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Initiation of Translation Can Occur Only in a Restricted Region of the *CYC1* mRNA of *Saccharomyces cerevisiae*

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The steady-state levels and half-lives of *CYC1* mRNAs were estimated in a series of mutant strains of *Saccharomyces cerevisiae* containing (i) TAA nonsense codons, (ii) ATG initiator codons, or (iii) the sequence ATA ATG ACT TAA (denoted ATG-TAA) at various positions along the *CYC1* gene, which encodes iso-1-cytochrome *c*. These mutational alterations were made in backgrounds lacking all internal in-frame and out-of-frame ATG triplets or containing only one ATG initiator codon at the normal position. The results revealed a "sensitive" region encompassing approximately the first half of the *CYC1* mRNA, in which nonsense codons caused Upf1-dependent degradation. This result and the stability of *CYC1* mRNAs lacking all ATG triplets, as well as other results, suggested that degradation occurs unless elements associated with this sensitive region are covered with 80S ribosomes, 40S ribosomal subunits, or ribonucleoprotein particle proteins. While elongation by 80S ribosomes could be prematurely terminated by TAA codons, the scanning of 40S ribosomal units could not be terminated solely by TAA codons but could be disrupted by the ATG-TAA sequence, which caused the formation and subsequent prompt release of 80S ribosomes. The ATG-TAA sequence caused degradation of the *CYC1* mRNA only when it was in the region spanning nucleotide positions −24 to +37 but not in the remaining 3′ distal region, suggesting that translation could initiate only in this restricted initiation region. *CYC1* mRNA distribution on polyribosomes confirmed that only ATG codons within the initiation region were translated at high efficiency. This initiation region was not entirely dependent on the distance from the 5′ cap site and was not obviously dependent on the short-range secondary structure but may simply reflect an open structural requirement for initiation of translation of the *CYC1* mRNA.

Mutationally-altered forms of the *CYC1* gene, which encodes iso-1-cytochrome *c* in the yeast *Saccharomyces cerevisiae* have been used to elucidate the major features of translation. The deficiencies of iso-1-cytochrome *c* in certain *cyc1* mutants and the corresponding deduced DNA sequences of *cyc1* mutations indicated that AUG was the only codon capable of initiating translation at appreciable levels (48, 49), a finding confirmed in yeast cells with other systems (7, 9). Also, early studies indicated that translation of *CYC1* mRNA initiated at the most upstream AUG codon (45–47) and that translation could initiate at a relocated AUG initiator codon within a 37-nucleotide region around the normal initiation site (45–49). These results and the results with deletion mutants (2) suggested the lack of any additional sequence requirements for initiation of translation. However, nucleotides preceding the AUG initiator codon at positions −1 and −3 slightly influenced initiation of translation, and the introduction of hairpin structures in the vicinity of the AUG initiator codon inhibited translation, with the degree of inhibition related to the stability and proximity of the hairpin (2, 9).

To further investigate whether translation can initiate at relocated AUG triplets, we have taken advantage of the instability of *CYC1* mRNA containing internal UAA nonsense codons, as illustrated in Table 1 for the *cyc1-91* mutant. While the instability of the *cyc1-91* mRNA could be rationalized, it was unclear why the *cyc1-133* initiator mutant contained a normal level of mRNA, because there are internally located open reading frames (Fig. 1). One interpretation was that the internally located open reading frames were not translated. Because of this difference in mRNA stability, we have systematically investigated the possibility that initiation cannot occur at some internal AUG triplets; these studies were carried out by constructing and analyzing the following three series of *cyc1* mutants and determining the levels and polyribosome distribution of the corresponding mRNA: (i) the TAA series, containing only one ATG codon at the normal initiating position and TAA nonsense codons at different positions along the *CYC1* mRNA; (ii) the ATG-TAA series, containing the sequence ATA ATG ACT TAA at different positions along the *CYC1* mRNA in a background lacking all other ATG triplets; and (iii) the ATG series, containing the sequence ATA ATG ACT at various positions along the *CYC1* mRNA in a background lacking all other ATG triplets. Our results suggest that there are a "sensitive" region and an initiation region. Upf1-mediated degradation of *CYC1* mRNAs occurred if premature nonsense codons were situated in the sensitive region, which constituted approximately the first half of the translated region. The initiation region is a region only in which relocated AUG initiator codon can initiate translation at appreciable efficiency, independently of the reading frame, and which encompasses 61 nucleotides from positions −24 to 37. This initiation region is not entirely dependent on the distance from the 5′ cap site and is not obviously dependent on the short-range secondary structure. Similar conclusions were made with a less extensive number of *cyc1* mutants that, however, contained all of the normal ATG triplets at internal sites (54).

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MATERIALS AND METHODS

Genetic nomenclature and yeast strains. *CYC1*$^+$ denotes the wild-type allele encoding iso-1-cytochrome *c*, whereas *CYC1* denotes the locus and is the generic symbol of any allele. Mutant alleles that produce either normal or decreased

TABLE 1. Instability of *CYC1* mRNA containing internal UAA nonsense codons

Allele	Sequence[a]					% of normal:	
						mRNA	Iso-1-cytochrome *c*
		(Met)	Thr	- Glu	- Phe-		
CYC1$^+$	ATA	ATG	ACT	GAA	TTC	100	100
cyc1-91	ATA	ATG	ACT	TAA	TTC	~2	<1
cyc1-133	ATA	ATA	ACT	GAA	TTC	100	<1

[a] The cleaved methionine residue is given in parentheses, and altered codons are underlined.

levels of iso-1-cytochrome *c* are designated *cyc1* followed by the allele number, e.g., *cyc1-1088*, *cyc1-1249*, etc. *CYC1* mRNA denotes normal or altered mRNAs transcribed from any *CYC1* allele.

Congenic yeast strains having the *CYC1* alterations listed in Table 2 were constructed as previously described by Baim and Sherman (2) and Li and Sherman (30). The desired alterations were made by oligonucleotide-directed mutagenesis by the method of Kunkel et al. (26) and with the plasmids and oligonucleotides listed in Table 2. The *cyc1-1249* allele was constructed by incorporating all six oligonucleotides in the same in vitro reaction. The alterations were verified by DNA sequencing (43). The *cyc1*::*CYH2*^{S} gene was replaced with the desired altered *CYC1* genes in the strain B-8242 (*MAT*α *cyc1*::*CYH2*^{S} *CYC7*$^+$ *ura3-52 leu2-3 leu2-112 cyh2*^{R}) by sequentially selecting for Ura$^+$ on uracil omission medium and Ura$^-$ on 5-fluoro-orotic acid (FOA) medium and by testing for Cyh^R, as previously described (2, 30). (Strain B-8242 was derived from strain B-7056 [or designated S260-11B; *MAT*α *cyc1*::*CYH2*^{S} *cyc7-67 ura3-52 leu2-3 leu2-112 cyh2*^{R}]) by replacing *cyc7-67* with the *CYC7*$^+$ allele [2, 30].) This procedure ensures that only a single copy of the altered gene is present at the *CYC1* chromosomal locus.

Oligonucleotides. Oligonucleotides used for oligonucleotide-directed mutagenesis (Table 2) Northern (RNA) analysis, and PCR were synthesized on an Applied Biosystems 380A DNA synthesizer and are listed in Table 3.

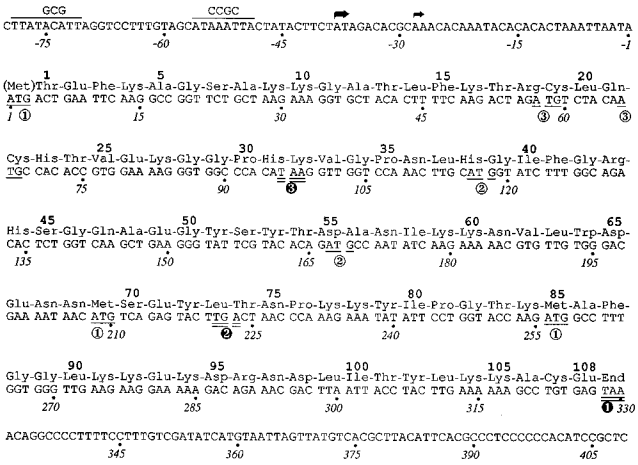

FIG. 1. The nucleotide sequence of the normal *CYC1*$^+$ gene and the corresponding amino acid sequence of iso-1-cytochrome *c* are shown with the numbering system beginning at, respectively, the ATG initiator codon and the amino-terminal threonine. The cleaved methionine residue is shown in parentheses. ATG triplets in the three frames are indicated by underlines and circled numbers. For example, ATG codons in frame 1 are at amino acid positions −1, 69, and 85, whereas ATG triplets in frame 2 are at nucleotide positions 116 and 167. Similarly, the chain-terminating triplets TAA, TGA, and TAG in all three frames are indicated by double underlines and white numbers in solid circles. Only the first terminating triplet following an in-frame ATG codon is shown. Six internal open reading frames are encompassed in the following nucleotide positions: 57 to 96, 66 to 96, 116 to 223, 167 to 223, 208 to 330, and 256 to 330. The *cyc1-944* allele and all of its derivatives (Table 4) contain a normal TATA element at position −123, an altered TATA element at position −178, and altered pseudo-TATA elements at positions −56, −78, and −93 (30), but only the replacements of elements at positions −56 and −78 are shown in the figure (above the normal *CYC1*$^+$ sequence). The *cyc1-944* major and minor transcriptional start sites at, respectively, positions −38 and −28 are indicated at the top of the figure.

Determination of cytochrome *c* content. Total amounts of cytochrome *c* were determined by spectroscopic examination of intact cells at −196°C (44) and by comparing the intensities of the c_α-bands at 547 nm to the c_α-bands of strains having known amounts of cytochrome *c*. A more accurate determination of cytochrome *c* content in intact cells was made by low-temperature (−196°C) spectrum recording with a modified Cary 14 spectrophotometer (17).

The preparation of total, nuclear, and cytoplasmic RNA from yeast cells. Total RNA was prepared according to the procedure of Li and Sherman (30), and nuclear and cytoplasmic RNA was prepared according to the procedure of Wise (53). Briefly, yeast cells were grown in 1 liter of YPD (1% Bacto-yeast extract, 2% Bacto-Peptone, 2% glucose) to an A_{600} of 1.0 to 1.2. The cells were harvested and washed with 40 ml of water containing 200 μl of 2-mercaptoethanol. Subsequently, the cells were resuspended in 40 ml of SB (1.2 M sorbitol, 10 mM EDTA [pH 8.0], 10 mM potassium phosphate [pH 7.5], 0.1% 2-mercaptoethanol). A total of 12 mg of Zymolyase 100T was added, and spheroplasts were formed by incubating the cell suspension at 30°C for 45 min. Subsequent steps were carried out on ice. Spheroplasts were harvested and washed with 30 ml of HMC (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.6], 5 mM MgOAC, 0.5 M sucrose) at 2,500 × *g* at 4°C for 1 min, and the pellet was resuspended in 24 ml of HMS (25 mM HEPES [pH 7.6], 5 mM MgOAC, 0.25 M sucrose) and 0.1% Nonidet P-40 and lysed in a prechilled Dounce homogenizer by six strokes with a loose pestle and six strokes with a tight pestle. The cytoplasmic fraction and nuclear fraction were separated by layering the lysate over 10 ml of an HMC cushion and centrifuging it at 8,000 × *g* at 4°C for 5 min in a swinging bucket rotor. The resulting supernatant was the cytoplasmic fraction, and the pellet was the nuclear fraction. Nuclear RNA was purified from the nuclear fraction by the procedure used for total RNA preparation. For cytoplasmic RNA preparation, 1 ml of the cytoplasmic fraction was first diluted with 2 ml of diethylpyrocarbonate (DEPC)-treated water, and then the solution was extracted once with phenol, once with phenol-chloroform-isoamyl alcohol (25:24:1), and once with chloroform-isoamyl alcohol (24:1) and then precipitated with 10 ml of 95% ethanol at −70°C. The precipitated RNA was collected and suspended in 100 μl of sterile water.

Determination of *CYC1* mRNA content. The relative amounts of *CYC1* mRNA were determined by standard Northern analysis, with the procedures previously described by Li and Sherman (30) that were based on the quantitative comparisons of the *CYC1* and *ACT1* mRNA band densities. Approximately 20 μg of total RNA was usually used for each analysis. The amount of *CYC1* mRNA was determined as a ratio of the densities of the *CYC1* and *ACT1* mRNA bands. An LKB 2222-010 UltroScan XL laser densitometer was used to determine the relative densities, which were presented by the computer as relative area of each band on the autoradiograph film of the Northern blot. The amount of *CYC1* mRNA in each mutant was determined by reference to the standard dilution curve, defining the normal *CYC1* allele in the derepressed growth condition as 100%.

The decay rates of *CYC1* mRNA were determined with yeast cells grown in YPD medium to an A_{600} of 0.75. Thiolutin (3 μg/ml) was added to the culture, 8-ml samples were collected every 3 min, and total RNA was prepared and analyzed as previously described (16).

Fractionation of polyribosomes. Yeast polyribosomes were fractionated by sucrose gradient centrifugation according to the procedure of Baim and Sherman (1) and Warner et al. (52) with minor modifications. Instead of growing the yeast strains in 100 ml of YPD medium and layering a portion of the supernatant liquid corresponding to 30 U of A_{260} over a 16-ml linear sucrose gradient, yeast strains were grown in 300 ml of YPD medium and all of the buffers used for preparing the supernatant were tripled, a portion of supernatant liquid corresponding to 90 U of A_{260} was layered over 36-ml linear sucrose gradient (10 to 50% [wt/vol]), and the sucrose gradients were centrifuged at 20,000 rpm at 4°C for 5 h in an SW27 rotor (Beckman Instruments, Inc.).

Treatments with 50 mM EDTA were carried out when dissociated polyribosomes were required.

Distribution of *CYC1* mRNA in the polyribosomal fractions. The distribution of *CYC1* mRNA along the polyribosomal gradient was determined by isolating RNA from the sucrose gradient fractions (1), subjecting approximately one-half of the RNA from each fraction to gel electrophoresis, and subsequently hybridizing the separated RNA fractions with *CYC1* and *ACT1* probes as described by Li and Sherman (30). The top fractions of the *CYC1* mRNA polyribosomal fractions were defined as the level of radioactivity in the top four fractions compared with the total level of radioactivity. The appropriate regions of the Northern blot membranes were cut, and the corresponding radioactivity levels were determined with a Beckman LS 6000SC liquid scintillation counter. The radioactivity levels were used to infer the efficiencies of translation initiation as discussed in the text.

Disruption of the *UPF1* gene. The yeast chromosomal *UPF1* gene was disrupted by transforming yeast cells with DNA fragments that were prepared by digesting the plasmid pPL51 with *Bam*HI and *Eco*RI. pPL51 is a derivative of YIp5 containing the *upf1*-Δ allele, which was constructed by deleting approximately 60% of the *UPF1* coding sequence (nucleotides −187 to +1726) between two adjacent *Hin*dIII sites and subsequently inserting a 1.1-kb *Hin*dIII fragment carrying the *URA3* gene (29). The transformants were selected on uracil omission plates, and the correct disruption was confirmed by the size of a PCR-amplified fragment.

TABLE 2. Construction of *CYC1* strains

Allele	Strain	Oligonucleotide[a]	Plasmid	Starting allele	Alteration[b]
cyc1-1088	B-8254	OL89-224	pAB870	*cyc1-944*	1 ATG → ATA
cyc1-1249	B-8883	OL92-162	pAB1105	*cyc1-1088*	57 ATG → GTG
		OL92-162			65 ATG → GTG
		OL92-163			116 ATG → ACG
		OL92-164			167 ATG → ACG
		OL92-165			208 ATG → CTG
		OL92-166			256 ATG → ATC
cyc1-1250	B-8884	OL92-182	pAB1106	*cyc1-1249*	1 ATA → ATG
cyc1-1253	B-8887	OL92-207	pAB1109	*cyc1-1250*	7 GAA → TAA
cyc1-1261	B-8895	OL92-196	pAB1117	*cyc1-1250*	31 AAA → TAA
cyc1-1262	B-8896	OL92-197	pAB1118	*cyc1-1250*	61 CTA → TAA
cyc1-1263	B-8897	OL92-198	pAB1119	*cyc1-1250*	91 CCA → TAA
cyc1-1264	B-8898	OL92-199	pAB1120	*cyc1-1250*	121 ATC → TAA
cyc1-1265	B-8899	OL92-200	pAB1121	*cyc1-1250*	151 GGG → TAA
cyc1-1266	B-8900	OL92-201	pAB1122	*cyc1-1250*	181 AAA → TAA
cyc1-1267	B-8901	OL92-202	pAB1123	*cyc1-1250*	211 TCA → TAA
cyc1-1268	B-8902	OL92-203	pAB1124	*cyc1-1250*	241 ATT → TAA
cyc1-1269	B-8903	OL92-204	pAB1125	*cyc1-1250*	271 TTG → TAA
cyc1-1270	B-8904	OL92-206	pAB1126	*cyc1-1250*	301 ATT → TAA
cyc1-1251	B-8885	OL90-244	pAB1107	*cyc1-1249*	−27 AACACAAATACA → ATAATGACTTAA
cyc1-1252	B-8886	OL90-247	pAB1108	*cyc1-1249*	−18 ACACACACTAAA → ATAATGACTTAA
cyc1-1253	B-8887	OL92-207	pAB1109	*cyc1-1249*	−3 ATAATAACTGAA → ATAATGACTTAA
cyc1-1254	B-8888	OL90-248	pAB1110	*cyc1-1249*	7 GAATTCAAGGCC → ATAATGACTTAA
cyc1-1255	B-8889	OL92-178	pAB1111	*cyc1-1249*	34 GGTGCTACACTT → ATAATGACTTAA
cyc1-1298	B-8970	OL93-254	pAB1047	*cyc1-1249*	58 TGTCTACAGTGC → ATAATGACTTAA
cyc1-1256	B-8890	OL90-249	pAB1112	*cyc1-1249*	79 GAAAAGGGTGGC → ATAATGACTTAA
cyc1-1257	B-8891	OL92-177	pAB1113	*cyc1-1249*	154 TATTCGTACACA → ATAATGACTTAA
cyc1-1258	B-8892	OL90-251	pAB1114	*cyc1-1249*	229 CCAAAGAAATAT → ATAATGACTTAA
cyc1-1259	B-8893	OL90-252	pAB1115	*cyc1-1249*	322 TGTGAGTAAACA → ATAATGACTTAA
cyc1-1260	B-8894	OL90-253	pAB1116	*cyc1-1249*	415 GAAAAGGAAGGA → ATAATGACTTAA
cyc1-1292	B-8964	OL93-230	pAB1041	*cyc1-1249*	−27 AACACAAAT → ATAATGACT
cyc1-1291	B-8963	OL93-229	pAB1040	*cyc1-1249*	−18 ACACACACT → ATAATGACT
cyc1-1295	B-8967	OL93-233	pAB1044	*cyc1-1249*	34 GGTGCTACA → ATAATGACT
cyc1-1294	B-8966	OL93-232	pAB1043	*cyc1-1249*	79 GAAAAGGGT → ATAATGACT
cyc1-1293	B-8965	OL93-231	pAB1042	*cyc1-1249*	88 GGCCCACAT → ATAATGACT
cyc1-1296	B-8968	OL93-173	pAB1045	*cyc1-1249*	7 GAA → TAA
cyc1-1297	B-8869	OL93-253	pAB1046	*cyc1-1294*	Replacements (Fig. 11)
cyc1-1299	B-8871	OL93-255	pAB1048	*cyc1-1294*	Deletion 13–72 (Fig. 11)
cyc1-1304	B-9000	OL94-27	pAB1061	*cyc1-1299*	GGC → TAA (Fig. 11)
cyc1-1305	B-9001	OL94-32	pAB1062	*cyc1-1297*	CAA → TAA (Fig. 11)

[a] Oligonucleotide sequences are listed in Table 3.
[b] The position of the first nucleotide of the alteration is indicated, as numbered in Fig. 1.

PCR amplification of the *UPF1* gene fragment. The *UPF1* gene fragment was amplified by using approximately 1 μg of chromosomal DNA in 100 μl of a standard reaction mixture. The reaction mixture also contained 2 μl of 10 mM deoxynucleoside triphosphate mixture, 2 pmol of each primer (OL94-28 and OL94-29), 2.5 U of *Taq* DNA polymerase, and 10 μl of 10× buffer (Boehringer Mannheim GmbH, Mannheim, Germany). The samples were placed in the PCR thermocycler (Perkin-Elmer Cetus Corp.) and cycled 25 times with denaturation at 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 3 min. The sizes of the PCR products were determined by electrophoresis and staining the agarose gels with ethidium bromide.

RESULTS

***CYC1* mRNA levels in mutants deficient in translation of iso-1-cytochrome *c*.** As mentioned above, previous work revealed that the levels of *CYC1* mRNAs were greatly diminished in nonsense mutants, whereas the levels remained approximately normal in mutants having altered initiator codons, even though both types of mutants lack iso-1-cytochrome *c* because of a deficiency in translation. Although the diminished levels of *CYC1* mRNA by premature translational termination are consistent with the findings with other yeast genes (15, 19, 28, 31, 39, 40, 55), the normal level of *CYC1* mRNA in initiator mutants is unexpected, especially because the *CYC1* mRNAs contain internal open reading frames (Fig. 1).

In order to systematically investigate the role of ATG initiator codons and TAA terminating codons in mRNA stability, we have constructed a series of mutants lacking internal ATG triplets or all ATG triplets. The key *cyc1* mutants are listed in Table 4. The *cyc1-944* allele contains only one TATA element, resulting in initiation of transcription within a restricted region (30). This *cyc1-944* allele was chosen because of the diminished number of *CYC1* mRNA species with different 5′ ends. The *cyc1-1088* allele was derived from *cyc1-944* by changing the normal ATG initiator codon to ATA, whereas the *cyc1-1249* allele was derived from *cyc1-944* by altering the normal ATG initiator codon and all six internal in-frame and out-of-frame ATG triplets (Table 2). The *cyc1-1250* allele was derived from *cyc1-1249* by changing the ATA codon back to the normal ATG initiator codon. These *cyc1* alleles subsequently were used to generate three series of mutants. The TAA nonsense series mutants were derived by introducing nonsense TAA codons at different nucleotide positions along the *cyc1-1250* mRNA. Similarly, the ATG-TAA series of mutants were de-

TABLE 3. Oligonucleotides used for site-directed mutagenesis, Northern blot analysis, or PCR[a]

Oligonucleotide	Sequence
OL89-224	−12 ACTAAATTAATAATAACTGAATTCAAG
OL92-162	45 TTTCAAGACTAGGTGTCTTACAGTGCCACACCGTG
OL92-163	105 TCCAAACTTGCACGGTATCTTTGGC
OL92-164	156 TTCGTACACAGACGCCAATATCAAG
OL92-165	196 GACGAAAATAACCTGTCAGAGTACT
OL92-166	246 TGGTACCAAGATCGCCTTTGGTGGG
OL90-244	−37 TAGACACGCAATAATGACTTAACACACTAAAT
OL90-247	−28 AAACACAAATATAATGACTTAATTAATAATAA
OL90-248	−4 AATAATAACTATAATGACTTAAGGTTCTGCTA
OL92-178	24 TGCTAAGAAAATAATGACTTAATTCAAGACTA
OL90-249	69 CCACACCGTGATAATGACTTAACCACATAAGG
OL92-177	144 AGCTGAAGGGATAATGACTTAAGACGCCAATA
OL90-251	219 CTTGACTAACATAATGACTTAAATTCCTGGTA
OL90-252	312 GAAAAAAGCCATAATGACTTAAGGCCCCTTTT
OL90-253	405 CGCTCTAACCATAATGACTTAAGTTAGACAAC
OL92-182	−15 CACACTAAATTAATAATGACTGAATTCAAG
OL92-207	−6 TTAATAATGACTTAATTCAAGGCCGGT
OL92-196	19 GGTTCTGCTAAGTAAGGTGCTACACTT
OL92-197	49 AAGACTAGGTGTTAACAGTGCCACACC
OL92-198	79 GAAAAGGGTGGCTAACATAAGGTTGGT
OL92-199	109 AACTTGCACGGTTAATTTGGCAGACAC
OL92-200	139 GGTCAAGCTGAATAATATTCGTACACA
OL92-201	169 GCCAATATCAAGTAAAACGTGTTGTGG
OL92-202	199 GAAAATAACCTGTAAGAGTACTTGACT
OL92-203	229 CCAAAGAAATATTAACCTGGTACCAAG
OL92-204	259 GCCTTTGGTGGGTAAAAGAAGGAAAAA
OL92-206	289 AGAAACGACTTATAAACCTACTTGAAA
OL88-68	426 TCTTGGTCTACCGACGATAGATGGGAAGACAGCA 393
OL93-208	157 AATACCCTTCAGCTTGACCAGAGTGTCTGCCAAA 124
OL93-209	350 ACAAAGGAAAAGGGGCCTGTTTACTCACAGGCTT 317
OL93-210	396 GGGAGGGCGTGAATGTAAGCGTGACATAACTAAT 363
OL93-173	−6 TTAATAATAACTTAATTCAAGGCCG
OL93-229	−28 AAACACAAATATAATGACTAAATTAATAATAA
OL93-230	−37 TAGACACGCAATAATGACTACACACACTAAAT
OL93-231	75 CGTGGAAAAGGGTATAATGACTAAGGTTGGTC
OL93-232	69 CCACACCGTGATAATGACTGGCCCACATAAGG
OL93-233	24 TGCTAAGAAAATAATGACTCTTTTCAAGACTA
OL93-253	49 AAGCTAGGTGTCTAAAACACTGCGTGACCATAATGACTCCAGGCGTTCATAAGGGTCCAAACTTGCAC
OL93-254	46 TTCAAGACTAGGATAATGACTTAACACACCGTGGAA
OL93-255	−6 TTAATAATAACTGAATTCACCGTGATAATGACTGGC
OL94-28	−258 CCCTTTGCTTACTTGATTTGGGAGGGACAC −229
OL94-29	1781 ATAACTTGTTTCGCACCTTTAACGATTGGG 1752
OL94-27	73 ACCGTGATAATGACTTAACCACATAAGGTTGGT
OL94-32	GTGACCATAATGACTTAAGGCGTTCATAAGGGT (see Fig. 11)

[a] The position of the first nucleotide is indicated either at the front or the end of the sequence, depending on whether the oligonucleotide corresponds to the upper or lower strand, respectively.

rived by generating the sequence ATA ATG ACT TAA at various sites in the *cyc1-1249* mRNA. The ATG series of mutants were derived by generating the sequence ATA ATG ACT at various sites in the *cyc1-1249* mRNA.

The *CYC1* mRNA levels in key *cyc1* mutants, presented in Table 5, were qualitatively equivalent to *cyc1* mutants containing internal ATG triplets. As expected, the *CYC1* mRNA level was greatly diminished in the *cyc1-1253* mutant, which contains the normal ATG initiator codon and a TAA terminating codon at nucleotide position 7. In contrast, the *CYC1* mRNA level was normal in the *cyc1-1249* mutant lacking all ATG triplets, even though nonsense triplets are present at various sites in the mRNA (Fig. 1), including at position 7 in the *cyc1-1296* mutant (Table 5).

The position of the nonsense codon influences the *CYC1* mRNA levels. The almost complete absence of *CYC1* mRNA in the *cyc1-1253* mutant containing a TAA terminating codon at position 7 and the normal level of *CYC1* mRNA in the *cyc1-1250* mutant containing the normal terminating codon at position 328 (Table 5) indicated that the position or context of the TAA codon markedly influenced the mRNA level, as previously observed in other yeast genes (31, 40). The results with the 11 mutants of the TAA nonsense series, shown in Fig. 2 and 3, indicated that the levels of *CYC1* mRNA approached and equaled the normal level when the nonsense codon was closer to the normal TAA codon at nucleotide position 328.

TABLE 4. Key *cyc1* mutants

Allele	Position 1 codon	Description
CYC1$^+$	ATG	Wild-type, two TATA elements
cyc1-944	ATG	One TATA element
cyc1-1088	ATA	One TATA element
cyc1-1249	ATA	One TATA element, no ATG triplets
cyc1-1250	ATG	One TATA element, no internal ATG triplets

TABLE 5. *CYC1* mRNA levels in certain key mutants

Allele	Sequence[a]					No. of ATG triplets	% of normal mRNA
			1	2	3		
		(Met)	Thr	- Glu	- Phe-		
cyc1-944	ATA	ATG	ACT	GAA	TTC	7	100
cyc1-1088	ATA	ATA	ACT	GAA	TTC	6	100
cyc1-1250	ATA	ATG	ACT	GAA	TTC	1	100
cyc1-1253	ATA	ATG	ACT	TAA	TTC	1	5
cyc1-1249	ATA	ATA	ACT	GAA	TTC	0	100
cyc1-1296	ATA	ATA	ACT	TAA	TTC	0	100

[a] The cleaved methionine residue is given in parentheses, and altered codons are underlined.

***CYC1* mRNAs with nonsense mutations rapidly decay but are stabilized by *upf1*-Δ mutations.** We have directly demonstrated that the low abundance of *CYC1* mRNA containing premature TAA codons was due to enhanced degradation. The half-lives in the representative *cyc1-1261* and *cyc1-1265* mutants were determined by measuring the loss of *CYC1* mRNA after inhibiting transcription with thiolutin. The results, presented in Fig. 4 and Table 6, revealed that the steady-state levels and half-lives of the *CYC1* mRNAs were directly related.

Mutations of the *UPF1* gene originally were isolated on the basis of their ability to act as allosuppressors of the *his4-38* frameshift mutation (10). Subsequent studies revealed that Upf1 is required for the degradation of mRNA containing premature nonsense codons (28, 40) and that *upf1* mutations act as omnipotent suppressors (29).

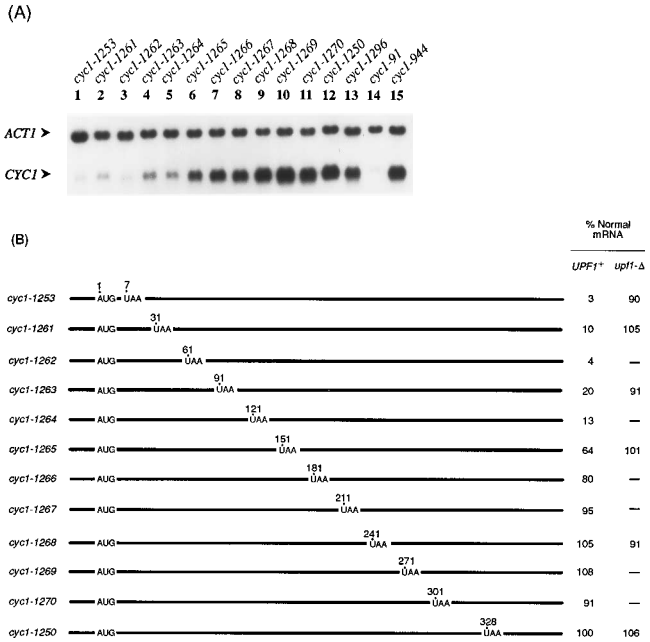

FIG. 2. (A) Northern blot analysis of *CYC1* mRNA in the control strain *cyc1-1296*, *cyc1-91*, and *cyc1-944* (Tables 4 and 5) and in the TAA series of mutants (see below). A total of 20 μg of RNA was denatured, electrophoresed in agarose, transferred to a nitrocellulose membrane, and cohybridized to the ^{32}P-labeled probes OL93-210 for the *CYC1* mRNA and OL88-68 for the *ACT1* mRNA. (B) Levels of the *CYC1* mRNA in the TAA series of mutants containing *UPF1*$^+$ and *upf1*-Δ alleles. The numbers above the codons denote the nucleotide positions of the A of the AUG initiator codons and the nucleotide positions of the U of the most proximal 5′ in-frame UAA codons.

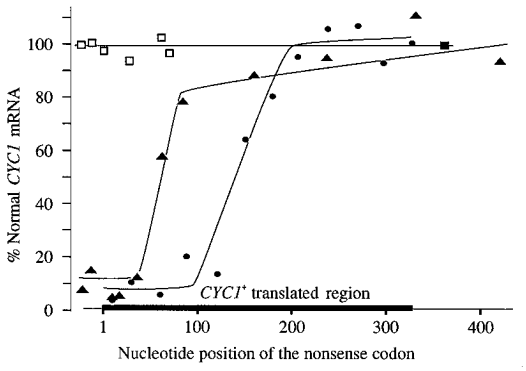

FIG. 3. Relative amounts of *CYC1* mRNA in the normal (■), TAA series (●), ATG-TAA series (▲), and ATG series (❒) of strains and the nucleotide position of the TAA codon. The translated region (nucleotide positions 1 to 330) of the normal *CYC1*$^+$ mRNA is indicated by the solid bar at the bottom of the figure.

We have also demonstrated that *upf1* mutations prevent the degradation of mRNA containing premature nonsense codons. The *UPF1* gene was disrupted in representatives of the *cyc1* mutants, and PCR amplification was used to identify the correct *upf1*-Δ disruptions. The PCR product of the wild-type *UPF1* gene was a 2-kb fragment, whereas the PCR product was a 1.2-kb fragment for the gene containing the correct *upf1*-Δ disruption (data not shown). Northern analysis (Fig. 5) indicated that the low amounts of *CYC1* mRNA in representative *cyc1 UPF1*$^+$ strains were restored to the normal level in the corresponding *cyc1 upf1*-Δ strains (Fig. 2B).

Levels of *CYC1* mRNA in the ATG-TAA mutants. The normal level of *CYC1* mRNA in, for example, the *cyc1-1088* mutant, which lacks the normal ATG initiator codon (Table 5) but which contains internal open reading frames (Fig. 1), and the almost complete deficiency in the *cyc1-1253* mutant (Table 5) indicate that short open reading frames cause diminutions of *CYC1* mRNA only when they are in certain positions in the mRNA. We have systematically investigated these positions with an ATG-TAA series of mutants, *cyc1-1251* through *cyc1-1260* (Table 2), containing the ATA ATG ACT TAA sequence at various sites along the *CYC1* mRNA, including the 5′ and 3′ untranslated regions. This ATA ATG ACT TAA sequence is

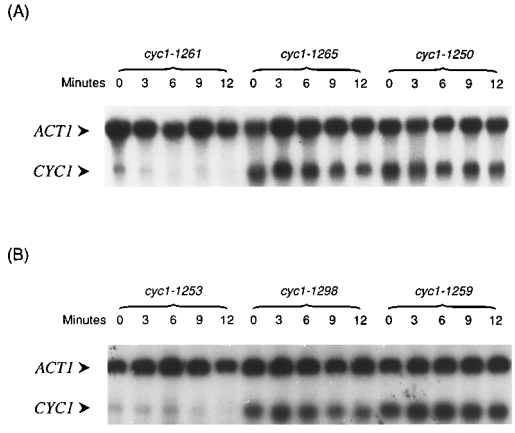

FIG. 4. Northern blot analysis of *CYC1* mRNA in representatives of the TAA (A) and ATG-TAA (B) series of mutants after various times of inhibition of transcription with 3 μg of thiolutin per ml. The probes were as described in the legend to Fig. 2. Quantitative estimates of the half-lives are presented in Table 6.

TABLE 6. *CYC1* mRNA levels and half-lives

Allele	% of normal mRNA	Half-life (min)[a]
cyc1-1261	10	1.5
cyc1-1265	64	8.5
cyc1-1250	100	12
cyc1-1253	5	1.5
cyc1-1298	58	7
cyc1-1259	110	~13

[a] The time before the start of mRNA decay was used as the first time point for calculating the half-lives.

found in the *cyc1-91* (see the introduction) and *cyc1-1253* (Table 5) nonsense mutants, which are almost completely deficient in *CYC1* mRNA. Because the *cyc1-1249* mutant lacked all in-frame and out-of-frame ATG triplets, each of the various ATG-TAA mutants, which were derived from *cyc1-1249*, only contained the single ATG triplet of the ATA ATG ACT TAA sequence. Furthermore, the ATA triplet in front of the potential ATG initiator codon provides a maximal context for efficient translation in yeast cells (2, 9).

The results of quantitative Northern analysis (Fig. 3 and 6) indicated that there were various levels of *CYC1* mRNA and that the deficiency depended on the proximity to the position of the normal ATG initiator codon. As mentioned above, the *cyc1-1253* mutant of the ATG-TAA series, which is equivalent to the first mutant of the TAA series, almost completely lacked *CYC1* mRNAs, as did all of the other *cyc1-1251*-through-*cyc1-1255* mutants. However, unexpectedly, the ATA ATG ACT TAA sequence did not cause pronounced diminutions of *CYC1* mRNA after approximately nucleotide position 40, a result which is in contrast to the results with the nonsense series, in which the TAA sequence did not cause pronounced diminutions after approximately nucleotide position 130 (Fig. 2 and 3). As discussed below, we believe this difference is due to the lack of initiation of translation after approximately nucleotide position 40.

CYC1 mRNA measurements of the representative mutants *cyc1-1253*, *cyc1-1298*, and *cyc1-1259* revealed a correspondence between the steady-state levels and the half-lives (Table 6 and Fig. 4). In addition, *upf1*-Δ disruption of the *cyc1-1251*, *cyc1-1253*, *cyc1-1255*, and *cyc1-1256* mutants (Fig. 5) restored the *CYC1* mRNA levels. These results demonstrated that the diminished levels of *CYC1* mRNA in the ATG-TAA mutants are the result of Upf1-dependent degradation, similar to the results with the TAA series of mutants.

Distribution of *CYC1* mRNA along polyribosomal gradients. We have investigated the translatability of *CYC1* mRNA from its distribution along the polyribosomal gradient by Northern analysis of RNA purified from the sucrose gradient fractions (Fig. 7). The control *ACT1* mRNAs from the *cyc1-944*, *cyc1-1250*, *cyc1-1088*, and *cyc1-1249* strains were distributed in the same polyribosomal position. The bulk of the *CYC1* mRNA in the *cyc1-944* mutant was associated with two to six ribosomes (data not presented). A similar *CYC1* mRNA distribution was also observed for the *cyc1-1250* mutant (Fig. 7 and Table 5) and with the normal *CYC1*$^+$ strain (1), even when the iso-1-cytochrome *c* was rapidly degraded because of the lack of heme attachment (13). However, a distribution indicating one to four ribosomes previously was observed when the rate of translation was diminished because of a hairpin structure in the mRNA (1). Thus, the distribution of a specific mRNA along the polyribosomal gradient reflects not only the length of the mRNA but also the rates of initiation, elongation, and termination of translation of the corresponding protein product.

Surprisingly, the *CYC1* mRNA from the *cyc1-1088* initiator mutant (Table 5) gave an unusual distribution covering essentially the entire gradient, as shown in Fig. 7. A similar distribution was also observed with the *cyc1-1249* initiator mutant (data not presented). At least portions of the *CYC1* mRNA in the polyribosomal fraction from *cyc1-1249* initiator mutants were presumed to be associated with 40S ribosomal subunits because of the lack of an ATG triplet on the mRNA. These results suggested that ATG triplets at positions 57 and 66 were not used for translation initiation on the *CYC1* mRNA from the *cyc1-1088* mutant, because there is a TAA triplet at position 96 for these two ATG triplets in reading frame 3 (Fig. 1). A heavy polyribosomal complex would not be expected to form along the *CYC1* mRNA from the *cyc1-1088* mutant with this translated short open reading frame. This result suggested that ATG triplets located at least at some positions (e.g., nucleotide positions 57 and 66) were not efficient for translation initiation. It was reasonable to speculate that 40S ribosomal subunits and

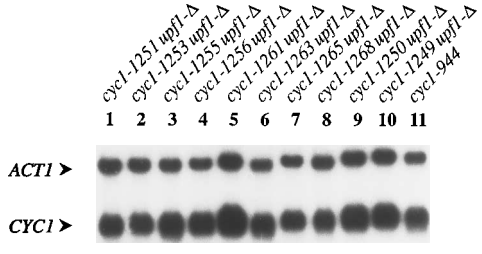

FIG. 5. Northern blot analysis of *CYC1* mRNA in representatives of the *cyc1 upf1*-Δ mutants from the TAA and ATG-TAA series. The probes were OL93-210 for the *CYC1* mRNA and OL88-68 for the *ACT1* mRNA (see the legend to Fig. 2).

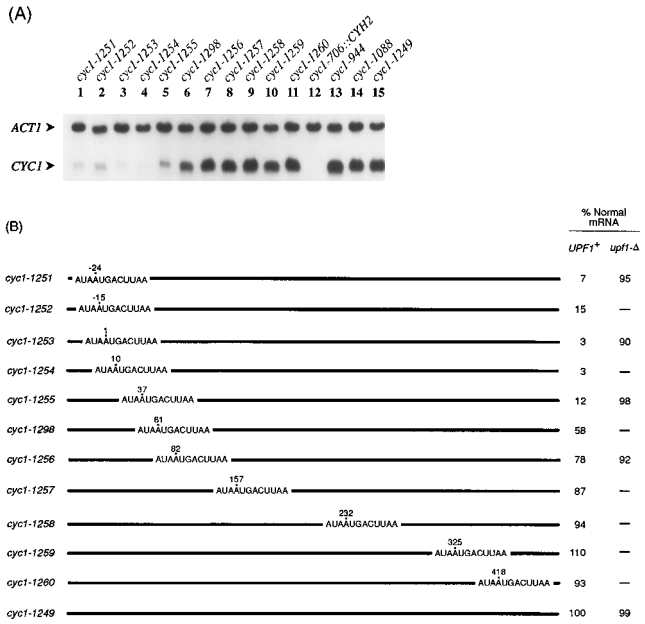

FIG. 6. (A) Northern blot analysis of *CYC1* mRNA in the control strain *cyc1-706*::*CYH2*, *cyc1-944*, *cyc1-1088*, and *cyc1-1249* (Tables 4 and 5) and in the ATG-TAA series of mutants. The probes were as described in the legend to Fig. 2. (B) Levels of the *CYC1* mRNA in the ATG-TAA series of mutants containing *UPF1*$^+$ and *upf1*-Δ alleles. The numbers above the codons denote the nucleotide positions of the A of the AUG initiator codons.

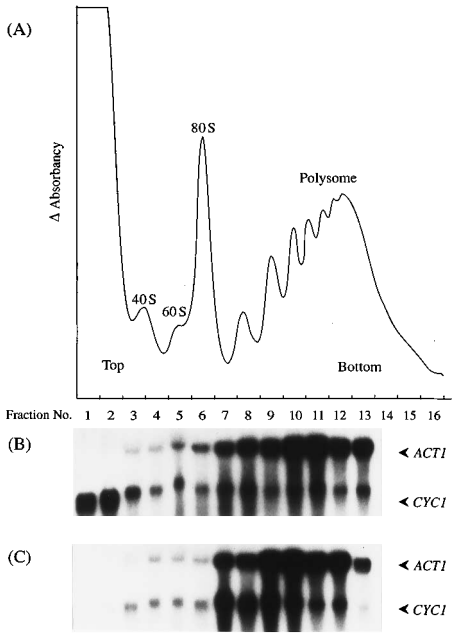

FIG. 7. Absorbancy profile of the fractionated polyribosomes and the distribution of *CYC1* mRNA in the initiator mutants *cyc1-1088* and *cyc1-1249*, as well as that from the control mutants *cyc1-944* and *cyc1-1250*. (A) A_{260} profile of polyribosomes sedimented in 10 to 50% sucrose is shown for *cyc1-1088*, which was similar to the profiles of other *cyc1* mutants. The sucrose gradient was centrifuged at 20,000 rpm for 5 h in an SW27 rotor. The positions of the fractions from which RNA was examined were aligned with the lanes of the gels and are indicated on the abscissa. (B) Approximately one-half of each fraction of total RNA from the *cyc1-1088* strain (Table 5) was separated and analyzed as described in the legend to Fig. 2. A similar result was obtained with RNA from the *cyc1-1249* strain (Table 5). (C) A similar analysis performed with *cyc1-1250* indicated that the bulk of the mRNA was associated with two to six ribosomes.

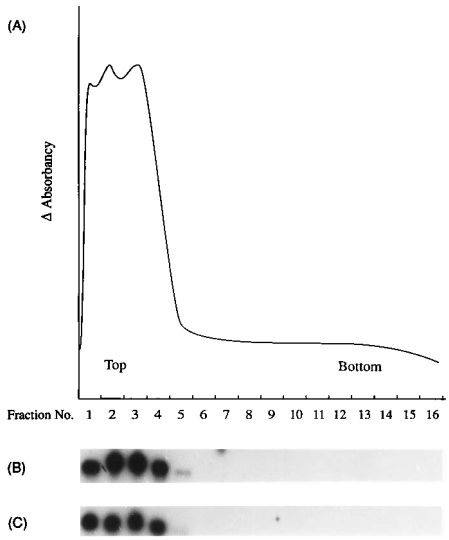

FIG. 8. Absorbancy profile of the fractionated polyribosomes after dissociation with 50 mM EDTA. (A) A_{260} profile of polyribosomes as described in the legend to Fig. 7. Approximately one-half of each fraction of total RNA from the *cyc1-1249* (B) and *cyc1-1250* (C) strains (Table 5) was separated and analyzed as described in the legend to Fig. 2.

not 80S ribosomes may be responsible for the peculiar distribution of *CYC1* mRNA from initiator mutants *cyc1-1249* and *cyc1-1088*. According to the scanning hypothesis, 40S ribosomal subunits are believed to bind initially at the 5′ end of mRNA and then migrate until the first favorable AUG codon is encountered; translation initiates at this site by the formation of an 80S ribosomal complex (22–25). If the initiator mutant lacks the normal AUG codon, one would anticipate an extended region of scanning by 40S ribosomal subunits. As elaborated in the Discussion, the normal level of *CYC1* mRNA in *cyc1-1088* and *cyc1-1249* initiator mutants could be explained by protection with 40S subunits.

We have confirmed that the *CYC1* mRNAs are truly associated with ribosomes by examining their distribution after dissociation with EDTA, as shown in Fig. 8.

Distribution of *CYC1* mRNA from ATG series mutants along polyribosomal gradients. We have provided an independent means for testing the hypothesis that translation cannot initiate if the ATG triplet is located after approximately nucleotide position 40, a conclusion that was originally reached by comparing the *CYC1* mRNA stabilities in the TAA and ATG-TAA series of mutants (Fig. 3). As shown above, the proportion of *CYC1* mRNA in the top fractions can be used as a measure of the degree of translation. For example, 5 and 37% of the normally translated *cyc1-1250* mRNA and the completely untranslated *cyc1-1249* mRNA, respectively, are found in the top five fractions (Fig. 9B). For this analysis, an ATG series of mutants were constructed by generating the sequence ATA ATG ACT at various sites in a *CYC1* mRNA that lacked all other ATG triplets. As expected, the results from Northern analysis indicated that *CYC1* mRNAs from all of the ATG series of mutants were stable, regardless of the positions of ATG triplets (Fig. 9). Apparently, the *CYC1* mRNAs in the ATG series of mutants were protected by 80S ribosomes or 40S ribosomal subunits if *CYC1* mRNA was efficiently translated or poorly translated, respectively, resulting in stability of *CYC1* mRNA in all mutants. The distributions of *CYC1* mRNA along the polyribosomal gradient were determined by Northern analysis of RNA purified from the sucrose gradient fractions (Fig. 10). Control *ACT1* mRNA molecules from all of the ATG series of mutants were distributed in the same polyribosomal position (data not presented). In contrast, *CYC1* mRNA molecules from these ATG series mutants were distributed differently (Fig. 10). The *cyc1-1291* and *cyc1-1295* mutants, which have ATA ATG ACT sequences at nucleotide positions −15 and 37, respectively, had the lowest proportion of *CYC1* mRNA in the top fractions, 6 and 14%, respectively, as shown in Fig. 10. In contrast, the *cyc1-1293* and *cyc1-1294* mutants, which have an ATA ATG ACT sequence at nucleotide positions 91 and 82, respectively, had high proportions of *CYC1* mRNA in the top fractions, 31 and 39%, respectively, similar to the value of 37% found in the *cyc1-1249* mutant, which lacked all in-frame and out-of-frame ATG triplets. These results indicated that translation initiation occurred efficiently if ATG was located inside the restricted initiation region, which was originally defined by *CYC1* mRNA stability in the ATG-TAA series of mutants.

The initiation region may simply reflect an open structural requirement for initiation of translation of the *CYC1* mRNA. We have made *cyc1* alterations (Fig. 11) that could reveal whether the initiation region was dependent on any putative short-range secondary structures or on the distance between the AUG initiation codon and the 5′ cap site. The *cyc1-1297* mutant contains multiple nucleotide replacements around the position 82 AUG site, the *cyc1-1305* mutant contains these alterations plus a premature TAA termination codon but retains the same codon usage, and the *cyc1-1299* and *cyc1-1304* mutants were derived from the *cyc1-1294* and *cyc1-1256* mu-

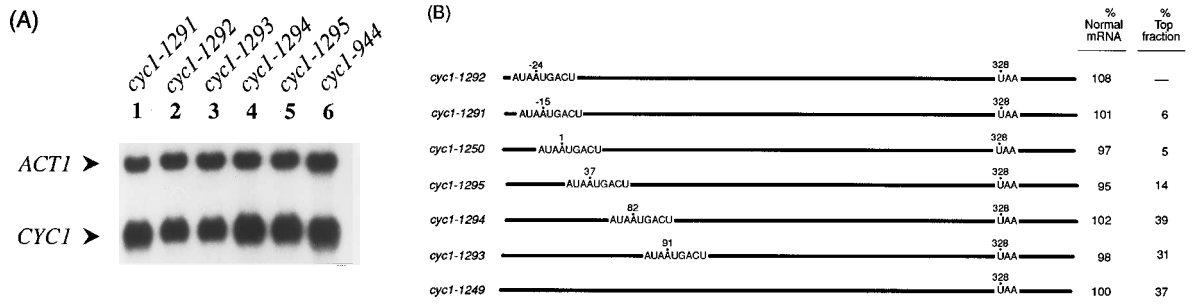

FIG. 9. (A) Northern blot analysis of *CYC1* mRNA in the control strain *cyc1-944* (Tables 4 and 5) and in the ATG series of mutants. The probes were as described in the legend to Fig. 2. (B) Levels of the *CYC1* mRNA in the ATG series of mutants. The numbers above the codons denote the nucleotide positions of the A of the AUG initiator codons.

tants, respectively, by deleting 60 nucleotides (Fig. 11 and 12). Although *CYC1* mRNAs in both the *cyc1-1297* and *cyc1-1299* strains were stable, they had different proportions of *CYC1* mRNAs in the top fractions, 29 and 18%, respectively (Fig. 10). Thus, both *cyc1-1297* and *cyc1-1299* appeared to be partially translated, in contrast to the related *cyc1-1294* mRNA, which did not appear to be translated. Similarly, the *cyc1-1304* deletion mutant was less stable than the *cyc1-1256* control strain, but not to the degree that would be expected if the stability was based simply on the distance between the cap site and the AUG initiator codon (Fig. 12). These results suggested that the translation initiation region was not entirely dependent on either putative short-range secondary structures in the vicinity of the AUG initiator codon or the distance between the AUG initiator codon and the 5′ cap site. We suggest that the translation initiation region may simply reflect an open structure required for the initiation process.

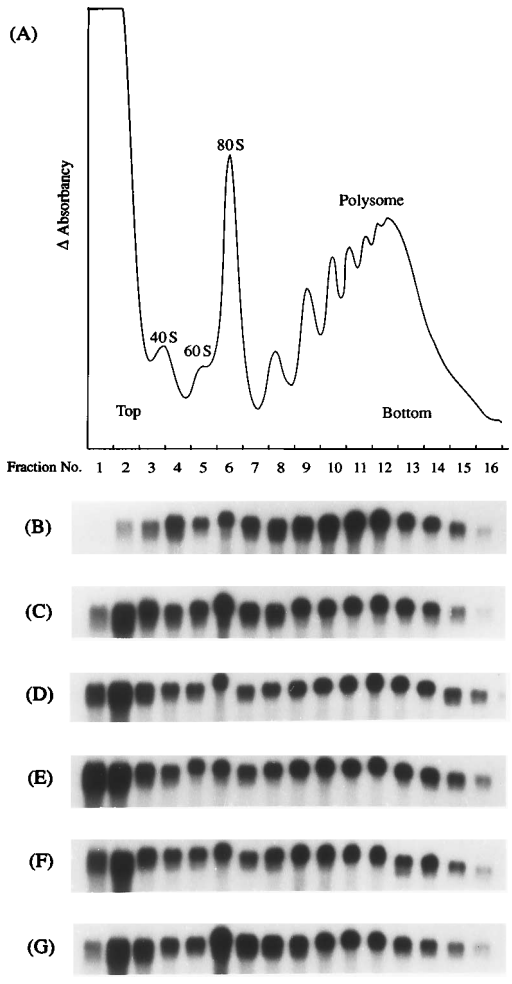

FIG. 10. (A) Absorbancy profile of the fractionated polyribosomes and the distribution of *CYC1* mRNA in the *cyc1* mutants *cyc1-1291* (B), *cyc1-1295* (C), *cyc1-1293* (D), *cyc1-1294* (E), *cyc1-1297* (F), and *cyc1-1299* (G). The experiment was performed in the same way as that described in the legend to Fig. 2, except that only the OL93-210 probe for the *CYC1* mRNA was used for the Northern blot analysis.

The initiation region is not dependent on the reading frame. Other *cyc1* mutants having AUG initiator triplets in different frames revealed that the translation initiation efficiency within the initiation region was not dependent on the translational reading frame (results not presented).

Normal distribution of *CYC1* mRNA between nuclei and cytoplasm in initiator mutants. In order to investigate whether untranslatable *CYC1* mRNAs are retarded in nuclei and are therefore unavailable for cytosolic degradation, *cyc1-944*, *cyc1-1088*, *cyc1-1250*, and *cyc1-1249* cells were fractionated into nuclear and cytoplasmic fractions (53) and the *CYC1* mRNA levels in both fractions were determined by Northern analysis (Fig. 13). The results presented in Table 7 showed that *CYC1* mRNAs in ATG initiator mutants *cyc1-1249* and *cyc1-1088* were stable and were distributed in the nuclei and cytoplasm in the same way as in the *cyc1-944* and *cyc1-1250* strains. These data suggested that translation of *CYC1* mRNA is not required for the stability or nuclear export of *CYC1* mRNA.

DISCUSSION

Degradation of *CYC1* mRNAs with premature nonsense codons. Early work with *Escherichia coli* clearly established a relationship between the premature termination of translation and the destabilization of mRNA (18, 32, 34, 37). These results suggested that prokaryotic ribosomes passively protect mRNA molecules against endonucleotic attack (4). Similarly, premature termination codons in a number of eukaryotic genes apparently caused increased rates of mRNA decay, including the human β-globin (3, 14, 33) and triosephosphate isomerase (11) genes and the yeast *URA1* (39), *URA3* (31), *PGK1* (19), *CYC1* (55), *HIS4* (15, 28), and *LEU2* (28) genes. Lacroute and coworkers (31) demonstrated directly that nonsense mutations increased the rate of mRNA degradation without affecting the rates of transcription synthesis. On the other hand, translational termination codons of certain other genes have the opposite effect by diminishing degradation (38). In these in-

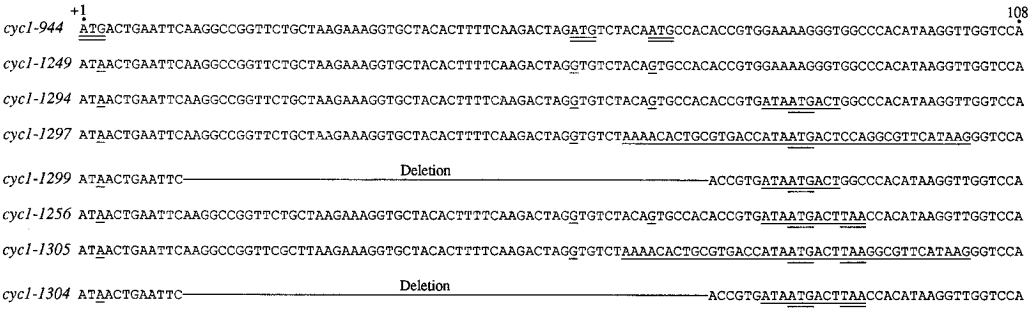

FIG. 11. Sequences of certain *cyc1* mutants showing alterations between nucleotides 3 and 102. ATG and TAA codons are double underlined. Alterations are single underlined.

stances, mRNA decay is dependent on translation of a short specific sequence that can be prevented by termination codons. Also, terminating mutations in higher eukaryotes may lead to mRNA deficiencies for other reasons, such as defective RNA processing (5, 20, 50, 51).

Although various mechanisms may exist, premature termination of translation of most yeast mRNAs, including *CYC1* mRNAs, causes degradation that requires Upf1. The loss of Upf1 function restores wild-type decay rates to mRNAs that would otherwise have been susceptible to the increased decay rates promoted by premature termination codons (28, 40, 41).

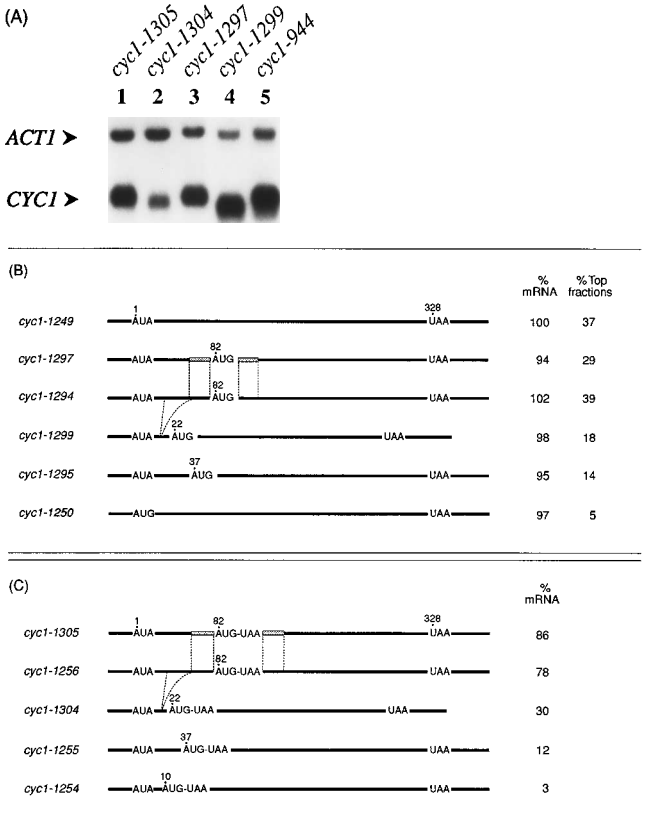

FIG. 12. (A) Northern blot analysis of *CYC1* mRNA in the strains listed in Fig. 11, including those having replacements and deletions in the vicinity of the AUG codon in *cyc1* mutants. The probes were as described in the legend to Fig. 2. (B) Levels of the *CYC1* mRNA and the percentage of the top fraction in the altered ATG mutants. (C) Levels of the *CYC1* mRNA in the altered ATG-TAA mutants. The altered regions are indicated by stippled bars.

Although required for nonsense-dependent degradation, so far *UPF1* has not been shown to encode an endonuclease, either directly or in conjunction with other genes.

The positions of the terminating codons determine the rates of mRNA decay, and all of the yeast genes, *URA3* (31), *URA1* (39), *PGK1* (40), *HIS4* (15), and *CYC1* (Fig. 3), that have been systematically investigated showed almost complete deficiencies of mRNA when the nonsense mutations were in the 5′ regions and normal or nearly normal levels when the nonsense mutations were in the 3′ regions. This steplike response suggests that premature terminations before sensitive elements are responsible for mRNA degradation. In this regard, a series of deletions in the *PGK1* translated region revealed a 106-nucleotide segment, 3′ to nonsense codons, that was necessary for the Upf1-mediated mRNA decay (40). Insertion of a 106-nucleotide segment downstream of a nonsense codon produced rapid decay of the mini-*PGK1* mRNA that was otherwise stable. Moreover, deletion of just this 106-nucleotide segment did not stabilize a *PGK1* mRNA containing a nonsense codon in the 5′ region. Thus, there appear to be more than one element that can promote nonsense codon-mediated decay of *PGK1* mRNA (40).

Furthermore, regions encompassing this 106-nucleotide segment, as well as encompassing other translated regions, did not enhance degradation when inserted 3′ to a nonsense codon situated in the 3′ region, past the sensitive region (40). These results indicated that the *PGK1* mRNAs with 3′ proximal nonsense codons are resistant to decay for reasons other than the lack of the 106-nucleotide segment or other specific elements. Analysis of other constructs with still other inserts suggested that the lack of enhanced decay of *PGK1* mRNAs with 3′

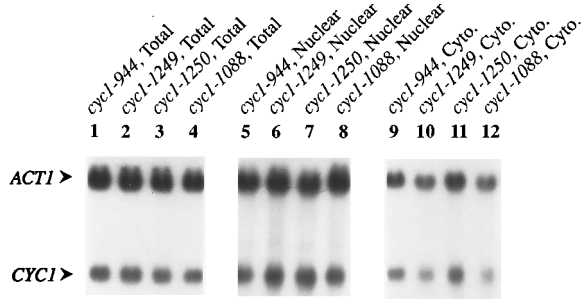

FIG. 13. Northern blot analysis of *CYC1* mRNA from total, nuclear, and cytoplasmic (Cyto.) fractions. Twenty micrograms of RNA was denatured, electrophoresed in agarose, transferred to a nitrocellulose membrane, and cohybridized to the ^{32}P-labeled probes OL93-208, OL93-209, and OL93-210 for the *CYC1* mRNA and OL88-68 for the *ACT1* mRNA. Estimates of the relative amounts of the *CYC1* mRNAs are presented in Table 7.

TABLE 7. Relative amounts of *CYC1* mRNA in nuclear and cytosolic fractions[a]

Allele	Relative amt (%) of *CYC1* mRNA	
	Cytosolic	Nuclear
cyc1-944	100	100
cyc1-1249	103	94
cyc1-1250	105	97
cyc1-1088	98	99

[a] The total relative amount of *CYC1* mRNA was 100% for each mutant.

proximal nonsense codons was not due simply to the increased distance of ribosomal travel but rather to the inactivation of the nonsense codon-mediated decay pathway when a specific region was translated. Thus, the extensive studies with altered *PGK1* mRNAs suggested that at least two types of elements were critical for nonsense codon-mediated decay: (i) the 106-nucleotide segment and other redundant elements that enhance rapid decay when not translated (sensitive elements) and (ii) elements normally in the last half of the *PGK1* mRNA that prevent decay when translated (stabilizing elements) but that are not in themselves sensitive elements (40).

Peltz et al. (40, 41) suggested that the role of 106-nucleotide segment in nonsense codon-mediated decay of *PGK1* mRNA was to promote translational reinitiation or pausing, a conclusion based on the following observations. (i) The 106-nucleotide segment encompasses three AUG codons, and deletion of two of these codons resulted in the loss of its ability to cause degradation of nonsense codon-containing *PGK1* mRNAs. (ii) A hairpin structure that inhibited initiation and reinitiation of translation also stabilized an otherwise unstable *PGK1* mRNA when inserted downstream of a nonsense codon. (iii) A nine-nucleotide sequence surrounding the two pertinent AUG codons was shown to be complementary to a segment of yeast 18S rRNA. Our results clearly do not support the notion that the *CYC1* mRNA contains such an element with these properties. The *cyc1* mRNAs used in the studies lacked internal AUG codons (Fig. 3), and, furthermore, similar degradation responses were obtained with *cyc1* mRNAs containing the normal AUG codons (54). Also, *CYC1* mRNAs do not contain any segments that are complementary to 18S rRNA (data not presented). Furthermore, as discussed below, we believe that initiation of translation cannot efficiently occur in the region past approximately nucleotide position 37. It remains to be seen whether *PGK1* and *CYC1* mRNAs are degraded by two different mechanisms, even if they are both Upf1-dependent, or whether translational reinitiation or pausing plays no role in nonsense-mediated decay of either mRNA.

Muhlrad and Parker (35) and Hagen et al. (15) have presented evidence that Upf1-dependent (or nonsense-mediated) mRNA decay proceeds by decapping and subsequent degradation by the action of the Xrn1 5′→3′ exoribonuclease. Furthermore, studies of initiation of translation (21, 36) and the presence of circular polyribosomes in electron micrographs (6) have suggested that the 5′ cap (or 5′ untranslated region) and the poly(A) tail are associated. This interaction between the 5′ cap and the poly(A) tail may in fact prevent decapping (35). One simple mechanism for nonsense-mediated mRNA decay is that nonsense codons in the 5′ region cause release of the 80S ribosomes and expose a sensitive element situated at the 3′ end of the sensitive region, and this sensitive element disrupts the association between the 5′ cap and the poly(A) tail, leading to decapping and degradation by the Xrn1 5′→3′ exoribonuclease. This sensitive element could be any of a number of sequences that interact with regions near either the 5′ cap of the poly(A) tail when not covered by 80S ribosomes. As discussed below, 40S ribosomal subunits and ribonucleoproteins (RNPs), as well as 80S ribosomes, may serve to prevent interactions with these hypothetical sensitive elements.

Furthermore, the sensitive elements may be redundant, and other elements outside of the sensitive region may also influence degradation, as indicated by extensive study of *PGK1* (40) and *HIS4* (15) mRNAs.

***CYC1* mRNAs are protected by 40S ribosomal subunits and RNPs.** The prevention of translation, and therefore the absence of 80S ribosomes, does not necessarily result in decay of mRNAs. The *cyc1-1249* mutant, lacking ATG triplets (Table 5), contains approximately the normal level and approximately the normal size of *CYC1* mRNA (Fig. 6). This *cyc1-1249* mRNA and related mRNAs are abnormally distributed along the polyribosomal gradient (Fig. 7) and are assumed to be associated primarily with 40S ribosomal subunits, as predicated by the scanning hypothesis (24), and with possible RNPs.

Similarly, mRNAs that contained hairpin structures preventing scanning of 40S ribosomal subunits, initiation of translation, or elongation of 80S ribosomes, may be protected from degradation because of their association with 40S ribosomal subunits and the retention of RNPs. Laso et al. (27) examined the translatability and steady-state levels of a series of yeast mRNAs differing only by the insertion of a stable hairpin structure in the 5′ untranslated region. Consistent with earlier experiments by Cigan et al. (8), these workers found that translation could be inhibited up to 96% by these structures without changing the levels and therefore, by inference, the stability of the mRNA. The untranslated mRNAs containing these 5′ hairpin structures accumulated in 43S preinitiation complexes (42). Furthermore, as discussed above, a hairpin structure that inhibited initiation and reinitiation of translation also stabilized an otherwise unstable *PGK1* mRNA when inserted downstream of a nonsense codon (41).

The simplest interpretation is that wild-type mRNAs and mRNAs lacking effective ATG initiator codons or containing secondary structures are transcribed and exported as large RNP particles (12). The mRNAs form 43S preinitiation complexes, and the RNPs are probably sequentially replaced by 40S subunits during the scanning process and by 80S ribosomes during the elongation process. If the *CYC1* mRNAs lack an effective ATG initiator codon, the resulting mRNAs may be covered and protected by 40S ribosomal subunits or RNPs. The abnormal distribution in a polyribosomal gradient is difficult to reconcile with the apparently normal size and level. However, the properties of mRNA associated with 40S subunits may differ from the more homogeneous distributions observed with mRNA associated with 80S ribosomes.

Thus, we propose that the lack of degradation of these mRNAs lacking ATG triplets is due to their association with RNPs and 40S ribosomal subunits. Likewise, mRNAs containing inhibitory secondary structures may be blocked in the translational process but still retain 43S preinitiation complexes, 40S ribosomal subunits, 80S ribosomes, or RNPs, depending on the site of the hairpin structure. On the other hand, termination of translation and release of the 80S ribosomes also may free the RNPs distal to the site of the nonsense codon. If a critical sensitive element is appropriately situated 3′ to the nonsense codon, the mRNA will be degraded by the Upf1 system.

Although 40S ribosomal subunits presumably are not terminated or released solely by chain-terminating codons, the scanning could be terminated in a controlled fashion by the formation and subsequent termination of 80S ribosomes, which

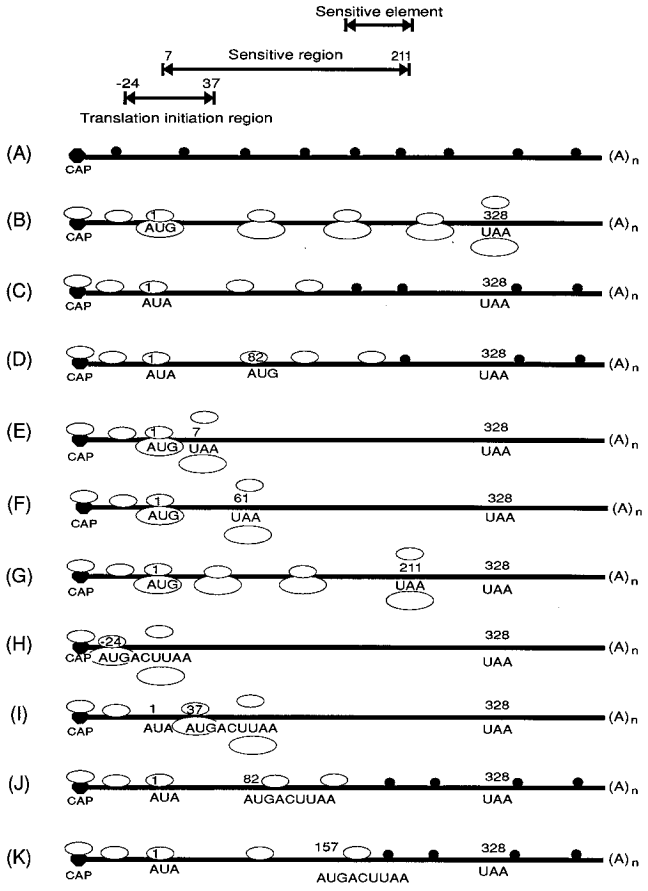

FIG. 14. Schematic representation of the translation initiation model, depicting the distribution of 80S ribosomes and 40S ribosomal subunits, as well as that of the proteins (solid circles) associated with RNP particles. Translation occurs efficiently only in the initiation region, either because the initiation process cannot occur or because 40S ribosomal subunits cannot scan past approximately nucleotide position 37. The sensitive region contains one or more sensitive elements that promote Upf1-dependent degradation unless protected by 80S ribosomes, 40S ribosomal subunits, or RNPs. Termination and 80S ribosome release occur at any UAA site that is preceded by an in-frame functional AUG initiation codon. Short open reading-frames (AUG ACU UAA) result in the exposure of sensitive elements, and therefore degradation, only if they lie within the initiation region. See the text for more detailed explanations.

occur by introducing the ATA ATG ACT TAA sequence. For example, the *cyc1-1251*-through-*cyc1-1255* mRNAs are almost completely lost by Upf1-mediated degradation because of the lack of protection by either 80S ribosomes or 40S ribosomal subunits and RNPs (Fig. 6).

Translation appears to initiate only in a restricted region—a proposal. The levels of Upf1-mediated degradation of *CYC1* mRNA differ in the TAA series and ATG-TAA series of mutants when comparisons are made with TAA codons at the same corresponding sites (Fig. 3); mRNA is not appreciably degraded when the TAA codons are 3′ to approximately nucleotide position 150 in the TAA series and approximately nucleotide position 50 in the ATG-TAA series.

We have interpreted these results by the model schematically presented in Fig. 14. *CYC1* mRNA in the wild-type and initiator mutants is transcribed from the chromosomal *CYC1* gene and transported as an RNP particle from the nuclei to the cytoplasm (Fig. 14A). Normally iso-1-cytochrome *c* is translated by initiation and termination at the wild-type AUG and UAA codons, respectively (Fig. 14B). When the normal AUG initiator codon is altered, translation does not initiate (Fig. 14C), even when there is an AUG codon at the internal site of the *CYC1* mRNA (Fig. 14D). These mRNAs are stabilized by the 40S ribosomal subunit and RNPs distributed along the mRNAs (Fig. 7).

Premature termination can occur at any UAA mutation (Fig. 14E, F, and G). The mRNA is degraded if the UAA termination codon lies inside the sensitive region (Fig. 14E and F) but not if it is distal to approximately nucleotide position 211, past this sensitive region (Fig. 14G). This degradation occurs by exposing one or more sensitive elements within the sensitive region; we propose that at least one sensitive element is located at the 3′ end of the sensitive region, depicted at the top of Fig. 14.

The scanning of the 40S ribosomal subunits can be disrupted by insertion of ATG-TAA codons, because of the formation and subsequent prompt release of 80S ribosomes (Fig. 14E, H, and I). These premature terminations also cause degradation of the mRNA because of exposure of one or more sensitive elements. However, no premature termination or destabilization occurs when the AUG-UAA sequence is inserted outside of the initiation region (Fig. 14J and K). Thus, we suggest that efficient initiation of translation occurs at the AUG codon only within this hypothetical initiation region.

The ability to initiate translation only within the initiation region was confirmed with the polyribosomal distributions of *CYC1* mRNAs having ATG triplets at various sites but with all having only the normal in-frame TAA terminating codon at position 328. As expected, *CYC1* mRNAs in all of the ATG series of mutants were stable (Fig. 9). Because of the lack of premature termination, *CYC1* mRNAs having an effective ATG codon are protected by 80S ribosomes and *CYC1* mRNAs having an ineffective ATG codon are protected by 40S ribosomal subunits and RNPs. However, the *CYC1* mRNAs were distributed differently in the polyribosomal gradients. Only a small proportion of the *cyc1-1291*, *cyc1-1250*, and *cyc1-1295* mRNAs were found in the top fractions of the gradient, indicative of translation, and all of these mRNAs contained ATG triplets within the initiation region. In contrast, high proportions of 30 to 40% of the *cyc1-1293* and *cyc1-1294* mRNAs were found in the top fractions of the gradient, indicative of the lack of translation and comparable to mRNAs that are untranslatable, and these mRNAs contained ATG triplets 3′ to the initiation region (Fig. 10).

As shown in Fig. 12, the results of the *cyc1* mutants described in Fig. 11 indicated that the initiation region could be altered by multiple nucleotide replacements and deletions. Changes in the levels of translation were estimated from comparisons of the *cyc1-1256*, *cyc1-1304*, and *cyc1-1305* mRNA stabilities and from comparisons of the *cyc1-1294*, *cyc1-1297*, and *cyc1-1299* mRNA polyribosome distributions. The 60-nucleotide deletion in *cyc1-1299* and *cyc1-1304* that diminished the distance between the cap site and the AUG initiator codon enhanced the level of translation, but not to the level found in the undeleted AUG mutants with equivalent distances. On the other hand, multiple replacements surrounding the AUG initiator codon, without altering the distance from the cap site (*cyc1-1297* and *cyc1-1305*), slightly diminished the level of translation. Because deletions also alter secondary structures, the results are difficult to interpret. It is possible that the initiation regions simply are open structures that allow high efficiencies of initiation of translation. It is also possible that the lack of initiation of translation outside the initiation region is due to the inability of 40S ribosomal subunits to scan past approximately nucleotide position 37 because of a secondary

structure. We anticipate that a weak hairpin structure would have such properties. RNPs would still be retained on an mRNA containing such a secondary structure preventing scanning of 40S ribosomal subunits, but the secondary structure would still allow elongation of 80S ribosomes. However, because *cyc1-1249* mRNAs are distributed throughout the gradient (Fig. 7), we favor the view that at least a fraction of the mRNAs may be covered with 40S ribosomal subunits.

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