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Interference of nonsense mutations with eukaryotic messenger RNA stability

[mapping/mRNA synthesis/oligo(dT)-cellulose/RNA·DNA hybridization]

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ABSTRACT **The fine structure map of the yeast *URA 3* gene was established by meiotic recombination, and amber nonsense mutations were located at different points on the map. The effect of the length of the labeling time on the specific radioactivity of *ura 3* messenger RNA and on its repartition between poly(A)-RNA and RNA not containing poly(A) has been followed in nonsense mutants. Nonsense mutations reduce the messenger level without lowering its instantaneous rate of synthesis. The strength of the reduction depends on the position of the nonsense codon within the locus and concerns essentially the accumulation of polyadenylylated *ura 3* mRNA.**

Yeast is a convenient organism for studying basic biochemical and genetic features specific to the eukaryotic cell. We have used the facts that fine structure mapping is possible in yeast and that the yeast *URA 3* gene coding for orotidine monophosphate decarboxylase has been cloned (1), allowing measurement of the corresponding mRNA, to investigate the effect of amber nonsense mutations, and therefore the effect of an arrest of the translational machinery, on eukaryotic RNA metabolism. In bacteria, studies of the mechanism of transcription polarity show clearly that ribosomes and the translation process play a role in the decay of mRNA and probably a passive role of protection (2–4). The work described here extends to eukaryotes the observation that a mRNA can be protected by ribosomes in spite of the fact that transcription is not directly coupled to translation as in bacteria.

MATERIALS AND METHODS

Strains and Media. All strains used in this study were derived from two isogenic *Saccharomyces cerevisiae* strains: FL 100 (ATCC 28283, wild type of mating type a) and FL 200 (ATCC 32119, wild type of mating type α). The media for growth and the procedure for the positive selection of *ura 3* mutants have been described (5, 6).

Genetic Analysis. Methods described by Mortimer and Hawthorne (7) were followed.

Mapping. Mitotic recombination was chosen to approximate the position of the *ura 3* mutants. Heteroallelic diploids for *ura 3* were grown from very small inocula on complete medium for 24 hr and then replicaplated onto minimal medium. The plates were irradiated with UV light for 10 sec and incubated at 28°C for 3 days before the prototrophic clones were counted.

A more accurate position of the mutants was determined by the method of meiotic recombination. Heteroallelic diploids for *ura 3* were grown for 24 hr on complete medium. They were transferred onto presporulation medium supplemented with uracil (20 μg/ml) for 24 hr and then onto sporulation medium for 24 hr. After suspension in distilled water (about 0.3 ml), the number of asci per ml was determined by hemocytometer (asci containing one, two, three, or four spores were recorded). Appropriate samples (about 10^6 asci) were then spread on minimal medium. Wild-type recombinants were counted after 3 days of incubation at 28°C. The number of prototrophs per 10^5 asci was routinely used to evaluate map distance.

RNA Labeling. To label the RNA we used the following procedure (1). Cells were labeled with [^{3}H]adenine (20 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) at a specific activity of 25 Ci/mmol. After a defined labeling time, the cultures were arrested directly by addition of 2 vol of cold ethanol. The cells were centrifuged, washed with the RNA extraction buffer (acetate buffer containing 1 mM sodium acetate, 5 mM NaCl, and 0.1 mM magnesium acetate at pH 5.1), and kept at −80°C until extraction. In steady-state labeling experiments, the cells were left for at least three generations in medium supplemented by 20 μCi and 10 μg of adenine per ml.

Extraction of RNA. The procedure of Waldron and Lacroute (8) was followed except that no carrier RNA was added and that no sodium dodecyl sulfate was used for the second ethanol precipitation. The RNA were lyophilized after the second ethanol precipitation and taken up in hybridization solution. The RNA concentration was calculated from the absorbance at 260 nm, assuming that 1 A_{260} unit equals 40 μg of yeast RNA per ml, the ratio of A_{280}/A_{260} being 2.1 (9). DNA is only 2% of the total nucleic acid in yeast (8) and, thus, did not significantly interfere in this RNA assay.

Oligo(dT)-cellulose fractionation of total RNA into RNA containing a poly(A) sequence [poly(A)-RNA] and RNA lacking a poly(A) sequence was carried out according to Fraser (10).

Preparation of Plasmid DNA. The *Escherichia coli* pBR 322 and pBR 322-6 (*ura 3*) plasmids (1, 11) were prepared by the procedure of Clewell (12).

Hybridization Reaction. The methods for preparing DNA filters and the RNA·DNA hybridization have been described in detail (1). Nitrocellulose filters with plasmid DNA were prepared by filtration according to the method of Gillespie and Spiegelman (13). For hybridization, the radioactive RNA was dissolved in an appropriate volume of hybridization solution (40% formamide/0.02% bovine serum albumin/0.02% Ficoll/0.5% sodium dodecyl sulfate) (14). The DNA filters (0.5 μg of DNA per filter) were incubated for 2 days at 37°C with shaking in 0.2 ml of the dissolved RNA (100 μg/ml). The filters were then washed, treated by pancreatic RNase, and dried. Radioactivity was measured in toluene/5% diphenyloxazole.

RESULTS

Fine Structure Map of *URA 3* Gene. Mutants of the *URA 3* locus (532 isolated) were characterized with respect to revertibility, braditrophy, and suppressibility. Among these *ura 3* alleles, 479 were not leaky and, of those, 118 had low reversion rates; 54 were suppressible by an amber suppressor character-

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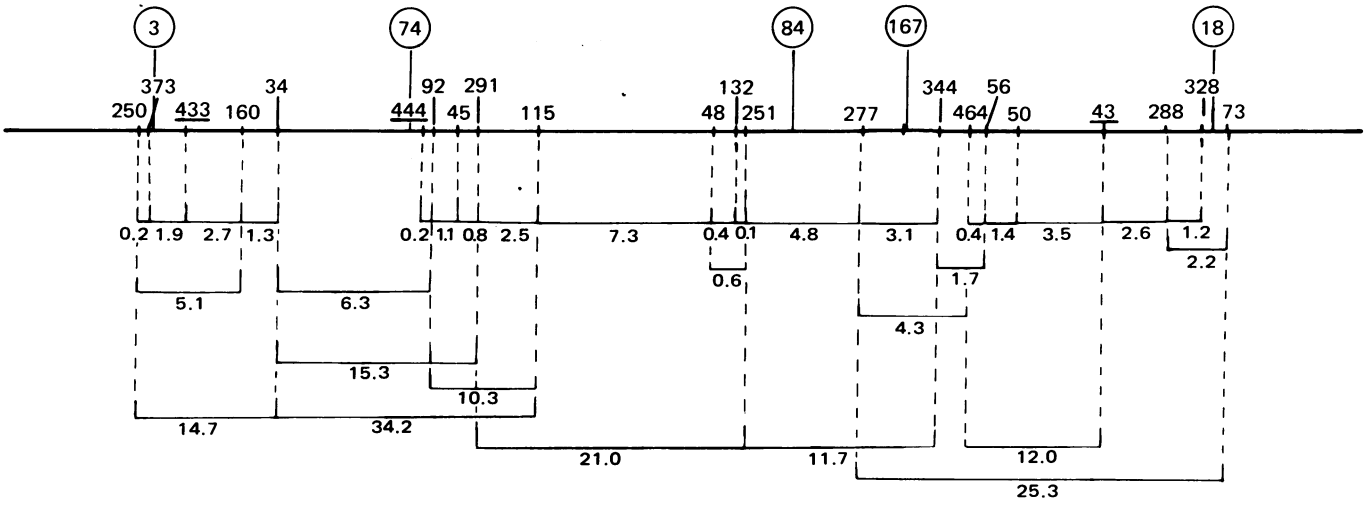

FIG. 1. Meiotic map of the *URA 3* locus. Numbers above the line refer to the allele number. Complementing alleles are underlined. Circles indicate the *ura 3* mutants that were found suppressible by an amber suppressor. Distances between the mutations are indicated below the line and represent the frequencies of meiotic heteroallelic recombination expressed in prototrophs per 10^5 asci. Values given represent an average of four different measurements.

ized by D. C. Hawthorne (personal communication) and very likely correspond to this nonsense codon.

The fine structure of the *URA 3* gene was constructed by using only the low reverting mutants. As a first step, mutants were distributed among several small groups by using the qualitative mitotic recombination test (see *Materials and Methods*); then a more precise order of the mutations inside each group was determined on the basis of the meiotic recombination frequencies in heteroallelic diploids. Fig. 1 presents the data that were used to establish the map of the *URA 3* locus. Local effects on recombination do not allow a precise map by this technique, as has been shown for iso 1 cytochrome *c* by Moore and Sherman (15). Nevertheless, the errors of the recombination mapping technique occur only on a relatively small scale and, therefore, do not challenge our general conclusion.

The following points can be noted. (*i*) The meiotic map of the *URA 3* gene shows a significant tendency to expansion, the recombination frequency in a large interval being higher than the sum of the corresponding short intervals (see Fig. 1). This result is a well-characterized feature of fungal meiotic maps (16). (*ii*) Several complementing *ura 3* alleles were found. The occurrence of this intragenic complementation suggests a multimeric structure for the enzyme orotidine monophosphate decarboxylase. This agrees with the biochemical data of Yoshimoto on this enzyme, which show that it is a dimer of two identical subunits of 26,000 molecular weight (17). (*iii*) The distribution of the sites is relatively homogeneous. The longest space without mutation has a length of 7.3 $rec^+/10^5$ asci—i.e., about a tenth of the map. Due to the map expansion in this locus, the width of this mutationless zone may be overestimated. Nevertheless, it might correspond to a region in which mutations do not give auxotrophy. (*iv*) Nonsense alleles were found throughout the locus. The effect of these nonsense mutations on *ura 3* mRNA metabolism is presented below.

***ura 3* mRNA Levels in Nonsense *ura 3* Mutants.** Table 1 presents the amount of specific *ura 3* mRNA measured by pulse or steady-state labeling in the wild-type strain and in two amber mutants. These two nonsense mutations were chosen at the two extremities of the *URA 3* gene locus (Fig. 1). The mutation *ura 1* was introduced into each strain in order to permit the induction of higher levels of *ura 3* mRNA (1).

A 2-min pulse gave similar RNA levels in the different strains, but with increasing labeling time the rate of *ura 3* RNA production in the mutant *ura 3-3* decreased more quickly than in the two other strains. To see if this effect was really linked to the position of the nonsense codon on the gene, the *ura 3* messenger level was measured in amber mutants with nonsense codons situated at intermediate gene positions. The effect on *ura 3* RNA level of a mutation *ura 3-373*, very likely a missense mutation in view of its low reversion rate and its lack of suppression by known suppressors, situated close to the *ura 3-3* site, was also studied in order to verify that the decrease in *ura 3* mRNA level seen in the *ura 3-3* strain was due to the nonsense nature of the mutation and not only to its position. Table 2 presents the results. The *ura 3-373* mutant had a normal wild-type *ura 3* mRNA level, strengthening the idea that the low level in *ura 3-3* is a nonsense effect. This was reconfirmed by the recovery of a nearly normal level of *ura 3* mRNA in a *ura 3-3* mutant when a UAG suppressor was added (Table 2). Intermediate levels were found for the nonsense mutations situated at intermediate positions on the map; that is, progressively higher specific mRNA levels were obtained with increasing distance from the *ura 3-3* mutation site. The effect was not linear; the mutants in the first half of the map all had a very low level.

Kinetics of Labeling of Nonpolyadenylylated *ura 3* mRNA and *ura 3* Poly(A)-mRNA Species. Because the addition of a

Table 1. *ura 3* mRNA levels in nonsense *ura 3* mutants

Strains*	Amount of *ura 3* mRNA†		
	2-min pulse	10-min pulse	Steady state
ura 3-3	7.74×10^{-5}	2.72×10^{-5}	Undetectable
ura 3-18	8.27×10^{-5}	8.39×10^{-5}	7.05×10^{-6}
+	9.00×10^{-5}	10.24×10^{-5}	8.40×10^{-6}

* Each strain possesses the mutation *ura 1-21*.

† After whole cell labeling, [^{3}H]RNA was isolated and hybridized with DNA from pBR 322 and pBR 322-6 plasmids. The radioactivity in RNase-resistant hybrids was determined by scintillation counting. Values of [^{3}H]RNA hybridized to pBR 322 DNA were subtracted from values of [^{3}H]RNA hybridized to pBR 322-6 DNA. The differences, expressed as a fraction of the total RNA radioactivity, represent the amount of specific *ura 3* mRNA. Each value is an average of three to six independent determinations. After 10 min of labeling, the specific binding of *ura 3-3* RNA to pBR 322-6 was about 50% of the total hybridized.

Table 2. Effect of position of nonsense codons on *ura 3* mRNA level

Strains*	Amount of *ura 3* mRNA†
ura 3-373‡	8.02×10^{-5}
ura 3-3	2.72×10^{-5}
ura 3-74	2.92×10^{-5}
ura 3-84	3.05×10^{-5}
ura 3-167	5.61×10^{-5}
ura 3-18	8.39×10^{-5}
ura 3-3 SUP§	8.55×10^{-5}
ura 3-18 SUP	10.45×10^{-5}

* Each strain possesses the mutation *ura 1-21*.

† After 10 min of labeling with [^{3}H]adenine (0.1 μg/ml; 25 Ci/mmol), RNA was isolated and hybridized with DNA of plasmids pBR 322 and pBR 322-6.

‡ Strain *ura 3-373* is a missense mutant. The other strains are amber mutants (see the position of nonsense mutations in Fig. 1).

§ A UAG suppressor was added in the strains *ura 3-3* and *ura 3-18*.

poly(A) tract to the 3′ end of mRNA is an important maturation step (18, 19), we determined the amount of polyadenylylated and nonpolyadenylylated *ura 3* mRNA in the *ura 3-3* and in the *ura 3-18* nonsense mutants. Fig. 2 shows the kinetics of labeling of *ura 3* mRNA after fractionation with oligo(dT)-cellulose.

In mutant *ura 3-18*, the majority of *ura 3* labeled RNA was found in the RNA fraction that did not contain poly(A) at shorter pulse times (less than 5 min), whereas at longer times the majority was polyadenylylated. After 15 min of labeling, about 75% of the *ura 3* RNA appeared to contain poly(A). In mutant *ura 3-3*, nonpolyadenylylated *ura 3* mRNA labeling followed roughly the same time course as in mutant *ura 3-18*, but there was only half as much. The specific activity of *ura 3* poly(A)-mRNA in strain *ura 3-3* very quickly approached a low steady-state value. Half-saturation of the pool of this *ura 3* poly(A)-mRNA with label occurred in approximately 2 min; this value, determined from the kinetics of incorporation by the method of Greenberg (20), includes the precursor pool saturation times and thus is probably overestimated.

The same calculation for the *ura 3* poly(A)-mRNA from strain *ura 3-18* gives a half-life of 10 min, the same as that found for wild-type *ura 3* mRNA (1).

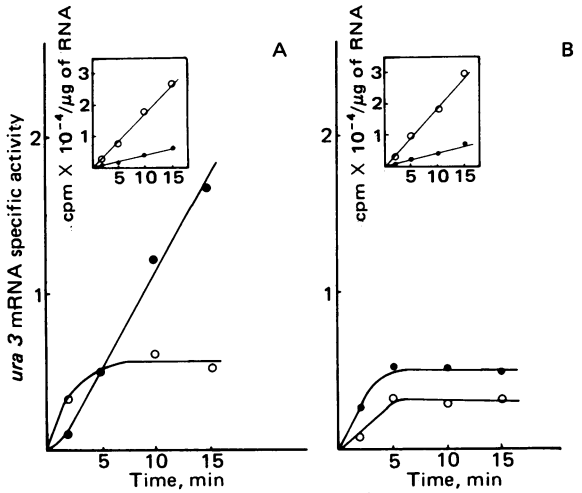

FIG. 2. Kinetics of labeling of nonpolyadenylylated *ura 3* mRNA and *ura 3* poly(A)-mRNA species. Cultures of mutants *ura 1-21 ura 3-18* (*A*) and *ura 1-21 ura 3-3* (*B*) growing at 30°C were labeled with [^{3}H]adenine (1 μg/ml; 1.3 Ci/mmol). Samples of each culture were removed at times indicated and RNA was extracted, then fractionated on oligo(dT)-cellulose and hybridized to DNA of pBR 322 and pBR 322-6 plasmids. Changes in specific activity of *ura 3* mRNA are expressed as cpm specifically hybridized with DNA of clone 6 per μg of total RNA fractionated. ●, *ura 3* poly(A)-mRNA; ○, *ura 3* mRNA not containing poly(A). (*Insets*) Incorporation of [^{3}H]adenine into total poly(A)-RNA (●) and RNA not containing poly(A) (○).

DISCUSSION

The results we present here show clearly that nonsense mutations interfere with the stability of the corresponding mRNA according to their position in the gene. The *ura 3-3* mutation is probably in the early part of the gene and the *ura 3-18* mutation in the terminal part of the gene. Our results, obtained with *ura 3-18*, very likely correspond to the normal processing of the mRNA, with the synthesis of a mRNA not containing poly(A) transformed later into a poly(A)-mRNA which accumulates in polysomes. The rapidly obtained steady-state level for mRNA not containing poly(A) would be due to its rapid maturation into poly(A)-mRNA. Our data give a half-life of 2 min for this maturation step. The length of the *ura 3* mRNA can be estimated to be in the range of 700 nucleotides because it codes for a protein subunit of 26,000 molecular weight (17) and the average amino acid molecular weight in yeast protein is 110 once 1 H_2O is removed. The elongation rate of yeast RNA at 30°C is about 29 nucleotides per sec (21), which means that the *ura 3* mRNA must be completed in about 25 sec. Thus, the maturation time seems much longer than the elongation time. Bossinger and Cooper (22, 23) have reached the same conclusion for the elongation and the maturation of allophanate hydrolase mRNA according to the kinetics of appearance of the enzymatic activity in the presence of different inhibitors.

The results obtained with *ura 3-3* are more difficult to interpret. The lack of accumulation of *ura 3* poly(A)-mRNA clearly implies that the stability of this mRNA is greatly decreased. Note that at the early time of 2 min there is more *ura 3* poly(A)- mRNA in *ura 3-3* than in *ura 3-18*, the sum of *ura 3* poly(A)-RNA and RNA not containing poly(A) being the same in the two strains. One way to interpret this result would be that the degradation of the *ura 3* mRNA after the nonsense codon occurs very fast but first produces large fragments still able to be elongated with poly(A) and to hybridize to DNA filters. As there are more fragments, if the probability of poly(A) addition is proportional to the number of extremities, there would be a faster transit from the nonpolyadenylylated to the poly(A) form in *ura 3-3*. This explanation, difficult to verify with the present sensitivity of the techniques, postulates a lack of specificity for poly(A) addition, which has been effectively observed in yeast. M. R. Chevallier has indeed shown in our laboratory that the RNA transcribed in yeast from the *E. coli* plasmid pBR 322 is polyadenylylated (unpublished results).

Direct biochemical evidence has recently been obtained by G. Loison in our laboratory showing that *ura 3-18*, but not *ura 3-3*, contains crossreacting material immunologically related to the wild-type orotidine monophosphate decarboxylase (unpublished data). Fowler and Zabin (24) have shown that strains with β-galactosidase nonsense mutations in the early part of the cistron had no crossreacting material, whereas those with mutations in the terminal part of the cistron had crossreacting material. Correlation between the quantity of crossreacting material and the position of the mutation along the locus is not strict in the middle of the cistron. Our immunological results confirm the position we assign to *ura 3-3*, and *ura 3-18* mutations with respect to the direction of translation.

In conclusion, our data show clearly that *ura 3* mRNA stability is radically altered by amber nonsense mutations entailing a lack of poly(A)-mRNA accumulation. It will be interesting

to specify whether the nonsense effect results in an acceleration of the normal mRNA degradation or in the setting in motion of another mechanism of messenger processing.

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