Ribonucleoprotein Particles Containing Heterogeneous Nuclear RNA in D.discideum^I

Ribonucleoprotein Particles Containing Heterogeneous Nuclear RNA in *D. discoideum*[1]

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キイロタマホコリカビのヘテロジニアス核RNA
を含むリボ核タンパク粒子

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SUMMARY

The nuclear pulse-labeled RNA in the cellular slime mold *Dictyostelium discoideum* exists as small-sized (10 to 50 S) ribonucleoprotein (RNP) particles in contrast to large-sized nuclear RNP particles in higher eukaryotes. These small-sized RNP particles are not artificial products due to a ribonuclease digestion of large particles, nor ribosomal and its precursor particles. It seems certain that the pulse-labeled RNA contained in the nuclear RNP is heterogeneous nuclear RNA, because it was heterogeneous in size and resistant to actinomycin D and because it exhibited the same sedimentation pattern as that of poly(A)-containing nuclear RNA. The proteins included in the RNP particles are composed of several major proteins and heterogeneous minor ones.

In mammalian nuclei there exist ribonucleoprotein (RNP) particles with sedimentation coefficients of 40 S to 250 S (1). The RNP particles contain the so-called heterogeneous nuclear RNA (HnRNA) which is supposed to be the precursor to cytoplasmic mRNA, although almost all of HnRNA degrades within the nuclei. It has been also demonstrated that HnRNA in mammalian cells is fairly large in size, as compared with cytoplasmic mRNA. In contrast, some of lower eukaryotes do not have such large-sized HnRNA as found in mammalian cells (2-4). In fact, HnRNA in the cellular slime mold *Dictyostelium discoideum* has been reported to be only 20 % larger on the average than cytoplasmic mRNA (2).

Then, a question arose as to whether or not such small HnRNA in this organism exists as RNP particles. In this paper, it will be reported that *D. discoideum* possesses nuclear RNP particles containing HnRNA, although they are small in size (10 S to 50 S) relative to those in higher eukaryotes.

The amoeba cells of *D. discoideum* NC-4 which were grown as reported previously (5) were
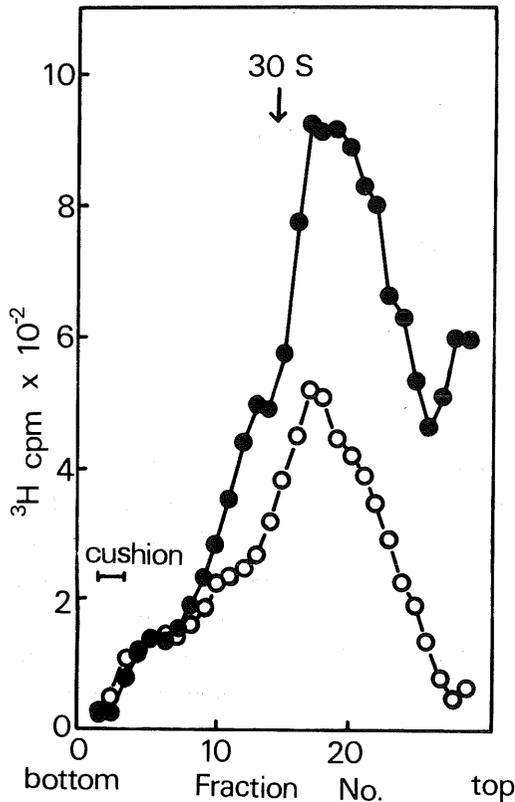

Fig. 1. Sedimentation analysis of the nuclear extracts prepared at pH 7.0 (a) and pH 8.0 (b) after labeling of cells with [^{3}H]uracil for 30 min at the early aggregation stage during morphogenesis. Amoeba cells grown by using dead *Escherichia coli* cells as nutrients (5) were harvested in a late log-phase of growth, washed several times by centrifugation with TKM buffer (15 mM Tris-HCl (pH 7.6), 20 mM KCl and 5 mM MgCl$_2$) and suspended in the same buffer containing streptomycin sulfate (150 μg/ml) at a cell density of 3 x 10^8/ml. In order to obtain a high degree of synchrony of morphogenesis, definite volumes of the cell suspension were dispensed on non-nutrient agar plates containing streptomycin (150 μg/ml), followed by incubation at 23°C. The cells in the early aggregation stage of morphogenesis (5 to 6 h after the onset of incubation) were treated with 120 μg/ml of actinomycin D for 1 h and then labeled with 500 μCi/ml of [^{3}H]uracil for 30 min in the presence of the drug. After labeling, cells were rapidly chilled and washed with ice-cold TKM buffer by centrifugation. The preparation of the nuclei, as described below, was carried out at 0°–4°C. The labeled cells were disrupted in a Potter-type homogenizer at a concentration of 2 x 10^8 cells/ml in 5 % sucrose containing 0.2 % Triton X-100, 3 mM magnesium acetate and 10 μg/ml polyvinylsulfate. After 10 min incubation, an equal volume of 22 % sucrose containing 3 mM magnesium acetate and 10 μg/ml polyvinylsulfate was added to the cell homogenate. The nuclei were pelleted by centrifugation at 1,000 x g for 10 min and washed twice with 13.5% sucrose containing 0.1% Triton X-100, 3 mM magnesium acetate and 10 μg/ml polyvinylsulfate. The crude nuclei were further purified through a solution of 1.95 M sucrose, 3mM magnesium acetate and 10 μg/ml polyvinylsulfate by centrifugation at 17,000 rpm for 45 min at 4°C in an SW 25.1 rotor of a Spinco L ultracentrifuge. The purified nuclei were suspended in TNM buffer (pH 7.0) containing 10 μg/ml polyvinylsulfate, stirred for 10 min at 4°C and centrifuged at 7,000 x g for 10 min. The resultant supernatant fluid was termed pH 7.0 nuclear extract, and the residual pellet was resuspended in TNM buffer (pH 8.0), stirred for 30 min at 4°C and centrifuged at 10,000 x g for 10 min to remove nucleoli and nuclear debris. The supernatant fluid so obtained was named pH 8.0 nuclear extract. The pH 7.0 or 8.0 nuclear extract was mixed with a trace amount of ^{14}C-labeled 30 S ribosomal subunits of *E. coli* Q13 as a reference to determine the sedimentation coefficient and then centrifuged using a Hitachi RPS 27 rotor at 26,000 rev./min for 14 h at 4°C in a 10–25% (w/v) linear sucrose gradient containing 2 M sucrose cushion in the bottom which was made with TNM (pH 8.0) containing 10 μg/ml polyvinylsulfate. After centrifugation, an appropriate number of fractions of the gradient were taken, an aliquot (0.05 ml) of each fraction was placed on glass-fiber filters (Whatman GF/C) and the radioactivity was measured in a Beckman liquid scintillation spectrometer as described previously (7). ○– –○, pH 7.0 nuclear extract; ●——●, pH 8.0 nuclear extract.
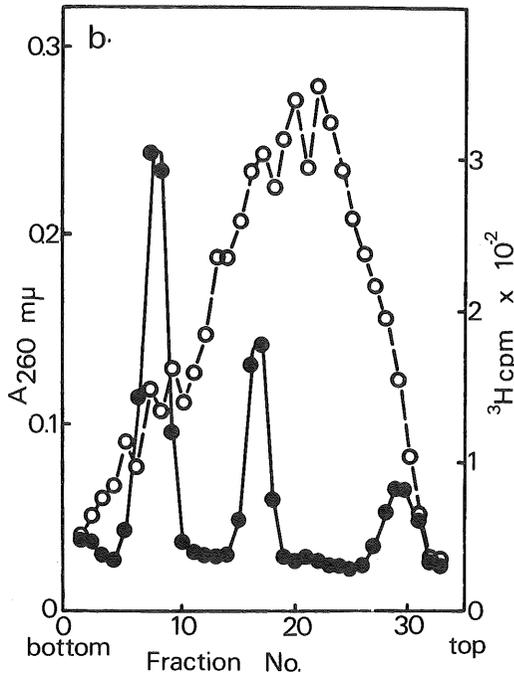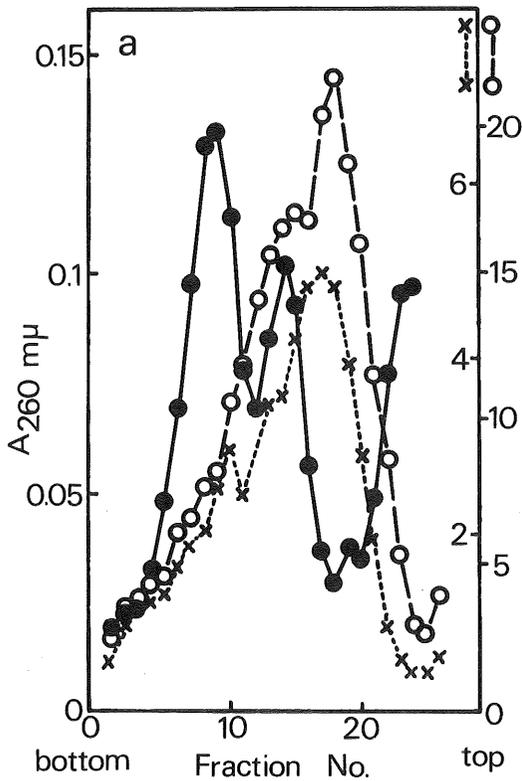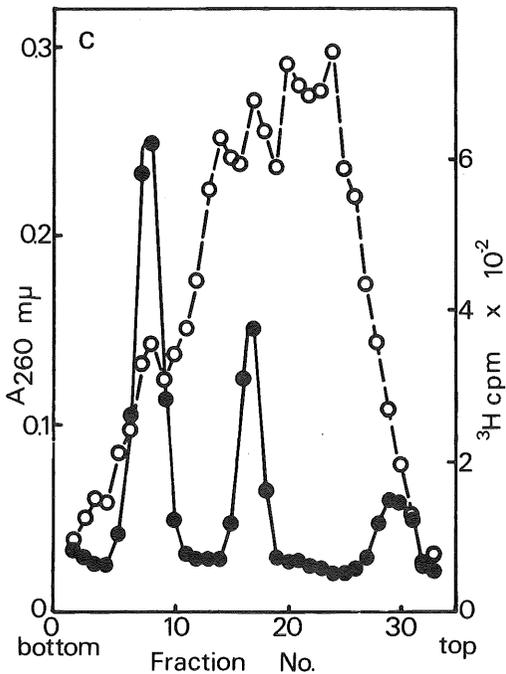
Fig. 2. Sedimentation analysis of RNA obtained from crude nuclei, the pH 7.0 and 8.0 nuclear extracts. The cells were labeled with [³H]uracil for 30 min in the presence of 120 μg/ml actinomycin D as described in the legend of Fig. 1.

a; RNA from crude nuclei. The crude nuclei were prepared from cell homogenate by centrifugation at 1,000 x g for 10 min for several times. They were suspended in 25 mM Tris-HCl (pH 7.6) containing 5 mM sodium EDTA and 2.6% sodium dodecylsulfate, followed by a treatment with 1% diethylpyrocarbonate and RNA was then extracted with a solution of phenol-chloroform-isoamylalcohol (49:49:2) (2) and precipitated in 95% cold ethanol. The RNA sample was dissolved in a solution consisting of 10 mM Tris-HCl (pH 7.6), 0.1 M NaCl and 0.05% sodium dodecylsulfate, mixed with unlabeled 26 S and 17 S rRNA's of *D. discoideum* as markers and analysed on 4.8 ml of a 15—30% (w/v) linear sucrose gradient in the above solution by centrifugation for 2 h at 48,000 rev./ min and 22°C in a Spinco SW 50 rotor. After centrifugation, an appropriate number of fractions of the gradient were taken and one-half portion of each fraction was measured for absorbance at 260 mμ and radioactivity. For the assay of poly(A)-containing RNA, the remaining half portion was adjusted at the final concentration to 10 mM Tris-HCl (pH 7.6), 0.12 M NaCl, 0.1 mM sodium

EDTA and 0.5% sodium dodecylsulfate and passed through poly(U)-filters prepared according to the procedure described by Sheldon et al.(8). The filters were then washed with the same buffer followed by washing with 0.3 M ammonium acetate in 50% ethanol, dried and counted as described by Firtel et al.(9).

  b and c; RNA from the pH 7.0 (b) and 8.0 (c) nuclear extract. The extraction of RNA of the pH 7.0 and 8.0 nuclear extract was performed by the sodium dodecylsulfate-phenol procedure as described previously (7). The RNA prepared from pH 7.0 and 8.0 nuclear extract was mixed with unlabeled 26 S and 17 S rRNA's of D. discoideum and centrifuged in 10−25% (w/v) linear sucrose gradients in 20 mM Tris-HCl (pH 7.6) containing 0.1 M NaCl, 2 mM sodium EDTA and 20 μg/ml polyvinylsulfate at 26,000 rev./min and 4°C for 15 h using a Hitachi RPS 27 rotor. After centrifugation, the gradient was fractionated, water was added to each fraction, and absorbance was measured at 260 mμ. The RNA was precipitated in 10% cold trichloroacetic acid containing 5% acetone. The precipitated material was collected on a glass-fiber filter (Whatman GF/C) and the radioactivity was measured as described previously (7).

  ●——●, absorbance at 260 mμ ; ○−−○, ^{3}H-radioactivity of RNA precipitated with trichloroacetic acid; ✕ ·····✕, ^{3}H-radioactivity of RNA bound to poly(U)-filters.

labeled pulsely with (^{3}H)uracil at an early aggregation stage during morphogenesis, following a pretreatment with actinomycin D (120 μg/ml) for 1 h. Since it is known that in this organism the rate of synthesis of rRNA decreased remarkably in the early stage of morphogenesis and the actinomycin D concentration used here selectively inhibits rRNA synthesis, it should be noticed that no precursor molecules of rRNA were synthesized under the above conditions for labeling. After the labeling of cells, the nuclei were isolated as described in the legend of Fig. 1 and nuclear extracts were then prepared from intact nuclei with TNM buffer (10 mM Tris-HCl, 0.1 M NaCl and 1 mM magnesium acetate) under two different pH conditions (pH 7.0 and 8.0) according to the procedure of Georgiev and Samarina (6). Preliminary experiments indicated that when the nuclear extract was prepared at pH 8.0, approximately 34 % of ^{3}H-labeled materials in nuclei were extracted, while the extraction efficiency at pH 7.0 was very low (about 5 %).

  Figure 1 shows the radioactivity profiles after a sucrose gradient centrifugation of nuclear extracts prepared at pH 7.0 and 8.0 from labeled cells. It can be seen that labeled materials sedimented heterodispersedly with a broad peak in the 20 S region. The sum of the radioactivity of the labeled material sedimenting in the region from 10 S to 50 S comprises approximately 88 % and 80 % of the total radioactivity in the pH 7.0 and 8.0 nuclear extracts, respectively. There were few labeled materials sedimenting faster than 50 S in both nuclear extracts.

  To know if small particles containing pulse-labeled RNA found in this experiment result from the partial degradation of large particles by ribonuclease digestion during the process of isolation of nuclei or if they are intrinsical entities in this organism, RNA prepared from crude nuclei, the pH 7.0 and pH 8.0 nuclear extracts of labeled cells were examined by sucrose gradient centrifugation. In this experiment, RNA obtained directly from the crude nuclei is expected to be larger in size than that from the pH 7.0 or pH 8.0 nuclear extract, if large particles containing labeled RNA really exist in the nuclei of this organism but are degraded during the course of the purification of nuclei or the extraction of labeled materials from nuclei. Figure 2 shows that no significant difference with the sedimentation profile of labeled RNA was observed between the RNA's of three different sources; almost all of the labeled RNA was heterodispersedly distributed from about 5 S to 30 S with a broad peak in the 15 S region, although the amount of labeled RNA sedimenting in the region heavier than 15 S was not so much in the RNA sample from the crude

nuclei relative to that from either pH 7.0 or pH 8.0 nuclear extract. This result suggests that the small nuclear particles containing pulse-labeled RNA inherently exist in this organism, but are not artificial particles due to the ribonuclease digestion.

It is also clear in Figs. 2b and 2c that the pH 7.0 or pH 8.0 nuclear extract does not contain [³H] labeled ribosomal precursor particles, because the actinomycin-resistant RNA prepared from both nuclear extracts is small and considerably heterogeneous in size in contrast to rRNA or its precursors. Then, are the small nuclear particles obtained here HnRNA? To elucidate this point, the sedimentation pattern of polyadenylic acid (poly(A))-containing RNA in the RNA sample prepared from crude nuclei was examined by the binding assay of poly(A)-containing RNA to polyuridylic acid (poly(U))-filters (poly(U) attached to glass-fiber filters), since in *D. discoideum* as well as in other eukaryotes HnRNA has at least one sequence of poly(A) at the 3'-end (2). As shown in Fig. 2a, the sedimentation pattern of poly(U)-binding RNA closely resembles that of actinomycin D-resistant RNA from the nuclei. Similar results were obtained also with the pulsely labeled RNA contained in nuclear particles prepared at pH 7.0 or pH 8.0 (data not shown). Thus, the above data clearly indicate that the pulsely labeled RNA in nuclear particles contains HnRNA.

Figure 3 shows the electrophoretic patterns of proteins extracted from the nuclear particles containing HnRNA in sodium dodecylsulfate polyacrylamide gels. When proteins were prepared from the pooled fractions containing 15 S to 20 S nuclear particles, they were separated into several major bands in addition to heterogeneous minor ones. The molecular weights of reproducible major protein bands were estimated to be about 42,000 and 60,000 daltons. These proteins are surely different from ribosomal (10) and chromatin (11) proteins of *D. discoideum,* and also
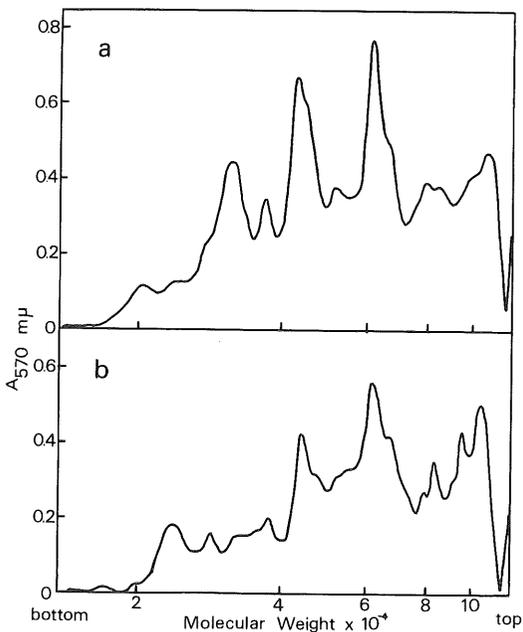

Fig. 3. Densitometric tracings of stained gels after electrophoresis of proteins from the nuclear RNP particles of *D. discoideum* on sodium dodecylsulfate-polyacrylamide gels. The pH 7.0 and 8.0 nuclear extracts were centrifuged in a sucrose gradient as described in Fig. 1. After centrifugation, the fractions corresponding to 15—20 S were pooled and 0.1 volume of 100% (w/v) cold trichloroacetic acid was added. The precipitated RNP particles were collected by centrifugation at 12,000 x g for 20 min at 0°C. The pellet was dried and then dissolved in 0.1ml of 0.1 M sodium phosphate (pH 7.0) containing 1% sodium dodecylsulfate, 1% 2-mercaptoethanol and 4 M urea. It was incubated for 3 h at 37°C and then electrophoresed on 10% polyacrylamide gels containing 1% sodium dodecylsulfate for 12 h at a constant current of 5 mA/gel. For the determination of the molecular weights of proteins, several marker proteins were parallely run in separate gels. After electrophoresis, the gels were stained with coomassie blue, destained with 7% acetic acid and densitometrically scanned at 570 mμ as described previously (10).
a, proteins of RNP extracted at pH 7.0 ; b, proteins of RNP extracted at pH 8.0.

different from cytoplasmic total and nuclear soluble proteins with respect to their molecular weights (unpublished data).

The results obtained in this study suggest that in *D. discoideum* HnRNA exists as comparatively small-sized (10 S to 50 S) RNP particles. Very recently, however, Firtel and Pederson (11) have reported 55 S nuclear RNP particle containing mRNA precursor in the same organism. They have obtained the RNP particles from cells in the log-phase of growth in which rRNA synthesis is much more active than in the morphogenetic phase. It is not clear that the difference between their data and the data in this paper is due to the difference of the method used for the preparation of RNP particles or to the difference of the developmental stage during the life cycle of this organism. Further experiments are now in progress to clarify the cause for the difference.

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