HTS1 Encodes Both the Cytoplasmic and Mitochondrial Histidyl-tRNA Synthetase of *Saccharomyces cerevisiae*: Mutations Alter the Specificity of Compartmentation

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ABSTRACT

Genetic and biochemical evidence shows that a single nuclear gene *HTS1* encodes both the mitochondrial and cytoplasmic histidyl-tRNA synthetases (Hts). The gene specifies two messages, one with two in-frame ATGs (−60 and +1) and another with only the downstream ATG (+1). We have made a new set of mutations that enables us to express only the mitochondrial or the cytoplasmic form and compared the subcellular distribution of the Hts1 protein in these mutants and wild type, using an antibody that interacts with both the mitochondrial and cytoplasmic Hts1 as well as Hts1::LacZ fusions. Mutations in the upstream ATG (−60) or frameshift mutations in the presequence affect only the mitochondrial enzyme and not the cytoplasmic enzyme. Mutations in the downstream ATG (+1 ATG to ATC) destroy the function of the cytosolic enzyme, but do not affect the function of the mitochondrial enzyme. Overexpression of this construct restores cytoplasmic function. Cells expressing a truncated form of Hts containing a deletion of the first 20 amino-terminal residues (Htsc) produce a functional cytoplasmic enzyme, which does not provide mitochondrial function. Overexpression of this truncated cytoplasmic protein provides mitochondrial function and produces detectable levels of the synthetase in the mitochondrion. These experiments suggest that Hts1 contains two domains that together allow efficient localization of Htsm to the mitochondrion: an amino-terminal presequence in the mitochondrial precursor that is likely cleaved upon delivery to the mitochondrion and a second amino-terminal sequence (residues 21–53) present in both the precursor and the cytoplasmic form. Neither one by itself is sufficient to act as an efficient mitochondrial targeting signal. Using our antibody we have been able to detect a protein of increased molecular mass that corresponds to that of the predicted precursor. Taken together these studies show that the specificity of compartmentation of the Hts protein depends upon both the primary sequence and the concentration of the protein in the cell.

In yeast cells tRNA synthetases charging a particular amino acid are found in two compartments–the cytosol and the mitochondrion. The two forms can each be encoded by two different nuclear genes, or they can be encoded by a single gene. Many of the mitochondrial synthetases are encoded by nuclear genes distinct from those encoding their cytoplasmic counterparts. Strains carrying null alleles of the genes encoding the mitochondrial forms are viable and affect only the ability of cells to respire (MYERS and TZAGOLOFF 1985; PAPE and TZAGOLOFF 1985; PAPE, KOERNER and TZAGOLOFF 1985; TZAGOLOFF and MYERS 1986; TZAGOLOFF *et al.* 1988; HERBERT *et al.* 1988).

Other mitochondrial synthetases appear to be encoded by the same nuclear gene that encodes the cytoplasmic synthetase. For example, the two histidyl-tRNA synthetases seem to be encoded by a single nuclear gene, *HTS1*, because various mutations in the *HTS1* gene can lead to either lethality or mitochondrial insufficiency (NATSOULIS, HILGER and FINK 1986). The two valyl-tRNA synthetases were also postulated to be encoded by a single gene, *VAS1* (CHATTON *et al.* 1988). The structural organizations of the two genes, *HTS1* and *VAS1*, are similar: two in-frame methionine codons at the amino-terminal end of the gene and two sets of transcripts, a longer set initiating upstream of both ATGs and a shorter set initiating between the two ATGs. This structural organization is not confined to genes encoding aminoacyl tRNA synthetases. Examples of nuclear genes encoding enzymes that are localized to more than one subcellular or extracellular compartment include *SUC2* (CARLSON and BOTSTEIN 1982), *LEU4* (BELTZER *et al.* 1986), *TRM1* (ELLIS, HOPPER and MARTIN 1989), and *FUM1* (WU and TZAGOLOFF 1987).

In the case of *HTS1*, several lines of evidence suggest that the upstream ATG is the translation start codon for the mitochondrial form, and the downstream ATG is the translation start site for the cyto-

plasmic form. A mutation that destroys the upstream (−60) ATG, *hts1-150*, leads to a respiratory deficient, Pet⁻ phenotype, but does not affect either the level of cytoplasmic histidyl-tRNA synthetase or viability. Mutations downstream from the second ATG lead to lethality, loss of cytoplasmic synthetase function, and respiratory deficiency (for example, *hts1-1*). Complementation studies with these two alleles showed that they are indeed in the same cistron, because *hts1-1* was not able to rescue the respiratory defect of *hts1-150* in a *hts1-150/hts1-1* diploid. These results strongly suggest that the −60 ATG is the initiation codon for the mitochondrial form of histidyl-tRNA synthetase (NATSOULIS, HILGER and FINK 1986).

A prediction from this one-gene, two-enzyme model is that the *HTS1* gene products should localize to both the cytosol and the mitochondria. Translation initiation from the −60 and +1 ATG further implies that the longer primary translation product would contain an amino-terminal presequence. We present genetic and biochemical evidence that support these contentions. Further, we tested the stringency requirements for successful localization of Hts to the mitochondria, and identified two components necessary for this process. The first component is the amino-terminal sequence encoded between the two ATGs, present only in the mitochondrial primary translation product. The second component is present in both mitochondrial and cytoplasmic primary translation products Htsp and Htsc, between residues 21 and 53. Both components are required for efficient targeting of Hts to the mitochondria, but neither one alone is normally sufficient.

MATERIALS AND METHODS

Media and culture conditions: Yeast media were prepared essentially as described in SHERMAN, FINK and LAWRENCE (1979). Rich medium consisted of 1% yeast extract (Difco Laboratories) and 2% Bacto-Peptone (Difco) supplemented with 2% galactose (YPA). Synthetic medium (S) consisted of 0.15% yeast nitrogen base (without amino acids and ammonium sulfate), 0.5% ammonium sulfate and 2% of the appropriate carbon source (D, glucose; GE, glycerol ethanol; A, galactose). For example, SD represents synthetic medium with 2% glucose. Synthetic complete (SC) media were the same as above but in addition were supplemented with all amino acids plus uracil and adenine. Supplements were omitted from SC media as specified. For example, SCD-Ura represents synthetic medium containing 2% glucose and all amino acids and adenine, but no uracil. Growth conditions were at 30° unless otherwise specified. The 5-fluoro-orotic acid (5-FOA) containing medium was described by BOEKE *et al.* (1987). For growth in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), cells were grown at 30° on rich medium containing 2% galactose (YPA) to early exponential phase. CCCP was then added to a final concentration of 2, 5, 10 or 20 μM. Growth was continued for 7–8 hr before harvesting the cells.

Yeast strains: Yeast strains used in this work are listed in Table 1 along with their genotypes. All strains designated HS and IC were constructed for this study; HS strains are haploid and IC strains are diploid. The other strains were obtained from our lab collection. Plasmids used to transform yeast strains are designated in parentheses.

Complementation tests for *hts1* alleles: Complementation tests were performed with new mutations of *HTS1* to determine their effects on cytoplasmic and mitochondrial Hts function. In these studies, failure to suppress respiratory deficiency of *hts1-150* or *hts1-6* strains is interpreted as a defect on mitochondrial Hts function, and failure to suppress lethality of *hts1-3* or *hts1-8* strains is interpreted as a defect in cytoplasmic Hts function.

Complementation of hts1-3 and hts1-8: hts1-3 and *hts1-8* are internal deletions of the structural gene that can be maintained only in cells containing a functional *HTS1* gene, because cells carrying the *hts1-3* or *hts1-8* alleles are inviable. To test any new allele for complementation with *hts1-3* or *hts1-8*, a two-step "plasmid-shuffle" procedure is used (BOEKE *et al.* 1987). For example, strain HS42 (*hts1-3 ura3-52 lys2-201*) is viable because of the presence of a Lys⁺ plasmid encoding *HTS1* (pGN611). The allele to be tested was subcloned into a plasmid encoding *URA3* as a selectable marker, and HS42 is transformed into URA⁺ with this plasmid. The URA⁺ LYS⁺ transformants are then transferred onto plates containing α-aminoadipate (CHATTOO *et al.* 1979) to select for strains that have lost the *2μ::LYS2::HTS1* plasmid. Only transformants containing a *URA3::hts1* plasmid able to complement *hts1-3* viability can grow on α-aminoadipate plates. Failure to obtain α-aminoadipate resistant strains means that the *hts1* allele in question provides insufficient cytoplasmic Hts function to support growth. Those transformants that were capable of segregating α-aminoadipate resistant (and therefore provide cytoplasmic function) were printed onto glycerol-ethanol medium (SCGE-Ura) to assay for their mitochondrial Hts function. Failure to grow on this medium was taken as evidence that the plasmid contained an *HTS1* allele that was unable to provide mitochondrial function. The complementation test of the *hts1-4* allele on a plasmid (pIC275) was carried out with *hts1-8* on the chromosome after transformation of the *HTS1/hts1-8 ura3-52/ura3-52* diploid (IC234) to Ura⁺, to avoid the potential for confusion created by recombinants between the two populations of plasmids differing only in one base pair (ATG in pGN611 and ATC in pIC275) in the *hts1* locus.

Complementation of hts1-150: hts1-150 is a linker insertion at −58 which replaces the −60 ATG with ATC. Strains carrying *hts1-150* have a Pet⁻ phenotype, unable to grow on glycerol-ethanol containing medium. Plasmids carrying new *hts1* alleles were transformed into strain HS8 (*hts1-150 ura3-52*) to test their ability to complement *hts1-150* for growth on glycerol-ethanol. The test was complicated by the instability of [*rho*⁺] in *hts1-150*. Strains containing *hts1-150* consist of a mixture of [*rho*⁺] and [*rho*⁻] cells, the latter varying from 30 to 100% depending on growth conditions. Obviously, a *hts1-150* [*rho*⁻] strain will remain [*rho*⁻] regardless of the *HTS1* allele on the plasmid. To avoid this problem we devised the following test. The URA⁺ transformants are printed onto glycerol-ethanol media (YPGE, SCGE-URA). Only transformants containing *URA3::hts1* plasmids able to complement *hts1-150* for respiration and still possessing [*rho*⁺] mitochondrial DNA can grow on these plates. Those transformants that failed to grow on glycerol-ethanol were tested by mating them to a *HTS1* [*rho*⁰] tester lawn. If the resulting diploids fail to grow, the original transformants are considered to be [*rho*⁻] and therefore not suitable for the complementation test. Only those transformants capable of contributing [*rho*⁺] to the *HTS1* [*rho*⁰] strain were consid-

TABLE 1

Yeast strains

Strain	Genotype
L2336	*MATα hts1-1 ura3-52 leu2-2*
CSH48	*MATα met1 [rho⁺]* (derived from D273-10B)
HS8	*MATa hts1-150 ura3-52 lys2-201*
HS17	*MATa ura3-52 his3 leu2-3,112 trp1-1 cyh2 can1*
HS42	*MATa hts1-3 ura3-52 lys2-201* (pGN611)
HS219	*MATa ura3-52 his3 leu2-3,112 trp1-1 cyh2 can1* (pIC2420)
HS220	*MATa ura3-52 his3 leu2-3,112 trp1-1 cyh2 can1* (pIC231)
HS222	*MATa ura3-52 his3 leu2-3,112 trp1-1 cyh2 can1* (YEp24)
HS259	*MATa hts1-3 his3 leu2::pRY183::LEU2 ura3-52 lys2* (pGN611)
HS266	*MATa hts1-3 his3 leu2::pRY183::LEU2 ura3-52 lys2* (pGN17)
HS292	*MATa hts1-6 ura3-52 lys2* (pGN611)
HS295	*MATa hts1-8 ura3-52 leu2-3,112* (pGN671)
HS299	*MATa hts1-8 ura3-52 lys2 leu2-3,112 cyh2 GAL2* (pGN611)
HS300	*MATa hts1-3 his3 leu2::pRY183::LEU2 ura3-52 lys2* (pIC276)
HS304	*MATa ura3-52 his3 leu2-3,112 trp1-1 cyh2 can1* (pGN671)
HS305	*MATa ura3-52 his3 leu2-3,112 trp1-1 cyh2 can1* (pIC2408)
HS306	*MATa ura3-52 his3 leu2-3,112 trp1-1 cyh2 can1* (pIC295)
HS311	*MATa ura3-52 his3 leu2-3,112 trp1-1 cyh2 can1* (pGN163)
HS312	*MATa hts1-150 ura3-52 leu2-3*
HS313	*MATα hts1-150 ura3-52 leu2-3* (YCp50)
HS314	*MATα hts1-150 ura3-52 leu2-3* (pGN17-150)
HS315	*MATα hts1-150 ura3-52 leu2-3* (pIC2258)
HS319	*MATα hts1-150 ura3-52 leu2-3* (pIC275)
HS320	*MATα hts1-150 ura3-52 leu2-3* (pIC276)
HS323	*MATα hts1-150 ura3-52 leu2-3* (pGN17)
HS324	*MATα hts1-150 ura3-52 leu2-3* (pGN17) [*rho⁰*]
HS325	*MATα hts1-150 ura3-52 leu2-3* (YEp24)
HS326	*MATa hts1-8 ura3-52 lys2 leu2-3,112 cyh2 gal80-D::LEU* (pGN611)
HS333	*MATa hts1-8 ura3-52 lys2 leu2-3,112 cyh2 gal80-D::LEU2* (pGN611) (pTM267)
HS334	*MATa hts1-8 ura3-52 lys2 leu2-3,112 cyh2 gal80-D::LEU2* (pGN611) (pGN17)
HS360	*MATa hts1-6 ura3-52 lys2* (pGN611) (pIC275)
HS361	*MATa hts1-6 ura3-52 lys2* (pGN611) (pIC276)
HS372	*MATa hts1-8 ura3-52 lys2 leu2-3,112 cyh2 gal80-D::LEU2* (pGN611) (pIC306)
IC234	*MATa/MATα hts1-8/HTS1 ura3-52/ura3-52 lys2/lys2*

ered in the test. Plasmids containing an allele of *hts1* which have intact mitochondrial *HTS1* function produce a mixture of glycerol-ethanol positive and negative Ura⁺ transformants (*e.g.*, pGN17), whereas plasmids carrying defective *hts1* alleles for mitochondrial function produce 100% glycerol-ethanol negative Ura⁺ transformants.

Complementation of hts1-1: hts1-1 is a point mutation in the structural gene that results in a temperature-dependent histidine requirement. At 37°, *hts1-1* strains grow only on media containing histidine. Plasmids carrying the alleles to be tested were transformed into a *hts1-1 ura3-52* strain (L2336). Ura⁺ transformants were streaked onto the following plates: (i) SCD-Ura-His, (ii) SCD-Ura, (iii) SCGE-Ura-His and (iv) SCGE-Ura. The plates were incubated at 37°. Transformants that grow on all four media at 37° have normal Hts function (*e.g.*, *hts1-11*); those that grow on i, ii and iv but not iii. lack mitochondrial Hts function (*e.g.*, *hts1-150*).

DNA manipulations: Rapid isolation of plasmid DNA from *Escherichia coli* cells was carried out by the boiling lysis method of HOLMES and QUIGLEY (1981). Large scale isolations of plasmid DNA from *E. coli* were prepared by purification from cesium chloride gradients as described in MANIATIS, FRITSCH and SAMBROOK (1982). *E. coli* cells were transformed with plasmid DNA by the method of MANDEL and HIGA (1970). HB101, TG1 or DB6507 were used as recipient strains. Yeast transformations were performed by the lithium acetate method of ITO *et al.* (1983) using 100 μg of calf thymus DNA as carrier. DNA sequencing reactions were performed according to the dideoxy method (see New England Biolabs M13 sequencing manual).

Plasmid constructions: *General:* Plasmids used in this work are listed in Table 2. YCp50 was used as the low copy number cloning vector for expression in yeast. It is pBR322 containing *URA3*, *CEN4* and *ARS1*. Two high copy number vectors, YEp24 and pCGS42 (kindly provided by J. SHAUM and J. MAO, Collaborative Research), were used. Both are pBR322 derivatives containing *URA3* and *2μ* origin of replication, but the sites of insertion and the exact boundaries of these fragments differ. Conditions for ligations and restriction endonuclease digestions were as recommended by the suppliers (New England BioLabs, Boehringer Mannheim Biochemicals, Collaborative Research and BRL).

Mutations in HTS1: We created the +1 ATG-to-ACG mutation in *hts1-* by oligonucleotide-directed mutagenesis. The deletion allele *hts1-3* was constructed by replacing the 2.6-kb *Bam*HI (+377 *HTS1*) to *Sal*I (of vector) fragment of pGN17-118 with the 1.5-kb *Bam*HI (+1537) to *Sal*I (vector) fragment of pGN17-241. Similarly, deletion *hts1-8* was generated by ligating the *Bam*HI linker inserted at −60 (*hts1-150*) to the *Bam*HI linker inserted at +1537 (*hts1-241*). *hts1-6* was created by inserting the 3.1-kb *Bam*HI (−11) to

TABLE 2

Plasmids

Plasmid	Genotype	Source
YEp24	pBR322-*2μ-URA3*	Laboratory collection
YCp50	pBR322-*CEN-ARS-URA3*	Laboratory collection
pCGS42	pBR322-*2μ-URA3*	Collaborative Research
pB656	p*GAL1* in pCGS42	Collaborative Research
pGN17	Original *HTS1* clone in YCp50	Laboratory collection
pGN17-150	*hts1-150* in YCp50	Laboratory collection
pGN17-n	*hts1-n* in YCp50 (*n* = 118, 136, 241)	Laboratory collection
pGN17-n	*hts1-n* in YCp50 (*n* = 11, 13)	This work
pGN163	*HTS1* in YEp24	Laboratory collection
pGN611	*HTS1* in *2μ::LYS2* vector	Laboratory collection
pGN671	*hts1-241::lacZ* in YCp50	Laboratory collection
pIC201	*trpE::hts1-136* in pATH3	This work
pIC231	*hts1-136::lacZ* in pCGS42	This work
pIC275	*hts1-4* in YEp24	This work
pIC276	*hts1-4* in YCp50	This work
pIC282	p*GAL1::Htsc* (−11) in YCp50	This work
pIC289	*hts1-6* in YCp50	This work
pIC291	*hts1-6* in YEp24	This work
pIC295	*hts1-4,136::lacZ* in pCGS42	This work
pIC306	p*GAL1::Htsd* (+100) in pCGS42	This work
pIC2258	*hts1-150* in YEp24	This work
pIC2408	*hts1-11::lacZ* in pCGS42	This work
pIC2420	*hts1-11* (filled-in *Bam*HI)*::lacZ* in pCGS42	This work
pTM267	p*GAL1::Htsc* (−11) in pCGS42	This work
pTT804	*gal80-D::Leu2::gal80-D′-2μ-URA3*	J. HOPPER

*Sal*I (in vector) fragment of pGN17-11 into the *Bam*HI (−60) to *Sal*I vector fragment of pGN17-150. *hts1-11* was generated by a *Bam*HI linker insertion at the *Taq*I site at −11. The structure of these mutations were confirmed first by restriction mapping and then by DNA sequencing.

Fusion constructs: The *trpE::HTS1* recombinant plasmid pIC201 was constructed by inserting the 3.0-kb *Bam*HI (linker at +100 of *HTS1*) to *Sal*I (in vector) of pGN17-136 into the *Bam*HI-*Sal*I-digested *trpE* vector pATH3. The fusion protein expressed from this plasmid preserved the reading frames of both *trpE* and *HTS1*. All *lacZ* fusion constructs contain the 3-kb *Bam*HI fragment of pMR100 (gift of M. CASABADAN). pIC231 was generated by first inserting the 1.6-kb *Eco*RI (+1 of vector) to *Bam*HI (+100) fragment of pGN17-136 into *Eco*RI- and *Bam*HI-digested pCGS42 vector; the 3-kb *lacZ* fragment was then inserted into the *Bam*HI site of the intermediate construct. pIC295 was generated by substituting the 1.5-kb *Hin*dIII fragment of pIC231 (+29 of vector to +60 of *HTS1*) with a 0.7-kb *Hin*dIII fragment of pIC277 (+29 of vector to +60 of *hts1-4*). In both pIC231 and pIC295, fusion to the *E. coli lacZ* gene occurs at the *Bam*HI linker insertion at +100 of *HTS1*. pIC2408 was generated by replacing the 890-bp *Bst*EII (−790) to *Bam*HI (+100) fragment of pIC231 with the smaller 810-bp *Bst*EII (−790) to *Bam*HI (−11) fragment of pGN17-11; the structure of the new fusion was confirmed by DNA sequencing. pIC2420 was obtained by filling in the *Bam*HI site at the *hts1-lacZ* junction of pIC2408 thus altering the reading frame. pGN671 was created by inserting the 3-kb *lacZ* fragment into pGN17-241. The *GAL1::HTSc* promoter fusions were constructed by inserting the *Bam*HI (linker at −11) to *Sal*I fragment from pGN17-11 containing Htsc into the *Bam*HI (*pGAL1* junction) to *Sal*I (vector) of pB656 (*GAL1* promoter on *2μ* vector) or of L157-1 (*GAL1* promoter on *CEN-ARS* vector), respectively. pIC306, the *GAL1::HTSd* promoter fusion was made by inserting the *Bam*HI (+100) to *Sal*I of pGN17-136 into *Bam*HI- and *Sal*I-digested pB656.

Integration of the *gal80-D* allele into the chromosome: The *gal80-D* null allele (kindly provided by Dr. J. HOPPER on plasmid pTT804) contains a deletion of an internal 0.64-kb *Bgl*II fragment from *GAL80* that has been replaced by a 2.8-kb *Bgl*II fragment containing the *LEU2* gene from YEp13 (TORCHIA *et al.* 1984). The resulting *gal80-D* allele consists of an internal deletion of *GAL80* marked with *LEU2*. Digestion of pTT804 with *Hind*III releases a 5.3-kb fragment containing the *gal80-D* allele. Isogenic *gal80-D* derivatives of strains HS299 (HS326) and HS292 (HS327) were obtained by transforming each of the two *leu2-3,112* recipient strains with 1 μg of gel- purified *Hin*dIII fragment containing *gal80-D*. Leu$^+$ transformants were obtained which carry the mitotically and meiotically stable *gal80-D* allele on the chromosome instead of the original *GAL80* allele.

Anti-*HTS1* antibody: To produce an antigen to make antibody against *HTS1* we fused the structural gene of *HTS1* to the *E. coli trpE* promoter at position +100 of *HTS1*. The fusion construct, plasmid pIC201 was transformed into *E. coli*. Upon induction with indoleacrilic acid for 6 hours, large quantities of the *trpE::HTS1* fusion protein were isolated as described by KOERNER *et al.* (1991) and were purified from a preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel (LAEMMLI 1970). Polyclonal antiserum to *HTS1* was prepared by repeated immunization of adult mice with 250 μg of *trpE::HTS1* fusion protein antigen dissolved in 300 μl of Freund's complete adjuvant, followed by induction of ascites fluid by the injection of sarcoma cells.

Protein manipulations: Total cellular protein was extracted from yeast by the combined "new TCA method" of YAFFE and SCHATZ (1984) and LYONS and NELSON (1984).

TABLE 3

Alleles of *HTS1*

Allele	Description
hts1-1	Mutation in structural gene (*tsm*4572); ts histidine auxotroph
hts1-2	Deletion from *Bst*XI(+514) to *Xho*I(+1880; lethal
hts1-3	Deletion from *Bam*HI linkers (+377) to (+1537); lethal
hts1-4	Single point substitution at +1 ATG to ACG; lethal
hts1-6	Deletion from *Bam*HI linkers (−60) to (−11); viable; Pet$^-$
hts1-8	Deletion from *Bam*HI linkers (−60) to (+1534); lethal
hts1-11	*Bam*HI linker insertion, in-frame; at TaqI (−11); viable; Pet$^+$
hts1-13	Blunt-ended *Bam*HI linker of hts1-11, religated; viable; Pet$^-$
hts1-30	Tandem *Bam*HI linker insertions at TaqI (−11); lethal
hts1-118	*Bam*HI linker insertion at +377; frameshift; lethal
hts1-136	*Bam*HI linker insertion at +100; frameshift; lethal
hts1-150	*Bam*HI linker insertion, in frame; −60 ATG changed to ATC; viable; Pet$^-$
hts1-225	*Bam*HI linker insertion at −177; viable; Pet$^+$
hts1-241	*Bam*HI linker insertion at +1534, in frame; viable; Pet$^+$

Mitochondria were prepared as described by DAUM, BOHNI and SCHATZ (1982). Fractionation of mitochondria into intermembrane space (IMS), matrix (MX) and membranes (MEM) also followed published procedures (DAUM, GASSER and SCHATZ 1982). Samples from each fraction were monitored using marker enzyme assays or Western blotting analyses. Antibodies against the β subunit of F_1-ATPase or citrate synthase (matrix), the *RIP* gene product (known as the 23-kD subunit of bc_1 complex, an inner membrane protein) or glyceraldehyde-3-phosphate dehydrogenase (cytosol) were used to reveal the presence or absence of these proteins in each fraction.

Immunoblotting analyses: Polyacrylamide gel electrophoresis in the presence of SDS was carried out according to LAEMMLI (1970). Following electrophoresis, proteins were transferred to nitrocellulose paper as described by TOWBIN, STAEHELIN and GORDON (1979). Typically, mouse anti-*HTS1* antiserum was diluted 1000-fold. Incubations were carried out at room temperature for 6–16 hr, with gentle agitation. The filter was then washed three times in washing buffer (20 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.01% NaN_3 and 0.05% Nonidet P-40). ^{125}I-Labeled sheep anti-mouse immunoglobulins or ^{125}I-labeled, affinity-purified Protein A (Amersham Corp.) diluted 1000-fold in incubation buffer was added to the filter for 2 hr. The filter was again washed three times in washing buffer, air-dried and autoradiographed.

β-Galactosidase assays: Cell extracts were prepared by vigorous agitation of the cells with glass beads and assayed as described by LUCCHINI *et al.* (1984). Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories) according to the manufacturer's instructions (BRADFORD 1976), using bovine serum albumin as the standard. Enzyme activity is expressed in units/mg protein.

RESULTS

Previous work on *HTS1* identified the −60 ATG as the likely translational start site for the mitochondrial Hts (NATSOULIS, HILGER and FINK 1986) because mutation of this ATG led to a Pet$^-$ phenotype but had no effect on viability, and the +1 ATG as the start site for the cytoplasmic form because deletion of the ensuing reading frame was lethal. This evidence is largely inferential and so we pursued further mutational and biochemical studies to support these observations. In these studies we refer to the mitochondrial protein as Htsm and the cytoplasmic protein as Htsc.

Mutations in the presequence of *HTS1* affect Htsm but not Htsc: We tested the ability of *CEN-ARS* plasmids carrying mutant alleles of *HTS1* (Table 3 and Figure 1) to rescue the inviability, respiratory deficiency or conditional histidine auxotrophy of *hts1-3*, *hts1-150* or *hts1-1* strains, respectively (Table 4). As summarized below, mutations that delete or alter the reading frame prior to ATG at +1 affect only the mitochondrial and not the cytoplasmic Hts function.

hts1-6: This allele is a deletion between positions −11 and −60 of *hts1-150*; both the upstream ATG and most of the presequence is removed, but the +1 ATG remains. Plasmids carrying this mutation (pIC289) provide cytoplasmic but not mitochondrial function. They rescue the inviability of *hts1-3* but not the respiratory deficiency of *hts1-150*. Furthermore, *hts1-6* can rescue the conditional histidine requirement of *hts1-1* on glucose but not on glycerol-ethanol. In strains carrying *hts1-6* as in those with *hts1-150*, cytoplasmic Hts function appears intact even in the absence of the presequence.

hts1l-11: This mutation is a four codon in-frame insertion at position −11 of *HTS1* that introduces the peptide Arg-Asp-Pro-Ala between the 17th and the 18th amino acid of the putative 20 amino acid leader peptide. The insertion of this peptide has no effect on either the cytoplasmic or mitochondrial function of *HTS1*. These results suggest that codon insertions in-frame with the upstream ATG are tolerated and do not affect the function of either the cytoplasmic or the mitochondrial synthetases.

hts1-13: This allele is a derivative of *hts1-11*, created by cutting pGN17-11 at the *Bam*HI site and removing the 4-base overhang with mung bean endonuclease.

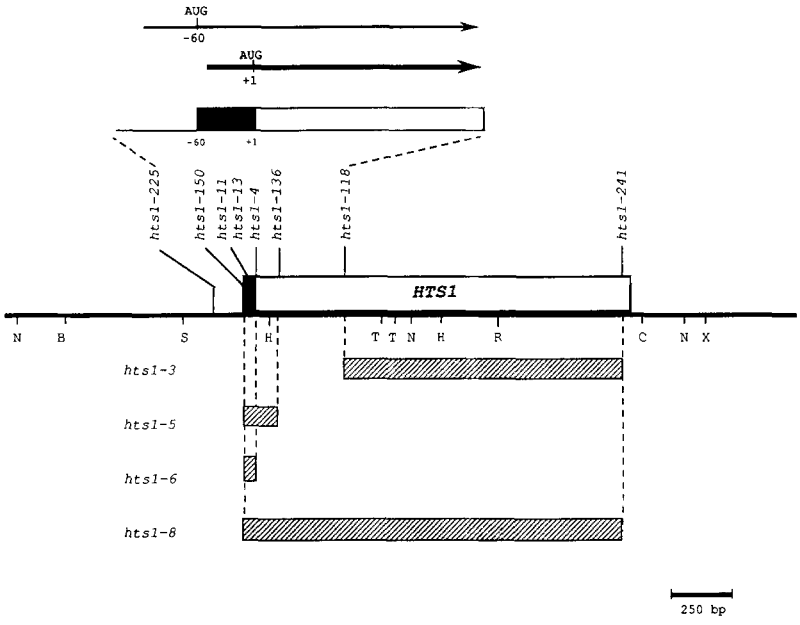

FIGURE 1.—Map of *HTS1* gene and flanking sequences showing the location of *hts1* mutations used in this study. The box above the line represents the *HTS1* coding region. The solid portion of the box represents the sequence encoding the mitochondrial presequence. The hatched bars below the box represent those portions of the gene that are deleted in each allele. Single-point mutations and linker-insertion alleles are shown above the box. See Table 3 for more detailed description of each allele. The letters below the line represent some of the restriction sites used in cloning experiments: H, *Hin*dIII; B, *Bst*EII; T, *Bst*XI; C, *Cla*I; N, *Nco*I; X, *Xho*I; R, *Eco*RI; S, *Ssp*I. The enlarged portion of the gene shows the transcripts initiating in the 5′ region: one set initiates between the two ATGs (cytoplasmic message, thick arrow), and a second set initiates upstream of the −60 ATG (mitochondrial message, thin arrow).

TABLE 4

Mutations in *HTS1*presequence and their phenotypes

	Allele[a] and medium[b]			
	hts1-1		*hts1-3*	*hts1-150*
Allele	Glucose[c]	Glycerol[c]	α-aminoadipate	Glycerol
HTS1	+	+	+	+
hts1-4	+	+	−	+
hts1-6	+	−	+	−
hts1-11	+	+	+	+
hts1-13	+	−	+	−
hts1-150	+	−	+	−

[a] Alleles listed across the top are integrated at *HTS1* in one copy on the chromosome. Alleles listed along the left are present on a *CEN* plasmid (YCp50).

[b] These are the media used to test the heterozygotes for growth. + means growth, − means no growth.

[c] *hts1-1* heterozygotes were tested at 37°.

This manipulation results in a frameshift with respect to the reading frame dictated by the −60 ATG, and, consequently, to a premature translational stop. The stop is prior to the +1 ATG so the cytoplasmic *HTS1* reading frame is intact. The *hts1-13* frameshift mutation has no effect on viability but is unable to restore the ability to grow on glycerol-ethanol to the *hts1-150* tester strain. Therefore, a frameshift in the presequence has no effect on the expression of the sequence following the +1 ATG.

These phenotypes confirm two important aspects of the proposed structure of *HTS1*. First, the observation that changing the reading frame of the presequence relative to the upstream ATG results in a Pet$^-$ phenotype, but an in-frame insertion does not, suggests that the presequence is translated and that it affects specifically the mitochondrial Hts function. Second, the fact that this frameshift mutation does not lead to inviability means that the reading frame of the cytoplasmic gene, dictated by the +1 ATG, is unaffected.

The ATG codon at +1 of *HTS1* is required for viability, but not for Htsm: The *hts1-4* mutation changes the +1 ATG (methionine) of *HTS1* to ACG. The effects of this mutation were assayed by transforming a low copy plasmid carrying *hts1-4* into each of the three tester strains *hts1-3*, *hts1-150* and *hts1-1*. Figure 2A shows the results obtained when *hts1-4::CEN-ARS* plasmid was transformed into a *hts1-3* strain, which requires the presence of a functional *HTS1* gene for viability, in this case provided by a *HTS1::2μ::LYS2* plasmid (pGN611). Cells transformed with the *hts1-4* allele grow well on the medium lacking lysine and α-aminoadipate, but fail to form colonies on medium containing α-aminoadipate. In contrast, cells transformed with the *HTS1* allele formed colonies on both kinds of media. Inability to lose the *HTS1::2μ::LYS2* plasmid on α-aminoadipate means that *hts1-4* is inviable.

To test the effects of the ATG-to-ACG mutation of *hts1-4* on mitochondrial function, we transformed the respiratory deficient strains *hts1-150* and *hts1-6* with plasmids carrying the *hts1-4* mutation both on a low copy (*CEN-ARS*) and a high copy (*2μ*) vector. The resulting transformants were streaked for single colonies on plates containing either glucose or glycerol-ethanol as the sole carbon source. Either on low or high copy vectors, *hts1-4* restores growth on nonfermentable carbon sources to the *hts1-150* or *hts1-6* recipient strains (Figure 2B). These findings show directly that the +1 ATG of *HTS1* is essential for Htsc function, and hence viability, but is not required for Htsm function.

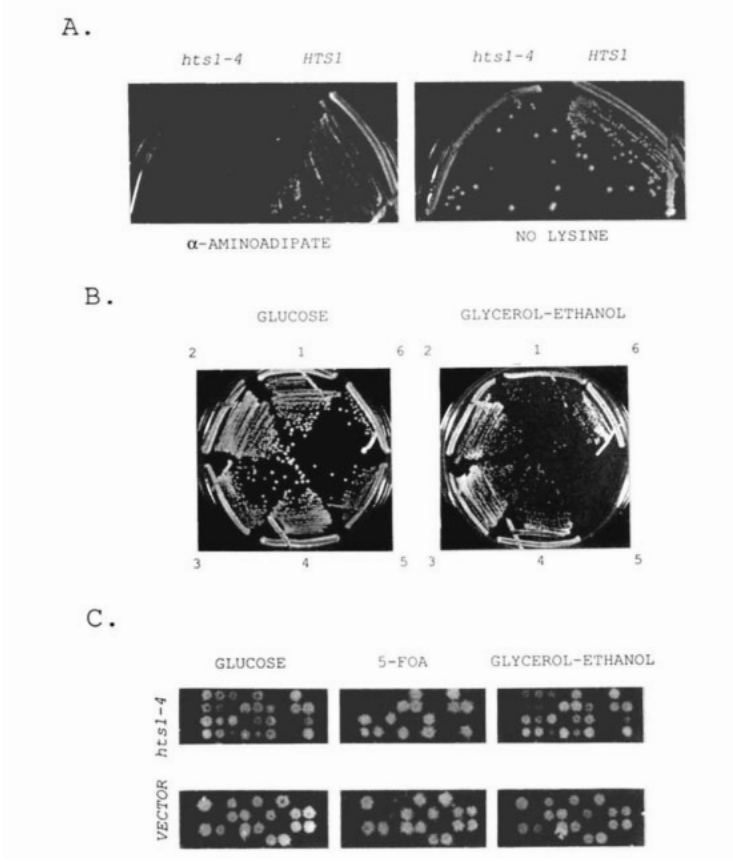

FIGURE 2.—Phenotypes of *hts1-4*, +1 ATG to ACG. (A) The *hts1-4* mutation leads to inviability in vegetative cells. Strain HS259 containing the null allele *hts1-3* requires the presence of the *HTS1::2μ::LYS2* plasmid (pGN611) for viability. This strain was transformed with the *CEN* plasmids bearing either the *hts1-4* (pIC276, left sector) or the *HTS1* (pGN17, right sector) allele. The resulting Ura$^+$ Lys$^+$ transformants (HS300, HS266) were tested for their ability to lose the Lys$^+$ plasmid. They were first grown on SCD-Ura for several generations, and then streaked for single colonies on α-aminoadipate medium (left) and on medium lacking lysine (right). (B) The *hts1-4* mutation does not affect Htsm function. *hts1-150* and *hts1-6* strains transformed with various plasmids were tested for their abilities to grow on nonfermentable carbon sources. The transformants were streaked for single colonies on plates lacking uracil and containing either glucose (left) or glycerol-ethanol (right) as the major source of carbon. Sector 1, *hts1-150/hts1-4::CEN-ARS::URA3* (HS320); 2, *hts1-150/hts1-4::2μ::URA3* (HS319); 3, *hts1-6/hts1-4::CEN-ARS::URA3* (HS361); 4, *hts1-6/hts1-4::2μ::URA3* (HS360); 5, *hts1-150/HTS1::CEN-ARS::URA3* [*rho*^{0}] (HS324); 6, *hts1-150/HTS1::CEN-ARS::URA3* (HS323). (C) Overexpression of *hts1-4* confers viability to *hts1-8* strains. The heterozygous *HTS1 ura3-52* × *hts1-8 ura3-52* diploid strain (IC234) was transformed with a multicopy plasmid bearing either *hts1-4* (pIC275, top) or no insert (YEp24, bottom). The Ura$^+$ transformants were induced to undergo sporulation, and the tetrads dissected onto YPD (GLUCOSE, left). The germinated spores were then printed onto medium containing either 5-FOA and glucose (5-FOA, middle) or YPGE (GLYCEROL-ETHANOL, right).

Anti-*trpE::HTS1* antiserum is specific to *HTS1*: Our conclusions concerning the behavior of our mutants were tested biochemically by cell fractionation and immunolocalization of Hts1. Antibodies against Hts1 were obtained using a fusion protein containing the *E. coli trpE* gene product fused to 493 amino acids of *HTS1*. The final antibody used was obtained from mouse ascites fluid as described in MATERIALS AND METHODS. The specificity of our antibody was determined in immunoblotting experiments using a functional *HTS1::lacZ* fusion. This *HTS1::lacZ* fusion (pGN671) contains most of the *HTS1* coding region (97%) fused to β-galactosidase and permits growth of strains deleted for the chromosomal copy of *HTS1*. The molecular mass of the hybrid protein predicted from the fusion construct is 180 kD.

Antisera against the TrpE::Hts1 fusion protein react with proteins whose size can be predicted by the particular *HTS1* DNA construct. Wild-type *HTS1* produces a protein of the size (55–58 kD) predicted from the DNA sequence (Figure 3A, lane 4); this protein is increased in cells overexpressing *HTS1* (lane 2). Strains containing the *HTS1::lacZ* hybrid produce a protein of ~180 kD, as predicted from the fusion construct (lane 1). Both the 180-kD and the 55–58-kD species are detected in a *HTS1* strain transformed with the *HTS1::lacZ* fusion plasmid (lane 3). The fact that one can predict the molecular mass of the protein recognized by the antiserum and that no other proteins were recognized suggests that the antiserum is highly specific for *HTS1*.

***HTS1* gene product is present both in the cytoplasm (Htsc) and in the mitochondrion (Htsm):** A prediction central to the one-gene, two-enzyme model of *HTS1* is that *HTS1* gene products should localize both to the cytoplasm and to the mitochondrion. If the hypothesis were correct, the anti-*HTS1* antibody should cross-react with proteins extracted from both the cytoplasmic and mitochondrial fractions. Fractionation of cell extracts into cytoplasmic and mitochondrial fractions (Figure 3B) shows that indeed, the anti-*HTS1* antibody reacts with proteins of the predicted mobility both in the cytosolic fraction and in isolated mitochondria. The reactivity with the mitochondrial fraction is not diluted upon successive cycles of careful washing. This latter treatment washes away traces of cytosolic contaminants such as glyceraldehyde-3-phosphate dehydrogenase. The β-subunit of F_1-ATPase, a well characterized mitochondrial protein, cofractionated with the mitochondria through the cycles of washes, as did the *HTS1* gene product.

Previous characterization of histidyl-tRNA synthetase activity established that about 5% of the activity resides in the mitochondrion; 90–95% of total activity isolated from yeast is cytosolic (BOGUSLAWSKI *et al.* 1974). The results with the anti-*HTS1* antibody are consistent with this finding; there is considerably more *HTS1* reactive material in the cytosol than there is in the mitochondria when equal proportions of each subcellular component were examined on the gel. Mitochondria prepared from *HTS1* cells (HS17) were further fractionated into IMS, MX, and mitochondrial

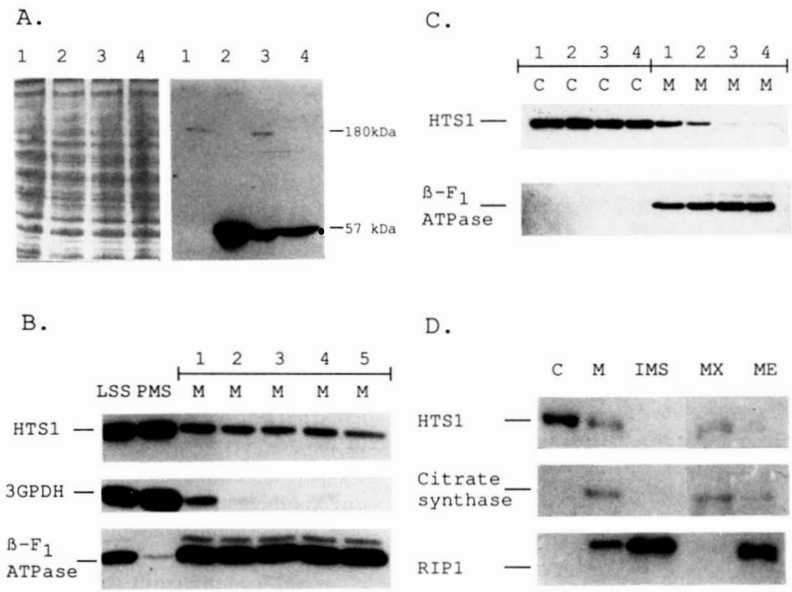

FIGURE 3.—Cellular distribution of *HTS1* gene products. (A) Immunological reactivity of a mouse antiserum raised against *E. coli TrpE::HTS1* fusion protein. Total cellular proteins were extracted from yeast cultures grown on SCD-URA as described in MATERIALS AND METHODS. Samples were dissociated for 5 min in SDS sample buffer prior to loading. A 25-μg aliquot of each sample was resolved by SDS-polyacrylamide gel electrophoresis (10% gel), then either stained directly with Coomassie blue (left) or transferred to nitrocellulose filter. The filter was incubated with a 1:1000 dilution of mouse antiserum raised against the TrpE-Hts1 fusion protein and decorated with I^{125}-labeled sheep anti-mouse IgG (right). Lane 1, *hts1-8/hts1-241::lacZ::CEN* (HS295); lane 2, *HTS1/HTS1::2μ* (HS311); lane 3, *HTS1/hts1-241::lacZ::CEN* (HS304); lane 4, *HTS1/YEp24* (HS222). The approximate molecular masses of the immunologically reactive bands are shown. (B) Hts1 is present in both the cytoplasmic and mitochondrial fractions. Subcellular fractions of the *HTS1* strain (HS17) were analyzed by SDS-polyacrylamide electrophoresis. Steady-state levels of proteins reacting with antiserum against *HTS1* (top panel), glyceraldehyde-3-phosphate dehydrogenase (a cytoplasmic marker, middle panel) or β-subunit of F_1-ATPase (a mitochondrial marker, bottom panel) in each fraction were measured by immunoblotting followed by incubation with I^{125}-labeled sheep anti-mouse IgG and autoradiography as described in MATERIALS AND METHODS. LSS, low spin supernatant, containing both cytoplasmic and mitochondrial fractions; PMS, post-mitochondrial supernatant, containing the cytoplasmic fraction; M, mitochondrial pellet. The numbers above each mitochondrial fraction (1–5) refer to the number of times the crude mitochondrial pellet has been purified by cycles of careful washing, centrifugation and resuspension in MT (0.6 M mannitol, 10 mM Tris-HCl, pH 7.4) buffer. (C) Cellular fractionation of Hts in *hts1-150* cells. The *hts1-150* strain HS312 was transformed with various plasmids. Transformants were grown to logrithmic phase in SCA-URA medium and then fractionated into cytoplasmic (C) and mitochondrial (M) components. Samples of 40 μg of each cytoplasmic fraction and 20 μg of each mitochondrial fraction were analyzed by SDS-polyacrylamide electrophoresis and immunoblotting using antiserum against Hts1 (top) and β-subunit of F_1-ATPase (bottom). Lane 1, *hts1-150/HTS1::CEN* (HS323); lane 2, *hts1-150/hts1-150::2μ* (HS315); lane 3, *hts1-150/hts1-150::CEN* (HS314); lane 4, *hts1-150*/no insert::*CEN* (HS313). (D) Htsm is localized to the mitochondrial matrix. Extracts of *HTS1* cells (strain HS17) were subfractionated into cytoplasmic (C) and mitochondrial (M) fractions as described. The crude mitochondria were further subfractionated into intermembrane space (IMS), matrix (MX), and mitochondrial membranes (ME). Samples were analyzed by SDS-polyacrylamide gel (10%) electrophoresis and immunoblotting using antibodies against Hts1 (top), citrate synthase (middle) and Rip1, the 23-kD subunit of the bc_1 complex (bottom).

membranes by selective osmotic shock treatment. Cross-contamination of contents from each fraction was monitored by immunoblots using antibodies against marker enzymes: the inner membrane marker was the 23-kD subunit of the bc_1 complex, and citrate synthase was the matrix marker protein. Upon fractionation, Htsm cofractionates with the matrix marker enzyme, citrate synthase (Figure 3D). This result is consistent with earlier studies suggesting a matrix localization for the mitochondrial histidyl-tRNA synthetase (BOGUSLAWSKI *et al.* 1974).

Mitochondria of *hts1-150* cells are deficient in Htsm: *hts1-150* is a mutation in the upstream ATG of *HTS1* which exhibits a Pet^- phenotype but has apparently intact Htsc function. The Pet^- phenotype is interpreted to result from either abnormal or reduced Htsm. Analysis of the proteins in mitochondria isolated from *hts1-150* shows that these mitochondria have reduced levels of Htsm (Figure 3C, lanes 3M and 4M), compared to the levels of Htsm present in strains carrying *HTS1* (lane 1M). In contrast, the level of Htsc (lanes 2C, 3C and 4C) is undiminished. These findings exactly mirror the physiological phenotype of this mutant and show directly that mutations in the upstream ATG lower the level of the mitochondrial but not the cytoplasmic form.

Evidence for a mitochondrial precursor, Htsp: Immunoblotting experiments with anti-*HTS1* antibody showed that under steady-state growth the Htsm and the Htsc species have similar mobilities. One prediction of our model of the *HTS1* locus is that the mitochondrial primary translation product should possess a 20-amino acid presequence not present in the cytoplasmic primary translation product. We sought to detect the putative precursor *in vivo* by inhibiting mitochondrial import with CCCP, an un-

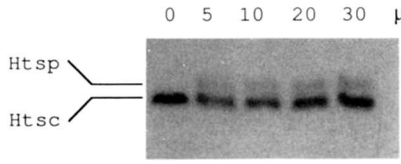

FIGURE 4.—Evidence for a mitochondrial precursor form of Hts, Htsp. *In vivo* accumulation of Htsp, the precursor form of Htsm, in CCCP-treated [*rho*-] cells. The [*rho*$^-$] strain CSH48 [*rho*^{0}] was grown at 30° on rich medium containing 2% galactose (YPA) to early logarithmic phase. The culture was then divided into five samples and CCCP was added to four of them at final concentrations of 5, 10, 20 and 30 μM. Growth was continued for 7.5 hr after addition of CCCP. Total protein homogenates were fractionated into mitochondrial and cytoplasmic fractions. The figure shows only the cytoplasmic fractions. Aliquots of 50 μg of each sample were boiled in SDS sample buffer and subject to analysis by polyacrylamide gel electrophoresis, followed by immunoblotting using an anti-Hts1 antiserum. The filter was decorated with ^{125}I-labeled sheep anti-mouse IgG and autoradiographed. The position of the precursor species Htsp and Htsc are marked.

coupler of oxidative phosphorylation. Previous work in *S. cerevisiae* showed that in the presence of CCCP, cytoplasmic pools of F_1-ATPase β-subunit precursor accumulates in large amounts during growth of a [*rho*$^-$] strain. Following inactivation of the uncoupler, the precursor could be post-translationally imported into the mitochondria where it was processed to the mature form (REID and SCHATZ 1982). Respiratory deficient, [*rho*$^-$] strains are used in such experiments because mitochondrial import is much more sensitive to CCCP treatment in respiratory deficient strains than in isogenic [*rho*$^+$] strains.

Growth of cells in CCCP permitted detection of the precursor form of Htsm, designated Htsp. Figure 4 shows the *in vivo* accumulation of Htsp in CCCP-treated, [*rho*$^-$] cells. Several concentrations of CCCP were added to independent cultures of exponentially growing [*rho*$^-$] cells. Total protein homogenates were fractionated into cytoplasmic and mitochondrial extracts by differential centrifugation. Samples were then analyzed by SDS-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose filter, and immunoblotted with anti-*HTS1* antiserum and ^{125}I- labeled anti-mouse IgG. A higher molecular mass species in CCCP-treated but not in untreated cells is present in the cytoplasmic fractions. The accumulation of this higher molecular mass form in the presence of CCCP and its apparent molecular mass (58 kDa) are consistent with this species being the precursor of Htsm.

Hts::β-galactosidase fusions localize to both the cytoplasm and the mitochondria: A second approach to the biochemical analysis of the subcellular distribution of *HTS1*-encoded proteins involved the use of gene fusions. Total cell homogenates or cytoplasmic and mitochondrial fractions were compared for the distribution of β-galactosidase activity (Figure 5).

We constructed an in-frame Hts1::lacZ fusion containing the upstream promoter region, both ATGs and the first 100 bp of the *HTS1* structural gene fused to the *E. coli lacZ* gene (pIC231, Figure 5, construct 1). Total β-galactosidase activity detected in cell homogenates reflects the additive expression of both Htsm and Htsc. Upon fractionation, the enzymes can be resolved from each other in their respective compartments. Consistent with the localization of native Hts proteins detected with anti-*HTS1* antibody, the β-galactosidase activities cofractionated mainly with the cytosol; only 7% of total activity was associated with the mitochondria. Of course, one could argue that the β-galactosidase that cofractionated with the mitochondria was simply contamination from the cytoplasmic form. To avoid the complication of cytoplasmic contamination we examined the localization of β-galactosidase in cells carrying pIC295, which is identical to the construct in pIC231 except that pIC295 lacks the translation start, the ATG at +1 (Figure 5, construct 2), and therefore reflects expression solely from the −60 ATG translation start site. In this construct there should be no contaminating cytoplasmic β-galactosidase activity because no fusion proteins should initiate at +1. The bulk of the β-galactosidase activity cofractionated with the mitochondrial pellet.

Immunoblots of pIC231 extracts incubated with a monoclonal antibody specific to β-galactosidase give fractionation patterns that are in agreement with the β-galactosidase enzyme assays (not shown). These data support the conclusion that initiation of the Hts1::lacZ fusion from the upstream ATG at −60 delivers β-galactosidase to the mitochondria, whereas initiation of the Hts1::lacZ protein fusion from the downstream ATG at +1 localizes β-galactosidase to the cytoplasm.

In-frame β-galactosidase fusions to the presequence of *HTS1*: pIC2408 encodes a fusion protein which contains the first 17 amino acids of the *HTS1* presequence fused to β-galactosidase (Figure 5, construct 3). Cells carrying this plasmid (HS305) were assayed for β-galactosidase and found to express 28 units of activity. This activity is completely abolished in cells carrying a frameshift between the ATG at −60 and the structural gene for *lacZ* (pIC2420, a plasmid derivative of pIC2408 in which the *Bam*HI site at the *hts1::lacZ* fusion junction is filled-in to create a frameshift (Figure 5, construct 4). In this frameshift construct, the coding sequence for β-galactosidase is no longer in frame with the −60 ATG. These results show that the −60 ATG and the reading frame following it are both required for expression of the fusion protein and presumably Htsm.

Interestingly, the first 53 amino acids of the mitochondrial primary translation product localized β-galactosidase to the mitochondrion (construct 1), but the first 17 amino acids did not (construct 3). These

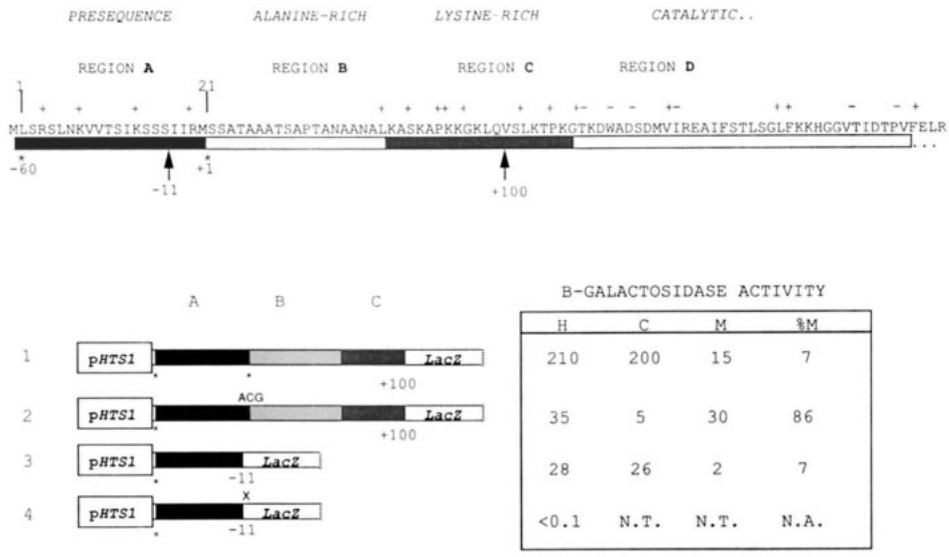

FIGURE 5.—The amino-terminal portion of Htsp directs β-galactosidase to the mitochondria. The figure describes the structure of four *hts1::lacZ* fusion constructs and their expression in a *HTS1* strain (HS17). All four plasmids contain the 2μ origin of replication (high copy number), the *URA3* gene, and amino-terminal segments of either a *HTS1* or *hts1* allele fused to the *E. coli* gene encoding β-galactosidase. 1, pIC231, which contains sequence from −1107 bp to +100bp of the *HTS1* region; 2, pIC295, which contains sequence from −1107 to +100 of *hts1-4*, +1 ATG to ACG; 3, pIC2408, which contains sequence from −1107 to −11; and 4, pIC2420, which contains sequence from −1107 to −11, except that the *Bam*HI site at the *hts1::lacZ* junction has been destroyed by a filling-in reaction using Klenow, followed by the religation of the blunt ends. This filling-in reaction resulted in an alteration of the reading frame for *lacZ* at −11 with respect to the ATG at −60 of *HTS1*, marked with ×. The constructs include sufficient upstream sequence from *HTS1* to direct expression of the fusion constructs, represented as *pHTS1* (promoter of *HTS1*). The top of the figure diagrams the amino-terminal region of *HTS1* in more detail. The amino acid residues predicted from the DNA sequence for the region are represented in single letter code; "+" and "−" represent charged residues. The boxes represent domains in Hts1: region A defines the region between the −60 and +1 initiator methionine codons, the presequence; region B contains an alanine-rich region with no charged residues; region C represents a lysine-rich domain with no negative charges and region D refers to the rest of the Hts protein. Numbers above the boxes denote amino acid residues; those below denote nucleotide base pairs relative to the +1 ATG. The breakpoint sites used in the fusion constructs are marked with arrows, and the two initiator methionines marked with asterisks. *HTS1* cells (HS17) harboring each of the four *hts1::lacZ* plasmids were grown on glycerol-ethanol media lacking uracil (SCGE-Ura) to logrithmic phase and fractionated into homogenate (H), cytoplasmic supernatant (C) and mitochondria (M) as described in MATERIALS AND METHODS. Samples were used in β-galactosidase enzyme assays and their activity is expressed as units/mg protein. Numbers under %M represent the value M/(C + M). "N.T." means not tested and "N.A." means not applicable.

results suggest that the mitochondrial presequence is necessary but not sufficient for localizing Hts to the mitochondrion, and reveal an important role for amino acids 21–53, present in both Htsp and Htsc, in this process.

Overexpression of Htsc restores respiration to strains defective in Htsc: Consistent with the notion that Htsc contains amino acid sequence(s) important for mitochondrial targeting, strains overexpressing Htsc (lacking the presequence) can provide mitochondrial Hts function. An *hts1-150* strain carrying the *hts1-150* allele on a *CEN* plasmid (HS314) is Pet$^-$. However, the same strain containing *hts1-150* on a 2μ-based, multicopy vector is Pet$^+$ (HS315, Figure 6A, sector 3). High copy suppression of the Pet$^-$ phenotype is observed with both orientations of *hts1-150* in YEp24 (data not shown). Fractionation of protein extracts from HS315 into cytoplasmic and mitochondrial components reveal cross-reactive material to the anti-*HTS1* antiserum in both the cytoplasmic and mitochondrial fractions of these cells, but not in the mitochondria of those of cells harboring either a control vector or the *hts1-150* allele on a low copy plasmid (Figure 3, lane 2).

Overproduction of Htsc using the *GAL1* promoter also leads to suppression of mitochondrial defects in *hts1* mutants. We constructed promoter fusions in which the DNA fragment encoding only the cytoplasmic form, Htsc, without the presequence, was fused to the inducible yeast *GAL1-10* promoter. This fusion, called p*GAL::HTSc*, was subcloned into both a multicopy plasmid (pTM267) and a low copy plasmid (pIC282) (see Figure 6B). Immunoblots show that these promoter fusion constructs overproduce Htsc when the culture is induced with galactose (data not shown).

Isogenic *hts1-8* strains containing either the wild-type *GAL80* allele or the null *gal80-D* allele (see MATERIALS AND METHODS) were tested for growth on glycerol-ethanol. *gal80-D* was used instead of galactose to control induction of Hts in these experiments because the presence of galactose in the glycerol-ethanol medium would permit growth whether or not the strains could grow on glycerol-ethanol, and therefore obscure the test. Null mutations of *gal80* are repressed by glucose and highly induced in its absence so *hts1-8*, *hts1-6* or *hts1-150* strains carrying the p*GAL::HTSc*

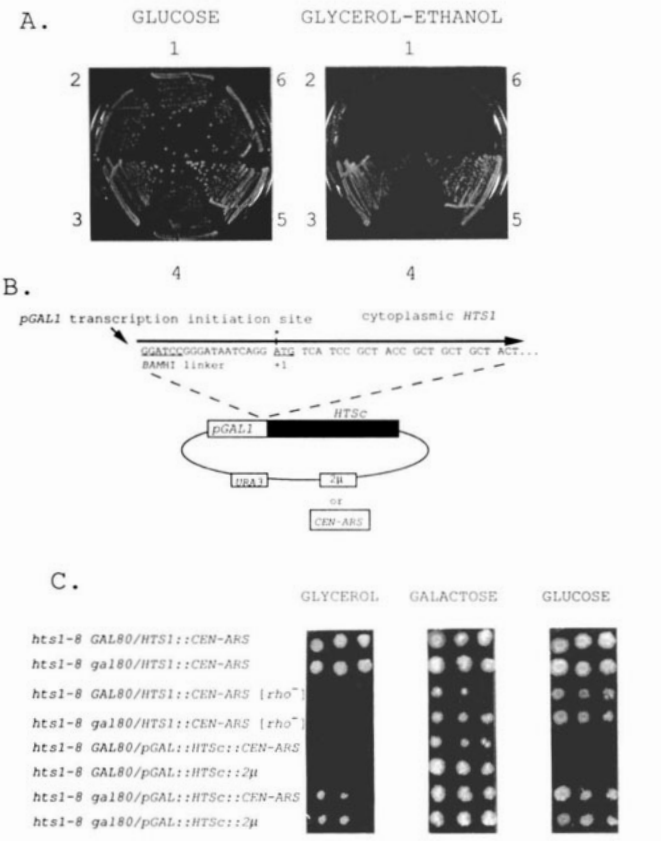

FIGURE 6.—Overexpression of cytoplasmic Hts suppresses respiratory deficiency in *hts1* mutants. (A) *hts1-150* on a multicopy plasmid restores growth on glycerol-ethanol to strains with *hts1-150* on the chromosome. The haploid strain HS312 (*hts1-150 ura3-52*) was transformed with the plasmids described below, and the resulting Ura$^+$ transformants were streaked onto either SCD-Ura (GLUCOSE, left) or SCGE-Ura (GLYCEROL-ETHANOL, right) plates and incubated at 30° for 3 days. Sector 1, the low copy *CEN-ARS* vector with no insert (YCp50); 2, *hts1-150::CEN-ARS* (pGN17–150); 3, *hts1-150::2μ* (pIC2258); 4, the multicopy 2 μ-based vector with no insert (YEp24); 5, *HTS1::CEN-ARS* (pGN17); 6, a [*rho*$^-$] derivative of the transformant shown in sector 5. (B) *GAL1-10* promoter fusion construct for overexpression of Htsc. *Htsc* was fused to the *GAL1-10* promoter on a *2μ* multicopy plasmid (pTM267) or on a low copy *CEN-ARS* plasmid (pIC282). Both constructs contain the 810-bp *Eco*RI to *Bam*HI fragment of *pGAL1-10* fused to the *Bam*HI linker at −11 of *HTS1*. All sequences upstream of −11 of *HTS1*, including the presequence, are absent from the plasmid constructs. The *pGAL1-10* DNA fragment provides the *GAL1* transcription start site for initiation of the Htsc messages in these plasmids. (C) Overproduction of Htsc suppresses respiratory deficiency in *hts1-8*. The *hts1-8* deletion is a null allele deficient for both cytoplasmic and mitochondrial Hts functions. *GAL80* and *gal80-D* derivatives of *hts1-8* were transformed with the Ura$^+$ plasmids listed by a two-step "plasmid-shuffle" method as described in the text. The figure shows serial dilutions of *hts1-8* transformants pre-grown in SCA-Ura, washed twice with H_2O and spotted onto SCGE-Ura (GLYCEROL-ETHANOL, left), SCA-Ura (GALACTOSE, middle) and SCD-Ura (GLUCOSE, right) plates. The dilution factor increases from left to right. The plates were incubated at 30° for 3 days.

plasmids produce high levels of Htsc without galactose.

We transformed the p*GAL::HTSc* overproducing plasmid (pTM267) into a *hts1-8 GAL80* strain as well as into the isogenic *gal80-D* derivatives, to test whether overproduction of Htsc by the plasmid can restore respiration to the Pet$^-$ recipient *hts1* strains. Figure 6C shows serial dilutions of the transformants (Ura$^+$, Lys$^-$ segregants from plasmid-shuffle) spotted onto glycerol-ethanol-, galactose- or glucose-containing media (SC-Ura). The [*rho*$^+$] transformants carrying the wild-type *HTS1* allele on a plasmid (pGN17) served as positive control, and [*rho*$^-$] derivatives of the same strains served as a negative control for growth on glycerol-ethanol.

The growth phenotypes observed can be explained in the following way. On galactose, both low (pIC282) and high (pTM267) copy number plasmids containing the p*GAL::HTSc* construct express Htsc, which is essential for viability. Therefore, all transformants tested, whether *GAL80* or *gal80-D*, were viable on galactose. On glucose, the p*GAL::HTSc* constructs in a *GAL80* background are subject to glucose repression, reducing Htsc expression to that below the minimum required for viability. In a *gal80-D* background, glucose repression is not as severe as in *GAL80* and Htsc is expressed at levels sufficient for viability.

The most critical results from this experiment came from the growth patterns of *GAL80* and *gal80-D* strains on glycerol-ethanol. In *gal80-D* strains the levels of Htsc overexpressed by both the low-copy number plasmid (pIC282) and the high copy number plasmid (pTM267) are sufficient to rescue not only Htsc function in the cytoplasm but also Htsm function in the mitochondria, leading to a Pet$^+$ phenotype. Critical to our analysis, these same plasmids in the *GAL80* background fail to confer growth on glycerol-ethanol, indicating that the restoration of mitochondrial Hts is dependent upon high level expression of Htsc. These results strongly suggest that Htsc can functionally replace the missing Htsm, in the absence of the mitochondrial presequence.

Mitochondrial targeting sequence in Htsc: The *lacZ* fusion studies described above identify amino acids 1–53 as necessary and sufficient for directing passenger proteins to the mitochondria. The localization of a truncated *HTS1* construct (Htsc) to the mitochondrion upon overexpression further points to amino acids 21–53 (the first 32 amino acids in Htsc) as essential for this process. We explored this possibility by constructing a p*GAL::HTS* fusion that is deleted for these amino acids, by removing all *HTS1* sequence upstream of +100 bp (pIC306). The first ATG encoded by the *GAL1*-driven transcripts in this fusion construct is at position +145 of the *HTS1* structural gene (Figure 7). Overexpression of this novel construct should produce an altered Hts initiating at the +145 AUG and lacking residues 1–63 (both the mitochondrial presequence and the first 48 residues encoded in the cytoplasmic Hts). This p*GAL::HTSd* construct on *2 μ* plasmid was tested in strains containing *hts1-8 gal80-D* on the chromosome.

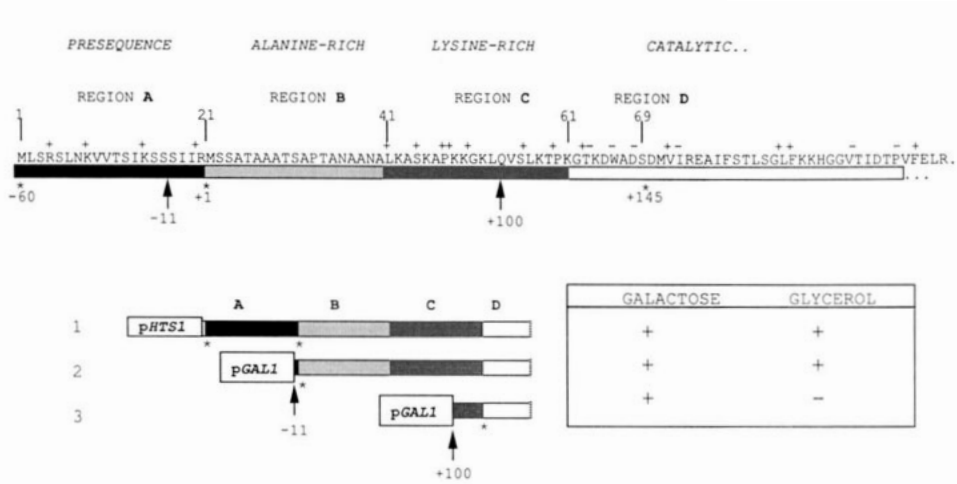

FIGURE 7.—Identification of mitochondrial targeting sequences in *HTS1*. The figure depicts the structure of *HTS1* in three plasmid constructs. 1, *HTS1::CEN-ARS* (pGN17); 2, p*GAL1::HTSC::2 μ* (pTM267) and 3, p*GAL1::HTSd::2μ* (pIC306). The table on the right shows the growth phenotypes of transformants obtained when each plasmid was introduced into a *hts1-8 gal80-D* strain (HS326) by the plasmid-shuffling technique described in MATERIALS AND METHODS. "+" represents growth and "−" represents no growth on media containing either galactose or glycerol-ethanol as the sole carbon source. The top of the figure represents the amino-terminal region of *HTS1* described in detail in Figure 5.

The *hts1-8 gal80-D* strain transformed with the p*GAL::HTSd* construct lacking residues 1–68 (pIC306) grows well on galactose, but does not grow on plates containing glycerol-ethanol as the sole carbon source (Figure 7). By contrast, the same strain transformed with the p*GAL::HTSc* construct lacking residues 1–20 (pTM267) grows well on both galactose and glycerol-ethanol plates.

These results can be explained in the following way. First, overproduction of the altered Hts lacking residues 1–68 provides sufficient Htsc for cytoplasmic protein synthesis. Assuming the the first amino acid of this truncated Hts is the methionine at +145 bp, these data imply that residues 1–68 of the mitochondrial Hts primary translation product (including the first 48 residues of the cytoplasmic Hts) are not essential for the charging reactions of histidyl-tRNA synthetase in the cytoplasm. Second, in comparison to the ability of the p*GAL::HTSc* (pTM267) construct to provide growth on glycerol-ethanol in a *hts1-8* strain upon overexpression, the failure of p*GAL::HTSd* (pIC306) to do so suggests that residues 21–68 contain either a region required for mitochondrial but not cytoplasmic Hts function, or a secondary mitochondrial targeting sequence. Since the charging reactions of the cytoplasmic and mitochondrial synthetases are indistinguishable *in vitro*, we favor the notion that residues 21–68 contain a secondary mitochondrial targeting sequence. A third alternative could be that the shorter protein (Htsd) is less stable than Htsc, accumulating to insufficiently high levels to effect a significant amount of import into the mitochondrion. Further biochemical analysis is necessary to distinguish among these possibilities.

Overexpression of *hts1-4* confers viability to *hts1-8* strains: Since *hts1-4* cells produce Htsm but lack the translation start site for Htsc at +1, we wondered if overexpression of *hts1-4* could suppress a null allele of *HTS1*, such as *hts1-8*. The complementation test was carried out between *hts1-4* on a plasmid (pIC275) and *hts1-8* in the chromosome after transformation of the *HTS1/hts1-8 ura3-52/ura3-52* diploid (IC234) to Ura$^+$ (see MATERIALS AND METHODS). The diploid was induced to undergo meiosis, and the resulting tetrads were dissected onto rich medium (YPD). After germination, viable spores were replicated (Figure 2C) onto media containing glucose (YPD, left column), 5-FOA (center column) or glycerol-ethanol (right column). As a control, the diploid IC234 was also transformed with the 2μ-vector (YEp24) without any insert.

The bottom panels show the growth phenotype of the diploid carrying the control plasmid, YEp24. Upon germination, only the two *HTS1* spores per tetrad survive to form colonies, because YEp24 cannot rescue the inviability of the *hts1-8* spores. All the viable spores are genotypically *HTS1*, grow well on glycerol-ethanol medium and, as they are Hts$^+$, can lose the Ura$^+$ plasmid in the presence of 5-FOA.

The *hts1-4::2μ* construct confers viability to strains containing *hts1-8*. Many of the tetrads derived from transformants of IC234 contain three and four viable spores. In these tetrads two of the spores can lose *hts1-4::2μ::URA3* plasmid on 5-FOA medium. In the three and four spored tetrads, one or two of the spores respectively fail to grow on 5-FOA. We assume that the 5-FOA positive spores are genotypically *HTS1*, and the 5-FOA negative spores are *hts1-8*. Not surprisingly, all the spores which germinate also grow on glycerol-ethanol, because either the *HTS1* allele or the *hts1-4::2μ* plasmid can provide mitochondrial Hts function.

By contrast, *hts1-4* on a low copy plasmid (pIC276) cannot provide viability to strains carrying either *hts1-3* (Figure 2A) or *hts1-8* (not shown). These results provide genetic evidence that low level expression of *hts1-4* is sufficient only for mitochondrial Hts func-

tion, whereas high level expression provides cytoplasmic Hts function as well. The simplest interpretation of these results is that overexpression of Htsm causes some of the protein to localize to and function in the cytoplasm. Further biochemical analysis is necessary to establish the molecular basis for this suppression.

DISCUSSION

We have presented genetic and biochemical evidence that *HTS1*, the nuclear gene for histidyl-tRNA synthetase in yeast, encodes both a mitochondrial and a cytoplasmic form of the enzyme. Mutational analyses of the amino-terminal presequence region of *HTS1*, expression of *hts1::LacZ* fusions and cell fractionation experiments with an antiserum specific to *HTS1* confirm our previous assignment of the −60 ATG as the translational start codon for the mitochondrial form, and the +1 ATG as the translational start for the cytoplasmic form. The mitochondrial form must be derived by cleavage of a longer precursor. The putative higher molecular mass precursor of the mitochondrial Hts accumulates in cells grown in the presence of the oxidative uncoupler CCCP. The existence of this precursor is supported by the observation that two distinct *in vitro* translation proteins were produced from an *SP6* fusion upstream of the −60 ATG, one the size of the precursor and one the size of Htsc (data not shown).

One striking result is that overexpression of Hts appears to overcome the compartmentation found in wild type cells. Overexpression of the cytoplasmic form (Htsc) lacking an amino-terminal presequence (*hts1-150* as well as deletions of the presequence) can functionally replace Htsm in the mitochondria. Overexpression of Htsc seems to restore mitochondrial functions including mitochondrial protein synthesis and successful maintenance of [*rho*$^+$] mitochondrial genomes (data not shown). Analysis of mitochondria from strains overexpressing the cytoplasmic form show that they have detectable Hts protein. This observation supports the notion that Htsc can physically be targeted and taken up into the mitochondria without the amino-terminal presequence.

The ability of the Htsc to substitute for Htsm when overexpressed suggests that Hts1 may have a mitochondrial targeting signal sequence present in both the mitochondrial and cytoplasmic primary translation products. Several additional experiments support this notion. First, a *hts::lacZ* fusion construct that contains the amino-terminal presequence (residues 1–17) fused to the *E. coli lacZ* gene (pIC2408) produced functional β-galactosidase but the activity failed to localize to the mitochondrion. However, a second *hts::lacZ* construct (pIC295) that includes both the amino-terminal presequence and residues 21–53 fused to the *lacZ* gene localized β-galactosidase to the mitochondrion efficiently (Figure 5). These results suggest that both the mitochondrial presequence (1–20), and the sequence downstream from it (21–53) containing the hydrophobic alanine stretch together with most of the basic lysine-rich sequence, function to target Hts to the mitochondrion. This conclusion is supported by the p*GAL::HTSd* construct, which lacks residues 1–68 and fails to provide mitochondrial function (Figure 7). Like the mitochondrial presequence, the region from residues 21–53 in the protein is not sufficient to target Hts to the mitochondrion by itself. Mutations that abolish the expression of the amino-terminal presequence in Hts (such as *hts1-150*, *hts1-6*, *hts1-13*) but produce the cytoplasmic Htsc, containing residues 21–53, result in respiratory deficiency and absence of Hts in the mitochondrial fraction (Figure 3C). Taken together, our data indicate that both the presequence and the region encoded by residues 21–53 are required to serve as an efficient targeting signal.

Some mitochondrial proteins of yeast, such as the F_1-ATPase β-subunit (BEDWELL, KLIONSKY and EMR 1987) and the mitochondrial malate dehydrogenase (THOMPSON and MCALISTER-HENN 1989) contain redundant targeting signals at their amino termini. Truncated versions of these proteins lacking the mitochondrial presequence both target to and function in the mitochondrion. Hts1 differs phenotypically from these examples in that mitochondrial function is abolished in strains carrying only Htsc, and is restored only upon overexpression of the truncated protein. In this respect Htsm resembles subunit V_a of cytochrome *c* oxidase (DIRCKS and POYTON 1990).

The requirement for both the presequence and residues 21–53 to localize passenger proteins to the mitochondrion resembles that described for *TRM1*, the gene encoding both cytoplasmic and mitochondrial tRNA dimethyltransferases, by ELLIS, HOPPER and MARTIN (1989). These workers found that amino acids 1–17 of *TRM1* were not able to target a passenger protein to the mitochondria but served to augment the targeting signal encoded in amino acids 18–48. Thus, amino acids 1–48 of *TRM1* was more efficient at targeting than 18–48 alone.

The import sequence (21–53) present in both Htsm and Htsc lies within the alanine-rich (21–40) and basic (41–62) segments. Alignment of the protein sequences of human, hamster, yeast and bacterial histidyl-tRNA synthetases (TSUI and SIMINOVITCH 1987; NATSOULIS, HILGER and FINK 1986; FREEDMAN *et al.* 1985) further raises speculation about the biological role of these domains. The segments containing residues 1–20 (presequence) and 21–41 (alanine-rich stretch) are conspicuously absent from the *E. coli* histidyl-tRNA synthetase, which is a smaller protein than Hts1. MIR-

ANDE and WALLER (1988) noted that when one compares the amino acid sequence of each of the known tRNA synthetases from *E. coli* with their yeast homologs, the yeast sequence is always longer and contains an additional basic amino-terminal extension (with the exception of the seryl-tRNA synthetase, which has a small carboxy-terminal extension instead). These extensions are very basic, consisting of clusters of lysines and arginines, and in the case of yeast lysyl-tRNA synthetase have been shown to be responsible for binding to polyanionic carriers such as heparin sulfate (CIRAKOGLU and WALLER 1985). The human and hamster histidyl-tRNA synthetases share large blocks of amino acid identity with yeast beginning at residue 55, but do not have the amino-terminal-basic hydrophobic-basic motif present in *HTS1*. To date, there is no evidence that these mammalian genes encode a mitochondrial form of the enzyme.

In these studies we also found that low level expression of *hts1-4* (+1 ATG to ATC) is sufficient for mitochondrial Hts function, but high level expression provides cytoplasmic function as well. The simplest interpretation of these results is that overexpression of Htsm results in some cytoplasmic localization of the protein, perhaps due to saturation of components of the mitochondrial import machinery. An alternative explanation could be that when the normal initiation codon at +1 is altered, other AUGs are used as initiation sites by a leaky scanning mechanism. The next in-frame AUG, at position +145, would encode a protein, which upon overexpression, could provide cytoplasmic function (Htsd). Although a formal possibility, we think this scenario unlikely because in the *hts1-4* construct ribosomes scanning the shorter transcripts would encounter two AUGs which are out-of-frame for Hts at positions +44 and +53 before reaching the AUG at +145, and ribosomes scanning the longer transcripts would encounter the additional AUG at −60. Ultimately, the most direct way to ascertain what form of Hts is functioning in the cytoplasm of cells overexpressing *hts1-4* is to determine the amino-terminal sequences of the Hts proteins in these cells and compare them with those in wild-type cells.

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