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In Vitro Nuclear Transport of Ribosomal Ribonucleoprotein: Temperature Affects Quantity but Not Quality of Exported Particles

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The in vitro export of ribosomal ribonucleoprotein (rRNP) from *Tetrahymena* nuclei was investigated at the optimal growth temperature of 28°C and at the nonlethal temperature of 8°C. At both temperatures, nuclei exported ribosomal precursor particles that revealed the same physical qualities of size, appearance in negative-staining electron microscopy, sedimentation coefficient, buoyant density, and rRNA pattern. Surprisingly, fewer rRNP particles were exported at 8 than at 28°C, as was revealed by a lower saturation plateau in the export kinetics from nuclei prelabeled with [^{3}H]uridine. Upon a temperature increase from 8 to 28°C, additional rRNP particles were exported. We conclude that nuclei export only a defined portion of rRNP particles at a given temperature, although enough potentially transportable rRNP particles are present in nuclei. Obviously, the reactivity of at least one of the reactants involved directly or indirectly in rRNP export changes with temperature.

In eucaryotes the transport of the different species of ribonucleoprotein (RNP) from nucleus to cytoplasm is an important posttranscriptional event, poorly understood to date (for recent reviews, see 28, 36, 37). One of the most enigmatic phenomena is the long-known fact that much more RNP is synthesized in nuclei than eventually appears in the cytoplasm. Cells obviously possess selective control mechanisms which sort out RNP to be exported to the cytoplasm from RNP remaining in the nucleus. These sorting mechanisms seem to be perturbed in cancer cells (e.g., 1, 4, 8, 24, 30, 32, 34).

For a long time, temperature was used to get more insight into the mechanisms underlying RNP transport. As early as 1965, Bier (6) showed that in the ovaries of *Musca* sp., quick cooling dramatically reduces the overall RNP transport, whereas overall RNA synthesis continues, albeit at lower rates, thus causing an accumulation of RNP in the nuclei. Similarly, a decrease in temperature induces a decrease in the nucleocytoplasmic overall RNP transport in whole cells of the unicellular eucaryote *Tetrahymena pyriformis* (22, 38). This cannot be explained as a reduction of the overall RNA transcription; rather, temperature influences a posttranscriptional event. The disadvantage of such in vivo studies, however, is that they do not resolve whether temperature affects the RNP transport indirectly (e.g., via RNA processing or possible nucleocytoplasmic feedback mechanisms) or directly (i.e., by affecting the virtual transport of already processed RNP particles).

The virtual processes of RNP transport can be approached only in vitro, i.e., in isolated nuclei. Recently, we also developed a new cell-free system with *T. pyriformis* (15). This system operates with nuclei which can be induced to export ribosomal precursor particles, equivalent to the in situ situation (18, 19), without any detectable rRNA processing. Using these nuclei, we here address the question of the effect of temperature on the export rate, the quantity, and the quality of the exported particles.

MATERIALS AND METHODS

Buffers. The buffers used in this study were as follows. PPY: 0.75% Difco Proteose Peptone no. 3 (Difco Laboratories, Detroit, Mich.), 0.75% yeast extract (Difco), 1.5% glucose (Roth, Karlsruhe, Federal Republic of Germany), 1 mM $MgSO_4$, 0.06 mM $CaCl_2$, 0.1 mM ferric citrate. HRM: 0.2 M sucrose, 0.56 mM $CaCl_2$, 0.37 mM $MgCl_2$, 20 mM Tris-hydrochloride (pH 7.4). RM: 0.2 M sucrose, 0.75 mM $CaCl_2$, 0.5 mM $MgCl_2$, 20 mM Tris-hydrochloride (pH 7.4). SA: 0.5% sodium dodecyl sulfate, 5 μg of polyvinyl sulfate per ml, 10 mM sodium acetate (pH 5.0). SM: 0.2 M sucrose, 8.4 mM $CaCl_2$, 5.6 mM $MgCl_2$, 20 mM Tris-hydrochloride (pH 7.4). ST: 0.2 M sucrose, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 20 mM Tris-hydrochloride (pH 7.4). TK: 0.9 mM $CaCl_2$, 0.6 mM $MgCl_2$, 50 mM KCl, 20 mM triethanolamine hydrochloride (pH 7.4).

Cultures and labeling with [^{3}H]uridine. Axenic 31 cultures of the ciliate protozoan *T. pyriformis* (amicronucleate strain GL) were grown in the logarithmic

growth phase (15,000 to 50,000 cells per ml) at 28°C in PPY medium containing a few drops of antifoam emulsion. These cultures were labeled at 28°C with 1 to 2 mCi of [5,6-^{3}H]uridine (specific activity, 50 Ci/mmol; NEN Chemicals, Frankfurt, Federal Republic of Germany). After 30 to 60 min, about 700 g of crushed ice was added to the culture, and the cells were harvested by centrifugation at 3,000 rpm and about 4°C for 10 min (4 by 1-liter SB rotor, Christ cryofuge, Heraeus Christ GmbH, Osterode, Federal Republic of Germany). The cells were next recentrifuged at 3,500 rpm for 5 min (4 by 100-ml SB rotor, Christ junior centrifuge).

Isolation of nuclei. After the cells were disrupted with glycerol (11, 40), nuclei were isolated and purified on two-step sucrose gradients by our previous method (41) with the modification described by Herlan et al. (15).

In vitro assay for ^{3}H-labeled RNP export. The conditions of RNP export were recently described in detail (15). In brief, nuclei were prelabeled with [^{3}H]uridine, suspended in ST buffer, kept at 0 to 4°C, and pipetted into the export media RM and HRM, respectively, pre-equilibrated at the respective temperatures. Under these conditions, the final concentrations of Ca^{2+}-Mg^{2+} (3:2) were lowered from 5 mM to 1.5 and 1.2 mM, respectively. RNP export was stopped by adding ice-cold SM buffer, i.e., by raising the bivalent ion concentration again to 5 mM. After centrifugation of the samples, the radioactivity in the nuclear pellets and the corresponding supernatants was counted. The RNP export was expressed as the trichloroacetic acid-precipitable radioactivity in supernatants taken as a percentage of the total trichloroacetic acid-precipitable radioactivity in the sample. The same procedure was also performed at time zero, i.e., nuclei were pipetted into RM and HRM media at 0 to 4°C, and these suspensions were immediately made 5 mM again with respect to the concentration of Ca^{2+}-Mg^{2+} (3:2). The original values were plotted on the curves without any background subtraction.

RNP export from unlabeled nuclei. Given fractions of unlabeled nuclei were pipetted into 12 ml of RM buffer at 28 or 8°C. After 10 min, the samples were cooled to 0 to 4°C, and nuclei were removed by centrifuging at 800 × *g* for 7 min and recentrifuging the supernatant at 2,200 × *g* for 15 min. The supernatants were further processed as described below.

Density gradient centrifugation. Supernatants either were fixed with 6% formaldehyde (26) or remained unfixed before they were dialyzed against TK buffer overnight and concentrated to 0.5 to 1.0 ml by polyethylene glycol. For rate zonal density gradient centrifugation, fixed or unfixed samples were layered over 27 ml of continuous 10 to 40% (wt/wt) sucrose gradients buffered with TK and were centrifuged at 21,000 rpm for 15 h (RPS 25 swinging-bucket rotor, Hitachi ultracentrifuge). For rapid equilibrium density gradient centrifugation, fixed samples were layered over step CsCl-gradients (7) and centrifuged at 38,000 rpm for 18 h (RP 65 rotor, Hitachi ultracentrifuge). Absorbance at 260 nm was continuously recorded in a flow cuvette. The refractive index was measured with a Zeiss refractometer. Sedimentation coefficients (23) and buoyant densities (17) were determined as described previously (15).

Extraction and separation of RNA. Supernatants were mixed with 1% sodium dodecyl sulfate and 0.1 M NaCl (final concentrations) before adding 2 volumes of ethanol. The samples were stored at −20°C overnight. RNA extraction with proteinase K and phenol was performed as detailed previously (15). RNA was separated by 2.2% polyacrylamide gel electrophoresis by the method of Loening (20), and the cylindrical gels were continuously scanned at 260 nm as described previously (15).

Electron microscopy. Negative staining was performed by the method of Valentine et al. (35). Carbon films made by evaporation of carbon onto freshly cleaved mica sheets were floated on the particle suspension for 1 min, then transferred to distilled water and finally to 1% uranyl acetate for 1 min and dried. The specimens were observed in a Siemens Elmiskop 101.

RESULTS

Quantity of exported RNP. Nuclei isolated from *T. pyriformis* GL can be induced to export RNP by lowering the final concentration of Ca^{2+}-Mg^{2+} (3:2) from 5 to 1.5 mM (15). Lowering the concentration to 1.2 mM even induces the export of additional RNP in a given nuclear fraction. This export can be quantitatively followed in nuclei prelabeled with [^{3}H]uridine. Immediately after the bivalent ion concentration was lowered, the nuclei released radioactivity (Fig. 1). This release was linear, at least during the first 3 min, and reached saturation at 28°C after 30 to 60 min, depending on the nuclear fraction used. The maximum level did not decline at 28°C for 3 h. This indicates that the exported RNP is not degraded by possible endogenous RNases. At 8°C, however, nuclei from the same nuclear fraction exported less RNP: both the initial rate and the saturation plateau decreased significantly. The plateau did not equilibrate with that at 28°C, even after 3 h (Fig. 1). Obviously, nuclei export maximally only a defined portion of RNP at a given temperature.

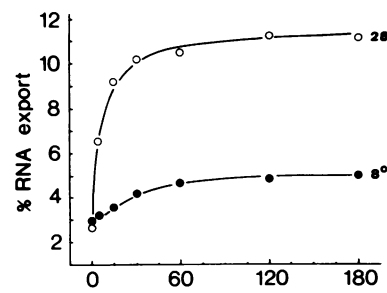

FIG. 1. Kinetics of RNP export from *Tetrahymena* nuclei prelabeled with [^{3}H]uridine at a final Ca^{2+}-Mg^{2+} (3:2) concentration of 1.5 mM at 28 and 8°C. The saturation plateaus do not equilibrate even after 3 h.

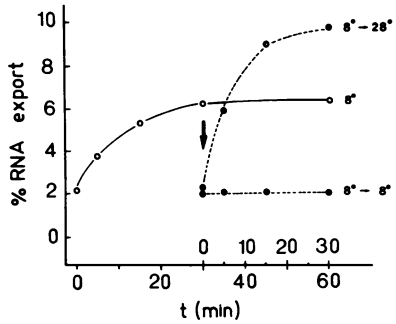

FIG. 2. RNP export kinetics at 8°C. After 30 min, export was stopped in one sample (arrow) which was subdivided in half; export was induced again at 8 and 28°C.

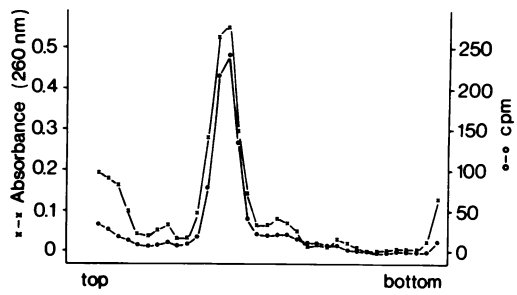

FIG. 4. Sedimentation profile of total unfixed material exported from *Tetrahymena* nuclei prelabeled with [^{3}H]uridine in continuous 10 to 40% sucrose gradients.

This finding could indicate that nuclei become inactivated at low temperature or that there is a temperature-dependent equilibrium between nuclear RNP and RNP exported from nuclei. To test this possibility, RNP export was induced at 8°C. After 30 min, export was stopped in a sample which was then halved. In one half, export was induced again at 8°C, but nuclei did not export RNP in comparison with the corresponding 8°C control (Fig. 2). In the other half, export was induced at 28°C, and these nuclei exported an additional portion of RNP (Fig. 2). Obviously, nuclei export only a defined quantity of RNP at 8°C, even if they contain enough potentially transportable RNP.

To examine the dependence of the initial rate of export on the maximum export at a given temperature, we induced RNP export at 28 and 8°C in nuclei from the same fraction. After 5 min, the export was stopped and reinduced again at the same temperatures. Figure 3 shows the corresponding export kinetics. As expected, the preincubated nuclei had lower export plateaus at both temperatures. Also, the initial rates were significantly decreased. This means that the initial export rate is directly proportional to the maximum export at a given temperature.

Quality of exported RNP. Figure 4 shows that the radioactivity released from nuclei at 28°C for 30 min under export conditions could be almost completely recovered in one fraction sedimenting at approximately 60S in 10 to 40% sucrose gradients. Negative-staining electron microscopy reveals that the 60S fraction contained particles about 27 nm in diameter (Fig. 5). Besides the prominent 60S fraction, three other minor

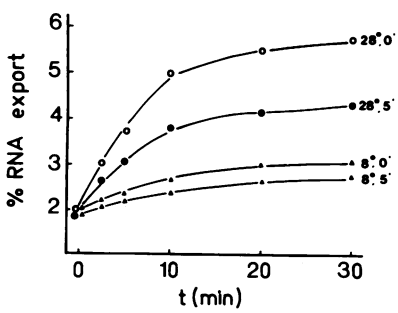

FIG. 3. RNP export kinetics at 28 and 8°C after preincubation of the nuclei under export conditions for 0 or 5 min.

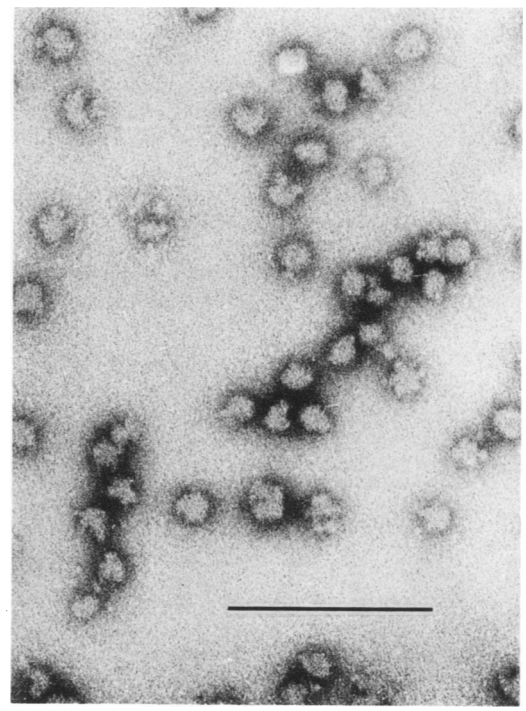

FIG. 5. Negative-staining electron microscopy of the 60S fraction recovered from 10 to 40% sucrose gradients. Magnification ×135,000. Bar, 0.2 μm.

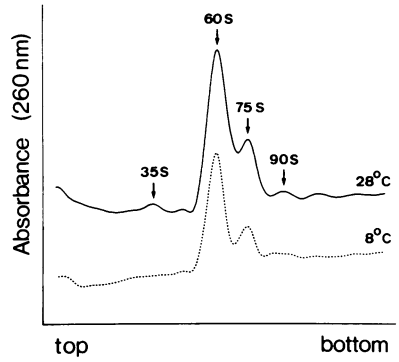

FIG. 6. Sedimentation profile (10 to 40% sucrose gradients) of total formaldehyde-fixed material exported from nuclei at 28 and 8°C.

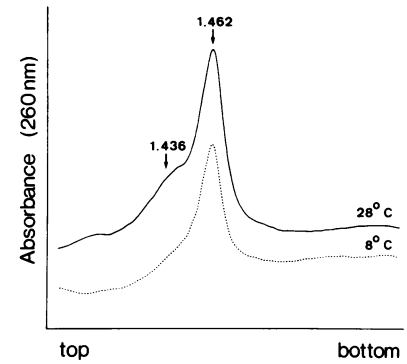

FIG. 8. Sedimentation profile of total formaldehyde-fixed material exported from nuclei at 28 and 8°C in CsCl gradients.

fractions sedimented at 75, 90, and 35S (Fig. 6). The sedimentation coefficients varied slightly from experiment to experiment. Figure 7 shows the RNA pattern of the exported material in 2.2% polyacrylamide gel electrophoretograms. Three fractions could be identified, corresponding to the pre-26S rRNA (M_r, 1.34×10^6), the 26S rRNA (M_r, 1.27×10^6), and the 17S rRNA (M_r, 0.66×10^6). In isopycnic CsCl gradients, the exported material was always separated in at least two fractions. The major fraction had a buoyant density of approximately 1.46 g/cm^3 (Fig. 8). These densities were much lower than those of ribosomes and ribosomal subunits isolated from the cytoplasm. Indeed, we have previously shown that the buoyant densities of the 70S monosomes and the 50 and 30S ribosomal subunits are 1.563, 1.580, and 1.556 g/cm^3, respectively (15). Thus, nuclei export ribosomal precursor particles rather than ribosomal subunits.

At 8°C, nuclei also exported ribosomal precursor particles. Negative staining electron microscopy did not reveal any significant change in the size and appearance of the particles in comparison with the particles exported at 28°C from nuclei. Moreover, sedimentation coefficients, rRNA patterns, and buoyant densities did not significantly change (Fig. 6–8).

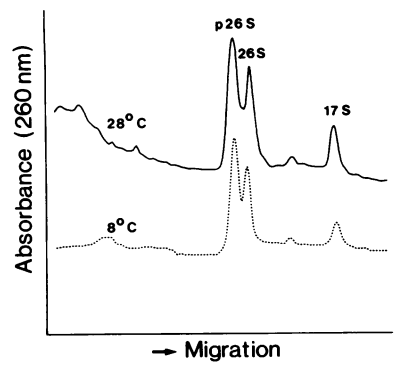

FIG. 7. Electrophoretograms in 2.2% polyacrylamide gels of the RNA extracted from total material exported from nuclei at different temperatures.

DISCUSSION

In recent years, several groups have analyzed the temperature dependence of RNP transport in different cell-free systems. Interest has been focused for the most part only on the export rates of overall RNP (e.g., 2, 9, 14, 25, 29, 33). The most intense study so far was performed by Clawson and Smuckler (9). They found that the ATP-driven release of overall RNP from rat liver nuclei proceeds much more slowly at low temperature than at higher temperature, but the maximum release was the same at the different temperatures. The conclusiveness of this study, however, remains limited since this in vitro system contains endogenous RNase activity, causing a decline in the percentage of exported RNP after maximum export. Our present data indicate that in vitro ribosomal RNP (rRNP) export from *Tetrahymena* nuclei obviously does not interfere with active RNases. In addition, this system allows the effect of temperature on RNP transport to be specified in more detail, i.e., it allows a distinction between the quantity and the quality of the RNP species exported.

Tetrahymena nuclei export ribosomal precursor particles in vitro (15). In addition, in situ ribosomal precursor particles are transported from nucleus to cytoplasm, where they mature to become functionally active ribosomal subunits (e.g., 18, 19, 27). A definite portion of the large ribosomal precursor particles exported in vitro contains p-26S rRNA. Whether this also holds true in situ is not yet known. In this context, it is noteworthy that the final process-

ing of the large rRNA species was previously reported to occur in the cytoplasm. First a small 5.8 or 7S rRNA segment is split off, and then a central hidden break is introduced into this large rRNA species (10; see, however, 16). Our data show that the particles exported from nuclei at 28 and 8°C appear to be alike, at least in size and appearance in negative-staining electron microscopy, sedimentation coefficient, buoyant density and rRNA pattern. At 8°C, however, fewer rRNP particles are exported from nuclei than at 28°C. This is puzzling since a good number of potentially transportable rRNP particles are present in nuclei at 8°C and can be exported upon raising the temperature to 28°C. This finding excludes the possibility that the nuclear capability of exporting rRNP particles becomes irreversibly inactivated at low temperatures, owing, for example, to the loss of a factor necessary for export. Thus, there obviously exists a mechanism inherent in nuclei determining the maximum number of rRNP particles that can be exported at a given temperature.

The temperature dependence of the quantity of the exported rRNP particles is important for several reasons. (i) It indicates that the in vitro export of rRNP particles from *Tetrahymena* nuclei is not driven by leakage; for thermodynamic reasons, leakage would always result in an equilibration of the maximum export levels at different temperatures. (ii) It indicates a temperature-dependent change in the reactivity of at least one of the reactants involved directly or indirectly in rRNP export (see also 21, 31); this change in reactivity could be due (for instance) to alterations in the nuclear rRNP particles or in possible transport sites or in the interactions of rRNP particles with transport sites or in nuclear structural elements such as the nuclear matrix, possibly mediating the intranuclear translocation of the rRNP particles (for reviews, see 3, 5, 37, 39). (iii) It indicates that the effect of temperature on the export rate cannot be unequivocally evaluated; the observed direct proportionality between the initial export rate and the maximal portion of exported particles makes the initial rate invalid as a measure for the export rate. Only by assuming that the export follows first-order reaction kinetics could we evaluate export rates as rate constants in terms of initial rate normalized to the corresponding maximum export.

Finally, it is noteworthy that nuclei isolated from *Tetrahymena* are structurally intact at the transport level, i.e., they are surrounded by a well-preserved nuclear envelope still revealing typical in situ properties under export conditions (11, 40; see also 12). Therefore, it is an attractive speculation that the nuclear envelope represents the temperature-dependent barrier for the export of rRNP particles that are "free" in nuclei. The pore complexes, for example, could control the number of rRNP particles which can be maximally exported from nuclei. In contrast, however, our data are also compatible with the view that the potentially transportable rRNP particles are not free inside nuclei but rather are somehow bound—for example, to the nuclear matrix (13). For instance, there could be different pools of bound nuclear rRNP particles, of which some are releasable at 8°C and others at 28°C. We are currently testing these possibilities.

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