Mitochondrial DNA synthesis is a thermosensitive process in the yeast Saccharomyces cerevisiae. We found that restoration of mtDNA synthesis following heat treatment of cells is dependent on reactivation of the mtDNA polymerase Mip1p through the action of a mitochondrial bichaperone system consisting of the Hsp70 system and the Hsp78 oligomeric protein. mtDNA synthesis was inefficiently restored after heat shock in yeast lacking either functional component of the bichaperone system. Furthermore, the activity of purified Mip1p was also thermosensitive; however, the purified components of the mitochondrial bichaperone system (Ssc1p, Mdj1p, Mge1p, and Hsp78p) were able to protect its activity under moderate heat shock conditions as well as to reactivate thermally inactivated Mip1p. Interestingly, the reactivation of endogenous Mip1p contributed more significantly to the restoration of mtDNA synthesis than did import of newly synthesized Mip1p from the cytosol. These observations suggest an important link between function of mitochondrial chaperones and the propagation of mitochondrial genomes under ever-changing environmental conditions.

Dividing eukaryotic cells must faithfully replicate and transmit their mtDNA to ensure the respiratory competence of their progeny. Although the mitochondrial genomes of most organisms contain multiple copies of mtDNA, they encode only a small number of proteins that are essential for mitochondrial function. In the yeast Saccharomyces cerevisiae, mtDNA encodes eight major proteins. All other mitochondrial proteins, including those functioning in replication and maintenance of mtDNA, are encoded by the nuclear genome, synthesized in the cytosol, and imported into mitochondria.

Maintenance of mtDNA in S. cerevisiae cells is not essential for survival; and under laboratory conditions, cells lacking mitochondrial genomes are viable as long as they are provided with a fermentable carbon source (e.g. glucose, galactose). However, these cells are unable to respire, grow slowly, and are not able to compete with cells harboring intact mtDNA in their natural environment.

Biochemical mechanisms of replication and maintenance of mtDNA are not well understood (for review, see Ref. 1); however, extensive genetic and biochemical studies have revealed a number of proteins that are important for inheritance of mitochondrial genomes. These include proteins directly involved in synthesis of mtDNA: the mtDNA polymerase Mip1p (2); a single-stranded DNA-binding protein, Rim1p (3); the mitochondrial RNA polymerase Rpo41 (4); as well as other proteins with yet unknown functions in mtDNA propagation. Mitochondrial chaperones belong to this latter group.

The involvement of chaperones in the process of DNA replication is well known (for review, see Ref. 5). Generally speaking, chaperones either can play very specific roles in triggering replication machineries or can fulfill their general chaperone function by protecting proteins participating in DNA synthesis against stress-dependent inactivation. For example, DnaK, DnaJ, and GrpE are required for activation of a bacteriophage λ prepriming complex, and ClpX functions in activation of bacteriophage Mu DNA synthesis (5). In both cases, genetic and biochemical studies have established an essential role for chaperones in the propagation of these genomes. In contrast, the physiological importance of a general chaperone function in the propagation and maintenance of chromosomal DNA is much less clear. Biochemical studies have shown that bacterial RNA polymerase can be protected against thermal denaturation and inactivation by DnaK and its cochaperones (6). In addition, a bacterial DNA initiator protein, DnaA, can be protected and reactivated by DnaK in vitro (7, 8). Even less is known about chaperone functions in eukaryotic DNA replication. Mammalian homologs of DnaK and DnaJ have been shown to protect purified DNA polymerase ε against heat inactivation (9); however, the physiological relevance of this result remains to be elucidated.

One of the first indications that general chaperone function might be important for genome propagation in eukaryotes came from studies of yeast mitochondria. It was recently shown that inactivation of Mdj1p, the mitochondrial DnaJ homolog, results in loss of mitochondrial genomes from cells grown at high temperature (10, 11). Mdj1p is a co-chaperone in the mitochondrial Hsp70 system, consisting of Ssc1p, a homolog of DnaK, and Mge1p, a homolog of bacterial GrpE. The Hsp70 system is known to play an important role in mitochondria biogenesis, participating in protein folding, protein degradation, and in the protection of proteins against heat stress (12–16). Another mitochondrial chaperone protein, termed Hsp78p, is a member of the Clp/Hsp100 class of proteins that exerts chaperone functions that overlap with those of the mitochondrial Hsp70 system (17). Recent evidence suggested that Hsp78p is required for the maintenance of mitochondrial genome integrity under severe temperature stress (18). Biochemical studies using purified proteins indicated cooperation between the Hsp70 system and Hsp78p, as refolding of a model substrate protein in the presence of the bichaperone Hsp70-Hsp78 system is more efficient than in either system alone (19). However, very little is known about the physiological function of this mitochondrial bichaperone system.

Studies conducted on homologous systems in bacteria
(DnaK-ClpB) and in the yeast cytosol (Hsp70-Hsp104) provided some hints regarding the possible functions of the bichaperone system in mitochondria. DnaK and ClpB play key roles both in preventing and reversing aggregation of the bulk of bacterial proteins under severe stress conditions (20–23). Biochemical experiments have also confirmed the requirement for both DnaK and ClpB in the refolding of thermally denatured protein substrates (24–26). Similarly, in the yeast cytosol, protein aggregates formed under heat stress conditions are rapidly eliminated in an Hsp104-dependent manner during recovery under moderate temperature (27). Furthermore, purified components of the bichaperone Hsp70-Hsp104 system are able to efficiently reanimate denatured luciferase (28). Thus, bichaperone systems seem to be responsible for the refolding of aggregation-prone proteins. However, the contribution of chaperone-mediated protein refolding in the recovery of metabolic processes, inactivated under heat shock conditions, is still unclear.

In this study, we investigated the role of the mitochondrial bichaperone Hsp70-Hsp78 system in the maintenance and restoration of mtDNA synthesis under heat shock conditions in S. cerevisiae. We found that mtDNA synthesis was a thermosensitive process and that its restoration, following severe heat shock, was dependent on chaperone-mediated reactivation of Mip1p polymerase. To elucidate the biochemical mechanism of protection and reactivation of mtDNA polymerase, we purified Mip1p and four mitochondrial chaperones (Ssc1p, Mdj1p, Mge1p, and Hsp78p). We found that Mip1p activity was thermosensitive and that the mitochondrial bichaperone Hsp70-Hsp78 system was able to protect its activity under moderate heat shock conditions. In addition, these chaperones were able to reactivate thermally inactivated Mip1p.

**EXPERIMENTAL PROCEDURES**

**Strains, Growth Conditions, and Isolation of Mitochondria—**Standard genetic techniques were used for the growth and manipulation of yeast strains (29). The conditional *mdj1-5* mutant strain (30), the *Δhsp78* mutant strain (17), and their isogenic wild-type strains were described previously. The BY4742 strain (his3, leu2, lys2, ura3, *hsp78*-KAN, kindly provided by M. Boguta, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland) was used to test the effects of overproduction of Mip1p.

For construction of the *pYesMIP1* plasmid, the entire MIP1 coding region was cloned into the pYES2.0 vector (Invitrogen) using a procedure described previously (31) and plasmid YEpTT-3 (kindly provided by F. Foury, Université Catholique de Louvain, Louvain, Belgium) (32). Mitochondria were isolated as described (33).

**Purification of mtDNA Polymerase Mip1p—**Yeast cells harboring the MIP1-overexpressing plasmid *pYesMIP1* were grown to stationary phase in 400 ml of 2% glucose-containing synthetic complete medium without uracil (29). This culture was diluted 40-fold into 10 liters of 2% galactose-containing synthetic complete medium to induce the expression of Mip1p. When the culture reached an A600 = 4, the cells were harvested (~100 g of cells). Spheroplasts were generated by incubation of the cells with zymolase 20T (ICN) as described (33). Spheroplasts were isolated by centrifugation and resuspended in 100 ml of cold lysis buffer (40 mM HEPES-KOH (pH 8.0), 15% glycerol, 2 mM EDTA, 2 mM 2-mercaptoethanol, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 12 μg/ml pepstatin, and 7 μg/ml leupeptin). Spheroplasts were lysed using a French press (Aminoce) at 18,000 g.s.i. After a clarifying spin (15,000 × g, 20 min), proteins were precipitated with ammonium sulfate (0.28 g/ml), resuspended, dialyzed against 1 liter of buffer A (20 mM HEPES-KOH (pH 8.0), 20% glycerol, 5 mM EDTA, 2 mM 2-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride) containing 0.15 mM NaCl, and loaded onto a DEAE-Sepharose column (Amersham Biosciences). Proteins were eluted with a linear salt gradient (0.15–0.6 M NaCl). Fractions containing mtDNA polymerase activity were applied to a heparin-agarose column (Amersham Biosciences) equilibrated with buffer A containing 0.35 mM NaCl. Mip1p was eluted with a linear salt gradient (0.35–1.2 M NaCl), dialyzed against buffer A with 0.2 mM NaCl, and loaded onto a 2-ml double-stranded DNA-agarose column (Amersham Biosciences). The protein was eluted with a linear salt gradient (0.2–1 M NaCl). Fractions containing active Mip1p were pooled, dialyzed against buffer A containing 0.15 mM NaCl, and stored at −70 °C.

**Purification of Other Proteins—**Purification protocols were used for purification of Hsp72p (19) and Ssc1p (34). Purified Mdj1p and Mge1p were kindly provided by Frank King (University of Göttingen). Protein concentrations were determined with the Bio-Rad Bradford assay system using bovine serum albumin as a standard. Molar concentrations are given on the basis of a hexameric structure for Hsp72p (19) and of a monomeric structure for the other proteins.

**Measurements of mtDNA Polymerase Activity—**The activity of purified Mip1p was measured in 25 μl of buffer B (20 mM HEPES-KOH (pH 8.0), 20% glycerol, 2 mM 2-mercaptoethanol, 0.1 mM EDTA (pH 8.0), 10 mM MgCl2, 5 mM ATP, 10 mM creatinine phosphate, and 100 μM creatinine kinase) supplemented with 25 μM concentrations each of dATP, dCTP, dTTP, and [methyl-3H]dTPP (100–140 cpm/pmol dNTP). Either salmon sperm double-stranded DNA activated by DNase I treatment (prepared as described (32) at 0.13 mg/ml) or M13mp18 (kindly provided by M. Boguta, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland) was used as a template as indicated. The reaction was started by addition of purified Mip1p, and the samples were incubated at 30 °C for 10 min. The rate of incorporation of radioactive dTTP into DNA was measured by precipitating the labeled DNA with 750 μl of 10% trichloroacetic acid and 100 mM sodium pyrophosphate. The precipitates were collected by glass-fiber (Whatman GF/C) and then washed three times with 1 ml of 1 M hydrochloric acid and 100 mM sodium pyrophosphate and once with 2 ml of ethanol. The filters were dried, transferred to scintillation fluid, and counted.

**Separation of Monomeric and Aggregated Mip1p by Centrifugation—**Purified Mip1p was incubated for 10 min at 42 °C in 25 μl of buffer B. Samples were then layered on 50 μl of buffer B supplemented with 25% (w/v) sucrose in 500-μl microcentrifuge tubes. The samples were centrifuged in a Ti-50 rotor (Beckman Instruments) at 30,000 × g for 1 h at 4 °C. 25 μl of the upper layer was removed, and the remaining liquid was discarded. The protein pelleted at the bottom was resuspended in 25 μl of Laemmli sample buffer. An aliquot of the top fraction was used to measure replication activity. Both top and bottom fractions were subjected to SDS-PAGE analysis.

**Measurements of mtDNA Synthesis in Isolated Mitochondria—**For analysis of mtDNA synthesis in organelle, isolated mitochondria (50 μg of total protein) were incubated in 25 μl of medium containing 50 mM HEPES-KOH (pH 7.2), 500 mM sorbitol, 4 mM KH2PO4 (pH 7.2), 80 mM KC1, 10 mM MgCl2, 3% (w/v) bovine serum albumin, 10 mM creatinine phosphate, 100 μg/ml creatinine kinase, 2 mM ATP, 10 μM GTP, 10 μM CTP, 10 μM UTP, 25 μM dATP, 25 μM dCTP, 25 μM dGTP, 25 μM dTTP, 25 μM [methyl-3H]dTPP (100–140 cpm/pmol dNTP), and 50 μg/ml aphidicolin (Sigma). The rate of mtDNA synthesis was measured after incubation for 30 min at 30 °C by counting the radioactivity present in acid-insoluble material as described above.

**RESULTS**

**The Bichaperone Hsp70-Hsp78 System Is Responsible for Restoration of mtDNA Synthesis following Severe Heat Shock—**Our goal was to investigate a possible role of the mitochondrial bichaperone Hsp70-Hsp78 system in the protection and reactivation of mitochondrial DNA synthesis under heat shock conditions. To perform such a study, we first had to establish conditions under which the activity of mtDNA synthesis was severely reduced, but the survival rate of the cells was high, to avoid isolation of mitochondria from dead cells. We found that incubation of cells at 39 °C for 30 min (to induce thermotolerance), followed by an increase in temperature to 46 °C for another 30 min, did not affect the survival rate, as 90% of the cells gave rise to colony-forming units on plates (data not shown). However, this treatment resulted in a significant decrease in mtDNA synthesis. In mitochondria isolated from the wild-type cells immediately after the heat shock, we observed an 85% reduction in the rate of incorporation of radioactive deoxyribonucleotides into mtDNA (Fig. 1), indicating that mtDNA synthesis is a thermosensitive process.

Next, we investigated whether mtDNA synthesis could be restored when cells were allowed to recover for 2 h at 25 °C following the heat treatment. However, this restoration could be due to either reactivation of endogenous mitochondrial pro-
proteins or the import of newly synthesized proteins from the cytosol. To distinguish between these possibilities, we set up experimental conditions under which protein synthesis was inhibited. Cycloheximide, a cytosolic protein synthesis inhibitor, was added just before the recovery phase to prevent import of newly synthesized proteins into mitochondria. Under these conditions, the level of mtDNA synthesis could be restored, as mitochondrial proteins were isolated from the wild-type cells demonstrated 57% of the activity measured before the heat shock (Fig. 1). This result indicates that reactivation of thermolabile endogenous mitochondrial proteins involved in DNA synthesis occurs during the recovery phase after heat shock.

It is well documented that chaperones are able to protect proteins against heat-induced denaturation, but are also capable of dissociating protein aggregates and catalyzing protein refolding. To study the involvement of the bichaperone Hsp70-Hsp78 system in the restoration of mtDNA synthesis, we performed the experiment described above using mutants that inactivate each component of the system separately. To inactivate the Hsp70 system, we used the conditional mutant mdj1-5, which functions as a cochaperone of mitochondrial Hsp70. It was shown previously that mdj1-5p is inactive at elevated temperature, both in vivo and in organelle (10, 30, 35). Cells harboring the mdj1-5 allele subjected to severe heat stress exhibited a decrease in mtDNA synthesis in organelle, similar to the wild-type strain (Fig. 1). However, mdj1-5 cells demonstrated no significant increase in mtDNA synthesis following the recovery phase (Fig. 1). From this result, we concluded that active Mdj1p is essential for the restoration of mtDNA synthesis. Likewise, we used cells harboring a deletion of the HSP78 gene to determine the role of Hsp78p in the restoration of mtDNA synthesis. In Δhsp78 cells, mtDNA synthesis decreased significantly under severe heat stress conditions (Fig. 1). But, similar to the mdj1-5 strain, a significant restoration of mtDNA synthesis was not observed following the recovery phase (Fig. 1). From these results, we concluded that a bichaperone (Hsp70-Hsp78) system is essential for reactivation of mitochondrial proteins involved in mtDNA synthesis following severe heat shock.

mtDNA Polymerase Mip1p Is a Native Substrate for the Mitochondrial Bichaperone System—mtDNA propagation is a complex process dependent on many cellular activities. However, incorporation of deoxynucleotides into mtDNA is catalyzed by only one enzyme present in the mitochondrial matrix, Mip1p polymerase (1). We have shown previously that the activity of Mip1p polymerase under heat stress conditions depends on the function of Mdj1p, a cochaperone of the Hsp70 system (10). Thus, Mip1p is a likely candidate for a protein substrate that is reactivated during the heat shock recovery phase by the bichaperone Hsp70-Hsp78 system. It is also possible that the rate of mtDNA synthesis was limited by another protein involved in the mitochondrial replication complex. This hypothetical protein might also be inactivated at high temperature and reactivate during the recovery phase.

If inactivation and restoration of mtDNA synthesis are dependent on Mip1p polymerase activity, then the rate of mtDNA synthesis should be directly correlated with the cellular levels of Mip1p polymerase. Therefore, we repeated the experiments described above using cells harboring a plasmid that overproduces Mip1p (Fig. 2B). We found that 30-fold overproduction of Mip1p (data not shown) resulted in a 4-fold increase in the rate of mtDNA synthesis in organelle compared with an isogenic
strain without the plasmid (note the different levels of mtDNA synthesis in Fig. 2, A versus B). Moreover, in yeast cells overproducing Mip1p, the activity of mtDNA synthesis decreased following heat shock to 20% of the control value and increased after the recovery phase to 60% of the control value (Fig. 2B). Thus, regardless of the higher level of mtDNA synthesis measured in the strain that overproduces Mip1p, the relative values of inactivation-reactivation remained the same as in the control strain. We also observed that the steady-state levels of overproduced Mip1p did not change throughout the experiment (Fig. 2C), although the synthesis of a new polymerase was completely inhibited during the recovery phase by the presence of cycloheximide (Fig. 2, D and E). Thus, we concluded that the pool of inactive endogenous Mip1p polymerase is a substrate for the chaperone-mediated reactivation. Furthermore, this result suggests that Mip1p is the limiting factor for restoration of mtDNA synthesis and that changes in the levels of mtDNA synthesis in organelle are good indicators of the changes in Mip1p polymerase activity in vivo.

Contributions of Reactivation and Import of Newly Synthesized Mip1p to Recovery of mtDNA Synthesis after Heat Shock—As shown above, mtDNA synthesis from heat-treated cells efficiently recovered to 60% of the value of untreated cells in the absence of new protein synthesis. Next, we attempted to assess what contribution the import of newly synthesized Mip1p polymerase would make to the restoration of mtDNA synthesis following severe heat stress. To evaluate this contribution, we compared the levels of mtDNA synthesis in mitochondria isolated from cells that recovered in the presence of the protein synthesis inhibitor cycloheximide with the analogous values measured in mitochondria isolated from cells that recovered in the absence of cycloheximide. Two possible outputs of this experiment were expected: first, if the contributions of reactivation and import are similar, then mtDNA synthesis in mitochondria isolated from cells that recovered in the absence of cycloheximide should be significantly higher than in mitochondria isolated from cells that recovered in the presence of the protein synthesis inhibitor; and second, if the contribution of import to restoration of mtDNA synthesis is negligible, then mtDNA synthesis in mitochondria isolated from cells that recovered in either the presence or absence of cycloheximide should be similar.

We found little difference in the levels of mtDNA synthesis in wild-type cells that recovered in the presence or absence of cycloheximide (Fig. 3), indicating that import of newly synthesized Mip1p does not significantly contribute to the recovery process. If the rate of Mip1p synthesis is a limiting factor for Mip1p import and thus for restoration of mtDNA synthesis,
Chaperones Restore Mitochondrial DNA Synthesis

Fig. 3. Contributions of reactivation and import of Mip1p to recovery of mtDNA synthesis after heat treatment. Yeast strains were grown as described in the legend to Fig. 2, except that, after heat shock, each culture was divided, and at the beginning of the low temperature recovery phase, cycloheximide (150 μg/ml) was added to one-half (reactivation) and absent in the other half (reactivation + import). No cell division occurred during the recovery phase in either the presence or absence of cycloheximide. mtDNA synthesis in organelles was measured as described under “Experimental Procedures.” Triplicate samples were analyzed for two independent mitochondrial preparations. Bars represent restoration of mtDNA synthesis calculated by subtracting the values measured directly after heat shock from the results obtained following the recovery phase. Ranges of obtained results are indicated. The values of mtDNA synthesis measured in mitochondria isolated from cells grown at 25 °C before the heat shock treatment were set as 100%.

Fig. 4. Thermal inactivation of purified Mip1p polymerase and protection of its activity by chaperone proteins. Purified Mip1p (0.04 μM) was either added directly to the reaction mixture (No heat treatment) or incubated for 10 min at the indicated temperatures in 25 μl of buffer B (see “Experimental Procedures”) in the presence of the indicated factors: 2.7 μg of primed M13 single-stranded DNA, 4.8 μg of bovine serum albumin (BSA), Triton X-100 at a 0.01% (v/v) final concentration, and purified chaperone proteins (Ssc1p, 0.25 μM; Mdj1p, 0.05 μM; Mge1p, 0.025 μM; and Hsp78p, 0.1 μM). Next, samples were transferred to 30 °C for 10 min and supplemented with a radioactive dNTP mixture and primed M13 single-stranded DNA template (if not present during the heat treatment). DNA synthesis was measured as described under “Experimental Procedures.”

then increasing the contribution of import by overproducing Mip1p should increase recovery of mtDNA synthesis. Indeed, when we repeated the experiments described above using a wild-type strain harboring the plasmid that overproduces Mip1p, we observed that the level of mtDNA synthesis was 2-fold higher in the cells that recovered in the absence of the protein synthesis inhibitor (Fig. 3). These results demonstrate that the contribution of import of newly synthesized Mip1p polymerase to the restoration of mtDNA synthesis can be observed only when the rate of Mip1p synthesis is significantly increased.

As shown in Fig. 1, cells harboring a deletion of HSP78 are very inefficient in reactivating Mip1p polymerase. We took advantage of this fact to further study the contributions of reactivation and import of Mip1p to recovery of mtDNA synthesis. We compared the levels of restoration of mtDNA synthesis in mitochondria isolated from the Δhsp78 strain that recovered after heat shock in either the presence or absence of the protein synthesis inhibitor. In the presence of cycloheximide, the restoration of mtDNA synthesis in the Δhsp78 strain was at the very low level of 5% of the value measured for mitochondria isolated from the same strain grown at 25 °C. Moreover, a very limited 11% increase in mtDNA synthesis was observed in the absence of the protein synthesis inhibitor (Fig. 3). This result further supported our conclusion that import of newly synthesized Mip1p polymerase was very ineffective in restoring mtDNA synthesis, even when reactivation of endogenous Mip1p was inhibited by lack of Hsp78p. In contrast, a high level of mtDNA synthesis (47% of the control value) was measured following the recovery phase in the absence of cycloheximide in the Δhsp78 strain overproducing Mip1p (Fig. 3). From these results, we concluded that, during the recovery from severe heat shock, the reactivation of Mip1p polymerase mediated by the mitochondrial bichaperone system is faster and more efficient than import of newly synthesized proteins, underscoring the physiological importance for the protein reactivation process.

Mitochondrial Chaperones Protect Mip1p Polymerase in Vitro—To directly test whether Mip1p is indeed a thermolabile protein that can be protected against heat inactivation by the bichaperone Hsp70-Hsp78 system, we purified Mip1p and the mitochondrial chaperones Mdj1p, Mge1p, Ssc1p, and Hsp78p. The activity of purified Mip1p polymerase was tested by measuring incorporation of [3H]dTTP into a DNA template (see “Experimental Procedures” for details). We found that Mip1p was indeed thermosensitive (Fig. 4). Incubation of purified Mip1p at elevated temperatures resulted in rapid loss of DNA synthesis activity. After a 10-min incubation at 42 °C, the enzyme lost 98% of its activity compared with the same amount of protein assayed without the heat treatment. Next, we tested whether mitochondrial chaperones are able to protect Mip1p against thermal denaturation. We added purified mitochondrial chaperones to the reaction mixtures containing Mip1p protein and incubated them at elevated temperatures. Following this incubation, DNA synthesis activity was measured at 30 °C as described under “Experimental Procedures.” After testing different combinations of Mdj1p, Mge1p, Ssc1p, and Hsp78p (data not shown), we found that the most efficient protection was observed when a mixture of all four chaperones was present together with Mip1p (Fig. 4) and that such treatment did not affect processivity of the polymerase (data not shown). These results indicated that the bichaperone Hsp70-Hsp78 system was able to protect Mip1p polymerase under
It is well documented that chaperones are able to maintain mtDNA polymerase in its native conformation. However, under moderate heat stress conditions (42°C), but was not very efficient when the temperature was raised to 46°C (15% of activity) and was completely inactive at 50°C (data not shown). To confirm that molecular chaperones play a specific role in the protection of Mip1p against heat inactivation, we also tested how the presence of other factors, including DNA template, bovine serum albumin at concentrations equal to the concentration of chaperone proteins, and Triton X-100, affects polymerase activity. We found that none of these factors was able to protect Mip1p against heat inactivation.

In an effort to understand the mechanism underlying Mip1p inactivation, we tested whether protein aggregation is responsible for the loss of Mip1p activity. Following incubation at the elevated temperature, the reaction mixture containing Mip1p was centrifuged through a 20% (w/v) sucrose solution to separate the monomeric soluble fraction from the aggregated proteins that formed a pellet. We found that untreated Mip1p localized predominately in the fraction containing soluble protein (top) (Fig. 5A). In contrast, incubation for 10 min at 42°C resulted in extensive aggregation, as most of the Mip1p was found in the pellet. In the presence of the four purified chaperones, Mip1p was distributed equally between the soluble and aggregated fractions (Fig. 5A). The presence of Mip1p in the soluble fraction correlated well with its level of DNA synthesis activity (Fig. 5B), indicating that mitochondrial chaperones are able to maintain mtDNA polymerase in its native conformation under moderate heat stress conditions.

Bichaperone-mediated Reactivation of Aggregated Mip1p Polymerase in Vitro—It is well documented that chaperones are not only able to protect proteins against heat-induced denaturation, but are also capable of dissociating protein aggregates and catalyzing protein refolding (for review, see Ref. 36). To test whether the mitochondrial bichaperone Hsp70-Hsp78 system is able to dissociate aggregates of Mip1p and to restore its DNA synthesis activity, purified Mip1p was incubated for 10 min at 42°C, and then the purified chaperone proteins, listed above, were added to the reaction mixture in the presence of ATP. After a recovery incubation for 10 min at 30°C, DNA synthesis was measured at 30°C (see “Experimental Procedures”). The presence of the complete bichaperone Hsp70-Hsp78 system resulted in reactivation of Mip1p to 37% of the value without the heat treatment (Fig. 6). Lack of either Hsp78p or chaperones of the Hsp70 system resulted in a very significant decrease in the efficiency of the Mip1p reactivation reaction. These results correlated well with our observations in vivo. They also indicated that Mip1p polymerase is a native substrate for mitochondrial chaperones and that its enzymatic activity can not only be protected under heat shock conditions, but may be regained when the temperature drops below the heat shock threshold.

**DISCUSSION**

Several lines of evidence indicate that Mip1p is a native substrate of the mitochondrial bichaperone system. In *vivo* experiments show that Mip1p is indeed a thermosensitive protein and that, under moderate heat stress conditions, mitochondrial chaperones are able to prevent its inactivation. These results correlated well with *in vivo* experiments showing that, in a wild-type strain, moderate heat stress conditions do not affect the level of mtDNA synthesis *in organelle* (10). However, in strains harboring either an *mdj1-5* mutant (10) or a deletion of the *HSP78* gene,^1^ levels of mtDNA synthesis decrease significantly at elevated temperature. These results indicate that the mitochondrial bichaperone system is required to maintain

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^1^ A. Germaniuk and J. Marszalek, unpublished data.
Mip1p activity. The thermosensitivity of Mip1p polymerase is not surprising considering its large size (143.5 kDa). Analysis of thermolabile proteins in Escherichia coli reveals that large proteins (>90 kDa) make up 80% of the thermosensitive protein fraction (22). Furthermore, the heat sensitivity of partially purified Mip1p has been previously reported (37).

However, when the temperature rises above 42 °C, reaching values characteristic for severe heat stress conditions, purified mitochondrial chaperones are not able to protect Mip1p polymerase against inactivation. A similar effect was observed in vivo, as the levels of mtDNA synthesis dropped significantly in mitochondria isolated from wild-type cells subjected to severe heat stress. When the same cells were allowed to recover for 2 h at moderate temperature, the level of mtDNA synthesis was restored up to 60% of the value measured before heat treatment. Moreover, this restoration was observed when import of mitochondrial proteins from the cytosol was prevented by incubating cells in the presence of the cytosolic protein synthesis inhibitor cycloheximide. These results indicate that restoration of mtDNA synthesis is dependent on the reactivation of endogenous mitochondrial proteins. Further analysis using cells harboring either mdj1-5 or Hsp78 mutations indicated that the bichaperone Hsp70-Hsp78 system is essential for the reactivation of mtDNA synthesis in vivo. Results of in vitro experiments using purified proteins confirmed the ability of the mitochondrial bichaperone system to reactivate aggregated Mip1p polymerase. Specifically, both the Hsp70 and Hsp78 components of the bichaperone system are required for the efficient reactivation of Mip1p. However, a distinction between protection and reactivation of Mip1p polymerase is probably artificial, as one can imagine that, under changing environmental conditions, both protection and reactivation take place at the same time. This is supported by the fact that the bichaperone Hsp70-Hsp78 system was involved in both functions.

Because we identified Mip1p polymerase as a native substate of the mitochondrial bichaperone system, we were able to assess the significance of both the reactivation of thermally inactivated endogenous Mip1p and the import of newly synthesized Mip1p to the restoration of mtDNA synthesis. By manipulating the level of Mip1p protein synthesis and by inhibiting the reactivation of endogenous Mip1p using a Hsp78 strain, we have demonstrated that the reactivation of endogenous Mip1p is more efficient in its contribution to the restoration of mtDNA synthesis than to import of newly synthesized Mip1p when Mip1p is produced at physiological levels. The contribution of imported Mip1p is comparable to that provided by the reactivation of endogenous Mip1p only when Mip1p is overproduced. To our knowledge, these are the first experiments to directly estimate the relative contributions of the reactivation of a protein versus protein synthesis to the restoration of a physiologically important process following heat shock. It was shown previously that other cellular processes, e.g., mRNA splicing (38) and mitochondrial translation (18, 39), are disrupted upon severe heat shock conditions and are reactivated in chaperone-mediated processes. However, in these cases, it was impossible to determine the protein substrates that were reactivated by chaperones and thus difficult to estimate the physiological importance of protein reactivation to the restoration of splicing and translation.

What is the molecular mechanism of the bichaperone-mediated reactivation of Mip1p in vivo? One possible scenario is that, under severe heat stress, Mip1p dissociates from the mtDNA and forms a large protein aggregate. When the temperature drops below the heat shock threshold, the mitochondrial bichaperone system may dissociate the aggregate and refold Mip1p. This mechanism is in agreement with our in vitro observations that Mip1p incubated at high temperatures forms aggregates large enough to pellet in 20% sucrose solution and that purified chaperone proteins are able to restore Mip1p activity. The ability of the mitochondrial bichaperone Hsp70-Hsp78 system to dissociate protein aggregates was shown previously using aggregated model substrate proteins (19). Moreover, formation of protein aggregates under heat stress conditions was observed both in bacteria (20, 21) and in yeast (27). In these organisms, molecular chaperones were shown to be responsible for elimination of protein aggregates upon recovery at moderate temperature.

An alternative scenario is based on the recent observations that several molecular chaperones are present in the isolated protein-mtDNA complexes called nucleoids (40). These are believed to be units of replication and segregation of mtDNA. We have also been able to isolate nucleoids and to detect the presence of Scs1p, Mdj1p, Mge1p, and Mip1p in the protein-mtDNA complex. What is the function of chaperones in the nucleoid? One possibility is that chaperones are attracted to the nucleoid by the presence of their natural substrates, like Mip1p, and probably other proteins involved in mtDNA metabolism. Thus, the presence of chaperones in the nucleoid protein-DNA complex might explain why reactivation of Mip1p is so efficient. If the thermal inactivation of mtDNA polymerase occurs within the scaffold of the nucleoid, then the rapid reactivation by chaperones that are present in the near vicinity would be possible as soon as the temperature drops below the heat shock threshold. Indeed, we have observed that preformed complexes of purified Mip1p with primed M13 single-stranded DNA template remained intact during heat shock treatment, even though the DNA synthesis activity was lost. This attractive hypothesis will be a subject of further research in our laboratory.

Is the role of mitochondrial chaperones in mtDNA synthesis restricted to their chaperone function? From our work, we can conclude that mitochondrial chaperones play a critical role in maintaining the activity of at least one important replication protein; however, this does not exclude the involvement of chaperones in more specific mechanisms of mtDNA replication. For example, we have shown previously that deletion of the MDJ1 gene results in the transition from a functional mitochondrial genome (rho–) into its nonfunctional, deleted form (rho−) (10). The molecular mechanism of this phenomenon remains to be elucidated. We would like to stress, however, that anybody who studies the specific role of molecular chaperones in mtDNA metabolism should not forget about their general chaperone function and should keep in mind that, because of the thermal sensitivity of mtDNA synthesis, the use of temperature-sensitive mutants would be significantly limited.

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REFERENCES

1. Lercrenier, N., and Fourny, F. (2000) Gene (Amst.) 246, 37–48
2. Geng, A., Bianchi, L., and Fourny, F. (1986) J. Biol. Chem. 261, 9328–9332
3. Difley, J. F., and Stillman, B. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7864–7868
4. Greenleaf, A. L., Kelly, J. L., and Lehman, I. R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 3391–3394
5. Konieczny, I., and Zylstra, M. (1999) Genet. Eng. 21, 95–111
6. Skowrya, D., Georgopoulos, C., and Zylstra, M. (1999) Cell 92, 939–944
7. Baneci, B., Kaguni, J. M., and Marszalek, J. (1998) Biochim. Biophys. Acta 1442, 39–48
8. Hwang, D. S., Crooke, E., and Kornberg, A. (1990) J. Biol. Chem. 265, 19244–19248
9. Ziemienowicz, A., Zylstra, M., Frolo, C., and Huber, U. J. (1995) J. Biol. Chem. 270, 15479–15484
10. Duchniewicz, M., Germaniak, A., Westermann, B., Neupert, W., Schwarz, E., and Marszalek, J. (1999) Mol. Cell. Biol. 19, 8201–8210
11. Rowley, N., Prup-Buus, C., Westermann, B., Brown, C., Schwarz, E., Barrell, B., and Neupert, W. (1994) Cell 77, 249–259

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