Unusual distribution of mitochondrial large subunit rRNA in the cytosol during conjugation in *Tetrahymena thermophila*

Takashi Kobayashi* and Hiroshi Endoh

Department of Biology, Faculty of Science, Kanazawa University, Kakuma, Kanazawa 920-1192, Japan

(Received 21 July 2004, accepted 4 November 2004)

The distribution of mitochondria during conjugation of the ciliated protozoan *Tetrahymena thermophila* was surveyed using a mitochondrial stain and fluorescence in situ hybridization (FISH). When the mitochondria-specific stain, MitoTracker, was used, the majority of mitochondria were detected in the cortex; their distribution was not changed during conjugation. On the other hand, FISH using mitochondrial large subunit (LSU) rRNA as a probe showed an unusual distribution of signals during conjugation. Unexpectedly, the signals were detected throughout the cytoplasm of conjugating cells. These signals were not observed in pre-mating cells and in exconjugants. The cytosolic localization of mitochondrial rRNA was supported by northern blot analysis using post-mitochondrial RNA fraction at the later stages of conjugation. These observations suggest selective mitochondrial breakdown or transport of LSU rRNA into cytosol. The biological significance of the conjugation-specific appearance of the cytosolic mitochondrial rRNA is discussed.

Key words: mitochondria, RNA localization, rRNA, sexual cycle, stem mitochondria

INTRODUCTION

Ciliated protozoans are characterized by the presence of two morphologically and functionally distinct nuclei within a single cell. One nucleus is a transcriptionally active polyploid macronucleus comparable to the "soma", while the other is an inactive diploid micronucleus that represents the "germ-line". Although they are unicellular, ciliates spatially distinguish between the soma and germ-line.

Conjugation of *Tetrahymena thermophila* begins with the interaction of complementary mating types. Conjugating cells undergo meiosis, nuclear selection of one of the haploid meiotic products, reciprocal nuclear exchange, diploid synkaryon formation (fertilization), and nuclear differentiation into new macro- and micro-nuclei, followed by resorption of the parental macronucleus (Martindale et al., 1982). In the process of macronuclear differentiation, selective elimination and rearrangement of micronuclear DNA, and a large-scale amplification of the DNA occur (see review by Prescott, 1994). Both types of nuclei are differentiated from the same synkaryon following two postzygotic divisions, which result in the production of four nuclei. Nuclear differentiation is predestined by nuclear position in the cytoplasm; the two nuclei located in the anterior region become the macronuclear anlagen, while those localized posteriorly become the micronuclei, without any structural change (Nanny, 1953). The molecular mechanism of the positional control of the nuclear differentiation has not been elucidated.

In our previous study, we detected a UV-sensitive period during conjugation in *Tetrahymena* (Kobayashi and Endoh, 1998). When conjugating cells were irradiated at meiotic prophase, especially at pachytene and diplotene, post-meiotic nuclear events were abolished and the subsequent processes of conjugation were aborted. Although the UV-target molecule has not been identified, organelles such as mitochondria are reasonable candidates. UV irradiation is known to cause swelling and vacuolation of mitochondria in *Smittia* (Kalthoff et al., 1975) and *Xenopus* (Ikenishi et al., 1974). Likewise, involvement of mitochondria in other UV-irradiation responses has been reported. For example, UV irradiation inhibits a pole cell formation in *Drosophila melanogaster* (Geigy, 1931; Jura, 1964; Okada et al., 1974). Mitochondrial rRNA molecules are released into the cytoplasm at an early stage of development and the degradation of the RNA by UV is responsible for the loss of pole...
cells (Kobayashi and Okada, 1989; Kobayashi et al., 1993). A similar distribution of mitochondrial rRNA in the cytoplasm has been reported in diverse species of animals, including Xenopus (Kobayashi et al., 1998), ascidians (Oka et al., 1999), sea urchin (Ogawa et al., 1999), and Planaria (Sato et al., 2001). Recently, it was reported that apoptosis is induced by the release of cytochrome c protein from mitochondria into the cytoplasm when the mitochondria are damaged by UV (Reviewed by Kulms and Schwarz, 2000).

In the present experiments investigating the distribution of mitochondria during conjugation, an unusual behavior of mitochondrial rRNA was observed. Mitochondrial LSU rRNA did not localize solely within the mitochondria as expected, but appeared to be dispersed in the cytosol during conjugation. A possible biological significance of this conjugation-specific change in the distribution pattern of mitochondrial LSU rRNA is considered.

MATERIALS AND METHODS

Strains, cell culture and mating induction Tetrahymena thermophila strains WA6 and WD6 were obtained from T. Sugai, Ibaraki University (Ibaraki, Japan). Conditions for cell culture and mating induction have been described previously (Kobayashi and Endoh, 1998); all experiments were performed at 26°C.

Cloning of mitochondria large subunit rRNA Total mitochondrial DNA was isolated as described by Morin and Cech (1986). The oligonucleotides used as PCR primers to clone the mitochondria large subunit rRNA (mt LSU rRNA) gene were mtLSU-1 (5'-CGGAATTCC-GTCCCTAGGTAAGACAAAT-3'), mtLSU-2 (5'-GTAGAAT-TCCCTATTTGCCACATAAGT-3'). PCR was performed under the following conditions: 45 sec at 92°C, 45 sec at 50°C, 60 sec at 72°C. A 371-bp partial fragment of mt LSU rRNA was isolated from agarose gels and cloned into pBluescript II SK- (Stratagene Inc.). The fragment was sequenced using a Thermosequenase Cycle Sequencing Kit (Amersham Bioscience AB) and a SQ-5500 DNA sequencer (Hitachi).

Staining of mitochondria Cells were fixed with 3.7% formaldehyde for 15 min at room temperature and washed three times with PBS. The cells were incubated for 30 min in 100 μM Mito-Tracker Green (Molecular Probes Inc) in PBS at room temperature and observed using a fluorescence microscope with a blue filter. DAPI (4,6-diamino-2-phenylindole) was used for staining of nuclei.

Preparation of probes and whole mount in situ hybridization For in situ hybridization, single stranded RNA probes were transcribed from the above mentioned sequence using the Digoxigenin (DIG) RNA labeling kit (Roche Diagnostics GmbH) and T3 RNA polymerase.

Whole mount in situ hybridization was performed using a modified method of Curtenaz and Beisson (1996). Cells were handled in 1.5 ml microtubes throughout. The hybridization solution (HS) contained 50% formamide, 1.5 x SSC, 50 μg/ml heparin, 100 μg/ml sonicated and denatured salmon sperm DNA and 0.1% Tween 20. Hybridization was performed for 3 h at 37°C with approximately 0.5 μg/ml of probe in HS containing 10% dextran sulfate.

To detect probes, the cells were incubated for 45 min at room temperature with 8 μg/ml fluorescein-labeled anti-DIG-Fab fragment (Roche Diagnostics GmbH). Counterstaining of nuclei was completed with DAPI. Micrographs were prepared using Adobe Photoshop 7.0.

Northern blot analysis The vegetative cells or conjugating cells were harvested by centrifugation at 1,000 x g for 5 min. Then the cells were resuspended in a cold mitochondria isolation buffer (MIB) [0.35 M sucrose, 10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 3 mM CaCl₂, 25 mM KCl and 10 mM vanadyl ribonucleoside complexes], and homogenized using a Polytron homogenizer. To remove nuclei and unbroken cells, the homogenate was centrifuged twice at 2,000 x g for 5 min at 4°C. To sediment the mitochondria, the supernatant was centrifuged at 10,700 x g for 10 min at 4°C. The precipitate (mitochondria) was resuspended in MIB. Cytosolic RNA (post-mitochondrial RNA) was isolated from the remaining supernatant as described by Burger (1987). Mitochondrial RNA extraction was carried out using Sepasol-RNA super (Nacalai).

Mitochondrial and cytosolic RNA (10 μg) were separated by electrophoresis in 1% (w/v) agarose-formaldehyde gels and stained with ethidium bromide. Then the RNAs were transferred to the Positively Charged Nylon Membrane (Roche Diagnostics GmbH) after soaking in a transfer buffer [0.01 M NaOH and 3 M NaCl] for 20 min. AlkPhos DIRECT Labeling and Detection System with CDP-star (Amersham Bioscience AB) was used in labeling of RNA probe, hybridization and detection according to the manufacture’s protocol.

RESULTS

Mitochondrial localization in vegetative and conjugating cells Conjugation process in Tetrahymena thermophila is diagramed in Fig. 1. To determine whether mitochondrial localization changes during conjugation, vegetative and conjugating cells were stained with a mitochondrial-specific fluorescent dye (Mito-Tracker) (Fig. 2). Cortical mitochondria were predominantly identified along the ciliary row, while few mitochondria were observed in the cytoplasm. This observation is con-
Unusual localization of MtLSUrRNA in *Tetrahymena*

**Fig. 1.** The conjugation process in *Tetrahymena thermophila*. **A.** Pre-conjugant. Pair formation by complementary mating types. **B.** Meiotic metaphase. **C.** Meiosis II. The micronucleus undergoes meiosis and four haploid meiotic products are formed. **D.** Gametic nuclear selection. One of the meiotic products is selected and divides mitotically once to produce gametic nuclei. At this stage, the remaining three meiotic products degenerate in the posterior region of the cell. **E.** Fertilization. One of the gametic nuclei is exchanged between partners. Stationary and migratory nuclei fuse and form a synkaryon (fertilized nucleus). **F.** The second post-zygotic division (PZD II). The synkaryon undergoes postzygotic division twice and yields four nuclei, two of which are located posteriorly and the other two anteriorly. **G.** Nuclear differentiation. The anterior nuclei differentiate into new macronuclei (macronuclear anlagen), and the posterior nuclei remain micronuclei (presumptive micronuclei). **H.** Macronuclear development II (Mac IIp). Macronuclear anlagen are developed and the old parental macronucleus degenerates in an apoptosis-like fashion. **I and J.** Mac IIe. After pair separation, one of the micronuclei is eliminated, while the remaining micronucleus divides once. The macronucleus and micronucleus are distributed in daughter cells called caryonides. **M**, macronucleus; **m**, micronucleus; **MA**, macronuclear anlage.

**Fig. 2.** Mitochondrial distribution in the *Tetrahymena* cell. Nuclei are stained with DAPI (A, C) and mitochondria are stained with Mito-Tracker (B, D). **A, B.** A pre-conjugant (upper) and a conjugant at meiotic metaphase (5 h after mixing). **C, D.** A conjugant at nuclear differentiation stage (8 h after mixing). **M**, macronucleus; **m**, micronucleus; **MA**, macronuclear anlage. Scale bar = 10 µm.
curred with a previous study that the majority of mitochondria are located in the cortical ridges, where they are anchored in position just below the cilia (Lynn and Small, 1991). When the distributions of mitochondria were compared between vegetative and conjugating cells, no conspicuous differences were observed (Fig. 2B and D). No change in mitochondrial distribution could be observed at any stages of conjugation (Fig. 2B and D, data not shown). These observations confirmed that mitochondrial position remained constant, even during conjugation.

**Distribution of mitochondrial LSU rRNA in cytosol**  On the other hand, when fluorescence in situ hybridization (FISH) was used to detect the distribution of mitochondrial LSU rRNA (Fig. 3), no signal was observed in the cytosol of pre-conjugants (Fig. 3A). As conjugation progressed, cytosolic signals began to appear and were randomly distributed throughout the cytoplasm (Fig. 3B). By 8 h after mixing, during nuclear differentiation, one parental macronucleus, two macronuclear anlagen, and two presumptive micronuclei were observed (Fig. 3C). A small degenerating meiotic product was still visible between the two presumptive micronuclei. At 12 h after mixing, when conjugation was near completion, the parental macronucleus had undergone condensation in the posterior region (Fig. 3D). In the lower exconjugant, the parental macronucleus was degenerating, with faint signals remaining (Fig. 3E). When a sense-RNA probe was used, no signal was detected (Fig. 3F). The abbreviations used in the figures are: M, macronucleus; dM, degenerating macronucleus; m, micronucleus; dm, degenerating meiotic product; MA, macronuclear anlage. Scale represents 10 µm.
ization (FISH) was carried out at various stages of conjugation, unexpected distribution of signals was observed during conjugation. In contrast to the pattern of mitochondria detected with Mito-Tracker, an anti-mt LSU rRNA probe gave a more random distribution of the signals (Fig. 3). In pre-mating cells, little or no such signals were detected (Fig. 3A). In conjugating cells, numerous signals of mt LSU rRNA began to appear at meiotic metaphase (Fig. 3B) and persisted through the stage of nuclear differentiation into new macro- and micronuclei (Fig. 3C). After nuclear differentiation, the signals gradually decreased in number and in intensity (Fig. 3D and E). When resorption of the parental macronucleus subsequent to nuclear differentiation was complete in the exconjugants, the strong signals were no longer observed (see the cell in the left side in Fig. 3E). The signals observed in this experiment might correspond to those of mt LSU rRNA, but not to nonspecific signals, since a sense probe of mt LSU rRNA did not confer the same strong signals as those with the anti-sense probe (Fig. 3F). In addition, specificity of the probe used here was confirmed by northern blot analysis to total RNA and mitochondria-enriched RNA, prior to FISH analysis (Fig. 4A). In hybridization to total RNA, only a faint band was detected on mt LSU rRNA (23S), suggesting no non-specific hybridization to other RNA species such as cytoplasmic 17 S and 26S rRNAs, whereas the probe gave a strong signal on 23S RNA in mitochondria-enriched RNA. This indicates that an artefact is not responsible for the detection of cytosolic mt rRNA in FISH. It is worth noting that signals representing the cortical mitochondria were usually detected along the ciliary rows, when cells were pretreated with 50 µg/ml proteinase K for 5 min at 37°C (data not shown). Under the present experimental condition where cells were not pretreated with proteinase K, the probe apparently did not enter the mitochondria, which resulted in the detection of cytosol-localized mt rRNA. Judging from this information, the present result implies that mt LSU rRNA might appear in the cytosol in a conjugation-specific manner.

To obtain further evidence for the presence of mitochondria rRNA in the cytosol during conjugation, northern blot analysis was carried out by isolating RNA from post-mitochondrial fraction after removing nuclear and mitochondrial fractions. As shown in Fig. 4B, no hybridization signal was detected on the RNA isolated from cells before mating or conjugants at earlier stages of conjugation (0 to 8 h), whereas a faint signal was identified during later stages of conjugation after a longer exposure (12 h). Although we can not completely deny the possibility that artificial breakdown of mitochondria occurred during the procedure of removing mitochondrial fraction, the timing of appearance of the mt rRNA in cytosol is consistent with the result detected with FISH. This coincidence favors the idea that this phenomenon is a programmed process, rather than an accidental consequence. Thus, these two observations suggest that the random distribution reflect the localization of mt LSU rRNA in the cytosol, but not in mitochondria. The mt rRNA might appear specifically in the cytosol during conjugation.

**DISCUSSION**

We previously demonstrated that UV irradiation at meiotic prophase induced the loss of all meiotic products and abortion of the subsequent conjugation process in *Tetrahymena* (Kobayashi and Endoh, 1998). The pre-
sent study was undertaken to investigate the potential role of mitochondria as a UV target in this process. Unexpectedly, mitochondrial LSU rRNA was distributed in the cytosol of conjugating cells. It is not clear whether the presence of mt rRNA in the cytosol is due to an active transport from the mitochondria or the result of mitochondrial breakdown. The presence of cytosolic mt LSU rRNA raises questions about functions of the free rRNAs. We discuss the following possible functions.

Nuclear differentiation is one of the interesting events during conjugation. A developmental fate of nuclei is determined, depending upon their intracellular positions. Prior to nuclear differentiation, four nuclei derived from a fertilized nucleus via two postzygotic divisions transiently locate anteriorly and posteriorly (Fig. 1F). Further development of the two anterior nuclei produces the new macronuclei, while the two posterior nuclei remain to be the presumptive micronuclei (Fig. 1G). Although the posterior region of the cell is the germinal micronucleus-positioned site at this stage, there is also a resorption site of the degenerating nuclei. Nuclear differentiation is sandwiched between two nuclear degradation events: the reduction of meiotic products and the resorption of the parental macronucleus (Fig. 1D and H). Especially, the degradation of extra meiotic products is observed near the presumptive micronuclei during nuclear differentiation stage (Fig. 3C). In fact, using "apofluor" staining, it was demonstrated that many lysosomes are distributed in the posterior portion of conjugating cells (Santos et al., 2000). These observations lead us to the idea that there should be a protective mechanism for the posteriorly-located presumptive micronuclei from degradation. It is known that autophagosome formation occurs before nuclear pyknosis of the meiotic products and the parental macronucleus (Weiske-Benner and Eckert, 1987). Thereafter, lysosome fusion with the autophagosome takes place, resulting in the final digestion and destruction of the nuclei. Taking these facts into consideration, a hypothetical protection mechanism to prevent degradation of non-targeted nuclei is naturally assumed. This may be supported by the following observation: UV irradiation in the postzygotic period led to the loss of the presumptive micronuclei, but not that of the macronuclear anlagen (Watanabe, 1991; Sugai, T., pers. commun.). If the cytosolic mt LSU rRNA was degraded by UV irradiation and was associated with the presumptive micronuclei, the breakdown of the mt LSU rRNA could permit the digestion of the new micronuclei through the fusion of the lysosome and micronuclei, which transiently occupy a position in the posterior of the cell. In this respect, the cytosolic RNA may function to block autophagosome formation or prevent the fusion of lysosomes and the presumptive micronuclei, thereby the micronuclei located in the posterior region of the cell could avoid degradation. Unfortunately, no clear association of the RNA with the micronuclei has been identified, although in some instances such a potential association seemed to be observed.

Alternatively, the results obtained in this study may relate to the release of apoptosis factor such as cytomegalo c or AIF (apoptosis inducing factor) from mitochondria to the cytosol, which is crucial to apoptosis (Kroemer, 1999; Kroemer and Reed, 2000; Ferri and Kroemer, 2001). During conjugation in *Tetrahymena*, it is well known that the parental macronucleus degrades via an apoptotic-like process (Davis et al., 1992; Mpoke and Wolfe, 1996). Caspase inhibitors block this degradation (Ejercito and Wolfe, 2003), and caspase-like activity has been confirmed in vitro at this stage (Kobayashi and Endoh, 2003). In addition, mitochondrial association with this process is suggested: Many of mitochondria are taken into the "large" autophagosome (autophagosome surrounding the old macronucleus) during conjugation where the degenerating macronucleus and mitochondria are co-localized within the restricted space (Kobayashi and Endoh, 2003; our unpublished results). Prior to migration into the large autophagosome, mitochondria are thought to be incorporated into many small autophagosomes. This may be responsible for the cytosolic localization of mt LSU rRNA during conjugation. If the breakdown of mitochondria in the autophagosome is a programmed process responsible for the release of mitochondrial factors and final activation of caspase-like activity, nuclear death may be triggered in a manner similar to apoptosis in higher organisms. Endonuclease G is also released from mitochondria and is responsible for DNA breakdown during animal apoptosis as a caspase-independent pathway (Li et al., 2001; Parrish et al., 2001). In fact, *Tetrahymena* retain Endonuclease G-like activity in mitochondria (our unpublished result). Protozoan apoptotic-like processes are likely to be more primitive, so that the release of mitochondrial factors could occur in autophagosomes triggered by the simple breakdown of mitochondria, without the need for a specific transporter as in higher organisms. The present observation of mt LSU rRNA during conjugation may reflect one such process.

With reference to this interpretation, the cytosolic mt LSU rRNA may be relevant to the turnover of old mitochondria during conjugation. Ciliates maintain numerous mitochondria in the large cells. Nevertheless, no clear heteroplasmy is usually observed. Conjugation is a sexual process that may include the active reduction of heterogeneous mitochondria. Evidence supporting this hypothesis was supplied by an elegant study that suggested the presence of stem cortical unit, which can permanently duplicate, in *Paramecium trichium* (Takahashi et al., 1998). Before cell division, the cortical units of cilia duplicate. This study implicates that the cortical mitochondria associated with the units also duplicate, so
that each cortical unit of cilia and cortical mitochondria shares an identical fate. In fact, cell size in the later stage of conjugation remarkably decreases, probably accompanied with the reduction of the number of the cortical units and cortical mitochondria, resulting in the bottle-neck effect, as seen in early development of some animals. Ciliates may use the mechanism, in which the breakdown of aged mitochondria during conjugation simply serves as an adaptive process to maintain the homogeneity of mitochondrial DNA in each generation.

Finally, the localization of mt LSU rRNA in the cytosol may be involved in germ-line determination in ciliates. In relation to this idea, a similar phenomenon has been reported in animals, e.g. pole cell formation in the early development of Drosophila is inhibited by UV irradiation (Geigy, 1931; Jura, 1964; Okada et al., 1974). One of the target molecules was mitochondrial large rRNA (mtlrrNA), which is located in the cytoplasm and functions as a determinant of pole cell formation (Kobayashi et al., 1993). Subsequently, it was directly elucidated by the observation using electron microscopic in situ hybridization and immunohistochemical analysis that mtlrRNA is localized outside the mitochondria in the germ plasm of Drosophila embryos in the form of ribosomes (Amikura et al., 2001). Signals of mt LSU rRNA shown in this study may reflect such a localization in the cytosol. In the case of Tetrahymena, nuclear differentiation is the process in which the destiny of germ-line and soma is determined. If mt LSU rRNA is selectively transported from mitochondria to the cytosol, this may be equivalent to the process of pole cell formation in Drosophila. If this is the case, it is reasonable to postulate that a germ-line differentiation system involving mitochondrial rRNA has been evolutionarily preserved since the time of unicellular protozoa. Unfortunately, now we have obtained no direct evidence, so our future task is to find more valid evidence.

We thank T. Sugai for a kind supply of Tetrahymena strains used in this study, and S. Kobayashi and M. Okada for encouragement to accomplish this study.

REFERENCES


