The EMBO Journal vol.3 no.11 pp.2467-2471, 1984

# Transcription from maize storage protein gene promoters in yeast

**P.Langridge[1], H.Eibel, J.W.S.Brown and G.Feix**

Institut für Biologie III, Albert Ludwigs Universität Freiburg, Schänzlestrasse 1, D-7800 Freiburg, FRG
[1]Present address: Waite Agricultural Research Institute, Glen Osmond, South Australia 5064, Australia

Communicated by H.Kössel

**Genes coding for zein storage proteins of maize of the 19 000- and 21 000-dalton size classes were integrated into chromosome II of the yeast *Saccharomyces cerevisiae*. Using the transformed yeast strains as an *in vivo* transcription system it was shown that promoter regions of the zein genes of both size classes were accurately recognized. In particular, the zein mRNA synthesis in yeast starts at the same nucleotide positions used in the maize endosperm. A difference in the promoter activity between the 21 000- and the 19 000-dalton zein genes was observed.**

*Key words:* promoter/transcription starts/yeast/zein

## Introduction

The zeins are the alcohol-soluble, prolamin type storage proteins of maize consisting of two major size classes with mol. wts. of 21 000 and 19 000 daltons (Burr and Burr, 1976; Gianazza *et al.*, 1976). The zeins are coded for by a complex multigene system probably in excess of 100 genes although whether all of them are active remains to be established (Wienand and Feix, 1980; Soave and Salamini, 1982). The zein genes are exclusively expressed in the maize endosperm (Jones *et al.*, 1977) where zein proteins are synthesized by endoplasmic reticulum-bound polysomes and incorporated into protein bodies (Burr and Burr, 1976). The expression of zein genes is under the control of several regulatory loci and studies of the interactions between genetic mutations at these loci suggest that there exists at least one regulatory pathway for each of the 19 000- and the 21 000-dalton zein genes (Di Fonzo *et al.*, 1980). Analysis of the genetic organization of cloned zein genes has shown them to be preceded by at least two widely separated promoter regions, one promoter (P1) lying ~1000 bp upstream while the other (P2) is situated directly before the coding sequence (Langridge and Feix, 1983). As a homologous transcription system in maize has not yet been developed, several studies on the regulatory sequences preceding zein genes have been performed using heterologous test systems. The promoters on two zein genomic clones coding for the 21 000-dalton proteins were found to be active in *in vitro* transcription systems prepared from either HeLa cells or *Xenopus* oocytes. However, no genes coding for the 19 000-dalton zeins (four were tested) were active (P.Langridge and G.Feix, unpublished data).

To develop a system which would allow the *in vivo* expression and analysis of zein genes from both classes we attempted to study their expression in yeast. As an eukaryotic system, yeast is easily amenable to genetic manipulation and the expression of foreign genes has already been carried out successfully using either their own or yeast promoter sequences (Henikoff *et al.*, 1981; Valenzuela *et al.*, 1982; Rothstein *et al.*, 1984).

## Results and Discussion

From the maize clones pML1 (Langridge and Feix, 1983) and pMS1 (Wienand *et al.*, 1981), containing genes for the 21 000- and 19 000-dalton zein proteins, respectively, the maize inserts were transferred to the *Eco*RI site of the yeast integrative vector YIp35C (Eibel and Philippsen, 1983) to yield the hybrid plasmids YIpL1 and YIpS1 as shown in Figure 1. This integrative vector, carrying the *URA3* gene as selectable marker in *Saccharomyces cerevisiae* and an internal segment of the yeast *LYS2* gene, was used in preference to episomal vectors to avoid problems of instability during propagation in yeast. YIpL1 and YIpS1 were linearized with *Xho*I at the unique *Xho*I site inside the internal *LYS2* segment. Subsequent yeast transformation (Hinnen *et al.*, 1978) allowed the integration of the plasmids into the chromosomal *LYS2* gene of the *S. cerevisiae* host strain VB2-20A via homologous recombination (Orr-Weaver *et al.*, 1981; Eibel and Philippsen, 1983) (Figure 2A). The transformants selected for YIpL1 and YIpS1 (TL cells and TS cells) exhibited the predicted lys$^-$ phenotype due to the disruption of the chromosomal *LYS2* copy. The integration of YIpL1 and YIpS1 into the *LYS2* gene was confirmed as follows. Firstly, the transformants were unable to complement known *lys2* mutations in *lys2* tester strains. Secondly, Southern hybridizations (Southern, 1975) showed that YIpL1 and YIpS1 sequences were covalently linked to the *LYS2* locus on chromosome II. DNA from the TL and the TS transformants was cleaved with *Pst*I and transferred to nitrocellulose after electrophoretic fractionation. Their hybridization patterns using YIpL1, YIpS1, pML1 and pMS1 separately as radioactive probes (Figure 2B), show that the joining fragments between the chromosomal *LYS2* and the integrated plasmid sequences gave weaker signals when compared with those derived from internal fragments of the plasmids. This indicates that multiple copies of each plasmid have integrated tandemly into the *LYS2* gene. Similar events were reported from other loci when high amounts of linear plasmid DNA were used in yeast transformation (Winston *et al.*, 1983). From the differences in intensity between the single copy flanking sequences and the multiple integrated plasmid sequences, it is estimated that 5-10 copies had integrated into chromosome II after the transformation.

### *Transcription of the 21 000-dalton zein genes*

Transcription from the zein gene promoters in the transformed yeast lines was studied initially by Northern analyses (Thomas, 1980) hybridizing radioactively labeled pML1 and pMS1 probes against total yeast RNA. Signals were detected only with the TL and the TS transformants but not with RNA from the untransformed VB-20A cells (data not shown). The starts of the zein-specific transcripts were mapped more

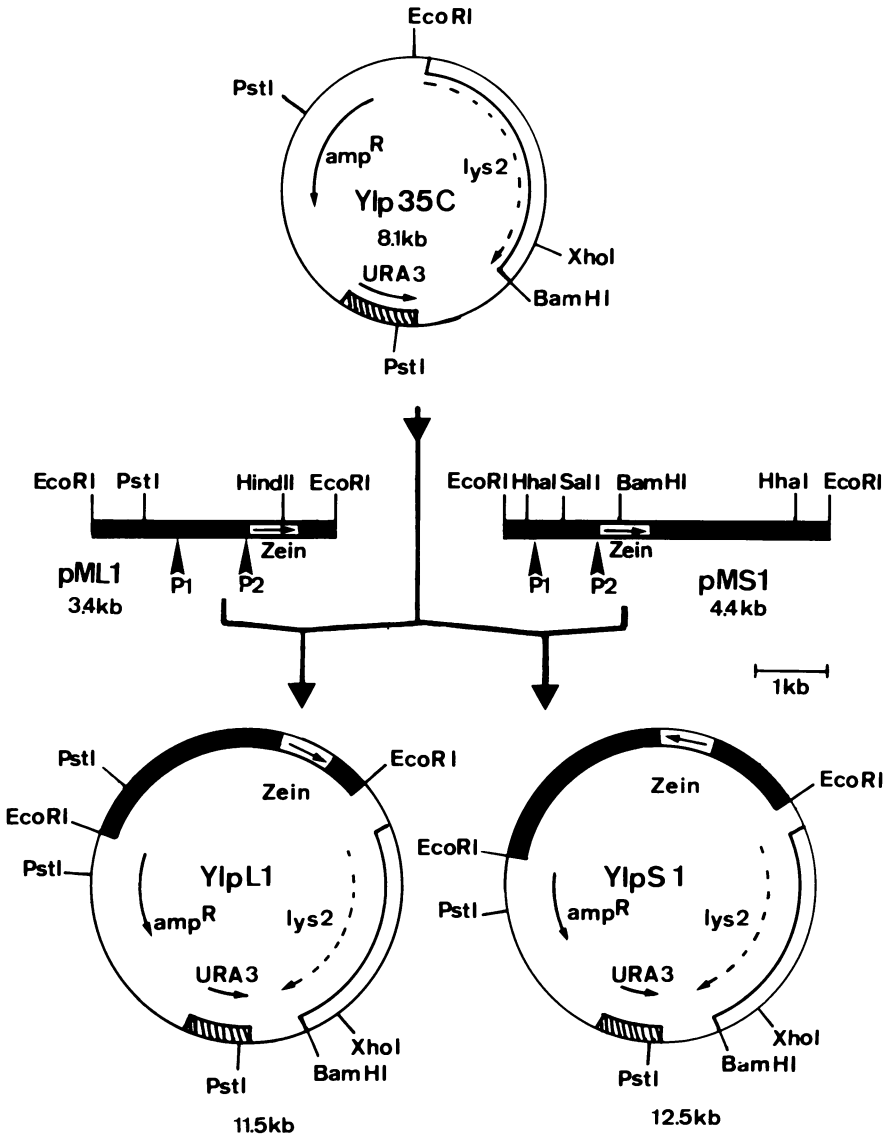



**Fig. 1.** Construction of the hybrid yeast-maize DNA plasmid vectors. The 3.4-kb *Eco*RI fragment from pML1 (Langridge and Feix, 1983) and the 4.4-kb *Eco*RI fragment from pMS1 (Wienand *et al.*, 1981) were subcloned separately into the *Eco*RI site of YIp35C (Eibel and Philippsen, 1983). The filled in boxes represent maize sequences, the localization of the zein genes and their transcription direction is indicated. The open boxes in YIp35C, YIpL1 and YIpS1 represent the internal segment of the *S. cerevisiae LYS2* gene, the hatched box the *URA3* gene. Arrows denote directions of transcription from 5′ to 3′. The arrows at P1 and P2 indicate the positions of the promoter regions of both types of zein genes.

precisely by S1 nuclease digestions (Sharp *et al.*, 1980). For the 21 000-dalton zein gene present in the YIpL1 transformed yeasts a *Pst*I – *Hind*II fragment of pML1 (Figure 1) containing both the P1 and the P2 promoter and the start of the coding sequence was used (Langridge and Feix, 1983). The end-labeled, radioactive DNA probe was denatured and hybridized to RNA prepared from TL yeast cells or to RNA isolated from maize endosperm. Unhybridized nucleic acids were digested with the single-strand specific nuclease from mung bean and the length of the nuclease-resistant DNA/RNA hybrid was measured on a polyacrylamide gel alongside appropriate size markers. The results (Figure 3A) indicate that with maize endosperm RNA three fragments are obtained (lane a). The upper band corresponds to the full-length *Pst*I-*Hind*II fragment (1800 bp) that has survived nuclease digestion by hybridization to its complementary DNA strand. The signals at 1500 bp and 660 bp represent DNA/RNA hybrids formed from transcripts starting at the P1 and the P2 promoters. The hybridization of the *Pst*I-*Hind*II probe against yeast RNA from the TL transformant shows no signal at the position corresponding to transcription from the P1 promoter. Instead, a 660-bp long RNA/DNA hybrid was obtained (Figure 3, lane b). This corresponds to a transcript faithfully initiated in yeast from the P2 promoter of the large zein gene.

*Transcription of the 19 000-dalton zein gene*

Similarly, transcription from the zein promoters of the YIpS1 transformed TS yeast (containing the genes for the 19 000-dalton zein proteins) was also studied by the mung bean nuclease digestion method. Here the DNA probe was prepared using the *Hha*I-*Bam*HI fragment of pMS1 (Figure 1) covering both promoters P1 and P2 as well as the start of the gene. Since cleavage by *Hha*I yields 3′ protruding ends which are labeled only poorly with polynucleotide kinase compared with the 5′ extensions produced by BamHI scission, the radioactive label is predominantly found at the *Bam*HI site. As with pML1, both promoters are used in maize endosperm

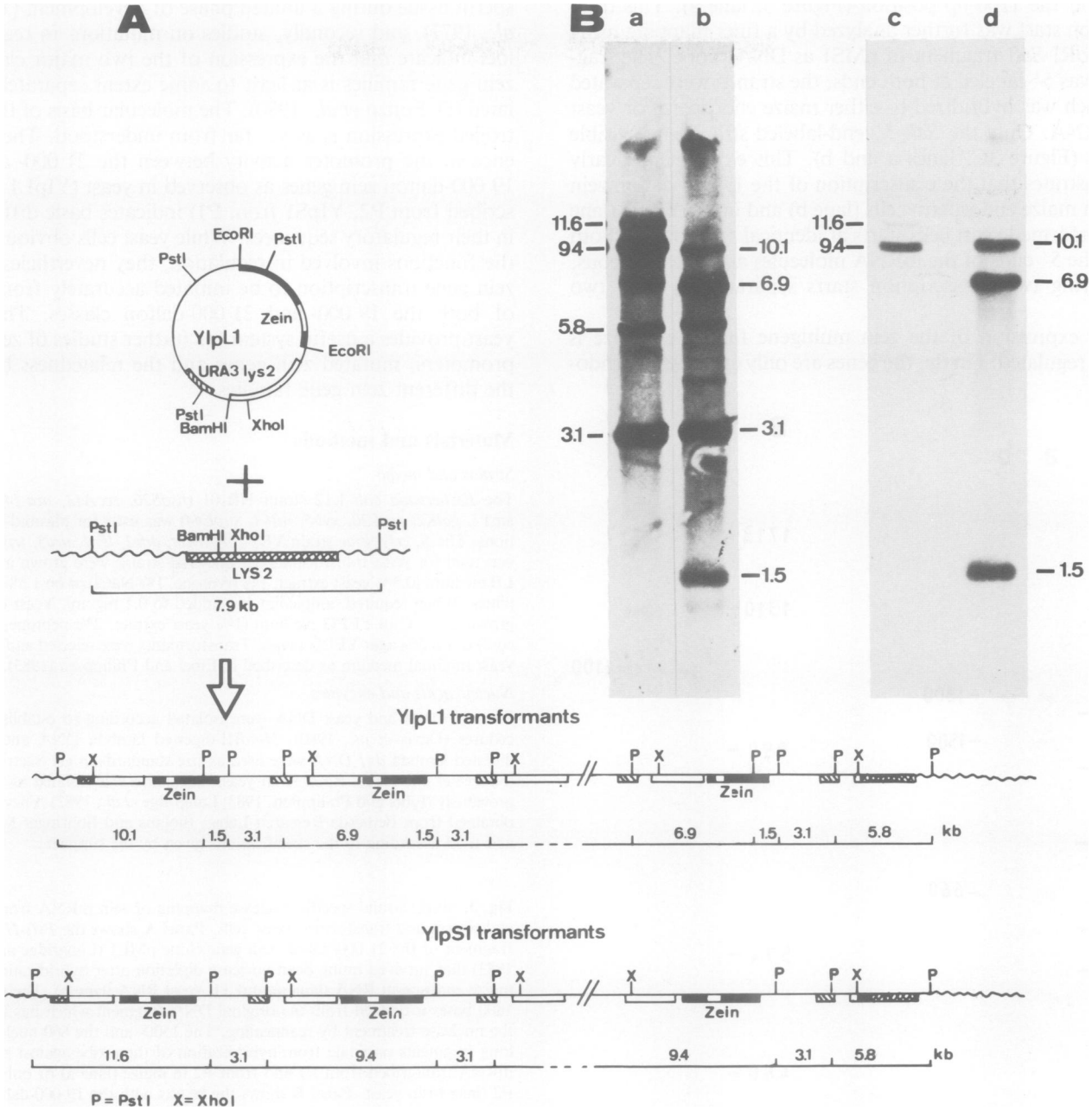
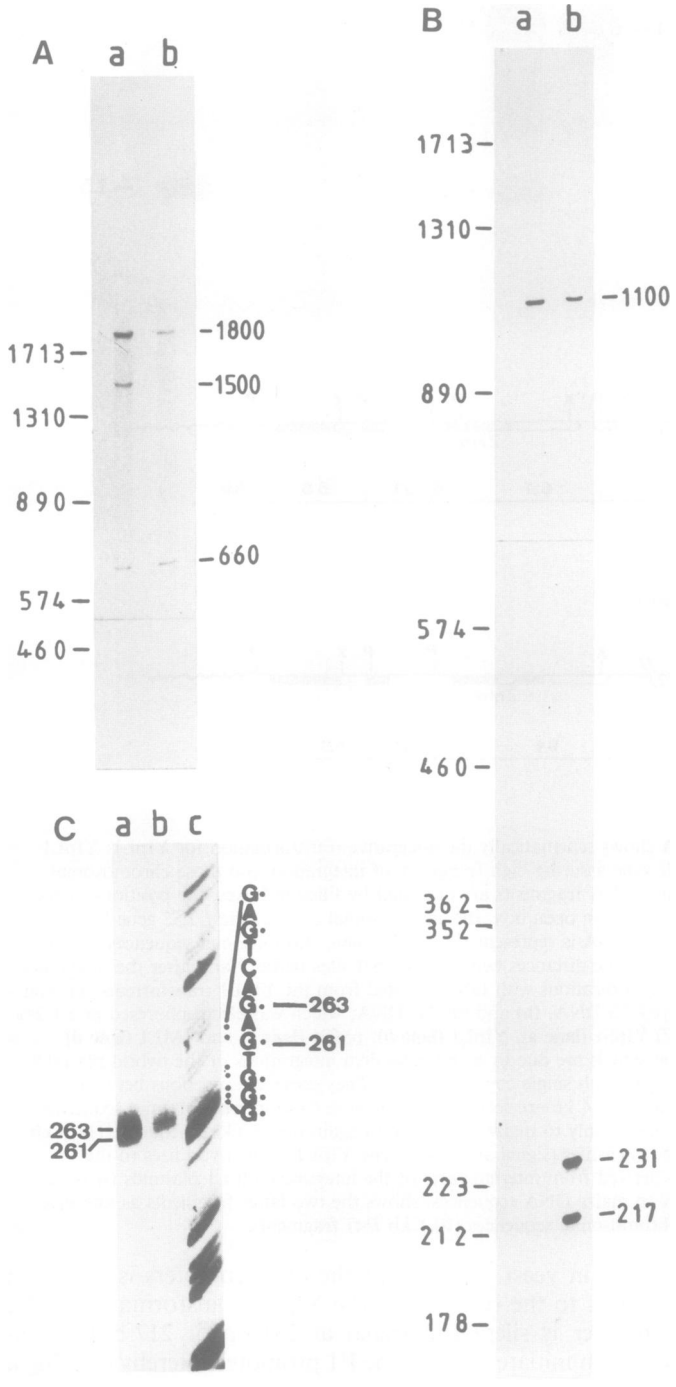



**Fig. 2.** Integration of the zein genes into chromosome II via *lys2* homology. **Panel A** shows schematically the integrative transformation for YIpL1: YIpL1 and YIpS1 were linearized by *Xho*I cleavage within the internal segment of the *LYS2* gene assuring high frequency of integration into those chromosomal sequences homologous to the free plasmid DNA ends (Orr-Weaver *et al.*, 1981). Maize DNA fragments are indicated by filled-in boxes, the positions of the zein genes are marked. The internal *lys2* gene segment carried by the plasmids is shown by an open box, the chromosomal copy of the *LYS2* gene by a double hatched box, the selective marker (*URA3*) by a hatched box. Integrated pBR322 DNA is represented by a thin line, chromosomal sequences by a wavy line. The positions of the relevant restriction sites are given: P, *Pst*I sites; X, *Xho*I sites. The distances between the *Pst*I sites before[11] and after the integration of YIpL1 and YIpS1 are marked by brackets. **Panel B.** Autoradiogram of Southern hybridizations with DNA isolated from the YIpL1 transformant TL and the YIpS1 transformant TS after *Pst*I cleavage. **Lanes (a)** and **(c)** represent *Pst*I-cleaved TS DNA, **(b)** and **(d)** TL DNA, which was electrophoresed in a 1.2% agarose gel and hybridized separately against radioactively labeled (Rigby *et al.*, 1977) YIpS1 **(lane a)**, YIpL1 **(lane b)**, pMS1 **(lane c)** and pML1 **(lane d)**. These probes are also shown in Figure 1. The different intensities of the hybridization signals are due to multiple tandem integrations of the hybrid plasmids at the *LYS2* locus. The YIpS1 probe shows *Pst*I fragments of 11.6 kb and 5.8 kb lengths with single copy intensities. They cover the junctions between integrated plasmid and chromosomal sequences. In addition stronger signals at 3.1 kb and 9.4 kb are seen. These originate from internal plasmid sequences which are present in multiple tandemly inserted copies. The pMS1 probe, which hybridizes only to maize DNA, shows again one flanking sequence (11.6 kb signal) with lower intensity and the amplified plasmid sequences of YIpS1 with higher intensities (signal at 9.4 kb). The YIpL1 probe hybridizes to the junction fragments (weaker signals at 10.1 kb and 5.8 kb) and to the *Pst*I fragments derived from internal parts of the integrated YIpL1 plasmids (stronger signals at 3.1 kb, 1.5 kb and 6.9 kb). The pML1 probe, which shares homology only to maize DNA sequences, shows the two latter fragments as intensive signals and one of the single copy fragments joining integrated plasmid DNA with chromosomal sequences (10.1 kb *Pst*I fragment).

to transcribe the pMS1 type genes (Figure 3B, lane b). The signal at 1100 bp is derived from RNA/DNA hybrids employing the P1 promoter and the two bands at 231 bp and 217 bp represent the transcripts starting at the P2 promoter. Again, in yeast only one of the two promoters is active: in contrast to the result from the YIpL1 transformant, the P2 promoter is silent (no signal at 231 bp or 217 bp). Transcription initiates only at the P1 promoter, thereby creating a

signal at the 1100-bp position (Figure 3, lane a). This transcription start was further analyzed by a finer mapping using the *Eco*RI-*Sal*I fragment of pMS1 as DNA probe. The fragment was 5′-labeled at both ends, the strands were separated and each was hybridized to either maize endosperm or yeast (TS) RNA. Only the *Sal*I 5′ end-labeled strand gave visible signals (Figure 3C, lanes a and b). This experiment clearly demonstrates that the transcription of the 19 000-dalton zein gene in maize endosperm cells (lane b) and in the YIpS1 (lane a) transformed yeast cells starts at identical positions. In both cases the 5′ ends of the mRNA molecules are heterogeneous, employing two transcription starts separated by only two bases.

The expression of the zein multigene family in maize is highly regulated. Firstly, the genes are only expressed in endosperm tissue during a limited phase of development (Jones *et al.*, 1977), and secondly, studies on mutations in regulatory loci indicate that the expression of the two major classes of zein gene families is at least to some extent separately regulated (Di Fonzo *et al.*, 1980). The molecular basis of this controlled expression is as yet far from understood. The difference in the promoter activity between the 21 000- and the 19 000-dalton zein genes as observed in yeast (YIpL1 is transcribed from P2, YIpS1 from P1) indicates basic differences in their regulatory sequences. While yeast cells obviously lack the functions involved in regulation, they nevertheless allow zein gene transcription to be initiated accurately from genes of both the 19 000- and 21 000-dalton classes. Therefore yeast provides a useful system for further studies of zein gene promoters, mutated zein genes and the relatedness between the different zein gene families.




## Materials and methods

*Strains and media*

The *Escherichia coli* K12 strain HB101 (*hsdS20, recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1, supE44*) was used for plasmid constructions. The *S. cerevisiae* strain VB2-20A (*Mat, ade2, leu2, ura3, trp1, LYS2*) was used for yeast transformations. Bacterial strains were grown at 37°C in LB medium (0.5% yeast extract, 1% tryptone, 1% NaCl) or on 1.5% agar LB plates. When required, ampicillin was added to 0.1 mg/ml. Yeast cells were grown at 30°C in YEPG medium (1% yeast extract, 2% peptone, 2% glucose) or on 2% agar YEPG plates. Transformants were selected and grown in yeast minimal medium as described in Eibel and Philippsen (1983).

*Nucleic acids and enzymes*

Plasmid DNA and yeast DNA were isolated according to established procedures (Davis *et al.*, 1980). *Hind*III-digested lambda DNA and *Hae*III-digested lambda *dv1* DNA were used as size standards in gel electrophoresis (Sanger *et al.*, 1982). RNA from yeast and maize was isolated as described previously (Eibel and Philippsen, 1983; Langridge *et al.*, 1982). Enzymes were obtained from Bethesda Research Labs., Biolabs and Böhringer Mannheim and used according to the specifications given by the suppliers.

**Fig. 3.** Single strand-specific nuclease mapping of zein mRNA from maize endosperm and transformed yeast cells. **Panel A** shows the *Pst*I-*Hind*II fragment of the 21 000-dalton zein gene clone pML1 (Langridge and Feix, 1983) that survived mung bean nuclease digestion after hybridization with maize endosperm RNA **(lane a)** and TL yeast RNA **(lane b)**. The signal at 1800 bases is derived from the original DNA fragment which has survived the nuclease treatment by reannealing. The 1500- and the 660-nucleotides-long fragments originate from hybridization of the probe against zein mRNA transcribed from P1 and from P2 in maize **(lane a)** or only from P2 **(lane b)** in yeast. **Panel B** shows the results with the 19 000-dalton zein gene using the *Hha*I-*Bam*HI fragment of pMS1 (Wienand *et al.*, 1981) as a probe. Three nuclease-resistant RNA/DNA hybrid fragments are observed when the hybridization was performed with maize endosperm RNA **(lane b)**: the signal at 1100 nucleotides corresponds to zein transcripts initiated at P1, the two signals at 231 and 217 bases represent the double start at P2. Hybridization against zein RNA synthesized in TS yeast cells **(lane a)** shows only one signal of 1100 nucleotides in length, indicating that zein transcription starts only at promoter P1. **Panel C.** Precise mapping of the transcription starts at the P1 promoter of the 19 000-dalton zein gene. The radioactively-labeled and strand-separated *Eco*RI-*Sal*I fragment of pMS1 was hybridized to RNA isolated from YIpS1 transformed yeast cells **(lane a)** and to maize endosperm RNA **(lane b)**. In both cases only the strand carrying the radioactive label at the *Sal*I-end gave nuclease-resistant hybrids. **Lane (c)** shows a DNA sequencing reaction (G track) carried out with this fragment which was run in parallel on the same gel. As the samples migrated on the gel with a 'smile', the positions of the G residues around the transcription start are marked by points. The double starts of the zein transcripts at the 263-bp and the 261-bp position are indicated and the actual DNA sequence (Langridge and Feix, unpublished results) is given to the right of the autoradiogram. The resulting signals of 263 and 261 bases in length are seen with both yeast and maize endosperm RNA, indicating that zein transcription is accurately intiated at the promoter P1 in yeast cells as well as in maize. The samples were electrophoresed on 6% polyacrylamide DNA sequencing gels alongside an end-labeled lambda *dv1 Hae*III-digested size marker.

*Transformations*

*E. coli* transformations were performed according to Davis *et al.* (1980), yeast transformations as described by Hinnen *et al.* (1978) with minor modifications (Eibel and Philippsen, 1983): VB2-20A (*ade2, trp1, leu2, ura3, LYS2*) was transformed with 10 μg of YIpL1 or YIpS1 DNA linearized with *Xho*I. Transformants were selected in supplemented minimal medium lacking uracil and tested for lysine auxotrophy caused by the disruption of the intact chromosomal *LYS2* gene upon plasmid integration (Orr-Weaver *et al.*, 1981). Transformants of the phenotype ura$^+$, lys$^-$ were further tested for stable mitotic plasmid inheritance (Stinchcomb *et al.*, 1979) which was observed with all isolates.

*Hybridization, DNA sequencing and radioactive labeling methods*

Hybridizations with nick-translated DNA probes (Rigby *et al.*, 1977) against restriction enzyme-digested yeast DNA which was transferred to nitrocellulose filter were performed according to Southern (1975).

The transcripts initiated at the different promoters were mapped using mung bean nuclease according to a modified version of the method of Sharp *et al.* (1980). The DNA 5′ end-labeling reactions were performed with polynucleotide kinase (Richardson, 1965), DNA sequences were determined by base-specific cleavage reactions (Maxam and Gilbert, 1980).

## Acknowledgements

The excellent technical assistance of Mrs E.Brutzer and Mrs H.Jonas is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 206 and FE 62/6-1).

## References

Burr,B. and Burr,F.A. (1976) *Proc. Natl. Acad. Sci. USA,* **73**, 515-519.
Davis,R.W., Botstein,D. and Roth,J. (1980) *Advanced Bacterial Genetics: A Manual for Genetic Engineering,* published by Cold Spring Harbor Laboratory Press, NY.
Di Fonzo,N., Fornasari,E., Salamini,F., Reggiani,R. and Soave,C. (1980) *J. Hered.,* **71**, 397-402.
Eibel,H. and Philippsen,P. (1983) *Mol. Gen. Genet.,* **191**, 66-73.
Gianazza,E., Righetti,D.G., Pioli,F., Galante,E. and Soave,C. (1976) *Maydica,* **21**, 1-17.
Henikoff,S., Tatchell,K., Hall,B.H. and Nasmyth,K.A. (1981) *Nature,* **298**, 33-37.
Hinnen,A., Hicks,J.B. and Fink,G.R. (1978) *Proc. Natl. Acad. Sci. USA,* **75**, 1929-1933.
Jones,R.A., Larkins,B.A. and Tsai,C.T. (1977) *Plant Physiol.,* **59**, 733-737.
Langridge,P. and Feix,G. (1983) *Cell,* **34**, 1015-1022.
Langridge,P., Pintor-Toro,J.A. and Feix,G. (1982) *Mol. Gen. Genet.,* **87**, 432-438.
Maxam,A.W. and Gilbert,W. (1980) *Methods Enzymol.,* **65**, 499-560.
Orr-Weaver,T.L., Szostak,J.W. and Rothstein,R.J. (1981) *Proc. Natl. Acad. Sci.,* **78**, 6354-6358.
Richardson,C.C. (1965) *Proc. Natl. Acad. Sci. USA,* **54**, 158-165.
Rigby,P.W., Dieckmann,M., Rhodes,C. and Berg,P. (1977) *J. Mol. Biol.,* **113**, 237-251.
Rothstein,S.J., Lazarus,C.M., Smith,W.E., Baulcombe,C.D. and Gatenby, A.A. (1984) *Nature,* **308**, 662-665.
Sanger,F., Coulson,D.R., Haig,G.F., Hill,D.F. and Petersen,G.B. (1982) *J. Mol. Biol.,* **162**, 729-773.
Sharp,P.A., Berk,A.J. and Bergert,S.M. (1980) *Methods Enzymol.,* **65**, 750-768.
Soave,C. and Salamini,F. (1982) *Qual. Plant., Plant. Foods Hum. Natr.,* **31**, 191-203.
Southern,E.M. (1975) *J. Mol. Biol.,* **98**, 503-517.
Stinchcomb,D.T., Struhl,K. and Davis,R.W. (1979) *Nature,* **282**, 39-43.
Thomas,P.S. (1980) *Proc. Natl. Acad. Sci. USA,* **77**, 5201-5205.
Valenzuela,P., Medina,A., Rutteer,W.J., Ammerer,G. and Hall,B.D. (1982) *Nature,* **298**, 347-350.
Wienand,U. and Feix,G. (1980) *FEBS Lett.,* **116**, 14-16.
Wienand,U., Langridge,P. and Feix,G. (1981) *Mol. Gen. Genet.,* **182**, 440-444.
Winston,F., Chumley,F. and Fink,G.R. (1983) *Methods Enzymol.,* **101**, 211-223.

*Received on 7 July 1984*