A Rapid Method for the Quantitative Study of RNA from Canine Distemper Virus Infected Cells

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SUMMARY

Centrifugation through CsCl was used to isolate $^{32}$P-labelled RNA in a one-step purification procedure. The method is suitable for quantitative as well as preparative studies and appears to have considerable advantages over conventional methods of RNA extraction. We have used this procedure to investigate the RNA synthesized in Vero cells infected with canine distemper virus (CDV). We show that the combination of CsCl centrifugation and affinity chromatography on poly-U Sepharose provides a rapid method for isolating messenger RNA from virus infected cells.

Recently, Glisin, Crkvenjakov & Byus (1974) reported a method for the isolation of RNA from disrupted cells by direct centrifugation through a CsCl cushion and they outlined the advantages of this one-step separation procedure over other methods of RNA extraction. We have assessed the suitability of this CsCl centrifugation procedure for the study of virus-induced RNA of CDV infected Vero cells. We find that the method is rapid and convenient and is more suitable for quantitative work than the conventional phenol extraction procedures. For work with $^{32}$P it has numerous advantages over other methods of RNA extraction. The RNA pellet obtained by centrifugation through the CsCl cushion is essentially free of residual low mol. wt. phosphate contamination which is never the case when phenol extraction is used. Hence, there is no need for secondary purification steps such as gel-filtration or repeated precipitation with ethanol. Also, transfer RNA which is generally very highly labelled with $^{32}$P, is not obtained by this method and this allows the high mol. wt. RNA to be studied directly. Glisin et al. (1974) have shown that close to 100% of ribosomal RNA is extracted by the CsCl procedure and the present studies indicate that the $^{32}$P-labelled RNA preparations are free from other $^{32}$P-labelled components such as DNA, lipid or protein. Another main advantage of the CsCl procedure is that the cell cultures are treated directly on harvesting with 4% sodium lauryl sarcosinate and can then be frozen. This is preferable to alternative procedures which require immediate processing of the samples such as with the preparation of cytoplasmic extracts. Also, cell fractionation procedures inevitably expose RNA to nuclease action, the effects of which may only become evident after denaturation of the purified RNA.

Monolayers of Vero cells were grown in supplemented Eagle's medium containing 10% foetal calf serum and antibiotic (100 units penicillin and 100 µg streptomycin/ml). Stock cultures were grown in Roux flasks but in all the experiments reported here monolayers were made in 75 cm² plastic Falcon flasks. For the preparation of radioactive RNA, monolayers were rinsed with phosphate-free medium. The medium was then replaced with 10 ml phosphate-free medium and 1 mCi of $^{32}$P added. $^{32}$P-orthophosphate in phosphate-free solution (purchased from Amersham-Buchler Gm 6H, 3300 Braunscheveig) was diluted to 1 mCi/ml with phosphate-free Eagle's medium prior to use. When required, actinomycin D (purchased from Serva Ltd, Heidelberg) at 5 µg/ml was present during both the pre-incubation period and throughout the labelling period.
After incubation for various periods the radioactive medium was poured off the cell monolayers and the flasks rinsed twice with 20 ml of ice-cold phosphate-buffered saline (PBS) and drained well by inserting a disposable paper plug into the neck of the flask and inverting it for 2 to 3 min. It is advantageous to remove as much non-incorporated radioactivity and excess liquid at this stage as possible. The monolayers were treated with 6 ml of sarkosyl buffer containing 4% (w/v) sodium lauryl sarcosinate (Serva Ltd, 6900 Heidelberg) in 0.1 M-tris-HCl, pH 8.0. The cells after a few minutes could be removed from the plastic surface by gentle swirling and transferred by pouring the jelly-like solution into a thick-walled glass tube. Complete disruption of the cells was achieved by forced-pipetting via a disposable 5 ml syringe fitted with a long needle. This procedure avoids contamination of homogenizers with 32P and also losses which would occur during further transfers. The suspension can be stored at −20 °C without affecting the nature of the RNA isolated. Solid CsCl (3 g) was dissolved in 3 ml of the cell homogenate by mixing at room temperature and layered on to 1.2 ml of a 5.7 M-CsCl-0.1 M-EDTA cushion (pH 6.5) in a 5 ml polyallomer centrifuge tube. The tubes were balanced by topping-up with sarkosyl buffer (0.25 ml) and centrifuged at 35,000 g for 16 h at 25 °C in a Beckmann SW 50.1 rotor. After centrifugation the DNA banding at the interface of the two CsCl solutions was removed with a Pasteur pipette and also the gelatinous layer at the top of the tube. The tube was inverted and all but the bottom 1 cm was cut off. The small visible pellet was readily dissolved in 1 ml SDS buffer (0.12 M-NaCl-0.02 M-tris-HCl (pH 7.5) − 0.5% SDS w/v) and precipitated with 2 vol. ethanol and 1 mg carrier RNA at −20 °C. The RNA used as carrier and marker was BHK RNA isolated by the phenol:SDS procedure as described by Clements & Martin (1970).

The recovery of radioactivity in the pellets obtained by centrifugation of the ethanol suspensions at 3500 rev/min for 30 min was consistently over 99.5%. This is in marked contrast to the difficulties of removing extraneous 32P-labelled contaminants after phenol extraction procedures. Hydrolysis with 0.1 N-NaOH at 37 °C for 1 h solubilized over 99.9% of the radioactivity in high mol. wt. RNA indicating that an insignificant amount of 32P-labelled DNA or protein contaminants were present. Analysis of the RNA isolated from duplicate monolayers labelled for the same period showed that the amount of radioactivity incorporated did not vary by more than ±3% in the two samples.

Samples of RNA were dissolved in 0.1 ml electrophoresis buffer containing 15% sucrose and analysed on 20% polyacrylamide gels containing 0.4% agarose as described by Clements & Martin (1971). The gels were subjected to electrophoresis for 2.5 h at 7 mA/gel at 4 °C. After electrophoresis the gels were sliced with a 60 unit razor blade cutter into 1.5 mm sections. The gel slices were transferred to plastic vials, 2 ml water added and the radioactivity determined utilizing Cerenkov radiation in a Packard Liquid Scintillation counter. The position of internal marker RNA (BHK RNA) was determined either by scanning at 260 nm in a Zeiss spectrophotometer or by fixing the gel with 10% trichloroacetic acid and observing the appearance of the RNA bands (opaque lines) against a blue background. This latter method provides a convenient and accurate assessment of the position of the main ribosomal RNA peaks, provided more than 50 µg RNA is applied to the gel. This distribution of 32P after electrophoresis on acrylamide gels is shown in Fig. 1. After long periods of labelling (20 h) the radioactivity was associated mainly with the 28 S and 18 S ribosomal components. In all these experiments very little 32P was associated with the solvent front indicating that the RNA isolated was free of low mol. wt. contaminants and also that transfer RNA was not extracted by this method. After short periods of labelling (5 h) the distribution of 32P was predominantly associated with lower mol. wt. RNA (20 to 10 S) which is probably cellular mRNA, as this will be labelled more rapidly under these labelling
conditions. Treatment with 2 µg RNase prior to electrophoresis degraded over 99.7% of the high mol. wt. RNA and there was no indication of any residual peaks that could be either RNase resistant RNA or DNA.

We have used this method for investigating the size of RNA molecules produced during the replication of canine distemper virus (CDV) in Vero cells. A stock preparation of the Onderstepoort strain of CDV which was kindly given to us by Dr M. Appel, was made by passing virus at a dilution of 10⁻⁴ in Vero cells and storing the harvest at -70 °C. For radioactive labelling experiments monolayers of Vero cells in Falcon flasks were infected with 5 ml of a 10⁻⁶ or 10⁻⁴ dilution of the stock virus. Under these conditions maximum c.p.e. was generally seen between 25 to 30 h post-infection (p.i.) depending on the age of the culture and over 90% of the cells were floating in the medium by 38 h p.i.

Infected monolayers were labelled with ³²P in the presence or absence of actinomycin D at 5 µg/ml from 17 h until 25 or 30 h p.i. RNA was isolated by the CsCl centrifugation procedure and the clear pellets dissolved in 5 ml CSB (50 mM-tris, 10 mM-EDTA, 0·7 M-NaCl and 25% formamide (Merck Ltd.) adjusted to pH 7.5 with HCl). The sample was mixed with a slurry of poly U-Sepharose-4B (0·3 g in 3 ml CSB) and after equilibration at room temperature for 30 min the Sepharose was transferred to a Pasteur pipette plugged tightly with glass wool. The Sepharose was allowed to settle and drain and the column washed with 20 ml CSB or until the radioactivity of the eluted fractions had decreased to background. The eluting solution was changed to 90% formamide in 10 mM-K₂PO₄, 7 H₂O, 10 mM-EDTA and 0·2% sodium lauryl sarcosinate, pH 7.5. The radioactivity which was eluted as a sharp peak by the 90% formamide was taken as poly A-containing RNA and was precipitated with 2 vol. ethanol after a fivefold dilution with water and addition of carrier RNA. Approx. 0·2% of the total radioactivity of the RNA pellet remained attached to the Sepharose even after prolonged washing with 90% formamide. However, this is most likely residual non-specific binding of ³²P-labelled contaminants as a considerable
proportion, although not all, could be removed by washing with 0.3 M-sodium phosphate at pH 7.5 (Firtel & Lodish, 1973).

The distribution of $^{32}$P on acrylamide gels of total RNA and poly A-containing RNA from CDV-infected cells is shown in Fig. 2. The profile for total RNA contained a peak at mol. wt. approx. $6 \times 10^6$, relative to ribosomal RNA. Although this probably represents the CDV RNA, the heterogeneous nature of the peak suggests that aggregates may be present. However, these high mol. wt. components do not contain accessible poly A segments. The poly A-containing RNA from CDV-infected cells showed the presence of seven peaks in the mol. wt. range 0.25 to $4 \times 10^6$. However, poly-A containing RNA from non-infected, but actinomycin D-treated Vero cells, also showed $^{32}$P activity in the high mol. wt. position and hence not all the above peaks may be of virus origin.

In conclusion we have found that the isolation of RNA by CsCl centrifugation of detergent-disrupted cells provides a convenient method for the quantitative investigation of virus induced RNA. We suggest that the CsCl centrifugation procedure, especially when it is combined with the direct solubilization of the RNA pellet in formamide buffer and fractionation in non-aqueous systems such as DMSO gradients of formamide-acrylamide gels, would provide the best base-line for the quantitative characterization of the molecular sizes of RNA species present.

Fig. 2. Polyacrylamide gel electrophoresis of $^{32}$P-labelled total and poly A-RNA from cells infected with CDV and labelled in the presence of actinomycin D at 5 µg/ml from 17 to 30 h p.i.: ---, total RNA; ---, poly A-containing RNA; . . . . , poly A-containing RNA from uninfected Vero cells.
Short communications

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REFERENCES


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