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# *In vivo* transcription of a eukaryotic regulatory gene

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**The *PPR1* gene encodes the positive regulator of the *URA1* and *URA3* genes in yeast. Its transcription product is a 2.9-kb polyadenylated RNA with an extremely short half-life of 1 min. The induced or non-induced cell contains ~0.1 molecules of *PPR1* RNA, a constitutive level which is not altered by changing the number of structural genes to be regulated. The DNA sequence of a 399-bp *AccI-BglII* fragment including 180 nucleotides of the 5'-flanking region of the gene *PPR1* has been determined. By S1 mapping we present evidence that the 5' non-coding region of the *PPR1* mRNA is heterogeneous in length. The 5' termini of the different RNAs map 20, 36, 45 and 50 nucleotides upstream from the translation start codon. The sequence indicates that the only open translation phase begins with an AUG codon that is preceded by two out-of-frame AUG triplets. This particular structure of the 5'-terminal sequence of the transcripts of *PPR1* is dicussed in relation to both their stability and translational efficiency.**

*Key words:* Northern blotting/nuclease S1 mapping/5' nucleotide sequence/RNA synthesis/yeast regulatory gene *PPR1*

## Introduction

In *Saccharomyces cerevisiae*, the *PPR1* gene product selectively stimulates the rate of transcription of the two unlinked structural genes *URA1* and *URA3*, which encode two inducible enzymes of the pyrimidine biosynthetic pathway, dihydroorotic acid dehydrogenase and orotidine 5'-phosphate decarboxylase, respectively. The gene *PPR1* can exist in a number of allelic forms: *PPR1*$^+$, the wild-type allele, which permits induction of the genes *URA1* and *URA3* by dihydroorotic acid (DHO) (Lacroute, 1968); *PRR1*$^-$, the non-inducible allelic form (Losson and Lacroute, 1981) and *PPR1*$^c$, the constitutive allelic form, which no longer requires DHO for induction (Loison *et al.*, 1980). We have recently demonstrated that the product of the *PPR1* gene plays a positive role in regulation (Losson and Lacroute, 1983).

The gene *PPR1* was cloned by complementation of a non-inducible *ppr1*$^-$ yeast strain (Losson and Lacroute, 1981). Both strands of *PPR1* have been separately recloned in phage M13 mp7 and used to characterize the *PPR1* RNA product. It was of interest: (i) to investigate the size and the extent of polyadenylation of the *PPR1* transcript; (ii) to study the parameters of its metabolism (i.e., the rate of synthesis and degradation) and (iii) to identify the site of initiation of transcription by S1 mapping. In addition, we present and discuss the nucleotide sequence of the *PPR1* 5'-flanking region.

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## Results

### *Identification of the PPR1 transcripts*

A restriction endonuclease map of the region of the yeast DNA containing the gene *PPR1* is given in Figure 1. The 1.3-kb *BglII-SalI* fragment characterized as internal to the *PPR1* gene (Losson and Lacroute, 1981) was used as a DNA probe for Northern blotting. The resulting autoradiograph is given in Figure 2. Track A shows the pattern obtained with total RNA from a wild-type strain. The single band visible in this track is significantly more dense when the DNA probe is hybridized against poly(A) DNA (track B). The position of this band corresponds to a length of 2.90 ± 0.05 kb.

The temperature-sensitive yeast mutant $RNA_2$ is defective in RNA processing at the non-permissive temperature (Rosbash *et al.*, 1981). To determine whether high mol. wt. precursors of PPR1 RNA exist, the Northern blot assay was carried out with RNA from the $RNA_2$ mutant and the wild-type strain at both the permissive and non-permissive temperatures. No precursors of the PPR1 RNA were detected using $RNA_2$ for 1 h at the non-permissive temperature (data not shown).

The above results indicate that the transcript of the gene *PPR1* is a polyadenylated species with an average size of 2.9 kb and without any precursor.

### *Direction of transcription*

Isolation of the gene *PPR1* permits assay of the rate of its *in vivo* transcription by DNA excess hybridization to pulse-labeled RNA. By using single-stranded *PPR1* DNA cloned in phage M13 mp7 as probe, the direction of transcription can additionally be determined.

Table I shows the extent of hybridization of labeled RNA from the wild-type strain to each cloned strand of *PPR1* DNA fixed to nitrocellulose filters. The results indicate that

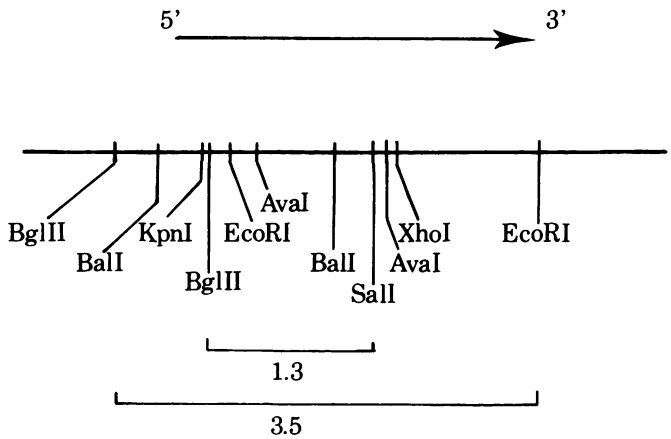


**Fig. 1.** Restriction endonuclease map of the *PPR1* gene region. The arrow indicates the direction of transcription of the *PPR1* gene. The original 3.1-kb *BglII-Sau3A* DNA fragment whose isolation is reported in Losson and Lacroute, 1981) was extended in the distal part of the gene by subsequent recloning to give the 3.5-kb *BglII-EcoRI* DNA fragment. The internal 1.3-kb *BglII-SalI* fragment was used as a DNA probe for Northern blotting.

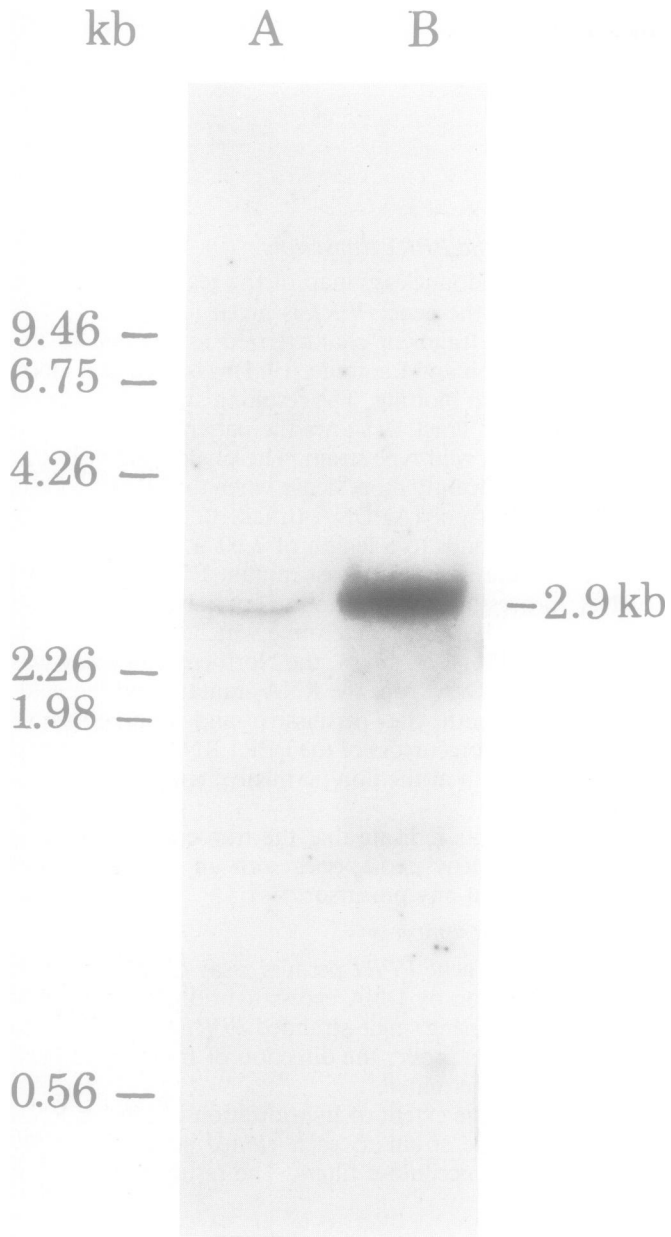


**Fig. 2.** Identification of the *PPR1* RNA by Northern blotting. 100 μg of total RNA **(track A)** and 10 μg of poly(A) RNA **(track B)** from a wild-type strain were run on a 1% agarose gel. Hybridization was carried out with 0.2 μg of the $^{32}$P-labelled 1.3-kb *Bgl*II-*Sal*I DNA fragment of *PPR1* (sp. act. 2 x $10^8$ c.p.m./μg) or with the coding strand of the same fragment. The *Hind*III restriction fragments of the λ DNA were used as size markers.

transcription of the gene is asymmetrical although occurring to some degree on both strands. About 87% of the RNA molecules were complementary to the strand arbitrarily named plus, 13% to the minus strand. The orientation of the yeast insert in each type of phage was determined by restriction analysis of the corresponding replicative forms (not shown). The polarity of the virus strand being known (Herrmann *et al.*, 1980) and the template strand for *PPR1* transcription determined to be the plus strand, the direction of transcription of the *PPR1* gene as indicated in Figure 1 could be deduced to proceed from the internal *Bgl*II site towards the *Sal*I site.

The hybridization data in Table I demonstrate that, in the wild-type strain, the gene *PPR1* is transcribed at a rate 5- to

**Table I.** Transcription of the gene *PPR1*[a]

| Strains | Amount of RNA hybridized to the plus strand of *PPR1* DNA[b] | Amount of RNA hybridized to the minus strand of *PPR1* DNA[b] |
|---|---|---|
| Wild-type FL100 | 0.32 x $10^{-5}$ (87) | 0.04 x $10^{-5}$ (13) |

[a]To determine which strand served as template for the transcription of *PPR1*, the 1.3-kb *Bgl*II-*Sal*I fragment of *PPR1* was cloned into the replicative form of phage M13 mp7 completely digested by *Bam*HI and partially cleaved by *Sal*I. Only phages carrying the yeast DNA in one direction (i.e., the *PPR1* DNA plus strand) were obtained from the infected cells. The 1.3-kb *Bal*I-*Bal*I fragment of *PPR1* (see Figure 1) was chosen for cloning into the *Hinc*II site of the replicative form of M13 mp7, to select a phage carrying *PPR1* DNA in the opposite orientation (the minus strand). DNA from each type of phage was used to assay *PPR1* RNA in wild-type cells labeled with [$^3$H]adenine (20 μCi/ml, 17 Ci/mmol) for 10 min (see Materials and methods).
[b]Expressed as c.p.m. specially retained by the DNA probe *versus* the input c.p.m. Each value is an average of six independent hybridizations. The number in brackets indicate the percentage of hybridization to each strand relative to total *PPR1* transcription.

**Table II.** *PPR1* RNA level in yeast transformed with a plasmid carrying *URA3*

| Strains | Transforming plasmid[b] | Amount of RNA hybridized to the plus strand of *URA3* DNA[a] | Amount of RNA hybridized to the plus strand of *PPR1* DNA |
|---|---|---|---|
| Wild-type FL100 | – | 1.5 x $10^{-5}$ | 0.27 x $10^{-5}$ |
| *ura3*-373-251-328 | pFL2 | 54.5 x $10^{-5}$ | 0.29 x $10^{-5}$ |
| Ratios[c] | | 36.3 | 1.1 |

[a]Cells were labeled with [$^3$H]adenine for 10 min. RNA was extracted and hybridization carried out as described in Materials and methods. The RNA levels were expressed as the fraction of radioactivity specifically retained by the DNA probe *versus* the input. Construction of the *URA3* single-stranded DNA probe has been previously reported (Hubert *et al.*, 1980).
[b]pFL2 is a chimeric bacterial-yeast plasmid containing *E. coli* plasmid pBR322, part of the yeast 2 μm plasmid and the yeast gene *URA3* (Chevallier *et al.*, 1980).
[c]Ratios of RNA levels in transformed strain relative to wild-type.

10-times lower than the structural genes *URA1* and *URA3* (Losson and Lacroute, 1981; Jund and Loison, 1982). *PPR1* RNA was also assayed in a strain transformed by a self-replicating plasmid carrying the structural gene *URA3*, under conditions where *URA3* is present at ~50 copies per cell. These transformed cells showed *PPR1* RNA levels similar to that of the wild-type strain (Table II), indicating that transcription of *PPR1* is not modulated by the number of gene copies to be regulated.

### *Half-life of the PPR1 RNA*

Figure 3 shows the kinetics of synthesis of the *PPR1* RNA determined by hybridization of RNA pulse-labeled with [$^3$H]-adenine at various intervals. The specific activity of *PPR1* RNA in the wild-type strain very quickly approaches a steady-state value. Half-saturation of the pool of this RNA with [$^3$H]adenine occurred in ~2 min, a value determined by the Greenberg's equation (Greenberg, 1962) which includes the precursor pool saturation time. The time required to saturate the intracellular nucleoside triphosphate pools used for RNA synthesis with label is directly expressed as the lag in incorporation of label into stable RNA and, from the insert in Figure 3, can be estimated to be 1 min. Thus, from these results, we conclude that the *PPR1* mRNA decays with a very short half-life of ~1 min.

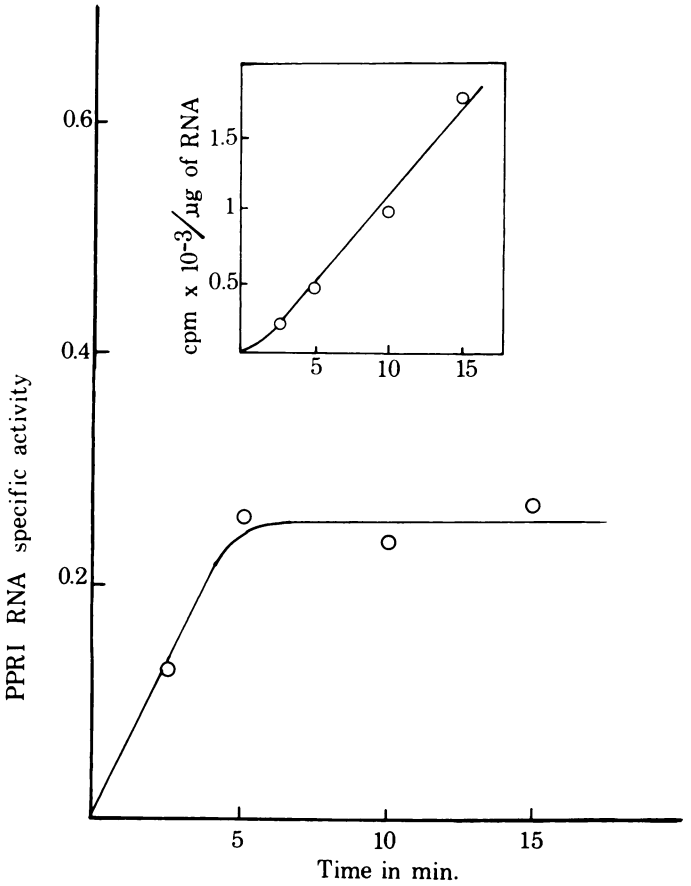


**Fig. 3.** Half-life of *PPR1* RNA. Cells of strain FL100 (+) were labeled with [$^3$H]adenine (1 µg/ml; 1.7 Ci/mmol). At various intervals, samples of the culture were removed and the RNA extracted and hybridized to DNA of phages M13 mp7 and M13 mp7 carrying the *PPR1* plus strand. Changes in specific activity of *PPR1* RNA were expressed as c.p.m. specifically retained by the *PPR1* plus strand per µg of total RNA extracted. **Insert**: incorporation of [$^3$H]adenine into total RNA.

### *The 5′ end of the PPR1 RNA*

The 5′ end of the *PPR1* RNA was mapped by hybridizing the poly(A) RNA from a wild-type strain with a 399-bp 5′ end-labeled *Acc*I-*Bgl*II complementary DNA strand (Figure 4). Hybrids were trimmed using S1 nuclease and the protected DNA strand subsequently analysed on sequencing gels. The DNA fragment was sequenced in parallel by the method of Maxam and Gilbert (1977). Autoradiographs are shown in Figure 4. Figure 5 summarizes the data. The pattern of S1-resistant DNA fragments indicates the presence of 5′ leader extensions of various lengths. After over-exposing the gel, three bands, with lengths of 265, 256 and 240 bases, and a minor band 270 bases long, appeared in the tracks corresponding to the DNA protection by the RNAs from the wild-type strain (Figure 4A). When the *PPR1* gene is introduced into yeast on an autonomously replicating plasmid, essentially the same transcripts are synthesized along with some longer transcripts (Figure 4A and B). We do not know whether the heterogeneity of 5′ termini is due to multiple transcription initiation events or to processing of a primary transcript.

### *5′ Nucleotide sequence of PPR1*

The 5′-flanking region of the gene *PPR1* was determined by the Maxam-Gilbert technique (Figure 5). The sequence presents only one open reading frame that could potentially encode the *PPR1* protein, extending the nucleotide + 1 to the end of the determined sequence. The various size classes of *PPR1* transcripts have their 5′ ends 20, 36, 46 and 50 nucleotides upstream from the putative translation start codon. A near-perfect Goldberg-Hogness box (Benoist and Chambon, 1980) can be found 59 bp upstream from the transcriptional start site −50 (5′-ATAAAATA-3′). An interesting feature within the 5′ non-coding region of the *PPR1* transcripts is the presence of two AUG codons at nucleotides −14 and −11 followed by an in-phase UGA stop codon at position +2, overlapping the *PPR1* AUG initiation codon.

## Discussion

In this study, we have used pulse-labelling techniques to investigate the metabolism of *PPR1* RNA. Data indicate that the transcript of the positive regulatory gene *PPR1* is a large (2.9 kb) message that is probably made constitutively: the level of *PPR1* transcription is not affected by the addition of the inducer DHO (Losson and Lacroute, 1981) or by changing the number of gene copies to be regulated. The half-life of 1 min of the *PPR1* message was determined by using the equation developed by Greenberg (1972):

$$A_t/A\infty = 1 - e^{-\ln 2(1/T_{1/2} + 1/T_D)t}$$

where $A_t$ is the specific activity of the RNA at time $t$ and $A\infty$ is the specific activity at equilibrium; $T_D$ is the cell doubling time and $T_{1/2}$ is the half-life of the RNA tested. The value $T_{1/2}$ of 1 min appears to be extremely short when compared with the average half-life of 17 min for the majority of yeast mRNAs (Hynes and Philipps, 1976). After a 10 min labeling period, the steady-state value of the *PPR1* message was found to represent a fraction equal to $6.7 \times 10^{-6}$ of the total labeled RNA. Since poly(A)-containing RNA in yeast cells labeled for 10 min represents 20% of the total labeled RNA (Losson and Lacroute, 1979), it follows that the *PPR1* message accounts for a fraction equal to $3.3 \times 10^{-5}$. At 10 min the total poly(A) RNA has not reached its steady-state but will increase by a factor of 2.7 as derived from Greenberg's formula by using a half-life value of 17 min. The fraction of the *PPR1* message at equilibrium would therefore be equal to $1.2 \times 10^{-5}$. Taking into account the poly(A) RNA content of a yeast cell (0.016 pg, Waldron and Lacroute, 1975) and the size of the *PPR1* message (2.9 kb), it follows that there are $0.12 \pm 0.1$ *PPR1* mRNA molecule per cell.

We have defined the 5′-flanking region of the *PPR1* gene by mapping the 5′ end of *PPR1* gene transcripts at the nucleotide level and have determined the sequence of this adjacent DNA. S1 nuclease mapping reveals a length heterogeneity at the 5′ end of the transcripts. The nucleotides coding for the 5′ end of the three major *PPR1* RNAs were localized at positions −20, −36 and −45 of the DNA (Figure 5). A minor RNA species was also observed, the 5′ end of which maps at position −50. Multiple 5′ ends have often been described in yeast (Bennentzer and Hall, 1981; Faye *et al.*, 1981; Nasmith *et al.*, 1981): they reflect numerous starting points for transcription or processing steps for the leader of a primary transcript.

The nucleotide sequence of the 5′-flanking region of *PPR1* has several striking features which may be of particular interest with respect to RNA stability and translation efficiency. The position of AUGs and termination codons within the entire sequence of the gene *PPR1* (data not shown) reveals only one reading frame which could encode the *PPR1* protein: it

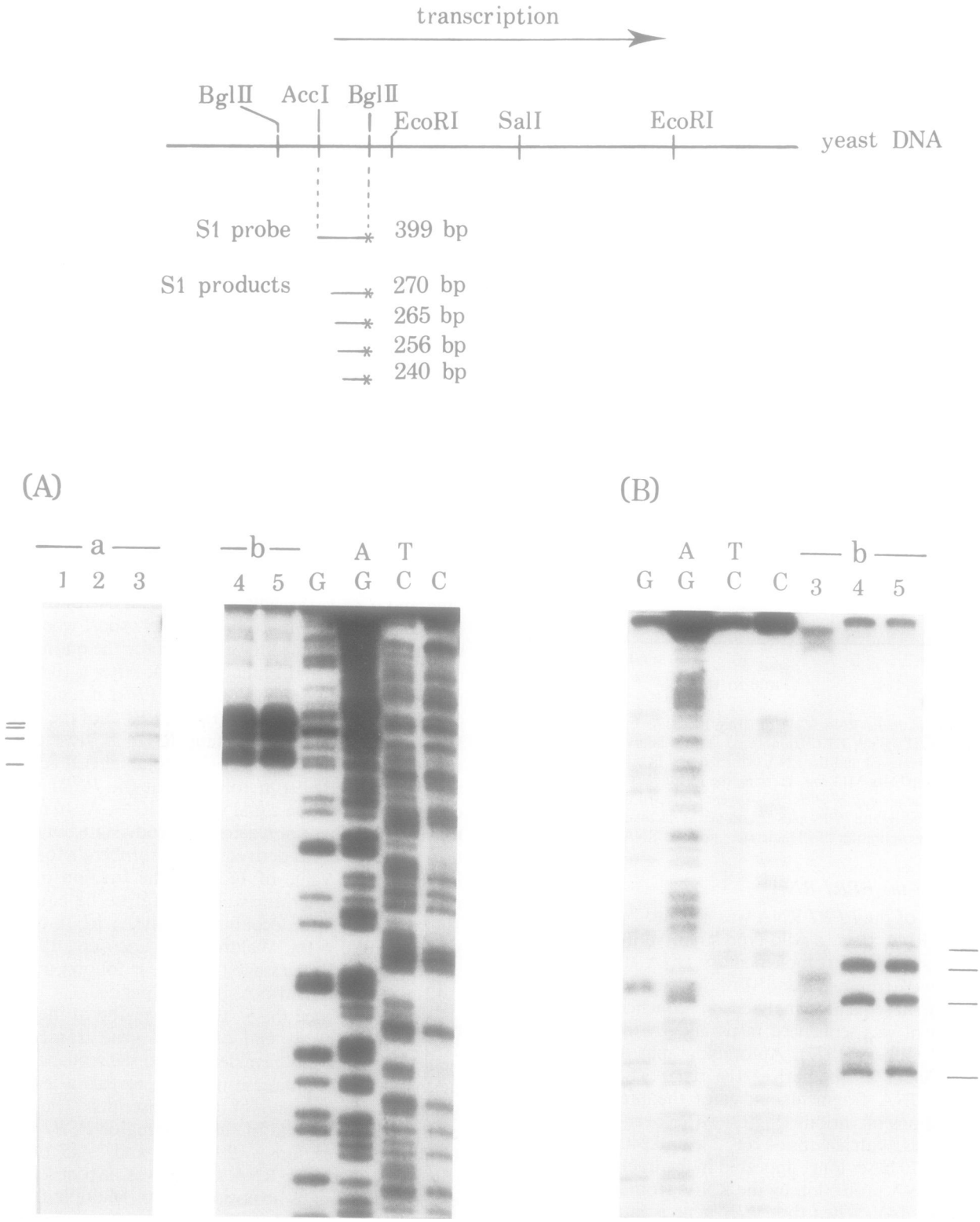


**Fig. 4.** S1-nuclease mapping of the 5′ termini of the *PPR1* RNA. The **upper part** of the figure shows the 399-bp *Acc*I-*Bgl*II 5′-labeled fragment used as DNA probe (sp. act. 7 x $10^5$ c.p.m./pmol). The **lower part** of the figure shows the S1 products of *PPR1* RNAs isolated from the wild-type strain (**-a-**, in an overexposed track) and from cells transformed with an autonomously replicating plasmid carrying *PPR1* (**-b-**). DNA-RNA hybrids were digested with 25 **(1)**, 50 **(2)**, 500 **(3)**, 3000 **(4)** or 6000 **(5)** units of S1-nuclease for 45 min at 37°C, then denatured and loaded onto an 8% DNA sequencing gel. **-b-** in **(B)**, same as in **(A)**, but the electrophoresis time was increased in order to map more precisely the 5′ ends. Horizontal bars indicate the uncorrected 5′ terminus map positions: the fragments generated by the S1 nuclease digestion migrate 1.5 bases slower than the corresponding cleavage fragment generated by the Maxam and Gilbert reactions.

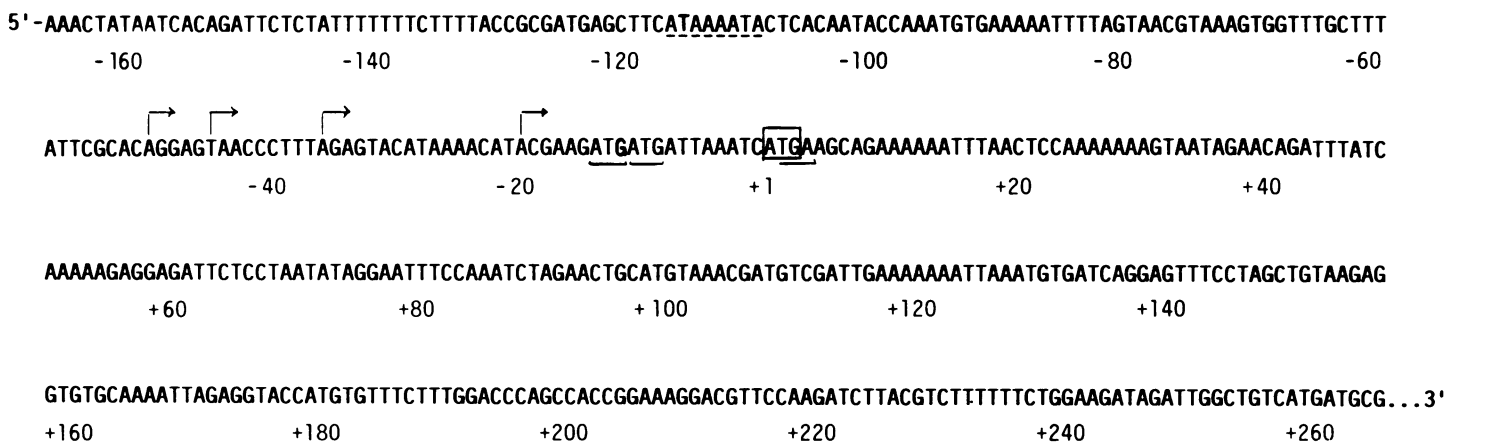


**Fig. 5.** 5′ nucleotide sequence of *PPR1*. Only the non-coding strand is shown. Position +1 is defined as the first nucleotide of the putative translation start codon (boxed ATG). The 5′ ends of *PPR1* RNAs are shown by arrows in the direction of transcription. The two first ATG codons are underlined, as is the corresponding terminator triplet TGA. Dots indicate the potential Goldberg-Hogness box.

begins with an AUG triplet located 20 nucleotides downstream from the nearest 5′ end of the *PPR1* RNA and preceded by two out-of-frame AUG codons. These upstream AUG codons are followed closely by a terminator codon UGA. As the majority of eukaryotic mRNAs initiate translation at the AUG triplet nearest the 5′ end of the message, these results, by demonstrating that yeast ribosomes can initiate at an internal AUG triplet, stimulate questions regarding the mechanism of translation initiation in eukaryotes. Kozak (1981) has proposed a modified scanning model to account for the few known cases in which translation does not start at the first AUG. This involved attachment of the 40S ribosomal subunit at the 5′ end of the transcript in the 3′ direction until the first AUG is reached. If the AUG is flanked by the optimal sequence $\overset{-3}{A}XXAUG\overset{+4}{\underset{Y}{R}}$ or $\overset{-3}{G}XXAUG\overset{+4}{R}$ (R = purine; Y = pyrimidine), all 40S subunits will stop at this first AUG, and the 60S subunits will join to form complete ribosomes and subsequently initiate translation at that site. The model admits that ribosomes can initiate at a downstream site, if the upstream AUGs are flanked by unfavourable sequences (GXXAUGY or YXXAUGX) such that only some migrating 40S subunits recognize the AUG as a 'stop signal' and initiate at this site, while the others continue to scan the message until the functional initiation translation site is found. This hypothesis involves a reduction of the translation efficiency of the mRNAs at internal AUG codons. Consistent with this model is the observation that nucleotides flanking upstream AUG triplets examined so far are different from those found adjacent to most functional initiation codons (Kozak, 1981). The *PPR1* mRNA is an exception to this rule since it possesses two potential translation initiation sites ($^{5'}$ AXXATGA$^{3'}$), as defined by the modified scanning model, situated upstream from the functional *PPR1* initiation codon. According to Kozak, both these upstream AUGs would be a near-absolute barrier against downstream initiation, therefore providing an even stronger translational attenuation for the *PPR1* expression. A terminator codon UGA follows these three codons further downstream. We have previously shown that non-translated mRNAs are degraded very quickly in yeast cells: nonsense mutations in the proximal region of the gene *URA3* greatly reduce the stability of the *URA3* messenger, the distal part of which is no longer protected by the ribosomes (Losson and Lacroute, 1979). The occurrence of the premature translation stop codon UGA in the *PPR1* gene at position +2 may therefore be correlated with the low stability of its RNA product. The UGA stop codon overlaps the putative functional initiation AUG of *PPR1*. It appears therefore that the structure surrounding the *PPR1* initiator codon, as well as its internal location, represents highly 'unfavorable' conditions for an efficient initiation of translation. By placing the biosynthesis of the *PPR1* protein molecule under the control of such an AUG initiation codon, the cell will very likely produce the protein only in low amounts. A limited synthesis of the *PPR1* protein is suggested by the semi-dominance of the *PPR1* deletion mutations showing a regulatory circuit with a strict dosage effect (i.e., one or less than one *PPR1* protein molecule per gene to be regulated; unpublished results). Note that a low cellular concentration of the *PPR1* protein would be consistent with its regulatory function and moreover might be necessary to avoid possible physiological disadvantages resulting from higher levels of the protein. Expression of the gene *PPR1* would therefore be an example of a correlation between the abundance of the protein synthetized and the half-life of the corresponding mRNA. Selective modification of different nucleotide sites within the *PPR1* sequence will provide a particularly useful approach to directly test our hypothesis and to investigate the rules governing metabolism and translation of a eukaryotic mRNA.

## Materials and methods

### *Strains*

All *S. cerevisiae* strains were isogenic derivatives of wild-type strain FL100 (ATCC 28583) except the mutant *rna2* (ts 368) (Hartwell *et al.*, 1970). Two *Escherichia coli* strains were used: BJ5183 F$^-$ *recBC sbcB endoI Gal met str thi bio hsdR*, for transformation and amplification of plasmid DNA, and JM103 *lacpro thi strA endA sbcB15 hsdR4 supE f'traD36 proAB lac* $I^QZ$ *M13*, for cloning in the phage M13 mp7.

### *Cloning in phage M13 mp7*

This was performed in the replicative form of the phage following the procedure given by the supplier (Biolabs Inc., Beverly, USA).

### *RNA extraction, transfer and mapping of the 5′ end*

Yeast cells were grown in YEPD (1% yeast extract, 2% peptone, 2% glucose) at 30°C. Spheroplasts were prepared as described by Chevallier *et al.* (1980) and lysed in acetate buffer (1 mM sodium acetate, 5 mM NaCl,

0.1 mM magnesium acetate, pH 5.1) containing 0.5% (w/v) SDS and 0.2% diethylpyrocarbonate. Total cellular RNA was purified from the lysed spheroplasts by three extractions with an equal volume of buffer-saturated phenol (Waldron and Lacroute, 1975). The poly(A) fractionation of RNA was carried out according to Fraser (1975).

Transfer of denatured RNA from agarose gels to nitrocellulose papers and hybridization with [$^{32}$P]nick-translated probe were performed as described by Thomas (1980).

Analysis of single-stranded DNA protected against nuclease S1 by hybridization with RNA was carried out according to Berk and Sharp (1977). Specific single-stranded DNA probes were obtained by labeling the 5′ ends of an appropriate restriction digest with T4 polynucleotide kinase. The relevant restriction fragment was isolated and its strands separated as described (Maxam and Gilbert, 1977).

*RNA labeling*

Cells grown in minimal medium (yeast nitrogen base Difco 6.7 g/l, 2% glucose) were labeled with [$^{3}$H]adenine (20 $\mu$Ci/ml). Following a specific labeling time, the cultures were arrested directly by addition of 2 volumes of cold ethanol. The cells were centrifuged, washed with RNA extraction acetate buffer and disrupted by vortexing with glass beads. Total labeled RNA was extracted using the method of Waldron and Lacroute (1975). Hybridization to DNA immobilized on nitrocellulose filters was carried out as described by Losson and Lacroute (1979): labeled RNA was incubated for 2 days at 37°C with shaking in a glass vial containing the DNA filters loaded with either 0.5 $\mu$g M13-*PPR1* plus strand DNA or 0.5 $\mu$g M13-*PPR1* minus strand DNA or 0.5 $\mu$g M13 mp7 DNA. To evaluate the specific hybridization to each strand of the yeast DNA, the counts retained on a M13-DNA filter were subtracted from the counts retained on a M13-*PPR1* DNA filter. The amount of hybridization was expressed as the fraction of radioactivity specifically retained *versus* the radioactivity in the input.

*DNA sequence analysis*

DNA fragments were sequenced by the method of Maxam and Gilbert (1977).

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