The Sizes of RNA Subunits Isolated from High and Low Leukaemogenic Friend Virus

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SUMMARY

The sizes of non-covalently linked RNA subunits isolated from highly leukaemogenic Friend virus derived from the plasma (PV, plasma virus) of leukaemic mice were compared to the RNA subunits isolated from low leukaemogenic Friend virus grown in tissue culture (TCV, tissue culture virus). Histograms derived from electron microscope measurements showed that about one-half of the plasma virus RNA was 1.4 to 2.5 μm in length, corresponding to a mol. wt. range from 1.8 × 10^6 to 3.2 × 10^6 and the other half less than 1.4 μm. In contrast, approx. 50% of the TCV RNA was only 0.7 to 1.6 μm in length (mol. wt. 0.9 × 10^6 to 2.0 × 10^6) and the remainder less than 0.7 μm in length regardless of whether the virus RNA was isolated from 3, 9, 36 or 72 h cultures. The histograms suggest size classes for both TCV and PV derived RNA subunits. Data obtained from sucrose gradient sedimentation of heat-denatured FLV RNA agreed with the electron microscope length measurements. The smaller sizes of the TCV RNA subunits are hypothetically related to the limited biological activity of Friend leukaemia virus produced from leukaemic cells in culture. Comparable results were obtained using two different RNA extraction procedures. Contamination of TCV nucleic acid preparations by cellular DNA was observed even when the virions were harvested from short term cultures and purified by isopycnic sucrose gradient centrifugation. Consequently, preparations of intact virus were treated with DNase prior to sucrose gradient purification of the virions.

INTRODUCTION

Several studies (Barski & Youn, 1965; Friend et al. 1966; Evenson et al. 1975a) have shown that the biological activity of murine leukaemia viruses produced in cell culture was significantly less, even on an electron microscope particle count basis (Barski et al. 1973), than that of virus isolated from the plasma of leukaemia virus infected mice. Our observations (Evenson et al. 1975a) on Friend leukaemia virus have shown that the leukaemogenic and spleen focus forming (SFF) activities of TCV (tissue culture-derived virus) were respectively 10^8 and 10^4 lower than those of PV (plasma-derived virus) on a particle count basis. TCV also had 10^4 less helper activity (S^+L^- test; Bassin et al. 1971) than PV, although the reverse transcriptase level was only tenfold less. Since the fine structure of both TCV and PV was preserved to approximately the same extent as seen in thin sections under the electron microscope, a study was undertaken to determine whether a difference in the size of the RNA subunits could be demonstrated which might correlate with the attenuated biological activity of TCV as compared to PV. Previous reports have indicated that a size
difference exists between the genome subunits of related but functionally different viruses. For example, Duesberg & Vogt (1970, 1973) have shown that the RNA subunits from competent Rous sarcoma viruses were about 10% larger than the size of the RNA subunits isolated from the avian leukemia viruses. Likewise Maisel et al. (1973) have observed an approximate 20% difference in size between two RNA subunits isolated from the Ostertag strain of Friend virus; the larger subunit may correspond to lymphatic leukemia activity and the smaller to spleen focus forming activity. We report here that the sizes of the non-covalently linked RNA subunits isolated from TCV are significantly smaller than those isolated from PV.

The presence of contaminating DNA in virus RNA preparations has been observed previously (Weber et al. 1974; Evenson et al. 1975) and may have led to misinterpretations about the structure of the virus genome (Granboulan et al. 1966). Although very little contaminating DNA was found in blood plasma-derived Friend virus samples, significant amounts were found in tissue culture-derived Friend virus samples. We have recently extended this observation to the case of Rauscher leukemia virus (RLV) isolated from JLS-V5-RLV cell cultures and believe that this is a common problem in isolating virus RNA from tissue culture systems.

**METHODS**

**Cells.** Friend leukaemia cells (FLC), clone 745, were grown as a suspension culture in Eagle's medium plus Earle's salts and supplemented with 15% foetal calf serum (BME 15 FCS), 100 g/ml streptomycin and 100 International units (i.u.)/ml of penicillin as previously described (Friend et al. 1966). These cells were originally isolated from Friend leukaemia solid tumours in DBA/2J mice and were shown to be chronically infected with FLV (Friend et al. 1966). Logarithmic phase growing cells were seeded at a concentration of 1 to 2 × 10^6 cells/ml into 1 to 3 l spinner flasks containing BME 15 FCS (Evenson et al. 1975a) and harvested at the times indicated. For virus collection from 3 and 9 h samples only, logarithmic phase growing cells in 6 l of BME 15 FCS were gently pelleted (1000 g for 5 min) in 500 ml bottles in a GS3 Sorvall rotor and immediately resuspended in 3 l of BME 15 FCS at 37 °C. These cultured cells had a viability of 98 to 100% as determined by exclusion of trypan blue dye.

**Isotope labelling.** For 3 h samples, logarithmic phase growing cells in 4 l of growth medium were concentrated as above and resuspended in 2 l of fresh growth medium containing 2.5 μCi 5-3H-uridine/ml (New England Nuclear, Boston, Mass., 30 Ci/mmol). For 72 h samples, the same isotope was added to freshly seeded 2 l cultures (as above) at a concentration of 0-3 μCi/ml.

**Virus concentration**

**Tissue culture virus.** Virions were harvested and concentrated as previously described (Evenson et al. 1975a). In brief, cells and cellular debris were removed by differential centrifugation in a Sorvall GS3 rotor. The supernatant was then filtered through 0.6 μm Nuclepore filters (General Electric, Pleasanton, Calif.) under 1 to 2 lb positive pressure. The viruses were pelleted in a GS3 rotor at 8500 rev/min (13000 g max) for 3 h. Electron microscopy of thin sections of the resulting pellets showed well-preserved and purified virions of type C morphology (Evenson et al. 1975a). Pelleted virions were resuspended and incubated in a solution containing 10 μg DNase/ml (chromatographically purified; Worthington Biochemical Corp., Freehold, N. J.) for 10 min at 37 °C. The DNase was pre-treated with iodoacetate according to Zimmerman & Sandeen (1966) to eliminate
possible residual RNase activity. The virus suspensions were then layered on to 12 ml 15 to 55% (w/v) linear sucrose (Mann Ultralipure, RNase free) gradients in NET buffer (0.15 M-sodium chloride, 0.01 M-tris and 0.001 M-EDTA) and centrifuged for 4 h at 40000 rev/min, 4 °C in a Spinco SW41 rotor. The visible virus band was collected with a Pasteur pipette and the RNA isolated as described below.

Plasma virus. Blood was collected with siliconized (Siliclad, Clay Adams, Parsippany, N.J.) 26 g needles and syringes by direct puncture of surgically exposed hearts from 75 to 100 Swiss Taconic mice (Taconic Farms, Germantown, N.Y.) 7 days after infection with Friend virus as previously described (Evenson et al. 1975a) and at which time splenomegaly was evident in 90 to 95% of the animals. Pooled plasma was filtered by positive pressure through 0.6 µm Nuclepore filters. The virions were pelleted from the filtrate at 27000 rev/min (34000 g max) for 1 h in a Sorvall SS34 rotor, resuspended in NET buffer and purified by sedimenting in sucrose gradients as described above. All procedures for virus purification were performed at 0 to 4 °C.

Extraction of RNA

Phenol procedure. The sucrose solution containing the isopycnically banded virus was diluted 1:2 with NET buffer and the viruses were pelleted by sedimentation in a Spinco SW50.1 rotor at 15000 rev/min for 1 h and then resuspended in 1 ml of NET buffer. Recrystallized sodium dodecyl sulphate (SDS) was added (Loll & Stutz, 1968) to a concentration of 0.5% (w/v) and the preparation was incubated for a few seconds at 37 °C. Immediately upon clearing of the virus suspension, RNA was extracted with 2 vol. of 0.05 M-tris buffer-saturated phenol containing 0.1% (w/v) 8-hydroxyquinoline (Scherrer & Darnell, 1962) at 4 °C. The phenol phase was removed with a pipette and the extraction procedure repeated once again. The phenol solution was prepared from a fresh supply of crystals (BDH Chemicals, Ltd, Poole, England) and used immediately. The aqueous phase was transferred to a test tube and the residual phenol was extracted with ether. Nitrogen gas was gently bubbled through the solution to remove the ether. The extracted RNA was precipitated from the solution by adding 2 vol. of absolute ethanol and storing overnight at −20 °C.

Guanidine-HCl (Gu-HCl) procedure. The RNA was isolated as described initially by Cox (1968) and modified by Pavlovec et al. (1978). The sucrose solution containing the isopycnically banded virus was diluted with an equal volume of NET buffer and the solution was weighed. The sample was chilled in a dry ice-acetone bath to about −5 °C and an equal weight of Gu-HCl crystals was added with continuous and rapid stirring. Following dissolution of the Gu-HCl, the mixture was stirred for an additional 30 min while still at about −5 °C. A volume of ethanol equal to that of the NET buffered virus suspension was admixed prior to storage at −20 °C overnight. The RNA precipitate was centrifuged for 1 h at 17000 rev/min in a Sorvall SS34 rotor. The pellet was washed twice with 70% ethanol and 2% potassium acetate, drained dry and then dissolved in 50 to 100 µl of 1 or 5 mM-dithiothreitol (DTT). The results obtained by the Gu-HCl procedure were essentially identical to those obtained after the phenol extraction and represent, therefore, a significant control. One difference between phenol and Gu-HCl extraction is the observation that although the recovery of 60 to 70S RNA is the same, a lesser amount of small mol. wt. RNA (4S) is recovered by the Gu-HCl technique (Pavlovec et al. 1978).

Heat denaturation of virus RNA. Virus RNA suspended in NET buffer containing 0.5% SDS was heated at 80 °C for 3 min followed by quick cooling to 20 °C.

Rate zonal centrifugation of RNA. Virus RNA preparations, layered on to 20.0 to 42.7%
(w/v) isokinetic sucrose gradients in NET buffer, pH 7, and 0.2% SDS, were centrifuged for 3 h or 5 h at 20°C in a SW41 Spinco rotor at 40,000 rev/min. The absorbance (at 254 nm) of the gradients was monitored with an ISCO UA5 spectrophotometer (Lincoln, Nebraska) either by continuous flow or monitoring single fractions. Radioactive fractions were admixed with an equal volume (0.4 ml) of 10% trichloroacetic acid (TCA). The acid-insoluble precipitate from each sample was collected on 0.2 µm Millipore filters (Millipore Corporation, Bedford, Mass.) and assayed for radioactivity by liquid scintillation counting. Ribosomal RNA isolated from 3H-uridine labelled Friend cells was used for sedimentation coefficient markers.

Electron microscopy. Within 1 min after dissolving the RNA pellet in 5 mM-DTT, a 5 µl sample of the solution was prepared for electron microscopy according to the method of Robberson et al. (1971); the remaining RNA solution was rapidly frozen in a dry-ice-acetone bath. The final spreading solution contained: 1 to 5 µg/ml of RNA, 50 µg/ml of cytochrome c (horse heart, type VI; Sigma, St. Louis, Mo.), 4 M-urea, 3 mM-tris buffer, pH 8.5, 1 mM-EDTA and 80% fresh or recrystallized formamide (v/v, Matheson Coleman Bell, Norwood, Ohio). Fifty µl of this mixture were heated in a 53°C waterbath for 30 s prior to spreading on a hypophase of pre-chilled (4°C) 0.15 M-tris buffer (pH 8.5). The samples were transferred on to 2.9% Parlodion coated grids, stained with 0.2% ethanolic uranyl acetate and rotary shadowed with Pt:Pd (Kleinschmidt & Zahn, 1959; Evenson, 1977). The samples were observed with a Siemens 101 electron microscope operating at 100 kV and equipped with a 50 µm objective aperture. This procedure did not produce significant numbers of breaks in single stranded vesicular stomatitis virus RNA or single stranded circular fd phage DNA which were co-spread or spread immediately after the FLV samples. Therefore, this method should not have induced significant numbers of breaks in FLV RNA.

Alternatively, the RNA was incubated in 4 M-urea, which produces a lesser degree of denaturation, the method being essentially that described by Weber et al. (1974). In brief, the RNA was first diluted 1:10 into 4 M-urea and to 25 µl of this dilution, 20 µl of 1 M-ammonium acetate and 5 µl of cytochrome c (1 mg/ml) were added. This mixture was spread on to a 15 mm-ammonium acetate hypophase and processed as described above.

Molecule length measurements. For each RNA sample, molecules prepared on a single grid were photographed at a magnification ranging from ×9500 to ×9800 and immediately afterwards the magnification of the electron microscope was checked by photographing a crossed lines grating replica. The negatives were printed on 20 × 24 in paper at a final magnification of about 75,000 ×. The molecule lengths were traced with the arm of a Numonics graphics calculator (North Wales, Pa.) having a sensitivity of 0.25 mm. At least 1000 molecules were measured for each experiment.

**RESULTS**

Characteristics of Friend virus RNA

**Zone velocity sedimentation**

RNA isolated from Friend virus by the Gu-HCl method sedimented in isokinetic sucrose gradients at 60 to 70S as shown in Fig. 1. According to the absorbance (at 254 nm) of the sucrose gradients, about 15% of the TCV material and 25% of the PV material sedimented at less than 30S and partially corresponds to cellular ribosomal RNA. It is difficult to completely remove all ribosomal RNA from virus preparations especially in view of the fact that complete ribosomes have been recognized entrapped in budding virions (Sato et al. 1971).
Heat-denatured TCV RNA isolated from 3 and 72 h harvests sedimented in isokinetic sucrose gradients as shown in Fig. 2. Both of the profiles show a rather broad distribution of molecules with sedimentation coefficients ranging from about 0 to 40S. The distribution suggests that several size classes of RNA may be present in the samples. The 3 h harvest sample contained significantly more RNA sedimenting at about 35S than was found in the 72 h sample. Denatured PV RNA sedimented with a peak at about 35S (Pavlovec et al. 1978).

**Length measurements of denatured RNA**

Since the relationship between mol. wt. and sedimentation coefficient can differ widely for various single stranded RNA molecules (Kolakofsky et al. 1974) we have measured by electron microscopy the sizes of denatured subunits of the RNA isolated from TCV and PV. Fig. 3(a and b) show typical electron micrographs of PV and TCV RNA from which length measurements were made. Fig. 4 shows the sizes of denatured TCV RNA. The data are presented as weight average histograms which have the advantage that the mass distribution of the various size classes can readily be seen. The size profiles of RNA isolated from 3, 9, 36 and 72 h samples were approximately the same, although the earlier times appear to have some larger molecules. The histograms show that the lengths of RNA subunits appear to belong to different size classes which are similar for all time
periods studied. The origin of the small differences observed from one time period to another is currently being investigated. The average sizes of each of these classes are approx. 0.4, 0.7 and 1.1 μm. Using fd phage single stranded circular DNA as a reference marker, these class sizes correspond to mol. wt. of 0.5 × 10⁶, 0.9 × 10⁶ and 1.4 × 10⁶, respectively.

Fig. 5 shows the sizes of denatured PV RNA prepared by exactly the same method as that used for TCV RNA. Observations from several experiments consistently showed that PV RNA subunits were significantly larger than those of TCV RNA. The weight distribution of length histograms of total PV RNA (Fig. 5) shows that the majority of the non-covalently linked RNA subunits ranged from 1.4 to 2.5 μm in length which correspond to mol. wt. ranging from 1.8 × 10⁶ to 3.0 × 10⁶. The maximum length was about 3.5 μm. The histogram suggests that PV RNA may have size classes also, but they are not as evident as those seen for TCV RNA.
Fig. 4. Weight average histograms of lengths of denatured TCV RNA isolated from virus samples harvested at the indicated times and prepared for electron microscopy by the method of Robberson et al. (1971).

Fig. 5. Weight average histogram of lengths of denatured PV RNA isolated from plasma-derived virus and processed for electron microscopy by the method of Robberson et al. (1971).

Contamination of RNA samples with cellular DNA

Tissue culture virus

Fig. 6 illustrates total nucleic acid isolated from virus treated as described in the legend. All samples, prepared for electron microscopy after denaturation with 4 M-urea, showed 60 to 70S complexes containing a high degree of secondary structure (Fig. 6a). Our recent observations, to be reported at a later date, indicate that the 60 to 70S complexes prepared
Fig. 6. Electron micrographs of nucleic acid isolated from isopycnic sucrose gradient purified TCV (72 h harvest) and prepared by the 4 M-urea technique. (a) Virus sample not filtered or treated with DNase; (b) virus sample filtered (0.6 μm) but not treated with DNase; (c) virus sample filtered (0.6 μm) and treated with DNase.
EM of Friend virus RNA

Table 1. Amount of $^3$H-thymidine labelled component retained on filters after enzyme treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment no.</th>
<th>Average ct/min for triplicate samples</th>
<th>Percent of radioactivity retained relative to buffer control</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase†</td>
<td>1</td>
<td>125</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>107</td>
<td>2</td>
</tr>
<tr>
<td>RNase‡</td>
<td>1</td>
<td>4615</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4975</td>
<td>109</td>
</tr>
<tr>
<td>Buffer§</td>
<td>1</td>
<td>3954</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4551</td>
<td>100</td>
</tr>
</tbody>
</table>

* Two $\mu$Ci/ml methyl-$^3$H-thymidine (20 Ci/mmole, New England Nuclear) were added to 500 ml cultures of log phase growing cells. Sixteen hours later the cells and cellular debris were removed by a low speed spin and intermediate spin as described in Methods. Sixty-five ml samples of supernatant were then filtered through 25 mm diam. Nuclepore filters (0.6 μm average pore size). The filters were then rinsed by aspirating 5 ml Earle's buffer through the filters, followed by treating the filters with either DNase, RNase or buffer.

† DNase: 0.01 % (w/v) DNase I (Sigma) in 0.1% (w/v) MgSO₄•7H₂O, 0.05 M-Na₂HPO₄ and 0.01 M-KH₂PO₄, pH 6.9 was added to the filter holders with the filters held in place and incubated for 1 h at 37 °C. The DNase solution was then aspirated through the filters and the filters washed with 5 % TCA followed by 70 % ethanol. The filters were air dried and measured for radioactivity by standard scintillation counting techniques.

‡ RNase treatment was the same as for DNase except that the RNase solution contained 0.05 % (w/v) RNase, 0.01 M-Na₂HPO₄, 0.01 M-KH₂PO₄, pH 6.9.

§ Buffer control: the buffer treatment was the same as DNase except that the DNase was omitted.

by this technique have a central core with a number of coiled branching arms (D. P. Evenson, unpublished observations). In contrast, double stranded DNA prepared under identical conditions remained as a relaxed duplex (Fig. 6a). To determine the nature and origin (i.e. intraviral or extraviral) of the DNA-like material seen in samples prepared as described in the legend for Fig. 6(a), the virus suspensions were filtered and/or treated with nucleases. As shown in Fig. 6(b), the DNA-like material was partially removed by filtration of the virus suspensions through 0.6 μm pore filters and was almost completely removed by DNase treatment of intact virions (Fig. 6c). These data strongly suggest that the contaminating DNA-like filaments were in fact extracellular DNA. These results are supported by the data shown in Table 1. Friend leukaemia cells were labelled with $^3$H-thymidine and the growth media filtered as described in Methods. The amount of radioactivity retained on the filters (0.6 μm porosity) after treatment with DNase, RNase and buffer alone are shown in Table 1. Virtually all of the radioactivity was removed by DNase treatment and therefore the material that was removed by filtration was probably cellular DNA, confirming previous reports (Weber et al. 1974; Evenson et al. 1975b).

Plasma virus

In contrast to the TCV preparations, total nucleic acid isolated from plasma virus contained virtually no DNA as determined by the same techniques except for the $^3$H-labelling experiments.

DISCUSSION

We have compared the size and structure of the RNA subunits isolated from a highly leukaemogenic Friend virus (PV) with that isolated from a low leukaemogenic variant (TCV). The structures of the 60 to 70S RNA complexes from both TCV and PV appear
similar after incubating and spreading the RNA with 4 M-urea. Electron microscope observations of these macromolecules indicate that they contain a considerable amount of secondary structure which agrees with the results obtained by enzyme digestion (Leis & Hurwitz, 1972) and ethidium bromide intercalation (Cavaliere, 1974).

The sizes of the non-covalently linked subunits of RNA isolated from PV are significantly larger than those isolated from TCV. Most of the denatured PV RNA has an apparent mol. wt. of about 2 to $3 \times 10^6$ whereas most of the TCV RNA is only about half that size. The data suggest that both TCV and PV contain size classes of RNA which are very similar to the size classes observed by Heine et al. (1975) for avian myeloblastosis virus RNA. Since one can expect that an intact, large subunit is needed for maximum biological expression, the difference in leukaemogenic potential observed (Evenson et al. 1975a) between TCV and PV could be related to RNA subunit size. Previous studies by Bader & Steck (1969) have suggested that the infectivity of RNA tumour viruses is proportional to the amount of 30 to 40S virus RNA rather than to that of 60 to 70S RNA.

The size of RNA subunits does not seem to be directly related to the time which elapsed between virus budding and harvest, i.e., the ‘age’ of the virion. Viraemia in the mouse is demonstrable as early as 4 days after infection with Friend virus (Friend et al. 1966); therefore, when the viruses are harvested at 6 days, many of the virions are likely to be at least 48 h ‘old’. Plasma-derived virions were compared for biological activity with TCV harvested at 72 h. In our initial study, which compared RNA subunit lengths from 6 day-infected mice with 72 h tissue culture samples, we showed that the TCV RNA subunits were significantly shorter than PV RNA subunits (Evenson et al. 1975b). However, somewhat unexpectedly, the TCV RNA was about the same size regardless of whether the virions were harvested from 3, 9, 36 or 72 h cultures. This observation is somewhat at variance with other studies, e.g., those of Mangel et al. (1974) which have indicated that denatured 60 to 70S RNA from early harvest virus (e.g. 3 h) has a narrow distribution of molecules around 35S in a sucrose gradient, while later harvests (e.g. 12 to 24 h) result in a wide distribution of molecules sedimenting between zero and 40S. Although it remains possible that TCV RNA has inherently shorter lengths than PV RNA, the smaller sizes could also be the result of nuclease nicking. A random nick in a region of secondary structure of a 60 to 70S virus RNA complex would not change the mass of the complex but would create two free ends which, when the complex is dissociated, would decrease the mass of the non-covalently linked subunits. For example, Bader & Steck (1969) found that incubation of virions for 4 h at 37 °C in growth media resulted in a significant fragmentation of the RNA which was detected only after denaturation, thus suggesting that the nuclease damage occurs quite soon after virus production. McClain & Kirsten (1972) found that the degradation was related more to the media than to the temperature or time; both fresh and spent media contained this degradative activity which was hypothetically related to nucleases. Riggin et al. (1974) found that heat-denatured RNA from 5 min harvests of Moloney murine leukaemia virus contained a major component with an apparent mol. wt. of $3.4 \times 10^6$ and a minor component of $1.7 \times 10^6$. Denatured RNA from the 3 h harvest contained both components; however, the $1.7 \times 10^6$ component predominated. Denatured RNA from 16 h virus was much more extensively degraded.

When PV were incubated in fresh BME 15 FCS or NET for 3 h at 37 °C, no loss of biological activity was observed (Evenson et al. 1975a). Thus, assuming that nuclease attacks on PV RNA would reduce biological activity, either BME 15 FCS contained no RNase activity or the PV were not susceptible to significant intraviral or extraviral nuclease digestion. The latter possibility is supported by the data of East et al. (1973) who showed that
treatment of murine sarcoma viruses with exogenous micrococcal ribonuclease had no effect on the subunit pattern of RNA isolated from the virions.

As previously reported (Evenson et al. 1975a), observations on thin sections of TCV (72 h harvest) and PV pellets indicated that TCV envelopes appeared to be somewhat less structurally intact. Therefore, it seems possible that cellular derived nucleases liberated into the tissue culture media might permeate the virus envelope and partially digest the RNA, thus giving rise to the smaller sized subunits; alternatively the nucleases could be entrapped by the budding virions. These hypotheses could be more appropriately tested if we could compare molecular lengths or sucrose gradient sedimentation profiles of denatured RNA obtained from very early harvest (e.g. 5 min) and later harvest virions. Unfortunately, because clone 745 Friend cells are only moderate producers of virus and grow only in suspension, it is not possible to obtain such very early harvests for comparison. Although we found some degradation in later samples relative to the 3 h samples, the bulk of the RNA was about the same size at all time points tested. Thus, any degradation that occurred was evidently rapid and quite limited.

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