Translation efficiency of zein mRNA is reduced by hybrid formation between the 5'- and 3'-untranslated region

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The secondary structure of zein mRNA affects its translational potential. Here we show that in a cell-free system the translation efficiency of zein mRNA containing inverted repeats in the 5'- and 3'-untranslated regions is reduced. This translational block is released after deletion of the 3'-inverted repeat. We conclude that the translational block is caused by hybrid formation between the two inverted repeats. The translational efficiency of zein mRNAs, is also affected by varying the length or the primary structure of the 5'-untranslated region.

Key words: in vitro transcription-translation/zein mRNA/inverted repeats/5'- and 3'-untranslated regions/translational potential

Introduction

In prokaryotes, the secondary structure of mRNA influences gene expression at the transcriptional and translational levels (for review, see Gold et al., 1981; Kozak, 1983). In eukaryotes, where several additional regulatory steps can intervene between transcription and translation, similar evidence is still missing. To investigate the possible role of mRNA structure in the regulation of eukaryotic translation we have used a recently described in vitro transcription-translation system (Stueber et al., 1984), and have chosen a cDNA and a genomic zein mRNA as a basis for our experimental model.

Zea is the major storage protein of Zea mays and consists of a family of related hydrophobic polypeptides synthesised in the seed endosperm from 14 to 55 days after fertilization. Zeins are synthesised on polysomes attached to a specialised part of the endoplasmic reticulum (ER) and the transfer of the nascent peptide across the ER membrane is accompanied by the proteolytic cleavage of a signal peptide of ~2000 daltons (Larkins et al., 1979).

Zea mRNAs have an interesting feature in their 5'- and 3'-untranslated regions. When folded back, the two ends of the transcript have a discrete degree of inverted homology which could allow intra- and/or intermolecular base pairing (Spena et al., 1982). This peculiar feature is not unique to the zein mRNAs, other plant mRNAs such as A-gliadin, phascolin, patatin, soybean actin and wheat histone H4 have short inverted repeats involving their leader and trailer sequences and in one case, the termination codon (Anderson et al., 1984; Slighsom et al., 1983; Mignery et al., 1984; Shah et al., 1982; Tabata et al., 1983). The functional significance of these inverted homologies is not clear but base pairing between 5' and 3' ends of an eukaryotic mRNA molecule could affect its translational potential.

Results

Zein genes contain inverted repeat structures between their 5'- and 3'-untranslated regions (Figure 1A,B). To test the effect of these inverted repeat structures on the translation efficiency of zein mRNAs, the zein zA1 gene (Spena et al., 1982) and the zein cM1 cDNA (Viotti et al., 1982) were cloned into the recently described expression plasmid pDS-6 (Stueber et al., 1984) (Figure 2). The DS family of expression plasmids is designed to allow the efficient transcription of a cloned DNA sequence in vitro. The transcription-coupled capping of RNA permits the direct translation of the mRNA by eukaryotic extracts such as those from wheat germ, HeLa cells or reticulocytes (Stueber et al., 1984; Hurt et al., 1984).

In the plasmid DS-6, a gene of interest can be inserted between the strong coliphage promoter Pν and the phage λ terminator τ. When transcribed in vitro by Escherichia coli RNA polymerase a monocistronic and bicistronic mRNA will be pro-

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Fig. 1. (A.I) Schematic drawing of the zA1 zein genomic clone represented as a BamHI-EcoRI fragment of ~2.45 kb. The EcoRI site flanks its 3' end while the BamHI site is ~1 kb upstream from the initiation codon. The TAG stop codon at position 236 has shortened its coding capacity to 235 amino acids. The imperfect inverted repeats present in its untranslated regions are indicated. (A.II) DNA sequence of the putative stem structure of the zA1 clone. The ΔG value of ~20.48 kcal was calculated according to Sailer (1977). Asterisks indicate regions of potential base pairing. (B.I) Schematic drawing of the cM1 cDNA clone, indicating (i) the zein cM1 coding sequence of 798 bp (266 amino acids), (ii) the 3'-untranslated region containing the natural part of its inverted repeat ( []) as well as the artificial one introduced during the cDNA cloning ( []), (iii) the 3'-untranslated region containing the inverted repeat and 31 adenine residues from the poly(A) tail. The flanking EcoRI sites, the two DraI sites and the unique HpaII site just preceding the 3'-inverted repeat are also indicated. (B.II) DNA sequence and putative stem structure of the cM1 cDNA clone. The ΔG value of ~76.73 kcal was calculated according to Sailer (1977).
duced. All transcripts will contain identical nucleotides from the pDS6 vector at their extreme 5' end. In an eukaryotic translation system only the 5'-proximal cistron will be efficiently translated (Rosenberg and Paterson, 1979; Stueber et al., 1984).

**Zein A2 gene**

The zein gene in pDS-A2 is derived from the genomic clone zA1 previously characterized (Spena et al., 1982). It has imperfect inverted repeats in its 5'- and 3'-untranslated regions matching for 20 bases as shown in Figure 1A and AII. Zein A2 consists of a DNA fragment of ~1500 bp containing 143 bases upstream from the ATG initiation codon, all the zein coding region, and 560 bases downstream from the second TAG termination codon (Figure 1A). The pDS-A2 clone, and all its derivatives, code for a truncated zein pre-protein of 235 amino acids because it contains a point mutation in its coding region which has changed a CAG codon at position 236 into a TAG stop codon. This does not, however, reduce the methionine content as compared with the cM1 full length zein pre-protein (see below). In plasmid pDS-A2Hpa, the 3'-inverted repeat region was deleted by a HpaII cleavage (Figure 3a). After transcription, mRNAs contain 31 nucleotides from the pDS-6 vector at their extreme 5' end (see Figure 6). To evaluate the effect of the zein A2 5'-untranslated region on the efficiency of translation, we have selected 5' deletion derivatives of pDS-A2. pDS-A250 has ~50 bases in front of the ATG initiation codon, while pDS-A28 and pDS-A22 have only eight and two bases before the initiation codon, respectively (Figure 3a). After transcription, mRNAs contain 31 nucleotides form the pDS-6 vector at their extreme 5' end (see Figure 6).

All plasmids were transcribed in vitro and translated in a wheat germ system as described in Materials and methods. Comparison of the protein profiles, obtained by transcription-translation of

**Fig. 2.** Schematic representation of plasmid pDS-M1 containing zein M1 cDNA. The T5 promoter is indicated by P, and t1 is the terminator of the Rnrb operon of E. coli. The 5'- and 3'-inverted repeats (I.R.) are indicated. For further details concerning the original vector DS6, see Stueber et al. (1984).

![Fig. 3](image-url) (a) Schematic drawing of the pDS-A2 clone and its deletion derivatives. Indicated are, +1: start of transcription; thick black line: 31 nucleotides from pDS6. The imperfect inverted repeats are boxed. The pDS-A2 clone is a Bal31 derivative of pA1, starting 143 bases upstream of the AUG initiation codon, while this region in pDS-A250, pDS-A28 and pDS-A22 is shortened to ~50, 8 and 2 bases, respectively. (b) Translation of mRNA transcribed from the plasmids outlined in a. The peptide profiles encoded by plasmids indicated above the lanes are shown. The position of pre-M1 zein and of pre-A2 zein are indicated. (c) Northern blot analysis of mRNA derived from pDS-A2 and pDS-A2Hpa.
plasmids pDS-A2 and its 3' deletion derivative pDS-A2Hpa, shows that deletion of the 3' inverted repeat results in a 2-fold increase in the level of translation of the zein preprotein (Figures 3a, b, 5). This result is not due to a difference in zein mRNA level (Figure 3) and one can conclude therefore that the 3' deletion of 515 bases including the inverted repeat is responsible for the observed increase in translational efficiency.

The translational potentials of the 5' deletion derivatives of pDS-A2 are shown in Figure 3b. mRNA derived from pDS-A2S0, contains practically all the inverted repeat and translates with an efficiency similar to pDS-A2, which contains 143 bp in its 5'-untranslated region. Translation of pDS-A2.8 and pDS-A2.2 mRNA is significantly lower than that from pDS-A2S0. These results indicate that a deletion very close to the ATG initiation codon negatively affects zein mRNA translation. It appears that a minimal length of the 5' leader sequence is critical for efficient translation of zein mRNA.

**Zein M1 cDNA**

The fact that inverted repeats in the 5'- and 3'-untranslated regions in zein mRNA can fold back and base pair with each other is also supported by a cloning artifact obtained in clone pCM1 (Figure 1B). It encodes a heavy chain zein polypeptide of 266 amino acids (Spena et al., 1982). Its 3'-untranslated region consists of 83 nucleotides followed by a tail of 31 adenosine residues. Its leader sequence of 141 bases begins with 71 thymidine residues followed by a perfect inverted repeat of the last 11 bases present at the 3' end of the RNA (Spena, Viotti and Pirrotta, unpublished results). This structure probably resulted from base pairing with the 3'-inverted repeat structure and priming of DNA polymerase at this site. The extreme 3' end thus appears to have been copied onto the 5' end (Figure 1B). mRNA copied from
this cDNA clone could base pair extensively (61 bases) to form a stem and loop structure as shown in Figure 1 BI (2). The cM1 cDNA clone is ~96% homologous to the zein zA1 gene (Spena et al., 1982).

To test the effect of the stem and loop structure on the translational efficiency of cM1 mRNA, we have cloned the cM1 cDNA into the DS-6 plasmid vector (pDS-M1) (Figure 4a). For comparison, we also constructed a plasmid in which the 72 bp comprising the inverted repeat sequence at the 3' end of cM1 have been removed by cleavage with HpaII (pDS-M1Hpa) (Figure 4a). To correlate the efficiency of the translational block with the stability and/or length of the stem structure, we have built two other derivatives of pDS-M1 plasmids. Plasmid pDS-M1 Dra was obtained by DraI cleavage. It has imperfect inverted repeats matching for 30 bases (Figure 4a). In plasmid pDS-M1Hpa/Dra the 3'-inverted repeat and the 71 thymidine residues at the very 5' end are deleted (Figure 4a).

Plasmids pDS-M1 and pDS-M1Hpa (Figure 4a) were transcribed in vitro and translated in a wheat germ system. The protein profiles obtained are shown in Figure 4b. The mobilities of pre-β-lactamase (pre-β-1), and pre-zein M1 are indicated. Efficient pre-zein synthesis is obtained with pDS-M1Hpa, as is evident by comparing the peptide profile of pDS-M1 (lane 2) with that obtained using pDS-M1Hpa (lane 1). The clone lacking the 3'-inverted repeat directs the synthesis of at least 50 times more pre-zein M1 than the pDS-M1 clone. Since equal amounts of zein mRNAs are transcribed from the two plasmids, as verified by Northern blot analysis (Figure 4c), this difference must be due to a translational block. We conclude, therefore, that the presence of the inverted repeats causes a drastic reduction in the translation efficiency of the zein M1 mRNA.

When the stretch of poly(T) residues in the 5'-non-coding region is removed, as in pDS-M1Hpa/Dra, translation efficien-

Fig. 5. Quantitation of the amount of labelled zein translated from mRNA derived from 0.5 μg of the respective plasmids indicated on top of the graph. The amount of pre-M1 zein obtained from pDS-M1Hpa/Dra has arbitrarily been taken as 100%. Quantitation was done by densitometric analysis of the autoradiographic film.

Fig. 6. Alignment of the 5'-untranslated regions of mRNA transcribed from pDS-A2Hpa and pDS-M1Hpa. Only those bases in mRNA-M1Hpa different from mRNA-A2Hpa are indicated. Base deletions are indicated by an asterisk. Start of the zein A2 derived leader sequences in mRNA A250, A28, A22, M1Hpa and Dra. The pDS-6 derived 31 nucleotides of the leader sequence and the AUG translation start site are underlined.

Fig. 7. (a) Putative stem structures between the 5'- and 3'-untranslated regions of five zein genes and their thermodynamic stabilities (Salser, 1977). zA1, and s4 (Kriid et al., 1984) are members of one zein homology class (opaque-2 dependent). zE19 (Spena et al., 1983). zG99 (Pedersen et al., 1982) and z7 (Hu et al., 1982) are members of another zein homology class (opaque-2 independent). (b) Putative stem structures between the untranslated regions of five plant genes and their thermodynamic stabilities.
efficiency. A2Hpa observed constraint regions. Previous studies is one of the features that affect translational efficiency (Kozak, 1980; Pavlakis et al., 1980; Pelletier and Sonenberg, 1985; Gough et al., 1985). Our results show that, in an eukaryotic environment, a mRNA containing inverted repeats in its untranslated region is translated less efficiently than a homologous mRNA in which the inverted repeat at the 3' end has been deleted. Most probably, the reduced translation efficiency is the result of the observed base pairing between the inverted repeat sequences in the 5'- and 3'-untranslated regions. This reduction varies inversely with the thermodynamic stability of the predicted stem structures.

Intramolecular RNA base pairing plays a central role in the regulation of bacterial protein synthesis (Hall et al., 1982; Horinouchi and Weisblum, 1980; Gheyten et al., 1982; Tressier et al., 1984; Gordon et al., 1984) but no such examples of control mechanisms have been found in eukaryotes. However, several plant gene transcripts could form stem structures between their 5' leader and 3' trailer regions as shown in Figures 1, 7a,b. The range of free energies would vary (see Figure 7).

The current view of eukaryotic translation is that eukaryotic ribosomal subunits bind at or near the 5' end of mRNAs, using the 'CAP' as attachment site. They then move along the RNA, scanning the sequence for an AUG codon where initiation of translation takes place (Kozak, 1983). Our experimental cases A2 and M4, as well as most of the other naturally occurring inverted repeats, do not include the AUG initiation codon in their putative stems. Since the stem structures are located 34 bases upstream from the initiating AUG codon, they would be stable enough to prevent the initiation complex to travel efficiently along the RNA chain. Consequently, a different level of expression among genes of the same homology class could be obtained through a post-transcriptional mechanism of gene regulation. In a gene family like that of zein, different mRNAs could be translated with different efficiencies, thus modulating gene family expression.

The zein mRNA inverted repeats could affect mRNA translational efficiency and/or mRNA stability in vivo. This is not, however, the only mechanism by which translation could be regulated. Stem structures, for example, could form within the 5'-untranslated regions and affect translation (Gough et al., 1985; Pelletier and Sonenberg, 1985). Another feature which affects translation could be the length of the 5'-untranslated regions. Our data suggest that there might exist a minimal length requirement for efficient translation. Moreover, the different translational efficiency of pDS-A2/Hpa and pDS-M1/Hpa transcripts in wheat germ extracts suggests the existence of other features in mRNA which affect the translational potential of zein mRNA.

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Northern blot analysis

In vitro transcribed mRNAs were separated on agarose/formaldehyde gels according to Seed and Goldberg (Maniatis et al., 1982), transferred on to nitrocellulose filters and hybridized to nick-translated probes. The 470-bp TaqI fragment from the zein zA4 coding region or the EcoRI fragment of the cM1 clone were used in the respective hybridizations.

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