# CHARACTERIZATION OF $T_4$ DOUBLE-STRANDED RNA BY EQUILIBRIUM CENTRIFUGATION IN $Cs_2SO_4$- AND ALKALINE IODIDE GRADIENTS

Dag E. HELLAND
*Department of Biochemistry, University of Bergen, Årstadveien 19, 5000 Bergen, Norway*

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## 1. Introduction

During the infection period of bacteriophage $T_4$ some of the RNA is transcribed from overlapping regions of the genome [1–3]. These overlapping or symmetrical transcripts form double-stranded RNA upon annealing with RNA isolated from cells at different times after infection.

We have shown that symmetrically transcribed RNA is associated with the polysomes, indicating a possible role of this RNA in translation [4].

To further characterize this RNA, centrifugation in isopycnic gradients was employed. This method is a powerful tool in analyzing heterogeneity in base composition as well as in distinguishing between single- and double-stranded nucleic acids. Here, the isolated double-stranded $T_4$ RNA has been centrifuged in different media used for density separations, and the density is compared to pulse-labelled $T_4$ RNA and to the product after annealing of labelled and unlabelled RNA when nuclease treatment is omitted.

These results show that the pulse-labelled $T_4$ RNA was homogeneous in $Cs_2SO_4$ and heterogeneous in KI and NaI density gradients while double-stranded $T_4$ RNA was heterogeneous in all density gradients.

Nevertheless it was possible to distinguish clearly between single-stranded and double-stranded RNA in $Cs_2SO_4$ and KI gradients. When labelled RNA was annealed to unlabelled RNA the density of the product was definitely lighter than non-annealed labelled RNA and heavier than isolated double-stranded RNA. It is concluded from these results that all species of both early and late labelled RNA contain regions complementary to early RNA.

## 2. Materials and methods

All chemicals used were of the highest purity commercially available. [5-$^3$H]Uracil was from the Radiochemical Centre, Amersham; Suprapure $Cs_2SO_4$, NaI and KI were from Merck, Darmstadt. The bacteriophage $T_4D$ and its host *Escherichia coli* B were gifts from Dr O. Skøld.

RNA was isolated from *E. coli* B that had been grown at 30°C with vigorous aeration in M-9 medium [5], containing 0.2 g glucose/100 ml, and infected at 3–5 × $10^8$ bacteria/ml (multiplicity of infection = 10). The survival rate was always <1%. Infection was stopped at the specified time by adding 1.3 ml 1 M $NaN_3$/100 ml culture, mixing, then pouring the culture immediately onto crushed ice. RNA was labelled using pulses of [5-$^3$H]uracil (3–4 μCi/ml infected bacteria) 3–6 min or 18–21 min after infection.

RNA was extracted using the hot phenol method [6]. In addition, each preparation was treated with DNase I (20 μg/ml, 37°C, 30 min) and re-extracted twice with hot phenol. RNA concentrations were determined spectrophotometrically [6]. The $A_{260}/A_{280}$ was 2.1–2.2.

Double-stranded RNA was formed by annealing unlabelled early RNA (2000 μg/ml) with labelled late RNA (2–3 μg/ml) at 70°C for 3 h in 0.01 M Tris–HCl (pH 7.3) (at 25°C) and 0.5 M KCl. For isolation of double-stranded RNA, the mixture was digested with 20 μg/ml pancreatic RNase, 20 units/ml $T_1$ RNase, and 20 μg/ml DNase I in 0.4 M KCl, 0.01 M Tris–HCl (pH 7.3 at 25°C) and 0.02 M $MgCl_2$ at 37°C for 30 min, then chromatographed on a BioGel P-60 column equilibrated with 0.1 M NaCl, 0.01 M Tris–HCl and 0.01 M EDTA to remove degraded RNA from nuclease-resistant RNA. Hybrids between melted $T_4$ DNA and late labelled $T_4$ RNA were

formed in solution [7] and single-stranded RNA and DNA not forming a complex was removed by incubation with $S_1$-nuclease at 45°C [8].

The ionic density media $Cs_2SO_4$, NaI and KI were all dissolved in 10 mM Tris–HCl (pH 7.3 at 25°C) and 1 mM EDTA. In the NaI and KI solutions 1 mM $Na_2SO_3$ was present to prevent the oxidation of $I^-$. The nucleic acids were added to the salt solutions and the initial density was adjusted by adding salt crystals. All centrifugations were done in an International B-60 Ultracentrifuge using the swing-out rotor no. 498 (3.5 ml/gradient). The initial density is given in the figure legends. At the end of the run gradient fractions were collected from the bottom of the centrifuge tube, using a peristaltic pump. The density throughout the gradient was immediately determined by measuring the refractive index of the fractions and the density was estimated from a standard curve between density and refractive index. The radioactivity was determined by mixing each fraction with 0.5 ml 1% β-mercaptoethanol to prevent formation of free iodine and with 5 ml Triton X-100 toluene-based scintillator. All experiments were carried out in polyallomer tubes washed carefully before use to remove nucleases and other impurities as heavy metal ions which could interfere with the centrifugations. The recoveries were 70–90% in all cases.

## 3. Results and discussion

### 3.1. *Centrifugation of RNA in $Cs_2SO_4$*

The distribution in $Cs_2SO_4$ gradients of the isolated nuclease-resistant RNA obtained after annealing late-labelled $T_4$ RNA with excess early RNA gives further evidence for the double-stranded nature of this RNA (fig.1) since it bands at the same density (1.614 g/cm$^3$) as that reported for the double-stranded Reovirus RNA [9]. The two types of double-stranded RNA have nearly the same base composition [10]. The band profile in fig.1B is asymmetrical, thus indicating heterogeneity of the isolated double-stranded $T_4$ RNA. Although the mixture, after annealing late-labelled RNA with an excess early RNA, is treated with both RNase's and DNase I, the shoulder at the right side in fig.1B indicates the presence of an RNA–DNA hybrid since it bands at slightly <1.5 g/cm$^3$. A pure hybrid is 1.485 g/cm$^3$ in $Cs_2SO_4$ under the conditions used here (table 1). The density

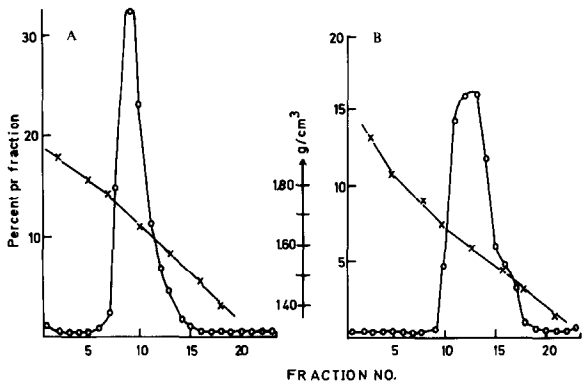


Fig.1. Isopycnic banding of late-labelled $T_4$ RNA and double-stranded $T_4$ RNA in $Cs_2SO_4$: (A) 1 μg 20–30 min $T_4$ [$^3$H] RNA (500 000 cpm/μg); (B) isolated double-stranded RNA (3000 cpm), were centrifuged in $Cs_2SO_4$ gradients containing 10 mM Tris–HCl (pH 7.3 at 25°C) and 1 mM EDTA at 45 000 rev./min for 60 h at 15°C in the 498 rotor, $\rho_i$ = 1.6130 g/cm$^3$.

of the $T_4$ RNA–DNA hybrid reported here is not far from that of the synthetic hybrid φX174 DNA–RNA (1.491–1.510 g/cm$^3$) reported [11].

The presence of hybrids in the nuclease-resistant RNA may be the result of small amounts of DNA present in the unlabelled RNA used in this experiment. In some preparations of double-stranded RNA the shoulder observed at the right side of the peak in fig.1B was present. The presence of a hybrid after annealing an excess of early-unlabelled RNA with late-labelled RNA may therefore give an over-estimation of the amount of symmetrically transcribed RNA present in the labelled RNA. This may also explain why the amount of symmetrical transcription, when assayed as trichloroacetic acid-precipitable radioactivity resistant to RNase after annealing, differs from one RNA preparation to another [3]. The

Table 1
Buoyant densities of $T_4$ nucleic acids in $Cs_2SO_4$ and KI

| | $Cs_2SO_4$ g/cm$^3$ | KI g/cm$^3$ |
|---|---|---|
| 20–30 min $T_4$ RNA | 1.693 | 1.625<br>1.588 (shoulder) |
| Double-stranded $T_4$ RNA | 1.613 | 1.571<br>1.557 (shoulder) |
| $T_4$ RNA–DNA hybrid | 1.485 | 1.552<br>1.526 (shoulder) |

buoyant density of the pulse-labelled $T_4$ RNA (1.693 g/cm$^3$) is high compared to other RNA of either viral or cellular origin [12] and as seen from fig.1A the density distribution is much more homogeneous than that for the double-stranded RNA.

### 3.2. *Centrifugation in alkaline iodide gradients*

The separations of nucleic acids in alkaline iodide solutions is different from that observed in $Cs_2SO_4$ [13,14]. For this reason it was of interest to analyze the double-stranded $T_4$ RNA in alkaline iodide solutions and to compare the buoyant density distribution with other nucleic acids. Isopycnic centrifugation of $T_4$ RNA in these media has not been reported. In the alkaline iodide media it appears that the buoyant density of both RNA and DNA is more dependent on the secondary structure than on the G+C content of the nucleic acids [14].

In NaI gradients (fig.2A–C) the density distribution of $T_4$ RNA, $T_4$ RNA–DNA hybrid as well as the double-stranded RNA covers nearly the whole density range. One possibility is that the heterogeneity is caused by RNA aggregates which form easily in the presence of divalent cations such as $Mg^{2+}$ [14]. Here, however, NaI of highest purity available was used, and in addition the solution was passed through Chelex-100 resin prior to use. It is thus unlikely that in these experiments divalent cations in the NaI solution were responsible for the observed heterogeneity. The reason for the heterogeneity of the RNA in NaI is unknown, but it may well be that it is caused by a combined effect of base composition and secondary structure. If this is the case, NaI gradients should provide a method useful for the separation of mRNA species. The density profiles of the same nucleic acids in KI are, however, not as complex as in

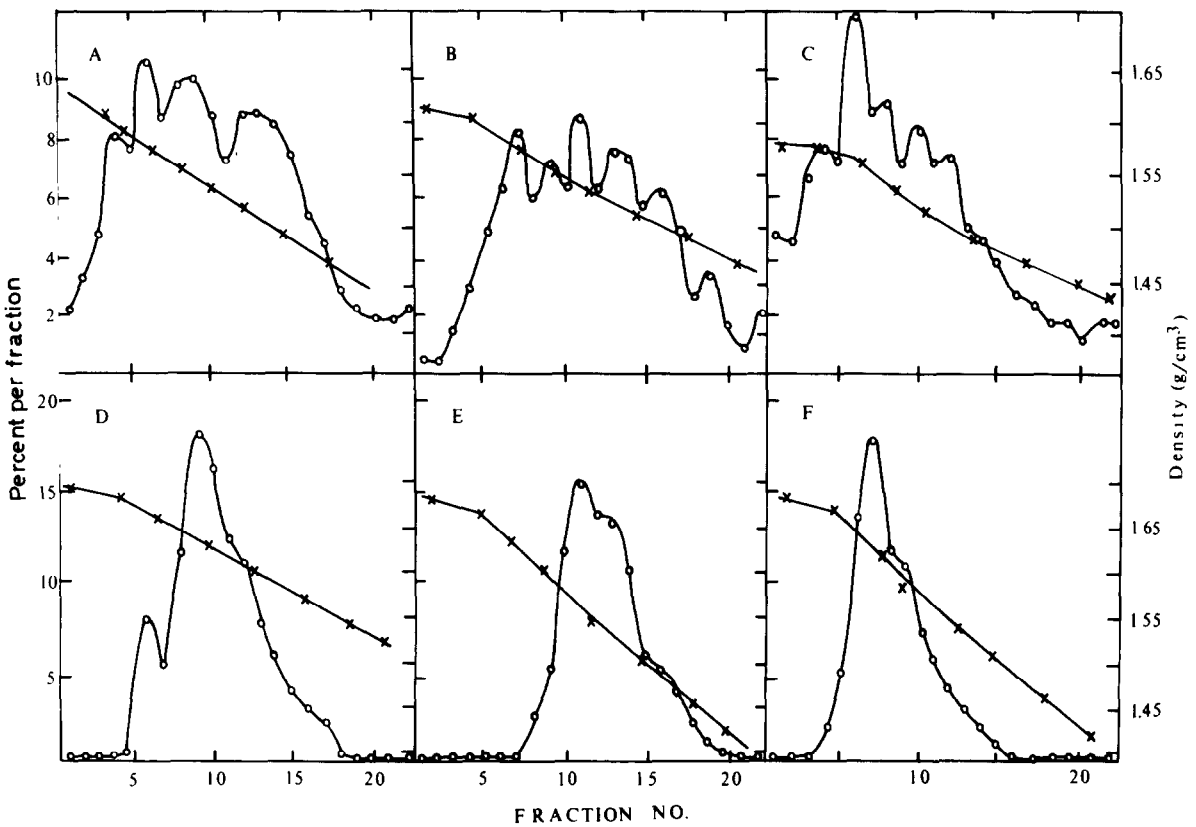


Fig.2. Isopycnic banding of late-labelled $T_4$ RNA, $T_4$ RNA–DNA hybrid and double-stranded $T_4$ RNA in NaI (A–C) and KI (D–F) gradients. $\rho_i$ (NaI) = 1.554 g/cm$^3$ and $\rho_i$ (KI) = 1.578 g/cm$^3$. The centrifugation was at 45 000 rev./min for 50 h at 20°C in the 498 rotor. (A,D) isolated double-stranded $T_4$ RNA, 3000 cpm; (B,E) $T_4$ RNA–DNA hybrid, 5000 cpm; (C,F) 20–30 min labelled $T_4$ RNA, 1 μg (50 000 cpm/μg).

NaI and for 3 different nucleic acid preparations shown in fig.2D–F, there appears to be 2–3 main bands. The double-stranded RNA (fig.2D) bands at 1.571 g/cm$^3$ which is in good agreement with the 1.57 g/cm$^3$ reported for double-stranded Reovirus in KI [13]. The small one-point peak at the left in this fig.2 is digested single-stranded RNA. The shoulder in fig.2D with a band density of 1.557 g/cm$^3$ may be an RNA–DNA hybrid since the $S_1$-treated hybrid bands at 1.552 g/cm$^3$ with two shoulders at lower densities (fig.2E). The late-labelled $T_4$ RNA gives one main band in KI at 1.625 g/cm$^3$ (fig.2F) corresponding well with the density of single-stranded yeast messenger- and ribosomal RNA in [13]. Since KI does not separate RNA according to the G+C-content [14], the shoulder in fig.2F at 1.588 g/cm$^3$ could be $T_4$ messenger RNA with a high degree of secondary structure. The presence of secondary structures in $T_4$ RNA is further supported by our studies on the chromatographic behavior of the same RNA on hydroxyapatite [10]. As expected, the density distribution profile of pulse-labelled early RNA was similar to that of pulse-labelled late RNA in the density media used here (not shown).

### 3.3. *Centrifugation of annealed early and late RNA*

When the mixture obtained after annealing late-labelled $T_4$ RNA with an excess of unlabelled early $T_4$ RNA, or early labelled $T_4$ RNA with an excess of unlabelled late $T_4$ RNA, was analyzed by density gradient centrifugation, without any prior nuclease digestion to remove single-stranded or non-complementary RNA, labelled RNA gave a density profile similar to that of the late-labelled RNA prior to annealing (cf. fig.1A and 3B,E). All the labelled RNA, however, banded at a lighter density after annealing than before, indicating a higher degree of secondary structure as a result of the annealing reaction. In $Cs_2SO_4$ an almost symmetrical peak banding at 1.65 g/cm$^3$ was obtained (fig.3B,E) and this is somewhat lighter than for the non-annealed labelled RNA (1.695 g/cm$^3$) (fig.1A). In a KI gradient the annealed labelled RNA banded at 1.595 g/cm$^3$ and, as with the non-annealed labelled RNA, it had a shoulder on the side of lower density making the results more complex to interpret (not shown). As expected, incubation of labelled RNA in the absence of unlabelled RNA did not change the density of the labelled RNA. Symmetrically transcribed RNA is always assayed for in the presence of a rather high concentration of unlabelled RNA [1]. As seen from fig.3 the unlabelled RNA which contributes most to the absorbance bands at the density obtained for single-stranded labelled RNA.

If the mixture, after the annealing, was incubated at 100°C for ~5 min to melt double-stranded regions prior to the isopycnic centrifugation, the labelled

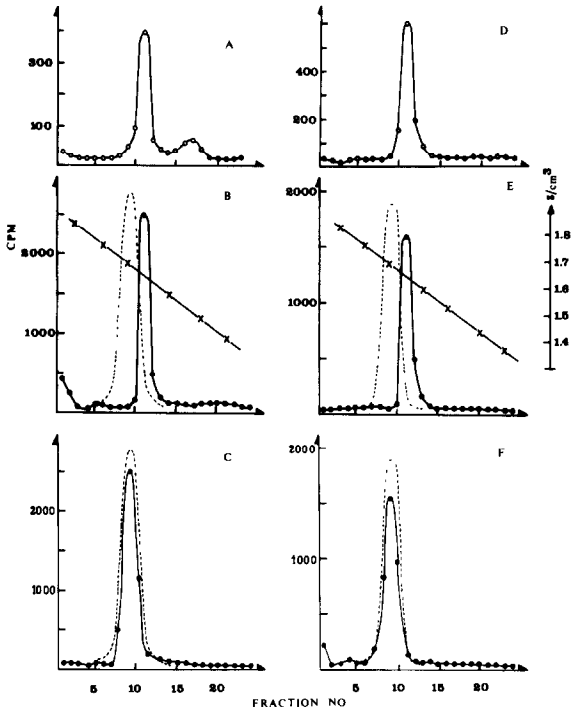


Fig.3. Isopycnic centrifugation of labelled $T_4$ RNA annealed to excess unlabelled $T_4$ RNA. (A–C) 5.3 μg 3–6 min labelled $T_4$ RNA was annealed to 1760 μg 20 min unlabelled RNA; (D–F) 5.0 μg 20–30 min labelled $T_4$ was annealed to 1845 μg 6 min unlabelled RNA. The total reaction volumes were 500 μl. Incubation was at 70°C in 0.5 M KCl, 10 mM Tris–HCl (pH 7.3 at 25°C) for 3 h. The reaction mixture (300 μl) was then mixed with $Cs_2SO_4$ (A,B,D,E) and the salt concentration was adjusted to give an initial density of 1.6500 g/cm$^3$; the final volume was 4.65 ml. 150 μl were heated to 100°C for ~5 min before being mixed with the $Cs_2SO_4$ (C,F). The gradients were centrifuged at 20°C for 58 h and 42 000 rev./min in a Beckman SW-50.1 rotor; they were harvested from the bottom and the $A_{254}$ was determined directly (– – –) using a Zeiss recording system. Each fraction in (A,B,D,E) (194 μl) was divided into two. One half was precipitated with 5% trichloroacetic acid onto glass filters (•——•) and the other half was diluted and treated with nucleases as in section 2 for the determination of double-stranded RNA (o——o). In (C,F) the fractions were precipitated with 5% trichloroacetic acid onto glass filters.

RNA banded at the same density as single-stranded (non-annealed) RNA (fig.3E,F), and the behaviour of the labelled and unlabelled RNA is similar. Since no peaks possessing the density of labelled single-stranded RNA were seen, it must be concluded that the total RNA population had a larger degree of secondary structure after annealing and furthermore that the complementary RNAs do not exist as single species, but are part of RNA molecules containing both complementary and non-complementary regions. All RNA molecules have more or less complementary regions forming double-stranded RNA upon annealing with excess unlabelled RNA, since there is no labelled RNA at the density of single-stranded RNA in these experiments. The suggestion that the complementary RNA represents only regions on the phage RNA molecules is further supported by the experiment in which the fractions from the experiment just described are treated with the nuclease mixture. As seen from fig.3A,D (open circles) the double-stranded RNA has the same distribution in the gradient as has the total labelled RNA after annealing. Similar data has been obtained by chromatography on hydroxyapatite and centrifugation on sucrose gradients [10]. If, however, fractions from the experiment in fig.3C,F were treated with the nuclease mixture, no resistance to the nucleases was seen.

## 4. Conclusion

The data obtained by isopycnic analyzes of the symmetrically transcribed $T_4$ RNA support the observations obtained by chromatography on hydroxyapatite and centrifugation on sucrose gradients [10], that most, if not all, $T_4$ RNA contain complementary regions. These findings explain why the polysomes contain the symmetrically transcribed RNA [4].

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