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Effect of Temperature on Nuclear Membranes and Nucleo-Cytoplasmic RNA-Transport in *Tetrahymena* Grown at Different Temperatures

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Summary. The effect of temperature on the nuclear envelope structure and the transport of total RNA and ribosomal subunits from nucleus to cytoplasm was examined in *Tetrahymena* cells propagated at two different temperatures. Freeze-etch electron microscopy of cells grown at 23 and 18 °C detects the emergence of smooth areas on the fracture faces of the nuclear membranes upon lowering the temperature below ~15 and ~12 °C, respectively. Coincident with these freeze-etch changes, a discontinuous decrease is observed in the nucleocytoplasmic RNA-transport; this is probably not due to a cease in RNA-synthesis. Below the thermotropic discontinuity observed in the transport of total RNA in 18°-cells the nucleocytoplasmic transport of the small and large ribosomal subunits is equally retarded. Recent temperature studies on the endoplasmic reticulum membranes of *Tetrahymena* suggest that the freeze-etch changes in the nuclear membranes are induced by a thermotropic clustering of the membrane lipids. We conclude that this lipid clustering induces the permanent protein constituents in the nuclear envelope pore complexes to change from a relatively "open" into a relatively "closed" state thus causing the observed decrease in RNA-transport.

In eukaryotic cells, the nuclear envelope separates the main site of transcription, the nucleus, from the main site of translation, the cytoplasm. The nuclear envelope is therefore often invoked to be critically involved in the regulation of nucleocytoplasmic exchanges (for review *see*, e.g., [8, 27, 33]). The preferred passageways through the nuclear envelope for macromolecules including RNA are the nuclear pore complexes [7, 28]. These complexes might, therefore, play an important role in the regulation of gene expression, in particular under conditions where they might be "closed", i.e., rate limiting in nucleocytoplasmic RNA-transfer.

Feldherr [9] has recently found that the nuclear envelope represents a barrier to the cytoplasm→nucleus transfer of colloidal gold particles injected into the cytoplasm of the amoeba *Chaos chaos* at lower tempera-

tures. Moreover, we have recently suggested that the rate of the nucleus→cytoplasm transport of RNA in *Tetrahymena* can possibly be limited at the level of the nuclear envelope pore complexes by temperature lowering [32]. *Tetrahymena* cells propagated at their optimal growth temperature, 28 °C, reveal a sharp decrease in nucleocytoplasmic RNA-transport when the temperature is dropped below ~ 18 °C. Coincidently, the freeze fracture appearance of the nuclear envelope membranes changes in a manner suggestive of lipid-protein segregation. It was suggested, therefore, that thermotropic lipid "transitions" might cause nuclear pore complexes to change from a relatively "open" to a relatively "closed" state [32].

One way to support this proposal is to demonstrate that the structural and functional transitions of *Tetrahymena* nuclear envelopes change coherently with lipid composition. To effect this we have induced *Tetrahymena* cells to shift their lipid "transition" temperatures by maintaining them in nutrient medium at two different suboptimal growth temperatures, i.e., at 23 °C and 18 °C. We have then used freeze-etch electron microscopy to determine the variation with temperature of nuclear membrane structure and have simultaneously measured the effect of cooling on the nucleocytoplasmic transport of total RNA and ribosomal subunits.

Materials and Methods

Cultures

Static cultures of the ciliate protozoon *Tetrahymena pyriformis* (amicronucleate strain *GL*) were axenically grown either at 23 °C or 18 °C in a medium consisting of 2% proteose peptone and 0.1% liver extract. The generation times of the 23°-cells and 18°-cells were ~4 and ~7 hr, respectively. We used cultures that had reached population densities of 20,000–30,000 cells/ml. Cell counts were performed in a Fuchs-Rosenthal-counting chamber. The diameters of macronuclei were measured by bright field microscopy (magnification: 1000×; 60 cells per sample) after fixation with 2% glutaraldehyde and staining with 0.05% methylgreen. Means were provided with standard deviations.

Freeze-etch Electron Microscopy

Cell cultures grown at 23 and 18 °C, respectively, were divided in five 100-ml aliquots. The 23°-aliquots were equilibrated for 10 min at 23 °C, 19 °C, 15 °C, 11.5 °C and 8 °C, while the equilibration temperatures of the 18°-aliquots were 18°C, 15 °C, 11.5 °C, 8 °C and 5 °C. 50-ml samples were removed from the equilibrated aliquots and fixed at the equilibration temperatures for 10 min with 2% glutaraldehyde buffered with 0.05 M Na cacodylate (pH 7.2). The remaining 50-ml samples were kept at their equilibration temperatures for a further 30 min, and then fixed. After 10 min, the cells were twice washed with the cacodylate buffer at 1,000×*g* for 5 min, transferred into 25% glycerol in stepwise

fashion, and the frozen on cardboard disks in Freon 22, cooled by liquid nitrogen, as in reference [35]. Fracturing, etching (1 min at −100 °C) and replicating was performed on a Balzers model 360 M [22]. The replicas were studied in a Siemens Elmiskop I A. We evaluated the percentage of fracture faces with smooth areas and the number of nuclear pore complexes on cut-outs of calibrated positives derived from at least 20 nuclear membrane fracture faces of at least three different replicas per sample. Means are provided with standard deviations.

Incorporation of (^{14}C) Uridine

The 23°- and 18°-cells were labelled at their growth temperatures for 30 min with 0.03–0.05 μCi/ml (^{14}C) uridine (U) (spec. act.: 469 mCi/mmole; NEN-chemicals). Then the cultures were divided in several aliquots. One aliquot was always kept at 23 °C or 18 °C, respectively. The others were incubated at different lower temperatures for further 30 min (temperature equilibration was accomplished within maximally 5 min). At the beginning and the end of this 30-min period we measured the incorporation of (^{14}C) uridine into whole cells and the corresponding cytoplasmic RNA at each temperature.

Incorporation of (^{14}C) uridine into whole cells was measured by a modification of the method of Leick [19]. For each temperature four 2.5 ml portions were removed from the culture flasks, precipitated, and washed five times with 5% trichloroacetic acid (TCA) at 0–4 °C. The processing of these TCA-precipitates and final counting in a Packard liquid scintillation spectrometer model 3380 was carried out as described recently [32].

(^{14}C) uridine incorporation into cytoplasmic RNA was monitored as follows. The cytoplasmic fraction was isolated by a modification of the method of Leick and Plesner [21]. Three 10-ml cell samples were removed from the culture flasks at each temperature, poured into an ice-salt bath for 3 min, pelleted at $2{,}000 \times g$ for 3 min, and then frozen and stored in liquid nitrogen. The frozen cell pellets were thawed in 10 ml of 5 mM Mg acetate, 2.5 mM KCl, 5 mM Tris/HCl (pH 7.2), 0.4 M sucrose, 0.1 mg/ml polyvinylsulfat, and 0.01% glutaraldehyde. This cell suspension was hand-homogenized in a loosely fitting Teflon-Potter-Elvejhem grinder until about 90% of the cells were broken according to light microscopical observation. The homogenate was diluted with 1.5-fold volume of the above buffer without glutaraldehyde and then centrifuged at $10{,}000 \times g$ for 15 min. Bovine serum albumin (0.15 mg/ml) was added to the supernatant and this precipitated and washed five times with TCA at 0–4 °C. The TCA-precipitate was suspended in 5% TCA, heated to 90 °C for 20 min and then centrifuged at $3{,}000 \times g$ for 15 min. In the supernatant RNA was measured by the orcinol method [24] and aliquots were assayed for radioactivity as above.

Isolation of Ribosomal Subunits

Frozen cell pellets were thawed in 20 ml of 10 mM Tris/HCl (pH 7.2), 0.25 M sucrose, 5 mM Mg acetate, 2.5 mM KCl. This buffer had been treated overnight with 2.0 mg/ml bentonite (Serva, Heidelberg, West Germany) and centrifuged to clearness at $22{,}000 \times g$ for 10 min. All subsequent steps were performed at 0–4 °C. The thawed cells were homogenized as above and centrifuged twice at $10{,}000 \times g$ for 10 min. After addition of Triton X-100 in a final concentration of 0.03% to the supernatant, 20 ml of this were layered over 5 ml of a 1.33 M ribonuclease-free sucrose solution, buffered as above. This two-step sucrose was centrifuged at $140{,}000 \times g$ for 4 hr (angle rotor, Weinkauf ultracentrifuge, Brandau, West Germany). The transparent pellet was resuspended in 1 ml 10 mM Tris/HCl (pH 7.4), 0.50 mM EDTA, 50 mM KCl. After 10 min, the suspension was layered over

a 27-ml continuous 15–30% (w/v) sucrose gradient buffered with 10 mM Tris/HCl (pH 7.4), 50 mM KCl and 0.1 mM EDTA. This gradient was centrifuged at 75,000 × *g* for 15 hr (swing-out rotor, Weinkauf ultracentrifuge). Fractions were collected from bottom of the gradient with an ISCO-fraction collector. One aliquot of the fractions was measured at 260 nm, and another aliquot was mixed with 10 ml instagel (Packard) and assayed for radioactivity as above.

Results

Nuclear Membrane Structure

— The nuclear envelope of *Tetrahymena* macronuclei consists of an outer (i.e., cytoplasmic) and inner (i.e., nucleoplasmic) nuclear membrane. Both inner and outer nuclear membranes are fractured internally along their hydrophobic core, thus exposing two faces per membrane [25, 29]. The inner nuclear membrane then reveals a face bordering the nucleoplasm (*IPF*) and a face oriented toward the nuclear envelope cisterna (*IEF*). The outer nuclear membrane reveals a cisternal face (*OEF*) and the opposite (*OPF*) adjacent to the cytoplasm. The freeze-etch appearance of the *IEF*-faces resembles that of the *OEF*-faces, and the *IPF*-faces look like the *OPF*-faces. In 23°-cells and 18°-cells incubated for 10 min at 23 °C and 19 °C, and at 18 °C and 15 °C, respectively, the two *PF*-faces bear numerous, uniformly distributed 75-Å particles, whereas the two *EF*-faces show few particles, but numerous, uniformly distributed depressions (Fig. 1). However, in 23°-cells and 18°-cells equilibrated for 10 min at 15 °C and 11.5 °C, respectively, small smooth areas, i.e., fracture face domains largely devoid of particles or depressions with diameters of about 100–200 nm (*cf.* Fig. 2 in [32]), can be detected on about 4% of the fracture faces (both *PF* and *EF*) of the nuclear membranes. The size of these smooth areas as well as the percentages of fracture faces showing these smooth areas increase at the lower temperatures. Thus, in the case of the 23°-cells incubated for 10 min at 11.5 °C and 8 °C, we observe that 37% and 65%, respectively, of the fracture faces show smooth areas. In the 18°-cells, 33% and 55% of the *PF*- and *EF*-faces show particle-depleted areas at 8 °C and 5 °C, respectively. The diameters of these domains can occasionally reach 0.8 μm at the lowest incubation temperatures, as can be seen in Fig. 2. The frequencies of fracture faces revealing particle-depleted areas did not change significantly with a further 30-min period at equilibration temperatures.

The *Tetrahymena* cells grown at 23 °C and 18 °C exhibit about the same number of nuclear pore complexes per square micron nuclear envel-

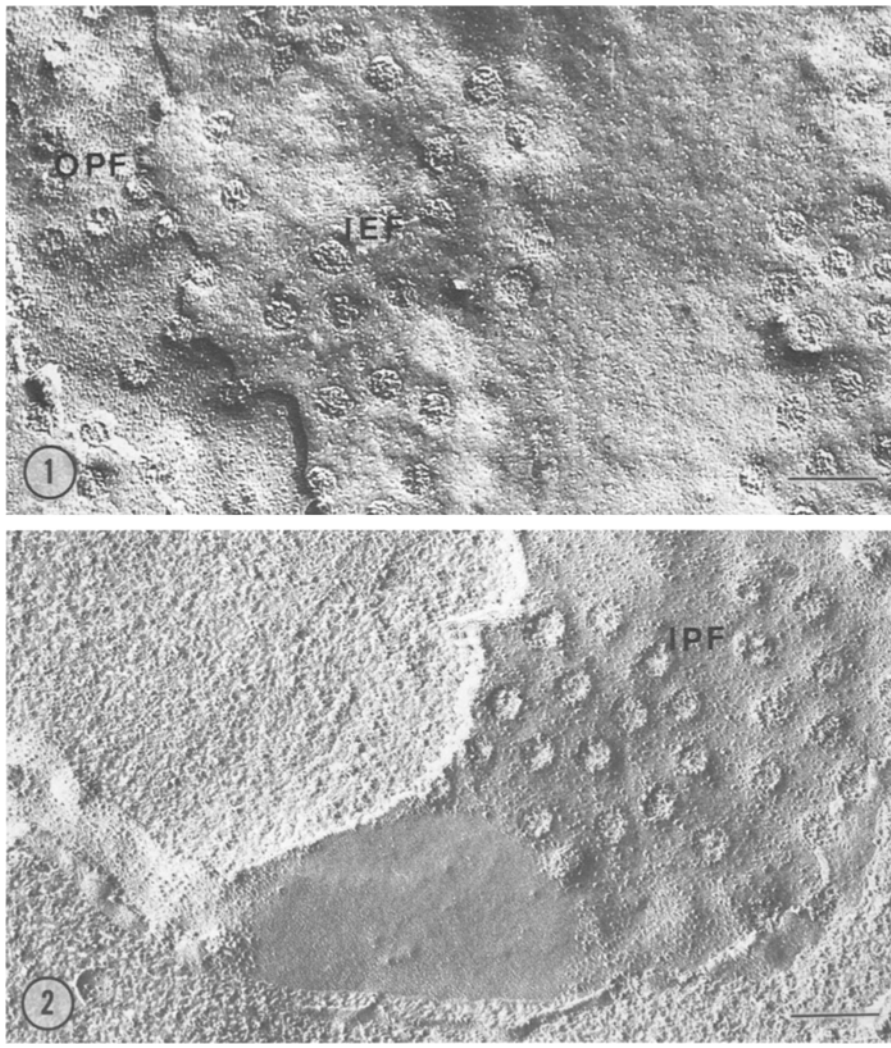

Fig. 1. Freeze-fractured nuclear envelope of a 18 °C-*Tetrahymena* cell incubated at 18 °C for 10 min. Membrane-intercalating particles are uniformly distributed on the outer nuclear membrane fracture face adjacent to the cytoplasm (*OPF*). The inner nuclear membrane fracture face bordering the nuclear envelope cisterna (*IEF*) reveals uniformly distributed "depressions" and only few particles. Note the large area free of pore complexes. Shadowing direction from left below. Bar represents 0.2 μm ×60,000

Fig. 2. Freeze-fractured nuclear envelope of a 18 °C-*Tetrahymena* cell incubated at 5 °C for 10 min. The inner nuclear membrane fracture face oriented towards the nucleoplasm (*IPF*) shows a large area largely devoid of the typical membrane-intercalating particles. The particles in the particle-bearing areas are not aggregated. Shadowing direction from right. Bar represents 0.2 μm ×60,000

ope area (23°-cells: 34.0±3.4; 18°-cells: 34.2±3.8)[1] as well as about the same diameter of the macronuclei (23°-cells: 10.7±1.8 μm; 18°-cells: 10.4±1.7 μm). Neither the nuclear diameter nor the nuclear pore complex frequency change significantly with temperature.

Transport and Synthesis of Total RNA

The 23°-cells and the 18°-cells which have been prelabelled and then incubated with (^{14}C) uridine at their growth temperatures for a further 30 min reveal linear or slightly exponential rates of (^{14}C) uridine incorporation into whole cells and the corresponding cytoplasmic RNA during this 30 min period. Table 1 shows the values of (^{14}C) uridine incorporation into whole cells and the cytoplasmic RNA at the beginning and the end of this period.

Both the 23°-cells and the 18°-cells incubated at lower temperatures have accumulated less (^{14}C) uridine into whole cells and cytoplasmic RNA after the 30 min periods we examined (Figs. 3 and 4). The 23°-cells incorporate less (^{14}C) uridine into whole cells than into cytoplasmic RNA in the temperature range between 23 °C and ~15 °C, while the

Table 1. (^{14}C) uridine incorporation into whole cells and cytoplasmic RNA of 23°- and 18°-*Tetrahymena* cells at the beginning and the end of a 30 min incubation period with (^{14}C) uridine at 23 °C and 18 °C, respectively

Growth temperature	Experiment number	(^{14}C) uridine incorporation into			
		whole cells (cpm/ml)		cytoplasmic RNA (cpm/μg RNA)	
		0 min	30 min	0 min	30 min
23 °C	1	497	975	63.2	106.8
	2	531	1066	73.8	142.5
	3	512	949	59.8	111.7
18 °C	1	124	245	7.7	16.8
	2	197	518	8.8	20.4
	3	377	704	28.8	55.4

1 Recently we observed a higher pore frequency (38.7±1.4/μm^2) in *Tetrahymena* cells grown at 28 °C [32]. We assume that this is not a response of the 28°-cells to growth temperature, but rather to the growth medium, which was supplemented with 0.4% liver extract. Interestingly, the number of nuclear pore complexes per cubic micron nuclear volume is about the same in these 28°-cells and in the 23°- and 18°-cells.

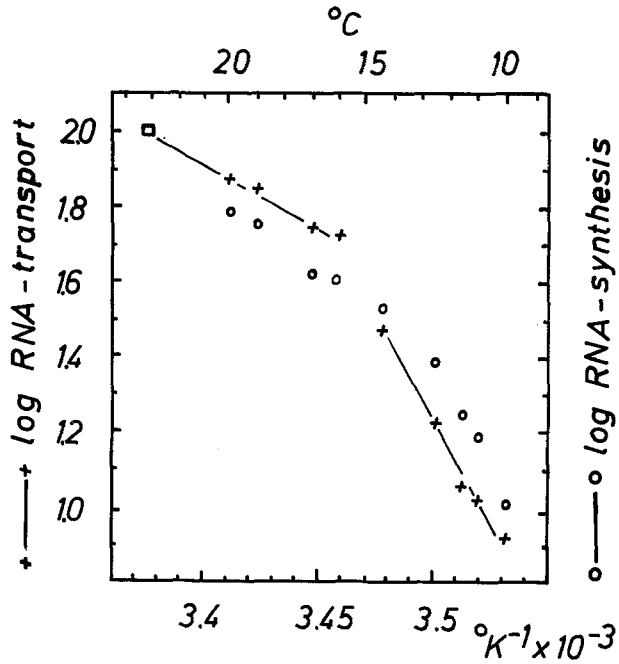

Fig. 3. Nucleocytoplasmic transport and synthesis of overall RNA in 23 °C-*Tetrahymena* cells as a function of temperature. In three experiments, cultures prelabelled with (^{14}C) uridine at 23 °C for 30 min were divided in several aliquots. A reference aliquot was always kept at 23 °C, while the other were incubated at different lower temperatures for further 30 min. During this period the incorporation of (^{14}C) uridine into whole cells (RNA-synthesis) and cytoplasmic RNA (RNA-transport) proceeds approximately linearly at the different temperatures as examined in control experiments. The cpm accumulated during this 30 min period in whole cells and cytoplasmic RNA at the different temperatures were normalized to the respective 23 °C-values, which can be taken from Table 1

reverse occurs below ~15 °C (Fig. 3). The 18°-cells also accumulate more (^{14}C) uridine into the whole cells than into the RNA of the cytoplasmic fraction below a critical temperature of ~12 °C (Fig. 4).

Under the conditions used it is reasonable to assume that the incorporation of (^{14}C) uridine into whole cells and total RNA of the cytoplasmic fraction reflects the synthesis of total cellular RNA and the appearance of nuclear-synthesized RNA in the cytoplasm, respectively. The appearance of RNA in the cytoplasm, which we term nucleocytoplasmic RNA-transport, is preceded by a series of reactions at the pretranscriptional, transcriptional and posttranscriptional level, which are actually not understood in detail. Thus, our findings that RNA-synthesis and nucleocytoplasmic RNA-transport behave in reverse above and below a critical temperature of ~15 °C and ~12 °C in 23°- and 18°-cells, respectively, can be most plausibly interpreted at the moment by stating that the

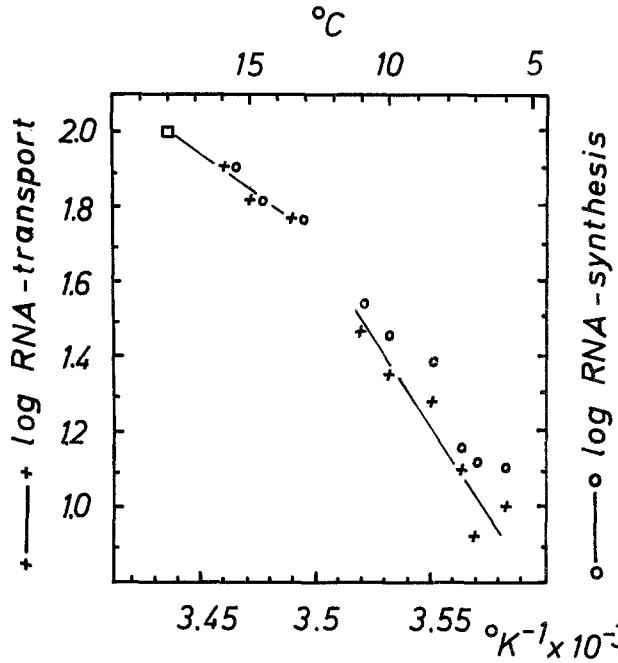

Fig. 4. Nucleocytoplasmic transport and synthesis of overall RNA in 18 °C-*Tetrahymena* cells as a function of temperature. Performance and evaluation of experiments was as described in the legend to Fig. 3

nucleocytoplasmic RNA-transport is rate limited at or even before the transcriptional level above the discontinuity and at the posttranscriptional nuclear level below the discontinuity. The latter level includes reactions such as RNA-processing, packaging of RNA with proteins, and the final transport of ribonucleoproteins through the nuclear envelope pore complexes into the cytoplasm. This interpretation is consistent with previous findings also found in other cells, i.e., that upon temperature lowering, the transport of RNA from nucleus to cytoplasm becomes more reduced than does RNA-synthesis; consequently, RNA accumulates within the nucleus [5, 12, 13].

Transport of Ribosomal Subunits

The ribosomal RNA accounts for nearly 90% of the total cellular RNA [18]. Formation, nuclear processing, and final appearance of the ribosomal RNA, i.e., the ribosomal subunits, within the cytoplasm occur within several minutes [6, 17, 20].

Fig. 5 shows the sucrose gradient sedimentation profile of large and

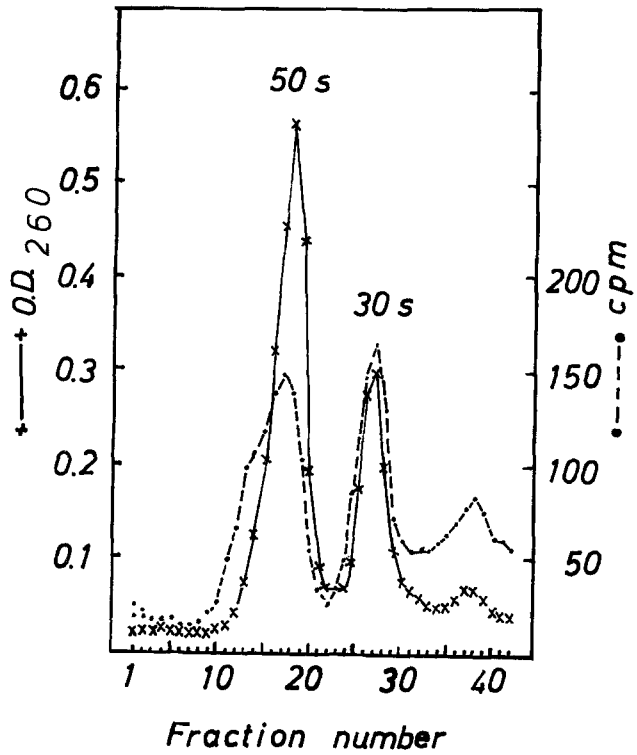

Fig. 5. Sucrose gradient sedimentation profile of the ribosomal subunits isolated from the cytoplasmic fraction of 18 °C-*Tetrahymena* cells prelabelled with (^{14}C) uridine at 18 °C for 20 min. The large and small ribosomal subunits were nominally designated as 50 *s* and 30 *s*, respectively (*cf.* ref. 17, 21)

small ribosomal subunits isolated from 18°-cells that had been labelled with (^{14}C) uridine for 20 min at 18 °C. The smaller subunit (30 *s*) reveals a higher specific activity than the larger one (50 *s*). When these labelled *Tetrahymena* cells were incubated at 15 °C for a further 30 min period, the specific activities of the 50 *s* and 30 *s* ribosomal subunits increased almost linearly and parallel to each other (Fig. 6). In accord, previous findings have also shown such uridine labeling kinetics for the 25 *s* and 17 *s* RNA (i.e., the main RNA constituents of the large and small ribosomal subunits, respectively) in 28°-cells [20]. The parallel increase of the specific activities indicates that both the small and large subunits are transported from nucleus to cytoplasm at the same rate. Despite equal transport and synthetic rates, a difference is found between the specific activities being due to the smaller nuclear pool-size of the small subunit.

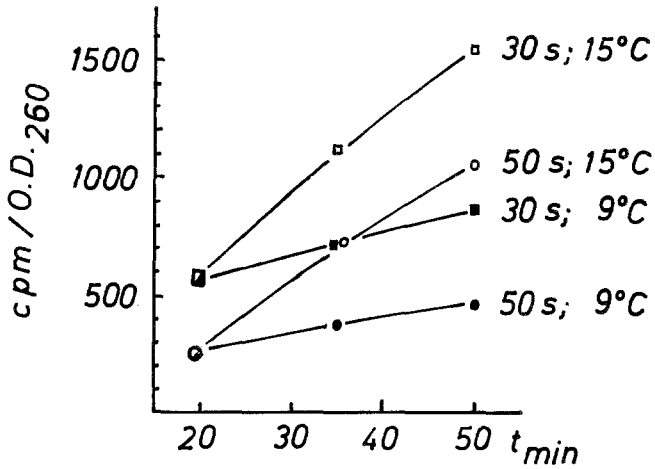

Fig. 6. Specific activities of the cytoplasmic ribosomal subunits isolated from 18 °C-*Tetrahymena* cells which were prelabelled with (^{14}C) uridine at 18 °C for 20 min and then incubated for further 30 min at 15 °C and 9 °C, respectively. As specific activity we evaluated the cpm/O.D.$_{260}$ of the three peak fractions of the 50 *s* and 30 *s*-subunits separated on sucrose gradients

At 9 °C, the linear increase in the specific activities of the small and large units is delayed in comparison to 15 °C, but proceeds again in a parallel fashion. This indicates that temperature lowering to 9 °C equally affects the nucleocytoplasmic transport rates of the large and small ribosomal subunits.

Discussion

Plots of nucleocytoplasmic RNA-transport *vs.* temperature for *Tetrahymena* cells grown at 23 °C and 18 °C, reveal discontinuities at ~15 °C and ~12 °C, respectively. At these temperatures, we also observe emergence of smooth areas on the fracture faces of the nuclear membranes. We must now enquire whether the discontinuities in nucleocytoplasmic RNA-transport are causally or fortuitously related to the temperature sensitive changes of nuclear membrane morphology.

Our results must be evaluated in terms of the thermotropism of *Tetrahymena* endoplasmic reticulum [34, 35], a membrane type closely related to nuclear envelope membranes. *Tetrahymena* cells grown at 28 °C show the appearance of particle-depleted regions on endoplasmic reticulum fracture faces at temperatures below ~17 °C [35]. Studies using electron spin resonance, fluorescence spectroscopy, differential thermal

calorimetry, and proton nuclear magnetic resonance [35] indicate that these alterations are not induced by a thermal liquid crystalline⇌crystalline phase transition of the membrane lipids as observed, e.g., in plasma membranes of prokaryotes [10, 14, 16, 26, 30] but rather by a thermotropic lipid phase segregation, i.e., clustering of "rigid" liquid crystalline lipid domains within "fluid" liquid crystalline lipid environments [35]. This lipid clustering also apparently induces changes in membrane function, as indicated by a discontinuity in the Arrhenius plot of the activity of endoplasmic reticulum glucose-6-phosphatase [35]. When cells are grown at 18 °C, the lipid clustering shifts to ~ 12 °C; this is accompanied by a corresponding displacement of the discontinuity in the Arrhenius plot of glucose-6-phosphatase activity to this temperature [34]. The reason for the altered thermotropism of cells grown at 18 °C is apparently a replacement of more saturated membrane phospholipids with more unsaturated ones [34; *cf.* also 23, 37].

The same mechanism can be expected to operate also in nuclear membranes. It is not surprising therefore that the 23°- and 18°-cells exhibit lipid clustering in nuclear membranes at temperatures lower than found with cells grown at 28 °C [32]. Provided that lipid clustering in nuclear membranes has an effect on that step of the nucleocytoplasmic RNA-transport which is located at the level of the nuclear envelope then one should expect our present finding that, in comparison to 28°-cells [32], the 23°-cells and 18°-cells have shifted the discontinuity in the overall RNA-transport to the corresponding lipid clustering temperatures.

In *Tetrahymena*, the nucleocytoplasmic transport of RNA proceeds exclusively through the nuclear pore complexes [36]. Our results indicate that their number per nucleus does not significantly change in both the 23°-cells and 18°-cells during the temperature treatment. Thus the most plausible explanation for the decreased rates of RNA-transport is that lipid clustering induces the existing nuclear pore complexes to change from a relatively "open" into a relatively "closed" state. This open→closed transition (not detectable by electronmicroscopy) is specifically thought to occur in the permanent protein constituents of the nonmembraneous pore complex material [31], which is widely believed to be responsible for nucleocytoplasmic exchanges of macromolecules (for review *see*: e.g., [8, 27, 33]. At least some of these permanent protein constituents are components of the nuclear structural framework termed nuclear protein matrix [3], which was recently demonstrated in rat liver nuclei [3, 4; *cf.* also 1] and *Tetrahymena* macronuclei [11]. Such a lipid-

induced transition in gating of nuclear pore complexes could also explain observations that temperature lowering blocks the cytoplasm→nucleus transfer of proteins in chick embryo fibroblasts [2, 12] and in mouse ascites tumor cells [15], and of colloidal gold particles in *Chaos chaos* [9]. Moreover, it could explain why the transport of molecules of different sizes is equally influenced, as is found for the nucleocytoplasmic transport of the large and small ribosomal subunits in *Tetrahymena* and for cytoplasm→nucleus transfer of differently sized colloidal gold particles in *Chaos chaos* [9]. Finally, we realize that it is difficult *in vivo* to discriminate unequivocally between RNA-transport and RNA-synthesis, since both processes are very probably linked together via the nuclear protein matrix by a feedback mechanism [33]. To overcome these difficulties, we have since studied the RNA-release from, and the protein entry into isolated macronuclei of *Tetrahymena*. These transport processes also show thermotrophic discontinuities (Nägel and Wunderlich, *in preparation*).

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