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HSP78 Encodes a Yeast Mitochondrial Heat Shock Protein in the Clp Family of ATP-Dependent Proteases

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The *Saccharomyces cerevisiae* nuclear gene for a 78-kDa mitochondrial heat shock protein (hsp78) was identified in a λgt11 expression library through immunological screening with an hsp78-specific monoclonal antibody. Sequencing of *HSP78* revealed a long open reading frame capable of encoding an 811-amino-acid, 91.3-kDa basic protein with a putative mitochondrial leader sequence and two potential nucleotide-binding sites. Sequence comparisons revealed that hsp78 is a member of the highly conserved family of Clp proteins and is most closely related to the *Escherichia coli* ClpB protein, which is thought to be an ATPase subunit of an intracellular ATP-dependent protease. The steady-state levels of *HSP78* transcripts and protein varied in response to both thermal stress and carbon source with an approximately 30-fold difference between repressed levels in cells growing fermentatively on glucose at 30°C and derepressed levels in heat-shocked cells growing on a nonfermentable carbon source. The response to heat shock is consistent with the presence of a characteristic heat shock regulatory element in the 5′-flanking DNA. Submitochondrial fractionation showed that hsp78 is a soluble protein located in the mitochondrial matrix. Cells carrying disrupted copies of *HSP78* lacked hsp78 but were not impaired in respiratory growth at normal and elevated temperatures or in the ability to survive and retain mitochondrial function after thermal stress. The absence of a strong mitochondrial phenotype in *hsp78* mutants is comparable to the nonlethal phenotypes of mutations in other Clp genes in bacteria and yeast. *HSP78* is the third gene, with *SSC1* and *HSP60*, known to encode a yeast mitochondrial heat shock protein and the second gene, with *HSP104*, for a yeast ClpB homolog.

Cells exposed to physiological stress, such as an abrupt elevation in temperature, synthesize a subset of highly conserved cellular proteins collectively known as the heat shock or stress proteins. Many members of the heat shock protein (HSP) families are expressed in the absence of stress and function as molecular chaperones in the folding of newly synthesized polypeptide chains and in the assembly of oligomeric structures (for recent reviews, see references 2 and 13). Heat shock proteins also appear to be involved in the degradation of unfolded or damaged polypeptides, either by catalyzing proteolysis or by presenting polypeptides to the active proteases. Well-known examples of heat-shock-inducible proteins involved in protein degradation are ubiquitin in eukaryotes (55) and the ATP-dependent La (Lon) and ClpP proteases in prokaryotes (4, 30). In addition, members of the newly discovered family of Clp proteins appear to combine an involvement in proteolysis with many of the characteristics of molecular chaperones, i.e., several are heat shock proteins, they are highly conserved in both prokaryotes and eukaryotes, and in eukaryotes, multiple forms or subfamilies appear to be partitioned in different cellular compartments.

The wide distribution of the Clp family of proteins became apparent when the sequence of the ClpA subunit of the two-component ClpA-ClpP ATP-dependent protease (18), also known as the Ti protease (21), was found to be related to a number of previously described proteins with known sequences but unknown functions. These proteins, now referred to as Clp proteins, share two large, highly conserved sequence blocks, each centered around a characteristic nucleotide-binding domain (17, 59). The sequences at the amino- and carboxy-terminal ends are not conserved, and the two highly conserved ATP-binding domains are separated by a highly variable spacer sequence, the length of which is a diagnostic characteristic for distinguishing members of the ClpA, ClpB, and ClpC subfamilies.

Most of our current understanding of the function of the Clp proteins comes from biochemical and genetic studies of *Escherichia coli* ClpA. The 87-kDa ClpA polypeptide associates with the 21-kDa ClpP peptidase in a stoichiometry of 6 ClpA to 12 ClpP to form a proteolytically active complex of approximately 750 kDa (37). ClpP by itself can hydrolyze small peptides, but it must be complexed with ClpA in order to attack larger substrates. Since ClpA is a protein-stimulated ATPase with no intrinsic proteolytic activity, it is considered to be a regulatory subunit in the ATP-dependent protease. The primary function of the ClpA-ClpP complex appears to be degradation of unfolded or abnormal proteins (37). In this regard, the Clp proteases may function in concert with the chaperones to provide alternative folding or degradation pathways for newly synthesized proteins. Although *clpA* function is required for the specific degradation of proteins by the N-end rule in bacteria (63), *clpA* mutants are not impaired either in growth rate or in the degradation of abnormal canavinine-containing proteins (27).

The ClpB protein in *E. coli* is larger than ClpA (857 versus 756 amino acids), has a longer spacer region between the two nucleotide-binding sites (124 versus 5 amino acids), and is induced by heat shock whereas *clpA* is not (16, 28, 60). *E. coli* also expresses a truncated ClpB′ protein by using an alternate translational initiation site in *clpB* at codon 149 (60). Null mutants of *clpB* are defective in growth and

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survival at high temperatures (60). The ClpB protein was purified recently from heat-shocked *E. coli* cells that carried the *clpB* gene on a multicopy plasmid (65). Purified ClpB, like ClpA, has protein-stimulated ATPase activity, but the two purified proteins differ significantly in their structural and biochemical properties. It is not known whether ClpB forms complexes with ClpP in vivo, but in vitro, ClpB does not substitute for ClpA in activating the ClpP peptidase (65).

ClpB proteins in *Trypanosoma brucei*, *Bacteroides nodosus*, and *Saccharomyces cerevisiae* are also heat shock induced (59). Of these, the best-characterized is the 908-amino-acid hsp104 protein of yeast, which is found in the nucleus and the cytoplasm and appears to be necessary for induced thermotolerance (44, 52).

The ClpC subfamily is characterized by a spacer region of approximately 60 amino acids and contains four known members from plants and one from bacteria (59). The nuclear gene-encoded plant ClpC proteins have N-terminal sequences that resemble transit sequences for import into chloroplasts (18), and the pea Clp protein has been localized to the soluble fraction of chloroplasts (41). It is significant that several chloroplast genomes contain a sequence for a ClpP protein (39), suggesting that chloroplasts might contain ClpC-ClpPATP-dependent proteases. ATP-dependent proteolytic activity has previously been observed in chloroplast extracts (36).

To date, relatively few proteolytic activities have been identified in mitochondria (for reviews, see references 15 and 49). The best-characterized are the processing peptidases in the mitochondrial matrix that cleave mitochondrial targeting presequences (46). In addition, three inner membrane proteolytic activities that act on cytochrome *c* have been found in *Saccharomyces cerevisiae*, and matrix enzymes resembling the La (Lon) protease have been isolated from rat liver and bovine adrenal mitochondria (15).

Here we describe the identification and initial characterization of a yeast nuclear gene (*HSP78*) for a 78-kDa, heat-shock-induced, catabolite-repressed mitochondrial protein in the ClpB subfamily. Thus, yeast cells maintain at least two nuclear genes, *HSP78* and *HSP104*, for the expression of ClpB-like proteins that function in the mitochondrial and nuclear-cytoplasmic subcellular compartments, respectively.

MATERIALS AND METHODS

Immunological reagents and procedures. During the production of murine monoclonal antibodies to proteins in the small subunit of the yeast mitochondrial ribosome, we isolated antibodies against mitochondrial proteins that were nonribosomal and heat shock induced. Two of these monoclonal antibodies recognized the 64-kDa molecular chaperone hsp60 (23). A third antibody reacted with the subject of this study, a previously unidentified 78-kDa protein that we have named hsp78. These monoclonal antibodies were used in the form of culture supernatants from the corresponding hybridoma cell lines.

Rabbit antisera to yeast cytochrome *c* peroxidase and subunit II of cytochrome *c* oxidase were kindly provided by G. Schatz (Biozentrum, Basel, Switzerland), and the rabbit antiserum to citrate synthase was kindly provided by P. Srere (VA Hospital, Dallas, Tex.). Immune complexes were decorated with ^{125}I-labeled sheep anti-mouse immunoglobulins (Igs) (Boehringer-Mannheim Biochemicals) for monoclonal antibodies and ^{125}I-labeled protein A (Sigma) for rabbit antibodies. These reagents were iodinated according to the method of Hunter and Greenwood (20). In some cases, as indicated in the text, immune complexes were visualized with either biotinylated sheep anti-mouse Igs (Amersham) or biotinylated donkey anti-rabbit Igs (Amersham) in conjunction with streptavidin-conjugated horseradish peroxidase (Amersham) and the chromogenic substrate 4-chloro-1-naphthol (Sigma).

Immunoblotting was performed as described elsewhere (9). The polypeptides in whole-cell extracts and subcellular fractions (6) were resolved by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) (32). Protein concentration was determined by the method of Lowry et al. (35).

The yeast genomic library in λgt11 (prepared by Michael Synder) was screened with the anti-hsp78 monoclonal antibody as described by Young and Davis (68). Two immunopositive recombinants (designated λ3 and λ5) were identified in a screen of approximately 250,000 plaques and were purified through several cycles of subcloning.

***S. cerevisiae* strains and media.** The *S. cerevisiae* strains used were 22-2D [*rho*$^+$] (MAT**a** *ura3-52 trp1 leu2-3,112 cyh2 can1*) and SB9882-4CR [*rho*$^+$] (MAT**a**/α *ura3-52/ura3-52 trp1-289/trp1-289 leu2-3,112/+ his4-519/+ can1 cry*^{r}) from J. Carbon. Cells were grown in the following media: YPD containing 1% yeast extract, 2% Bacto Peptone (Difco Laboratories), and 2% glucose; YPGE containing 1% yeast extract, 2% Bacto Peptone, 2% glycerol, and 2% ethanol; SD minimal medium containing 0.67% yeast nitrogen base without amino acids (Difco), 2% glucose, and nutritional supplements as required (56).

DNA manipulations and hybridizations. Standard procedures were used for *E. coli* transformation and in vitro manipulation of DNA (51), and for the growth and maintenance of phage stocks and the preparation of phage DNA (57). Yeast transformations were performed by the lithium acetate procedure of Ito et al. (22). Enzymes were used according to the specifications given by the supplier. DNA restriction fragments were purified from agarose gels with the Qiaex gel extraction kit (Qiagen). DNA hybridization probes were ^{32}P labeled by random priming as described by Feinberg and Vogelstein (11). For Northern (RNA) blot analysis, total yeast RNA was isolated as described previously (62) and the concentration of RNA was estimated from the A_{260}. The RNAs were resolved by electrophoresis in a 6% formaldehyde–1% agarose gel with 0.2 M MOPS (3-[*N*-morpholino]propanesulfonic acid), pH 7.0, as the running buffer and transferred to GeneScreen Plus (Dupont). The blots were prehybridized and hybridized according to protocols provided by the manufacturer. After hybridization, the blots were washed once in 2× SSC (20× SSC is 3 M sodium chloride plus 0.03 M sodium citrate)–1% SDS at 60°C for 1 h and once in 0.2× SSC–0.1% SDS at 60°C for 1 h. Southern blot analysis was performed essentially as described by Southern (58) with total yeast DNA isolated as described by Sherman et al. (56).

Primer extension analysis. Primer extension analysis was essentially as described by Kuechler et al. (31). A 25-μg amount of RNA was treated with proteinase K (50 mg/ml) at 37°C for 30 min before hybridization with 10 ng of a ^{32}P-labeled (5′ end) oligonucleotide complementary to nucleotides +3 to +23 in the *HSP78* sequence (see Fig. 2). The hybridized DNA primer was extended with 200 U of Superscript RT reverse transcriptase (GIBCO-Bethesda Research Laboratories) in a 20-μl reaction mixture volume at 50°C for 90 min.

DNA sequence analysis. The sequence of *HSP78* was

determined from both DNA strands by the dideoxy chain termination method (54) with single-stranded template from subclones in M13 (40). The National Biomedical Research Foundation Protein Data Base, the SwissProt Data Base, and GenBank-EMBL were searched by the method of Pearson and Lipman (45). Protein sequences were aligned with the PILEUP program in the University of Wisconsin Genetics Computer Group Sequence Analysis software package, version 7.1 (8).

Isolation of yeast mitochondria and protease protection. Mitochondria were prepared essentially as described by Daum et al. (6). Spheroplasts were washed twice in ice-cold mannitol buffer (0.6 M mannitol, 20 mM Tris-HCl [pH 7.4], 1 mM EDTA) and resuspended to 500 mg (wet weight)/ml in the same buffer containing protease inhibitors (24). After being chilled on ice, the suspension of spheroplasts was equilibrated with N_2 gas (300 lb/in^2) in a Parr bomb for 15 min and the spheroplasts were lysed by a quick release of the pressure (69). Mitochondria used in the preparation of mitoplasts were resuspended in freezing medium (29), frozen at −80°C, and thawed just before use. Mitoplasts were prepared by osmotic shock treatment of freeze-thawed mitochondria and treated with protease essentially as described by Glick et al. (14). Membrane and soluble fractions were prepared from whole mitochondria as described previously (6), except that ultrasonic irradiation was done in a buffer containing 100 mM NaCl, 20 mM K^+-HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.5), 1 mM EDTA, and 1 mM PMSF (phenylmethylsulfonyl fluoride). Supernatant (soluble) and pellet (membrane) fractions were obtained by centrifugation at 100,000 × *g* for 20 min.

Nucleotide sequence accession number. The sequence for *HSP78* has been assigned GenBank number L16533.

RESULTS

Cloning and sequencing of *HSP78*. In the preparation of monoclonal antibodies to yeast mitochondrial ribosomal proteins, we identified antibodies to two different mitochondrial, nonribosomal, heat shock proteins (10). Two of these antibodies recognized the molecular chaperone hsp60 (23); a third antibody reacted with a 78-kDa polypeptide, which we have named hsp78. Since a 78-kDa mitochondrial heat shock protein had not been described previously for *S. cerevisiae*, we used the anti-hsp78 antibody to immunologically screen a yeast genomic DNA library in λgt11 for clones with potential hsp78 coding sequences (68). Two immunopositive clones, designated λ3 and λ5, were obtained and shown by Southern hybridization analysis to contain overlapping segments of DNA derived from a single copy sequence in the yeast genome (data not shown). By assuming the epitope recognized by the hsp78 monoclonal antibody was encoded within the overlapping region of the λ3 and λ5 DNA inserts, it was possible to deduce the orientation and position of the hsp78 coding region in the cloned yeast DNA fragments (Fig. 1A). The presence of the predicted open reading frame for hsp78 was confirmed by sequencing appropriate subclones of yeast DNA derived from λ3 and λ5 (see Materials and Methods).

The complete nucleotide sequence of *HSP78* with its flanking regions is shown in Fig. 2. The sequence contains a single long open reading frame of 2,433 nucleotides capable of encoding an 811-amino-acid polypeptide with a pI of 9.28 and a calculated molecular mass of 91,262 Da. Although the size of the predicted polypeptide is significantly larger than the size of hsp78 estimated from its electrophoretic mobility in SDS-PAGE, this discrepancy could be the result of

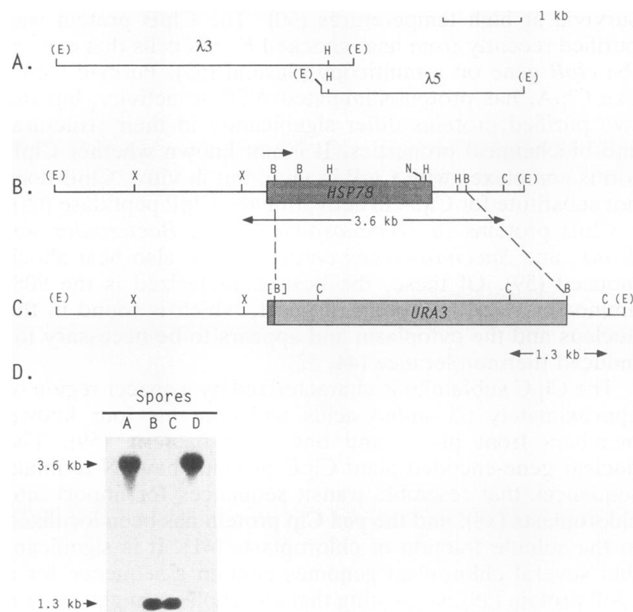

FIG. 1. Restriction maps of *HSP78* and the structure of the *hsp78::URA3* allele. (A) Partial restriction maps of the λgt11 clones λ3 and λ5, showing the region of overlap. Since the monoclonal antibody reacted with the polypeptides expressed from both λ3 and λ5, the epitope is most likely encoded within the overlap sequence. The *Eco*RI sites introduced during cloning in λgt11 are in parentheses. (B) Restriction map of *HSP78*. The *HSP78* open reading frame is indicated by a shaded box. The arrow above the box indicates the direction of transcription. (C) Structure of the *hsp78::URA3* deletion-insertion mutation. The linear 6.8-kb *Eco*RI fragment was used to replace the genomic copy of *HSP78* in the haploid strain 22-2D and the diploid strain SB9882-4CR. The *Bgl*II site lost by the insertion of *URA3* is indicated by brackets. (D) Southern analysis of genomic DNA from spores in a tetrad from a Ura^+ transformant of SB9882-4CR. The DNA was digested with *Xba*I and *Cla*I and probed with a ^{32}P-labeled 1.1-kb *Nco*I-*Cla*I fragment derived from the *HSP78* coding region and 3′-flanking sequences. Spores A and D are *HSP78*; B and C are *hsp78::URA3*. Abbreviations: E, *Eco*RI; X, *Xba*I; B, *Bgl*II; H, *Hin*dIII; N, *Nco*I; C, *Cla*I.

proteolytic cleavage of a mitochondrial targeting presequence or anomalous electrophoretic migration, or both. The amino acid composition of the first 53 amino acids in the predicted hsp78 sequence is consistent with the properties of known mitochondrial leader peptides (46). Two TATA box-like sequences are present at positions −103 and −93 in the 5′ upstream sequence (61), and the polyadenylation signals TACATA (47) and AATAAA (25) are present in the downstream sequence. In addition, the sequence between positions −247 and −230 matches a characteristic heat shock regulatory element containing GAA segments repeated at 2-nucleotide intervals in alternating orientations (1, 66). Four pentamers of nGAAn lie within this sequence; a minimum of three such units is required for a competent heat shock regulatory element (1). The 5′-flanking region of *HSP78* contains two elements that resemble consensus MIG1-binding sites. These sites have been implicated in the glucose repression of several yeast genes (42) and are composed of an AT-rich stretch at the 5′ end of a GGGG motif (19). The first such sequence in *HSP78* begins at position −201 (5′-TgAATAGGGGG-3′), and the second is inverted at position −131 (5′-CCCCCTAgTTA-3′). The spacing and inverted

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-353                                                                                                 ACTGGAAGATCAACTTTTTGCTTTGGCCTTAT
-321   AGGTACTATAAATTTTAATAATTAATGTAACAGACAAATCAAGGTGTGATTCTGTTCTGATAAAATTTGTTATTTTCCAGAATGTTCTAGAAGTGTTCTTTGATCTA
-214   ATGGCATCGCGTTTGAATAGGGGGTGCACGGACTTTTTTTTTTGAATAAACCCCTGTGATTCATTGTATCTAAGCTCATCAAACCCCCTAGTTACTGACTCACGCAC
                                                                                    →   →   →
-107   ACACATATATATACATATATATGCATATGTAAGCATGAAAAGTTTGACTTCTATTATCTATTCCAGGAACCCTGAAACAAGCGAGTGAAAATCTTTCAAGGTTAAAT

+1     ATG TTA AGA CAA GCT ACA AAA GCA CCA ATA CAA AAA TAT TTA CAG AGA ACG CAA CTA TTA AGG CGA AGT ACT CCA CGA ATA
+1      M   L   R   Q   A   T   K   A   P   I   Q   K   Y   L   Q   R   T   Q   L   L   R   R   S   T   P   R   I

+82    TAC ACT ATT GTA CAG TGT AAA AGA TCT ATT TGC AGT TTT AAT GCA AGA CCT CGA GTA GCC AAT AAA CTT CTA TCA GAT ATA
+28     Y   T   I   V   Q   C   K   R   S   I   C   S   F   N   A   R   P   R   V   A   N   K   L   L   S   D   I

+163   AAA ACA AAT GCA TTA AAT GAA GTC GCT ATT TCA ACC TGC GCC CTC AAG AGC TCA TAT GGA TTG CCC AAT TTC AAA AGA ACG
+55     K   T   N   A   L   N   E   V   A   I   S   T   C   A   L   K   S   S   Y   G   L   P   N   F   K   R   T

+244   TAC GTC CAA ATG AGG ATG GAT CCC AAT CAG CAA CCG GAA AAG CCA GCT TTA GAA CAA TTT GGT ACA AAC TTG AAA AAA TTA
+82     Y   V   Q   M   R   M   D   P   N   Q   Q   P   E   K   P   A   L   E   Q   F   G   T   N   L   K   K   L

+325   GCG CGC GAC GGT AAA TTA GAC CCT GTC ATT GGC AGA GAC GAA GAA ATC GCA AGA GCT ATT CAA ATT TTA TCA AGA AGA ACA
+109    A   R   D   G   K   L   D   P   V   I   G   R   D   E   E   I   A   R   A   I   Q   I   L   S   R   R   T

+406   AAA AAT AAT CCA TGT TTG ATC GGT CGA GCT GGT GTC GGT AAA ACC GCC CTT ATT GAT GGG TTA GCT CAA AGA ATT GTT GCT
+136    K   N   N   P   C   L   I   G   R   A   G   V   G   K   T   A   L   I   D   G   L   A   Q   R   I   V   A

+487   GGG GAG GTC CCT GAC TCG TTG AAA GAT AAA GAT CTA GTA GCT TTG GAT TTG GGA TCT TTG ATT GCT GGC GCC AAA TAT AGA
+163    G   E   V   P   D   S   L   K   D   K   D   L   V   A   L   D   L   G   S   L   I   A   G   A   K   Y   R

+568   GGT GAA TTC GAA GAA CGT TTA AAA AAG GTT TTA GAA GAA ATT GAC AAG GCC AAT GGT AAG GTT ATT GTT TTC ATT GAT GAA
+190    G   E   F   E   E   R   L   K   K   V   L   E   E   I   D   K   A   N   G   K   V   I   V   F   I   D   E

+649   GTT CAT ATG CTG CTT GGC TTA GGT AAA ACT GAT GGT AGT ATG GAT GCT TCT AAC ATC TTA AAG CCA AAA CTG GCC CGT GGG
+217    V   H   M   L   L   G   L   G   K   T   D   G   S   M   D   A   S   N   I   L   K   P   K   L   A   R   G

+730   CTA GCT TGT ATC TCT GCA ACC ACT CTT GAT GAA TTC AAA ATC ATT GAA AAA GAT CCG GCT TTA TCG AGA AGG TTT CAG CCT
+244    L   A   C   I   S   A   T   T   L   D   E   F   K   I   I   E   K   D   P   A   L   S   R   R   F   Q   P

+811   ATC TTA TTG AAT GAG CCG AGC GTC TCC GAT ACG ATT TCT ATA CTA AGA GGT TTA AAA GAA AGG TAC GAG GTT CAT CAT GGT
+271    I   L   L   N   E   P   S   V   S   D   T   I   S   I   L   R   G   L   K   E   R   Y   E   V   H   H   G

+892   GTA AGG ATC ACT GAT ACA GCT TTA GTG TCT GCT GCA GTT CTT TCA AAT CGT TAT ATA ACA GAC AGG TTT CTA CCT GAT AAA
+298    V   R   I   T   D   T   A   L   V   S   A   A   V   L   S   N   R   Y   I   T   D   R   F   L   P   D   K

+973   GCC ATT GAT CTA GTT GAC GAA GCT TGT GCT GTA CTA CGT TTA CAA CAT GAA TCC AAA CCG GAT GAA ATA CAG AAA CTG GAT
+325    A   I   D   L   V   D   E   A   C   A   V   L   R   L   Q   H   E   S   K   P   D   E   I   Q   K   L   D

+1054  CGT GCT ATT ATG AAA ATA CAA ATA GAA CTG GAA TCT TTA AAG AAA GAG ACC GAC CCT GTT TCC GTG GAA AGA AGA GAA GCG
+352    R   A   I   M   K   I   Q   I   E   L   E   S   L   K   K   E   T   D   P   V   S   V   E   R   R   E   A

+1135  TTG GAA AAG GAC CTA GAA ATG AAA AAT GAT GAA TTA AAC AGA TTA ACA AAA ATA TGG GAC GCT GAA AGA GCT GAA ATT GAA
+379    L   E   K   D   L   E   M   K   N   D   E   L   N   R   L   T   K   I   W   D   A   E   R   A   E   I   E

+1216  TCT ATC AAA AAT GCT AAG GCA AAT TTG GAA CAA GCA AGA ATT GAG TTG GAA AAG TGT CAG AGA GAG GGT GTA TAC ACT AAA
+406    S   I   K   N   A   K   A   N   L   E   Q   A   R   I   E   L   E   K   C   Q   R   E   G   V   Y   T   K

+1297  GCA TCC GAG TTG CGA TAT TCC AGA ATT CCT GAC TTG GAG AAG AAG GTA GCT TTA AGC GAG AAG AGT AAG GAC GGA GAT AAA
+433    A   S   E   L   R   Y   S   R   I   P   D   L   E   K   K   V   A   L   S   E   K   S   K   D   G   D   K

+1378  GTA AAT TTA CTT CAT GAT TCA GTT ACT TCT GAC GAT ATT TCT AAG GTT GTT GCC AAA ATG ACT GGT ATT CCT ACA GAA ACG
+460    V   N   L   L   H   D   S   V   T   S   D   D   I   S   K   V   V   A   K   M   T   G   I   P   T   E   T

+1459  GTG ATG AAA GGC GAC AAG GAC CGC CTA CTG TAT ATG GAA AAT TCT TTA AAA GAA AGA GTC GTT GGC CAA GAC GAG GCC ATT
+487    V   M   K   G   D   K   D   R   L   L   Y   M   E   N   S   L   K   E   R   V   V   G   Q   D   E   A   I

+1540  GCT GCT ATT TCT GAT GCT GTA CGT CTT CAA AGG GCT GGT TTG ACC AGT GAA AAG AGA CCT ATT GCC AGC TTT ATG TTC TTA
+514    A   A   I   S   D   A   V   R   L   Q   R   A   G   L   T   S   E   K   R   P   I   A   S   F   M   F   L

+1621  GGT CCT ACT GGT ACT GGT AAA ACA GAA TTG ACC AAG GCT TTA GCT GAA TTC CTA TTT GAT GAC GAA TCA AAT GTG ATT CGT
+541    G   P   T   G   T   G   K   T   E   L   T   K   A   L   A   E   F   L   F   D   D   E   S   N   V   I   R

+1702  TTT GAC ATG TCA GAA TTT CAA GAG AAG CAT ACT GTC TCT CGT TTA ATC GGT GCA CCT CCA GGT TAC GTC TTA AGC GAA TCT
+568    F   D   M   S   E   F   Q   E   K   H   T   V   S   R   L   I   G   A   P   P   G   Y   V   L   S   E   S

+1783  GGT GGC CAA TTA ACT GAG GCT GTC AGA AGA AAG CCT TAT CCT GTC GTT TTA TTT GAT GAA TTT GAA AAA GCG CAC CCC GAT
+595    G   G   Q   L   T   E   A   V   R   R   K   P   Y   P   V   V   L   F   D   E   F   E   K   A   H   P   D

+1864  GTT TCC AAA CTA CTG TTG CAA GTG TTG GAT GAA GGT AAG CTG ACC GAT TCC CTA GGC CAT CAT GTT GAT TTC CGT AAC ACG
+622    V   S   K   L   L   L   Q   V   L   D   E   G   K   L   T   D   S   L   G   H   H   V   D   F   R   N   T

+1945  ATC ATT GTG ATG ACC TCA AAC ATT GGA CAA GAT ATT TTA TTA AAC GAC ACA AAG TTG GGT GAT GAT GGT AAG ATT GAC ACC
+649    I   I   V   M   T   S   N   I   G   Q   D   I   L   L   N   D   T   K   L   G   D   D   G   K   I   D   T

+2026  GCA ACC AAG AAT AAA GTT ATC GAA GCA ATG AAA AGA TCC TAT CCA CCT GAG TTC ATC AAT CGT ATT GAT GAT ATT CTA GTT
+676    A   T   K   N   K   V   I   E   A   M   K   R   S   Y   P   P   E   F   I   N   R   I   D   D   I   L   V

+2107  TTC AAT AGG CTA TCC AAG AAA GTT TTG AGG TCA ATT GTT GAT ATT AGA ATT GCA GAA ATT CAA GAT CGT TTG GCC GAA AAA
+703    F   N   R   L   S   K   K   V   L   R   S   I   V   D   I   R   I   A   E   I   Q   D   R   L   A   E   K

+2188  AGA ATG AAA ATT GAT TTA ACT GAT GAA GAT GCG AAG TGG CTA ACT GAC AAA GGC TAC GAT CAA TTA TAT GGT GCA AGG CCA
+730    R   M   K   I   D   L   T   D   E   D   A   K   W   L   T   D   K   G   Y   D   Q   L   Y   G   A   R   P

+2269  TTG AAT AGA TTA ATT CAT AGA CAG ATT TTG AAC TCC ATG GCA ACT TTC TTA TTA AAA GGT CAA ATT AGA AAC GGC GAA ACT
+759    L   N   R   L   I   H   R   Q   I   L   N   S   M   A   T   F   L   L   K   G   Q   I   R   N   G   E   T

+2350  GTT AGA GTG GTG GTT AAA GAT ACT AAG CTT GTC GTG CTA CCA AAT CAT GAA GAA GGC GAA GTT GTT GAA GAG GAA GCT GAA
+784    V   R   V   V   V   K   D   T   K   L   V   V   L   P   N   H   E   E   G   E   V   V   E   E   E   A   E

+2431  AAG TAA CTGCACAAGCGGTTTATGAATAATAAATTCCCAAGAAAATACATATATAAACATACACATTTCTCTTTTGTAAATAGAAGAATTAAAATAAAAGATTTT
+811    K   -

+2536  TGGTATCAAATAAACTGGATGAATGTTACATGTACCAAGTGCCCTGGCAATACAATAAAGCTATTGGTCTGGAAGTATTGATATTATTCAAATGTAATCTTCGAGAG
+2643  TTACTGCGGGTAATACACACATGTGTCACATATTTTTCTCTCGAGTATGAAAAAATTTCATTACCTTTGTAAAAGCTTAATACGATCATTTCAATATGACTAGTAAT
+2750  AAAGGGGAAAGTTTGCGAAAATGGATGATTTTTCAAGAGATACCGAAAAACTTTGTTTCTGGTTAAAAACTACCGCTGAAATTGAAGTATCCCCTAAGATAGAAATC
+2857  AAAGATCTT
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FIG. 2. Nucleotide sequence of the *HSP78* gene and the predicted amino acid sequence of the gene product. The sequence of *HSP78* was determined from both strands by the dideoxy chain termination method (54). Four pentamers of nGAAn, corresponding to the heat shock consensus element (1, 66), are underlined in the 5′ noncoding region. Arrows indicate transcriptional start sites as determined by primer extension. Underlined in the 3′-flanking sequence are the putative transcription termination signals. Underlined within the deduced protein sequence are two putative nucleotide-binding sites that are characteristic of all Clp proteins. Nucleotides and amino acids are numbered on the left, with +1 indicating the putative translational start site.

orientation of these elements closely resemble the arrangement of MIG1-binding sites in *SUC2* (42).

Primer extension analysis of total RNA from cells grown in YPGE at 30°C revealed three predominant 5′ ends for *HSP78* transcripts corresponding to positions −31, −36, and −41 with respect to the ATG codon (indicated with arrows in Fig. 2; data not shown). The same 5′ ends were detected with RNA isolated from cells that had been heat shocked for 1 h at 42°C.

A comparison of the predicted amino acid sequence of hsp78 with the sequences in the current National Biomedical Research Foundation and SwissProt data bases revealed a high degree of similarity between hsp78 and members of the Clp family of proteins (16). Overall, hsp78 is most similar to ClpB of *E. coli* (70% similarity, 50% identity) and to other members of the ClpB subfamily. An alignment of the sequences for hsp78, yeast hsp104, and the ClpA and ClpB proteins of *E. coli* is shown in Fig. 3. hsp78 is 65% similar and 44% identical to hsp104 and 62% similar and 43% identical to the ClpA protein of *E. coli*. Underlined in Fig. 2 are the two nucleotide-binding motifs consisting of GXXGXGKT followed by a stretch of at least four hydrophobic amino acids and ending with aspartate about 60 amino acids downstream (12). hsp78 is somewhat shorter than either ClpB (857 amino acids) or hsp104 (908 amino acids), mainly because hsp78 is truncated in the region between the N terminus and the first nucleotide-binding domain. Interestingly, the predicted size of mature hsp78 (approximately 758 amino acids) is similar to the size of the *E. coli* ClpB′ protein (709 amino acids), which is believed to originate from internal translational initiation on the *clpB* mRNA (60). The carboxy-terminal end of the predicted hsp78 contains an acidic sequence EEAE followed by a lysine at the C terminus. Although we do not know whether this feature is functionally important in hsp78, it is strikingly similar to the 4-amino-acid sequence DDLD found at the C termini of hsp104 and several of the eukaryotic hsp70 and hsp90 proteins (34).

Since the λ3 and λ5 clones both expressed an epitope recognized by the anti-hsp78 monoclonal antibody, the approximate location of this epitope in hsp78 can be deduced from the DNA sequence shared by these two clones (Fig. 1A). The region of overlap spans nucleotides 979 to 1320 and encodes amino acids 327 to 440. The fact that this part of the protein is not highly conserved among the Clp proteins is consistent with our observation that the anti-hsp78 monoclonal antibody does not cross-react with hsp104, the other known yeast ClpB homolog (data not shown).

hsp78 is a matrix protein. The localization of hsp78 was determined by subcellular and suborganellar fractionation and immunoblot analysis. A mitochondrial localization for hsp78 is consistent with the results of the subcellular fractionation experiment shown in Fig. 4A; both hsp78 and cytochrome *c* oxidase subunit II (COXII), a marker for the mitochondrial inner membrane, were highly enriched in the mitochondrial fraction and were not detected in the postmitochondrial supernatant. When mitochondria were disrupted by ultrasonic irradiation, separated into soluble and membrane fractions by centrifugation, and subjected to immunoblot analysis, more than 85% of both hsp78 and citrate synthase, a soluble matrix marker (67), were recovered in the soluble fraction, pointing to a localization of hsp78 in either the intermembrane space or the matrix. To distinguish these two possibilities, mitoplasts were prepared and subjected to protease protection analysis. As shown in Fig. 4B, hsp78 was retained in mitoplasts (lane 2), whereas the intermembrane space protein cytochrome *c* peroxidase was lost. hsp78 and citrate synthase were protected from attack by proteinase K in intact mitoplasts (Fig. 4B, lanes 3 and 4) and were accessible when the inner membrane was solubilized with detergent (lane 6). Taken together, these results indicate that hsp78, like the two other mitochondrial heat shock proteins hsp60 and Ssc1p (3, 26), is a soluble protein in the mitochondrial matrix.

***HSP78* expression is controlled by heat shock and carbon source.** The steady-state levels of *HSP78* transcripts and protein were determined in cells incubated under a variety of conditions. The Northern analysis given in Fig. 5A shows that *HSP78* transcripts increased approximately 10-fold when cells grown at 30°C on either fermentable (YPD) or nonfermentable (YPGE) carbon sources were heat shocked for 1 h at 42°C. Moreover, the *HSP78* transcripts were three- to fivefold more abundant in cells grown in YPGE irrespective of whether the cells had been heat shocked. Thus, the levels of *HSP78* transcripts varied approximately 30-fold between cells grown fermentatively in glucose-containing media at 30°C (Fig. 5A, lane 1) and heat-shocked cells grown on a nonfermentable carbon source (lane 4). In comparison, the levels of the mRNA for hsp60, the mitochondrial GroEL homolog, increased only slightly upon heat shock (Fig. 5A, compare lanes 1 and 3 with lanes 2 and 4).

The immunoblot analysis shown in Fig. 5B shows that the accumulation of hsp78 is roughly proportional to the levels of *HSP78* mRNA determined by Northern analysis (Fig. 5A). A possible exception to this is the roughly equal levels of hsp78 detected in heat-shocked cells regardless of whether growth was on a fermentable or nonfermentable carbon source (Fig. 5B, compare lanes 2 and 4), whereas *HSP78* transcripts were clearly more abundant in heat-shocked cells grown on a nonfermentable carbon source (Fig. 5A, compare lanes 2 and 4). The relative amounts of hsp60 were proportional to the levels of *HSP60* mRNA under the various conditions (compare Fig. 5A and B). The results indicate that *HSP78* is regulated mainly at the level of transcription or mRNA stability in response to both carbon source and thermal stress.

***HSP78* is not essential for mitochondrial function.** To examine the function of *HSP78*, we created a gene disruption by deleting approximately 3.1 kb of DNA from the *HSP78* coding region and inserting a 3.8-kb *Bam*HI-*Bgl*II fragment containing *URA3*. A 6.8-kb *Eco*RI fragment containing the *hsp78*::*URA3* disrupted gene was used to replace chromosomal copies of *HSP78* in the haploid strain 22-2D and the diploid strain SB9882-4CR by homologous recombination (50). Ura^+ transformants were isolated from both strains, and integration at the *HSP78* locus was confirmed by Southern analysis of genomic DNA, as shown in Fig. 1D for the spores in a tetrad derived from a Ura^+ transformant of SB9882-4CR. The absence of the 78-kDa hsp78 polypeptide in a representative *hsp78*::*URA3* mutant was verified by immunoblot analysis of proteins in subcellular fractions (Fig. 4A).

Haploid *hsp78* mutants are viable; hence, hsp78 is not essential for cell growth. This distinguishes *HSP78* from *SSC1* and *HSP60*, which encode the yeast mitochondrial homologs of *E. coli* DnaK and GroEL, respectively, and are required for cell viability (3, 5). The viability of *hsp78* mutants is consistent, however, with the nonlethal phenotypes associated with mutations in genes for other ClpB-like proteins in *S. cerevisiae* and bacteria.

In *E. coli*, mutations in the *clpB* gene result in a slower growth rate at 44°C, the upper limit for cell growth. We

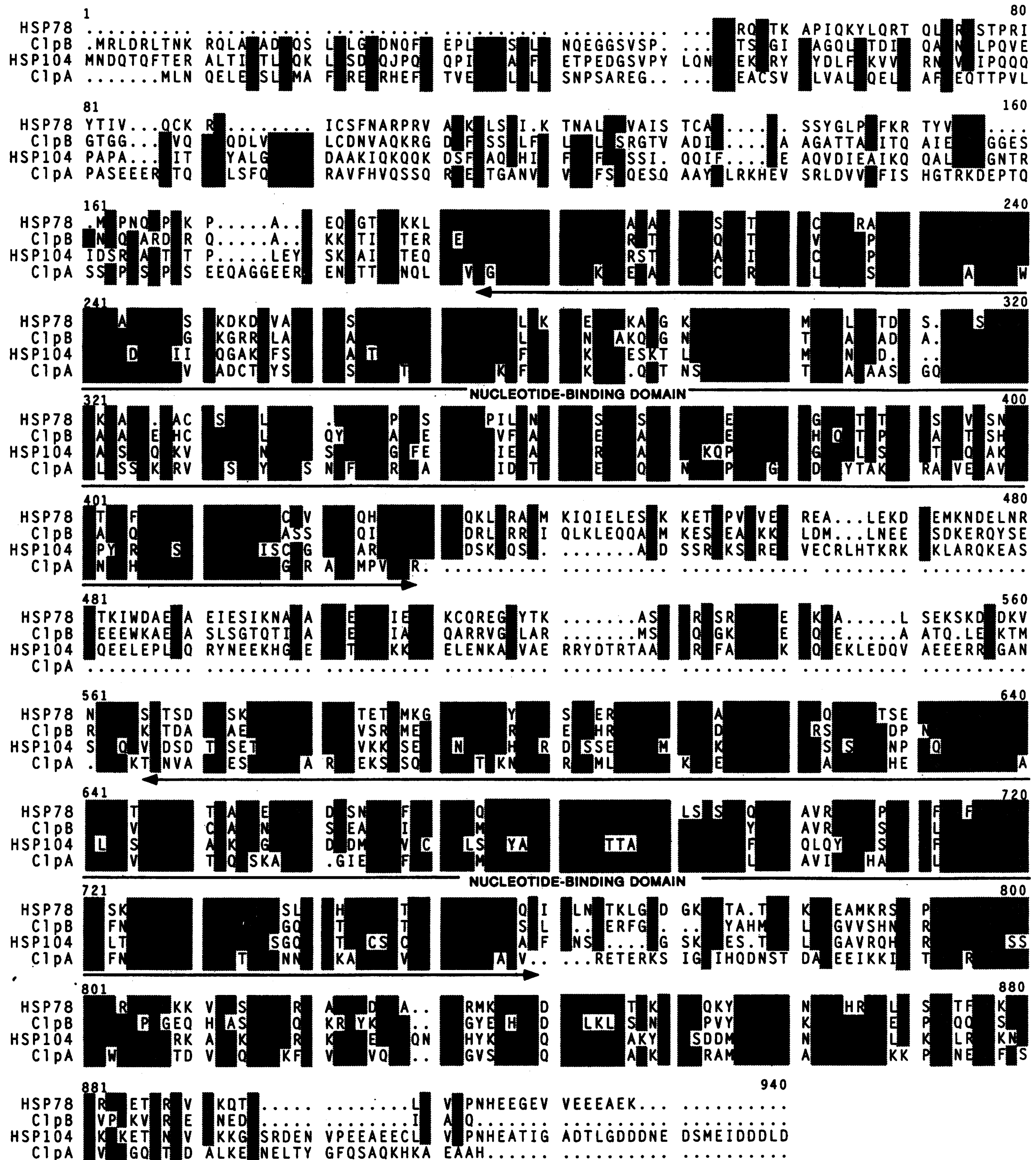

FIG. 3. Alignment of yeast and *E. coli* Clp proteins with the predicted sequence of hsp78. The amino acid sequences of hsp78, hsp104 (44), ClpB (18), and ClpA (18) are aligned. Gaps introduced to maximize homology are shown as dots. Identical and conserved residues (ILMV, KHR, DENQ, ST, AG, and FWY) that are present in at least three of the four sequences are shaded. The two highly conserved sequence blocks containing the nucleotide-binding domains are underlined. The internal valine (position 161) believed to be at the amino terminus of ClpB' is boxed in the ClpB sequence (60).

therefore examined the growth of *hsp78::URA3* [*rho*+] strains on fermentable and nonfermentable carbon sources at 30 and 37°C. Incubation of *S. cerevisiae* at 37°C is known to induce the heat shock response while still permitting cell growth (43). In contrast to the growth rates for bacterial *clpB* mutants, the growth rates for *hsp78* mutant strains were not significantly different from those of the wild type under any of the conditions we tested (data not shown). In addition,

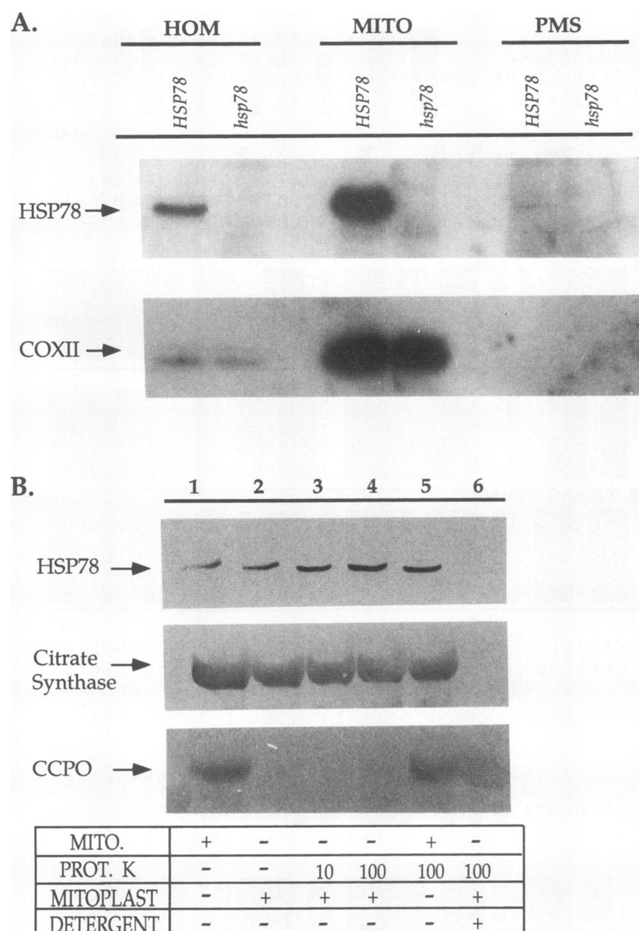

FIG. 4. hsp78 is localized to the mitochondrial matrix. (A) Subcellular fractionation. Wild-type (*HSP78*) and mutant (*hsp78*::*URA3*) strains were grown to mid-exponential phase in YPGE (2% glycerol, 2% ethanol). Whole cell homogenate (HOM), mitochondrial (MITO), and postmitochondrial supernatant (PMS) fractions were prepared from spheroplasts (see Materials and Methods). Proteins from each fraction (120 μg per lane) were subjected to immunoblot analysis with the anti-hsp78 monoclonal antibody and rabbit antisera against cytochrome *c* oxidase subunit II (mitochondrial marker). The immune complexes were decorated with ^{125}I-labeled anti-mouse Ig or protein A. (B) Submitochondrial fractionation. Freeze-thawed mitochondria were resuspended in 0.6 M sorbitol–20 mM K^+-HEPES (14) at 2.0 mg/ml, and 0.1-ml aliquots were treated as follows. Lane 1 contains mitochondria without further treatments; lane 2 contains mitochondria diluted ninefold with 20 mM K^+-HEPES (pH 7.4) and incubated on ice for 20 min to generate mitoplasts; lanes 3 and 4 are as lane 2 except that mitoplasts were prepared in the presence of 10 and 100 μg of proteinase K per ml, respectively; and lane 5 contains mitochondria incubated on ice with 100 μg of proteinase K per ml for 20 min. Samples in lanes 1 through 5 were adjusted to 1 mM PMSF, centrifuged again, resuspended in 0.6 M sorbitol buffer, and precipitated with trichloroacetic acid. Lane 6 is as lane 4 except that protease digestion was done in the presence of 1.0% (wt/vol) Triton X-100, and following treatment with PMSF, trichloroacetic acid was added directly, without a prior centrifugation step to remove proteinase K. All samples were subjected to SDS-PAGE, and the proteins were transferred to nitrocellulose filters. The filters were cut into strips to separate the polypeptides being tested, and each strip was incubated with the appropriate immunological reagent, either the monoclonal antibody to hsp78 or antisera to citrate synthase (matrix marker) or to cytochrome *c* peroxidase (CCPO) (intermembrane space marker) (48). Filter-bound Igs were visualized with the biotin-streptavidin detection system (Amersham) and a chromogenic substrate. The diffuse band that cross-reacts with the cytochrome *c* peroxidase antibody in lane 6 is apparently due to the presence of proteinase K, which was not removed by centrifugation prior to analysis.

hsp78 [*rho*^{0}] haploid strains grew normally on fermentable carbon sources, and *hsp78*/*hsp78* [*rho*$^+$] diploids sporulated and the haploid spores germinated. Thus, *HSP78* function is not essential for mitochondrial energy metabolism, sporulation, spore germination, or growth at moderately elevated temperatures.

Another phenotype associated with mutations in genes for ClpB-like proteins is a decreased tolerance to extreme thermal stress. For example, in comparison with the wild type, *clpB* mutants of *E. coli* are more susceptible to cell death following a shift from 30 to 50°C (60), and although *hsp104* mutant and wild-type yeast cells die at the same rate when shifted from 25 to 50°C, the mutants are 1,000-fold more sensitive to killing when the cells are preincubated at 37°C prior to thermal stress at 50°C (52). Thus, hsp104 appears to play an important role in the phenomenon of induced thermotolerance. More recently, hsp104 has been implicated in tolerance to other stress conditions, including poisoning by ethanol or arsenite and prolonged incubation at low temperatures (53).

We found that *hsp78* mutants were not impaired in the ability to survive thermal killing when shifted from 30 to 50°C, either directly or after preincubation at 37°C for 1 h, irrespective of whether media containing fermentable or nonfermentable carbon sources were used for growth, treatment, and recovery of the stressed cells (data not shown). Thus, unlike *clpB* and *HSP104*, *HSP78* does not appear to play an important role in thermotolerance or induced thermotolerance as measured by cell viability. Moreover, we found that the *hsp78* mutants were not impaired in their ability to survive thermal stress when forced to recover at 30°C on a nonfermentable carbon source, either when plated as single cells on YPGE agar medium or when incubated in batch culture in YPGE liquid medium. It appears, therefore, that *HSP78* function is not required for preservation of the capacity for mitochondrial oxidative energy metabolism and macromolecular synthesis in heat-stressed cells.

DISCUSSION

On the basis of its size and heat shock regulation, hsp78 initially appeared to be a candidate for a new yeast hsp70 protein. However, subsequent cloning and sequencing established that *HSP78* specifies a member of the highly conserved family of Clp proteins. The Clp proteins are believed to be regulatory subunits that function with a catalytic subunit ClpP in two-component, ATP-dependent proteases implicated in the degradation of abnormal proteins (see references 15, 17, and 38 for reviews). *E. coli* contains multiple Clp proteins, ClpA, ClpB, and ClpB′, but so far only ClpA has been shown to be part of an active protease in vitro (reviewed in references 38 and 59). ClpA is produced constitutively, whereas heat shock increases the synthesis of ClpP and ClpB about 2-fold and 10-fold, respectively (30, 59). The separate regulation of the *clp* genes has led to the proposal that the stress-induced ClpB and ClpB′ proteins associate with ClpP to form a proteolytic complex similar to the 750-kDa ClpA-ClpP complex that would presumably function in the removal of abnormal proteins generated by

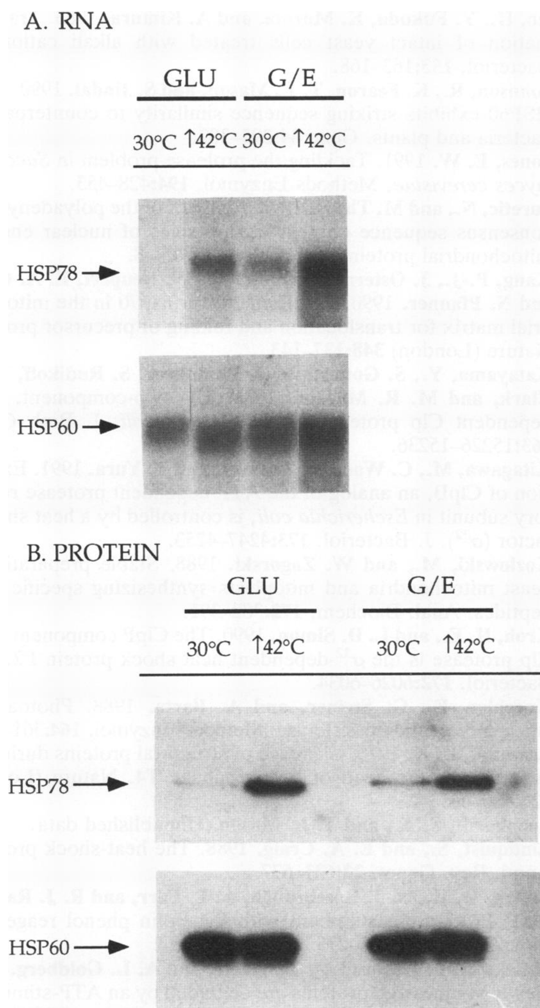

FIG. 5. Expression of *HSP78* is regulated according to temperature and carbon source. Strain 22-2D [rho^+] was grown to mid-exponential phase at 30°C in media containing either a fermentable (YPD) (GLU) or a nonfermentable (YPGE) (G/E) carbon source and subjected to heat shock at 42°C for 1 h. Samples of total RNA and total protein were prepared from these cells before (30°C) and after (↑ 42°C) heat shock treatment. (A) Total cellular RNAs (10 μg per lane) were separated by electrophoresis in a 6% formaldehyde–1% agarose gel containing 0.5 μg of ethidium bromide per ml, transferred to a nylon membrane, and hybridized with the ^{32}P-labeled 1.4-kb *Hin*dIII fragment from the *HSP78* coding region. The probe hybridizes to a single RNA band of 2.6 kb (RNA size standards not shown). For comparison, the blot was stripped and reprobed with the ^{32}P-labeled 1.2-kb *Stu*I-*Xba*I fragment from the *HSP60* coding region. (B) Immunoblot analysis of total cell protein (80 μg per lane) using monoclonal antibodies to hsp78 and hsp60. Immune complexes were decorated with ^{125}I-labeled sheep anti-mouse Igs.

thermal stress (37). Although the high degree of similarity between ClpA and ClpB (63% overall and 85% within the conserved regions) suggests that ClpB could be an activator of ClpP (18), there is currently no direct evidence for a ClpB-ClpP complex in vivo and in recent in vitro experiments, purified ClpB could not replace ClpA in activating ATP-dependent degradation of proteins by ClpP (65).

If hsp78 is part of a multisubunit complex, it could provide an explanation for why we obtained an anti-hsp78 monoclonal antibody from mice immunized with sucrose-gradient-purified 37S subunits from the mitochondrial ribosome. For example, we also recovered monoclonal antibodies to the chaperone hsp60, which sediments as a 20 to 25S particle in sucrose density gradients and is a detectable contaminant in our small subunit preparations. However, in contrast to hsp60, we found that virtually all of the immunologically detectable hsp78 remained at the top of the high-salt-concentration sucrose gradients used for the separation of ribosomal subunits (10). Apparently, small amounts of hsp78 associated with ribosomal subunits or other macromolecular complexes such as the hsp60 chaperone were sufficient to elicit an immune response to hsp78.

The discovery of a mitochondrial ClpB homolog raises the question of whether there are mitochondrial proteins related to ClpA and ClpP. ClpP coding sequences are present in the chloroplast genomes of several species (39), but comparable sequences are not found in any of the mitochondrial genomes characterized to date. Therefore, mitochondrial ClpP-like proteins, if they exist, are likely to be encoded by nuclear genes.

The presence in tomatoes of two different nuclear genes that encode Clp proteins with N-terminal sequences resembling transit peptides for import into chloroplasts (18) suggests that eukaryotic organelles might have multiple Clp regulatory subunits. The presence of more than one species of Clp protein in yeast mitochondria could explain in part our failure to detect a growth or thermotolerance phenotype for the *hsp78* knockout mutation. Another Clp protein, perhaps constitutively expressed, could mask the effects of a loss-of-function mutation in *HSP78*. Alternatively, unrelated proteins could provide functions that overlap those of hsp78. For example, mammalian mitochondria contain an ATP-dependent endoprotease that is biochemically related to protease La from *E. coli* (15), and a nuclear gene (*PIM1*) for a mitochondrial homolog of the protease La (Lon) polypeptide has been identified recently in *S. cerevisiae* (64). Also, since it has been argued that Clp proteins have many of the attributes of molecular chaperones (59), mitochondrial chaperonin proteins could conceivably compensate for the absence of hsp78.

Although it remains to be determined whether hsp78 is part of an ATP-dependent protease, certain properties of *hsp78* mutants are consistent with the involvement of hsp78 in the turnover of unassembled mitochondrial proteins. We have observed that mitochondrial ribosomal proteins overproduced from *GAL1* promoter constructs accumulated at significantly higher levels in *hsp78* mutants than in the wild type, and at least two mitochondrial ribosomal proteins that do not accumulate in *HSP78* [rho^0] cells, presumably because of degradation in the absence of ribosome assembly, appear to be stabilized in *hsp78* [rho^0] cells (7, 33). In particular, the Mrp2 ribosomal protein was found to be present at similar steady-state levels in [rho^+] and [rho^0] cells that have a disrupted chromosomal allele of *HSP78* (7). We are currently using biochemical and genetic approaches to further characterize the regulation of hsp78 and its role in mitochondrial protein metabolism.

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