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The naturally occurring silent invertase structural gene *suc2°* contains an amber stop codon that is occasionally read through

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Summary. The yeast invertase structural gene *SUC2* has two naturally occurring alleles, the active one and a silent allele called *suc2°*. Strains carrying *suc2°* are unable to ferment sucrose and do not show detectable invertase activity. We have isolated *suc2°* and found an amber codon at position 232 of 532 amino acids. However, transformants carrying *suc2°* on a multicopy plasmid were able to ferment sucrose and showed detectable invertase activity. Full-length invertase was found in gels stained for active invertase and in immunoblots. Therefore we concluded that the amber codon is occasionally read as an amino acid. The calculated frequency of read-through is about 4% of all translation events.

Key words: Nonsense mutation – Read-through – *Saccharomyces cerevisiae*

Introduction

The *SUC2* gene from *Saccharomyces cerevisiae* encodes both forms of invertase, the cytosolic enzyme and the glycosylated external invertase (Carlson and Botstein 1982). The latter form is responsible for cleavage of sucrose and raffinose, which cannot be taken up by yeast. Synthesis of external invertase is regulated by glucose repression (Gascón and Ottolenghi 1972). The internal invertase has no known function (Carlson and Botstein 1982).

Two natural alleles of *SUC2* are known: the active allele and an inactive allele designated *suc2°* (Carlson et al. 1981). Strains carrying *suc2°* do not ferment sucrose and raffinose and they show no detectable invertase activity (Carlson et al. 1981). However, such strains can ferment sucrose and raffinose provided they carry an additional invertase gene at any one of five known *SUC* loci besides *SUC2* (Carlson et al. 1985).

The silent invertase gene *suc2°* is transcribed (Carlson and Botstein 1982) and it can revert to an active *SUC* gene (Carlson et al. 1981; del Castillo Agudo and Zimmermann 1986). In Southern blots of genomic yeast DNA using the *SUC2* gene as probe a difference between *SUC2* and *suc2°* could be observed only after digestion with *Bam*HI (Carlson and Botstein 1983; Hohmann and Zimmermann 1986). However the mutated *Bam*HI site lies about 6.5 kb distant from the gene. Thus, *suc2°* should not exhibit larger rearrangements.

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Since *suc2°* is a naturally occurring silent allele (Carlson et al. 1981; Carlson and Botstein 1983) we were interested in its properties. We isolated *suc2°* and characterised its differences from the active allele *SUC2*. We found a stop codon within the first half of the coding region but transformants carrying *suc2°* on a multicopy plasmid were able to ferment sucrose.

Materials and methods

Yeast strains. The *suc2°* gene was isolated from strain 1.99.-9A *MATα ura3-52 suc0 mal0* and the fragment carrying the *SUC2* gene was isolated from strain 1.106.-2B *MATα trp1 ura3-52 SUC2 mal0*. As recipient for all further transformations strains 2.64.-3B and 2.64.-5A, both *MATα trp1 leu2-3/112 ura3-52 suc2Δ::URA3 mal0*, were used in which the entire coding region of *SUC2* had been substituted by the *URA3* gene from yeast as a *Hin*dIII fragment (Fig. 1; Hohmann 1987). Crude extracts from the following strains were used as controls in gel electrophoresis experiments: 2.64.-1A MATα *trp1 leu2-3/112 ura3-52 suc-2Δ::URA3 mal0*; 1.1.-6B *MATα trp1 leu2-3/112 ura3-52 suc2° mal0*; 1.106.-4D *MATα trp1 ura3-52 his4 SUC2 mal0*; 1.106.-1A *MATα trp1 leu2-3/112 SUC2 mal0*. The strain 3971-5B *MATa* $trp1\text{-}1_a$ *ura3* $ura4_a$. $met1\text{-}8_a$ $ade5\text{-}7_a$ $leu2_a$ $lys1\text{-}1_0$ *aro1-Da* $ilv1\text{-}1_0$ $can1_0$ *SUC mal* was obtained from the Yeast Genetic Stock Center, Berkeley, California, and crossed with 1.99.-9A to get the nonsense alleles of 3971-5B into a *suc2°* background. Nonsense suppressors were isolated from that strain by simultaneous selection for methionine and tryptophan prototrophy.

DNA manipulations. Standard techniques for the manipulation of DNA were used as described by Maniatis et al. (1982). DNA was purified from agarose gels following the method of Tautz and Renz (1983).

Yeast transformation. Yeast sphaeroplasts were prepared using 0.1 mg zymolyase 20T (distributed by ICN Biomedicals, Eschwege) per 10^8 cells and transformed by the method of Beggs (1978).

For the cotransformation experiments to map the possible mutation (Kunes et al. 1987) within the *suc2°* gene 1 μg of plasmid DNA of YRp7*suc2°* digested with *Bam*HI or *Kpn*I and 10 μg of the respective fragment of *SUC2* were used. Thus, the molar excess of the fragment was 70- to 130-fold.

Cloning of suc2°. Figure 1 shows the restriction map of the *SUC2* gene and its allele *suc2°*. *SUC2* has been isolated previously on a smaller fragment of yeast DNA (Hohmann and Zimmermann 1986). *suc2°* and a larger fragment with *SUC2* were cloned by plasmid eviction (Winston et al. 1983). The *Eco*RI-*Sal*I fragment 5′ from the *SUC2* coding region (Fig. 1) was subcloned into YIp5 (Struhl et al. 1979). The *Sal*I site is located within the tetracycline resistance gene of plasmid YEp13 in which *SUC2* was originally isolated (Hohmann and Zimmermann 1986). The resulting plasmid was linearised with *Xho*I and integrated into the yeast genome at the *SUC2* or *suc2°* locus. Integration was checked by stability test, tetrad analysis and by Southern analysis. Chromosomal DNA from these transformants was digested with *Bgl*II. The DNA was extracted twice with phenol:chloroform:isoamyl alcohol 50:50:1 to remove the restriction enzyme, precipitated with ethanol and ligated at low DNA concentration (0.05–0.1 μg/μl) to give preferentially circular DNA molecules. These were transformed into *Escherichia coli* and transformants were selected for ampicillin resistance.

RNA isolation and Northern blot analysis. Cells were grown on synthetic medium selective for maintenance of the plasmid of transformants or on YEP (1% yeast extract, 2% peptone) medium (untransformed cells) supplemented with 2% glucose overnight and invertase synthesis was derepressed for 2 h in fresh medium with 0.1% glucose. Total RNA was prepared according to Denis et al. (1981) and mRNA was enriched by chromatography on oligo(dT) cellulose (Boehringer Mannheim) as described by Aviv and Leder (1972). RNA was electrophoresed in 1% agarose gels (6.8% formaldehyde) and transferred to Hybond N filters (Amersham, Braunschweig). Transfer and hybridisation were performed as described in the product information. DNA probes were labelled by nick translation (Maniatis et al. 1982) with [α^{35}S] dATP.

Detection of active invertase in polyacrylamide gels. Yeast cells were grown in synthetic medium with 2% glucose overnight and transferred into fresh medium with 0.1% glucose and grown for 4 h to derepress invertase synthesis. Active invertase from yeast crude extracts was detected in nondenaturing polyacrylamide gels as described by Grossmann and Zimmermann (1979).

Western blot analysis. Growth conditions were as described above. Crude extracts were denatured and electrophoresed in 10% polyacrylamide gels (70:1). Proteins were transferred to nitrocellulose by electroblotting (Burnette 1981). An invertase antiserum (kindly provided by Prof. Sentandreu, University of Valencia, Spain) was incubated for 2 h with crude extracts from yeast cells deleted for the *SUC2* gene (see above) to remove all antibodies directed against the carbohydrates of the glycosylated invertase. Immunoblot analysis was performed according to Burnette (1981) using nonfat dry milk as blocking agent and alkaline phosphatase labelled secondary antibody (Sigma, München) as the detection system.

Invertase assay. Invertase activity in crude extracts (see above) was measured according to Goldstein and Lampen (1975) and protein was quantified by the microbiuret method (Zamenhoff 1957).

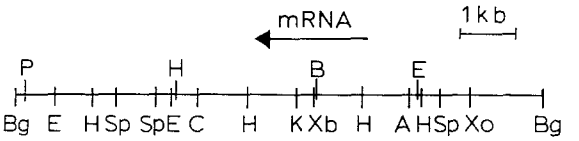

Fig. 1. Restriction map of *SUC2* and *suc2°*. A, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sp, *Sph*I; Xb, *Xba*I; Xo, *Xho*I. No site was found for *Sal*I

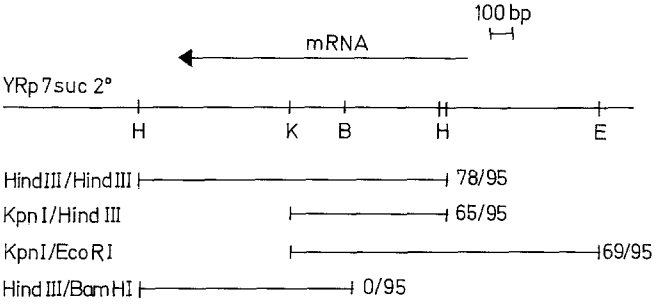

Fig. 2. Strategy and results of cotransformation experiments. Plasmid YRp7*suc2°* (only the *suc2°* gene is shown) was linearised with *Bam*HI or *Kpn*I so that the cotransformed fragment overlaped the gap. The experiments were performed as described in Materials and methods. First number, raffinose fermenters; second number, transformants tested

Results

Characterisation of the suc2° *gene*

Figure 1 shows the restriction map of *SUC2* and *suc2°* which is the same for all 12 restriction enzymes tested. Therefore we concluded, that both alleles differ only by minor changes in the DNA sequence. We attempted to map a region or regions within the gene which carry mutations inactivating *suc2°* by cotransformation of a gapped plasmid carrying *suc2°* and fragments from the active gene *SUC2* according to the method described by Kunes et al. (1987). The strategy is shown in Fig. 2. *suc2°* was subcloned into plasmid YRp7 (Struhl et al. 1979) on a *Bgl*II-*Sal*I fragment (the *Sal*I site is from the vector which was integrated in the *Xho*I site of the fragment shown in Fig. 1). The plasmid was linearised using either *Bam*HI or *Kpn*I. The fragments from *SUC2* cotransformed with the linearised plasmid should mend the gap and transformants were selected for tryptophan prototrophy. A strain deleted for the entire coding region of *SUC2* was used as recipient to prevent integration of the plasmid into the genome. Transformants were checked for their ability to ferment raffinose. From the four fragments used only the 3′ *Bam*HI-*Hin*dIII fragment was unable to give raffinose fermenting transformants (Fig. 2). Therefore we concluded that all mutations inactivating *suc2°* lie within the 5′ *Hin*dIII-*Bam*HI fragment.

We have sequenced the entire *suc2°* gene (S. Hohmann and D. Gozalbo, submitted to the EMBL data base) and found 16 nucleotide substitutions within the coding region (1596 nucleotides, 532 amino acids) and 5 amino acid substitutions (Table 1). At position 232 a glutamic acid codon is changed to give an amber stop codon. Thus, *suc2°* is a nonsense allele. This amber codon lies within the 5′ *Hin*dIII-*Bam*HI fragment shown to carry all mutations inactivating *suc2°*.

Table 1. Differences in the amino acid sequence of *SUC2* and *suc2°*

Position	Amino acid	
	SUC2	*suc2°*
83	Asn	His
87	Gln	Glu
232	Glu	Amber
408	Ala	Pro
432	Ala	Val

The entire coding region is 532 amino acids long

Table 2. Specific invertase activities of transformed and untransformed strains carrying *SUC2*, *suc2°* or hybrid genes

Strain	Plasmid	Invertase (mU/mg protein)
SUC2	None	4000
suc2°	None	<20
suc2Δ	None	<20
suc2Δ	YRp7*SUC2*	10000
suc2Δ	YRp7*suc2°*	200
suc2Δ	YRp7 *5′SUC2-3′suc2°*	4500
suc2Δ	YRp7 *5′suc2°-3′SUC2*	400
suc2Δ	pJDB207*SUC2*	55000
suc2Δ	pJDB207*suc2°*	700
suc2° SUP	None	400

The data are the average of at least 4 independent transformants. *suc2° SUP* is a strain carrying an amber suppressor. pJDB207 is a multicopy plasmid (50–200 copies per cell) described by Beggs (1981)

*Transformants with YRp7*suc2° *ferment sucrose*

Transformants carrying the plasmid YRp7*suc2°* were able to ferment sucrose. However, they did not ferment raffinose which is cleaved by invertase less efficiently and thus yeast cells require much higher specific invertase activities to ferment raffinose. Moreover, YRp7*suc2°* transformants showed significant invertase activity (Table 2). Therefore we asked, whether the truncated invertase protein consisting only of the first 231 amino acids has residual invertase activity or whether the stop codon was occasionally read as an amino acid. A first hint was given by the specific invertase activities of transformants carrying YRp7*suc2°*, YRp7*SUC2* and hybrid genes constructed using fragments from *suc2°* and *SUC2* (Fig. 3; Table 2). The hybrid *5′ suc2°-3′SUC2* gives about twice the specific invertase activity of *suc2°* alone while the *SUC2* gene gives about twice the activity of the hybrid *5′SUC2-suc2°*. This indicates that the 3′ half of *SUC2* has a positive effect on the activity of this hybrid invertase. This is only possible if the amber codon is read through rather than if the truncated invertase exhibits residual activity. Alternatively, the 3′ part of *SUC2°* could influence the specific invertase activity by increasing the mRNA stability or translational efficiency.

Detection of full-length invertase encoded by suc2°

Figure 4 shows a non-denaturing polyacrylamide gel stained for active invertase. In the crude extracts of YRp7*suc2°* transformants small amounts of glycosylated external invertase could be detected. The external invertase is about 15 times more abundant than the internal form in *SUC2* strains under derepressing conditions (Hohmann and Zimmermann 1986). In the crude extracts of transformants with

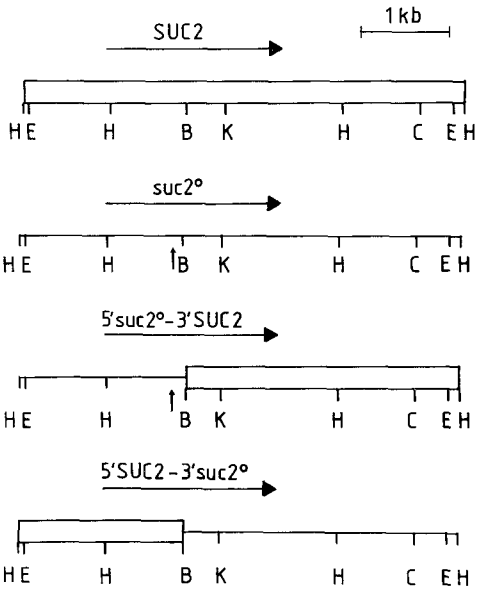

Fig. 3. Construction of hybrid genes from *SUC2* and *suc2°*. The constructions were cloned into YRp7. For restriction enzyme abbreviations see Fig. 1 legend. *Vertical arrows* show the amber codon within *suc2°*

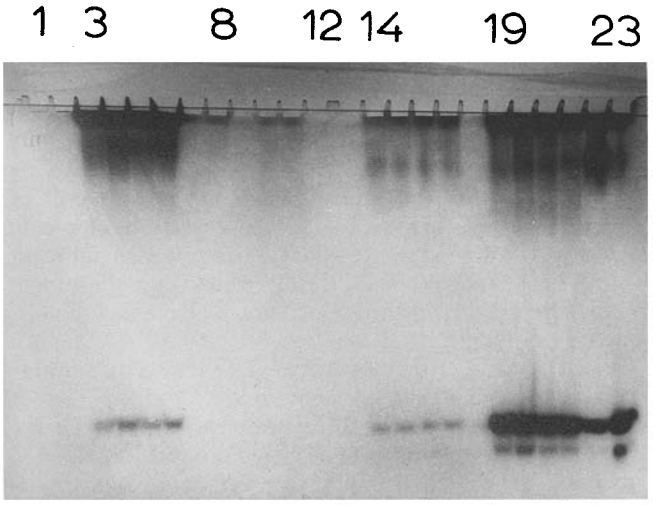

Fig. 4. Native polyacrylamide gel with crude extracts stained for active invertase. Bands correspond to the unglycosylated internal invertase, and the smear at the start to the glycosylated external form. The amount of protein (crude extract) applied is given below in parentheses for each lane. Lanes 1 and 2, *suc2Δ*, untransformed (140 µg); lanes 3–6, *suc2Δ*, YRp7 *5′SUC2-3′suc2°* (20–30 µg); lane 7, blank; Lanes 8–11, *suc2Δ*, YRp7*suc2°* (85–135 µg); lanes 12 and 13, *suc2°*, untransformed (150 µg); lanes 14–17, *suc2Δ*, *5′suc2°-3′SUC2* (80–100 µg); lane 18, blank; lanes 19–22, *suc2Δ*, YRp7*SUC2* (7–10 µg); lanes 23 and 24, *SUC2*, untransformed (100 and 200 µg); the additional signal at the bottom visible in lanes 19–24 may be due to a degradation product of the internal invertase and is usually found in overloaded gels

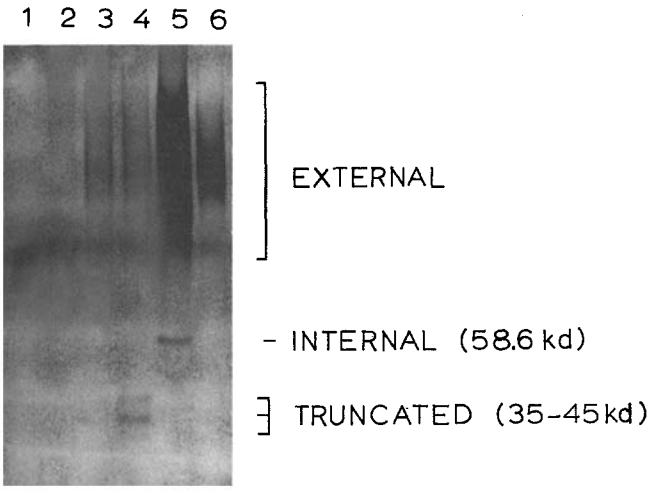

Fig. 5. Western blot analysis of crude extracts from transformed and untransformed strains. 130 µg of protein was applied to each lane. Lane 1, *suc2Δ*, untransformed; lane 2, *suc2°*, untransformed; lane 3, *suc2Δ*, YRp7*suc2°*; lane 4, *suc2Δ*, YRp7 *5′suc2°-3′SUC2*; lane 5, *suc2Δ*, YRp7*SUC2*; lane 6, *SUC2*, untransformed. External, internal and truncated refer to the forms of invertase

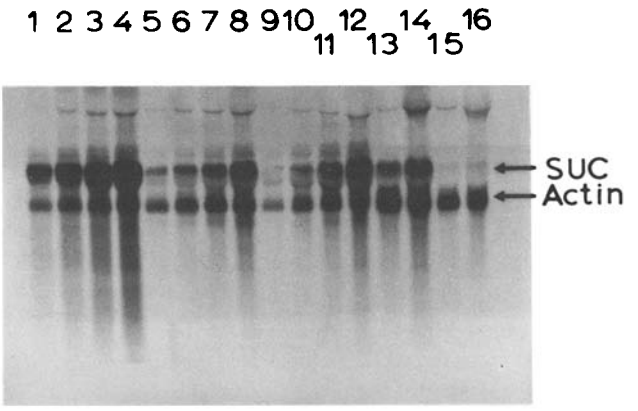

Fig. 6. Northern blot analysis of mRNA from transformed and untransformed strains. The amounts of mRNA applied were 1, 2, 5 and 10 µg for transformants and 5 and 10 µg for untransformed strains. The filter was hybridized with the *Hind*III fragment carrying the *SUC2* coding region and the *Kpn*I-*Bam*HI fragment carrying the actin gene from yeast (Gallwitz and Sures 1980). Lanes 1–4, *suc2Δ*, YRp7*SUC2*; lanes 5–8, *suc2Δ*, YRp7*suc2°*; lanes 9–12, *suc2Δ*, YRp7 *5′suc2°-3′SUC2*; lanes 13 and 14, *SUC2*, untransformed; lanes 15 and 16, *suc2°*, untransformed

the hybrid gene *5′suc2°-3′SUC2* which has a higher specific invertase activity, even internal invertase indistinguishable from the internal invertase of *SUC2* strains or transformants with the plasmid YRp7*SUC2* could be detected.

Figure 5 shows a Western blot analysis of an SDS-polyacrylamide gel. The glycosylated external invertase appears as a large smear while the internal invertase, only visible in the crude extract from transformants carrying YRp7*SUC2*, appears as a sharp band. Small amounts of external invertase are visible in the transformants with YRp7*suc2°*, YRp7 *5′suc2°-3′SUC2* and even in untransformed *suc2°* strains. These results show that the amber codon is actually read through to give detectable amounts of active full-length invertase.

In the crude extract of YRp7 *5′suc2°-3′SUC2* transformants and to a lesser extent in the crude extract of YRp7*suc2°* transformants three proteins smaller than the internal invertase (mol. wt. 58600 daltons; Taussig and Carlson 1983) of YRp7*SUC2* transformants could be detected. The molecular weight of these proteins of 35000 to 45000 daltons was higher than that expected for the truncated invertase encoded by *suc2°* (25500 daltons). Therefore we conclude that these proteins were partially glycosylated forms of the truncated invertase.

The mRNA of suc2° *is much less abundant*

Figure 6 shows a Northern blot analysis of the mRNA from *SUC2* and *suc2°* strains and from transformants carrying the plasmids YRp7*suc2°*, YRp7*SUC2* and the hybrid gene YRp7 *5′suc2°-3′SUC2*. Different amounts of mRNA were applied to the gel and the actin mRNA (Gallwitz and Sures 1980) was used as a control. The invertase mRNAs from *suc2°* and from the hybrid gene *5′suc2°-3′SUC2* are much less abundant than the mRNA of the *SUC2* gene. Quantitative measurements using slot blot analysis revealed that transformed and untransformed strains of *suc2°* have about 10% of the invertase mRNA of the corresponding *SUC2* strains (data not shown).

Discussion

Properties of the silent allele suc2°

The naturally occurring silent allele of the invertase structural gene *SUC2* differs only by 16 of 1596 nucleotides and 5 of 532 amino acids. The only amino acid difference inactivating the gene is the amber codon at position 232. This was supported by the isolation of nonsense suppressors carrying the *suc2°* gene (untransformed) which could ferment sucrose (Table 2). Transformants carrying the hybrid invertase gene *5′SUC2-3′suc2°*, which does not contain the amber codon, have about half the specific invertase activity of strains transformed with *SUC2*. Thus, the 3′ half of *suc2°* reduces the activity of this invertase. This region has two amino acid differences between the two alleles. Most probably the substitution of an alanine by a proline residue is responsible for the reduced function (Table 1). The proline residue may influence the secondary structure of the protein. Alternatively, the 3′ half of *suc2°* could decrease the mRNA stability or the translation efficiency. However, *SUC2* and *suc2°* differ only by 7 bp downstream of the *Bam*HI site to the end of the transcribed region (S. Hohmann and D. Gozalbo, submitted).

The amber codon in suc2° *can be read as an amino acid*

That the amber codon is actually read as an amino acid was shown in three ways: (1) The hybrid gene *5′suc2°-3′SUC2* gave a higher invertase activity than the *suc2°* gene alone, thus a region downstream of the amber codon seemed to influence the activity of the protein. (2) Full-length invertase was detected in gels stained for active invertase. (3) Full-length and truncated invertase polypeptides were found in Western blots.

The amber codon is read as an amino acid sufficiently often to enable the cells to ferment sucrose if the *suc2°* gene is present on a multicopy plasmid. The plasmid YRp7 is unstable and has a tendency to stay in the mother cell

during cell division (Murray and Szostak 1983) but the cells carrying the plasmid have about 20–50 copies. The invertase activity of untransformed *suc2°* strains is at the limit of detection (about 20 mU/mg protein). Thus, the 200 mU/mg protein found in the YRp7*suc2°* (Table 2) transformants represent an overproduction of at least tenfold. The stop codon is read through also in untransformed strains carrying the *suc2°* gene, because a very weak signal for external invertase could be detected in Western blots, which is completely absent in strains with a deletion of the *SUC2* gene.

Ways of read-through of stop codons

It has been found that the yeast $tRNA^{gln}_{cag}$ can suppress amber mutations when transformed into yeast cells on multicopy plasmids (Weiss and Friedberg 1986). The $tRNA^{gln}_{cag}$ should also suppress amber codons in untransformed cells, however the efficiency is too low to be detectable. We introduced, in contrast to the experiment of Weiss and Friedberg (1986), the amber allele on multicopy plasmids. If the $tRNA^{gln}_{cag}$ is not limiting in the cell and suppresses amber mutations with the same frequency regardless of the copy number of the nonsense allele, this suppression could lead to the observed sucrose fermentation of transformants carrying *suc2°* on the multicopy vectors YRp7 or pJDB207 (Table 2).

The context of a stop codon is important for its function in translation termination (Feinstein and Altman 1978; Bossi and Roth 1980). The context of the amber codon within the *suc2°* gene does not follow the preferences for eucaryotic stop codon context as compiled by Kohli and Grosjean (1981).

Internal stop codons and mRNA stability

We found in strains carrying *suc2°* only about 10% of the invertase mRNA of the corresponding *SUC2* strains. This may be due either to a reduced initiation of transcription or a decrease in mRNA stability. Revertants of *suc2°* reach invertase activities as high as those of *SUC2* strains (del Castillo Agudo and Zimmermann 1986) and *suc2°* strains with a nonsense suppressor can have invertase activities sufficient to ferment raffinose. These observations do not exclude that initiation of transcription is less efficient for *suc2°* than for *SUC2* but they favour the explanation of a reduced mRNA stability. A decrease in the stability rather than in the synthesis of yeast mRNA has been shown for nonsense alleles of the *URA1* and *URA3* genes (Losson and Lacroute 1979; Pelsy and Lacroute 1984). The half-life of the mRNA was reduced to one-tenth in a mutant allele carrying the stop codon in the 5′ region of the gene. It was suggested that the mRNA is usually stabilised by ribosomes. A reduced mRNA stability was also observed for nonsense alleles of the human β-globin gene (Baserga and Benz 1988). However, these authors also found a reduced accumulation of mRNA in the nucleus and suggested a feedback from translation in the cytosol to mRNA synthesis or stability in the nucleus.

Frequency of read-through

The specific invertase activity in transformants carrying YRp7*suc2°* is about 2% of the activity found in YRp7*SUC2* transformants. However, the 3′ half of *suc2°* reduces the activity in the construct 5′*SUC2*-3′*suc2°* to about 50%. Thus, the read-through frequency would be about 4% of all translation events. We assume in this calculation, that transcription initiation is the same for *SUC2* and *suc2°* and that the low steady state level of mRNA found in *suc2°* strains is due to degradation of not fully translated mRNA. We also assume no further differences in mRNA stability, translation efficiency and that the two amino acid substitutions within the 5′ half of *suc2°* do not have a severe effect on the activity of the enzyme. The estimated value for read-through of 4% is in agreement with the signals visible in the Western blot analysis where possible effects of these amino acid differences can be ignored. The level of read-through seems rather high and shows the importance of two consecutive stop codons at the end of most coding regions and further sequence requirements for proper termination of translation.

Physiological importance of read-through translation

Occasional suppression of stop codons has been observed to lead to the production of physiologically important products in some systems (reviewed in Ryoji et al. 1983). The *E. coli* phage Qβ produces two kinds of coat proteins the less abundant of which is the result of an occasional read-through of a UGA termination codon. Precursors of reverse transcriptase of murine leukaemia virus and a subunit of the replicase of tobacco mosaic virus are also synthesized by translation through a stop codon. Moreover, a capacity to read through amber and ochre stop codons has been observed in a variety of different organisms (Ryoji et al. 1983) and thus, this flexibility of the translational apparatus may be of general importance.

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