The *Neurospora crassa* *cyt-20* Gene Encodes Cytosolic and Mitochondrial Valyl-tRNA Synthetases and May Have a Second Function in Addition to Protein Synthesis

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The *cyt-20-1* mutant of *Neurospora crassa* is a temperature-sensitive, cytochrome *b*- and *aa*₃-deficient strain that is severely deficient in both mitochondrial and cytosolic protein synthesis (R. A. Collins, H. Bertrand, R. J. LaPolla, and A. M. Lambowitz, Mol. Gen. Genet. 177:73-84, 1979). We cloned the *cyt-20*⁺ gene by complementation of the *cyt-20-1* mutation and found that it contains a 1,093-amino-acid open reading frame (ORF) that encodes both the cytosolic and mitochondrial valyl-tRNA synthetases (valRSs). A second mutation, *un-3*, which is allelic with *cyt-20-1*, also results in temperature-sensitive growth, but not in gross deficiencies in cytochromes *b* and *aa*₃ or protein synthesis. The *un-3* mutant had also been reported to have pleiotropic defects in cellular transport process, resulting in resistance to amino acid analogs (M. S. Kappy and R. L. Metzenberg, J. Bacteriol. 94:1629-1637, 1967), but this resistance phenotype is separable from the temperature sensitivity in crosses and may result from a mutation in a different gene. The 1,093-amino-acid ORF encoding valRSs is the site of missense mutations resulting in temperature sensitivity in both *cyt-20-1* and *un-3* and is required for the transformation of both mutants. The opposite strand of the *cyt-20* gene encodes an overlapping ORF of 532 amino acids, which may also be functional but is not required for transformation of either mutant. The *cyt-20-1* mutation in the valRS ORF results in severe deficiencies of both mitochondrial and cytosolic valRS activities, whereas the *un-3* mutation does not appear to result in a deficiency of these activities or of mitochondrial or cytosolic protein synthesis sufficient to account for its temperature-sensitive growth. The phenotype of the *un-3* mutant raises the possibility that the valRS ORF has a second function in addition to protein synthesis.

The filamentous fungus *Neurospora crassa* has been a useful model organism for analysis of mutants deficient in mitochondrial protein synthesis. In *N. crassa*, such mutants are readily identified by screening strains deficient in cytochromes *b* and *aa*₃, which contain mitochondrially synthesized subunits, whereas in the yeast *Saccharomyces cerevisiae*, a facultative aerobe, mutants that are tightly deficient in mitochondrial protein synthesis are unstable and give rise to petite mutants at high frequency (52). Studies of *N. crassa* cytochrome *b*- and *aa*₃-deficient mutants (designated *cyt* mutants) have shown that deficiency in mitochondrial protein synthesis can result from a defect in the translational apparatus itself or in some process that impairs mitochondrial protein synthesis indirectly, such as energy transduction (17). A number of *cyt* genes have been cloned by complementation of the corresponding nuclear mutations and have been found to encode mitochondrial ribosomal proteins (e.g., *cyt-21* and *cyt-22*; 40, 43), aminoacyl-tRNA synthetases (aaRSs) (*cyt-18*; 3), or proteins required for mitochondrial RNA processing (*cyt-4*; 72).

In this work, we focused on the nuclear gene *cyt-20*. A temperature-sensitive mutation in this gene, *cyt-20-1*, was characterized in our laboratory (16). The *cyt-20-1* mutant is phenotypically wild type when grown at 25°C but is grossly deficient in cytochromes *b* and *aa*₃ when grown at 37°C (16,

62). At 37°C, the *cyt-20-1* mutant is severely deficient in both mitochondrial and cytosolic protein synthesis (16, 17). Further, when cells are shifted from 25 to 37°C, mitochondrial protein synthesis turns off rapidly in the mutant, suggesting that the defective component functions directly in this process (18).

A second temperature-sensitive mutation *un-3*, is allelic with *cyt-20-1*, but the *un-3* mutant is not grossly deficient in protein synthesis and was not known to be defective in mitochondrial function (35). The *un-3* mutant had also been reported to have pleiotropic defects in the transport of acidic and neutral amino acids, resulting in resistance to amino acid analogs (34, 35, 50), but this resistance phenotype is separable from the temperature sensitivity in crosses and may result from a mutation in a different gene (41).

To investigate how mutations in the same gene could result in these different phenotypes, we cloned the *cyt-20* gene by complementation of the *cyt-20-1* mutation. We found that the gene contains a 1,093-amino-acid (aa) open reading frame (ORF) that encodes both the mitochondrial (mt) and cytosolic valyl-tRNA synthetases (valRSs) and is the site of missense mutations resulting in temperature sensitivity in both *cyt-20-1* and *un-3*. The *cyt-20-1* mutation results in a gross deficiency of both cytosolic and mt valRS activities, whereas the *un-3* mutation does not appear to result in deficiency of these activities or of mitochondrial or cytosolic protein synthesis sufficient to account for its temperature-sensitive growth. The phenotype of the *un-3* mutant raises the possibility that the valRS ORF has a second function in addition to protein synthesis.

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FIG. 1. Map of the *cyt-20* gene. The map shows locations of restriction sites and the relative positions of the 1,093-aa ORF encoding the valRS and the 532-aa ORF on the opposite strand. Also shown are the locations of the *cyt-20-1* (♦) and *un-3* (↑) mutations, the *SpeI* linkers inserted in the *cyt-20⁺* gene (◆), and the stop codon inserted in the 532-aa ORF (*).

MATERIALS AND METHODS

Strains of *N. crassa* and growth conditions. The wild-type strain was 74-OR23-1A (designated 74A; FGSC 987). The mutant strains were *cyt-20-1a* (previously designated 289-56; 62), RAQ20 *cyt-20-1 qa-2 aro-9 inl a, un-3A* (55701; FGSC 636) (30), and RLM 5718A *un-3 qa-2 aro-9 nuc-2 inl* (gift of R. L. Metzenberg, University of Wisconsin). The strains used for mapping the restriction site polymorphisms were RLM47-34a *al-2 nuc-1 ad-2 nic-1*, Mauriceville-1c A (FGSC 2225), and progeny of a cross between these strains (4 to 35; obtained from R. L. Metzenberg).

The procedures for maintaining strains, carrying out genetic crosses, and growing conidia and mycelia were as described previously (20). Mycelia were grown in liquid culture for the following times: 74A (25°C, 14 h; 37°C, 10 h), *cyt-20-1* strains (25°C, 14 h; 30°C, 14 h; 37°C, 24 h), *un-3* strains (25°C, 18 h; 30°C, 24 h), and *cyt-20-1* and *un-3* transformants (37°C, 12 to 14 h).

Preparation of DNA. Plasmid and cosmid DNAs were prepared by the sodium dodecyl sulfate (SDS)-lysis method (48) or by the potassium acetate method (2). *N. crassa* whole-cell DNAs were prepared as described previously (2).

Transformation of *N. crassa*. *cyt-20-1* or *un-3* host strains were transformed by using spheroplasts made with Novozym 234 (Novo Laboratories, Wilton, Conn.) (2).

Cloning of the *cyt-20⁺* gene. The *cyt-20⁺* gene was cloned by the sib selection procedure (2), using a cosmid library related to that of Vollmer and Yanofsky (76) but assembled independently from the same ligation and packaging (gift of S. Vollmer and C. Yanofsky, Stanford University). The library consists of *Sau3A* partial-digest fragments of whole-cell DNA from *N. crassa* wild-type 74A cloned into pSV50, a pBR322-derived vector containing the λ *cos* site and the *N. crassa* benomyl resistance (*Bml^r*) gene (76). A double-selection scheme was used to select *cyt-20⁺* *Bml^r* transformants. Following transformation, the cells were suspended and plated in 3 to 5 ml of top agar containing 1× Vogel's salts (75), 0.5 M $MgSO_4$, 2% sorbose, 0.05% sucrose, 0.05% fructose, and 0.6% Difco agar on a bottom agar of 1× Vogel's salts, 2% sorbose, 0.05% sucrose, 0.05% fructose, 1.5% Difco agar, and 0.25 μ g of benomyl per ml. The top agar contained no benomyl, allowing the transformants to regenerate under nonselective conditions before benomyl diffused in from the bottom agar. The plates were incubated at 25°C for 4 to 12 h, to allow regeneration in the absence of selection for *cyt-20⁺*, and then transferred to 40°C, a temperature that gave a low background of untransformed *cyt-20-1* cells. One plate was left at 25°C to measure the efficiency of *Bml^r* transformation. Colonies were scored after 3 days at 40°C. Putative transformants were picked

onto slants (1× Vogel's salts, 2% sucrose, 0.25 μ g of benomyl per ml, 1.5% agar; Sigma Chemical Co., St. Louis, Mo.) and incubated at 40°C. Strains that grew rapidly, with conidiation, were considered stable transformants.

The sib selection procedure involves transforming the mutant strain with pools of DNA of successively decreasing complexity. In the case of *cyt-20-1*, for the first round of transformation, we used 5 μ g of CsCl-purified DNA and 2×10^8 cells in a final volume of 160 μ l. For all subsequent rounds, we used approximately 1 μ g of DNA prepared by the potassium acetate method (2) and 5×10^7 cells in a final volume of 40 μ l. The final clone, after four rounds of sib selection, was called pSV-*cyt-20⁺*.

Subcloning and sequencing of the *cyt-20⁺* gene. To localize the *cyt-20⁺* transforming activity, pSV-*cyt-20⁺* was cut with various restriction enzymes. The restriction digests plus intact pSV50 to provide *Bml^r* were used to transform competent *cyt-20-1* RAQ20 (*cyt-20-1a qa-2 aro-9 inl*) or *un-3* RLM 5718A. Transformants were scored by growth at 40°C in the presence of 0.25 μ g of benomyl per ml as described above.

Restriction fragments containing the *cyt-20⁺* transforming activity were subcloned from pSV-*cyt-20⁺*. pAK7C1 contains a 14-kb *ClaI* partial-digest fragment of pSV-*cyt-20⁺* cloned into the *AccI* site of pBS(+) (Stratagene, La Jolla, Calif.). pAK20P7 contains a 5-kb *PvuII* fragment of pAK7C1 cloned into the *HincII* site of pBS(+). Subsequent analysis revealed that the inserts of pAK7C1 and pAK20P7 contain a spurious 2.3-kb *ClaI* fragment of unknown origin linked to the 3.2-kb *PvuII*-*ClaI* *N. crassa* DNA fragment containing the *cyt-20⁺* gene (Fig. 1). To obtain a longer *cyt-20⁺* clone, a 5.0-kb *SalI* fragment that hybridizes to the 3.2-kb *PvuII*-*ClaI* fragment and to a cDNA clone containing the 3' end of the *cyt-20* gene was isolated from pSV-*cyt-20⁺* and cloned into the *SalI* site of pBluescript KS(−) (Stratagene). This plasmid, designated pAKSal6, contains all of the *cyt-20⁺* gene except for the first 22 codons and was used to obtain sequences downstream of the *ClaI* site. pBTC20 is a whole-gene clone consisting of a 3.6-kb *PvuII*-*NsiI* *N. crassa* DNA fragment from pSV-*cyt-20⁺* cloned between the *SmaI* and *PstI* sites of pBS(+).

DNA sequencing. For sequencing, gel-purified restriction fragments of pAK20P7 and pAKSal6 were subcloned into M13mp18 or M13mp19 (Pharmacia, Inc., Piscataway, N.J.) or pBluescript KS(−). Single-stranded phage or alkali-denatured plasmids were sequenced by the dideoxy method (37, 66). The sequences of both DNA strands were determined experimentally.

In vitro mutagenesis of the *cyt-20⁺* gene. *SpeI* linkers, which contain translation stop codons in all three reading

frames, were inserted after codons 13 and 926 of the 1,093-aa ORF of the *cyt-20⁺* gene. To insert the linkers, pBTC20 was partially digested with *Xho*I, and the linearized plasmid was isolated by electrophoresis in a 0.8% agarose gel. The 3' recessed ends of the plasmid were filled in with the Klenow fragment of DNA polymerase I, and the plasmid was then religated in the presence of the *Spe*I linker 5'-CTAGAC TAGTCTAG-3' (New England BioLabs, Beverly, Mass.). Clones were screened for the absence of one of the *Xho*I sites and for the presence of a new *Spe*I site at the appropriate position. The clones were designated *pcyt-20*[*Spe*I13] and *pcyt-20*[*Spe*I926].

To determine whether the 532-aa ORF on the strand opposite the 1,093-aa ORF is required for *cyt-20-1* transforming activity, we introduced a single base change (C-2275 to A) that gives a UGA termination codon at codon 9 of the 532-aa ORF but gives a silent change (UCC to UCA) at codon 541 of the 1,093-aa ORF on the opposite strand. To accomplish this, a 509-bp *Hind*III-*Sph*I fragment from the *cyt-20⁺* gene was cloned into M13mp18 and subjected to site-directed mutagenesis, using the oligonucleotide 5'-CTACTAAGTCATACAAGAGG-3' (44). Clones were screened by sequencing, and a 349-bp *Bgl*II-*Sph*I fragment containing the modification was replaced into pBTC20. This clone was designated *pcyt-20-mut532*.

Cloning of the mutant *cyt-20-1* and *un-3* genes. Whole-cell DNA from *cyt-20-1* or *un-3* was cut with *Pvu*II and *Nsi*I, and DNA fragments of approximately 3.6 kb were isolated following electrophoresis in a 0.8% agarose gel. The DNAs were ligated into pBS(+), which had been digested with *Sma*I and *Pst*I, and clones were identified by plaque hybridization. Plaques were transferred to nitrocellulose filters and baked at 80°C for 2 h (1). The filters were then probed with pAK20P7 1.4- and 1.5-kb *Xho*I restriction fragments, which had been gel purified and ³²P labeled by nick translation (64). Positive plaques were picked and replated for purification. The final *cyt-20-1* and *un-3* clones were called pBT289-56 and pBTun3, respectively.

Gel-purified restriction fragments from the clones described above were cloned into pBS(+) and sequenced by the dideoxy method, using Sequenase (United States Biochemical Corp., Cleveland, Ohio).

Southern hybridization. DNA was blotted to Hybond-N (Amersham, Arlington Heights, Ill.) and hybridized by the method of Southern (70), as modified by Smith and Summers (69). Recombinant plasmids used as probes were ³²P labeled by nick translation (64).

Cytochrome spectra. Reduced-minus-oxidized difference spectra of crude mitochondria were obtained as described previously (9), using a Beckman DU-7 spectrophotometer.

Assay of aaRS activity. Lysates were prepared from mitochondria purified by the modified flotation gradient method (45). The final mitochondrial pellets were lysed with 0.3% Triton X-100–10 mM Tris-HCl (pH 7.5)–1 mM β -mercaptoethanol–1 mM phenylmethylsulfonyl fluoride, and the lysates were centrifuged in a Beckman Ti50 rotor (100,000 $\times$ g, 90 min, 4°C) to pellet insoluble material (3).

Cytoplasmic lysates were prepared by a modification of the cell disruption method of Cramer et al. (19). Mycelial pads were disrupted in a Bead-Beater (Biospec Products, Bartlesville, Okla.) in 15% sucrose–10 mM Tricine-KOH–0.2 mM EDTA, pH 7.5, and nuclei and cell debris were pelleted at 1,000 $\times$ g. The supernatant was filtered through a 4.7-cm Whatman 934AH glass fiber filter and centrifuged first at 15,000 $\times$ g to remove organelles and then in a Beckman Ti50.2 rotor (100,000 $\times$ g, 90 min, 4°C) to pellet insoluble

material. Endogenous tRNAs were removed by adsorption to DEAE-Sephacel (63). The extract was then dialyzed overnight against 20 mM potassium phosphate (pH 7.5)–20 mM β -mercaptoethanol–1 mM MgCl₂–50% glycerol and stored at –20°C. Protein concentrations were measured by the method of Bradford (10).

Aminoacylation reactions were carried out essentially as described by Baldacci et al. (6). Reactions were in 20 μ l of solution containing 4 μ l of extract, 15 mM MgCl₂, 50 mM Tris (pH 8.8), 5 mM ATP, 1 μ Ci of radioactive amino acid (L-[2,3-³H]valine, 42 Ci/mmol; L-[4,5-³H]lysine, 20 Ci/mmol; or L-[3,5-³H]tyrosine, 42 Ci/mmol; ICN, Irvine, Calif.), and tRNA substrate. Mitochondrial synthetases were assayed with purified *Escherichia coli* tRNA^{Val} (6 μ g; 150 pmol of amino acid acceptor activity; Sigma), purified *E. coli* tRNA^{Tyr} (2.5 μ g; 200 pmol of amino acid acceptor activity; Subriden RNA, Rollingbay, Wash.), or unfractionated *E. coli* tRNAs (100 μ g; Sigma). Assays of cytosolic synthetases were the same except that the substrate was 100 μ g of yeast tRNA (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and 0.2 mM CTP was included in the reaction mixture (8). Aminoacylation reactions were initiated by addition of extract, incubated for 15 min at temperatures specified in Tables 3 and 4, and terminated by addition of 0.5 ml of ice-cold 10% trichloroacetic acid–0.1% SDS. After incubation on ice for at least 10 min, ³H-aminoacyl-tRNAs were collected on Whatman GF/C glass filters and washed 12 times with 2 ml of cold 2.5% trichloroacetic acid–0.1% SDS (21). Filters were dehydrated with 1 ml of 1:1 (vol/vol) mixture of ethanol and ether and counted in a Beckman LS 1801 scintillation counter, using Beckman Ready Protein⁺ liquid scintillation cocktail. Parallel incubations with boiled extracts were used to determine background counts per minute, which were subtracted from the final values.

Nucleotide sequence accession number. The GenBank accession number for the sequence presented in this report is M64703.

RESULTS

Cloning of the *cyt-20⁺* gene. The *cyt-20⁺* gene was cloned by complementation of the *cyt-20-1* mutation by the sib selection procedure as described in Materials and Methods. After subdivision of the library, we obtained a single transforming cosmid, pSV-*cyt-20⁺*, which has an insert size of ~40 kb.

The identity of the putative *cyt-20⁺* clone was confirmed by mapping its chromosomal location, using restriction fragment length polymorphisms as genetic markers (51). In a cross between RLM47-34a *al-2 nuc-1 ad-2 nic-1*, which contains linkage group I markers, and Mauriceville-1c A, the *N. crassa* chromosomal DNA insert in pSV-*cyt-20⁺* was tightly linked to mating type (18 of 18 progeny), in agreement with genetic mapping data showing that *cyt-20-1* is located within 1 map unit of mating type (15, 40).

The *cyt-20* transforming activity was localized within pSV-*cyt-20⁺* by cutting the cosmid with various restriction enzymes and cotransforming the *cyt-20-1* mutant with the cut cosmid plus the vector pSV50 to provide Bml^r as a second selectable marker. On the basis of the results, progressively smaller subclones were made from pSV-*cyt-20⁺* as described in Materials and Methods. The smallest subclone that has *cyt-20⁺* transforming activity is pAK20P7, which contains a 3.2-kb *Pvu*II-*Cla*I fragment of the *cyt-20⁺* gene cloned in pBS(+) (Fig. 1). Transformants obtained with

pAK20P7 had wild-type cytochrome spectra and growth rates (data not shown).

After the *cyt-20*⁺ gene was sequenced, it became apparent that pAK20P7 is missing the final 66 aa of the *cyt-20* ORF, which apparently are not required for *cyt-20*⁺ transforming activity. To obtain the 3' end of the gene, a 5.0-kb *SalI* fragment that hybridizes to the 3.2-kb *PvuII*-*ClaI* fragment and to a 3'-terminal cDNA clone was isolated from pSV-*cyt-20*⁺ and cloned into the *SalI* site of pBS(-). This clone, designated pAKSal6, begins with codon 23 of the ORF and extends for 1.7 kb downstream of the ORF. A whole-gene clone, designated pBTC20, was obtained by cloning the 3.6-kb *PvuII*-*NsiI* fragment of pSV-*cyt-20*⁺ (Fig. 1) between the *SmaI* and *PstI* sites of pBS(+).

cyt-20⁺ appears to be a single-copy gene, as determined by hybridization of pAK20P7 to *HindIII* digests of *N. crassa* whole-cell DNA at hybridization stringencies ranging from 10 to 50% formamide at 37°C (data not shown).

The *cyt-20*⁺ gene encodes a 1,093-aa ORF homologous to valRSs. Using DNA fragments of pAK20P7 and pAKSal6 subcloned in M13 phage or pBluescript KS(-), we determined the nucleotide sequence of both DNA strands of a 4,135-bp region that contains the *cyt-20*⁺ gene (Fig. 2). Analysis of the sequence showed that it contains a 1,093-aa ORF interrupted near its 5' end by a putative intron of 79 bp, identified by conformity to the *N. crassa* intron consensus sequences (25; Fig. 1 to 3). The ORF could encode a polypeptide as large as 123.4 kDa. The sequence presented here extends 573 bp upstream and 201 bp downstream of the 1,093-aa ORF.

A data bank search showed that the 1,093-aa ORF has strong similarity to both *S. cerevisiae* and *E. coli* valRSs (52.5 and 43.8% identity, respectively). Figure 4 shows alignment of the 1,093-aa ORF with the yeast valRS. Similarity extends throughout the proteins, with no large internal insertions in either sequence. The only sequences that do not have high similarity are at the amino termini of the ORFs and presumably correspond to mitochondrial targeting sequences. Both the HIGH and KMSKS sequences, which are characteristic of class 1 aaRSs (24), are present in the *cyt-20* ORF (indicated by over- and underlines in Fig. 4).

In yeast cells, both the cytosolic and mt valRSs are encoded by the same gene but are translated by using two different ATG initiation codons (12). The first ATG gives a protein that has an N-terminal leader required for targeting the protein to mitochondria, whereas the second ATG, which is located in frame, 47 codons downstream, gives a protein that lacks the N-terminal leader (12). Examination of the 1,093-aa ORF in the *cyt-20*⁺ gene showed two similarly positioned ATGs at codons 1 and 44 (Met residues underlined in Fig. 2). As for the yeast valRS, proteins initiated at the first ATG would have an N-terminal leader with characteristics expected for a mitochondrial targeting sequence (i.e., rich in basic, hydrophobic, and hydroxylated amino acids and lacking acidic amino acids; 4), whereas proteins initiated at the second ATG (codon 44) would lack this N-terminal leader. Potential processing signals for the N-terminal leader, which fit the consensus Arg-X, where X is often a small amino acid (39, 65, 68, 74), are at Arg-7-Leu-8, Arg-25-Val-26, and Arg-31-Pro-32.

Initiation codons in *N. crassa* nuclear genes are generally preceded by an A/C-rich region and the consensus sequence Pu(C/A/T)CA(A/C)(A/C) (Pu indicates purine) immediately upstream of the ATG (60). The first ATG in the *cyt-20*⁺ ORF, which is presumably used for the mt valRS, is preceded by a somewhat A/C-rich region (61% A/C in 54 bases)

and the sequence CTCTGG, which is a poor match to the consensus. The *N. crassa* mt tyrRS and mt leuRS genes also have initiation codons preceded by poor matches to the consensus sequence (3, 13), although otherwise the initiation contexts for the three mitochondrial synthetases have relatively little in common (see Fig. 7). It is possible that the mitochondrial synthetases use relatively weak initiation contexts as part of a mechanism for limiting or regulating the amount of protein at the level of translation. The second ATG in the 1,093-aa ORF, which is presumably used for cytosolic valRS, is preceded by a more A/C-rich region (74% A/C in 23 bp) and the sequence GTCATC, which matches the consensus at all but one position (see Fig. 7).

In *N. crassa*, highly expressed genes show a strong bias in codon usage such that pyrimidines are used almost exclusively in the third position, G is used over A where a purine must be used in the third position, and the codons AGN and TTPu are rarely used (36, 58). *N. crassa* nuclear genes encoding components involved in cytosolic protein synthesis (e.g., cytoplasmic ribosomal proteins, *crp-1* and *crp-2*, and the cytoplasmic leuRS) have codon biases that fit the pattern for highly expressed genes (14, 38, 73), whereas those encoding components involved in mitochondrial protein synthesis have a moderate codon bias expected for intermediate expressed genes (e.g., *cyt-18*⁺, *cyt-21*⁺, *cyt-22*⁺, and *leu-5*⁺; 3, 14, 40, 43). The 1,093-aa ORF of the *cyt-20*⁺ gene, which encodes both the cytosolic and mt valRSs, has only a moderate codon bias (Table 1).

The opposite strand of the *cyt-20*⁺ gene encodes a 532-aa ORF. Interestingly, the strand opposite that containing the 1,093-aa ORF also contains a long ORF of 532 aa interrupted by an apparent intron of 62 bp near its 5' end (Fig. 1 and 3; the first exon of the 532-aa ORF encompasses positions 2361 to 2354 and the second exon encompasses positions 2291 to 704). The first ATG in the 532-aa ORF (position 2361) is preceded by an A/C-rich region (78% A/C in 18 bases) and a three-of-six match (TCAAAG) to the consensus sequence found preceding initiation codons in *N. crassa* nuclear genes (see above). If we exclude the potential intron, the ORF on the opposite strand would have a length of 545 aa, beginning with an ATG in the intron. This ATG is preceded by 20 nucleotides (nt) that are only 50% A/C and by a poor match (AATGCG) to the initiation consensus sequence. The codon usage in the 532-aa ORF is less biased than that in the 1,093-aa ORF but is not random, as might be expected for a noncoding sequence (Table 1). Screening of the GenBank/EMBL data base with use of the UWGCG program (22) showed that the 532-aa ORF has 21.3% identity to a chromate transport protein of *Pseudomonas aeruginosa* (11), with the alignment requiring the introduction of 15 gaps.

An additional mutant allele of the *cyt-20* gene. The chromosomal location of the *cyt-20-1* mutation, which is tightly linked to mating type, raised the possibility that it might be allelic with a previously characterized temperature-sensitive mutation, *un-3*, which maps to a gene located in this same region of linkage group I (31, 56). The allelism of the two mutations was confirmed by genetic analysis carried out by R. L. Metzenberg. Crosses between *un-3A* and *cyt-20-1a* gave no recombinants that grew at 37°C among 21,200 viable progeny, and the *cyt-20-1* and *un-3* mutations, both of which are recessive, failed to complement in forced heterokaryons (49).

We were surprised to learn that *un-3* is allelic with *cyt-20-1*, since *un-3* was thought to be defective in a component affecting cellular transport and was not known to have impaired mitochondrial function (34, 35). The phenotypes of

XhoI
CTCGAGGGACTACAAAACGCTACAAGAAATATGACTGGGAAAGATCGATTCTGGGTCTCCCGTTCAGTTT 70

TCACCAGGAATCACGAAATGACTCGAACGGTCGCGCAGATACTGAGGCCACCCGAATGTAAGCCAACTGTTGGTGAGTCTAAACAAGCCAA 160

CAGGCAATAGCTAGGTACTTAGCGTACAGTGAAGTCTGTCATCCCTTCCCTTATATCGACGAAATACTTCTAGGTAAGGTAGGTTCATGT 250

ATCGAAGTCGGAAGATGGAATTAACCTCGACGCTATGGTGGTACTTCCCATAGGTGAAGGGAGCACCCCTGATGGAGGGTGGGAGAGGG 340

GCTTGGCCTCTGTGACCAGGTACCCCTCAAACTTGTACGTATGTAGTGTACGCGGCTGACAGGTACAGTGGGGCCCGTCGCTGGGAA 430

ATCTTTTGACTCATGGCCATGCTCCCGGCGGCTAGTCCGCTCTGCGCTCCGAAGGGCGGCGCAGAAATTCCTGTCAGCCTGACGGC 520

PvuII
CCGCCTATAACAATCAACCCCTCAGCTGCCCATTTGACCCCTATCGTCTCTGGATGCCGCTTTCGTGTGGCGATTGGCAATTGGAAGCT 610

MetProLeuSerLeuTrpArgLeuAlaIleGlySerS 13

XhoI Sali
CGAGAAGGCATTGCTTAAATCACGTCGACCCGCTACTTCTCTGTCGACCGTATTCGACCTACAGTAACAACAAGAAAGTCA 700

erArgArgHisSerLeuLeuLysIleThrSerThrArgValLeuProValProArgProTyrSerThrTyrSerAsnAsnLysLysValI 43

TCATGCCGGGAGAAGATCAACCTGCTGCCGCGCTGCCGCTGCGCGGCTGCGCAAGACGCGCTGGTGGCCCTGCCCGAAGAAGAACG 790

leMetProGlyGluAspGlnProAlaAlaAlaProAlaAlaAlaProAlaAlaGlnAspAlaProGlyAlaProAlaProLysLysAsnA 73

AccI
GTTCTGAGCCTCGTGGATCTGAACTCTCAATGTGCAATTGTGGCCACCAACTTACACCTGTTTCTTTGCTCTGTAGACAAGAAGGAG 880

spLysLysGlu 76

AAGGCCAAGGCCGACAAGGCCGCAAAATTCGCGCTAAGCAAGCCGCGCCAAAGCGAAGCAGCCTGCTGCTCAGTCCGCACCCAAAGAG 970

LysAlaLysAlaAspLysAlaAlaLysPheAlaAlaLysGlnAlaAlaAlaLysAlaLysGlnProAlaAlaGlnSerAlaProLysGlu 106

AAGAAGGAGAAAATCCTGCCCTGCCTCTTACGAAGATAGCACCCCGCTGGCGAGAAGAAGGTCAATCTTTTGGAGCACCCTCAC 1060

LysLysGluLysThrProAlaLeuProProTyrGluAspSerThrProAlaGlyGluLysLysValIleGlnSerPheGluHisProHis 136

PstI
TTCTCTGCATACAACCCAGCGCGCTCGAGGCGGCATGGTATCAATGTTGGGAGAAGGGCCGCTACTTCAAGCCCGAAAGCTGCAGGAAA 1150

PheSerAlaTyrAsnProSerAlaValGluAlaAlaTrpTyrGlnTrpTrpGluLysAlaGlyTyrPheLysProGluSerCysArgLys 166

CCCTCGGCTGGCAAGTTCGTCATCCCTCTGCCACCTCCGAACGTTACCGGCGCTCTCCACTGCGGCCATGCTCTCGCCAACTCCCTGCAA 1240

ProSerAlaGlyLysPheValIleProLeuProProProAsnValThrGlyAlaLeuHisCysGlyHisAlaLeuAlaAsnSerLeuGln 196

*
t KpnI g
GATACTTTGATTGGTACCGCATGAAGGGCTATGAGACCTTGTGGGTGCCCGGTTCGACCATGCGCGTATTTTCGACCCAGTCCGTC 1330

AspThrLeuIleArgTrpTyrArgMetLysGlyTyrGluThrLeuTrpValProGlyCysAspHisAlaGlyIleSerThrGlnSerVal 226

Cys

GTCGAGAAGATGCTCGGAAGAAGGAGAAGAAGATCCGTCAGAAGCTTGGCCGTGAGAAGTTCACAGACCTCGTCTGGGAGTGAAGGGT 1420

ValGluLysMetLeuTrpLysLysLysIleArgGlnGluLeuGlyArgGluLysPheThrAspLeuValTrpGluTrpLysGly 256

HindIII
GAATACCATCAGCGCATCAACAATGCCAGAAGCTTATGGGCGGCTCGATGGAGTGGAGCCGCGAGGCTTTCACCATGGATAAGAACCTC 1510

GluTyrHisGlnArgIleAsnAsnAlaGlnLysLeuMetGlyGlySerMetAspTrpSerArgGluAlaPheThrMetAspLysAsnLeu 286

*
c

ACCGTGCCACCATGGAGACTTTCTGCGCTCTCATGACGAGGGCTCATCTATAGGTCTAACCGCTTGGTGAAGTGGTGCACCCACTTG 1600

ThrAlaAlaThrMetGluThrPheCysArgLeuHisAspGluGlyLeuIleTyrArgSerAsnArgLeuValAsnTrpCysThrHisLeu 316

g
Pro

AACACCGCGCTCAGTGGTTTGGAGGTGAGACCAAGGAGATTACCGGCGGTACACTTTCGACGTTCCCGGTTACGACAAGAAGGTGAG 1690

AsnThrAlaLeuSerGlyLeuGluValGluThrLysGluIleThrGlyArgThrLeuLeuAspValProGlyTyrAspLysLysValGlu 346

c

TTCCGTGTCCTTACACACTTCAAGTACCAGATCGACGTTCTGAGGAGACGATCGAGGTGCCACCAACCGTCCCGAAACCATGCTTGGC 1780

PheGlyValLeuThrHisPheLysTyrGlnIleAspGlySerGluGluThrIleGluValAlaThrThrArgProGluThrMetLeuGly 376

c BamHI

GATACCGGTATTGCCGTCACCCGAGGATCCCGCTACACCCATCTTGTCCGCAAGTTCGCGCGCCACCCCTTGTGACCGTCTCCTT 1870

AspThrGlyIleAlaValAsnProGluAspProArgTyrThrHisLeuValGlyLysPheAlaArgHisProPheValAspArgLeuLeu 406

c

CCCATTGTCAACGCAACTACGTTGATAAGGAGTTCGGTACAGGTGCTGTCAAGTTGACCCCGGCCACGATTTCACGATTACCAGCTT 1960

ProIleValThrAspAsnTyrValAspLysGluPheGlyThrGlyAlaValLysLeuThrProAlaHisAspPheAsnAspTyrGlnLeu 436

XhoI
GGTCAGCGTCACAACTCGAGTTCATCAATATTCTGAACGAGAACGGAACCCCTCAACGCAATGCCGGCCCTTTTAAGGGACAGAAGCGC 2050

GlyGlnArgHisAsnLeuGluPheIleAsnIleLeuAsnGluAsnGlyThrLeuAsnAspAsnAlaGlyProPheLysGlyGlnLysArg 466

FIG. 2. Sequence of the *cyt-20* gene. The nucleotide sequence was determined by dideoxy sequencing of restriction fragments of pAK20P7 or pAKSal6 cloned into M13mp18 or -19 or pBluescript KS(-). The sequences of both DNA strands were determined experimentally. The putative intron at positions 791 to 869 was identified by conformity to the *N. crassa* splice site consensus sequences (25; see Fig. 3) and by the break in homology to yeast valRS. Nucleotide and amino acid changes in the mutant alleles are indicated above and below the sequence, respectively. The putative *cyt-20-1* and *un-3* mutations are as follows: *cyt-20-1*, Arg-201 to Cys; and *un-3*, Ser-306 to Pro (indicated by asterisks). Other sequence changes found only in *cyt-20-1* are indicated by lowercase letters, those found only in *un-3* are indicated by italicized lowercase letters, and those found in both *cyt-20-1* and *un-3* are indicated by capital letters. Recognition sequences for the *N. crassa* CPC-1 protein are underlined. Sequence elements shared with other *N. crassa* aaRSs (see Fig. 7) are boxed. Met residues corresponding to potential initiation codons are underlined. Arrowheads indicate major 5' RNA ends mapped in preliminary primer extension experiments (71).

TCGACGCCCCGTACACTGTTGTGGAGAACTGACCAAGCTTGGACTCTTCGTTAAGAAGGAACCCAAACCAATGAAGATTCCCCTTTGT 2140
 PheAspAlaArgTyrThrValValGluGluLeuThrLysLeuGlyLeuPheValLysLysGluProAsnProMetLysIleProLeuCys 496

GAGAAGTCTAAGGACGTTATTGAGCCCATGATGACCGAGCAATGGTGGGTTCGCATGAAGGAGATGGGTGAAGCTGCGCTCCAAGTTGTC 2230
 GluLysSerLysAspValIleGluProMetMetThrGluGlnTrpTrpValArgMetLysGluMetGlyGluAlaAlaLeuGlnValVal 526

BglII EcoRV
 GAGGAGGGCAAGGTCAAGATCTCCCCGAGAGTGTACTAAGTCTCTACAAGAGGTGGCTTGATATCCAGGATTGGTGTATTATCCCGT 2320
 GluGluGlyLysValLysIleSerProGluSerAlaThrLysSerTyrLysArgTrpLeuAlaAspIleGlnAspTrpCysIleSerArg 556

CAGCTCTGGTGGGGCCATCGCATTCCTGCTTACCGCGTCATCTTTGAGGGTGAGGAGGGCCAACTGAGAACGAGAAGTCCGAATGGGTG 2410
 GlnLeuTrpTrpGlyHisArgIleProAlaTyrArgValIlePheGluGlyGluGluGlyGlnArgGluAsnGluLysSerGluTrpVal 586

GTCGGCAGGACACAGGAGGAGGCTCAGGCAAAGGCTGAGGCCAAGTTCGCGCGCGCAAGTTCCTCTTGAGCAGGACCCCGACTGTCTC 2500
 ValGlyArgThrGlnGluGluAlaGlnAlaLysAlaGluAlaLysPheAlaGlyArgLysPheThrLeuGluGlnAspProAspCysLeu 616

GATACCTGGTTTCAGCTCCGGACTTTGGCCTATGGCCATTCTTGGCTGGCCCAACACTGAGAACCTCGATTTCAGAAGTTCTTCCCTACC 2590
 AspThrTrpPheSerSerGlyLeuTrpProMetAlaIleLeuGlyTrpProAsnThrGluAsnLeuAspPheLysLysPhePheProThr 646

SphI KpnI
 AGCATGCTCGAAACCGGCTGGGATATTTGTCTCTCTGGGTGACCGGTATGATTATGCTCAGTCTCAAGATGACCGGAGAGGTACCGTTC 2680
 SerMetLeuGluThrGlyTrpAspIleLeuPhePheTrpValSerArgMetIleMetLeuSerLeuLysMetThrGlyGluValProPhe 676

ACTGAGGTGTAAGTCCACAGTCTGATTCTGTTCCGAGGGCAGGAAAATGAGCAAGTCTTTGGGCAACGTCATTGACCTCTTGACATC 2770
 ThrGluValTyrCysHisSerLeuIleArgAspSerGluGlyArgLysMetSerLysSerLeuGlyAsnValIleAspProLeuAspIle 706

ATTCGCGGTATTGAACTAGAGGATCTCCACGCAAAGCTGCTGGTTCGTTAACCTGAAGGAGGAGGAAGTCGCGAGGCCACCAATACCAA 2860
 IleArgGlyIleGluLeuGluAspLeuHisAlaLysLeuLeuValGlyAsnLeuLysGluGluGluValAlaArgAlaThrLysTyrGln 736

AAGACTGCCTTCCCCGGCGGTATTCCCGAGTGGCGCGGACGCCATCGCTTTACCCCTTCTCTCTACACCACTGGCGGTGGCGATATC 2950
 LysThrAlaPheProGlyGlyIleProGluCysGlyAlaAspAlaMetArgPheThrLeuLeuSerTyrThrThrGlyGlyGlyAspIle 766

EcoRV
 AAGACTGCCTTCCCCGGCGGTATTCCCGAGTGGCGCGGACGCCATCGCTTTACCCCTTCTCTCTACACCACTGGCGGTGGCGATATC 2950
 LysThrAlaPheProGlyGlyIleProGluCysGlyAlaAspAlaMetArgPheThrLeuLeuSerTyrThrThrGlyGlyGlyAspIle 766

HindIII
 AACTTCGACATCAGGGTTATGCACGCTTACCGCAGATTCTGCAACAAGATTGGCAAGCCAGCAAGTACGTCCTTGGTAAGCTTCTCTCAG 3040
 AsnPheAspIleArgValMetHisAlaTyrArgArgPheCysAsnLysIleTrpGlnAlaSerLysTyrValLeuGlyLysLeuProGln 796

BamHI
 GACTTTATGCCCAAGGGTGAAGTTCACACCGCAACTTCTCCGTTCCCGAGAAGTGGATCCTTACCCGCATGAACGTGGCTGTTAAGGGC 3130
 AspPheMetProLysGlyGluLeuAspThrAlaAsnPheSerValProGluLysTrpIleLeuHisArgMetAsnValAlaValLysGly 826

SacI
 ATGAATGAGGCTCTTGAGGCCCCGAGTCTCCAGAGCCACCAAGGTGGCTTACCGATTCTTCTATGACGAGCTCTGCGACGCTCTTCATC 3220
 MetAsnGluAlaLeuGluAlaArgGluPheSerArgAlaThrLysValAlaTyrGlnPhePheTyrAspGluLeuCysAspValPheIle 856

KpnI
 GAGAACAGCAAGGGTATCCTTAGCGACGGTACCCCGAGGAGCAGCAGCGTCAACAGACACTGTACCATGCCCTTGATGTGGCGCTG 3310
 GluAsnSerLysGlyIleLeuSerAspGlyThrProGluGluGlnGlnSerValGlnGlnThrLeuTyrHisAlaLeuAspValAlaLeu 886

SacI
 CGCCTTCTCCACCCCATCATGCCCTACATTACTGAGGAGCTCTGGCAGCGTCTTCTCGCAAGCAGGGCGATGGCGAGACCATCATGCTT 3400
 ArgLeuLeuHisProIleMetProTyrIleThrGluGluLeuTrpGlnArgLeuProArgLysGlnGlyAspGlyGluThrIleMetLeu 916

XhoI
 GCTCCCTACCTGCTTTGAGTCTCAGCTCGAGTTTGGCAGCCGAAGCTGAGGACTATGAGCTTGGTCTCAAGTGCGCCGGCGGCATCCGC 3490
 AlaProTyrProAlaPheGluSerGlnLeuGluPheAlaThrGluAlaGluAspTyrGluLeuGlyLeuLysCysAlaGlyGlyIleArg 946

TCCCTGGCTGCCGACTATAACATCAAGTCGGATGGCAGAGCGTTTCAAGGCTACTACTCGGCAGACGCTTGCTACTGTTAGTGCTCAG 3580
 SerLeuAlaAlaAspTyrAsnIleLysSerAspGlyArgAlaPheIleLysAlaThrThrAlaAspSerLeuAlaThrValSerAlaGln 976

CTCGCCCCATCAGGACACTCTGCGGCAAGGGTGTAAAGGAGGTCAACGCTTGGGCGCTGATGAGGAGCTGCCAAGAGGCTGCGCCGTC 3670
 LeuAlaAlaIleArgThrLeuCysGlyLysGlyValLysGluValAsnValLeuGlyAlaAspGluGluLeuProArgGlyCysAlaVal 1006

ClaI
 TATGTCATCAATGCCGAGATCACCGTCTTCTCCAGGTTGGCGGTAGCATTAGCGACATCGATGCCGAGATCAAGAAGATCACCAACAG 3760
 TyrValIleAsnAlaGluIleThrValLeuLeuGlnValGlyGlySerIleSerAspIleAspAlaGluIleLysLysIleThrThrLys 1036

TTGCAGAAGACTGATCTCACCATCAAGAAGCAACAGGAGCTTCTGTCTAAGGATGGTTTCGAGAAGGTTCAGCGAGGCTGCCAGGAGTCT 3850
 LeuGlnLysThrAspLeuThrIleLysLysGlnGlnGluLeuLeuSerLysAspGlyPheGluLysValSerGluAlaValGlnGluSer 1066

DraI
 GAGAAGCAGAAGCTGGCCGATGCCAAGCGGCAAGGAGAACTACCAACGCCTCTGGAGGAGTTCAGCAAGCTCAAGATTAAATGAAA 3940
 GluLysGlnLysLeuAlaAspAlaGlnAlaAlaLysGluAsnTyrGlnArgThrLeuGluGluPheSerLysLeuLysIleEnd 1093

TTCGGAGTTGGCATCTGTTTAAAGCGTTTACTACGGTCCCTGAGTGTCCGAACAATGCTCAAAGCGTCAGAAACGTTGTAGCTAGTATTTT 4030
 TTTTCTCAATCAAAAACGGCGTAGTTCCGAATTTGAATCATCCCACTATAAACTTTGCGTGATAAGGATGCATGTAGTGTTCGACTTT 4120

HpaI
 CGACTTTCCGTTAAC 4135

FIG. 2—Continued.

INTRON SEQUENCES

consensus	GTAAGT C	-----	ACTAAC G G	-----	7-19	-----	CAG T
1093 aa ORF	791-GTTCGT	-----	ACCAAC	-----	23	-----	TAG-869
532 aa ORF	2353-GTAAGC	-----	GCTGAC ACCAAT	----- -----	26 8	----- -----	CAG-2292

FIG. 3. Comparison of sequences in potential introns interrupting the 1,093-aa ORF and the 532-aa ORF on the opposite strand. Consensus sequences shown are for the 5' splice site, putative branch point, and 3' splice site of *N. crassa* genes (25).

the two mutations were compared further. Both *cyt-20-1* and *un-3* are temperature sensitive. However, *cyt-20-1* cells grow similarly to wild-type cells at 25 and 30°C and grow slowly at 37°C (62), whereas *un-3* cells grow slowly at 25°C and do not grow above 30°C (61).

Kappy and Metzenberg (35) measured the rate of cellular protein synthesis in *un-3* cells by incorporation of ¹⁴C-lysine, whose transport is unaffected in the mutant, or ¹⁴C-phenylalanine into trichloroacetic acid-precipitable radioactivity. For both amino acids, they found that following a temperature shift from 24 to 35°C, the growth of *un-3* cells ceased immediately, but incorporation of the radioactive amino acid continued at about the same rate, which was 50 to 60% of the

wild-type rate at 35°C, for at least 5 h, suggesting that cessation of growth was not due to inhibition of protein synthesis (35).

To assess mitochondrial function in the mutant strains, we analyzed their cytochrome spectra (Fig. 5). When grown at 25°C, the *cyt-20-1* mutant had wild-type amounts of cytochromes *b* and *aa₃*. At 30°C, the amounts of cytochromes *b* and *aa₃* relative to cytochrome *c* were decreased approximately 60%, and at 37°C, the mutant was grossly deficient in cytochromes *b* and *aa₃*. By contrast, the *un-3* mutant grown at 25 and 30°C had 60 to 70% wild-type amounts of cytochromes *b* and *aa₃* relative to cytochrome *c*, even though its growth rate in liquid culture at 30°C was less than 13%

Nc	MPLSLWRLAIGSSRRRESLLKITSTRVLPVPRPYS---	TYSNK-KVI-MPG-EDQPAAPAAA-----	PAAQDAPGAPAPKNDKKEKAKAD	81
Sc	MNKWLNTLSKTFTR--LLNCHYRRSLPLCNFSLKSLTHNQVRFKMSDLDLPPVDPKTGEVITNPLKED--	GSPKTPKEIEKKEKKAE		88
Nc	KAAPFAAKQA---AAKAKQPAASAPKEKKEKTPALPPYEDSTPAGEKKVQSFEHPHSAYNPSAVEAAWYQWWEKAGYFKPE---	SCR-K		166
Sc	KLLKFAAKQAQAKNA--ATTGASQKKPKKKKEVEP-IPFIDKTVPGKKILVSLDDPALRGYNPANVESSYDWNWIKTGVEPEFTADGKVK			178
Nc	PSAGKFVIPLEPPNVGTALBEGHALANSLQDTLIRWYRMKGYSYETLVWPGCDHAGISTQSVVERKMLWKEKKIRQELGREKFTDLVWWEKGEY			258
Sc	P-EGVFCIPAPPPNVGTALBEGHALTIAIQDSLIRYRMKGKTVLPLGFDHAGIATQSVVEKQIWAQDKRTREDYGREAFVKGKVEWKEEY			269
Nc	HQRINNA--OKLMGSSMDWSREAFMDKNLTAAATMETTCLHDEGLIYRSLVNVWCTH--LNTALSGLEVETKEITGRLLDVPDGYDKKVEFG			348
Sc	HSRIKQIQKL--GASYDWSREAFTLSPELTSVEEAFVRLDEGVIRASRLVNVWSVLLNTAISNLEVENKDKVSRLLSVPGYDEKVEFG			360
Nc	VLTHFYKIDGSEETIEVATTRPETMLGDTGIANVPEDPRYTHLVGKFARHPFVDRLLPIVTD--NYVDKEFGTGAVKLTPAHDFNDYQLGQR			439
Sc	VLTSEFYVIGSDKELIATTRPETIFGDTAVAVHPDDRYKELHKGFIQHPFLPKIPITDKEAVDMEFGTGAVKITPAHDQNDYNTGKR			452
Nc	HNLEFINILNENGLTNDNAGP-FKGKRFDARYTVVEELTKLGLFVKKEPNFMKIPLCEKSKDVIEPMTEQWVVRKEMGEAALQVVEEGK			530
Sc	HNLEFINILTDDGLLNECEGPEWQGMKRFDAKKVIEQLKEKNLYVGQSDNEMTPTCSRSGDIIEPLKLPQWWSQSEMAKDAIKVVKDQG			544
Nc	VKISPEASATSKYKRWLADIQWVCISRLWNGHRIPAYRVIFEGEGQRENEKSEWVVGRTQEEAQAKAEAKFAGRKFTLEQOPDCDITWFS			622
Sc	ITITPKSEAEYFWLGNIQWVCISRLWNGHRCVYFIIIEGEEHEDRIDGY--WVAGRSMEAEKKAAYPNKFTLEQDEVDITWFS			635
Nc	GLWPMAILGWPN--TENLDFKKFPPTSMLETGWDILFFWVSRMIMLSLKMTEGEVFPFTEVYCHSLIRDSEGRKMSKSLGNVIDPLDIIRGIELE			713
Sc	GLWPFSTLWPEKTKDME--TFYFPFSLMETGWDILFFWVTRMILLGLKLTVGSPFKEVFCVSLVRDAQGRKMSKSLGNVIDPLDVTGKILD			725
Nc	DLEAKLLVGNLKEEVARATKY-QKTAFFGGIPECGADAMRFTLLSYTTGGGDINFDI--RVMEAYRRFCNKIWQASKYVLGKLPQDFMPKG-			802
Sc	DLEAKLLQGNLDPREVKA-KIGQKESYPNGIPQCGTDAMRFALCAYTTGGRDINLDILRV-EGYKFCNKIYQATKFLMRLGDDYQPPAT			815
Nc	-ELDTANFSVPKMWILHRMVAVKGMHEALEAREFSRATKVAYQFFYDELCDVFIENSKGILSDGTPKEQSSVQQLTYHALDVALRLLEPIM			893
Sc	EGL-SGNSLVEKWLKLTSTSKIYNEALDKRDLTSTSSIEYFWY-LICDVYIENSKYLIQEGSAIEKKSADTLTYILLDNALKLIHFFM			905
Nc	PYITEELWQLPRKQGD-GETIMLAPYPAFESQLEFATEARDYELGLKCAAGIRSAAADYINIKSDGRAFIKATTADSLATVSAQLAAIRTL			984
Sc	PFISEEMWQLPKRSTKAASIVKASYPVYVSEYDDVKSANAYDLVNLITKARSLLSEYNILKNGKVFVESNHEEYFKTAEDQKDSIVSLI			997
Nc	GRGVKEVNVGLADEELPRGCAVYVINAETIVLLQVGGSSISDIAEIKKITTQLQKTDLTIKK-----QQELLSKDGFE-KVSEAVQSEKQK			1070
Sc	-KAIDEVTVVRDASEIEGCVLQSVNPEVNVHLLVKGHV-DIAEIAKVKQKLEKA-----KSKNGIEQTINSKD-YETKANTQAKANKSK			1082
Nc	LADAQAAKENYQRTLEEFSEKLI			1093
Sc	LONTVAEIEGLEATTENLKRLLK			1105

FIG. 4. Alignment of the amino acid sequences of the 1,093-aa ORF (Nc) and the yeast valRS (Sc). The sequences were aligned by using the algorithm of Needleman and Wunsch (55). Dashed lines indicate identical amino acids, and colons indicate related amino acids. The sequence of yeast valRS is from Jordana et al. (33). The HIGH and KMSKS sequences characteristic of class 1 synthetases (24) are indicated by over- and underlines.

TABLE 1. Codon usage in the 1,093-aa and 532-aa ORFs

Codon	Amino acid	No. of codons		Codon	Amino acid	No. of codons		Codon	Amino acid	No. of codons		Codon	Amino acid	No. of codons	
		1093	532			1093	532			1093	532			1093	532
TTT	Phe	7	5	TCT	Ser	9	2	TAT	Tyr	8	0	TGT	Cys	3	4
TTC	Phe	36	9	TCC	Ser	13	4	TAC	Tyr	24	1	TGC	Cys	12	3
TTA	Leu	0	0	TCA	Ser	1	0	TAA	End	1	0	TGA	End	0	1
TTG	Leu	13	8	TCG	Ser	8	1	TAG	End	0	0	TGG	Trp	26	4
CTT	Leu	34	46	CCT	Pro	21	3	CAT	His	8	12	CGT	Arg	14	3
CTC	Leu	31	36	CCC	Pro	25	0	CAC	His	13	5	CGC	Arg	21	5
CTA	Leu	1	0	CCA	Pro	3	14	CAA	Gln	16	7	CGA	Arg	2	3
CTG	Leu	16	9	CCG	Pro	9	2	CAG	Gln	28	12	CGG	Arg	0	5
ATT	Ile	23	3	ACT	Thr	16	2	AAT	Asn	5	8	AGT	Ser	6	6
ATC	Ile	31	8	ACC	Thr	39	12	AAC	Asn	32	11	AGC	Ser	18	13
ATA	Ile	0	4	ACA	Thr	7	2	AAA	Lys	9	4	AGA	Arg	6	6
ATG	Met	27	6	ACG	Thr	2	11	AAG	Lys	85	13	AGG	Arg	8	9
GTT	Val	18	17	GCT	Ala	30	4	GAT	Asp	25	10	GGT	Gly	25	22
GTC	Val	31	14	GCC	Ala	48	15	GAC	Asp	30	15	GGC	Gly	37	21
GTA	Val	2	13	GCA	Ala	7	6	GAA	Glu	15	16	GGA	Gly	7	6
GTG	Val	9	8	GCG	Ala	14	13	GAG	Glu	79	14	GGG	Gly	0	17

that of the wild type. By comparison, the [*poky*] mutant, which has less than 15% wild-type cytochrome *aa*₃ as a result of deficiency in mitochondrial protein synthesis, has a substantially higher growth rate in liquid culture (approximately 33% of the wild-type level; 46). These results suggest

that the concentrations of cytochromes *b* and *aa*₃ are not rate limiting for growth in *un-3* cells.

The *un-3* mutant had been reported to have pleiotropic defects in cellular transport processes resulting in resistance to ethionine and other amino acid analogs (34, 35, 50).

FIG. 5. Cytochrome spectra of wild-type 74A and *cyt-20-1* strains at 25, 30, and 37°C and for the *un-3* strain at 25 and 30°C. Peaks at 550, 560, and 605 nm are the α peaks of cytochromes *c*, *b*, and *aa*₃, respectively.

TABLE 2. Transformation of *cyt-20-1* and *un-3* with pBTC20 digested with different restriction enzymes^a

DNA plus pSV50	No. of transformants	
	<i>cyt-20-1</i>	<i>un-3</i>
pBTC20	1,125	1,150
pSV50 alone	0	0
pBTC20 cut with:		
<i>EcoRI</i>	143	310
<i>BamI</i>	12	13
<i>HindIII</i>	0	0
<i>Sall</i>	36	35
<i>PstI</i>	6	7
<i>SacI</i>	4	3
<i>XhoI</i>	0	0
<i>SphI</i>	3	5

^a pBTC20 (5 µg) was cut with the indicated restriction enzyme and cotransformed into *cyt-20-1* or *un-3* with 1 µg of the vector pSV50 to provide Bml^r as a second selectable marker. Transformants were scored by growth at 40°C in the presence of benomyl (0.25 µg/ml). Values indicate the number of transformants per 5 µg of pBTC20 plus 1 µg of pSV50. Digestion of pBTC20 with *EcoRI*, which cuts in the vector 50 bp upstream of the ORF, resulted in a four- to ninefold decrease in transformation frequency with both mutants. In other experiments with a different *cyt-20*⁺ clone, pAK20P7, digestion with *XbaI* or *XmaI*, both of which cut elsewhere in the vector pBS(+), had no large effect on the transformation frequency (40). The experiment was repeated twice with essentially the same results.

However, in a cross between an *un-3*⁺ and an *un-3* strain, ethionine-resistant progeny that are not temperature sensitive were obtained at a frequency of approximately 0.1% (20 of 22,000 ascospore progeny), indicating that the temperature sensitivity and drug resistance result from two separate mutations (41). An average map unit in *N. crassa* is approximately 43 kb (57, 59). However, the mating-type locus and the temperature sensitivity mutation in *un-3* are separated by 12 kb, and this corresponds to a genetic map distance of only 0.04 map unit (26, 56). Although we cannot exclude the possibility that both the drug resistance and temperature sensitivity mutations are located in the same gene, the relatively large distance between the two mutations (i.e., a minimum of 0.1 map unit) suggests that they are in different genes.

Transformation of both *cyt-20-1* and *un-3* is dependent on the 1,093-aa ORF. The following experiments showed that transformation of the temperature-sensitive mutations in both the *cyt-20-1* and *un-3* mutants requires the 1,093-aa ORF and not the 532-aa ORF on the opposite strand. First, transformation with subclone pBTC20, which contains a 3.6-kb *PvuII-NsiI* fragment that just encompasses the 1,093-aa ORF, fully corrected the temperature-sensitive defects in both the *cyt-20-1* and *un-3* mutants. Transformants of *cyt-20-1* and *un-3* grown at 37°C had essentially normal growth rates and cytochrome spectra (not shown). An *un-3* clone isolated independently by Glass et al. (27), who used this marker to walk to mating type, also efficiently transformed *cyt-20-1* (data not shown). Second, as shown in Table 2, the ability of pBTC20 to transform the *cyt-20-1* and *un-3* mutants was inactivated by the same restriction enzymes. Digestion of pBTC20 with *Sall* or *SacI*, which cut near either end of the 1,093-aa ORF, strongly decreased *cyt-20-1* and *un-3* transforming activity, even though neither enzyme cuts within the 532-aa ORF (Fig. 1). Finally, as shown in Fig. 6, the insertion of *SpeI* linkers, which contain translation stop codons in all reading frames, after codons 13 or 926 of the 1,093-aa ORF strongly decreased *cyt-20-1* and *un-3* trans-

forming activities, even though neither *SpeI* linker is located in the 532-aa ORF (see also Fig. 1).

To investigate directly whether the 532-aa ORF is necessary for *cyt-20* transforming activity, we used in vitro mutagenesis to make a single base change, C-2275 to A, which places a UGA stop codon at codon 9 of the 532-aa ORF but results in a silent change in the 1,093-aa ORF on the opposite strand. As shown in Fig. 6, this construct, *pcyt-20-mut532*, transformed *cyt-20-1* and *un-3* with an efficiency similar to that of the wild-type clone, indicating that the 532-aa ORF is not necessary for the *cyt-20* or *un-3* transforming activity.

The *SpeI* linker inserted at codon 13 of the 1,093-aa ORF is located within the putative mitochondrial targeting sequence and introduces stop codons in all three reading frames between the two potential ATGs of the 1,093-aa ORF. This mutant gene, which should be able to produce the cytosolic but not mt valRS, did not transform *cyt-20-1* but consistently gave a small number of transformants with *un-3* (10/5 µg of transforming DNA). Most transformants in *N. crassa* result from integration of transforming DNA at ectopic chromosomal locations, with a small proportion resulting from integration at the homologous site. The low transformation frequency of *un-3* with the *pcyt-20*[*SpeI*-13] DNA (10/5 µg of transforming DNA) could reflect correction of the mutation by homologous recombination (5), although it is not clear why a similar low transformation frequency was not obtained for *cyt-20-1*. Taken at face value, these findings suggest that both the *cyt-20-1* and *un-3* mutants are dependent on the mitochondrial product, but it remains possible that the *SpeI* linker also interferes with synthesis of the cytosolic product or its localization to some other cellular compartment.

Analysis of the mutant alleles. The *cyt-20-1* and *un-3* mutant alleles were cloned in pBS(+) as 3.6-kb *PvuII-NsiI* fragments isolated from whole-cell DNAs of the mutant strains, as described in Materials and Methods. The resulting plasmids were designated pBT289-56 and pBTun3, respectively. To localize the mutations in *cyt-20-1* and *un-3*, we tested hybrids between the mutant and the wild-type genes for their ability to transform the *cyt-20-1* or *un-3* mutants. As shown in Fig. 6, these experiments localized both mutations to an internal *PstI-BglII* fragment of 1,102 bp, spanning amino acids 166 to 532 of the 1,093-aa ORF.

The 1,102-bp *PstI-BglII* fragments from *cyt-20-1* and *un-3* were sequenced to identify the mutations. A complication in the sequence analysis was that the *cyt-20*⁺ gene was cloned from a standard *N. crassa* library made from wild-type 74A, whereas the *cyt-20-1* and *un-3* mutants were isolated in strains having mating type *a*, which apparently have somewhat different alleles of *cyt-20*⁺. The different alleles have a number of presumably silent polymorphic changes, which complicate identification of the mutations.

Fortunately, comparison of sequences in this region of the *cyt-20-1* and *un-3* genes showed that each has a single unique nucleotide change that affects an amino acid in the 1,093-aa ORF and is presumed to correspond to the mutation. In the case of *cyt-20-1*, this is a C-to-T transition at nucleotide 1253, which changes Arg-201 to Cys, whereas in *un-3*, this is a T-to-C transition at nucleotide 1568, which changes Ser-306 to Pro (indicated by asterisks in Fig. 2). In addition to the presumptive mutations, the two mutant alleles have several base changes in common in this region, as well as a few unique base changes, which do not affect coding of amino acids. The common changes are T to C at position 1732 and A to C at position 1912, and the unique changes are C to G

FIG. 6. Transformation of *cyt-20-1* and *un-3* with *cyt-20* genes modified by in vitro mutagenesis or with hybrid genes containing different segments of the two mutant alleles. *pcyt-20*[SpeI13] and *pcyt-20*[SpeI926] are constructs with a *SpeI* linker (indicated by arrowheads) inserted after codons 13 and 926, respectively. *pcyt-20*-mut532 is a construct with a stop codon in the 532-aa ORF and a silent change in the 1,093-aa ORF (position indicated by an asterisk). Hybrid genes were constructed by exchanging the 1.1-kb *PstI*-*BglII* fragment between clones of the wild-type gene (pBTC20) and the mutant gene (pBT289-56 or pBTun3). Portions derived from the wild-type gene are in gray, portions derived from the *cyt-20-1* gene (pBT289-56) are speckled, and portions derived from the *un-3* gene are hatched. Transformants are number per 5 μ g of transforming DNA plus 1 μ g of pSV50. The experiment was repeated twice with essentially the same results.

at positions 1294 and 1627 in *cyt-20-1* and G to C at position 1804 in *un-3*. Since all these changes are silent, they are presumed not to correspond to the mutations.

Assay of mt and cytosolic valRSs in the *cyt-20-1* and *un-3* mutants. To determine whether the mutations in the *cyt-20* gene affect valRS activity, we assayed aminoacylation activities in mitochondrial and cytoplasmic extracts from wild-type 74A and mutants *cyt-20-1* and *un-3*. The extranuclear mutant [*poky*] was included as a control to assess changes in valRS activity due to deficiency in mitochondrial protein synthesis or energy metabolism. Since the assays were carried out with crude extracts, which may contain variable amounts of other proteins, we compared both the specific activities of valRS and the ratios of mt and cytosolic valRSs to unrelated aaRS activities (mt tyrRS and cytoplasmic lysRS, respectively), which should not be directly affected by mutations in the *cyt-20* gene.

As shown in Table 3, the *cyt-20-1* mutant grown at either 25 or 37°C had grossly decreased levels of both mt and cytosolic valRS activities assayed in vitro, consistent with the previously observed decrease in the rates of mitochondrial and cytosolic protein synthesis (16, 17). Compared with wild-type 74A, the *cyt-20-1* mutant grown at 37°C had 200-fold lower mt valRS activity, and the ratio of mt valRS to mt tyrRS was more than 150-fold lower. Even at 25°C, mt

valRS activity was already 100-fold lower than in wild-type 74A, and the ratio of mt valRS to mt tyrRS was more than 30-fold lower. Similarly for the cytosolic valRS activity, the *cyt-20-1* mutant grown at 25 and 37°C showed decreases of about 10- and 40-fold, respectively, with the ratio of valRS to lysRS activities decreased about 20-fold at either temperature (Table 4). The deficiency of mt and cytosolic valRS activities in lysates of cells grown at the permissive temperature presumably reflects instability of the mutant enzyme in vitro, as found previously for other temperature-sensitive enzymes (13). In control experiments, the [*poky*] mutant, which is strongly deficient in mitochondrial protein synthesis, showed at most a 4.5-fold decrease in the ratio of the mt valRS to mt tyrRS activities and essentially no change in the ratio of cytosolic valRS to lysRS activities, indicating that the observed decreases in valRS activities in the *cyt-20-1* mutant are not secondary effects of deficiency in mitochondrial protein synthesis or energy metabolism. The finding that the *cyt-20-1* mutation strongly decreased the activities of both the cytosolic and mt valRSs supports the conclusion that the *cyt-20* gene encodes both enzymes.

The *un-3* mutant also showed decreases in mt and cytosolic valRS activities, but both activities remained higher than in *cyt-20-1* cells (Tables 3 and 4). Thus, in *un-3* cells grown at 25 or 30°C, cytosolic valRS activity remained more

TABLE 3. Mt valRS and tyrRS activities from 74A, *cyt-20-1*, and *un-3* cells at different temperatures

Growth temp (°C)	Assay temp (°C)	Strain	Sp act (10 ⁻³ pmol of amino acid incorporated/min/mg of protein) ^a		ValRS/TyrRS
			ValRS	TyrRS	
25	25	74A	4.7 ± 0.02 (3)	2.4 ± 2.9 (4)	1.96
		<i>cyt-20-1</i>	0.04 ± 0.02 (2)	0.6 ± 0.03 (3)	0.07
		<i>un-3</i>	0.30 ± 0.02 (2)	0.13 ± 0.03 (3)	2.31
		[<i>poky</i>]	0.17 ± 0.01 (3)	0.29 ± 0.03 (3)	0.42
25	37	74A	0.34 ± 0.10 (3)	0.097 ± 0.035 (3)	3.50
		<i>cyt-20-1</i>	0.014 ± 0.005 (2)	0.048 ± 0.011 (2)	0.29
		<i>un-3</i>	0.31 ± 0.14 (2)	0.30 ± 0.09 (2)	1.03
		[<i>poky</i>]	0.25 ± 0.02 (3)	0.31 ± 0.012 (3)	0.80
37	37	74A	3.6 ± 0.7 (2)	0.73 ± 0.16 (2)	4.93
		<i>cyt-20-1</i>	0.017 ± 0.007 (2)	0.61 ± 0.07 (2)	0.03
30	37	<i>un-3</i>	0.24 ± 0.001 (2)	0.34 ± 0.03 (2)	0.71

^a Assayed in mitochondrial extracts, using purified *E. coli* tRNA^{Val} or tRNA^{Tyr} as substrates (see Materials and Methods). Numbers in parentheses indicate number of assays performed.

than 50% that of wild-type 74A, consistent with the rates of cellular protein synthesis measured by Kappy and Metzenberg (35). Mt valRS activity was 15-fold lower than in wild-type 74A at either 25 or 30°C, but the ratio of mt valRS to mt tyrRS was increased slightly at 25°C and was only 7-fold lower at 30°C, compared with 150-fold lower for *cyt-20-1* at 37°C.

DISCUSSION

Our results show that the *N. crassa cyt-20⁺* gene encodes both the mt and cytosolic valRSs. The *cyt-20⁺* gene contains an ORF of 1,093 aa having 52.5% identity to the *S. cerevisiae* valRS, and the *cyt-20-1* mutant has a missense mutation in this ORF (Arg-201→Cys), which results in gross deficiency of both mt and cytosolic valRS activities. The yeast *VAS1* gene had been shown previously to encode both the cytosolic and mitochondrial forms of the valRS (12).

As in the case of *VAS1* and other yeast genes that encode both the mitochondrial and cytosolic forms of the same enzyme (7, 12, 23, 53, 54), the *N. crassa cyt-20⁺* gene has two appropriately positioned in-frame ATGs that could be

used as initiation codons for the mt and cytosolic valRSs. Use of the upstream ATG would give a protein having an extra 43 aa with the characteristics expected for an N-terminal mitochondrial targeting sequence, whereas use of the downstream ATG would give a protein lacking the N-terminal targeting sequence, presumably the cytosolic valRS. Preliminary primer extension mapping of *cyt-20⁺* transcripts showed major 5' ends at three positions, one with two closely spaced bands about 55 nt upstream of the first ATG and the others between the two ATGs, 16 and 79 nt upstream of the second ATG (71; Fig. 3). Thus, the use of the two alternative ATGs in the *cyt-20* gene might be determined in part by the translation of mRNAs having different 5' ends, as suggested for the yeast *VAS1* gene (12).

The 5' noncoding region of the *cyt-20⁺* gene has two short sequence elements in common with the 5' noncoding regions of the genes encoding the mt tyrRS and leuRS (*cyt-18* and *leu-5*, respectively) and two different short sequences in common with the cytosolic leuRS gene (Fig. 7; sequences boxed in Fig. 2). These sequences may be involved in the regulation of synthesis of cytosolic and mitochondrial aaRSs at the transcriptional or posttranscriptional level. The *cyt-*

TABLE 4. Cytoplasmic valRS and lysRS activities from 74A, *cyt-20-1*, and *un-3* cells at different temperatures

Growth temp (°C)	Assay temp (°C)	Strain	Sp act (10 ⁻³ pmol of amino acid incorporated/min/mg of protein) ^a		ValRS/LysRS
			ValRS	LysRS	
25	25	74A	26.6 ± 6.5 (3)	86.8 ± 4.6 (3)	0.31
		<i>cyt-20-1</i>	2.2 ± 0.3 (3)	180 ± 9 (3)	0.012
		<i>un-3</i>	13.9 ± 5.5 (3)	149 ± 12 (3)	0.09
		[<i>poky</i>]	41.9 ± 4.2 (2)	105 ± 2.4 (2)	0.39
25	37	74A	8.4 ± 0.8 (3)	9.03 ± 0.6 (3)	0.93
		<i>cyt-20-1</i>	0.77 ± 0.03 (3)	15.3 ± 1.7 (3)	0.05
		<i>un-3</i>	7.1 ± 3.3 (3)	16.4 ± 1.7 (3)	0.43
		[<i>poky</i>]	45.7 ± 4.3 (2)	86.2 ± 17 (2)	0.53
37	37	74A	25.1 ± 4.2 (2)	21.3 ± 0.7 (2)	1.12
		<i>cyt-20-1</i>	0.67 ± 0.20 (2)	11.4 ± 0.8 (2)	0.06
30	37	<i>un-3</i>	17.8 ± 5.2 (2)	33.8 ± 0.7 (2)	0.53

^a Assayed in cytoplasmic extracts, using yeast tRNA as substrate (see Materials and Methods). Numbers in parentheses indicate number of assays performed.

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cyt-20:      CAAACACTTG--(74 bp)--GCTTCGGGCCG--(105 bp)---CTCTGG ATG C
cyt-18:      CAAACTCTTG--(63 bp)--GCTTCGGGTTG---(70 bp)---CATCAA ATG C
leu-5:       CAAAGACTGA--(65 bp)--GCTTCGGCTTG---(67 bp)---AACGCG ATG C

                                C
                                GACAAA ATG
                                AT  CC

                                C
                                GACAAA ATG
                                AT  CC

cyt-20:      GCTGACAGGTACA--(58 bp)--TCCGTCTGC--(209 bp)--GTCATC ATG C
N.c. leuRS:   GCTTGTAGGTACA--(27 bp)--TCAGTTTGC---(83 bp)--ACAAAC ATG G

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FIG. 7. Sequence elements upstream of *N. crassa* genes encoding aaRSs. Shown are sequences immediately preceding initiation codons and sequences in common between the upstream regions of the *cyt-20*⁺ gene, the *cyt-18*⁺ gene, which encodes the mt tyrRS (3), and the *leu-5*⁺ gene, which encodes the mt leuRS (13), and different sequences in common between the *cyt-20*⁺ gene and the cytoplasmic leuRS gene (8, 14). CONSENSUS is the consensus sequence immediately preceding initiation codons in *N. crassa* nuclear genes (60).

cyt-20⁺ gene also has three exact matches and two five-of-six base matches to the recognition sequence TGACTC for the *N. crassa* cross-pathway control protein CPC-1 (sequences underlined in Fig. 2). The presence of these sequences raises the possibility that transcription of the valRS gene is stimulated by amino acid starvation (60). Cross-pathway control sequences are also located upstream of the gene for the cytoplasmic leuRS but not the mt tyrRS or mt leuRS (3, 14).

An intriguing feature of the *cyt-20* gene is that the strand opposite the valRS ORF encodes a 532-aa ORF with 21.3% identity to a chromate resistance protein of *P. aeruginosa* (11). Interestingly, the *E. coli* valRS gene (28) has a similarly positioned opposite strand ORF of 759 aa, which has similarity to both the *N. crassa* ORF (19.2% identity over 532 aa) and the *P. aeruginosa* chromate transport protein (21% identity over 416 aa). In the yeast valRS gene, however, what would be a comparable ORF at this location is interrupted by stop codons at 12 positions. Although we did not detect a transcript from that strand that would express the 532-aa ORF (42), it is possible that it encodes a functional protein that is present in low abundance or is expressed only at certain developmental stages or under certain metabolic conditions. However, it remains possible that the ORF is maintained simply as a consequence of constraints on the valRS coding sequence and codon usage patterns in *N. crassa* and *E. coli*.

Our results demonstrate that both *cyt-20-1* and *un-3* have mutations in the 1,093-aa ORF encoding valRS. For *cyt-20-1*, the decreased growth rate at high temperature is clearly correlated with gross deficiencies of both mt and cytosolic valRS activities (Tables 3 and 4) and of mitochondrial and cytosolic protein synthesis (16, 17). This does not appear to be the case for *un-3*, however. A complication in the interpretation of the *un-3* phenotype is that the strain contains two closely linked mutations, one in the valRS ORF conferring temperature sensitivity and the other, probably in a different gene, affecting membrane transport (41). Keeping this caveat in mind, Kappy and Metzenberg (35) found that when the *un-3* mutant was shifted from 24 to 35°C, growth was inhibited immediately, but protein synthesis appeared to continue at the same rate, which was approximately 50 to 60% of the wild-type rate at 35°C, for at least 5 h. The rate of protein synthesis measured by Kappy and Metzenberg (35) agrees roughly with the levels of cytosolic valRS activity in *un-3* measured in our assays, which were also more than 50% of the wild-type level (Table 4). Further, we found that the *un-3* mutant grown at 30°C still contains substantial amounts of cytochromes *b* and *aa*₃ relative to cytochrome *c* (Fig. 5). Together, these findings suggest that the temperature sensitivity of *un-3*, although caused by the mutation in the valRS ORF, is not due to deficiency of cytosolic or mitochondrial protein synthesis.

There are several possible explanations for the temperature-sensitive growth of the *un-3* mutant in the absence of strong deficiency in protein synthesis. First, it may result from an interaction of the weak valRS mutation with the second closely linked mutation affecting membrane transport. However, we consider this less likely than other possibilities, since *un-3* transformants have essentially wild-type growth rates at 37°C and, at least in nonauxotrophic backgrounds, show no residual temperature sensitivity associated with the transport mutation. Second, temperature-sensitive growth of the *un-3* mutant may result from accumulation of abnormal proteins due to misacylation by the mutant valRS. However, this would require some additional assumptions to account for the rapid cessation of growth when the *un-3* mutant is shifted to a higher temperature (35), e.g., that the abnormal proteins that accumulate at 25°C are temperature sensitive. The third possibility, which we favor at present, is that the growth defect in the *un-3* mutant is not due to an effect on the valRS activity, but instead reflects some other function of the valRS or a derivative of this protein.

There is precedent for aaRSs having functions in addition to protein synthesis. Charged tRNAs are intermediates in synthesis of aminoacyl phospholipids, which are constituents of bacterial cell membranes (47), and charged tRNA^{Glu} is an intermediate in the synthesis of δ-aminolevulinic acid, which is an intermediate in chlorophyll biosynthesis (32). The *E. coli* alaRS regulates transcription of the alaRS gene by binding to its promoter, and the *E. coli* thrRS regulates its own translation by binding to the 5' end of its mRNA (reviewed in reference 67). Finally, two mitochondrial synthetases, the *N. crassa* mt tyrRS and the yeast mt leuRS, have been shown to function in splicing of mitochondrial group I introns, independently of their aminoacylation activity (3, 29).

Insight into the additional function of the valRS ORF will require further investigation of the temperature sensitivity mutation in *un-3* in strains in which it has been separated from the membrane transport defect.

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