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Heme regulates the expression in *Saccharomyces cerevisiae* of chimaeric genes containing 5′-flanking soybean leghemoglobin sequences

Erik Østergaard Jensen, Kjeld A.Marcker and Ingrid S.Villadsen[1]

Department of Molecular Biology and Plant Physiology, University of Aarhus, C.F.Møllers Allé 130, DK-8000 Aarhus C, and Department of Microbiology, The Technical University of Denmark, DK-2800 Lyngby, Denmark

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The TM1 yeast mutant was transformed with a 2 μm-derived plasmid (YEp24) which carries a chimaeric gene containing the *Escherichia coli* chloramphenicol acetyl transferase (CAT) gene fused to the 5′- and 3′-flanking regions of the soybean leghemoglobin (Lb) c_3 gene. Expression of the chimaeric CAT gene is controlled specifically by heme at a post-transcriptional level, most likely by regulating the efficiencies of translation. Expression of another chimaeric gene consisting of the neomycin phosphotransferase (NPTII) gene fused to only the 5′-flanking region of the Lbc_3 gene is regulated by heme in a similar way. Thus, in yeast, heme modulates the translation of the chimaeric mRNAs through interactions with the 5′ Lbc_3 non-coding region.

Key words: leghemoglobin/chimaeric genes/heme/translational control/yeast

Introduction

Leghemoglobins (Lbs) are monomeric hemoproteins synthesized exclusively in the root nodules which develop through the symbiotic association of *Rhizobia* with leguminous plants. It is not known how the Lb genes are activated but it is generally assumed that a signal of rhizobial origin is involved. A candidate for such a signal is heme which forms the prosthetic group of the Lbs. Biochemical evidence suggests that the heme necessary for functional Lb protein formation is synthesized by *Rhizobium* (Nadler and Avissar, 1977).

The synthesis of several hemoproteins in the yeast *Saccharomyces cerevisiae* is regulated by the level of intracellular heme which also forms the prosthetic group of these proteins. Transcription of the isocytochrome *c* gene (Guarente *et al.*, 1984) is heme dependent while for the catalase T_1 gene, heme control is exerted both at the transcriptional and the post-transcriptional level (Hamilton *et al.*, 1982).

It was therefore possible that the heme-specific regulatory mechanism in yeast would be able to recognize corresponding regions on the inducible Lb genes. To test this hypothesis a chimaeric gene consisting of the coding region of the *Escherichia coli* chloramphenicol acetyl transferase (CAT) gene fused to the 5′- and 3′-flanking region of a soybean Lb gene (Lbc_3) was constructed such that the expression of the CAT gene is under the control of the Lb promoter. The chimaeric gene was inserted into the yeast plasmid YEp24 which contains the yeast *URA3* gene as a selectable marker (Botstein *et al.*, 1979). The yeast strain TM1, a *ura3-52, hem1* mutant, which is unable to synthesize heme because of a mutation in the δ-aminolevulinate (δ-ALA) synthetase gene, was subsequently transformed with the resulting construction. The expression of the chimaeric CAT gene in yeast proved to be specifically regulated by heme at the post-transcriptional level.

Our data indicate that a heme-specific regulatory system in yeast is able to interact with the 5′-non-coding Lb sequence such that the translation of these mRNAs is affected. In view of the highly specific nature of this effect it is plausible that heme also controls the translation of Lb mRNAs in the soybean nodule. This implies that the molecular basis of this type of post-transcriptional control is identical or at least very similar in yeast and in plants.

Results

Construction of vectors

A 4-kb soybean DNA fragment containing the Lbc_3 gene (Wiborg *et al.*, 1982) including 2 kb and 1 kb of the 5′- and 3′-flanking regions was cloned in pBR322. The sequence stretching from seven bases upstream of the initiator ATG codon to five bases after the stop codon was deleted by a combination of restriction nuclease and *Bal*31 digestions and replaced by synthetic *Eco*RI linkers. A DNA fragment from pBR328 coding for the CAT enzyme was isolated and the ends were treated by a combination of restriction endonuclease and *Bal*31 digestion such that the resulting fragment contained only the coding sequence including five bases in front of the initiator ATG and 31 bases downstream from the stop codon. This fragment was then fused to the 5′ and 3′ Lb flanking sequences through the *Eco*RI site. Finally, this construct was inserted into the yeast plasmid YEp24 (Botstein *et al.*, 1979) through the *Bam*HI and *Sal*I sites present in this vector. The resulting vector is called YEpLbCAT.

Plasmid YEp5LbKm is a derivative of YEpLbCAT in which the CAT gene and the 3′-non-coding Lb sequence were replaced by a fragment of Tn*5* encoding neomycin phosphotransferase (NPTII). This fragment contains the NPTII coding sequence including two bases in front of the initiating ATG codon and 200 bases 3′ to the stop codon. A schematic representation of the two vectors is shown in Figure 1.

CAT activity in yeast transformed with the chimaeric gene

S. cerevisiae strain DBY747 was transformed with clone YEpLbCAT and CAT activity measured in several transformants grown under different physiological conditions. Appreciable CAT activity was present in all transformants tested indicating that the Lb promoter is recognized by yeast. However, during this investigation it was consistently noted that CAT activities were 4–6 times higher in yeast grown on a non-fermentable carbon source compared with a fermentable carbon source (Table I). The increased synthesis of the CAT enzyme in yeast grown on a non-fermentable carbon source correlates with an increased synthesis of several yeast hemoproteins. This increase is partly due to a higher intracellular concentration of heme, which regulates the expression of several hemoprotein genes in yeast. Heme regulates the transcription of the *CYC1* gene (Guarente *et al.*, 1984) while,

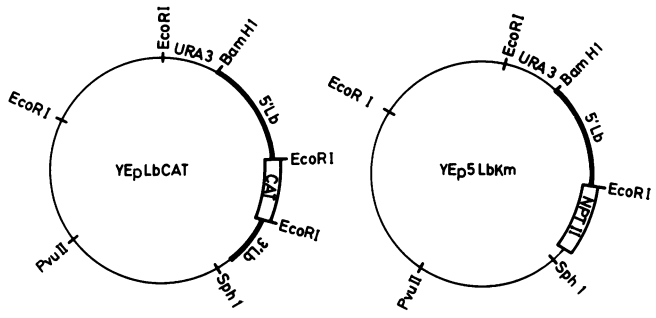

Fig. 1. Schematic presentation of the two vectors used in this investigation. The diagrams show the position of the cloned CAT gene, the NPTII gene, the 5'- and 3'-flanking Lbc_3 sequences (5' and 3' Lb) and of the *URA3* gene in the YEp24 vector. Several restriction endonuclease cleavage sites are also indicated.

Table I. Effect of carbon source on CAT gene expression in yeast strain DBY747 transformed with YEpLbCAT

	Rel. CAT activity %
Succinate	100
Glycerol	100
Glucose	28
Sucrose	18

S. cerevisae was grown in minimal medium + 2% carbon source. Cells were harvested at a cell density of 5 × 10^6/ml. Preparation of extracts and determination of CAT activity were as described in Materials and methods.

in the case of catalase T_1, control is exerted both at the transcriptional and the translational level (Hamilton *et al.*, 1982). The Lbs are also hemoproteins and since the CAT gene is flanked by Lb non-coding sequences it was suspected that the increased synthesis of the CAT enzyme in yeast grown on a non-fermentable carbon source was due to an effect of heme on the expression of the chimaeric gene.

To test this hypothesis a *hem*1 yeast mutant was transformed with YEpLbCAT. The *hem*1 mutation prevents the synthesis of the heme precursor, δ-ALA. CAT activities were then determined in transformants grown in the absence or presence of either δ-ALA, heme or the heme analog deuteroporphyrin IX. The results of such experiments are shown in Table II. There was 20 times more CAT activity in yeast grown in the presence of δ-ALA than in yeast grown in the absence of the heme precursor. Similar high CAT activities were found in yeast grown in the presence of heme or deuteroporphyrin IX. In other experiments CAT activity increased up to 40-fold by the addition of δ-ALA. Thus, the experiments recorded here clearly indicate that the expression of the chimaeric CAT gene in yeast is somehow regulated by intracellular heme.

Heme does not regulate the transcription of the chimaeric CAT gene

The experiments above do not indicate whether heme regulates the expression of the chimaeric CAT gene by a transcriptional or a post-transcriptional mechanism. Accordingly total RNA extracted from yeast grown in the presence or absence of heme precursors was subjected to Northern blotting analysis using CAT- or *URA3*-coding sequences as hybridization probes (Figure 2). The two minor transcripts A and B are not detected when using probes containing 5'-coding CAT sequences only. The amounts of CAT and *URA3* gene transcripts are unaffected by

Table II. The effect of heme precursors and heme analogs on CAT gene expression

	Rel. CAT activity %
Glucose + δ-ALA	100
Glucose + d.p.	84
Glucose + heme	19
Glucose	5

Heme-deficient cells were supplemented with Tween 60, ergosterol and methionine. Deuteroporphyrin (d.p.) and heme were added to 5 μg/ml and, δ-ALA to 50 μg/ml. All assays employed the *hem*1 strain TM1 transformed with YEpLbCAT.

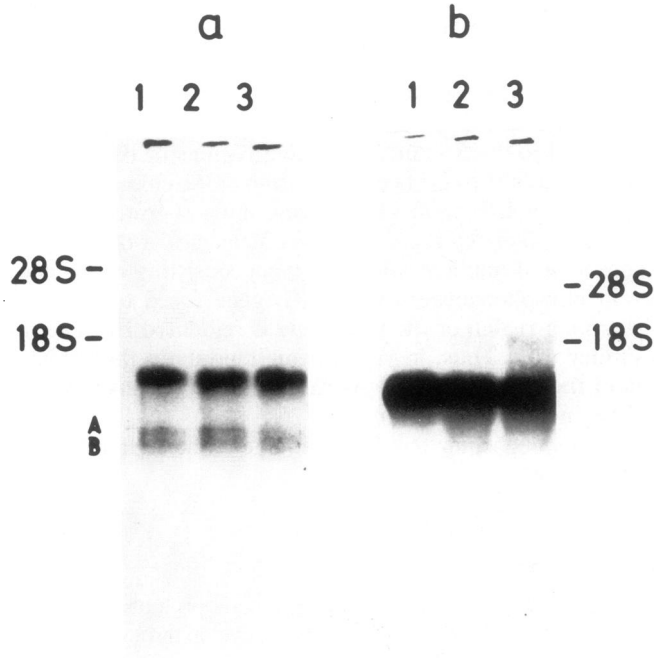

Fig. 2. Northern blotting analysis of total RNA extracted from yeast strain TM1 transformed with YEpLbCAT grown in the presence of 50 μg/ml δ-ALA **(1)**, 5 μg/ml deuteroporphyrin **(2)** and ergosterol, Tween 60, methionine **(3)**. The probes were a CAT gene-specific hybridization probe **(a)** and a *URA3*-specific hybridization probe **(b)**. Each lane contained 50 μg total RNA. 28S rRNA, 18S rRNA and the *URA3* transcript served as mol. wt markers. **A** and **B** denote minor transcripts which do not contain 5' CAT coding sequences.

alterations of the intracellular heme concentrations. Thus, the presence of heme does not increase the synthesis of CAT mRNA and consequently heme does not regulate the transcription of the chimaeric gene. In some yeast transformants, however, an increased amount of CAT mRNA was occasionally observed in cells with a high heme content but this effect was rather irreproducible. The possibility that heme to some extent regulates the transcription of the chimaeric gene cannot be ruled out. The 2 μm-derived vector YEp24 is a multicopy plasmid, which may obscure an effect on transcription due to imperfect interactions of the heme-specific regulatory elements with the Lb gene promoter. This might be an explanation for the effect of heme on transcription occasionally observed in some transformants. Nevertheless the results recorded here on the effect of heme on CAT gene expression cannot be explained by an increased transcription of the gene, but rather must be due to some post-transcriptional event.

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                                 +1                         *  *  *
a)--TATAAATAAGTATTGGATGTGAAGTTGTTGCATAACTTGCATTGAACAATTAATAGAAATAACAGAAAAGTAGAATTCTAAAATG GAG
b)                                                                    --------GTAGAAAAGAAATATG GGT
c)                                                                    --------GTAGAATTCGC--ATG ATT
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Fig. 3. The 5′ leader sequences of (a) the chimaeric CAT gene, (b) the Lbc_3 gene (c) the chimaeric NPTII gene. The position of the two additional thymidine nucleotides referred to in the text is boxed. Position +1 indicates the transcriptional start of the soybean Lbc_3 mRNA in the nodule. Asterisks refer to the transcriptional starts in yeast of the CAT mRNA determined by primer extension.

It was of interest to determine the transcriptional starts on the chimaeric gene. Accordingly primer extension of total RNA extracted from yeast grown with or without heme precursor was carried out using as a primer the oligonucleotide (5′ CAACG-GTGGTATATCCAGTG 3′) which is complementary to nucleotides 15 – 34 in the CAT-coding sequence (Walker *et al.*, 1983). The result of the primer extension assay was further confirmed by S1 nuclease protection analysis. There are several initiation sites, but all are located in the same region as the initiation site of the Lbc_3 mRNA used in soybean nodules (Figure 3). Since Lbc_3 mRNA and the yeast transcripts have corresponding transcription initiation starts it is concluded that yeast is able to recognize the Lb promoter. Other plant promoters such as the zein promoter are also recognized by yeast (Langridge *et al.*, 1984). The CAT mRNAs which contains the entire CAT coding region are ~1300 – 1350 bases long, and the plant transcription termination signal is therefore not recognized by yeast in which case the mRNAs would have been ~1000 bases long. Consequently, the CAT transcripts terminate at a site which is located ~300 – 350 nucleotides downstream from the plant termination signal.

Expression of the chimaeric CAT gene is regulated by a specific post-transcriptional heme effect

To determine whether heme stimulates general protein synthesis in yeast the activity of the *URA3* gene product, orotidine-5′-phosphate decarboxylase, was measured in extracts of the heme mutant grown with and without the heme precursor (Table III). The activity of this enzyme is independent of heme synthesis. In a TM1 mutant similar to the one used in this study the main proteins are synthesized with comparable efficiencies irrespective of the presence or absence of heme (Hamilton *et al.*, 1982). Only synthesis of certain hemoproteins is affected. Thus heme specifically regulates the expression of the chimaeric CAT gene in yeast. This effect is dependent on protein synthesis as shown in Figure 4. Addition of δ-ALA together with cycloheximide, which inhibits protein synthesis, to cultures grown in the absence of the heme precursor prevents the stimulation of CAT activity. Furthermore, the half-life of the CAT enzyme is independent of heme synthesis. *In vitro* addition of heme to a heme-deficient extract does not stimulate CAT activity. When extracts of heme-deficient cells were mixed in various proportions with those from cells supplemented with δ-ALA their capacities for synthesizing acetylchloramphenicol were additive. This indicates that the CAT enzyme is not affected by an unknown component, the activity of which is sensitive to heme.

There is no evidence for an inactive CAT enzyme precursor so the possibility that heme somehow activates a latent form of the enzyme is highly unlikely. Catalase mRNAs extracted from heme-deficient and heme-containing *hem*1 cells respectively were translated in a cell-free system prepared from heme-containing cells with equal efficiencies, while being poorly translated in a cell-free system prepared from heme-deficient cells (Hamilton *et al.*, 1982). In the latter system translation of the catalase mRNAs is at least partly restored by the addition of heme. Due

Table III. Heme does not control expression of the orotidine-5′-phosphate (OMP) decarboxylase gene

	CAT activity	OMP decarboxylase activity
Glucose	2	0.7
Glucose + δ-ALA	60	0.6

Transformed heme-deficient cells were grown in the presence of ergosterol, Tween 60 and methionine. δ-ALA was added to 50 μg/ml. All assays employ strain TM1 transformd with YEpLbCAT. The CAT activity is expressed as nmol chloramphenicol acetylated/min/mg protein and OMP decarboxylase activity as μmol orotidine-5′-phosphate decarboxylated/h/mg protein.

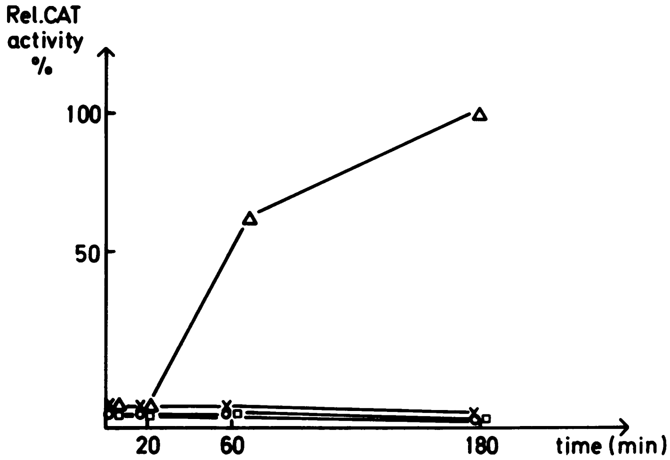

Fig. 4. Effect of cycloheximide on gene expression. Four cultures of heme-deficient cells were grown in glucose to a density of 2.5 × 10_6 cells/ml. At time 0 cycloheximide was added to two cultures (100 μg/ml). Three min later δ-ALA (50 μg/ml) was added to one of the cycloheximide-containing cultures and to one of the cultures without the antibiotic. The yeast strain was TM1 transformed with YEpLbCAT. CAT activity was measured before addition of cycloheximide, and 20, 60 and 180 min after the addition. Symbols: no addition (x), + cycloheximide (○), + δ-ALA(Δ), + cycloheximide and δ-*ALA* (□).

to the lack of a CAT antibody similar experiments have not been performed with CAT mRNAs extracted from cells grown under the two different conditions. The CAT mRNAs synthesized in yeast grown with and without heme synthesis have the same transcriptional starts and also terminate in the same region since only one size class of mRNAs containing the entire coding sequence was detected in Northern blotting analysis. Thus the CAT mRNAs present in cells grown under the two different conditions are apparently identical. Consequently, it is likely that the CAT mRNAs present in heme-deficient cells also are translatable. The evidence presented here therefore strongly suggests that the expression in yeast of the chimaeric CAT gene is specifically controlled by heme at the level of translation.

The expression in yeast of a chimaeric gene consisting of only the 5′-flanking region and the NPTII gene is affected by the presence of heme to almost the same extent as the expression

Table IV. The effect of heme precursor on the expression of the chimaeric NPTII gene

	NPTII activity
Glucose + δ-ALA	30
Glucose	3

Heme-deficient cells were grown in the presence of ergosterol, Tween 60 and methionine. The strain employed was TM1 transformed with YEp5LbKm. The NPTII activity is expressed as nmol kanamycin phosphorylated/mg protein/h.

of the chimaeric CAT gene (Table IV). Northern blotting analysis showed that the amounts of NPTII gene transcripts are unaffected by alteration of the intracellular heme concentration. This indicates that the coding sequences and the 3′ Lb region are without significant importance for the heme-specific increase in translation observed for the respective mRNAs. It also implies that the molecular mechanisms in yeast mediating the specific heme effect are able to recognize the 5′ Lb non-coding region with the result that translation of mRNAs containing such sequences are affected.

Discussion

Like some other plant promoters the Lb promoter is recognized by yeast. Transcription directed by the Lb promoter is apparently not controlled by intracellular heme. It is, however, conceivable that possible heme-dependent interactions with the Lb promoter are imperfect due to the origin of the Lb sequences. Such interactions might easily be obscured by a copy number effect such that no effect on transcription is detected. Evidence presented here clearly demonstrates that heme specifically controls the expression of the chimaeric CAT gene by a post-transcriptional event, which most likely occurs at the level of translation. A chimaeric gene consisting only of the 5′-flanking region of the Lbc_3 gene and the NPTII gene is regulated in a similar way. Consequently, the sequences responsible for the heme specific-regulation of the chimaeric genes reside in the 5′-non-coding region of the mRNAs. The chimaeric CAT gene contains five nucleotides in front of the initiating ATG which originates from the *E. coli* CAT gene, but the corresponding sequence in the chimaeric NPTII mRNA is quite different and still its translation is affected by heme. Due to the method of construction of the chimaeric genes the 5′-non coding ends of the mRNAs contain two adjacent thymidine nucleotides 6 and 7 bp upstream from the initiating ATG codon which are present in neither the *E. coli* genes nor the plant gene.

It is highly unlikely that the presence of the two additional Ts is responsible for the heme effect observed on the translation of the chimaeric mRNAs. It is therefore concluded that yeast contains a heme-dependent regulatory system which is able to recognize the 5′ Lb non-coding region in such a way that the translation of mRNAs containing such a sequence at the appropriate position is affected.

The molecular mechanism mediating the heme effect on the translation of some hemoprotein mRNAs in yeast is unknown. Neither is it known whether heme regulates initiation or a later stage of translation. It is questionable whether more than a formal analogy exists between the specific effect on hemoprotein mRNA translation in yeast and the unspecific heme control of translation observed in reticulocytes (Lenz and Baglioni, 1977). An intriguing point is whether heme also controls the translation of Lb mRNAs in the plant nodule. Govers *et al.* (1985) have recently demonstrated that Lb gene expression in pea nodules is regulated by a post-transcriptional event. In view of the highly specific nature of the heme effect noted here, it is conceivable that the post-transcriptional control of Lb expression observed in pea nodules is mediated by heme. This would imply that the regulatory mechanisms exerting heme control of the translation of several hemoproteins in yeast have been preserved through evolution into higher plants.

Materials and methods

Restriction endonucleases, T4 polynucleotide kinase, T4 DNA ligase, DNA polymerase and *Bal*31 were purchased from Amersham, New England Biolabs and Boehringer. ^{32}P-Labeled compounds were purchased from NEN and Amersham. ^{14}C-Labeled chloramphenicol and oritidine-5′-monophosphate were from NEN. The synthetic oligonucleotide was purchased from Cruachem, Scotland.

Strains

The yeast strains DBY747 (Mata, *his 3-Δ*^{1}, *leu*^{2}*-3, leu2-111, ura3-52*) and TM1 (Matα, *hem1, ura3-52*) were employed in this study.

Growth conditions and transformation

Yeast strains were grown in a medium containing 0.2% yeast nitrogen base without amino acids and ammonium sulfate, 0.2% urea, 2% carbon source and 40 μg/ml of required amino acids. The TM1 strain was supplemented with 0.1% Tween 60, 20 μg/ml ergosterol and 50 μg/ml methionine, or with 50 μg/ml δ-ALA. Transformation of yeast followed the procedure of Ito *et al.* (1983).

Preparation of yeast extracts

Cells were harvested at a cell density of 5×10^6 cells/ml by centrifugation and resuspended in 1/50 vol of extraction buffer (50 mM Tris-HCl pH 7.5, 400 mM NaCl, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride). The cells were disintegrated in a French Press and cell debris removed by centrifugation. The supernatants were immediately frozen in liquid nitrogen and stored at −80°C.

Enzymatic assays

CAT activity was determined according to Walker *et al.* (1983). The reaction time was 1 h. NPTII activity was assayed by the procedure of Haas and Dowding (1975) with the following modification. The ATP solution consisted of 3 mM ATP instead of 0.75 mM ATP. Orotidine-5′-phosphate decarboxylase assay was according to Wolcott and Ross (1966) using ^{14}C-labeled orotidine-5′-phosphate as a substrate.

Construction of vectors

A DNA fragment comprising 2100 bp upstream from the initiator codon of the soybean Lbc_3 gene and 100 bp of the coding sequence was treated with *Bal*31. The reaction was stopped after 2, 3 and 4 min, respectively, and the resulting fragments were cloned into pBR322 after addition of *Eco*RI linkers. The resulting clones were analysed by DNA sequencing. One of the clones contained an *Eco*RI fragment terminating 7 bp 5′ to the initiator codon and this was used for further manipulation. An *Xho*I fragment stretching from 3 bp 3′ to the Lbc_3 stop codon and 1 kb further downstream was linked to the 2-kb Lbc_3 5′-flanking regions after addition of *Eco*RI linkers to generate a promoter cassette in which structural sequences can be inserted through an *Eco*RI site.

The CAT gene from pBR328 was isolated on a fragment generated by an incomplete double digestion with *Alu*I and *Taq*I. The *Alu*I site is located 5 bp 5′ to the CAT initiator codon and the *Taq*I site is 100 bp 3′ to the CAT stop codon. Part of the 3′-untranslated sequence was removed by *Bal*31 treatment leaving 31 bp downstream from the stop codon. This fragment was fused to the Lb promoter cassette after addition of *Eco*RI linkers. Finally, this construct was inserted into the yeast plasmid YEp24 through the *Bam*HI and *Sal*I sites present in this vector. The resulting vector is called YEpLbCAT.

Plasmid YEp5LbKm is a derivative of YEpLbCAT where the *Eco*RI − *Pvu*II fragment containing the CAT gene and the 3′-non-coding Lb sequence was replaced by a *Sau*3A − *Pvu*II fragment from pKm2 (Beck *et al.*, 1982) encoding NPTII. This fragment contains the NPTII-coding sequence including 2 bp in front of the initiator codon and 200 bp 3′ to the stop codon.

Primer extension and S1 nuclease analysis

Primer extension was according to Boel *et al.* (1983). For each reaction 50 μg of total RNA was used. S1 nuclease mapping was according to Nasmyth (1983). The labelled DNA fragment was complementary to the chimaeric gene sequence stretching from 500 bp 5′ to the initiation codon to 34 bp downstream of the initiator codon.

RNA isolation and Northern blotting

The RNA was isolated essentially according to Broach *et al.* (1979) except that the cells were broken in the presence of 1 vol of phenol:chloroform:isoamyl alcohol (24:24:1).

The Northern blotting and hybridization were according to Marcker *et al.* (1984).

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