Synthetic oligonucleotide tails inhibit in vitro and in vivo translation of SP6 transcripts of maize zein cDNA clones

Gad Galili¹, Evelyn E. Kawata², Richard E. Cuellar¹, L. D. Smith² and Brian A. Larkins¹,²*

¹Department of Botany and Plant Pathology and ²Department of Biology, Purdue University, West Lafayette, IN 47907, USA

Received 26 September 1985; Revised 19 December 1985; Accepted 27 December 1985

ABSTRACT

A maize zein cDNA clone was used to synthesize mRNA with the SP6 in vitro transcription system. Although we obtained full-length transcripts of the cDNA sequence, these were inefficient templates for protein synthesis. Removal of the 5' oligo(G) sequence that was synthesized during the cDNA cloning procedure allowed efficient translation of the mRNAs in a wheat germ cell-free protein synthesis system or in Xenopus laevis oocytes. The alteration in translational efficiency did not result from an interaction of the 5' oligo(G) homopolymer tail with the 3' oligo(C) sequence, as transcripts with or without the oligo(C) tail were translated similarly in both protein synthesis systems. Ribosome interaction with the mRNA may be affected due either to the secondary structure of the oligo(G) sequence itself, or an unusual secondary structure between the oligo(G) sequence and another region in the mRNA. Synthetic oligonucleotides at the 5' end of cloned cDNA sequences may generally be inhibitory for translation of mRNAs transcribed in vitro.

INTRODUCTION

The storage proteins of maize seed consist of a group of alcohol-soluble polypeptides called zeins. These proteins are synthesized in the developing endosperm and accumulate in dense aggregates called protein bodies within the lumen of the rough endoplasmic reticulum (1). SDS polyacrylamide gel analysis of the proteins extracted from protein bodies reveals polypeptides with apparent mol wts of 27,000, 22,000, 19,000, 15,000, and 10,000 daltons (2). Sequence analysis of recombinant DNA clones corresponding to these proteins has shown that zeins consist of at least three structurally distinct types of proteins (3). The Mr 22,000 and Mr 19,000 zeins appear to be rod-shaped molecules that associate through hydrogen bonding and hydrophobic interactions (4), but in general the mechanisms by which zeins aggregate into protein bodies are unknown.

The SP6 vector system has proven very useful for the preparation of RNA probes for studying gene transcription (5) and RNA processing (6–8). In several studies it has also been used to transcribe RNAs functional for...
protein synthesis in vitro and in vivo (9-11). The latter capability makes it a particularly useful system to characterize proteins encoded by cDNA or genomic clones whose function is not understood. It can also be used to study proteins encoded by single genes, or groups of genes, and their interaction with one another or with various components of the cell (10). We have utilized this system to transcribe mRNAs corresponding to cDNA clones of maize zeins in order to study the properties of individual zein polypeptides. Although essentially full-length copies of zein mRNAs were transcribed with this system, they were ineffective templates for protein synthesis. Removal of the 5' oligo(G) sequence introduced during the cDNA cloning procedure resulted in mRNAs that were efficient templates. These mRNAs directed the synthesis of zein proteins that were processed into membrane vesicles in Xenopus oocytes.

MATERIALS AND METHODS

Restriction endonucleases and T4 DNA ligase were purchased from BRL, Bethesda, Md. The slow form of Bal 31 nuclease was from IBI, New Haven, Conn., and DNAse 1 was from Worthington, Freehold, N.J. 7m(5')Gppp(5')G was from P. L. Biochemicals, Milwaukee, Wis., [32P] dCTP (3000 Ci/mMole) and [3H] leucine were from ICN, Irvine, Calif. The pSP65 vector, SP6 polymerase, and RNAsin were purchased from Promega Biotech, Madison, Wis. A dideoxynucleotide DNA sequencing kit was purchased from Amersham, Arlington Heights, Ill.

Construction and transcription of SP6 plasmids.

The plasmid cZ19cl (3) contains a full length zein cDNA cloned into the polylinker of pUC8 (see Fig. 1). The oligo(G) homopolymer tail at the 5' end of the cDNA sequence was removed by Bal 31 nuclease digestion of cZ19cl following restriction digestion of the Eco Rl site in the pUC8 polylinker. The cDNA sequences were excised by restriction digestion with Hind III and transferred into Sma I, Hind III digested M13mpl1. To determine the extent of Bal 31 nuclease digestion, the DNA sequence of the 5' end of the cDNA was determined by the method of Sanger et al. (12). The cDNA inserts, with varying amounts of oligo(G) tails at their 5' ends, were excised from M13mpl1 with Eco Rl and Hind III and ligated into the Eco Rl and Hind III sites of pSP65.

The SP6 human β-globin clone was a gift from Dr. Stephen Spritz, Department of Genetics, University of Wisconsin, Madison, Wisconsin. This clone encodes the complete 5' non-coding region of the mRNA, the protein
In vitro transcription reactions contained 1.5 µg of a SP6-zein construct and 0.75 µg of a SP6-human -globin construct. The plasmids were prepared for run-off transcription by restriction enzyme digestion at the Hind III site in the pSP65 polylinker at the 3' end of the cDNA sequence or the Ava I site in the zein cDNA. The linearized templates were added to a transcription buffer that contained 40 mM tris-HCl, pH 7.5, 6 mM MgCl₂, 2 mM spermidine, 10 mM dithiothreitol, 1 unit/µl RNAasin, 100 µg/ml BSA, 0.5 mM ATP, TTP, and CTP, 0.1 mM GTP, 0.5 mM 7m(5')Gppp(5')G, and 15 units of SP6 RNA polymerase. After 30 min at 40°C, the GTP concentration was adjusted to 0.5 mM, and transcription was continued for 1.5 hr. Upon completion of the reaction, additional RNAasin was added to 1 unit/µl and the DNA template was removed by digestion with 20 µg/ml of DNase I for 15 min at 37°C. Based on dot hybridization analysis, approximately 0.2 µg of RNA was transcribed per µg of plasmid DNA. The size of the RNAs was determined by Northern blot hybridization. RNA transcripts were glyoxalated in 1 M glyoxal, 70% formamide, and 10 mM phosphate buffer, pH 7.0 for 15 min at 55°C. Following glyoxalation, the RNAs were separated by electrophoresis in 1.4% agarose gels containing 20 mM phosphate buffer at pH 7.0. Transfer of RNA to nitrocellulose paper and hybridization to a nick translated fragment of cZ19cl were as described by Melton et al. (5).

In vitro translation of mRNAs.

Zein mRNA was isolated from developing maize endosperm as previously described (13). The native mRNA or SP6 transcripts were translated in a standard wheat germ cell-free protein synthesis system (13), and the polypeptides were labeled with [³H] leucine. Following incubation at 25°C for 1 hr, the reactions were boiled in SDS sample buffer, separated by SDS-PAGE, and analyzed by fluorography (14).

Preparation of Xenopus laevis oocytes.

Adult Xenopus laevis females were obtained from South Africa Snake Farm, Fish Hoek, Cape Province, or NASCO, Fort Atkinson, Wisconsin, and maintained as previously described (15). Gravid females were anesthetized by hypothermia and ovarian tissue was surgically removed. Stage VI oocytes (16) were manually defolliculated with watchmaker's forceps and cultured in OR-2 (17) until after microinjection. Following microinjection of approximately 1–5 ng of SP6 transcripts or native mRNA, oocytes were incubated in 90 mM potassium phosphate, pH 7.2, 10 mM NaCl, 10 mM MgSO₄ for 0.5 hr and then maintained in 50% Leibovitz (Gibco), 15 mM HEPES, pH 7.8 for 17–18 hr. Oocyte proteins were
then labelled by injection of \([^{3}H]\) leucine (1.1 uCi, 120 pmoles leucine) and allowed to incubate for 75 min (incorporation of \([^{3}H]\) leucine increased in a linear fashion during this time). At the end of the labelling period oocytes were frozen on powdered dry ice.

**Extraction and analysis of protein from oocytes.**

Frozen oocytes were homogenized in 1.1 ml of ice cold 50 mM NaCl, 0.5 mM PMSF, 5 mM sodium metabisulfite, and 10 ug/ml leupeptin, with a glass dounce homogenizer. The resulting homogenate was divided in two halves and processed separately. Globin and zein were extracted together from one half of the homogenate by adding 2.3 volumes of absolute ethanol, and heating the mixture for 15 min at 60°C. These samples were spun in a Sorvall HB-4 rotor at 6000 rpm for 20 min at 20°C and the supernatant solutions were lyophilized. The dried residues were suspended in sample buffer and subjected to electrophoresis on 15% polyacrylamide gels (18). Gels were fixed and treated with En\(^3\)Hance (New England Nuclear, Boston, MA) according to the manufacturer's instructions. Films were developed in a Kodak M20 X-ray processor.

Globin was extracted separately from the zein protein in the following manner. The homogenate was spun in an Eppendorf centrifuge for 5 min at 4°C. The protein was precipitated by adding four volumes of absolute ethanol and storing the mixture at -20°C overnight. Samples were spun in an HL-4 rotor (GLC-1 Sorvall) at 3000 rpm for 10 min and the pellets were dried in vacuo. The dried pellets were suspended in sample buffer prior to separation by gel electrophoresis.

**RESULTS**

In order to study the synthesis and processing of a single zein polypeptide, we transferred a full length cDNA clone encoding a Mr 19,000 zein polypeptide into the Eco RI and Hind III sites of the plasmid pSP65 (see Fig. 1). Runoff transcripts of the zein cDNA insert were synthesized in vitro with SP6 polymerase (5). The mRNA was added to a wheat germ cell-free protein synthesis system or alternatively injected into Xenopus laevis oocytes. An analysis of the products revealed only a trace amount of zein from the wheat germ translation reaction, while none was isolated from mRNA-injected oocytes. This did not appear to result from inefficient transcription, since an analysis of the RNA showed that primarily full-length copies of the cDNA insert were transcribed and capped in vitro. Furthermore, mRNAs transcribed from a pSP6-\(\alpha\)globin construct were found to translate
efficiently in both systems. Because the zein cDNA clone was constructed by G/C-tailing (19), it seemed possible that translation of the mRNA was inhibited by the oligo(G) and oligo(C) tails flanking the cDNA insert.

To determine whether the homopolymer (G) tail on the 5' end of the cDNA insert affected translation, we removed varying amounts of this sequence by
Figure 2. Diagrams of selected 5' deletions of pCZ19cl in pSP65. pSP6.Z19cl.17 has a synthetic oligo(G) tail of 20 residues plus 41 nucleotides of non-translated sequence preceding the initiating methionine (ATG) at the 5' end of the cDNA. The mRNA encodes a mature protein (M) that is preceded by a signal peptide (S) of 21 amino acids. The 3' end of the cDNA has 101 non-translated nucleotides followed by an oligo (C) tail the length of which was not determined. pSP6.Z19cl.22 and pSP6.Z19cl.6 differ from pSP6.Z19cl.17 by having shorter 5' ends. pSP6.Z19cl.22 has an oligo (G) tail of only 8 residues, while pSP6.Z19cl.6 has no oligo(G) tail and is missing the next ten non-translated nucleotides. The SP6 promotor is indicated by the cross-hatched area and the polylinker by the thick black line. For in vitro transcription, the clones were linearized by restriction digestion at the Ava II site or the Hind III site at the 3' end of the polylinker in pSP65.

Bal 31 nuclease digestion (Fig. 1). The plasmid cz19cl was linearized at the 5' end of the cDNA by restriction enzyme digestion with Eco R1, and treated for varying periods of time with Bal 31 nuclease. To determine the extent of 5' sequence removal, the cDNA insert was excised by digestion with Hind III and cloned into the Sma I and Hind III sites of M13mpl1. Following DNA sequence analysis, three clones with varying amounts of 5' deletions were transferred to pSP65.

A detailed structural map of these clones is shown in Figure 2. The cDNA sequence in cz19cl (3) contains a portion of the 5' non-coding sequence of the mRNA, the protein coding region, and 101 nucleotides of 3' non-coding sequence. The 3' end of the clone may be incomplete, since it does not include a portion of the poly(A) tail. The cDNA is flanked by a 5' oligo(G) tail of 20 nucleotides and a 3' oligo(C) tail of undetermined length. The protein encoded by this sequence contains 219 amino acids and is preceded by a signal peptide of 21 residues. Clone pSP6.Z19cl.17 contains the entire oligo(G) tail of 20 residues. Clone pSP6.Z19cl.22 has an oligo(G) tail of eight residues, while pSP6.Z19cl.6 is missing the oligo(G) tail as well as 10 nucleotides of the mRNA 5' non-translated sequence.

Removal of the oligo(G) sequence preceding the cDNA insert had no effect on transcription (Fig. 3), but it was found to have a significant effect on the translation of the SP6-zein constructs in vitro. There were barely detectable levels of zein when the transcript containing 20 oligo(G) residues
Figure 3. Northern hybridization analysis of SP6-zein transcripts. Glyoxalated RNAs were separated on 1.4% agarose gels, transferred to nitrocellulose paper, and hybridized to an internal 864 nucleotide Hinf1 fragment of cZ19cl. a) pSP6.Z19cl.17 (20 G residues); b) pSP6.Z19cl.22 (8 G residues); c) pSP6.Z19cl.6 (no G residues) d) native zein mRNA. The SP6-zein transcripts are slightly shorter than the native zein mRNA due to the absence of a 3' poly(A) tail.

was added to the wheat germ translation system; the SP6-βglobin control was efficiently translated in this reaction (Fig. 4, lane b). Translation of the SP6-zein transcript was significantly increased upon removal of 12 oligo(G) residues (Fig. 4, lane c) or the entire oligo(G) sequence (Fig. 4, lane d). In the latter case, removal of an additional 10 nucleotides of 5' non-translated sequence did not affect translational efficiency. Translation of the SP6-βglobin mRNA was comparable in both of these reactions. The zein protein whose synthesis was directed by the SP6 transcript migrated similar to the precursors of the Mr 19,000 zein proteins in translations of native mRNAs (Fig. 4, cf., lane a and lanes c, d). A doublet of polypeptides was observed in some translation reactions (Fig. 4, lane b), which may result from altered initiation or termination of protein synthesis in vitro.

The oligo(G) tails were found to have a similar effect on mRNA translation in Xenopus oocytes. In oocytes co-injected with the SP6-βglobin transcript and the SP6-zein construct with 20 oligo(G) residues (Fig. 5, lanes b), only the globin protein could be detected. Removal of 12 of the 20 oligo(G) residues resulted in increased translational efficiency of the SP6-zein transcript (Fig. 5, lanes c), while translation efficiency was enhanced to an even greater extent by complete removal of the oligo(G) tail (Fig. 5, lanes d). These differences in protein synthesis were not related to RNA stability, as each of the SP6-transcripts was stable in oocytes,
Figure 4. SDS polyacrylamide gel analysis of in vitro translation products from native or SP6-derived zein mRNAs. The polypeptides in lane a were from total zein mRNA from maize endosperm. Products in lanes b-d were from SP6-derived transcripts of reactions containing 1.5 ug of a SP6-zein construct and a 0.75 ug of a SP6-globin construct. The zein constructs were as follows: lane b: pSP6.Z19cl.17 (20 G residues); lane c, pSP6.Z19cl.22 (8 G residues), lane d, pSP6.Z19cl.6 (no G residues); lane m, mol wt markers. The position of the zein (z) and globin (g) polypeptides are indicated.

provided they were capped. A comparison of the level of globin synthesis in oocytes co-injected with the various SP6-zein transcripts indicated that translational efficiency was comparable among the different groups of oocytes. Although globin is traditionally extracted from oocytes in saline buffers (20), we found it to be equally soluble in 70% ethanol. We determined by Northern hybridization analysis that the SP6-γ-globin and SP6-zein plasmids were transcribed in vitro with equal efficiency and thus comparable amounts of mRNAs were injected among different groups of oocytes (data not shown).

The zein protein synthesized in oocytes injected with SP6-zein transcripts migrated to the same position as the mature Mr 19,000 zein proteins (Fig. 5, lane a). It was previously shown that Xenopus oocytes
process the signal peptides of the zein proteins to yield polypeptides that correspond to the native proteins (13). To verify that proteins directed by SP6-zein transcripts were transported into membrane vesicles, we isolated the membrane fraction from mRNA-injected oocytes (Fig. 6). The membranes were divided into three aliquots. The first was extracted with 70% ethanol directly (Fig. 6, lanes a and d), the second was treated with Proteinase K in the presence of 0.1% SDS prior to ethanol extraction (Fig. 6, lanes b and e), while the third was treated with Proteinase K in the absence of SDS (Fig. 6, lanes c and f). Proteins whose synthesis was directed by either native zein mRNAs (Fig. 6, lanes a-c) or SP6-zein transcripts (Fig. 6, lanes d-f) were resistant to Proteinase K in the absence of SDS, indicating that they are sequestered in membrane vesicles.

The reduced translational efficiency of SP6-zein mRNAs containing 5' oligo(G) sequences may result from inefficient ribosome attachment.
Figure 6. Compartmentalization of zein proteins in oocyte membrane vesicles. Membranes were isolated from oocytes injected with native zein mRNA (a-c) or SP6-zein transcripts (d-f). The membranes were treated with proteinase K in the presence (b, e) or absence (c, f) of 0.1% SDS, or received no protease treatment at all (a, d). Following proteinase treatment, the membrane proteins were extracted with 70% ethanol and analyzed by SDS PAGE. The mol wts of the polypeptides are indicated in kilodaltons.

Alternatively, it may be due to base pairing of the 5' oligo(G) sequence with the 3' oligo(C) tail. Since an Ava I site in the 3' non-coding sequence precedes the oligo(C) tail by one nucleotide (Fig. 2), it was possible to prepare SP6-zein transcripts missing this sequence by restriction digestion with Ava I prior to in vitro transcription.

Removal of the oligo(C) tail from the 3' end of the SP6-zein transcript did not affect the pattern of protein synthesis observed following removal of the oligo(G) sequence alone. Zein synthesis was barely detectable in oocytes injected with the SP6-zein transcript containing the entire oligo(G) sequence, but missing the 3'oligo(C) tail (Fig. 7, lane a). With progressive removal of the 5' oligo(G) sequence, there was a proportional increase in the level of zein synthesis (Fig. 7, lanes b and c). The pattern of zein synthesis obtained with transcripts that did not contain oligo(C) tails was identical to that observed when the oligo(C) tail was present (Fig. 7, lanes d-f). The results presented in Figure 6 also indicate that more zein is synthesized in oocytes injected with SP6-zein transcripts containing oligo(C) tails. Although there were some differences in the level of globin synthesis among the group of oocytes shown in Figure 7, the higher level of zein synthesis by transcripts containing oligo(C) tails has been observed in other experiments.
Figure 7. Comparative translational efficiency of SP6-derived zein mRNAs following removal of 5' and 3' homopolymer tails. Oocytes were injected with mRNAs isolated following co-transcription of a SP6-α-globin clone and the various SP6-zein constructs. The polypeptides were labeled with $[^3H]$ leucine and extracted with 70% ethanol. The SP6-zein constructs in lanes a-c were linearized at the Ava I site to eliminate the oligo(C) tail, or at the Hind III site (lanes d-f) to include the oligo(C) tail at the 3' end of the mRNA. The SP6-zein constructs were as follows: lanes a and d, pSP6.Z19c1.17 (20 G residues), lanes b and e, pSP6.Z19c1.22 (8 G residues), lanes c and f, pSP6.Z19c1.6 (no G residues), lane m, mol wt markers.

DISCUSSION

We previously demonstrated that native endosperm mRNAs direct the synthesis and processing of zein storage proteins when injected into Xenopus laevis oocytes (13). Moreover, the zein proteins synthesized in oocytes accumulate in membrane vesicles that have physical characteristics similar to the protein bodies of maize endosperm (21). Since the zein fraction consists of a mixture of proteins that are encoded by multiple mRNAs (3), by transcribing mRNAs corresponding to different zein clones, it should be possible to study the association of specific proteins within oocyte membranes. Our results demonstrate that the SP6 system can be used to produce functional zein mRNAs that direct the synthesis and processing of zein proteins. This approach should also be useful to study the potential for improving the nutritional quality of zeins by adding lysine and tryptophan to the proteins via site-directed mutagenesis of the genes (22).

The zein cDNA clone used in these experiments was constructed by
inserting double stranded cDNA into the Pst I site of pUC8 (3) with a G/C-tailing procedure (19). This is a common method for cDNA cloning, and it facilitates the transfer of sequences into pSP65 for studies on the properties of the protein encoded by the clone (Fig. 1). However, the in vitro transcripts from such clones contain synthetic oligo(G/C) polymers preceding and flanking the mRNA sequence.

Stueber and co-workers (23) suggested a 5' oligo(G) sequence may reduce translation of synthetic mRNA, and this is clearly demonstrated by our results. It seemed possible that the oligo(C) sequence at the 3' end of the cDNA may have caused the mRNA to circularize and thereby become an inefficient template for protein synthesis. However, the pattern of zein synthesis with transcripts completely lacking the oligo(C) tail was similar to those with only the 5' oligo(G) sequence removed. This result demonstrates that the translational inhibition is a direct effect of the oligo(G) sequence at the 5' end of the mRNA. It is possible that the 5' oligo(G) sequence may cause the mRNAs to be more susceptible to ribonuclease, and thereby reduce the level of protein synthesis; however, this seems unlikely since the reduction of protein synthesis was similar in both the wheat germ cell free system and in Xenopus oocytes. Furthermore, we recovered similar amounts of mRNA from oocytes injected with SP6 transcripts that had 5' oligo(G) tails of different lengths (data not shown).

The reduced translational efficiency caused by the oligo(G) tail may result from either the presence of the polypurine sequence, or an altered mRNA secondary structure. Long homopolymers of oligo(G) are known to have an unusual secondary structure (24), and a sequence of 20 residues at the 5' end of a mRNA may significantly alter the formation of a ribosome initiation complex. It is also possible that the long oligo(G) sequence may introduce an unusual hairpin structure at the 5' end of the mRNA. It was previously shown that the introduction of potential hairpin structures at the 5' end of the SP6 transcripts can significantly reduce the level of their translation (25). We identified a sequence CTTCCCTTCTCCCCC that begins 98 nucleotides after the initiation codon in cZ19cl (3). This sequence could hybridize with 16 of the 20 nucleotides in the oligo(G) tail with a calculated G of -32.5 kcal. This structure would be further stabilized by two additional hybridizing regions in the loop which are of seven and 10 nucleotide pairs that have calculated G's of -17 and -19 kcal, respectively. A hairpin structure of this type might prevent ribosome attachment or affect translation of the mRNA. The potential for a 5' oligo(G) sequence to form a hairpin
structure with C/T rich regions of mRNAs could make this a generalized problem for a variety of cDNA sequences.

The absence of the oligo(C) tail did not have a significant effect on translation in the wheat germ cell-free system (data not shown), and it was not found to interact with the oligo(G) sequence in transcripts injected into Xenopus oocytes. The level of zein synthesis appeared to be slightly greater in oocytes injected with transcripts containing the oligo(C) tail. Since the SP6-zein transcripts lacked a 3' poly(A) tail, the oligo(C) sequence may have been a functional substitute. Preliminary experiments suggest that SP6-zein transcripts that contain the oligo(C) tail may be more stable in oocytes. However, the degree to which the oligo(C) sequence substitutes for a poly(A) tail will need to be verified with additional experiments.

The inhibitory effect of the 5' oligo(G) sequence seemed less pronounced in translations with the wheat germ system than with Xenopus oocytes, since transcripts containing a 5' sequence with eight G residues were translated almost as efficiently as those with no G residues in vitro (Fig. 4). This difference in sensitivity undoubtedly reflects a difference in the translational stringency of the two systems, and may also account for the appearance of the doublet zein bands in some of the wheat germ reactions (Fig. 4, lane c). In cZ19cl there is a second AUG within eight amino acids of the initiation codon (3), and initiation from both codons may account for the doublet peptide.

Acknowledgements.

We wish to express our thanks to Dr. Richard Spritz, Department of Genetics, University of Wisconsin, for providing us with a SP6 human β-globin clone that was used as a control in these experiments. We also thank Dr. P. T. Gilham for analysis and helpful discussion regarding mRNA secondary structure. The technical assistance of Steven Casper, Judith S. Lindell and Ms. E. C. Logan-Ashman in preparation of the manuscript was greatly appreciated. This research was supported by a grant from Agrigenetics to BAL. Journal Paper 10,486 of the Purdue Agriculture Experiment Station. G. G. is the recipient of a fellowship from the Weizmann Institute.

*To whom correspondence should be addressed

REFERENCES