Evidence for 30-40S RNA as Precursor of the 60-70S RNA of Rous Sarcoma Virus

(subunits of tumor virus RNA/RNA-dependent DNA polymerase/RNA primers)

E. CANAANI, K. V. D. HELM, AND P. DUESBERG

Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, Calif. 94720

Communicated by Robley C. Williams, November 26, 1972

ABSTRACT Rous sarcoma virus harvested from cells at intervals of 3 min has the same density, sedimentation coefficient, and DNA polymerase as virus harvested at hourly intervals. The RNA of the Prague strain-C consists of a minor class of 60-70S RNA, a major class 30-40S RNA, and a 4-12S class of RNA present at variable concentration. The RNA of the Schmidt-Ruppin strain-A contains more 60-70S than 30-40S RNA. Upon incubation of virus harvested at 3-min intervals at 40° in cell growth medium or Tris-saline, most of the 30-40S RNA is converted to 60-70S RNA.

The electrophoretic mobility of the 30-40S RNA of the Rous virus harvested at 3-min intervals is lower than that of the 30-40S subunits of completely dissociated 60-70S RNA; after heating, their mobilities are identical. Heating also releases some small RNAs from 30-40S RNA of virus harvested at 3-min intervals, but five times more 4S RNA is released if the 30-40S RNA is allowed to convert to 60-70S in the virus. The template activity for Rous virus DNA polymerase of the 30-40S RNA of Rous virus harvested at 3-min intervals is about five times lower than that of 60-70S RNA. It is suggested that association of 30-40S RNAs with some RNAs of the 4-12S class may take place simultaneously with their conversion to 60-70S RNA.

The RNAs of all known groups of RNA tumor viruses consist of a major 60-70S class and minor 4-12S class of polynucleotides (1, 2). The 60-70S RNA has a subunit structure (2). It can be dissociated into several major 30-40S RNAs (3-5) and several small heterogeneous and distinct RNAs (6, 7). There is also evidence that certain RNA tumor viruses contain, in addition to 60-70S RNA, free 30-40S RNA in the virion (8-10). Yet, free 30-40S RNA has not been observed in avian tumor viruses propagated under similar conditions. However, it was found recently that Rous sarcoma virus (RSV), an avian tumor virus, if harvested from cells at intervals of 5 min, contains heterogeneous RNA smaller than the 60-70S of virus harvested at hourly intervals (11). The RNA of such virus consisted of a principal 55-60S component, and several smaller components. Conversion of these RNA components to 60-70S RNA was not observed in purified virus (11).

These notions, and in particular the one that virus harvested at short intervals has an RNA different from virus harvested at long intervals (10, 11), led us to ask whether a precursor form of the 60-70S RNA complex can be demonstrated in the virion. We found that RSV harvested from infected cells at intervals of 3 min contains three to six times more 30-40S RNA than 60-70S RNA, and that most of the 30-40S RNA may be converted to 60-70S RNA in the virion. We

have also asked whether the viral 4-12S RNAs and the small RNAs associated with 60-70S RNA (6, 7) result only from partial degradation of 60-70S RNA in the virion as proposed by some (12), or whether at least a fraction of these small RNAs are present in virus freshly budded from infected cells. Recent studies in this laboratory indicated that in several strains of avian tumor viruses harvested at hourly intervals small RNAs were present, both free and associated with 60-70S RNA (unpublished). The relative concentrations of these small RNAs were only little decreased with decreasing age of the virus, making it unlikely that all of these small RNAs result from degradation of the 30-40S subunits. We report here that even virus harvested from cells at intervals of 3 min contains free 4-12S RNAs, and that the 30-40S RNA of this virus is associated with some small RNAs.

MATERIALS AND METHODS

Virus. Prague RSV of subgroup C (13) was used in all but one experiment (see Fig. 3). Propagation and purification of radioactive virus harvested at hourly intervals was by published procedures (14). [³H]RSV harvested at 3-min intervals was prepared by addition of 100-200 μ Ci of [³H]uridine (New England Nuclear Corp., 20-30 Ci/mmol) to a transformed culture of chick fibroblasts in 6 ml of complete medium (14) per 10-cm petri dish. After incubation for 5-12 hr, the medium was removed. Subsequent virus collections at 3-min intervals were with 3-4 ml of medium 199 (GIBCO) containing 2% tryptose phosphate broth, 1% chick serum, and no additional radioactive precursor. Media were cooled immediately to 0-4°, and all further operations with the virus were at this temperature.

RESULTS

Properties of Rous sarcoma virus harvested at short intervals

[³H]RSV in medium harvested at intervals of 3 min from infected cultures was concentrated and sedimented in a sucrose gradient, together with ³²P-labeled RSV harvested at intervals of 5 hr (Fig. 1). It can be seen in Fig. 1A that both viruses had the same sedimentation coefficient of about 600 S, based on a 200S marker of tobacco mosaic virus. The distribution of viral DNA polymerase activity after sedimentation was determined in parallel on another aliquot in a separate rotor tube, but without other viral markers. It is shown in Fig. 1B that peaks of poly(rA)·oligo(dT)-dependent DNA polymerase activity and [³H]uridine label of virus harvested at 3-min intervals coincide at about 600 S. The buoyant density

Abbreviation: RSV, Rous Sarcoma Virus.

FIG. 1. Velocity and equilibrium density gradient sedimentation of Rous sarcoma virus (RSV) harvested at intervals of 3 min. (A) [^3H]Uridine-labeled RSV harvested at 3-min intervals (see text) was precipitated from 100 ml of medium with an equal volume of saturated ammonium sulfate and redissolved in 10 ml of standard buffer (7). After centrifugation for 3 min at $3000 \times g$, the supernatant was reprecipitated and centrifuged at $30,000 \times g$ for 5 min. The pellet was redissolved in 1–1.5 ml of standard buffer. It was then mixed with an appropriate amount of ^{32}P -labeled RSV harvested at intervals of 3 hr and with a marker of tobacco mosaic virus, and layered on a preformed sucrose gradient (10–25%, w/v) in standard buffer containing 0.2 M NaCl. Centrifugation was for 70 min at 25,000 rpm in a Spinco SW25.3 rotor at 2° . Radioactivity was determined in a Packard spectrophotometer on a 100- μl aliquot of each fraction in 5 ml of toluene-based scintillation fluid containing 20% NCS (Nuclear Chicago). The tobacco mosaic virus marker was located by its absorbance at 260 nm. (B) [^3H]Uridine-labeled RSV harvested at 3-min intervals was purified from 170 ml of medium and sedimented as described for (A). A 100- μl aliquot was incubated with 100 μl of a solution containing 12 mM MgCl_2 , 10 mM dithiothreitol, 0.1 M Tris-HCl (pH 8.0), 0.2% Triton X-100, 2 μg of poly(rA), 1 μg of $(\text{dT})_{12-18}$, and 5 μM [^3H]dTTP for 2 hr at 40° . [^3H]Poly(T) was determined as described (17, 18), and was plotted after subtraction of the ^3H counts of the virus. (C) Identical aliquots of fractions 8–15 (Fig. 1B) were pooled and diluted with an equal volume of standard buffer and layered on a preformed sucrose density gradient prepared from 4 ml of 55% sucrose and 4 ml of 20% sucrose in standard buffer. Centrifugation was for 12 hr at 25,000 rpm in a Spinco SW25.3 rotor at 2° .

FIG. 2. Sedimentation analysis of the RNA of RSV harvested at 3-min intervals before and after incubation of the virus in cell growth medium at 40° . (A) 400 ml of medium containing [^3H]uridine-labeled RSV harvested at 3-min intervals was divided in half. One-half was purified and its RNA was extracted in the presence of carrier tobacco mosaic virus (3). Subsequently, an appropriate amount of 60–70S [^{32}P]RNA of RSV harvested at intervals of several hours was added. Centrifugation was in a 15–30% glycerol gradient containing standard buffer and 0.1% Na dodecyl sulfate for 90 min at 40,000 rpm in a Spinco SW41 rotor at 20° . Radioactivity was determined as described for Fig. 1. (B) The other half of the ^3H -labeled RSV harvested at 3-min intervals was incubated for 45 min at 40° . Subsequently, the RNA was analyzed as for (A).

of [^3H]RSV sedimenting at 600 S (Fig. 1B, fractions 8–15) in a preformed sucrose density gradient was about 1.17–1.18 g/ml (Fig. 1C). These experiments indicate that virus released from cells at intervals of 3 min has the same sedimentation coefficient and buoyant density as virus harvested at intervals of several hours (1), and that such virus particles contain viral DNA polymerase (15, 16).

The sedimentation distribution of the RNA of virus harvested at intervals of 3 min is seen in Fig. 2A. A major class of RNA sedimented at 30–40S, ahead of a 31S RNA marker from tobacco mosaic virus. Only 25% of the RNA sedimented with the 60–70S marker of [^{32}P]RSV harvested at intervals of 3 hr. In addition, a 4–12S peak of slowly sedimenting RNA was present at about the same relative concentration as in virus collected in periods of several hours. Storage of purified or unpurified virus at $0-4^\circ$ for 48 hr did not affect the sedimentation pattern of the viral RNA.

The RNA of Schmidt-Ruppin RSV of subgroup A (13) harvested at intervals of 3 min is seen in Fig. 3. In this and other experiments there was some more 60–70S RNA than 30–40S RNA in this virus strain.

Conversion in the virion of 30–40S to 60–70S RNA

To test whether the 30–40S RNA in freshly budded virus may be a precursor of 60–70S RNA, or perhaps the RNA of

Fig. 3. Sedimentation analysis of the RNA of Schmidt-Ruppin RSV-A harvested at 3-min intervals under conditions described for Fig. 2A.

abortive virus particles, the following experiments were done. Medium containing virus released at intervals of 3 min was divided into two aliquots. One aliquot was incubated for 45 min at 40°, another aliquot was kept at 0°. Virus was then purified from both aliquots, and the RNAs were extracted and analyzed. It is shown in Fig. 2A and B that in virus that was incubated at 40°, most of the 30-40S RNA had disappeared and that an approximately equivalent amount of radioactivity had appeared in the 60-70S region of the gradient. In addition, we observed in this and other experiments that RNA sedimenting faster than 60-70S was present in virus incubated at 40°. This result suggests that most 30-40S RNA is converted to 60-70S RNA, and some to even faster-sedimenting forms in the virion.

To determine whether components of the tissue culture medium are required to convert 30-40S RNA of virus harvested at intervals of 3 min to 60-70S RNA, purified virus was incubated for various times in standard buffer (7) containing 10-20% glycerol. The results (Fig. 4) showed that after 5-15 min at 40° most of the 30-40S RNA, and after 30 min practically all of the 30-40S RNA, had been converted to 60-70S and faster sedimenting RNA. The relative amount of 4-12 S RNA was not significantly affected by incubation of virus at 40°.

FIG. 4. Rate of conversion at 40° of the 30-40S RNA to 60-70S RNA in purified RSV harvested at intervals of 3 min. Purified [³H]-uridine-labeled RSV harvested at intervals of 3 min was concentrated in the presence of about 0.5 mg of carrier RSV harvested at intervals of 5 hr by sedimentation through a 2-cm column of 30% onto a cushion of 80% glycerol in standard buffer containing 1 mM mercaptoethanol. ³H-labeled virus was then diluted 5-fold in standard buffer and identical aliquots were incubated for 0 (A), 5 (B), 15 (C), and 30 (D) min at 40° before extraction of the RNA (see Fig. 2). Centrifugation of the RNA was for 55 min at 50,000 rpm in a Spinco SW50.1 rotor.

TABLE 1. Template activity of viral RNAs for RSV DNA polymerase

RNA (0.1 μg)	Treatment	[³ H]dTMP incorporated (cpm)
60-70S*	—	15,340
60-70S	100°, 45 sec	1,510
30-40S†	—	1,880
30-40S	100°, 45 sec	1,170
30-40S†	—	3,020
30-40S	100°, 45 sec	1,530
30-40S†	—	3,800
30-40S	100°, 45 sec	1,700
30-40S	+0.1 μg of (dT) ₁₂₋₁₈	36,000

Assays with partially purified RSV DNA polymerase were for 1 hr as described (7, 17), but precipitation of [³H]DNA synthesized was after incubation for 2 hr at 40° in 0.3 N KOH to solubilize viral [³H]RNA.

* 60-70S RNA was prepared from virus harvested at intervals of 5 hr.

† 30-40S RNA was prepared from [³H]uridine-labeled RSV harvested at intervals of 3 min.

In some virus preparations the rate of conversion was lower than that shown in Fig. 4 (experiment not shown). This slower rate may have been due to the presence in such preparations of virions in which the capacity to convert the RNA was lost or impaired during virus purification. Attempts to convert 30-40S RNA after disruption of the virus by detergents or phenol have not been successful so far, implying that intact structural components of the virus are required for this conversion.

FIG. 5. Electrophoresis of the 30-40S RNA of ^3H -labeled RSV, harvested at intervals of 3 min, before (A) and after (B) heating to 100° . (A) The RNA from RSV harvested at intervals of 3 min was analyzed as described for Fig. 4, and fractions (fractions 7-10, Fig. 4A) corresponding to the 30-40S peak were pooled. The RNA was precipitated with ethanol redissolved in 50 μl of electrophoresis sample buffer (3), and divided into two aliquots. A 25- μl aliquot was mixed with 5 μl of an appropriate amount of 60-70S RSV [^{32}P]RNA that had been heated to 100° for 45 sec in the same buffer. Electrophoresis was after the addition of a methylene blue marker for 6 hr at 7 V/cm in a polyacrylamide gel crosslinked with 2.1% bisacrylamide (14). Radioactivity of 1-mm gel sections was determined after incubation in 3 ml of toluene-based scintillation fluid containing 20% NCS and 2% H_2O at 60° for 2-12 hr. (B) Another 25- μl aliquot of the 30-40S [^3H]RNA was mixed with the above-described 60-70S RSV [^{32}P]RNA. The mixture was then heated to 100° for 45 sec and analyzed as for (A).

Electrophoretic analyses of 30-40S RNA of virus harvested at intervals of 3 min

It appeared from sedimentation analyses, such as those shown in Figs. 2 and 4, that the 30-40S RNA of virus harvested in 3-min periods sedimented slightly faster than the 30-40S RNA obtained by complete melting of 60-70S RNA from RSV. As shown in Fig. 5, the 30-40S [^3H]RNA of virus harvested at intervals of 3 min (see fractions 7-10, Fig. 4A) also had a lower electrophoretic mobility than a 30-40S [^{32}P]RNA marker obtained by complete melting of 60-70S [^{32}P]RNA from RSV. However, after they were heated to 100° the electrophoretic mobilities of both RNAs were identical. Therefore, we conclude that the primary structures of the 30-40S RNAs of virus harvested at 3-min intervals and of heat-dissociated 60-70S RNA are very similar or identical.

As shown in Fig. 6 the increase of the electrophoretic mobility of native 30-40S RNA after it was heated to 100° (shown in Fig. 5) is accompanied by the appearance of low amounts of small RNA species detectable by electrophoresis in a 6% polyacrylamide gel. Thus, the difference in electrophoretic mobility between native 30-40S RNA and heated 30-40S RNA may be due to the association of native 30-40S RNA with small RNAs, and/or to differences in the conformation of the two forms of 30-40S RNA. After heating, the ratio of 4S RNA relative to 30-40S RNA from virus harvested at 3-min intervals was about five times lower than that obtained previously after analysis of heated 60-70S viral RNA (7). This result implies that the 30-40S RNAs must associate with additional 4S RNAs as they are converted to 60-70S RNA in the virus at 40° . To test this assumption directly, we have incubated an aliquot of the virus harvested at 3-min intervals, used above, for 2 hr at 40° in cell growth medium to allow conversion of its RNA to the 60-70S form; we then determined the ratio of 30-40S to small RNAs

after heating 60-70S RNA to 100° . We found (Fig. 6B) that the ratio of 4S RNA to 30-40S had increased to about the same level as that found previously in 60-70S RNA (7). Since it was shown above that 30-40S RNA was not detectably degraded by the conversion to 60-70S RNA, it appears likely that these additional 4S RNAs were picked up from the viral pool of slowly sedimenting 4-12S RNA.

Template activity of 30-40S RNA RSV for RSV DNA polymerase

We have recently found that heating to 100° reduces the template activity of 60-70S RSV RNA for RSV DNA polymerase 5- to 10-fold (17, 18), and that this reduction in template activity correlates with the dissociation of a 4S RNA species that appears to function as a primer for DNA synthesis and represents but a minor fraction of the 4S RNA associated with 60-70S RNA (7). We asked, therefore, whether the 4S primer was present in 30-40S RNA of virus harvested at short intervals or whether it was picked up during the formation of 60-70S structures.

The template activity of 30-40S RNA of virus harvested at 3-min intervals was about five times lower than that of 60-70S RSV RNA (Table 1). Heating reduced the template activity of native 30-40S RNA by about 50% (Table 1). By contrast, the template activity of 60-70S RNA was reduced 10-fold under the same conditions of experiment. This result suggests that at most some (20%), but not all, of the primers of 60-70S RNA are already present in 30-40S RNA of virus harvested at short intervals. This result is compatible with the notion that native 30-40S RNA is associated with five times less 4S RNA than 60-70S RNA. If oligo(dT) was added to 30-40S RNA of virus harvested at short intervals, its template activity was increased 10-fold; by contrast, the template activity of 60-70S RNA is only increased 2- to 3-fold, as described previously (18). This result implies that native 30-40S RNA has more free binding sites for oligo(dT) than does 60-70S RNA, and that perhaps some of these sites are those that bind primers after conversion to 60-70S RNA.

DISCUSSION

The experiments described in this paper suggest that the 60-70S RNA of avian tumor viruses is probably the result of an aggregation of 30-40S subunits and of some small RNAs, most or all of which may occur in the virus shortly after it is released from the cell. The ratio of 30-40S to 60-70S RNA in 3-min virus appears to vary in different viral strains (see Figs. 2, 3, and 4). Such variations may reflect strain-specific rates of aggregation of RNA subunits or of release of virions from the cell surface. Our findings are in agreement with the notions of others that certain RNA tumor viruses contain in addition to 60-70S RNA free 30-40S RNA (8-10), possibly also as precursors of 60-70S RNA. Our data also confirm the finding of Cheung *et al.* (11) that free 30-40S RNA is detectable in virus harvested at short intervals. However, we find a principal 30-40S RNA component that is readily converted to 60-70S in such virus, whereas Cheung *et al.* (11) observed a principal 55-60S RNA component that is not converted to 60-70S RNA in partially purified virus. Further work will be required to explain this divergence.

The formation of 60-70S RNA from 30-40S and smaller subunits in the virion appears to rule out the argument that the subunit structure of 60-70S tumor virus RNA is the result

FIG. 6. Electrophoresis of the small RNAs associated with the large RNAs of RSV harvested at 3-min intervals before (A) and after (B) incubation of virus for 2 hr at 40°. (A) Medium containing [^3H]uridine-labeled RSV harvested at intervals of 3 min was divided in half. The 30-40S RNA of one half was prepared as described for Fig. 2, and heated at 100° as described for Fig. 5. It was then mixed with an appropriate amount of chick ^{32}P -labeled transfer RNA and with methylene blue and analyzed by electrophoresis for 2 hr at 15 V/cm in a 6-cm gel consisting of an upper 2-cm section of 2.5% and a lower 4-cm section of 6% polyacrylamide. Radioactivity was determined as described for Fig. 5. (B) The other half of the ^3H -labeled RSV collected at 3-min intervals was incubated in medium for 2 hr at 40°. The 60-70S RNA was then prepared from purified virus and analyzed as described for (A).

of post-transcriptional cleavage of a putative 60-70S polynucleotide. This conclusion is compatible with failures to find a single 60-70S polynucleotide in RNA tumor viruses (2) and in virus-infected cells (19, 20).

Our data are consistent with the view that the conversion of 30-40S to 60-70S RNA is a physical, rather than a chemical, event. For example, the primary structure of 30-40S RNA is not detectably changed after conversion to 60-70S RNA. In addition, the finding that there are fewer 4S RNAs associated with 30-40S RNA before than after conversion to 60-70S RNA is likely to be the result of an association reaction. Association of preexisting 30-40S and 4-12S RNAs may take place in the virion simultaneously with the conversion to 60-70S RNA. We suggest this because cleavage of 30-40S RNAs as a possible alternative source of small RNAs was not detectable, and cleavage of 4-12S RNAs was not determined. Nevertheless, the possibility exists that a minor cleavage of 30-40S RNA or of some of the 4-12S RNAs in the virion may accompany the conversion of 30-40S to 60-70S RNA. The role of small RNAs in the formation of 60-70S RNA cannot be deduced from our experiments. It is conceivable that some small RNAs function as linkers of 30-40S subunits in the 60-70S complex.

We find that native 30-40S RNA has five times lower template activity for RSV DNA polymerase than does 60-70S RNA, and it contains five times less 4S RNA. This result is in agreement with our findings (7) that the high template activity of 60-70S RNA complex is due to its containing 4S RNAs, with some of them serving as primers for the DNA polymerase.

The biological significance of the association of 30-40S subunits and small RNAs, in particular 4S RNA, to form the 60-70S RNA complex may be better understood by further study of the infectivity of the immature virus.

We thank Drs. Cheung, Smith, Stone, and Joklik for a preprint of their paper and Marie Stanley and Jeff Weber for their assistance. This study was conducted under Contract no. 71-2173 within the Special Virus-Cancer Program of the National Cancer Institute, NIH, PHS and by Public Health Service Research Grants CA 11426 from the National Cancer Institute and Deutsche Forschungsgemeinschaft (K.v.d.H.).

1. Robinson, W. S., Pitkanen, A. & Rubin, H. (1965) *Proc. Nat. Acad. Sci. USA* **54**, 137-144.
2. Duesberg, P. H. (1970) *Current Topics in Microbiology and Immunology* **51**, 79-104.
3. Duesberg, P. H. (1968) *Proc. Nat. Acad. Sci. USA* **60**, 1511-1518.
4. Erikson, R. L. (1969) *Virology* **37**, 124-131.
5. Montagnier, L., Golde, A. & Vigier, P. (1969) *J. Gen. Virol.* **4**, 449-452.
6. Erikson, E. & Erikson, R. L. (1971) *J. Virol.* **8**, 254-256.
7. Canaani, E. & Duesberg, P. (1972) *J. Virol.* **10**, 23-31.
8. Jarrett, O., Pitts, J. D., Whalley, J. M., Clason, A. E. & Hay, J. (1971) *Virology* **43**, 317-320.
9. Stone, L. B., Takemoto, K. K. & Martin, M. A. (1971) *J. Virol.* **8**, 573-578.
10. Lin, F. H. & Thormar, H. (1972) *J. Virol.* **10**, 228-233.
11. Cheung, K.-S., Smith, R. E., Stone, M. P. & Joklik, W. K. (1972) *Virology*, in press.
12. Bader, J. P. & Steck, T. L. (1969) *J. Virol.* **4**, 454-459.
13. Vogt, P. K. (1970) in *Comparative Leukemia Research 1969*, ed. Dutcher, R. M. (Karger, Basel), pp. 153-167.
14. Duesberg, P. H. & Vogt, P. K. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 1673-1680.
15. Temin, H. M. & Mizutani, S. (1970) *Nature* **226**, 1211-1213.
16. Baltimore, D. (1970) *Nature* **226**, 1209-1211.
17. Duesberg, P., Helm, K. V. D. & Canaani, E. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 747-751.
18. Duesberg, P., Helm, K. V. D. & Canaani, E. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2505-2509.
19. Leong, J.-A., Garapin, A.-C., Jackson, N., Fanshier, L., Levinson, W. & Bishop, J. M. (1972) *J. Virol.* **9**, 891-902.
20. Tsuchida, N., Robin, M. S. & Green, M. (1972) *Science* **176**, 1418-1420.