Stepwise Dissociation of High Molecular Weight Avian Myeloblastosis Virus RNA: 30–40S RNA Subunits – the Best Natural Template-Primer for Viral Reverse Transcriptase

By M. TRÁVNÍČEK, LUDMILA YU. FROLOVA* AND J. ŘÍMAN

Laboratory for Biochemical Investigation of Cancer,
Institute of Organic Chemistry and Biochemistry,
Czechoslovak Academy of Sciences, Prague, Czechoslovakia and
* Institute of Molecular Biology of the Academy of Science of the USSR, Moscow

(Accepted 8 October 1975)

SUMMARY

Controlled disruption of 60S AMV RNA with formamide was used to prepare 50–55S and 30–40S RNAs. When the activities of these RNAs as templates for AMV reverse transcriptase were compared it was found that 50–55S RNA was 1.5 times and 30–40S RNA 2 to 3 times more active than 60S RNA. The 30–40S RNA produced by heating, instead of formamide disruption, was inactive as a template but activity was restored by addition of oligo(dT). 40% of the 4S RNA initially associated with the 60S RNA remained associated with all the RNA species obtained by formamide treatment but was lost on heating. It is concluded that this RNA acts as resident primer whereas the other 60% of the 4S RNA is less firmly bound and appears to have little or no primer activity. Removal of the less firmly bound 4S RNA increases the template activity of the viral RNA.

INTRODUCTION

Genomic RNA of oncornaviruses is an 60–70S aggregate composed of two to four 30–40S main RNA subunits (Duesberg, 1968; Erikson, 1969; Mangel, Delius & Duesberg, 1974) and several low mol. wt. RNA species (Erikson & Erikson, 1971; Faras et al. 1973; Rosenthal & Zamecnik, 1973). This complex RNA structure is claimed to be a better natural template-primer for oncornaviral reverse transcriptase than any other natural RNA (Duesberg, Helm & Canaani, 1971 a; Faras et al. 1972). Disruption of the RNA aggregate by dimethylsulphoxide or by heat abolishes the template activity of the main RNA subunits formed, due to the loss of an 4S RNA primer (Duesberg, Helm & Canaani, 1971 b; Canaani & Duesberg, 1972; Leis & Hurwitz, 1972).

However, from a more detailed examination of the release of associated 4S RNA from the 60–70S RSV-RNA complex by thermal denaturation (Canaani & Duesberg, 1972) and from a comparison of this release with the loss of template activity it has been concluded that all but one of the associated 4S RNA species are released from the RSV-RNA aggregate at the temperature of 63 °C without reduction of template activity (Dahlberg et al. 1974; Faras et al. 1974). This 4S RNA species, identified as a cell-coded tRNA (Sawyer, Harada & Dahlberg, 1974) specific for tryptophan (Maugh, 1974), was shown to be a principal primer for RSV-RNA directed DNA synthesis in vitro (Dahlberg et al. 1974; Faras et al. 1974; Sawyer et al. 1974).
A stepwise release of associated 4S RNA performed by a controlled dissociation of the aggregate structure of 60S AMV RNA by formamide (Trávníček & Říman, 1973 b) permitted us to obtain intermediate RNA structures deprived of various portions of associated 4S RNA. Here we describe the isolation of such structures and demonstrate differences in their template-primer efficiency in a reconstituted reverse transcriptase system catalysed by a purified AMV enzyme.

**METHODS**

*Reagents.* [3H]-dGTP and [3H]-TTP were purchased from the Radiochemical Centre, Amersham. Unlabelled deoxynucleoside triphosphates and dithiothreitol (DTT) were from Calbiochem, poly(rA)-oligo(dT)$_{10}$ and oligo-(dT)$_{10}$ from P–L Biochemicals. Formamide was the product of Merck, AG., Microgranular DEAE cellulose DE52 and phosphocellulose P11 were from Whatman. Carrier-free [32P]-orthophosphate was purchased from ROTOP, Dresden, GDR.

*Virus.* BAI strain A avian myeloblastosis virus (AMV) was used in all experiments. For preparation of non-labelled viral RNA and virion DNA polymerase (reverse transcriptase) the virus was purified from blood plasma of leukaemic chicks (Leghorn White), exsanguinated in terminal stages of myeloblastic leukaemia, as previously described (Trávníček & Říman, 1973 a).

*Viral RNA.* Virus was purified from 300 ml of blood plasma as previously described (Trávníček & Říman, 1973 a) and total AMV RNA extracted with phenol-sodium dodecyl sulphate (Spiegelman et al. 1970). The 60S RNA, either native or after controlled formamide treatment, was sedimented through glycerol gradients (Trávníček & Říman, 1973 b). 300 to 500 µg portions of total AMV-RNA in TE (0.01 M-tris-HCl, pH 7.6; 0.005 M-EDTA; 0.1% SDS) were treated at 37 °C for 10 min in the total vol. of 0.2 ml with either 10% or 50% formamide and quickly chilled. After dilution to 0.75 ml with TE the samples were placed on the top of 10 to 30% glycerol-TNE (0.01 M-tris-HCl, pH 7.6; 0.1 M-NaCl; 0.005 M-EDTA) gradients and centrifuged in a Spinco SW41 rotor at 40000 rev/min for 4 h at 4 °C. Gradient fractions forming peaks of 60S RNA and its 50-55S and 30-40S RNA subunits were pooled and precipitated with ethanol. After repeated ethanol precipitation the dried RNA precipitates were solubilized in TN (0.01 M-tris-HCl, pH 7.6; 0.1 M-NaCl), final concentration approx. 1 µg/10 µl, divided into small samples and kept frozen at -20 °C. Each sample was melted only once.

*Viral DNA polymerase (reverse transcriptase).* AMV DNA polymerase was isolated from 50 mg of purified virus essentially by the method of Kacian et al. (1971), including DEAE-cellulose and phosphocellulose chromatography. The enzyme eluate from the phosphocellulose column was concentrated with Minicon B-15 (Amicon Corp. U.S.A.) and stored in samples at -70 °C in a buffer containing 0.05 M-tris-HCl, pH 8.0; 0.4 M-KCl, 2 mM-DTT, 10% glycerol and 0.02% Triton X-100. The enzyme retained more than 90% of its original activity for at least 2 to 3 months. Enzyme purification and testing were carried out as described by Kisselev et al. (1973).

*DNA polymerase (reverse transcriptase) assay.* The 0.1 ml assay mixtures of a reconstituted system for reverse transcriptase reaction directed by viral RNAs contained the following components (in µmol): 5 of tris-HCl (pH 8.3); 0.6 of MgCl$_2$; 6 of KCl; 0.4 of dithiothreitol; 0.02 each of dATP, dCTP, dTTP; 0.002 of [3H]-dGTP (sp. act. 700 to 900 ct/min/pmol) and 2 to 3 units of AMV reverse transcriptase. One unit of enzyme activity is defined as the amount of AMV reverse transcriptase catalysing the incorporation of 1 nmol of dTTP using poly(rA)-oligo(dT)$_{10}$ as a template under standard conditions. 60S AMV RNA and its
50–55S or 30–40S constituent RNA subunits were used as templates at levels from 0.25 μg to 0.50 μg per 0.1 ml assay. When oligo(dT) was used as an artificial primer, 0.1 μg of oligo-(dT)$_{10}$ was included. After incubation at 37 °C for 10, 20, 30 and 60 min, 20 μl samples were taken and precipitated with trichloroacetic acid (10%). The precipitates were collected on nitrocellulose filters, dried and the radioactivity was determined on a Packard scintillation spectrometer using BBOT-toluene scintillation fluid.

The results are expressed in ‘specific template activities’, as pmol of incorporated dGTP per 1 μg of viral RNA tested.

[^32P] labelling of virus. To grow[^32P]-labelled virus, 30 to 40 μCi/ml of carrier-free[^32P]-orthophosphate was added to virus-producing cultures of leukaemic myeloblasts, cultivated in suspension, as previously described by Říman & Beaudreau (1970). The cells were continuously labelled for 24 to 30 h. The virus was purified from medium collected every 5 h and 60S AMV RNA isolated (Trávníček & Říman, 1973 b).

Electrophoresis. Composite agarose (0.5 %)-bisacrylamide-crosslinked polyacrylamide (1.7 %) gels in a buffer containing 0.045 M-tris, 0.045 M-boric acid, 0.014 M-EDTA, 0.2 % SDS, pH 8.3 (TEB) were prepared essentially by the method of Tiollais et al. (1972). 20 μl samples of[^32P]-labelled viral RNA (8 to $10^4$ ct/min) in TE containing 10 % glycerol and bromophenol blue marker, were placed on the top of $10 \times 0.5$ cm cylindrical gels and electrophoresed in TEB at 8 V/cm for 75 min. The gels were then cut into 2 mm slices, dried on nitrocellulose membrane filters and their radioactivity was determined in BBOT-toluene scintillation fluid in a Packard scintillation spectrometer.

RESULTS

Release of 4S RNA associated with high mol. wt. AMV RNA

We have previously found (Trávníček & Říman, 1973 b) that high mol. wt. AMV RNA could be converted through a number of intermediate stages into its constituent RNA subunits by a controlled formamide treatment.

Utilizing the higher resolution of polyacrylamide-agarose gel electrophoresis we re-examined this question in order to determine whether the changes of the level of constituent RNA subunits were accompanied by a stepwise release of associated 4S RNA.

Gel electrophoretic analyses of[^32P]-labelled 60S AMV RNA in subsequent stages of its stepwise dissociation are summarized in Table 1. Formamide treatment released approx. 60 % of the 4S RNA (1.5 % of the 60S RNA) in a stepwise fashion. At the same time the 60S RNA was converted through a 50–55S intermediate to 30–40S RNA subunits. 40 % of the 4S RNA remained associated with the 30–40S RNA subunits after formamide treatment but could be released by heating.

Isolation of subunit forms of genomic AMV RNA as templates for reverse transcriptase

To test the influence of associated 4S RNA on the ability of subunit structures of genomic AMV RNA to act as templates for the viral reverse transcriptase the following stages were chosen for the isolation of RNA subunit structures deprived of various portions of associated 4S RNA.

Control

No treatment. Native 60S AMV RNA was isolated (Fig. 1a).
Fig. 1. Isolation of high mol. wt. 60S AMV RNA and its 50–55S and 30–40S RNA subunit structures. Samples of unlabelled total AMV RNA were treated with indicated formamide concentrations and centrifuged in a Spinco SW41 rotor at 40000 rev/min for 4 h at 4 °C in 10 to 30% glycerol-TNE gradients. Sedimentation is from left to right. Marked regions forming peaks of 60S RNA and its 50–55S and 30–40S RNA subunits were isolated. (a) Untreated total AMV RNA; (b) total AMV RNA treated with 10% formamide; (c) total AMV RNA treated with 50% formamide.

Table 1. Release of associated 4S RNA during stepwise transition of the aggregate structure of 60S AMV RNA*

<table>
<thead>
<tr>
<th>Gel</th>
<th>Treatment</th>
<th>60S-50S (%)†</th>
<th>40S-10S (%)†</th>
<th>4S (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>—</td>
<td>93</td>
<td>7</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>5% formamide, 37 °C, 2 min</td>
<td>71</td>
<td>28</td>
<td>6.5</td>
</tr>
<tr>
<td>C</td>
<td>10% formamide, 37 °C, 2 min</td>
<td>45</td>
<td>53</td>
<td>8.5</td>
</tr>
<tr>
<td>D</td>
<td>20% formamide, 37 °C, 2 min</td>
<td>20</td>
<td>79</td>
<td>9.5</td>
</tr>
<tr>
<td>E</td>
<td>40% formamide, 37 °C, 2 min</td>
<td>12</td>
<td>87</td>
<td>1.00</td>
</tr>
<tr>
<td>F</td>
<td>60% formamide, 37 °C, 2 min</td>
<td>—</td>
<td>98</td>
<td>1.50</td>
</tr>
<tr>
<td>G</td>
<td>no formamide, 100 °C, 2 min</td>
<td>—</td>
<td>98</td>
<td>2.00</td>
</tr>
<tr>
<td>H</td>
<td>20% formamide, 100 °C, 2 min</td>
<td>—</td>
<td>98</td>
<td>2.50</td>
</tr>
</tbody>
</table>

* Samples of [32P]-60S AMV RNA in TE (10⁵ ct/min in 15 μl total volume) were heated in sealed capillary tubes and analysed by gel electrophoresis.
† The total radioactivity in the 50–60S, 10–40S and 4S regions have been expressed as percentage of the radioactivity loaded on the gel.

Early stage

The early stage of the aggregate conversion (Fig. 1b) was performed by 10% formamide treatment. No native 60S RNA aggregate could be detected; approx. ⅓ of the aggregate was in the form of 50–55S intermediate RNA structures and ⅓ had already been converted to 30–40S main RNA subunits. At this stage about 30% of associated 4S RNA was released (Table 1). The high mol. wt. RNA structures formed, namely the 50–55S RNA and 30–40S₁ RNA, were isolated.
High template activity of AMV RNA subunits

**Fig. 2.** Comparison of template-primer efficiency for AMV reverse transcriptase of AMV RNA subunit structures isolated from three stages of dissociation of 60S AMV RNA. 0.25 to 0.5 μg amounts of indicated RNAs were tested as template-primers in the reconstituted reactions of purified reverse transcriptase of AMV. Kinetics of [H]-dGTP incorporation were as described in Methods. The results are expressed in “specific template activities” as pmol of dGTP incorporated per 1 μg of RNA. (a) RNA subunit structures isolated from the preparation of total AMV RNA documented in Fig. 1 were used as template-primers. (b) RNA subunit structures isolated from total AMV RNA of an independently prepared virus sample were used as template-primers for comparison. ○—○, 30–40S1 RNA; ●—●, 30–40S2 RNA; △—△, 50–55S RNA; ▲—▲, 60S RNA; ×—×, 60S RNA (heated).

**Late stage**

The late stage of the aggregate conversion (Fig. 1c) was performed using 50% formamide. Both the native 60S RNA aggregate and the 50–55S intermediate RNA structures were absent; the only products detected were 30–40S RNA subunits, which still contained approx. 40% of associated 4S RNA (Table 1). This RNA, designated as 30–40S2 RNA, was again isolated.

**Final stage**

The final stage of the aggregate disruption was obtained by heating samples of the 50–55S RNA, 30–40S1 RNA, 30–40S2 RNA and native 60S RNA at 100 °C for 2 min in sealed capillary tubes, followed by rapid cooling. All these RNA structures were converted to 30–40S RNA subunits free of all associated 4S RNA. However, free 4S RNA remained in the heated RNA samples in amounts characteristic for the given stage of aggregate conversion.

All these structures were tested as template-primers and oligo(dT)-stimulated templates in the reconstituted reaction of purified AMV reverse transcriptase.
Comparison of template activities of AMV RNA subunit structures in the reconstituted reaction of AMV reverse transcriptase

Naturally primed reaction

Fig. 2 shows a comparison of kinetics of DNA synthesis carried out with RNA template-primers prepared from two independently isolated samples of total AMV RNA. The native 60 S RNA aggregate and the high mol. wt. RNA products of its partial dissociation by formamide were found to be active as template-primers. They exhibited relatively high initial rates of DNA synthesis, decreasing during the assay period of 60 min. On the other hand, 30-40 S RNA subunits prepared by heating were almost inactive.

It appears that the partial dissociation of 60 S AMV RNA, connected with a stepwise removal of 60 % of associated 4 S RNA improves the template efficiency of RNA subunit structures formed. Template-primer activity of 50–55 S RNA, which represents a main product of the early stages of 60 S RNA aggregate transition, increased by 50 % as compared with the template-primer activity of native 60 S RNA (Fig. 2). This increase correlates with a loss of 30 % of associated 4 S RNA (Table 1).

Both 30–40 S₁ RNA and 30–40 S₂ RNA show a two- to threefold improvement in template-primer activity (Fig. 2) correlated with a loss of approx. 60 % of associated 4 S RNA (Table 1).

Heating of the native 60 S RNA aggregate or any RNA product of its partial dissociation abolishes the template activity of 30–40 S RNA subunits formed (Fig. 2). This corresponds to the loss of the last 40 % of most firmly bound 4 S RNA species.

Oligo(dT) stimulated reaction

As shown in Fig. 3, template activities of native 60 S RNA and RNA structures obtained by its moderate disruption with formamide were improved approx. 2 to 3 times by oligo(dT) artificial primer when compared with naturally primed DNA synthesis shown in Fig. 2.
High template activity of AMV RNA subunits

When these RNAs were heated prior to use as templates in oligo(dT) stimulated DNA synthesis, a similar improvement of template efficiency was observed (Fig. 3). It appeared that oligo(dT) restored the template activity of all heated RNAs to a slightly lower level than that of respective unheated samples with the exception of 30–40S RNA, where slightly higher template activity of heated RNA was detected.

DISCUSSION

The initiation of DNA synthesis catalysed by oncornaviral reverse transcriptase with oncornaviral 60–70S RNA or on its constituent RNA subunits as templates appears to occur at sites where 4S RNA primers are hydrogen-bonded (Duesberg, Helm & Canaani, 1971b; Faras et al. 1972; Leis & Hurwitz, 1972; Dahlberg et al. 1974), and/or a tpoly(rA) regions upon artificial priming with oligo(dT) (Baltimore & Smoler, 1971; Duesberg et al. 1971; Goodman & Spiegelman, 1971; Reitz et al. 1972).

We found that 50–55S RNA and especially both types of 30–40S RNA subunit structures, isolated after a controlled denaturation of the native AMV RNA by formamide, act as efficient template-primers. This suggests that all these RNA structures carry their own resident primer molecules, in spite of the loss of as much as 60% of associated 4S RNA. The complete loss of template-primer activity observed upon heating can either be due to a loss of the resident 4S RNA primers or, alternatively, to an inactivation or a destruction of the template. However, since the template activity of all heated RNAs could be restored by oligo(dT) priming, we may conclude that the last 40% of associated 4S RNA, which cannot be released by formamide treatment but which is removed by heating, accounts for the template-primer activity of oncornaviral RNA, whereas 60% of less firmly bound 4S RNA has little or no priming capacity.

These observations are consistent with the direct proof of the primer function of a single species of associated 4S RNA which was found (Faras et al. 1973; Dahlberg et al. 1974; Sawyer et al. 1974) to be more firmly bound to the RSV-RNA template than the rest of associated 4S RNA without primer function.

However, neither the preservation of resident 4S RNA primer molecules nor the possible interference due to an extensive secondary structure of oncornaviral genomic RNA (Trávníček & Říman, 1973c; Mangel et al. 1974) can account for the 50% increase of template-primer efficiency of 50–55S RNA structures and for two- to three-fold increase in the case of 30–40S RNA subunits when compared with the native 60S viral RNA aggregate.

There are two reasons why we think that these findings could be due to an involvement of a certain portion of associated 4S RNA species which has no primer function: (a) The increase of specific template activities of 50–55S RNA and especially of 30–40S RNA subunits correlates with the loss of increasing portions of less firmly bound 4S RNA species, both for naturally primed and oligo(dT)-stimulated DNA synthesis. (b) Upon heating, all isolated viral RNA structures acquire an identical secondary structure, being converted to 30–40S RNA subunits, free of 4S RNA. If only secondary structure interferes with the template activity, then all heated RNA samples should display the same oligo(dT)-primed template activity. However, this was not observed. In oligo(dT) stimulated DNA synthesis similar increases were obtained with heated templates and their unheated counterparts. The only difference which could be detected among all viral RNA structures upon heating, was the presence in the RNA samples of different amounts of released 4S RNA without a primer function. An increase in specific oligo(dT)-primed template activities of heated RNAs appears again to correlate with decreasing amounts of less firmly bound 4S RNA species
without primer function, as in the case of the respective unheated RNAs. Here, however, the 4S RNAs are not bound to the template, but are present in the RNA samples under investigation in a free state.

The observed increase in template activities of the AMV RNA subunit structures could be explained by the suppression of the inhibitory influence of less firmly bound portion of 4S RNA species without primer function, regardless whether bound to, or released from, the template. We may also speculate that such 4S RNA species might assume a regulatory function in the process of reverse transcription of oncornaviral genome.

We wish to thank Marie Buzková and Jiří Buzek for their expert assistance in propagation of cells and Eva Svobodová for technical aid.

REFERENCES


High template activity of AMV RNA subunits


(Received 6 May 1975)