The EMBO Journal vol.5 no.3 pp.459–465, 1986

Transcription and expression of zein sequences in yeast under natural plant or yeast promoters

I.Coraggio, C.Compagno[1], E.Martegani[1], B.M.Ranzi[1], E.Sala, L.Alberghina[1] and A.Viotti

Istituto Biosintesi Vegetali, C.N.R., Via Bassini, 15, and [1]Dipartimento Fisiologia e Biochimica Generali, Università, Via Celoria, 26, 20133 Milano, Italy

Communicated by S.Ottolenghi

Maize genomic fragments containing the regulatory and coding regions of a zein gene for a low size class 23-kd protein have been inserted in an interspecific *Escherichia coli–Saccharomyces cerevisiae* expression vector in different constructions. The presence of the inducible *GAL1–10* upstream activation site (UAS) allows us to regulate differentially by carbon sources the transcription of the zein gene both under the plant promoter and under the yeast *CYC-1* promoter. We found that the zein promoter region is properly recognized at the correct transcription start, while different termination points occur during transcription. The yeast UAS was also shown to function as a typical eukaryotic enhancer regardless of its distance or orientation with respect to the plant promoter. Yeast cells transformed by a plasmid containing a zein sequence fused to a short piece of the *CYC-1* gene produced a fused polypeptide, of expected mol. wt, in variable amount from 0.2 to 5% depending on the growth phase conditions.

Key words: yeast transformation/plant promoter/zein expression/modulation of expression

Introduction

The zeins, a group of hydrophobic polypeptides divided into two main size classes of 23 and 27 kd are coded by a multigene family of ~120 sequences localized on three chromosomes of the haploid maize complement (Viotti *et al.*, 1979, 1982, 1985; Hagen and Rubenstein, 1981; Soave and Salamini, 1984). The sequence analysis of several zein genes suggests the absence of introns from all the members of the multigene family, and in some cases the occurrence of anomalous genes that can produce truncated polypeptides or may be silent and not expressed as protein (pseudogenes) (Wienand *et al.*, 1981; Spena *et al.*, 1983; Kdril *et al.*, 1984; Viotti *et al.*, 1985).

The zein genes are mainly expressed in the endosperm tissue, where the protein precursors are matured by cleavage of the leader peptide and the mature zeins accumulate in the protein bodies (Larkins *et al.*, 1979; Hurkman *et al.*, 1981; Viotti *et al.*, 1978). The regulation of zein genes at the molecular level is far from clear, even though studies on several mutated loci (possibly regulatory) and on their interaction in different genetic combinations indicated that at least two different regulatory pathways for the 23- and 27-kd size classes or sequence prototypes exist (Di Fonzo *et al.*, 1979; Soave and Salamini, 1984; Viotti *et al.*, 1985). In one case, sequence analysis and studies on the expression in a heterologous system (Langridge and Feix, 1983; Langridge *et al.*, 1984) have shown that the two promoters at the 5′ upstream region of both high and low size class zein genes are differentially active in yeast cells.

To gain more insight into the zein system we started to clone the various zein genes with or without their own promoter, or with heterologous regulatory sequences in yeast or other eukaryotic vectors. Here we report the construction of some recombinants obtained by insertion of zein sequences in a yeast expression vector and evidence of variable transcription and expression of zein sequences in yeast under different growth conditions. The abundance of single zein polypeptides that can be obtained, and easily purified, opens the way to chemical–physical studies of modified zeins in order to define structural constraints (Viotti *et al.*, 1985) for this agronomically important seed storage protein.

Results

Construction of yeast plasmids

To determine whether the zein promoter functions in yeast and whether it is regulated from the upstream activator site (UAS) of *GAL1–10* (Guarente *et al.*, 1982), the 2000-bp *Sal*I–*Sal*I fragment containing the zein gene E19 and its flanking sequences was inserted in the *Xho*I site of the pLGSD5 vector in both orientations to yield the clones pL15A and pL15B (Figure 1). For other type of investigations (see below) and to compare the above constructions with recombinants bearing zein sequences but with an unmofidied UAS/*CYC-1* promoter distance, a zein gene fragment was inserted downstream the *CYC-1* promoter. The *Sma*I–*Hinc*II fragment of 1089 bp from the subclone pUE20, containing a piece of the zein gene E19, has been inserted by blunt end ligation in the *Bam*HI site of pLGSD5 after DNA polymerase I treatment (clone pL27, Figure 1).

During construction of pL27, another clone, called pL26, was selected and identified as a recombinant with a double insertion of the *Sma*I–*Hinc*II fragment, with the two fragments in the same orientation as in pL27. The frame of the zein sequence to the *CYC-1* sequence of pLGSD5 at the junction site was assessed by sequencing both strands (Figure 1).

The resulting plasmids contain the yeast 2 μm plasmid origin of replication and the yeast *URA-3* gene, which allows selection in *ura-3* cells growing in medium lacking uracil. For comparison of the transcription specificity under galactose induction, the *Sal*I–*Sal*I E19 fragment was also inserted in the single *Sal*I site of the YRp7 yeast vector (clone pY7Z).

Zein sequence transcription in yeast

Recombinant plasmids were used to transform the yeast auxotrophic strain X-4004. After plating on selective media some of the auxotrophic colonies from the different transformation experiments were tested for the presence of zein transcripts or for zein-immunoreactive translation product. About 85% of the auxotrophic clones were found to be positive for zein transcription and succesive plating analysis indicated that these transformants are stable for at least 70 generations under selective conditions (Alberghina *et al.*, 1985). For zein transcription analysis total

© IRL Press Limited, Oxford, England

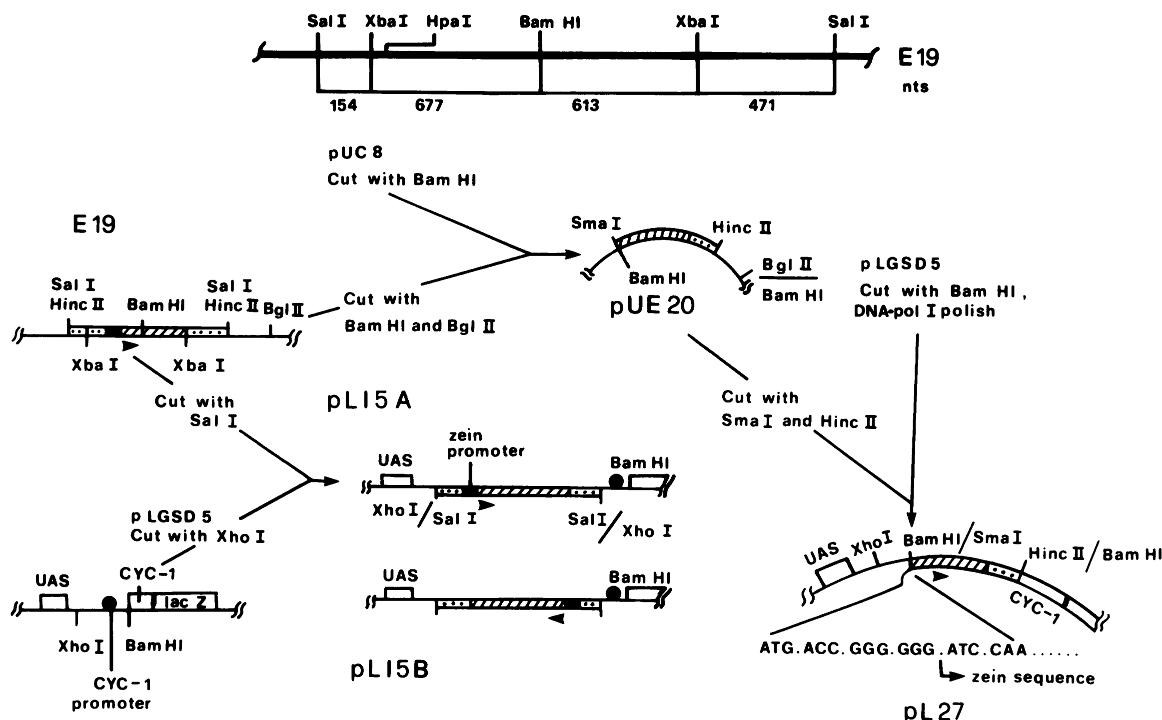

Fig. 1. Schematic presentation of yeast plasmid constructions. Fragments of the E19 zein gene (Spena *et al.*, 1983) have been inserted in different ways in the single *Xho*I or *Bam*HI sites of pLGSD5 yeast vector. The *Bam*HI–*Bgl*II fragment has been firstly inserted in the *Bam*HI site of pUC8 and then, by *Sma*I–*Hinc*II cuts, inserted by blunt end ligation in the *Bam*HI site of pLGSD5 treated by DNA polymerase I in the presence of only deoxyguanosine triphosphate. The sequence at the junction site is reported (clone pL27). The *Sal*I–*Sal*I fragment has been inserted in both orientations in the *Xho*I site giving the pL15A and pL15B constructions. The same fragment was inserted in the YRp7 *Sal*I site (not shown). The open boxes are relevant regions in pLGSD5. In the E19 sequence the dotted boxes are the flanking sequences of the gene, the closed and hatched boxes are, respectively, the promoter and the transcribed regions. Arrow heads indicate the directions of transcription from 5′ to 3′ in maize and in yeast. The *CYC-1* promoter is indicated by a closed circle. Drawings not to scale.

cellular RNA was isolated from the different transformants, fractionated on a denaturing agarose gel, transferred to a nitrocellulose filter and hybridized to radioactive zein E19 DNA probe (*Xba*I–*Xba*I fragment 1289 bp, Figure 2). The clone pL15A shows a transcript of expected mol. wt (~970 nucleotides) that co-migrates with zein mRNA from maize endosperm, suggesting a correct initiation and termination of transcription, while pL15B originated two transcripts of higher mol. wt of ~1100 and 1350 nucleotides (Figure 2A). In both cases the transcripts are present only in galactose-grown cultures, indicating that *GAL1–10* UAS is also able to regulate the transcription of heterologous promoter. The pY7Z clone that contains the same *Sal*I fragment in a non-expression vector gives variable amounts of non-specific transcripts (Figure 2A, c).

The specificity of transcription due to galactose induction is strengthened by analysis of the pL26 and pL27 clones. In this case higher amounts of transcripts containing zein sequences were obtained; one of ~1 kb is induced by galactose but it is also present at a lower level in glucose-grown cells, while the other is present only in galactose-induced cultures and shows, as expected from the plasmid constructions and assuming correct recognition of the termination site, a length of ~700 nucleotides. It is important to note that pL26, which contains two zein fragments, gives a similar amount of transcript with equal nucleotide length to that of pL27.

Analysis of transcripts

The nature of the different zein transcripts found in the various transformants was ascertained by S1 mapping and strand-specific hybridization experiments.

Radioactive SP6 probes, obtained from transcription of E19 sequences (from its ATG to polyadenylation site) inserted in pSP64 and pSP65 plasmids (Green *et al.*, 1983), were hybridized to total RNA from yeast transformants (pL15A, pL15B, pL26, pL27 and pLGSD5) and to total maize RNA as control. Only the radioactive probe obtained from the strand that is not transcribed in maize gave a positive signal (data not shown), indicating that only the correct strand is transcribed in yeast and that transcription in pL15B also occurs under the maize promoter, even though it is distally inserted with respect to *GAL*-UAS. However the Northern blot experiments show that the transcripts have different lengths and, as suggested from preliminary results, they probably derive from uncorrected terminations rather than from different transcriptional start points. This evidence was confirmed by the results of zein expression at the translational level (Figure 5) and from data of the S1 mapping experiment.

Figure 3 shows the protected fragment derived from the S1 mapping experiments of the transcription start in maize and yeast. It should be emphasized that in maize the E19 gene belongs to a subfamily of zein sequences more or less related to each other (Viotti *et al.*, 1985) that are expressed during endosperm development.

One predominant fragment of ~230 nucleotides is obtained and a minor one ~15 nucleotides shorter, indicating that in the maize line W64A, transcription starts at about −60 nucleotides from the first ATG as with other zein genes belonging to this subfamily and also expressed in other maize lines (Matzke *et al.*, 1984; Viotti *et al.*, 1985). In yeast, poly(A) RNA from pL15A and pL15B gives fragments of similar length, that are more visible when hybrid-selected mRNA is used in protection experiments.

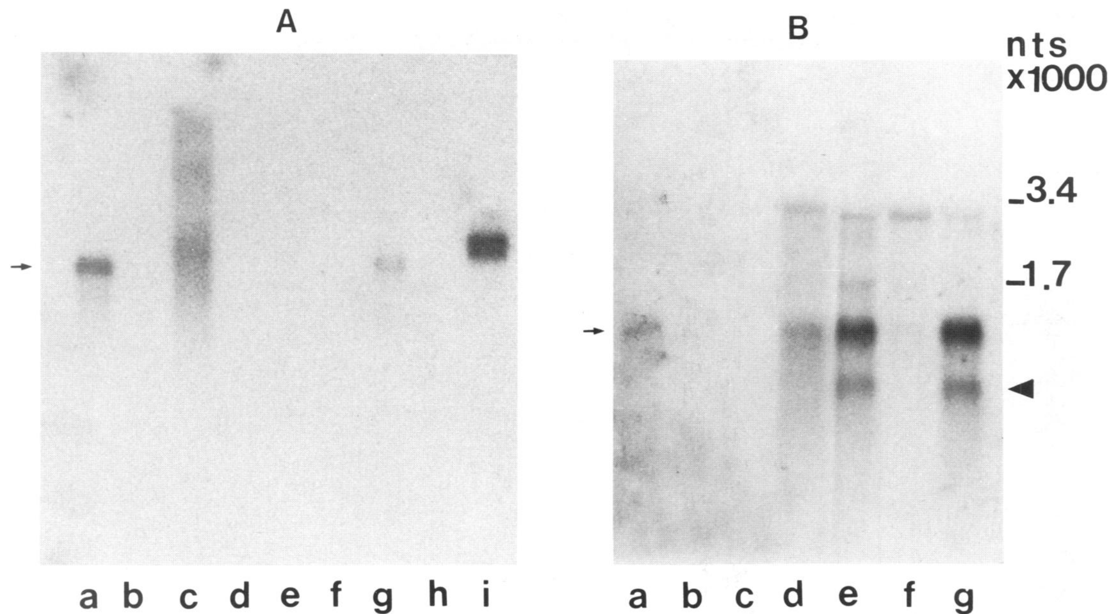

Fig. 2. Transcription of the zein sequence in yeast. Total RNA (20 μg) from the different yeast transformants grown in minimal medium supplemented with 2% carbon sources and harvested in exponential phase, was resolved on an agarose–formaldehyde gel, transferred to nitrocellulose and hybridized to a ^{32}P-labelled zein DNA restriction fragment. **Panel A.** Total RNA: from maize endosperm **(a)**; from YRp7 **(b)** and Y7Z **(c)** grown in glucose minimal medium; from pLGSD5 grown in glucose **(d)** or galactose **(e)**; from pL15A grown in glucose **(f)** or in galactose **(g)**; from pL15B grown in glucose **(h)** or in galactose **(i)**. **Panel B.** Total RNA: from maize endosperm **(a)**; from pLGSD5 grown in glucose **(b)** or galactose **(c)**; from pL27 grown in glucose **(d)** or in galactose **(e)**; from pL26 grown in glucose **(f)** or in galactose **(g)**. On the side the relative mobilities of yeast rRNAs (nucleotides) are indicated. The arrow indicates the zein mRNAs in maize. The arrowhead indicates the correct zein transcript specifically induced by galactose in yeast.

Other shorter protected fragments are visible in yeast pL15A and pL15B RNA, one of which corresponds to the minor fragment also present in maize. In protection experiments, in which hybrid-selected mRNA has been used, two radioactive bands of 596 and ~680 nucleotides are also visible; these probably correspond to DNA:DNA hybrids formed between the labelled fragments and some DNA molecules also selected by the filter-bound pUE01 zein clone (see lane 5, Figure 3).

Synthesis and accumulation of zein in yeast

The E19 gene, like some other zein genes (Spena *et al.*, 1982, 1983; Kridl *et al.*, 1984), contains an in-frame stop codon, in this case after 37 triplets from the first ATG. As a result, the E19 gene probably would not give any functional zein peptide in maize, or any immunoreactive protein in yeast. On the other hand, in preliminary experiments with modified E19 genes, with this stop codon mutated by site-directed mutagenesis into TAC or AAA codons, we noticed that pre-zein synthesized in yeast is only partially matured (cleavage of the signal peptide) and also partially degraded into three or four peptides.

To overcome these problems we constructed the pL27 clone (Figure 1) by fusing a coding fragment of the E19 gene, devoid of the signal coding part and of the stop codon, to the *CYC-1* gene of the pLGSD5 vector. The chimeric gene contains: the 5′ leader sequence of the *CYC-1*, the ATG, three codons derived from the construction, followed by the E19 fragment that codes for 178 amino acids of mature zein and the trailer part of the E19 gene (Figure 1). This construction allowed us to follow zein peptide synthesis and stability, independently from its maturation process: the system depends upon an unmodified promoter–transcription start distance, and on the occurrence of a homologous 5′ leader mRNA sequence for a correct mRNA–ribosome self recognition in yeast.

Transformants from pL27 and pL26 clones, selected by uracil auxotrophy, were replated individually on nitrocellulose filters layered on a minimal plate supplemented with galactose. Expression of zein polypeptides can be easily detected by colony immunoassay as reported in Figure 4. To better characterize the immunoreactive product we took advantage of the ethanol solubility of zein proteins. Total protein extracts and ethanol-soluble proteins were fractionated on polyacrylamide gels and subjected to Western blot analysis using antiserum to zein.

The pL27 and pL26 yeast transformants exponentially grown in galactose minimal medium contain an immunoreactive protein, absent from the extract of pLGSD5 transformants, of the expected mol. wt of ~19 500 (Figure 5).

To study the kinetics of induction of the expression of zein gene, pL27 transformants were grown in glucose minimal medium to 5×10^6 cells/ml (exponential phase) and then collected and transferred to galactose minimal medium. At intervals, aliquots of culture were removed and total protein or total RNA was extracted. Figure 6 shows the Northern blot analysis of zein transcripts at different times after induction. A detectable level of specific zein mRNA appears after 10 h of galactose induction with a continuous increase up to 24 h (early stationary phase, 2×10^7 cell/ml). A parallel analysis of protein extracts shows, in agreement with the mRNA levels, that zein polypeptide appears at the same time and reaches its maximum in the stationary phase of cell growth (Figure 7).

A quantitative analysis done on total protein extracts from pL27 cultures grown in galactose medium shows that in stationary phase zein-immunoreactive material reaches ~5% of total proteins, while in the exponential phase it represents only 0.2% (Figure 7). This increase does not depend upon the plasmid copy number, which was shown to be constant (~10 copies/cell) during the various growth phases (data not shown).

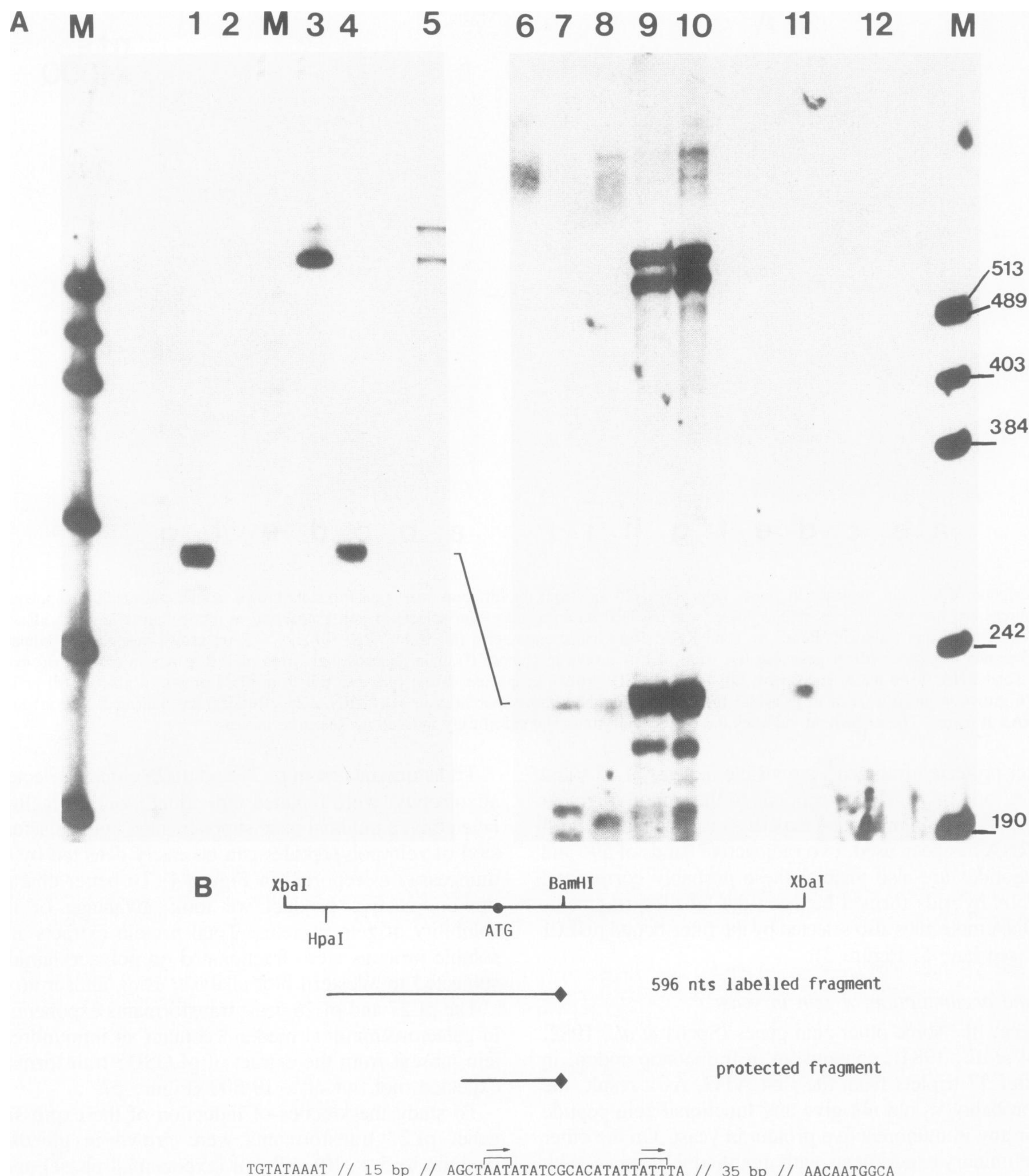

Fig. 3 (A) S1 nuclease mapping of the transcription start of the E19 gene in yeast and maize. Poly(A) RNA from maize and yeast or hybrid-selected mRNA from yeast hybridized to a *Hpa*I–*Bam*HI 596-bp fragment labelled at the 5′ *Bam*HI end. The DNA fragment was hybridized to RNAs for 18 h at 52°C in 60% formamide, Pipes 10 mM, NaCl 400 mM pH 6.8, treated with S1 nuclease at 30°C in 600 μl of sodium acetate 30 mM, NaCl 300 mM, $ZnCl_2$ 3 mM, 50 μg ssDNA, pH 4.5 for 2 h and then analyzed by electrophoresis on a 6% polyacrylamide/8 M urea sequencing gel. **Lane 3**, labelled fragment. **Lanes 2** and **12**, fragments treated with 100 and 200 units of S1 nuclease, respectively. The protected fragments were: from poly(A) RNA maize endosperm 1 μg **(lanes 1, 4)** or 0.1 μg **(lane 11)**; from poly(A) RNA yeast strains pL15A 1 μg **(lane 7)** or pL15B 0.3 μg **(lane 8)**; from hybrid-selected mRNA of yeast strain pL15A 3 μg **(lane 9)** or pL15B 1 μg **(lane 10)**. **Lane 5:** labelled fragment annealed to clone pUE01 and treated with 200 units of S1 nuclease. In **lane 6** the labelled fragment was annealed to 100 μg of total yeast RNA from pLGSD5. **Lane M** represents radioactive markers derived from pUC8/*Hpa*II fragments labelled with [α-^{32}P]dCTP by Klenow treatment. On the side the fragment size is reported in nucleotides. **(B)** Schematic presentation of labelled and protected fragments. In the sequence the transcription starts are indicated (arrows).

Discussion

Zein sequences have been cloned recently by chromosomal integrative vectors in yeast and sunflower (Langridge *et al.*, 1984; Matzke *et al.*, 1984). In both cases only transcription of zein sequences has been obtained without the production of any detectable translation product, even though correct initiation and the presence of translatable mRNA have been demonstrated.

In this paper we have reported for the first time the synthesis and accumulation of a zein polypeptide in a heterologous system.

Zein sequences borne by the episomal vector are also correctly transcribed, both from natural plant and yeast promoters. In the case of pL15A and pL15B constructions the start of transcription in yeast chiefly occurs at the same nucleotide as in maize, −60 from the first ATG. This suggest that *GAL*-UAS, at least

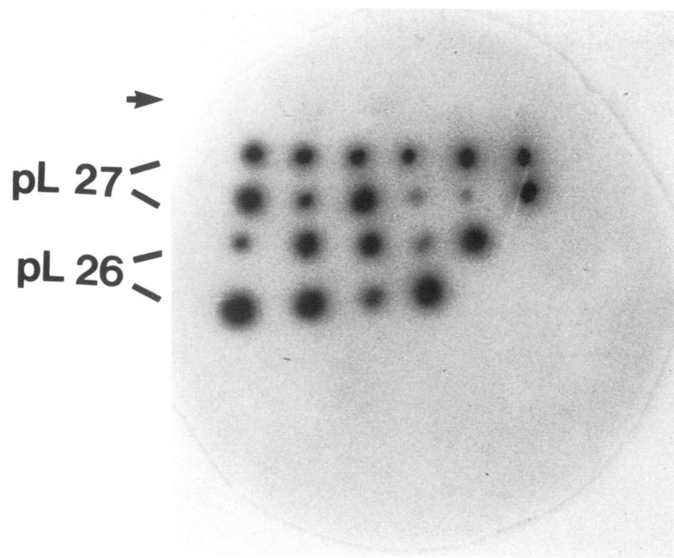

Fig. 4. Detection of zein polypeptide synthesis in yeast colonies containing pL27 and pL26 plasmids. Cells were grown on a nitrocellulose filter overlayered on a galactose minimal plate. Colonies were lysed and the filter decorated with zein antibodies and [^{125}I]protein A as described. As control, a row of yeast colonies transformed with pLGSD5 was applied (arrow).

in these constructions, shows all the typical features of the higher eukaryotic enhancer (Guarente, 1984; Gruss, 1984) providing the recognition of close or distant promoters also with inverted orientation.

However, in pL15B a slightly higher transcription is obtained than in pL15A and longer transcripts occur when the zein promoter is inversely orientated with respect to the *GAL*-UAS region (transcripts of 1100 and 1350 nucleotides). Probably the UAS, by destabilizing the chromatin structure in its vicinity, determines the recognition by polymerase II of different termination points. In this respect several yeast termination sequences -TAGT- or -TATGT- (Zaret and Sherman, 1982) followed by AT-rich and T-rich regions are present in the trailer part of the zein sequence. The length heterogeneity at the 3′ terminus, however, may also be due to different degrees of polyadenylation.

Yeast cells transformed with pL26 or pL27 plasmids express rather high levels of inducible zein mRNA and almost all ura^+ clones synthesize immunoreactive zein polypeptides as demonstrated by colony immunoassay. In our hands colony immunoassay represents a fast and sensitive method for screening directly in yeast for the expression of cloned heterologous genes.

The immunoreactive zein polypeptide is also extracted as ethanol-soluble material, demonstrating that it has the solubility properties of true zein; gel electrophoresis, followed by Western blotting, indicates the presence of a single polypeptide of the expected mol. wt, in agreement with the synthesis of a fused polypeptide that lacks the signal peptide and thus should not undergo subsequent processing steps. The amount of zein present in the transformed cells during exponential growth in galactose was rather low, $\sim 0.1-0.2\%$ of total protein, in the same range found by other authors for heterologous protein expressed in yeast under the *GAL1−10* promoter (Stepien *et al.*, 1983). However greater amounts of zein are obtained during the induction experiments and, surprisingly, a >20-fold increase of zein polypeptide was observed in stationary phase cultures grown in galactose, with $\sim 5 \times 10^6$ zein molecules per cell. Other plant

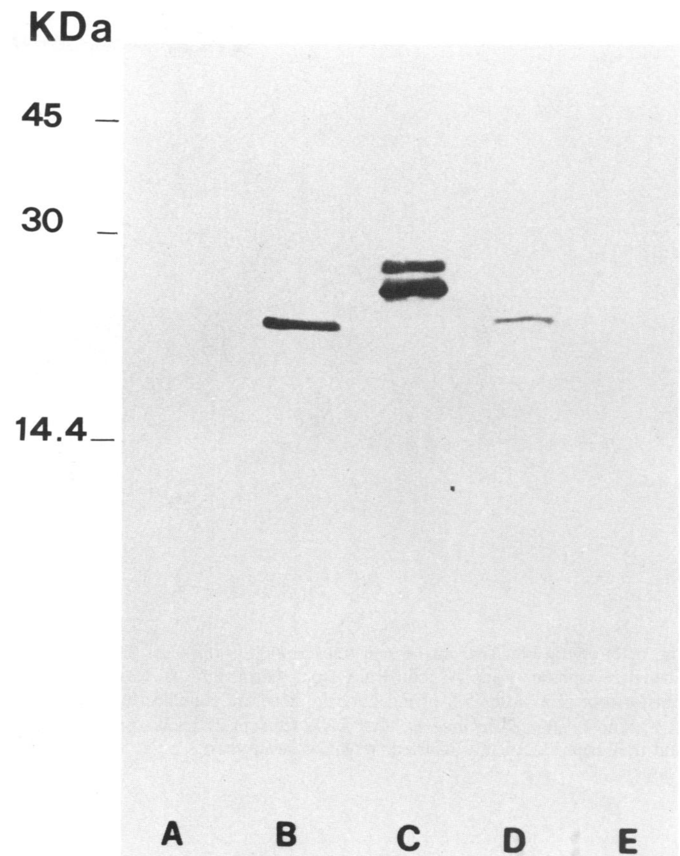

Fig. 5. Immunological detection of zein poypeptide synthesized in yeast transformants grown in galactose minimal medium and fractionated on SDS-PAGE. Ethanol-soluble protein extracted from pLGSD5 **(lane A)** or from pL27 **(lane B)**. **Lane C**, 2 μg of maize zein. Total yeast proteins from pL27 **(lane D)** or from pLGSD5 **(lane E)**. Standard proteins are: ovalbumin (45 kd); carbonic anhydrase (30 kd); lysozyme (14.4 kd).

proteins have recently been expressed in yeast with, however, a low level of synthesis probably depending on plasmid constructions and growth phase conditions (Edens *et al.*, 1984; Cramer *et al.*, 1985).

This finding underlines the relevance of the growth conditions for the optimization of the expression of heterologous proteins in yeast. Many factors can affect the amount of a heterologous product in yeast: e.g. the plasmid copy number, metabolic modulations of the rates of transcription and translation, mRNA stability and protein degradation, all of these can be affected by growth conditions (Mellor *et al.*, 1985).

Since the copy number of plasmids does not significantly change during the various growth phases, the observed zein accumulation in stationary phase is due to an increase of transcription, as shown in Figure 6, and probably also to a preferential or higher rate of zein mRNA translation and/or to a higher stability of the translated product.

Materials and methods

Strains, plasmids and media

The *Saccharomyces cerevisiae* strain X4004-3A (*a, lys5, trp1, met2, ura3*) was used for yeast transformation according to Hinnen *et al.* (1978). Transformants were selected for uracil auxotrophy by growth at 30°C on a minimal media containing 2% carbon source, 0.7% yeast nitrogen base (Difco) supplemented with 50 μg of amino acids per ml.

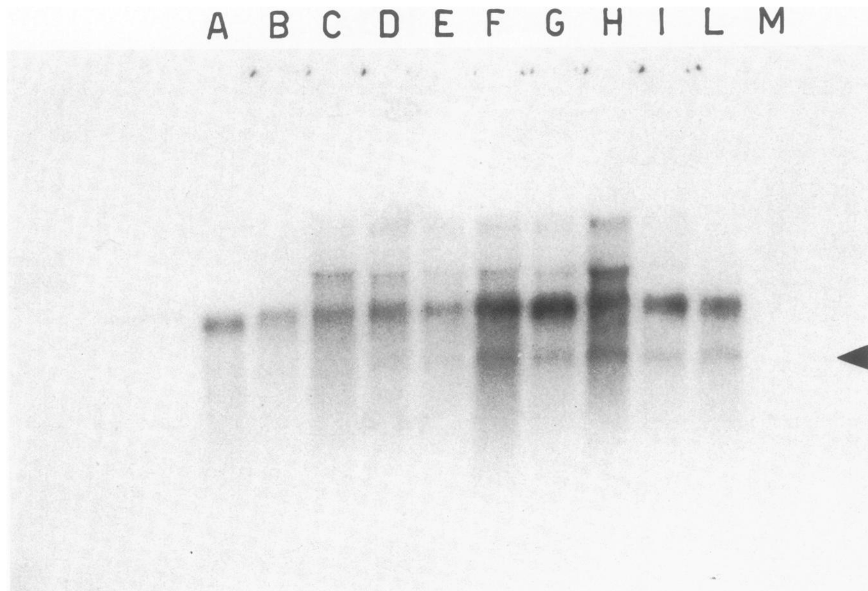

Fig. 6. Northern blot analysis of zein transcription at different time of induction. Yeast total RNA (20 μg) was resolved on the gel and hybridized to a ^{32}P-labeled zein probe. **Lane A**, total RNA from maize; **lane B**, total RNA from yeast transformant pL27 grown in glucose; **lane C**, total RNA from yeast transformant pL27 after 5 h of induction in galactose medium; **lane D**, after 7 h; **lane E**, after 10 h; **lane F**, after 15 h; **lane G**, after 18 h; **lane H**, after 21 h; **lane I**, after 24 h; **lane L**, total RNA from pL27 grown only in galactose for 24 h; **lane M**, total RNA from pLGSD5. Arrowhead indicates the correct zein transcript specifically induced by galactose in yeast.

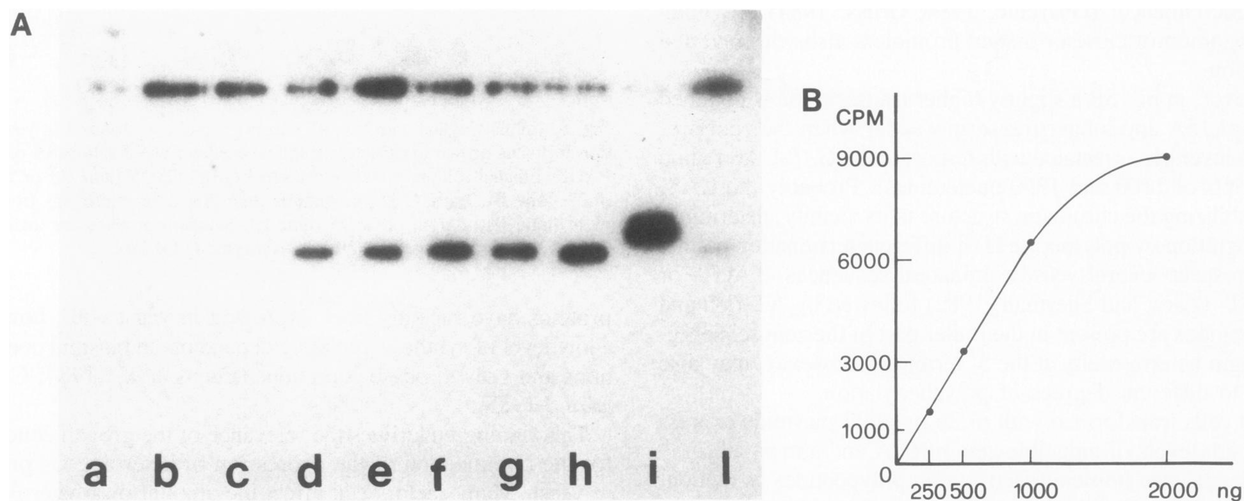

Fig. 7. (A) Analysis of protein extracts at different times of induction. Total yeast protein extracts (10 μg) were fractionated on SDS-PAGE and the zein polypeptide was detected by immunodecoration on electroblotted filters. **Lane a**, total yeast proteins from pLGSD5; **lane b**, total yeast protein from pL27 grown in glucose; **lane c**, total yeast protein from pL27 after 7 h of induction in galactose medium; **lane d**, after 15 h; **lane e**, after 18 h; **lane f**, after 21 h; **lane g**, after 24 h; **lane h**, after 40 h; **lane i**, 1 μg of maize zein; **lane l**, total yeast protein from pL27 exponentially grown in galactose. **(B)** Standard curve of immunoreacted zein polypeptide. Known amounts, as reported on the abscissa, of zein protein from maize were fractioned on SDS-PAGE and electroblotted on a nitrocellulose filter. The filter was treated with zein antibody and then immunodecorated with ^{125}I-labelled *S. aureus* protein A. The filter was exposed to X-ray film and the areas of the filter corresponding to the film image cut and counted in a liquid scintillator counter.

The plasmids pUC8, pLGSD5 (Guarente *et al.*, 1982) and YRp7 (Struhl *et al.*, 1979) were used for subcloning and for recombinant constructions using the 71-18 (*Δlac, pro^-, supE, thi^-/F′-lacI9, lac ZΔM15, pro B^+A^+*) and 79-02 (*r^-m^-, rec BC, str^r, lac Z^- Y^-, leu^-, thr^-, pro^-/F′-lac I^q ZΔM15, tra D36, proBA*) *Escherichia coli* strains as recipient cells. Recombinants were selected by phenotypic changes on X-gal plates or by colony hybridization using proper labelled fragments. Plasmids of putative colonies were subjected to restriction analysis to identify the correct orientation of fragment insertion. The sequence across the *CYC*−1−zein gene fusion junction was determined by chain termination sequencing according to Vieira and Messing (1982).

Colony immunoassay

Zein antibody was prepared by immunization of a New Zealand rabbit, according to Esen *et al.* (1983) with zein proteins (23 000−27 000 range) purified by SDS-gel electrophoresis. The whole serum was diluted 100- to 500-fold with TBSE (Tris-HCl 20 mM pH 7.5, NaCl 140 mM, EDTA 1 mM) for use in the immunobinding assay. Colony immunoassay of transformants was carried out according to Lyons and Nelson (1984).

RNA isolation, Northern gel and hybridization

Total yeast RNA was isolated according to Federoff *et al.* (1982). Total maize RNA extraction, fractionation of RNAs on denaturing agarose gel and hybridization of Northern filters were performed as previously described (Viotti *et al.*, 1982).

S1 mapping

The subclone pUE01, of the E19 gene (Figure 1) containing the *Sal*I−*Sal*I fragment in the pUC8 plasmid, was restricted with *Bam*HI, labelled at the 5′ end by the phosphatase-kinase reaction in the presence of [$\gamma^{32}P$]ATP and finally

digested with *Hpa*I. The fragment (596 bp) was electrophoresed on a strand separation gel and purified from the acrylamide slice according to Ofversted *et al.* (1984). The single end-labelled fragment was annealed to 0.3 – 1 μg of poly(A) RNA from yeast or to 0.1 – 1 μg of poly(A) RNA from maize endosperm. Annealing was also carried out on RNA selected by filter hybridization. About 200 μg of yeast RNA was hybridized to the nitrocellulose filter to which DNA of the pUE01 subclone had been attached (Viotti *et al.*, 1982) and the hybrid-selected mRNA was used as above. Annealing conditions, S1 treatment and analysis of protected fragments were carried out as previously described (Spena *et al.*, 1982).

Yeast extracts and protein gels

For the extraction of total yeast proteins, cell pellets (10^8 cells) were disrupted by vortexing with glass beads in 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF) and diluted in sample buffer to 2% SDS; 10% glycerol, 5% mercaptoethanol, 62.5 mM Tris-HCl pH 6.8. The samples were heated at 100°C for 5 min. The insoluble material was removed by centrifugation for 10 min in an Eppendorf microfuge.

For the extraction of ethanol-soluble yeast proteins, cell pellets (frozen to −20°C) were ground with alumina powder in cold mortars and extracted with 10 mM Tris-HCl pH 7.5, 1 mM EDTA. The mixture was vigorously shaken for 30 min and then centrifuged. The pellet was extracted first with 0.5 M NaCl and then with distilled water. The pellet was finally extracted with 70% ethanol for 2 h. After centrifugation, the supernatant was dried and resuspended in SDS-sample buffer. Samples were subjected to SDS-polyacrylamide gel electrophoresis according to Laemmli (1970).

Proteins were blotted to a nitrocellulose filter according to Towbin *et al.* (1979). The filter was extensively washed in TBS (0.01 M Tris-HCl pH 7.4, 0.9% NaCl) then in TBS + 0.05% Triton X-100 and finally saturated by 5% BSA in TBS. Blots were then incubated with antibody against zein diluted 1:500 in TBS, 5% BSA. After extensive washing the filter was incubated with peroxidase-conjugated mouse anti-rabbit IgG diluted 1:500 in TBS, 5% BSA and extensively washed. The visualization of immune complexes occurred by adding a solution containing 24 ml of TBS, 6 ml of 0.3% 4-chloronaphthol in methanol, 10 μl of H_2O_2 followed by incubation at room temperature for 30 min. Alternatively, immunocomplexes were detected with ^{125}I-labelled *S. aureus* protein A followed by autoradiography (Burnette, 1981). Zein in yeast extracts was quantitated either by dot immunoassay or after electroblotting and immunodecoration with ^{125}I-labelled *S. aureus* protein A, by cutting the nitrocellulose filter areas corresponding to each specific polypeptide, and by counting in a liquid scintillation apparatus. Different known amounts of pure zein were loaded onto the gel, and thus on the same electroblotted filter, in order to construct a standard curve.

Plasmid copy number

Purified DNA from transformed strains isolated by the method of Cryer *et al.* (1975) was completely digested with *Hin*dIII, fractionated on agarose gel and then blotted on nitrocellulose filter (Southern, 1975). The filter was hybridized with a *URA-3* probe labelled by nick translation and the copy number determined by densitometric scanning of autoradiographed X-Kodak films. The single copy chromosomal 1.1-kb *URA-3* fragment (Bach *et al.*, 1979) has been taken as internal standard.

Acknowledgements

This work was partially supported by the CEE-BEP grant no. GBI-4-106-I to L.Alberghina and A.Viotti and by the P.F. IPRA C.N.R. subproject 3, paper 750, no. 8401734 to B.M.Ranzi.

References

Alberghina,L., Martegani,E., Ranzi,B.M., Compagno,C., Coraggio,I. and Viotti,A. (1985) In *Plasmid Instability*, Abstracts CEE-BEP Meeting, Heraklion.

Bach,M.L., Lacroute,F. and Botstein,D. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 386 – 390.

Burnette,N.V. (1981) *Anal. Biochem.*, **112**, 195 – 203.

Cramer,J.H., Lea,K. and Slightom,J.L. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 334 – 338.

Cryer,D., Eccleshall,R. and Marmur,J. (1975) *Methods Cell Biol.*, **12**, 39 – 44.

Di Fonzo,N., Fornasari,E., Salamini,F., Reggiani,R. and Soave,C. (1979) *J. Hered.*, **71**, 397 – 402.

Edens,L., Bom,I., Ledeboer,A.M., Maat,J., Toonen,M.Y., Visser,C. and Verrips,C.T. 61984) *Cell*, **37**, 629 – 633.

Esen,A., Conroy,J.M. and Wang,S.Z. (1983) *Anal. Biochem.*, **132**, 462 – 467.

Federoff,H.J., Cohen,J.D., Eccleshall,T.R., Needleman,R.B., Buchferer,B.A., Giacolone,J. and Marmur,J. (1982) *J. Bacteriol.*, **149**, 1064 – 1070.

Green,M.R., Maniatis,T. and Melton,D.A. (1983) *Cell*, **32**, 681 – 694.

Gruss,P. (1984) *DNA*, **3**, 1 – 5.

Guarente,L. (1984) *Cell*, **36**, 799 – 800.

Guarente,L., Yocum,R.R. and Gifford,P. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7410 – 7414.

Hagen,G. and Rubenstein,I. (1981) *Gene*, **13**, 239 – 249.

Hinnen,A., Hicks,J.B. and Fink,G.R. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 1929 – 1933.

Hurkman,W.J., Smith,L.D., Richter,J. and Larkins,B.A. (1981) *J. Cell. Biol.*, **89**, 292 – 299.

Kdril,J.C., Vieira,J., Rubenstein,I. and Messing,J. (1984) *Gene*, **28**, 113 – 118.

Laemmli,U.K. (1970) *Nature*, **227**, 680 – 684.

Langridge,P. and Feix,G. (1983) *Cell*, **34**, 1015 – 1022.

Langridge,P., Eibel,H., Brown,J.W.S. and Feix,G. (1984) *EMBO J.*, **3**, 2467 – 2471.

Larkins,B.A., Pedersen,K., Handa,A.K., Hurkman,W.J. and Smith,L.D. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 6448 – 6452.

Lyons,S. and Nelson,N. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 7426 – 7430.

Matzke,M.A., Susani,M., Binns,A.N., Lewis,E.D., Rubenstein,I. and Matzke,A.J.M. (1984) *EMBO J.*, **3**, 1525 – 1531.

Mellor,J., Dobson,M.J., Roberts,N.A., Kingsman,A.J. and Kingsman,S.M. (1985) *Gene*, **33**, 215 – 226.

Ofversted,L.G., Hammarstrom,K., Balgobin,N., Hjerten,S., Pettersson,U. and Chattopadhyaya,J. (1984) *Biochim. Biophys. Acta*, **782**, 120 – 126.

Soave,C. and Salamini,F. (1984) *Phil. Trans. R. Soc. Lond. Ser. B*, **304**, 341 – 347.

Southern,E. (1975) *J. Mol. Biol.*, **98**, 503 – 517.

Spena,A., Viotti,A. and Pirrotta,V. (1982) *EMBO J.*, **1**, 1589 – 1594.

Spena,A., Viotti,A. and Pirrotta,V. (1983) *J. Mol. Biol.*, **169**, 799 – 811.

Stepien,P.P., Brousseau,R., Wu,R., Narang,S. and Thomas,D.Y. (1983) *Gene*, **24**, 289 – 297.

Struhl,K., Strinchcomb,D.T., Scherer,S. and Davis,R.W. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1035 – 1039.

Towbin,H., Staechelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350 – 4354.

Vieira,J. and Messing,J. (1982) *Gene*, **19**, 259 – 268.

Viotti,A., Sala,E., Alberi,P. and Soave,C. (1978) *Plant Sci. Lett.*, **13**, 365 – 375.

Viotti,A., Sala,E., Marotta,R., Alberi,P., Balducci,C. and Soave,C. (1979) *Eur. J. Biochem.*, **102**, 211 – 222.

Viotti,A., Abildsten,D., Pogna,N., Sala,E. and Pirrotta,V. (1982) *EMBO J.*, **1**, 53 – 58.

Viotti,A., Cairo,G., Vitale,A. and Sala,E. (1985) *EMBO J.*, **4**, 1103 – 1110.

Wienand,U., Langridge,P. and Feix,G. (1981) *Mol. Gen. Genet.*, **182**, 440 – 444.

Zaret,K.S. and Sherman,F. (1982) *Cell*, **28**, 563 – 573.

Received on 11 December 1985