Gel Electrophoresis of Avian Leukosis and Sarcoma Viral RNA in Formamide: Comparison with Other Viral and Cellular RNA Species

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Class *a* and class *b* 30 to 40S RNA subunits obtained by heat dissociation from the 60 to 70S RNA complex of avian tumor viruses were compared with several RNA standards by electrophoresis in formamide-polyacrylamide gels. Class *a* RNA was found to have a lower electrophoretic mobility and hence probably a higher molecular weight than class *b* RNA. The absolute molecular weight of class *a* and *b* RNA could not be determined with accuracy, because the relationship between logarithm of molecular weight and mobility of the RNA standards was not linear. The size of class *a* RNA fell into the range of 2.4×10^6 to 3.4×10^6 daltons and that of class *b* into the range of 2.2×10^6 to 2.9×10^6 daltons, depending on the standards used. The possible biological significance of this difference is discussed.

The 60 to 70S RNA of RNA tumor viruses can be dissociated by heat or dimethylsulfoxide into several molecules of 30 to 40S (7). Nondefective avian sarcoma viruses contain a 30 to 40S species, termed class *a* RNA, which has a lower electrophoretic mobility in aqueous buffer than the 30 to 40S RNA found in all transformation-defective (td) and leukosis viruses which is termed class *b* RNA (8, 12; P. H. Duesberg and P. K. Vogt, *Virology*, in press). Recently, oligonucleotide fingerprint and RNA-DNA hybridization analyses of class *a* RNA of nondefective avian sarcoma viruses and class *b* RNA of td or leukosis viruses of the same subgroup and strain have shown that class *a* RNA contains probably most, and perhaps all, of the sequences of class *b* plus some additional sequences (M. M.-C. Lai, P. H. Duesberg, J. Horst, and P. K. Vogt, *Proc. Nat. Acad. Sci. U.S.A.*, in press). The relation of class *a* and *b* RNA was proposed to be $a = b + x$.

The purpose of this study was to determine the size difference between class *a* and *b* RNA by electrophoresis in formamide. This method is thought to be independent of conformational factors influencing electrophoretic mobility of RNA in aqueous solution (14). It was found that class *a* RNA species are larger (perhaps by 12%) than class *b* species. The absolute size of the two RNA species could not be determined

accurately, because a linear relationship between the logarithm of molecular weight and the electrophoretic mobilities of the RNA standards was not obtained.

MATERIALS AND METHODS

Electrophoresis in formamide polyacrylamide gels. A modification of the procedures of Staynov et al. (14) and H. Boedtker (personal communication, 1972) was used. Formamide (99%, Matheson, Coleman and Bell, Inc.) was deionized by stirring with about 5% (wt/vol) mixed bed resin (Bio-Rad, Richmond, Calif.) for 1 h at room temperature and subsequently filtered through a glass fiber filter (Whatman GF/C). Phosphate-buffered (0.02 M, pH 7.0) formamide was prepared by adding 142 mg of Na_2HPO_4 and 138 mg of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ to 100 ml of deionized formamide and stirring until dissolved. Buffered or unbuffered formamide solutions were stored at -70°C . To prepare 3.5% (wt/vol) polyacrylamide gels (7 to 8 gels, 0.6 by 7 cm), 0.7 g of acrylamide and 0.122 g of bisacrylamide (Bio-Rad) were dissolved by stirring in 19.75 ml of buffered formamide. Then, 250 μl of ammonium persulfate (100 mg/ml of water) and 40 μl of tetramethylethylenediamine were added. The solution was then poured into Plexiglas tubes (0.6 by 12 cm), closed at the bottom with dialysis membrane, and overlaid with 25 to 50 μl of water. Gels (3.5% or 3%) polymerized in 30 min at room temperature. They were used directly or after sealing with Parafilm and storage under buffered formamide for several weeks in

a refrigerator. The final concentration of formamide in these gels was between 96 and 98%.

RNA samples (10–30 μ g) were prepared by adding, to ethanol-precipitated RNA, 25 μ liters of formamide containing 2 mM phosphate buffer (prepared from 1 vol of 0.02 M phosphate-buffered and 9 vol of deionized formamide) and then 15 μ liters of a solution containing 1 vol of deionized formamide, 1 vol of glycerol, and enough bromophenol blue to serve as tracking dye. Alternatively, RNA dissolved in 5 to 10 μ liters of aqueous electrophoresis buffer (6, 8; P. H. Duesberg and P. K. Vogt, *Virology*, in press) was mixed with at least 3 vol of formamide containing 2 mM phosphate buffer and 2 vol of the formamide-glycerol solution described above. The RNA solution was then layered on top of the gel with a capillary pipette under a 5-cm column of formamide containing 0.02 M phosphate. The 5-cm formamide column was freshly poured on top of the gel prior to electrophoresis and was left on top of the gel during electrophoresis. Upper and lower buffer reservoirs contained 100 ml and 250 ml, respectively, of 0.04 M phosphate buffer (pH 7.0) in water. Electrophoresis at room temperature was for about 16 h at 80 V in 3.5% gels or for 6 h at 100 V in 3% gels. After electrophoresis the gels were frozen in ethanol-dry ice. During this procedure the gels retained their original length. (Up to about 1 cm from the bottom of the gel, freezing was much slower presumably because aqueous buffer had diffused into the gel up to this point during electrophoresis. The electrophoretic mobility of RNAs was reduced upon migration into this region. RNAs appeared to have higher mobilities in pure [99%] formamide solution than in aqueous formamide solution. A similar observation was described for RNA in pure and aqueous dimethylsulfoxide [15].)

Gels were then sliced into 1-mm sections by stacked razor blades. Radioactivity was determined in a Tri-Carb spectrometer after incubation of each slice for 2 to 12 h at 60°C in 5 ml of toluene-based scintillation fluid containing 10% NCS (Nuclear Chicago) and 1% water. To detect unlabeled tobacco mosaic virus RNA, gels were incubated for 1 h in 1 M acetic acid containing 0.2% methylene blue and subsequently destained by stirring in a perforated plastic tube in 300 ml of 1 M acetic acid. RNA bands were then located by inspection and marked by insertion of a stainless steel needle into the gel. Thereafter, radioactivity was determined as described above. Correlation between the radioactive peaks and methylene blue-stained RNA components was by the position of the stainless steel needle and by measuring the position of the stained RNA component after slicing the gel.

RNA preparations. Propagation of the avian tumor viruses used was in gs^{-}/chf^{-} chick cells (17) as well as in cells which were not tested for these properties. The RNA of the viruses used was not detectably affected by these properties of the chick cell. Preparation of viral and cellular RNA was as described previously (1, 6, 8, 12; P. H. Duesberg and Peter K. Vogt, *Virology*, in press) except for Sendai virus RNA. Sendai virus was propagated in MDBK cells (5) or chicken embryo fibroblasts. MDBK cells were grown in Dulbecco modified Eagle (DME) medium containing 10% fetal calf serum, 100 U of

penicillin per ml, 50 μ g of streptomycin per ml, and 0.4% $NaHCO_3$. Cells were infected with 1 ml of allantoic fluid of Sendai virus-infected chicken embryos (2) in 9 ml of virus growth medium (DME, containing 2% calf serum and otherwise supplemented as above). After 10 h at 36°C, the medium was replaced by 5 ml of fresh growth medium, and 100 μ Ci of 3H -uridine was added. Subsequently, media were changed at 5-h intervals and pooled for virus purification (1, 2).

RESULTS

The 60 to 70S RNA of recently cloned Prague strain Rous sarcoma virus of subgroup C (PR RSV-C) and of its transformation-defective derivative td PR-C (16) was analyzed by electrophoresis in formamide. It was known that cloned PR RSV-C contains only class *a* RNA, and td PR-C only class *b* RNA (P. H. Duesberg and P. K. Vogt, *Virology*, in press). Figure 1 shows the distribution of PR RSV-C ^{32}P -RNA and td PR-C 3H -RNA in the presence of three RNA markers, Sendai 3H -RNA and 28S and 18S chick ribosomal 3H -RNA, after electrophoresis in 3.5% polyacrylamide containing phosphate-buffered formamide. The 60 to 70S RNA of tumor viruses migrated farther in formamide than the 57S RNA of Sendai virus but slower than the other RNA markers. This was expected because 60 to 70S tumor virus RNA consists of major 30 to 40S subunits and minor heterogeneous RNA species which dissociate under denaturing conditions (7) such as formamide. The position of tobacco mosaic virus (TMV) RNA is also indicated in Fig. 1. The position of TMV RNA was calculated from a parallel experiment, in which TMV RNA was located by staining with methylene blue (13), and RNA markers of PR RSV-C ^{32}P -RNA and chick ribosomal 3H -RNAs were located subsequently by determination of their radioactivity.

Figure 2 shows four different electrophoretic analyses of the above studied RNA species in 3% polyacrylamide containing phosphate-buffered formamide. In addition the 60 to 70S RNA of RAV-2, a leukemia virus containing only class *b* RNA (P. H. Duesberg and P. K. Vogt, *Virology*, in press) was analyzed (Fig. 2B). The reduction of polyacrylamide concentration from 3.5 to 3.0% increased the electrophoretic mobilities of all small RNA species tested by about the same factor, but the mobility of Sendai virus RNA was increased by a larger factor (cf. Fig. 1 and 2).

The relative electrophoretic mobilities of the four marker RNAs used were, as expected (14), inversely proportional to the molecular weights reported for these RNAs. These are 6×10^6 for Sendai RNA (1, 2), 2×10^6 for TMV RNA (3, 4)

FIG. 1. Electrophoresis of 60 to 70S PR RSV-C RNA (labeled PR-C in the figure), 60 to 70S td PR-C RNA, 57S Sendai virus RNA, and chick 28S and 18S rRNA in a 3.5% polyacrylamide-formamide gel. The position of TMV RNA is indicated by an arrow. It was determined from analysis of a parallel gel with PR RSV-C RNA, 28S and 18S rRNA markers as described in Materials and Methods. The td PR-C, Sendai and ribosomal RNAs used were not differentially labeled. To determine suitable concentrations of the respective radioactive ^3H -RNAs and the duration of electrophoresis required for electrophoretic resolution each of these RNAs was first subjected to electrophoresis on separate gels using a [^{32}P]PR RSV-C RNA standard. The insert shows two different logarithm molecular weight-electrophoretic mobility relations using 18S ribosomal RNA and 28S ribosomal RNA or TMV RNA as markers to draw calibration curves.

and 28S rRNA (11, 14), and 0.7×10^6 for 18S rRNA (11, 14). However it was not possible to place these RNA standards on a linear logarithm molecular weight-mobility calibration curve as described for smaller RNAs analyzed in similar conditions (14). Nevertheless, two tentative calibration curves were drawn using 18S rRNA and 28S rRNA or TMV RNA as markers (inserts Fig. 1 and 2). Using these calibration curves, Sendai RNA registers as a 3×10^6 to 4×10^6 dalton RNA rather than a 6×10^6 dalton RNA (1, 2) under these conditions and was not used as a standard to draw calibration curves (see Discussion).

Electrophoresis in aqueous solution of uncloned PR RSV-C which contains class *a* and *b*

RNA (8) in the presence of the RNA standards used above is shown in Fig. 3. A comparison of the analysis shown in Fig. 1, 2, and 3 indicates that the RNA species tested migrate in the same order in buffered aqueous or formamide solution. Again it was not possible to place the four RNA standards on a linear logarithm-molecular weight calibration curve. Yet two tentative calibration curves were drawn using 18S rRNA and 28S rRNA or TMV RNA as standards. In these conditions Sendai virus RNA registers as a 7.5×10^6 dalton RNA, if 28S and 18S rRNAs are used to draw a calibration curve, and as 4×10^6 dalton RNA if 18S rRNA and TMV RNA are used to draw the calibration curve.

The resolution of larger RNA species was better in aqueous solution than in formamide (cf. Fig. 1-3). In formamide better resolution was obtained at the higher concentration of polyacrylamide (cf. Fig. 1 and 2).

DISCUSSION

The results shown in Fig. 1 and 2 indicate that the class *a* RNA of Pr RSV-C has a lower electrophoretic mobility in formamide than class *b* RNA of td PR-C and RAV-2. The same differences in electrophoretic mobility in formamide were observed between class *a* RNA of B77 and SR RSV, and class *b* RNA of td PR-C, RAV(O), and RAV-2 (data not shown). Since the logarithm molecular weight of a given RNA is inversely proportional to its electrophoretic mobility in formamide (14), it is concluded that class *a* RNA of sarcoma viruses is larger than class *b* RNA of transformation-defective avian tumor viruses (8; P. H. Duesberg and P. K. Vogt, Virology, in press).

However the absolute molecular weights of class *a* and *b* RNA could not be determined accurately, because the relation between logarithm molecular weight and electrophoretic mobility was not linear for the RNA standards used. For example, the mobilities of two of the most commonly used RNA standards for 2×10^6 daltons, TMV RNA (3, 4) and 28S rRNA (11, 14), were found to be different both in buffered aqueous or formamide solution (Fig. 1-3). The apparent molecular weight of Sendai RNA also varied considerably if compared to different standards in the same gel. Because determination of the molecular weight of Sendai RNA (about 6×10^6) (1, 2) is less certain than that of the other RNA standards, it was not used as a quantitative marker to estimate the molecular weight of class *a* and class *b* RNA of tumor viruses. Nevertheless, it served as a qualitative

FIG. 2. Electrophoresis in 3% polyacrylamide-formamide gels. A, 60 to 70S PR RSV-C RNA (labeled PR-C in the figure), 60 to 70S td PR-C RNA and TMV RNA. B, 60 to 70S PR RSV-C RNA and RAV-2 RNA. C, 60 to 70S td PR-C RNA, TMV RNA, 28S and 18S rRNA. D, 57S Sendai virus RNA, 60 to 70S td PR-C RNA and TMV RNA. The TMV RNA markers in A, C, and D were located by staining with methylene blue prior to determination of the radioactivity in the gel (see Materials and Methods). The insert shows two different logarithm molecular weight-electrophoretic mobility relations using 18S ribosomal RNA and 28S ribosomal RNA or TMV RNA as markers to draw calibration curves. The distances moved by different RNA species in the four experiments depicted and in other experiments were interconverted on the basis of markers to obtain the calibration curve.

marker, which had a consistently lower electrophoretic mobility in formamide or in aqueous solution than the other RNAs investigated.

The molecular weights of class *a* RNA of PR RSV-C estimated from the calibration curves in Fig. 1 and 2 vary between 2.4×10^6 and 3.4×10^6 and those of class *b* RNA of td PR-C between 2.2×10^6 and 2.9×10^6 in formamide. Thus the difference between the average molec-

ular weights of class *a* and *b* RNA is about 350,000 (12% of the average *a* subunit) if determined by this method. In contrast, the difference between the average molecular weights of class *a* and *b* RNA can be calculated to be 950,000 (24% of the average *a* subunit), using the data obtained after electrophoresis in aqueous buffers (Fig. 3). Obviously a more accurate estimate of the absolute difference between *a*

FIG. 3. Electrophoresis of the 60 to 70S RNA of uncloned PR RSV-C, 57S Sendai virus RNA, TMV RNA, 28S and 18S rRNA in a 2% aqueous sodium dodecyl sulfate polyacrylamide gel (P. H. Duesberg and P. K. Vogt, *Virology*, in press). The RNA of uncloned PR RSV-C contains both class a and class b 30 to 40S RNA subunits (8). The RNAs were heated at 100 C for 30 s in 50 μ liters of electrophoresis sample buffer (P. H. Duesberg and P. K. Vogt, *Virology*, in press) and then quenched in melting ice prior to electrophoresis. TMV RNA was located by its opalescence after incubation of the gel for 12 h at 4 C in 5% trichloroacetic acid. Determination of radioactivity in the gel was as described for Fig. 1 and 2. The insert shows two different logarithm molecular weight-electrophoretic mobility relations using 18S and 28S rRNA or TMV RNA as markers to draw calibration curves.

and b subunits by this method depends on the availability of more suitable RNA standards and/or a better understanding of the physical properties of large RNA molecules in aqueous and formamide solution.

We have recently shown that the 60 to 70S RNA species of some sarcoma viruses contain only class a molecules, and that transformation-defective viruses contain only class b RNA (P. H. Duesberg and P. K. Vogt, *Virology*, in press). However, it is not yet clear whether the three to four physically indistinguishable species of class a or b RNA of the 60 to 70S RNA complex are chemically identical or different (7, P. H. Duesberg and P. K. Vogt, *Virology*, in press; P. H. Duesberg, E. Canaani, K. v.d. Helm, M. M.-C.

Lai and P. K. Vogt, IV Lepetit Colloquium 1972, North-Holland, in press; P. K. Vogt, IV Lepetit Colloquium 1972, North-Holland, in press). The genome may be polyploid or segmented haploid. If the 60 to 70S viral RNAs consist of identical subunits, the total difference between the RNAs of transforming and transformation-defective viruses may be only 350,000 daltons, corresponding to about 35,000 daltons of protein. This difference would be three to four times greater if the 60 to 70S RNA consists of three to four chemically distinct subunits.

A protein of 35,000 daltons may be too small to account for the numerous differences observed between transformed and nontransformed cells (9). Moreover, viral genes concerned with transformation by avian tumor viruses were shown to fall into two to four complementation groups (10; J. A. Wyke and M. Linial, *Virology*, in press). This observation would predict a larger genetic difference between sarcoma and transformation-defective viruses. Nevertheless, it is conceivable that the effect of transformation specific genes is pleiotropic and that some genetic information needed for virus replication also participates in the process of cell transformation.

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ADDENDUM IN PROOF

The molecular weight of Sendai virus RNA has recently been reported to be 2.3×10^6 on the basis of sedimentation analyses in DMSO gradients (D. Kolakofsky and A. Brusch, 1973, *J. Virol.* 11:615-620). However, this value appears too small if one assumes that the viral RNA is one molecule and if one considers that at least all known viral structural proteins (J. Content and P. H. Duesberg, 1971, *J. Virol.* 6:707-716) are coded for by viral RNA. Therefore the RNA of Sendai virus should be larger than 2.3×10^6 , perhaps in the range of 3×10^6 to 4×10^6 as may be estimated from our formamide-polyacrylamide gels.

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