridization mixtures with ^{32}P-labeled strands and unlabeled RNA were incubated at 65° for 2–5 days in a total volume of 0.2–1.0 ml. All samples for annealing contained 1 M NaCl, 0.4% sodium dodecyl sulfate, and 0.02–0.10 M phosphate buffer at pH 6.8. RNA·DNA hybrids were analyzed by hydroxylapatite chromatography as previously described (8).

Isolation of Double-Stranded RNA. RNA samples were incubated for 18–36 hr at 65° in 1 M NaCl or at 37° in 50% formamide, 0.6 M NaCl, all in 0.05 M Tris·HCl at pH 7.5. The mixtures were diluted and digested for 60 min at 37° with 20 μg/ml of RNase A (Worthington) and 20 U/ml of RNase TI (Worthington) in 0.05 M Tris·HCl at pH 7.5 with 0.15 M NaCl and 0.5 mM EDTA. Double-stranded RNA was isolated by cellulose chromatography as described by Franklin (16).

Determination of RNase Resistance after RNA·DNA Hybridization. Samples with cytoplasmic RNA labeled with [^{3}H]-uridine and unlabeled l- and h-strands were incubated at 65° for 18–36 hr. The reaction mixture contained 1 M NaCl, 1 mM EDTA, 0.01 M Tris·NCl at pH 7.9, and 50 μg of yeast RNA in a total volume of 0.1 ml. After incubation the samples were diluted 10-fold with 0.15 M NaCl in 0.01 M Tris·HCl at pH 7.9. Ribonuclease A and TI were added to final concentrations of 25 μg/ml and 25 U/ml, respectively. After 60 min at 37° sodium dodecyl sulfate was added to a final concentration of 0.5% and the samples were fractionated on Sephadex G-100 columns (Pharmacia Fine Chemicals, Uppsala, Sweden) with 0.15 M NaCl, 1 mM EDTA, 0.01 M Tris·HCl at pH 7.9.

RESULTS

Hybridization of late nuclear RNA to separated strands of ad2 DNA

Different amounts of unlabeled nuclear RNA were hybridized to ^{32}P-labeled l- and h-strands of ad2 DNA. The fraction of each strand in the hybrid was determined by chromatography on hydroxylapatite. Fig. 1 shows that late nuclear RNA could hybridize to about 85% of the l-strand and 10–15% of the h-strand. Cytoplasmic RNA present late after infection has previously been found to hybridize to 65–70% of the l-strand and 25% of the h-strand (8).

Hybridization of late nuclear RNA to separated strands of the six *Eco*RI fragments of ad2 DNA

Cytoplasmic RNA synthesized late after infection is complementary to two or more separate regions on both the complementary strands (13), and significant fractions of both the the l- and h-strands from fragments *Eco*RI-A, B, C have been found to hybridize with late cytoplasmic RNA (13). In order to compare late nuclear and cytoplasmic RNA, hybridizations were performed with unlabeled nuclear RNA and ^{32}P-labeled separated strands of fragments A–F. The results showed that late nuclear RNA can hybridize to 84–91% of the l-strand from all six *Eco*RI fragments (Table 1, Fig. 2A). A significant fraction of the h-strand of fragments *Eco*RI-A, *Eco*RI-B, and *Eco*RI-C also hybridized with late nuclear RNA. However, in each case a smaller fraction of the h-strand hybridized to late nuclear RNA when compared to cytoplasmic RNA (Table 1). When nuclear RNA was preincubated under annealing conditions before exposure to DNA, the amount of hybridization to the h-strand of fragments *Eco*RI-B and *Eco*RI-C was greatly reduced (Table 1). This suggests self-complementarity between RNA molecules. Furthermore, since late cytoplasmic RNA can hybridize to about 50% of the h-strand of fragments *Eco*RI-B and C and since nuclear RNA is derived from about 85% of the l-strand of these two fragments, over-

TABLE 1. *Hybridization of late nuclear and cytoplasmic RNA with ^{32}P-labeled complement-specific DNA from the six adenovirus 2 EcoRI cleavage fragments*

DNA probe	Fraction of the genome	Percent of DNA probe in hybrid with	
		Nuclear RNA*	Cytoplasmic RNA*
RI-Al	0.59	84	71
RI-Ah		13 (6)†	15
RI-Bl	0.12	88	52
RI-Bh		15 (1)	40
RI-Cl	0.10	87	31
RI-Ch		19 (3)	50
RI-Dl	0.07	91	82
RI-Dh		3	5
RI-El	0.06	88	82
RI-Eh		3	12
RI-Fl	0.05	87	84
RI-Fh		7	11

* Average of three experiments.

† Values in parentheses give the fraction of DNA probe in hybrid after hybridization with nuclear RNA which had been incubated under annealing conditions before hybridization.

lapping RNA sequences must be synthesized during adenovirus infection (Table 1, Fig. 2).

Demonstration of complementarity between late nuclear and cytoplasmic RNA

^{32}P-Labeled h-strand DNA of fragment *Eco*RI-C was used to directly demonstrate complementarity between late RNA preparations from nucleus and cytoplasm. Late cytoplasmic RNA was incubated with different amounts of late nuclear RNA under annealing conditions. After 48 hr, ^{32}P-labeled h-strands of fragment *Eco*RI-C were added to the incubation mixture and hybridization was continued. After another 2 days the samples were assayed by hydroxylapatite chromatography and the fraction of the DNA probe in hybrid was determined. After preincubation with nuclear RNA the late cytoplasmic RNA could hybridize to a smaller fraction of the h-strand of fragment *Eco*RI-C than without preincubation (Table 2). For example, late cytoplasmic RNA which was preincubated without nuclear RNA hydridized to 42% of the h-strand of fragment *Eco*RI-C, whereas the same amount of cytoplasmic RNA after preincubation with nuclear RNA hydridized only to about 10% of the same DNA (Table 2).

Isolation of double-stranded RNA from adenovirus infected cells

Cells were pulse-labeled with [^{3}H]uridine for different periods of time at 16 hr after infection and nuclear and cytoplasmic RNA was prepared. The RNA preparations were mixed with unlabeled nuclear or cytoplasmic RNA and incubated under annealing conditions. After digestion with ribonuclease, double-stranded RNA was isolated by chromatography on cellulose. The duplex nature of the RNA was verified by equilibrium centrifugation in Cs_2SO_4. Only 0.5–1.3% of late pulse-labeled nuclear RNA became double stranded after annealing (Table 3). Pulse-labeled late cytoplasmic RNA contained less than 0.1% of the radioactivity in double-stranded RNA after annealing. Mixtures of unlabeled

TABLE 2. *Hybridization of the h-strand of fragment EcoRI-C with late cytoplasmic RNA with or without prehybridization to late nuclear RNA*

RNA in preincubation mixture*	Percent of fragment *Eco*RI-Ch DNA in hybrid
Cytoplasmic RNA	42
Cytoplasmic RNA + nuclear RNA (61 μg)	29
Cytoplasmic RNA + nuclear RNA (122 μg)	15
Cytoplasmic RNA + nuclear RNA (305 μg)	10

* Samples which contained 118 μg of cytoplasmic RNA were incubated for 2 days under annealing conditions before the ^{32}P-labeled DNA probe was added.

cytoplasmic RNA and labeled nuclear RNA contained about 3% of the label in duplex RNA (Table 3). Since the major fraction of RNA which is synthesized late after infection is viral (17), the results suggest that only a small fraction of the viral RNA in the nucleus which has been synthesized late after adenovirus infection is self-complementary and no complementary RNA sequences are transported to the cytoplasm.

In order to determine the complexity of double-stranded RNA from adenovirus infected cells, samples of nuclear RNA were incubated under annealing conditions and double-stranded RNA was isolated as described above. The isolated RNA was heat denatured and hybridized in solution with fragmented ^{32}P-labeled separated strands of ad2 DNA. As shown in Table 4 double-stranded viral RNA can hybridize to about 60% of the genome. It is possible that the 60% figure is an underestimate since some sequences present in duplex RNA may be present at low concentrations and, therefore, fail to hybridize.

RNA synthesis from the h-strand at different time periods after infection

Late nuclear RNA contains sequences which are derived from almost the entire l-strand (Table 1). Although some of these

TABLE 3. *Percentage double-stranded RNA in pulse-labeled late nuclear and cytoplasmic viral RNA after exhaustive annealing**

RNA preparation	Period of label *in vivo* (min)	% labeled RNA in double strands†
Labeled nuclear + unlabeled nuclear (500 μg)	10	1.3
	30	1.0
	60	0.8
	180	0.5
Labeled cytoplasmic + unlabeled cytoplasmic (200 μg)	180	<0.1
Labeled nuclear + unlabeled cytoplasmic (200 μg)	180	3.1

* RNA preparations which were pulse-labeled at 16 hr after infection were incubated under annealing conditions in a total volume of 120 μl for 18 hr.

† Double-stranded RNA was estimated by chromatography on cellulose columns (16).

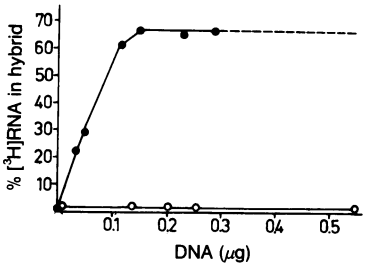

FIG. 3. The fraction of late pulse-labeled cytoplasmic RNA in hybrid after annealing with l-(●) or h-strand (○) DNA. Cytoplasmic RNA was isolated from cells which were pulse-labeled between 17 and 20 hr after infection with 20 μCi/ml of [^{3}H]-uridine. The reaction mixtures contained 0.22 μg of RNA and different amounts of l- and h-strand DNA. After incubation the samples were digested with RNase and fractionated on Sephadex G-100. RNA in hybrid was scored as the fraction of radioactivity in the void volume.

sequences are complementary to RNA sequences in late cytoplasmic RNA, only a small fraction of the RNA which is synthesized late becomes double-stranded after incubation under annealing conditions. This suggests that RNA sequences from the h-strand are synthesized at a lower rate than sequences from the l-strand or that RNA sequences from the h-strand turn over more rapidly. To investigate these possibilities, infected cells were pulse-labeled with [^{3}H]uridine for 3 hr at different times after infection. Cytoplasmic RNA was isolated by oligo(dT) cellulose chromatography and incubated with different amounts of unlabeled l- and h-strand DNA under annealing conditions. After digestion with ribonuclease the samples were chromatographed on Sephadex G-100 and the fraction of radioactivity eluting as hybrid with the void volume was determined. Less than 1% of RNA which was pulse-labeled at 17–20 hr after infection became ribonuclease resistant after hybridization with the h-strand, whereas 60–70% of the label became resistant to ribonuclease after incubation with the l-strand (Fig. 3). In contrast, cytoplasmic RNA from cells which were pulse-labeled 2–5 and 8–11 hr after infection contained significant amounts of RNA which hybridized both to l- and h-strand DNA (Table 5).

DISCUSSION

Late after adenovirus infection nuclear RNA can hybridize to a greater fraction of the l-strand of the viral DNA than can cytoplasmic RNA. This is compatible with previous reports on sequence differences between nuclear and cytoplasmic RNA, detected by competition hybridization experiments on filters (3, 5, 18). Late cytoplasmic RNA accounts for 90–95% of the coding potential of the adenovirus 2 genome, assuming that messenger RNA is asymmetric (8). Hybridization experiments with late nuclear RNA and separated strands of the six *Eco*RI fragments of ad2 DNA showed that the additional sequences present in nuclear RNA do not merely correspond to the remaining 5–10% of the ad2 genome, but also include sequences which are complementary to stable messenger RNA (Table 1). This was demonstrated in hybridization experiments between late nuclear or cytoplasmic RNA and separated strands of fragment *Eco*RI-B and *Eco*RI-C. RNA prepared from the cytoplasm is derived from about 50% of the h-strand of these two fragments whereas RNA from the nucleus hybridized to 85% or more of the l-strand from all

TABLE 4. *Hybridization of double-stranded RNA with ^{32}P-labeled h-strand DNA*

RNA sample	% h-strand DNA in hybrid
Denatured RNA*	63
Native RNA	4
Denatured and† hydrolyzed RNA	5

The hybridization mixture contained 0.002 μg of probe DNA and approximately 1 μg of double-stranded RNA estimated from the recovery of radioactivity. The same results were obtained with l-strand DNA as probe.

* Double-stranded RNA was denatured by boiling for 20 min in 0.01 M Tris·HCl at pH 7.5 with 1 mM EDTA.

† Double-stranded RNA was boiled for 20 min in 0.2 M NaOH, neutralized, and hybridized with DNA.

*Eco*RI fragments. Thus, overlapping RNA sequences are present late after adenovirus infection. Furthermore, complementarity between late nuclear and cytoplasmic RNA was demonstrated in experiments in which the h-strand of fragment *Eco*RI-C was hybridized to cytoplasmic RNA which had been preincubated with nuclear RNA (Table 2).

Although the cytoplasm late after infection contains RNA sequences which are complementary to 25% of the h-strand, our results suggest that little or no newly synthesized messenger RNA originates from the h-strand at 17–20 hr after infection (Table 5). This finding implies that most late messenger RNA from the h-strand is synthesized early and preserved until late after infection. Our results are at variance with those of Landgraf-Laurs and Green (7), who found that 10–15% of adenovirus specific RNA which was synthesized late after infection hybridized to the h-strand. Their results are, however, not strictly comparable to ours since they used total cellular RNA and filter hybridization.

TABLE 5. *Percent of pulse-labeled cytoplasmic RNA in RNase resistant hybrid after annealing with an excess of unlabeled l- and h-strand DNA*

Time period for pulse-labeling*	Percent ^{3}H-labeled RNA in hybrid after hybridization with†		
	l-strand DNA	h-strand DNA	Ratio l/h
2–5 hr	2.6	1.3	2
8–11 hr	38	3.7	10
17–20 hr	66	0.3	220

* Infected cells were labeled with 20 μCi/ml of [^{3}H]uridine at different time periods after infection. Cytoplasmic RNA was extracted by phenol (15) and fractionated twice by oligo(dT)–cellulose chromatography.

† RNA in hybrid was scored as the fraction of radioactivity recovered in the void volume after Sephadex G-100 chromatography of RNase-treated samples. Two or more different concentrations of DNA were used for each RNA sample to ascertain that all viral RNA sequences were saturated with DNA. The values represent an average of three to five determinations. Background radioactivity, recovered in the void volume after chromatography of RNA samples without hybridization to DNA, was subtracted in each case.

A preferential transcription of the l-strand late after infection would explain why only a negligible fraction of late pulse-labeled nuclear RNA can be recovered as duplex RNA after self-hybridization (Table 3), although the infected cells apparently contain complementary RNA sequences (Table 2). Because of the presence of complementary sequences, hybridizations between nuclear RNA and viral DNA will be competing reactions between the DNA probe and RNA, on one hand, and between complementary RNA molecules, on the other. Consequently liquid hybridization might not score all nuclear RNA sequences.

Transcription of complementary RNA sequences has previously been detected in eukaryotic systems. Aloni and coworkers have reported that mitochrondrial DNA and DNA from papovaviruses are transcribed in a manner which leads to synthesis of complementary RNA sequences (19–22). From studies on transcription of SV40 DNA Aloni (20) has proposed a model which postulates that both strands of SV40 DNA are completely transcribed. After addition of poly(A) at the 3′ termini, the transcripts are degraded from their 5′ ends so that messenger RNA is generated which contains no overlapping sequences. Adenovirus messenger RNA could be generated by a similar mechanism although late—after adenovirus infection messenger RNA is preferentially generated from the l-strand. Our results do not exclude, however, that both strands are transcribed late with the same frequency but that the h-strand transcripts become degraded so rapidly that they escape detection.

It is not possible to establish whether synthesis of complementary sequences during adenovirus infection reflects a specific mechanism to generate messenger RNA or if this is due to occasional errors in the control of transcription. Complementary RNA from substantial regions on fragments *Eco*RI-B and *Eco*RI-C can readily be demonstrated late during adenovirus reproduction. Duplex RNA, although it only represents a small fraction of the total RNA synthesized, corresponds to at least 60% of the sequences of adenovirus DNA. Altogether, these findings suggest that the synthesis of complementary RNA sequences is a consequence of the mechanism by which the adenovirus genome is transcribed.

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