MGG

Mitochondrial mutations restricting spontaneous translational frameshift suppression in the yeast *Saccharomyces cerevisiae*

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Summary. The +1 frameshift mutation, M5631, which is located in the gene (*oxi1*) for cytochrome *c* oxidase II (COXII) of the yeast mitochondrial genome, is suppressed spontaneously to a remarkably high extent (20%–30%). The full-length wild-type COXII produced as a result of suppression allows the mutant strain to grow with a "leaky" phenotype on non-fermentable medium. In order to elucidate the factors and interactions involved in this translational suppression, the strain with the frameshift mutation was mutated by $MnCl_2$ treatment and a large number of mutants showing restriction of the suppression were isolated. Of 20 mutants exhibiting a strong, restricted, respiration-deficient (RD) phenotype, 6 were identified as having mutations in the mitochondrial genome. Furthermore, genetic analyses mapped one mutation to the vicinity of the gene for tRNA[Pro] and two others to a region of the tRNA cluster where two-thirds of all mitochondrial tRNA genes are encoded. The degree of restriction of the spontaneous frameshift suppression was characterized at the translational level by in vivo ^{35}S-labeling of the mitochondrial translational products and immunoblotting. These results showed that in some of these mutant strains the frameshift suppression product is synthesized to the same extent as in the leaky parent strain. It is suggested that more than one +1 frame-shifted product is made as a result of suppression in these strains: one is as functional as the wild-type COXII, the other(s) is (are) nonfunctional and prevent leaky growth on non-fermentable medium. A possible mechanism for this heterogenous frameshift suppression is discussed.

Key words: Yeast – Mitochondria – Frameshift – Suppression – Restriction

Introduction

Triplet reading during translation involves a certain degree of inaccuracy, which is observable as spontaneous

frameshifting or spontaneous suppression of frameshift mutations. This inaccuracy often has a strong sequence-specific nature and in some mRNA contexts doublet or quadruplet decoding takes place with an efficiency of up to 50% (Spanjaard and van Duin 1988). In some instances site-specific frameshifting is required for expression of several genes, for example, for the γ subunit of DNA polymerase III (Tsuchihashi and Kornberg 1990), release factor 2 of *Escherichia coli* (Craigen et al. 1985) or reverse transcriptase of retroviruses (Jacks and Varmus 1985; Jacks et al. 1987; Jacks et al. 1988b). A series of experiments involving in vitro mutagenesis has revealed the importance of features of the mRNA for such shift events. Examples are the presence of Shine-Dalgarno sequences in a reading frame (Weiss et al. 1988), runs of a single base in the mRNA sequence (Brierley et al. 1987), or downstream hair-pin or pseudo-knot mRNA structures (Jacks et al. 1988a; Brierley et al. 1989). On the other hand, genetic approaches, in which several intergenic suppressors were isolated and characterized, have also shown that mutations in the translational apparatus (tRNAs, EF-Tu, ribosomes) can cause non-triplet decoding to some extent (Hughes et al. 1987; for review see Murgola 1985).

Translational inaccuracy, with regard to the spontaneous suppression of frameshift mutations, has also been detected in the mitochondria of the yeast *Saccharomyces cerevisiae* (Fox and Weiss-Brummer 1980; Weiss Brummer et al. 1984). In particular, the +1 frameshift mutation M5631, which results in a run of six instead of five Ts in the reading frame for cytochrome *c* oxidase subunit II (COXII), can be suppressed spontaneously to a significant extent to produce functional COXII (Fox and Weiss-Brummer 1980). As a result of the suppression, the mutant strain is able to grow on nonfermentable medium, thus exhibiting a "leaky" phenotype. An alteration in the tRNA gene *ser1* (Weiss-Brummer et al. 1989; Hüttenhofer et al. 1990) can enhance the frameshift suppression, which leads to an increased growth rate on non-fermentable medium. Conversely, a mutational change in the mitochondrial 15S rRNA gene (par^r_{434}) can reduce the efficiency of suppression, causing reduced production of the full-length COXII and thus restricting growth on non-fermentable medium (Weiss-Brummer and Hüttenhofer 1989). The influence of these

mutations indicates that frameshift suppression is a multifactor process and the RNA components of the translational system participate functionally in reading frame maintenance during protein synthesis.

For the purpose of understanding the multifactor process of frameshifting, we isolated mutant strains deriving from the leaky mitochondrial mutant strain 777-3A-M5631 that show reduced levels of suppression, i.e. they have lost or restricted ability to grow on non-fermentable medium. Six mitochondrial mutations were characterized genetically in this study to gain insight into the possible functional interaction of the RNA components during translation. The reason for the selection of yeast mitochondrial mutations is that all RNA components of the mitochondrial translational apparatus (tRNAs, rRNAs and mRNAs) are encoded by the mitochondrial genome, while the protein components, except for ribosomal protein Var1, are encoded by the nuclear genome (for review see Tzagoloff and Meyers 1986). Genetic localization of these mitochondrial mutations was achieved by rho^- deletion mapping. Their loci, which all mapped to regions of the mitochondrial genome encoding several tRNAs, revealed that more than one tRNA may interfere in one frameshifting event. Protein analysis of these mutants allowed us to construct a model for the +1 frameshifting in which heterogeneous translational products are synthesized.

Materials and methods

Strains and media. Genotypes and origins of yeast strains are given in Table 1. Yeast media were made up as follows: YD, 1% yeast extract, 3% glucose; YG, 1% yeast extract, 3% glycerol; WO, 1.7% Difco Yeast Nitrogen Base, 5.2% ammonium sulfate, 3% glucose. Solid media contained in addition 2% agar.

$MnCl_2$ mutagenesis and genetic crosses. Mutagenesis was performed as described by Schweyen et al. (1978), with a final concentration of 10 mg/ml $MnCl_2$.

Cytoduction (kar cross). Mitochondrial genomes were cytoducted through rho^0 *kar-1* strains as described by Lancashire and Mattoon (1979), and Weiss-Brummer et al. (1987).

Isolation of rho^- *strains.* Cells (KL-0-7, KL-0-M5631) grown in YD medium until early stationary phase were plated on YD medium as single colonies. Colonies that could not grow on YG medium after replica-plating were crossed further with several known *mit^-* strains (M5801, M2532, M7473, M4901) to map deletions of the mitochondrial genome as described by Schweyen et al. (1976). Retained regions of rho^- genomes that had arisen spontaneously were also characterized by means of DNA hybridization with specific probes isolated from well-mapped rho^- genomes (C7 for the 21S rRNA region, P2 for the 15S rRNA region and SEG15/S1 for the *var1* region) and DNA clone pYm424 for the tRNA^{phe} region. Mitochondrial DNA was isolated as described by Hudspeth et al. (1980).

For rho^- genome subcloning the strain K7-21-5 was treated with ethidium bromide as described by Weiss-Brummer and Hüttenhofer (1989). In order to characterize deleted regions, fragments from the mitochondrial DNA of K7-21-5 and C7 were used as hybridization probes. They were specific for the 5' region (2.8 kb *Mbo*I fragment) and the 3' region (1.0 kb *Ava*II fragment), of the 21S rRNA gene and for the region of the tRNA gene cluster (0.4 kb *Ava*II fragment) (see Sor and Fukuhara 1983; de Zamaroczy and Bernardi 1986 for the restriction map).

Growth rate determination. Spontaneous respiration-competent (RC) revertants ($oxi1^-$-M5631→$OXI1^+$) of the mitochondrial HC strains (diploids with KL-0) were isolated from cells plated on YG medium. RC cells were grown in YG medium until early stationary phase at 30° C. Then 0.5 ml of each culture was transferred to 5 ml YG medium and incubation for 6 h. Exponentially growing cells were inoculated in three batches of 150 ml YG medium to a titer of 1×10^5/ml and growth at 23°, 30° and 37° C was monitored by measurement of OD_{600}. The analysis was repeated at least three times with independently isolated RC revertants of each diploid HC strain.

Isolation of mitochondrial RNA and Northern analysis. Diploid HC strains crossed to KL-0 were grown at 30° C in YD medium to early stationary phase. Cells washed once with H_2O were then transferred to YG medium. After 6 h incubation at 30° C cells were spheroplasted by treatment with zymolyase 20000 (Seikagaku Kogyo, Tokyo, Japan; 0.4 mg/g cells) in 1.5 M sorbitol. Mitochondria were isolated from the lysed cells in 0.6 M sorbitol (40000 g, 20 min). SDS was added to the mitochondrial suspension (0.5%, end concentration) and the mitochondrial RNA was isolated by phenol/chloroform extraction, precipitation in ethanol/sodium acetate and DNase I treatment. Mitochondrial RNA was separated in 1.5% agarose/glyoxal gels. Blotting and hybridization were performed as described in the Genescreen protocol.

The tRNA-specific single-stranded DNA (ssDNA) probes were prepared from synthetic 15-mer oligonucleotides complementary to an appropriate tRNA. They were elongated for a further 20–30 nucleotides on the ssDNA template of a suitable M13 clone with α-[^{32}P]dATP through the labeling reaction of the dideoxy chain termination method (Sanger et al. 1977).

Analysis of mitochondrial translational products. Labeling and detection of mitochondrial translational products were carried out according to Haid et al. (1979), and Weiss-Brummer et al. (1987), except that preincubation with chloramphenicol was omitted.

Immunological identification of COXII. Mitochondria were isolated as described above for isolation of mitochondrial RNA. Samples (120 μg of protein per lane) were subjected to electrophoresis in a gradient SDS-polyacrylamide (10%–15%) gel and transferred to a nitrocellulose filter as described by Haid and Suissa (1983).

Table 1. Strains of *Saccharomyces cerevisiae*

Strain	Genotype		Remarks	Reference
	Nuclear	Mitochondrial		
777-3A-M5631	α *ade1, op1*	*rho⁺, mit⁻ (oxi1⁻*-M5631)	Derived from 777-3A	Weiss-Brummer et al. (1979)
777-3A	α *ade1, op1*	*rho⁺, mit⁺*		Kotylak and Slonimski (1977)
7-0	α *ade1*	*rho⁰*	*rho⁰* mutant of 777-3A/SH	Sang Ho Kim, unpublished
MS20/A1	α *kar1-1, leu2, canʳ*	*rho⁰*		A. Haid, unpublished
MS2-0-7	α *kar1-1, leu2, canʳ*	*rho⁺, mit⁺*	Cytoductant from cross MS20/A1 × JC8-0-7	
MS2-0-M5631	α *kar1-1, leu2, canʳ*	*rho⁺, mit⁻ (oxi1⁻*-M5631)	Cytoductant from cross MS20/A1 × JC8-0-M5631	
MS2-0-441	α *kar1-1, leu2, canʳ*	*rho⁺, mit⁻ (oxi1⁻*-M5631) *hc441*	Cytoductant from cross MS20/A1 × JC8-0-441	
KL14-4A	a *his1, trp2*	*rho⁺, mit⁺*		Wolf et al. (1973)
KL-0	a *his1, trp2*	*rho⁰*	*rho⁰* mutant of KL14-4A induced with ethidium bromide	
KL-0-7	a *his1, trp2*	*rho⁺, mit⁺*	Cytoductant from cross KL-0 × MS2-0-7	
KL-0-M5631	a *his1, trp2*	*rho⁺, mit⁻ (oxi1⁻*-M5631)	Cytoductant from cross KL-0 × MS2-0-M5631	
KL-0-441	a *his1, trp2*	*rho⁺, mit⁻ (oxi1⁻*-M5631) *hc441*	Cytoductant from cross KL-0 × MS2-0-441	
JC8/AA1	a *kar1-1, leu1*	*rho⁰*		Lancashire and Mattoon (1979)
JC8-0-7	a *kar1-1, leu1*	*rho⁺, mit⁺*	Cytoductant from cross JC8/AA1 × 777-3A	
JC8-0-M5631	a *kar1-1, leu1*	*rho⁺, mit⁻ (oxi1⁻*-M5631)	Cytoductant from cross JC8/AA1 × 777-3A-M5631	
JC8-0-441	a *kar1-1, leu1*	*rho⁺, mit⁻ (oxi1⁻*-M5631) *hc441*	Cytoductant from cross JC8/AA1 × HC441	
C7 (MH41-7B/AA12)	a *ade2, his1*	*rho⁻*		Wesolowski and Fukuhara (1979)
P2 (MH41-7B/P21)	a *ade2, his1*	*rho⁻*		Wesolowski and Fukuhara (1979)
C45s-1	a *his4*	*rho⁻*		Fox (1979)
SEG15/S1	a *his1, trp2*	*rho⁻, mfs-1*		Weiss-Brummer et al. (1989)

Further Western blotting procedures were performed according to the Blotto method (Johnson et al. 1984). All reactions were carried out with antiserum raised against yeast COXII, kindly provided by Prof. G. Schatz, Biozentrum Basel, and Dr. T.D. Fox, Cornell University, N.Y.

Immunoprecipitation. The mitochondrial translation products (ca. 10 μl; 5×10^5 cpm) labeled *in vivo* as described above were solubilized in 1% SDS by incubation for 10 min at 95° C and subsequently in 1% Triton, 1 M NaCl for a further 10 min at 23° C. After centrifugation (40000 g, 15 min) the supernatant was transferred in 200 μl TNET buffer (300 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5) and incubated with 20 μl antiserum for 18 h at 4° C. Precipitation was carried out with 2 mg protein A-Sepharose CL-4B (Pharmacia). The pellet was washed three times in 100 μl TNET buffer, then once in 10 mM Tris-HCl, pH 7.5 and suspended in Laemmli buffer.

Results

Isolation of HC mutants

The mitochondrial +1 frameshift mutation of the strain 777-3A-M5631, which is located in the gene *oxi1* coding

23°C   30°C   37°C   C

A

B

	777-3A-M5631	777-3A-M5631		
HC 31	HC 34	HC 35	HC 39	HC 63
HC 218	HC 248	HC 263	HC 288	
HC 301	HC 315	HC 388	HC 393	
HC 408	HC 427	HC 440	HC 441	HC 444 HC 478
HC 603				

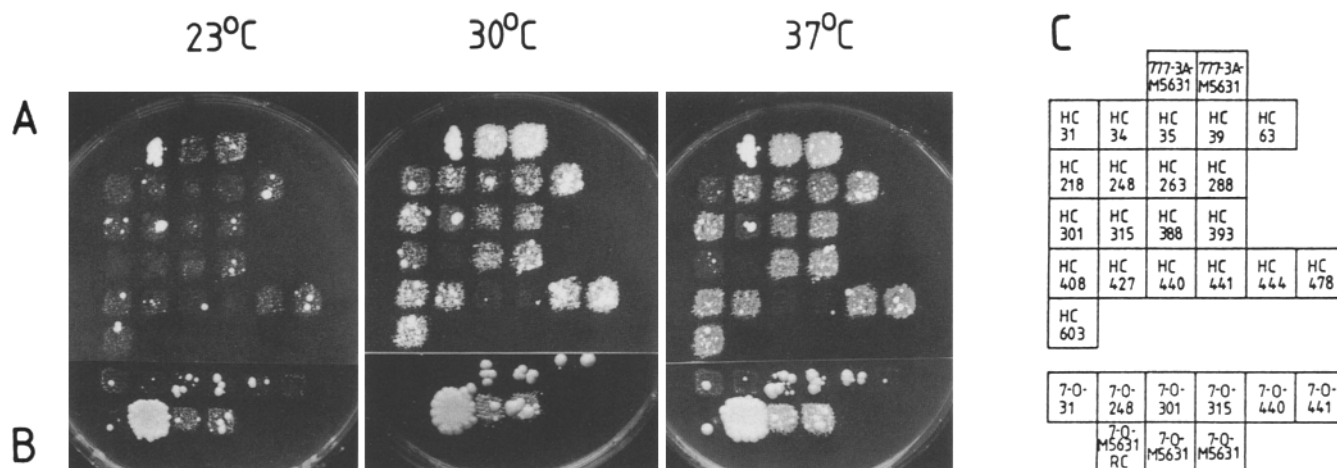| 7-0-31	7-0-248	7-0-301	7-0-315	7-0-440	7-0-441
7-0-M5631 RC	7-0-M5631	7-0-M5631			

Fig. 1 A–C. Phenotype of HC strains on medium (YG) containing the non-fermentable carbon source glycerol. Orientation of strains on the plate is shown in **C**. **A** HC strains were crossed with KL-0 for complementation of the recessive nuclear mutation *op1*. Diploid cells grown on WO medium were replica-plated on YG medium and incubated for 7 days at 23°, 30° and 37° C. For reference the growth of the identically treated parental strain 777-3A-M5631 is shown in the first row. **B** Phenotype of cytoductants (7-0-31, -248, -301, -315, -440, and -441) of HC strains, HC31, HC248, HC301, HC315, HC440, HC441. Cytoduction of mitochondria was carried out via the *kar* strain JC8/AA1 and to the strain 7-0 isogenic to the parental strain. Isolated cytoductants were crossed with KL-0 and plated as described above. In the last row the phenotype of the cytoductant of the "leaky" parental mitochondria (7-0-M5631) isolated and crossed in the same manner as the HC strains is shown beside the respiration-competent revertant (*oxi1⁻* → *OXI1⁺*)

for COXII can be suppressed spontaneously to a significant extent (Fox and Weiss-Brummer 1980). This translational suppression was observed originally as a leaky growth phenotype on non-fermentable medium. The suppressed phenotype is only apparent when the mutant strain is crossed with a strain possessing the wild-type nuclear alleles for respiratory function (*PET⁺*), which complement the recessive nuclear *op1* mutation (*pet⁻*) of 777-3A-M5631. The phenotype of the diploids on non-fermentable medium was used here as the first criterion for isolation of strains with restricted suppression of the frameshift mutation; single colonies of 777-3-M5631 that had been mutagenized with $MnCl_2$ were crossed with KL-0, which is a *PET⁺* strain and possesses no mitochondrial genome (*rho⁰*). The *rho⁰* state of the cross partner allowed the direct selection of the desired mutants based upon their phenotype, since all diploid progeny of the cross have acquired the mitochondrial genome of the mutated strains with the original frameshift mutation M5631. Colonies showing a more reduced respiration capacity or an RD (respiratory-deficient) phenotype on non-fermentable medium were selected.

In the following step, strains carrying second-site mutations specific for frameshift suppression were isolated. The selection was based on the criterion that a specific second-site mutation should have its effect only in combination with the first frameshift mutation; mutants which have lost the first frameshift mutation should grow normally on non-fermantable medium. For this purpose the mitochondrial frameshift mutation was replaced by the wild-type allele through a *rho⁻* cross with strain C45s-1 (see Fig. 2). This strain carries an extensively deleted mitochondrial genome (*rho⁻*) retaining only a short region of the gene *oxi1* with the wild-type allele of the M5631 mutation. A *rho⁺* recombinant ge-

nome resulting from the cross consequently possesses no +1 frameshift mutation in the *oxi1* gene.

Mutant strains that segregated respiration-competent recombinant progeny were selected, and 20 of them, showing distinct phenotypes, were isolated. These strains, in which second-site mutations specially reduce the level of spontaneous suppression of the mitochondrial frameshift mutation, were termed HC strains and their mutations HC mutations (see Fig. 1 A for phenotypes).

Selection of mitochondrial HC mutations

The method of selection used to obtain the HC mutations implies that either nuclear or mitochondrial loci could be involved in restriction of frameshift suppression. Nuclear HC mutations should all be dominant because selection occurred in the zygotic state. On account of our interest in identifying the mitochondrial (RNA) factors responsible for frameshift suppression, mitochondrial HC mutations were selected through the 'mitochondrial back-cross' method (Dujardin et al. 1980). Mitochondrially inherited mutations can be distinguished from nuclear ones by their ability to segregate during mitotic cell divisions. Each of the HC strains was crossed with the strain carrying the original mitochondrial genome bearing the frameshift mutation. A cross with a mitochondrial HC mutation should show mitotic segregation of two phenotypes on non-fermentable medium: the original leaky respiration phenotype (777-3A-M5631) and a further reduced respiration capacity (HC) phenotype. In contrast, the diploid progeny of a cross with a nuclear HC mutation will all show the reduced growth of the HC mutant strain.

The results of the genetic analyses are presented in Table 2. Through the cross 6 of 20 HC mutations were

Table 2. Mitochondrial backcross of HC strains

HC strain	Cross with KL-0-M5631						Number of colonies tested	Cross with KL-0						Number of colonies tested
	Phenotype							Phenotype						
	leaky (%)			restricted leaky (%)				leaky (%)			restricted leaky (%)			
	23° C	30° C	37° C	23° C	30° C	37° C		23° C	30° C	37° C	23° C	30° C	37° C	
	−	+ +	+ +	−	+/−	+/−		−	+ +	+ +	−	+/−	+/−	
HC31	57			43			230	0			100			250
HC34	0			100			235	0			100			250
HC35	0			100			226	0			100			250
HC39	0			100			239	0			100			250
HC63	0			100			215	0			100			250
HC218	0			100			242	0			100			250
HC248	42			58			220	0			100			250
HC263	0			100			255	0			100			250
HC288	0			100			218	0			100			250
HC301	52			48			220	0			100			250
HC315	61			39			204	0			100			250
HC388	0			100			227	0			100			250
HC393	0			100			201	0			100			250
HC408	0			100			245	0			100			250
HC427	0			100			235	0			100			250
HC440	58			42			226	0			100			250
HC441	65			35			210	0			100			250
HC444	0			100			245	0			100			250
HC478	0			100			233	0			100			250
HC603	0			100			216	0			100			250
777-3A-M5631	100			0			250	100			0			280

Strains were crossed with KL-0-M5631 and KL-0. Single colonies of the diploid cells grown on WO medium were replica-plated on YG medium and incubated for 7 days at 23° C, 30° C and 37° C.

As a standard wild-type growth was associated with + + + +; accordingly leaky growth with + +, reduced leaky growth with +/− and no growth with −.

identified as being mitochondrial. They are HC31, HC248, HC301, HC315, HC440 and HC441. Comparison of the mitochondrial and nuclear HC mutations revealed an interesting distinction between their phenotypes: the mitochondrial mutations all exhibit a stronger restricted phenotype (RD) than the nuclear ones ("semi-leaky") (see Fig. 1 A).

The mitochondrial localization of these six mutations was also confirmed by cytoduction (see Materials and methods). The mitochondria of these mutant strains were transferred to the *rho*⁰ strain (7-0) via double *kar* crosses. All of the newly constructed HC strains again showed the reduced growth phenotype in the zygotic state with *rho*⁰ strain (KL-0), whereas the similarly constructed parental strain exhibited the leaky growth phenotype (see Fig. 1 B).

Rho⁻ mapping of mitochondrial HC mutations

Mapping of the mitochondrial HC mutations was achieved through deletion mapping with *rho⁻* strains. This method relies on the ability of yeast mitochondrial genomes to be crossed in the zygotic state, to recombine with each other at high frequencies and finally to segregate quickly in a homoplasmic state during mitotic cell divisions (for review see Dujon 1981). In the situation described here, the cross with a *rho⁻* strain that retains the wild-type region corresponding to a given HC mu-
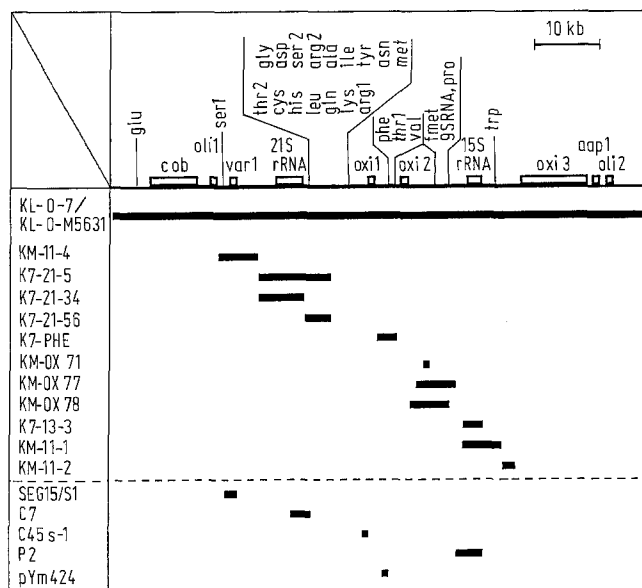

Fig. 2. Deletion diagrams of various mitochondrial DNAs. Mitochondrial DNA is shown here as *linear* for convenience. Genes are shown above the line, abbreviated according to de Zamaroczy and Bernardi (1986) (structural genes are *boxed*). *Horizontal lines* represent the retained DNA. KM strains were derived from K L-0-M5631 and K7 strains from KL-0-7. The 11 *rho⁻* strains shown in the upper part were used for deletion mapping of the mitochondrial HC mutations (see text). The five DNAs in the lower part are the mitochondrial DNAs used for selection of HC strains (C45s-1) and of appropriate *rho⁻* genomes

Table 3. Deletion mapping of the mitochondrial HC mutations

HC strain	Deletion strain											
	KM11-4	K7-21-5	K7-21-34	K7-21-56	K7-PHE	KM-OX71	KM-OX77	KM-OX78	K7-13-3	KM-11-1	KM-11-2	KL-0
HC31	−	−	−	−	−	−	−	−	−	−	−	−
HC248	−	−	−	−	−	−	−	−	−	−	−	−
HC301	−	−	−	−	−	−	+	+	−	−	−	−
HC315	−	+	−	+	−	−	−	−	−	−	−	−
HC440	−	+	−	+	−	−	−	−	−	−	−	−
HC441	−	+	−	+	−	−	−	−	−	−	−	−
777-3A-M5631	+	+	+	+	+	+	+	+	+	+	+	+

Segregation of the parental leaky phenotype in progeny colonies of a corresponding cross is give as +. The mitochondrial DNAs retained in rho^- strains are shown in Fig. 2. In the crosses of K7 strains the leaky phenotype was suppressed generally, so that the analysis was carried out after incubation for 2–3 weeks

Table 4. Mitochondrial recombination analysis of HC strains

HC strain	Cross with KL-0							Cross with KL-0-441						
	Phenotype						Number of colonies tested	Phenotype						Number of colonies tested
	leaky (%)			restricted leaky (%)				leaky (%)			restricted leaky (%)			
	23° C	30° C	37° C	23° C	30° C	37° C		23° C	30° C	37° C	23° C	30° C	37° C	
	−	+ +	+ +	−	+/−	+/−		−	+ +	+ +	−	+/−	+/−	
HC31	0			100			200	3.4			96.9			205
HC248	0			100			220	11.4			88.6			158
HC301	0			100			200	8.1			91.9			209
HC315	0			100			400	2.1			97.9			329
HC440	0			100			400	0			100			410
HC441	0			100			400	0			100			420

Strains were crossed with KL-0 and KL-0-441. See legend of Table 2 for further details. The frequency of occurrence of the parental leaky phenotype following the cross between a given HC strain and KL-0-441, which carries the HC441 mutation, is a measure of the genetic distance between the mitochondrial mutations in the two strains (see text for further details)

tant locus on the mitochondrial genome will segregate recombinants carrying the mitochondrial genome without the HC mutation. The mitochondrial genome of these recombinants is the same as that of the parental strain 777-3A-M5631, so that they can be observed as progeny colonies showing leaky growth on non-fermentable medium, i.e. an HC mutation maps to the region of the retained rho^- genome that can segregate leaky colonies as a significant proportion of progeny after the cross.

Prior to mapping, rho^- strains carrying different deletions on the mitochondrial genome were isolated initially from strains mitochondrially isogenic to the HC strains (KL-0-7, KL-0-M5631, see Materials and methods). Isogenicity of the mitochondrial genome was particularly necessary for the analysis here, in view of the polymorphism of the mitochondrial DNA sequence, especially that involving the genes for the rRNAs, which has been documented previously between several wild-type strains (de Zamaroczy and Bernardi 1986; Hüttenhofer et al. 1988).

Eleven rho^- strains (K7 and KM series) were used for mapping the HC mutations. They cover the regions of the 15S rRNA, 21S rRNA, *var1* or tRNA genes (see Fig. 2).

The results of these crosses are presented in Table 3. Three HC mutations (HC315, HC440 and HC441) were mapped to the region of the tRNA gene cluster that is retained in the mitochondrial genome of K7-21-56. One HC mutation (HC301) was mapped to the region of 9S RNA-tRNA^{Pro}, retained in KM-OX77 and KM-OX78 but not in OX71. Two other mitochondrial mutations (HC31 and HC248) were localized to regions that were not covered by these 11 rho^- genomes.

Recombination analysis of mitochondrial HC mutations

The allelism of the mitochondrial HC mutations was analysed by determining the genetic distance between HC441 and the other five HC mutations from the recombination frequency. Recombination between the HC441 mutation and a given other HC mutation results in a mitochondrial genome without any HC mutations. Since this recombined genome is the same as the parental leaky genome, the frequency of occurrence of the parental leaky phenotype corresponds to the recombination frequency between two HC mutations. As a partner in the cross the strain KL-0-441 carrying the HC441 mutation was constructed through cytoduction (see Table 1).
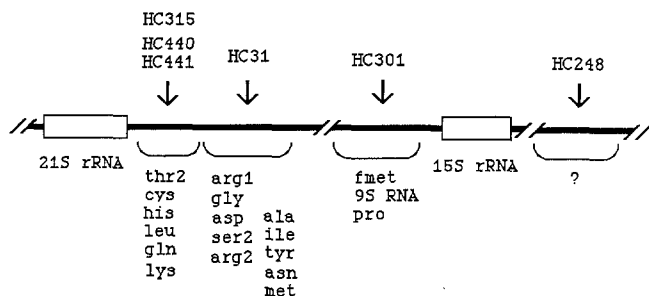
Fig. 3. Genetic map of the mitochondrial HC mutations. The mitochondrial genome is shown as a *bar*. Genetic loci are indicated under the bar (see text)

The results presented in Table 4 show the linkage of two mutations, HC31 and HC315, to the HC441 locus with recombination frequencies of 6.8% and 4.2%, respectively. As HC315 and HC441 map to the same region of the tRNA gene cluster, HC31 probably also lies in the vicinity of the above region, in the tRNA gene cluster. Mutation HC440 did not segregate leaky recombinants, indicating that it lies very near or is identical to HC441. Two other mutations, HC248 and HC301, showed a high output of leaky progeny (22.8% and 16.2%, respectively), which indicates that the corresponding loci are unlinked and separated by tens of kilobases in the yeast mitochondrial genome (Dujon 1981) (see Fig. 3 for a summary of the results shown in Tables 3 and 4).

Influence of HC mutations on growth rate

The HC mutant strains were selected such that they can grow on non-fermentable medium in the absence of the mitochondrial frameshift mutation M5631. In order to characterize the specificity of the restriction effect of the

Table 5. Growth rate of respiration-competent mitochondrial HC strains

Strain	Growth rate μ (h^{-1})		
	23° C	30° C	37° C
777-3A-M5631	0.20	0.24	0.23
	(1.00)	(1.00)	(1.00)
HC31	0.20	0.21	0.16
	(1.00)	(0.88)	(0.70)
HC248	0.20	0.18	0.17
	(1.00)	(0.75)	(0.74)
HC301	0.18	0.22	0.19
	(0.90)	(0.92)	(0.83)
HC315	0.20	0.21	0.18
	(1.00)	(0.88)	(0.78)
HC440	0.14	0.17	0.11
	(0.70)	(0.71)	(0.48)
HC441	0.18	0.23	0.19
	(0.90)	(0.96)	(0.83)

Growth rate (μ) is calculated from the doubling time (t_D); $\mu = \dfrac{\ln 2}{t_D}$.
The ratios of the rates to that of the parental strain at each corresponding temperature are shown in parentheses. All strains were crossed with KL-0 (see Materials and methods)

mitochondrial HC mutations on frameshift suppression more precisely, the growth rates of RC derivatives of these strains which had lost the original mutation M5631 were measured in non-fermentable rich medium. To allow comparison with the suppression phenotype on the plates, these strains were also crossed with the *rho*^{0} strain KL-0.

The growth rates were calculated from the OD$_{600}$ curves of logarithmically growing cells and are shown in Table 5. Compared with that of the RC revertant of the parental strain, the following effects of HC mutations could be seen: (a) the growth rate of HC440 is reduced by about 30%–50%; (b) the other HC mutations showed minimal effects. Specifically, HC315 and HC441 showed only a minor reduction (about 5%–10% at 30° C and 20% at 37° C).

Since the mitochondrial HC mutant strains do not show the level of growth characteristic of the original leaky phenotype on non-fermentable medium even after a 2 week incubation (data not shown), the phenotype of these strains is caused by the specific restrictive effect of the mutations on the degree of spontaneous suppression of the frameshift mutation M5631.

Protein analysis

The influence of the HC mutations on spontaneous frameshift suppression was analyzed directly at the translational level. The mitochondrial translation products were labeled in vivo with $^{35}SO_4^{2-}$ in the presence of cycloheximide and were subjected to polyarcylamide disc gel electrophoresis. As shown in Fig. 4A, two products could be related to COXII. Besides the truncated 12.5 kDa product synthesized by regular triplet translation of the +1 frameshift message, the 32.5 kDa product corresponding to full-length COXII was generated in the leaky strain. The 32.5 kDa product results from the spontaneous suppression of the frameshift mutation. The suppression rate (32.5 kDa/12.5 kDa + 32.5 kDa) is about 26%, based on densitometric measurements of the autoradiograms and taking into consideration the different numbers of radioactively labelled cysteine and methionine residues in the two products. Protein analysis of the mitochondrial HC mutant strains revealed that the 32.5 kDa suppression product was also synthesized in all of these strains to a considerable extent. The greatest reduction in the amount of the 32.5 kDa product was seen in strains HC440 and HC441, which had about one-half the amount seen in the leaky original strain (13%–14%). Strains HC301 and HC315 show a reduction of about one-quarter (18%–22%). HC31 and HC248 caused no significant reduction, rather they showed a slight increase in suppression rate (25%–30%). These results appear to contradict the simple expectation based on the restrictive phenotypic effect of the HC strains on frameshift suppression. To examine the possibility of artifacts caused by the use of cycloheximide during protein radiolabeling, the COXII suppression product was assayed by Western blotting of total mitochondrial proteins extracted from cells that had been
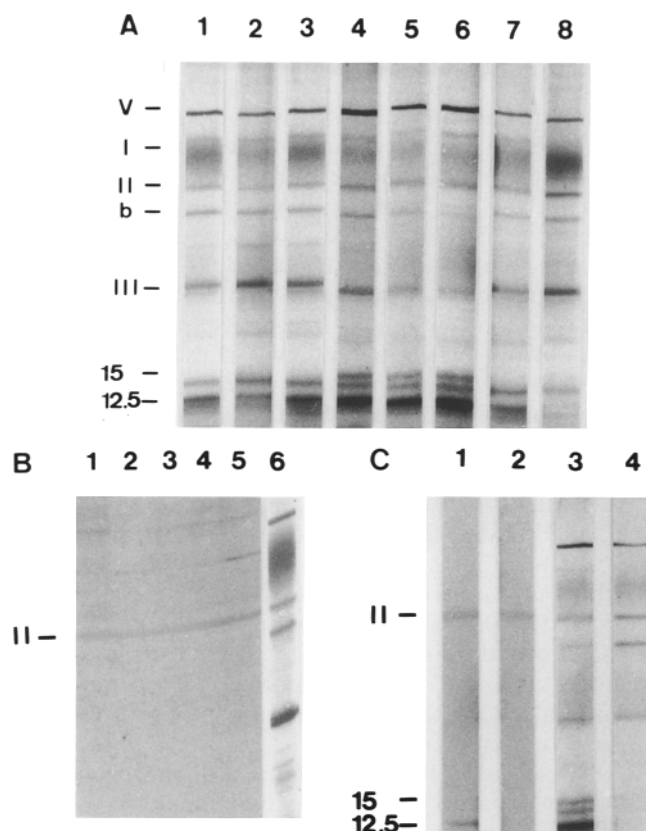
Fig. 4A–C. Protein analysis. A ³⁵S-labeled mitochondrial translational products: V, Var1; I, II, and III, cytochrome c oxidase, subunits I, II and III; b, apo-cytochrome b; 12.5 and 15 kDa, suppression products. Lane 1, HC31; lane 2, HC248; lane 3, HC301; lane 4, HC315; lane 5, HC440; lane 6, HC441; lane 7, 777-3A-M5631 leaky; lane 8, 777-3A-M5631 RC (OXI1⁺ revertant). B Immunoblot analysis of COXII. Lane 1, 777-3A-M5631 RC; lane 2, 777-3A-M5631 leaky; lane 3, HC301; lane 4, HC315; lane 5, HC441; lane 6, marker, radioactively labeled mitochondrial translational products of a wild-type strain. C Immunoprecipitation of COXII from HC440 with serum A (lane 1) and serum B (lane 2). Lane 3 shows the ³⁵S-labeled mitochondrial translational products of HC440 and lane 4 of HC440, an OXI1⁺ revertant

The results presented in Fig. 4C reveal that the 15 kDa protein could not be precipitated by either serum. This indicates that the 15 kDa protein does not possess any of the COXII antigenic determinants detected by the sera. The possible identity of the protein is discussed below.

Influence on mitochondrial RNA synthesis

Several mitochondrial HC mutations were mapped to regions adjacent to the genes for two rRNAs: HC301 to the upstream region of the 15S rRNA gene, and HC315, HC440 and HC441 to the down-stream region of the 21S rRNA gene. To examine their influence on rRNA synthesis, total mitochondrial RNA was isolated from cells adapted in a non-fermentable medium similar to plate growth conditions and was analyzed by means of Northern blotting. The strains tested were HC301, HC315 and HC441 together with the original leaky strain and its RC revertant. As shown in Fig. 5A, both rRNAs were processed in all HC strains examined just as in the original strain. No aberrant RNA bands were detectable. Comparison of the amount of each rRNA by densitometry did not reveal any deviation from that of the control.

The HC mutations, HC301, HC315 and HC441, were further analyzed for the synthesis of several tRNAs chosen according to the mapped positions of the mitochondrial HC mutations. Transcripts of tRNA^{pro}, tRNA^{thr2}, tRNA^{gln}, with tRNA^{ser1} as control, were analyzed with mitochondrial RNA prepared as described above. The intensity of the autoradiogram was standardized to the value of the leaky strain. The most notable change was
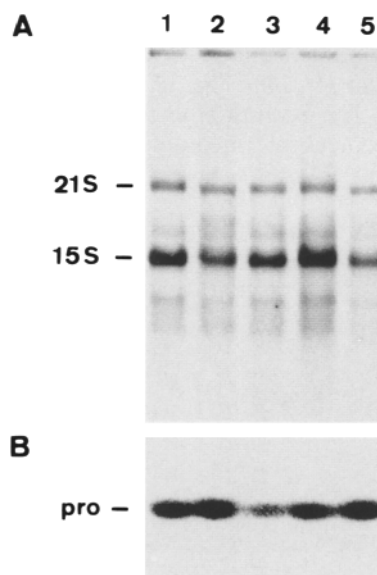
grown under similar conditions to those grown on non-fermentable medium. In all three HC strains (HC301, HC315 and HC441) analyzed, the 32.5 kDa suppression product was also detected at approximately the same level as in the leaky strain (see Fig. 4B).

In addition to the 32.5 kDa COXII band, a 15 kDa protein band that is specific to several HC mutant strains (HC315, HC440 and HC441) was detectable (see Fig. 4A). This band was not seen in OXI1⁺ revertants of the respective strains indicating that it is generated through translation of the oxi1-M5631 message (Fig. 4C, lane 4). To test this possibility, the mitochondrial translation products of HC440, which were radioactively labeled in vivo, were subjected to immunoprecipitation with antiserum for COXII. Two sera reacting with different antigenic determinants were available for the analysis: serum A precipitates the 12.5 kDa fragment of the M5631 frameshift product as well as mature 32.5 kDa COXII; serum B cannot precipitate the 12.5 kDa fragment but can precipitate mature COXII.

Fig. 5A and B. Northern analysis of mitochondrial rRNAs (A) and tRNA^{pro} (B). Lane 1, 7-0-M5631, RC (OXI1⁺ revertant); lane 2, 7-0-M5631 (leaky parental strain); lane 3, 7-0-301 (HC301); lane 4, 7-0-315 (HC315); lane 5, 7-0-441 (HC441). To obtain identical nuclear backgrounds, the strains used were all cytoductants of the respective strains (see Fig. 1 and text)

seen in tRNA^{pro} of HC301 which was reduced about 30% compared with that of the parental strain (see Fig. 5B). All strains showed little difference in the amounts of the other tested tRNAs. No transcript with an aberrant length was observed (data not shown).

Discussion

The mitochondrial +1 frameshift mutation M5631, which results in a sequence of six instead of five consecutive T residues in the *OXI* gene, is suppressed spontaneously to a significant degree of about 25%. Alterations in the translational apparatus, such as the par^r_{454} mutation of 15S rRNA, can influence the suppression (Weiss-Brummer and Hüttenhofer 1989). This indicates that the suppression takes place at the level of translation. To understand the requirements for spontaneous translational frameshifting, we attempted to isolate intergenic mutations that reduce the suppression. Six such mitochondrial mutations (mitochondrial HC mutations) were characterized and mapped to several tRNA gene regions.

The specificity of their effect on frameshift suppression was shown by their respiration-competent phenotype in the absence of the frameshift mutation M5631. Four mitochondrial HC mutations (HC31, HC301, HC315 and HC441) influence the respiration-competent growth rate to only a minimal extent (0%–10% reduction relative to the original $OXI1^+$ strain).

The restrictive effect of the mitochondrial HC mutations on suppression was further examined at the translational level. The results of the protein analysis appear not to correlate with the phenotype of the HC strains. All mitochondrial HC strains synthesize the suppressed product with the wild-type size of 32.5 kDa to a considerable extent, as assayed by radiolabeling in the presence of cycloheximide. The amount of the product is not reduced in strains HC31 and HC248. The 32.5 kDa protein was also identified by immunoblotting in these strains grown on non-fermentable medium. Thus, the

appearance of ^{35}S-labelled mitochondrial translational products is not an artifact resulting from, for example, labeling in the presence of cycloheximide. One reasonable explanation for all these results is that more than one kind of 32.5 kDa product might be synthesized as a result of the shift suppression of the *oxi1* message containing the +1 frameshift mutation. At least one suppressed 32.5 kDa product is functional as COXII, whereas the others are non-functional.

The region of the wild-type COXII sequence around the mutation site is important for its function, as becomes obvious when the known COXII protein sequences from different organisms are compared. This region – comprising 9 amino acid residues upstream and 11 residues downstream from the +1 mutation site – is characterized as a transmembrane domain strongly conserved during evolution (Capaldi et al. 1983). The first amino acid residue changed by the mutation is proline, which is totally conserved in COXII from the procaryote *Paracoccus denitrificans* to plants, fungi, and lower and higher animals (see Fig. 6). As in the case of bacteriorhodopsin, in which the proline residues play an important role in transmembrane proton pumping (Mogi et al. 1989), this residue should be subject to positive selection pressure. Hence, the translational suppression should cause the incorporation of the proline at this site in order to generate the functional COXII and the leaky phenotype. Thus, the prolyl-tRNA is the tRNA that probably decodes the first codon after the +1 mutation site and shifts in the direction +1. On the other hand, if some aminoacyl-tRNA(s) other than prolyl-tRNA could decode and shift +1 at the same site, non-functional COXII protein(s) with the same length as the wild-type COXII would be produced.

According to the model, prolyl-tRNA should possess a certain affinity for the 0-frame codon UCC normally read by tRNA^{ser1}. This aberrant pairing is possible if first codon U–G base-pair were allowed (see Fig. 7). Such first codon wobble has previously been reported in *E. coli* (Toth et al. 1988) and seen in initiation of translation by tRNA^{fmet}. The first altered 0-frame codon

	81																			99
oxi1–M5631	I	E	V	I	W	T	I	F	*S*	*S*	*C*	*N*	*F*	*I*	*N*	*Y*	*C*	*F*	*P*
S.cerevisiae	I	E	V	I	W	T	I	F	P	A	V	I	L	L	I	I	A	F	P
Hansenula saturnus	I	E	I	I	W	T	I	L	P	A	V	V	L	L	I	I	A	F	P
Neurospora crassa	I	E	I	I	W	T	I	L	P	A	V	I	L	I	L	I	A	F	P
Tritium aestivum	I	E	I	I	W	T	I	F	P	S	V	I	L	L	F	I	A	I	P
Zea mays	I	E	I	I	W	T	I	F	P	S	V	I	P	L	F	I	A	I	P
Glycine max	I	E	I	L	W	T	I	F	P	S	I	I	P	M	F	I	A	I	P
Pisum sativum	I	E	I	L	W	T	I	F	P	S	I	I	P	M	F	I	A	I	P
Drosophila melanogaster	I	E	M	I	W	T	I	L	P	A	I	I	L	L	F	I	A	L	P
Xenopus laevis	I	E	M	V	W	T	I	M	P	A	I	S	L	I	M	I	A	L	P
Bos taurus	V	E	T	I	W	T	I	L	P	A	I	I	L	I	L	I	A	L	P
Mus musculus	V	E	T	I	W	T	I	L	P	A	V	I	L	I	M	I	A	L	P
Homo sapiens	M	E	T	V	W	T	I	L	P	A	I	I	L	V	L	I	A	L	P
Paracoccus denitrificans	I	E	V	I	W	T	L	V	P	V	L	I	L	V	A	I	G	A	F

Fig. 6. Comparison of COXII amino acid sequence in the region of the frameshift mutation M5631. The conserved proline residue at the mutation site is indicated. References for sequences are given by Bairoch (1988). In the first row is shown the sequence of the mutant strain. The amino acid residues altered as a consequence of the +1 mutation are *italicized*
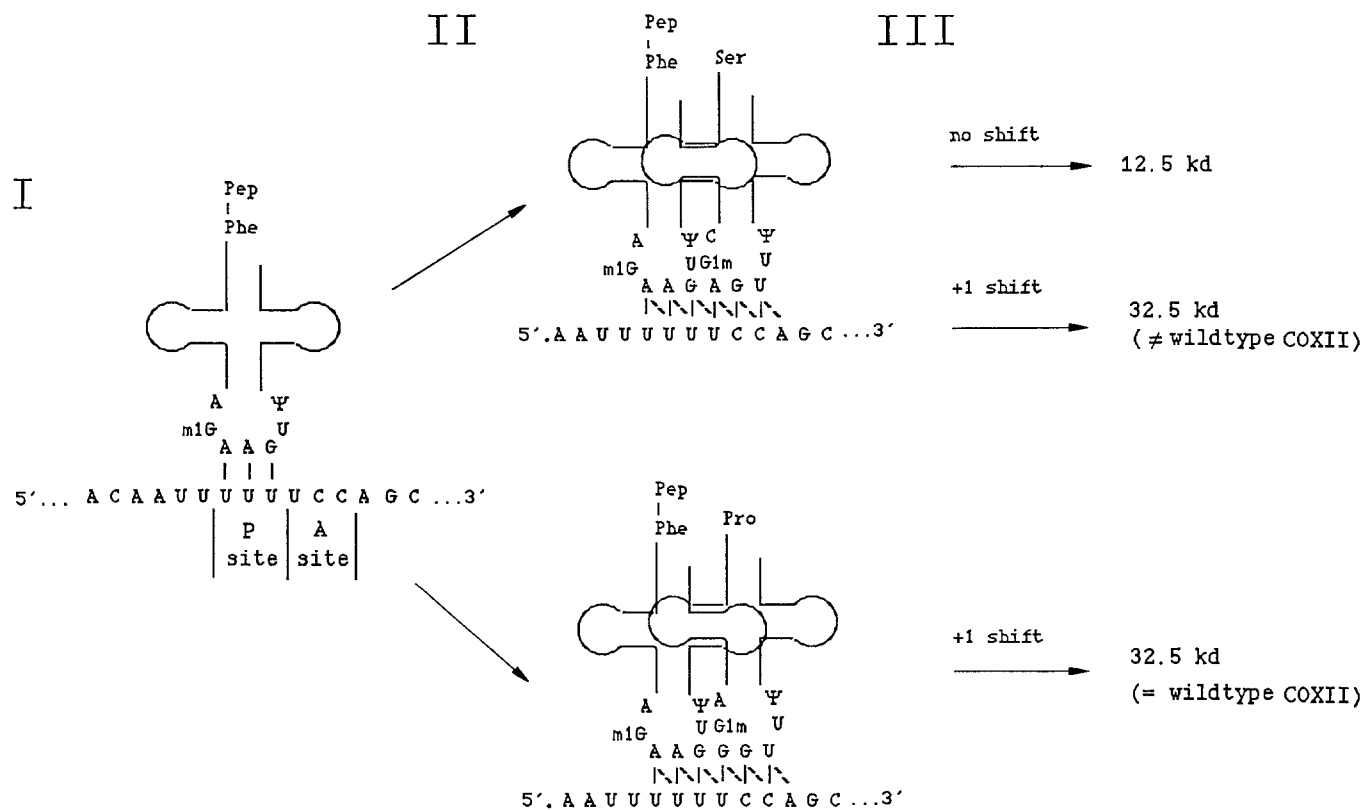
Fig. 7. A model for the frameshift suppression of the frameshift mutation M5631. I, UCC, the first codon altered by the +1 mutation, is positioned in the A site on a ribosome. II, codon recognition by the cognate seryl-tRNA (above) or the non-cognate prolyl-tRNA (below). III, shift events and their consequent translational products. After +1 shift the prolyl-tRNA in the A-site position encounters its cognate codon. Note that the peptidyl (phenyl)-tRNA positioned in the P-site interacts cognately both before and after the shift. Anticodon loop sequences are according to Sibler et al. (1986) and Hüttenhofer et al. (1990)

UCC is moreover quite rarely used in yeast mitochondria. In a total of 2167 codons in all except then unassigned reading frames UCC appears only once (Sibler et al. 1986). The tRNA^{ser1}, which is a unique tRNA cognate to UCC, prefers to read codons UCA (75 of 2167) and UCU (40 of 2167) indicating it is difficult to decode UCC. A certain correlation between rare codons in highly expressed genes and shift events has been demonstrated in *E. coli* (Curran and Yarus 1989). The rarity of the first mutated codon is probably one reason why the frameshift mutation is suppressed so efficiently.

Non-triplet decoding by prolyl-tRNA and some other(s) must compete with the cognate seryl-tRNA for the codon UCC. A reduction in the concentration of prolyl-tRNA in mitochondria will lead directly to restriction of the +1 shift that generates the functional COXII shift product. That a change in concentration of certain aminoacyl-tRNAs can cause a frameshift event has already been shown in vitro, as well as in vivo, in systems in which competition between a cognate and non-cognate aminoacyl-tRNA for a codon presented in the A-site on ribosomes has been demonstrated (Atkins et al. 1979; Weiss and Gallant 1986).

The nature of the HC301 mutation could fit this model, as it was mapped to the region of the gene for tRNA^{pro} in this work. However, further DNA sequence analysis has revealed that HC301 is not located in the area of the mapped region that is transcribed and en-

codes genes, which extends for 2 kb from 100 bp upstream of the gene for tRNA^{fmet} to 0.5 kb downstream of the gene for tRNA^{pro} (Sakai 1989). In the same work it was shown that the HC301 locus is linked with a recombination frequency of 8% to the par^r_{434} locus of the 15S rRNA gene located about 6 kb downstream of the tRNA^{pro} gene. This indicates that HC301 is situated in the 2 kb spacer region downstream of the gene for tRNA^{pro}. As shown here, HC301 affects the amount of transcription of tRNA^{pro}, reducing it to 70% of the original strain. The action of the mutation on the suppression is therefore probably through reduction of the concentration of the tRNA^{pro} that normally competes with tRNA^{ser1} and suppresses the frameshift.

Recently, a mutation in the gene tRNA^{ser1} has been isolated in our laboratory. This mutation, *mfs-1*, located in the anticodon stem affects the frameshift suppression positively, allowing the mutant to grow in a quasi respiratory-competent manner (Weiss-Brummer et al. 1989; Hüttenhofer et al. 1990). Interestingly, this mutation does not cause significantly increased synthesis of the 32.5 kDa COXII suppression product (H. Sakai, unpublished data). Previously, it was assumed that the mutation could facilitate the +1 shift by itself. On the basis of the above discussion it is, however, plausible that *mfs-1* may reduce the ability of tRNA^{ser1} to decode the rare codon UCC and this in turn allows the competitor tRNA^{pro} more opportunities to misread and shift at

UCC and generate the functional COXII with the conserved proline residue.

Furthermore, tRNA^{ser1} itself could also induce a $+1$ shift to generate the non-functional 32.5 kDa product. This is not only because tRNA^{ser1} decodes the 0-frame codon UCC very rarely, but it might also pair excellently with the $+1$ codon CCA through ^{33}U grapple pairing, as discussed by Hüttenhofer et al. (1990) (see Fig. 7). The finding that double mutants (HC301, *mfs-1*) show a leaky growth phenotype intermediate between that of both single mutants might support this possibility (H. Sakai, unpublished data).

The mapping of mitochondrial HC mutations to other tRNA gene regions further indicates the involvement of different tRNAs in interference with the suppression of one frameshift mutation. HC315, HC440 and HC441 especially, which were mapped to a region encoding several tRNAs, show one particular mitochondrial translation phenotype: appearance of a 15 kDa "sub"-product. Since the product is specific to the frameshift mutation, it should be generated through aberrant translation downstream of the mutation site. Considering all the reading frames of the downstream region, it is only possible if read-through takes place at the stop codon of the *oxi1*-M5631 message, keeping the same $+1$ shifted reading frame. In this way the 12.5 kDa fragment can be extended by 17 amino acid residues resulting in a total molecular weight of 15 kDa. This presumed 15 kDa protein consists of 134 amino acid residues, of which 46 residues, i.e. more than one-third, do not exist in the wild-type COXII sequence. This is perhaps the reason why the protein is not recognized by two different antisera. That these three HC mutations have the same translation phenotype is interesting, because HC315 has a genetic localization distinct from that of HC440 and HC441. This again suggests that multiple factors (tRNAs) could be involved in one suppression event. Further identification of the primary structure of these mutations would contribute to the clarification of the mechanism of their effects on the frameshift suppression.

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