Further Characterization of the Cellular DNA Hybridizing with the RNA of Rous Sarcoma Virus

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ABSTRACT Both 70S and 4S RNAs of Rous sarcoma virus (RSV) contain nucleotide sequences homologous to those in cellular DNA. Preparations of these RNAs were found to include some adventitious cellular sRNA. Nevertheless, both 70S and 4S RSV-RNAs were found by competition techniques to contain sequences distinct from those in cellular ribosomal RNA and 4S RNA. Competition experiments also reveal substantial homology between 70S and 4SRSV-RNA. In hybridization experiments performed in formamide at low temperatures, the melting profiles of hybrids between 70S RSV-RNA and cellular DNA, and 4S RSV-RNA and cellular DNA, are essentially superimposable. It is suggested that there are at least two origins of 4S RSV-RNA in RSV-RNA preparations: degradation of 70S RSV-RNA and inclusion of adventitious cellular 4S RNA. The possibility of a specific viral 4S RNA is not excluded.

The RNA of Rous sarcoma virus (RSV) hybridizes with eukaryotic and oncogenic viral DNAs. RSV-RNA hybridized to all of the DNAs analyzed has the same, or a closely similar, base composition; this evidence, coupled with the observation of close similarities in melting temperatures of the complexes with various DNAs, prompted the suggestion that a common base sequence in DNA functions in hybridization with RSV-RNA (1).

In this article, we take up two questions arising out of the previous work. First, how precise is the homology between RSV-RNA and eukaryotic DNA? As a consequence of the existence of partially related base sequences (2), molecular hybridization reactions of DNAs and RNAs of eukaryotes may not display locus specificity (3). However, the specificity of these reactions may be enhanced in formamide solutions at low temperatures (3). We have employed the formamide techniques to compare the melting profiles of hybrids between 70S RSV-RNA and cellular DNA, and 4S RSV-RNA and cellular DNA.

Second, what are the relations between the low molecular weight RSV-RNAs and the 4S RNA of the host cell? This question arises from a consideration of the finding (4) that in addition to the 70S RSV-RNA of the viral genome, the Rous virion contains 4S RNA, as well as cellular 18S and 28S RNAs. Moreover, avian myeloblastosis virus (AMV) appears to contain functional tRNA acquired from the host cell (5-7). We earlier showed that contamination with 18S and 28S ribosomal RNAs was not a factor contributing to our

previous findings (1). However, the presence of 4S RNA in the virion raises the question, to what extent does the homology between RSV-RNA and cellular DNA reflect homology between such an acquired low molecular weight RNA and cellular DNA, as distinct from homology between a true viral RNA and cellular DNA? We have answered this question by competition experiments, and by further characterizing the hybrids between 4S RSV-RNA and cellular DNA, which have in turn been compared with hybridized 70S RSV-RNA and cellular DNA. We conclude that, although our RSV-RNA contains some contaminating 4S RNA of cellular origin, the interaction of RSV-RNA with cellular DNA does involve DNA sequences other than those homologous to cellular low molecular weight RNA and ribosomal RNAs.

MATERIALS AND METHODS

[³²P]Orthophosphate, carrier-free, was purchased from New England Nuclear Corp. [³H]uridine (26.2 Ci/mmol) was obtained from Schwarz BioResearch. Formamide (Fisher Chemicals, reagent grade) was autoclaved before use. Salmon-sperm DNA was purchased from Nutritional Biochemicals Co., calf-thymus DNA and RNase B were bought from Worthington Biochemical Corp. RNase was treated as described (1).

Propagation of cells and virus; labeling with radioisotopes

Preparation of fibroblastic cells from 11-day-old chick embryos (avian leukosis virus-free eggs were obtained from SPAFAS, Inc., Norwich, Conn.), infection with a Bryan high-titer strain of RSV (maintained in our laboratory for several years), and virus assays were performed as described (1), as were ³²P labeling and purification and concentration of RSV.

Preparation of ³²P-labeled RSV-RNA

Purified and concentrated ³²P-labeled RSV was first dialyzed for 2-3 hr against a buffered solution containing 0.01 M potassium acetate-0.1 M NaCl-10 µg/ml polyvinyl sulfate (pH 5.0), after which bulk RNA was extracted by a sodium dodecyl sulfate-phenol method (8). The RNA was then fractionated by centrifugation through sucrose gradients (5-20% in the aforementioned buffer) in a Spinco preparative ultracentrifuge, an SW with 25.1 rotor (15,000 rpm, 18 hr, 4°C). The sedimentation pattern is shown in Fig. 1. Fractions 2-11, inclusive, were first mixed with an amount of 2.0 M potassium acetate sufficient to bring the final molarity of potassium acetate to 0.1, after which the RNA was precipitated with four volumes of absolute ethyl alcohol precooled to -20°C; the mixture was held at -20°C overnight. The pre-

Abbreviations: RSV, Rous Sarcoma Virus; SSC, 0.15 M NaCl-0.015 M sodium citrate.

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FIG. 1. Sucrose density gradient centrifugation of RSV-RNA. ^{32}P -labeled RSV-RNA was fractionated as described in *Methods*. After centrifugation, the radioactivity in 5 μl of each fraction was determined in a scintillation counter.

precipitate was collected as described (8). This fraction we designate as 70S RSV-RNA. Fractions 25–27, inclusive, were treated in the same fashion and are designated as 4S RSV-RNA.

Preparation of cellular DNA and RNAs

Cellular nucleic acids (DNA, ribosomal RNA, and low molecular weight RNA) were extracted from either 11-day-old chick embryos (less heads) or secondary monolayer cultures of

FIG. 2. Competition of labeled 70S and 4S RSV-RNAs and labeled cellular low molecular weight RNA with unlabeled cellular low molecular weight RNA. Filters containing 10–20 μg of chick-embryo DNA were incubated under standard hybridization conditions ($4 \times \text{SSC}$, 62°C , 12 hr) with 2 μg of either 70S or 4S ^{32}P -RSV-RNA (8×10^4 cpm/ μg and 1.5×10^5 cpm/ μg , respectively) or with 2 μg of cellular ^3H -RNA of low molecular weight (1×10^5 cpm/ μg) in the presence of varying amounts of unlabeled chick-embryo low molecular weight RNA. At 100% hybridization, the values were, for 4S RSV-RNA, 370 cpm; for 70S RSV-RNA, 220 cpm; and for low molecular weight RNA, 230 cpm.

FIG. 3. Competition of 70S RSV-RNA with 4S RSV-RNA. Filters containing 10–20 μg of chick-embryo DNA were incubated under standard conditions ($4 \times \text{SSC}$, 62°C , 12 hr) with 0.5 μg of 70S ^{32}P -RSV-RNA (1.1×10^5 cpm/ μg) or 0.5 μg of 4S ^{32}P -RSV-RNA (1.0×10^5 cpm/ μg) in the presence of 0.5 μg of chick-embryo low molecular weight RNA and varying amounts of unlabeled 4S RSV-RNA. As controls, 28S ^3H -rRNA (chick, 2.5×10^5 cpm/ μg) and unlabeled 28S rRNA (chick) were used. Values at 100% hybridization were 70S RSV-RNA, 200 cpm; 4S RSV-RNA, 1600 cpm; and 28S rRNA, 460 cpm.

11-day-old chick-embryo fibroblastic cells, and fractionated on methylated albumin-kieselguhr (MAK) columns. The 18S and 28S ribosomal RNAs were further fractionated by sucrose gradient centrifugation (8). In labeling studies, cultured fibroblasts were incubated for 2–3 days with 10 $\mu\text{Ci/ml}$ of ^3H -uridine; RNA and ribosomal RNA were prepared as just described.

Molecular hybridization

DNA-RNA hybridization was performed using DNA filters prepared by a modification (1) of the method of Gillespie and Spiegelman (9). Details of nucleic acid reassociations in formamide, as performed by the methods of McConaughy *et al.* (3), are described in the figure legends.

RESULTS

Competition of cellular low molecular weight RNA with RSV-RNA

Competition experiments show clearly that only a fraction of the RSV-RNA hybridizing with cellular DNA can be accounted for by the presence of cellular low molecular weight RNA in the Rous virion. In Fig. 2, only 10% of the hybridization between 70S RSV-RNA and cellular DNA is lost after competition with cellular low molecular weight RNA. Although there was some variation from experiment to experiment, the reduction never exceeded 20% under the conditions employed. Somewhat more variation was encountered when 4S RSV-RNA was used, with the reduction occasionally being as high as 40%. However, the findings presented in Fig. 2 are the most typical. In short, it seems likely that both 70S and 4S RSV-RNA preparations contain some cellular low molecular weight RNA, with the amount contained in the

FIG. 4. Saturation curves for ^{32}P -labeled 70S and 4S RSV-RNAs hybridized with chick-embryo DNA. Filters containing 20–30 μg of DNA were incubated with ^{32}P 70S (7.0×10^4 cpm/ μg , closed circles) or 4S (2.1×10^5 cpm/ μg , open circles) ^{32}P RSV-RNA in the presence of chick-embryo low molecular weight RNA and rRNA (1.0 and 0.5 mg, respectively). Counts were standardized to 100 μg of DNA.

4S fraction being higher, as would be expected. However, a reduction greater than 40% was never observed. In Fig. 2, for example, where a 20% reduction by low molecular weight RNA was maximal for RSV-RNAs, there was an 85–90% reduction in hybridization when cellular low molecular weight RNA was homologously competed.

Relation between 70S RSV-RNA and 4S RSV-RNA

The results just described indicate that the 4S RSV-RNA is not simply a contaminating 4S cellular RNA, but rather that it is truly viral. If so, how is it related to the 70S RSV-RNA? We have tried to gain some insight into this question by competition experiments with unlabeled 4S RSV-RNA as the "competitor" and ^{32}P -labeled 70S and 4S RSV-RNA in the final hybrids with cellular DNA (Fig. 3). The experiments were performed in the presence of unlabeled cellular sRNA (in both competition and final hybridization steps). As a control of the experimental conditions, 28S ribosomal RNA was homologously competed.

These experiments are less than fully satisfactory; because of the difficulty in obtaining large amounts of RSV-RNA, competition could not be studied over a broad range. Nevertheless, the findings do show that in the presence of cellular low molecular weight RNA, 4S RSV-RNA does compete significantly with 70S RSV-RNA. Thus, a highly significant component (at least half) of the 70S RSV-RNA that is homologous to chick-embryo DNA is also homologous to 4S RSV-RNA. We may say, therefore, that 4S RSV-RNA contains at least some sequences found in 70S RSV-RNA (suggesting that they are derived by degradation of 70S RSV-RNA) plus some cellular low molecular weight RNA. We cannot say whether there are any unique sequences in 4S RSV-RNA.

The saturation values for 70S and 4S RSV-RNA with chick-embryo DNA are the same, although the curves differ slightly (Fig. 4). Cellular low molecular weight RNA must be present in order to reach saturation in hybridizing 4S RSV-RNA and cellular DNA (about 1000 μg of cellular RNA for 50 μg of chick-embryo DNA). Although the saturation values for calf thymus, Chinese hamster, and salmon sperm DNAs differ from the value for chick embryo DNA, the pattern of the curves are similar to those in Fig 4.

FIG. 5. Time course of hybrid formation between chick-embryo DNA and RSV-RNA under standard (A) and stringent (B) conditions. (A) Filters containing 54 μg of DNA were incubated with 1 μg of ^{32}P -labeled 4S RSV-RNA (4.4×10^5 cpm/ μg , open circles) or with 0.5 μg of ^{32}P -labeled 70S RSV-RNA (1.4×10^5 cpm/ μg , closed circles), in $4 \times \text{SSC}$, in the presence of 1 mg of cellular low molecular weight RNA and 500 μg of cellular ribosomal RNA, at 62°C . (B) As in (A), except that a mixture of 30% formamide plus $2 \times \text{SSC}$ replaced the $4 \times \text{SSC}$, and incubation was performed at 37°C .

Effects of formamide on DNA-RNA hybridization

The precision of homology between RSV-RNA and several eukaryotic DNAs was studied by forming hybrids in formamide solutions. At the outset, two experimental protocols were employed: hybridization in increasing concentrations of formamide at a constant temperature, and hybridization in a constant amount of formamide (30%) at increasing temperatures. Although considerable cross-reaction was observed at concentrations of formamide under 30%, the extent of hybridization in formamide concentrations of from 30 to 70% at 37°C did not depart appreciably from the values under standard conditions ($4 \times \text{SSC}^\dagger$, 62°C). The optimum temperature in 30% formamide was observed to be 37°C , which corresponded to 59°C in aqueous solutions, a value very close to the 62°C previously employed. The kinetics of hybridization in aqueous medium ($4 \times \text{SSC}$, 62°C) and formamide (30%, in $2 \times \text{SSC}^\ddagger$, 37°C) were compared, with both 70S RSV-RNA and 4S RSV-RNA, in combination with 11-day-old chick embryo DNA (Fig. 5). No significant differences were observed, the reaction being half-completed by 2.5–3 hr; this result may be interpreted as suggesting that the DNA sequences homologous to RSV-RNA are contained in the "repeated sequences" (2).

The melting profiles of hybrids between 70S RSV-RNA and cellular DNA, and 4S RSV-RNA and cellular DNA, are essentially superimposable (Fig. 6). Earlier, we had shown that under standard conditions the T_m of the hybrid between 70S RSV-RNA and chick-embryo cellular DNA was 73°C . Hybrids formed under stringent conditions were used in the

† SSC, standard saline citrate; $4 \times \text{SSC}$, 0.60 M NaCl–0.06 M sodium citrate.

‡ 0.30 M NaCl–0.03 M sodium citrate.

FIG. 6. Thermal stabilities of the hybrids between chick embryo DNA and RSV-RNAs. Ten filters, containing 50–60 μ g of chick-embryo DNA, were incubated with 2 μ g of 32 P-labeled 4S RSV-RNA (3.0×10^6 cpm/ μ g) and ten with 5 μ g of 70S [32 P]RSV-RNA (1.2×10^6 cpm/ μ g) in 50% formamide–2 $\times$ SSC, containing chick embryo rRNA and low molecular weight RNA (0.5 mg and 1.0 mg, respectively, at 37°C).

present comparison. The T_m s for cellular DNA with 70S and 4S RSV-RNAs, respectively, are 80°C and 79°C. Similar curves were obtained with calf-thymus, salmon-sperm, and RSV-infected chick-embryo fibroblast DNAs.

DISCUSSION

Despite the considerable evidence for homology between oncogenic viral RNAs and cellular DNAs (1, 10–13), the possibility remained that the viral RNA–cellular DNA hybrids were, in fact, hybrids of a cellular RNA contained in the virus preparations with cellular DNA. We had earlier excluded the ribosomal RNAs as a source of such an artifact (1), but the observation that cellular low molecular weight RNA (or even functional tRNA), presumably acquired from the host cell during viral assembly, is present in RNA viruses (4–7) made it necessary to determine whether it might, in fact, account for our earlier observations. The present findings show that although both 70S and 4S RSV-RNAs contain some cellular low molecular weight RNA, they also contain viral-specific nucleotide sequences homologous to cellular DNA, and distinct from cellular low molecular weight RNA.

Bishop *et al.* (4) also suggested that Rous virus 4S RNA is not simply a contaminant, but no conclusive evidence was offered. Bishop *et al.* (4) have summarized earlier explanations of its origin: (a) that it is derived from 70S RSV-RNA by degradation during preparation; and (b) that it could be a contaminant derived from cellular debris. We believe that both explanations may be correct. Our competition experiments, in which competition with cellular low molecular weight RNA reduced the interaction of both 70S and 4S RSV-RNAs with cellular DNA, suggest that some cellular low molecular weight RNA may be included in viral RNA

preparations. In this conclusion we agree with Bishop *et al.* However, we also find significant competition between 70S and 4S RSV-RNAs in the presence of cellular low molecular weight RNA, consistent with the argument that one may be derived from the other. Bishop *et al.* (4) believe that their observations (electrophoretic homogeneity of 4S RSV-RNA and results of methyl-labeling experiments) rule out the degradation hypothesis. If they are correct, then to explain our observations it is necessary to postulate not only that the Rous virus contains at least two viral-specific RNAs, the 70S RNA of the viral genome and a 4S RNA (distinct from cellular 4S RNA), but that the 70S RNA contains substantial stretches of sequences identical to those in the 4S viral RNA.

Our own experiments do not exclude the possibility of a viral-specific 4S RNA. We are not inclined, however, to discard the degradation hypothesis as one source of this RNA.

In our earlier article (1), we took up the question of the size of the RNA recovered from hybrids between 70S RSV-RNA and cellular DNA. We considered the observation that they were in the 3–4S range to be meaningful. Now, however, Fry and Artman (14) have reported that even the RNA recovered from hybrids of DNA and ribosomal RNAs (prepared from *E. coli* under stringent conditions) sediments at 3–4S after RNase treatment. Since RNase treatment was part of our procedure, our interpretation must now be doubted. We can offer no meaningful estimate of the fraction of the 70S RNA involved in the hybrid. However, the difference in base composition between complete 70S RNA and the RNA in the hybrids, and the rather broad melting profiles of the hybrids, suggests that something considerably less than the complete 70S RNA is involved. Whatever the size of these sequences, the facts that these hybrids are specifically formed under stringent conditions, and that both the cellular ribosomal RNAs and low molecular weight RNAs are eliminated as possible sources of contamination, have encouraged us to continue to explore their function.

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