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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.

Zein 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).

Zein 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, phaseolin, 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; Slightom *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 zA_1 gene (Spena *et al.*, 1982) and the zein cM_1 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 $P_{N25}x/_0$ and the phage λ terminator t_0. When transcribed *in vitro* by *Escherichia coli* RNA polymerase a monocistronic and bicistronic mRNA will be pro-

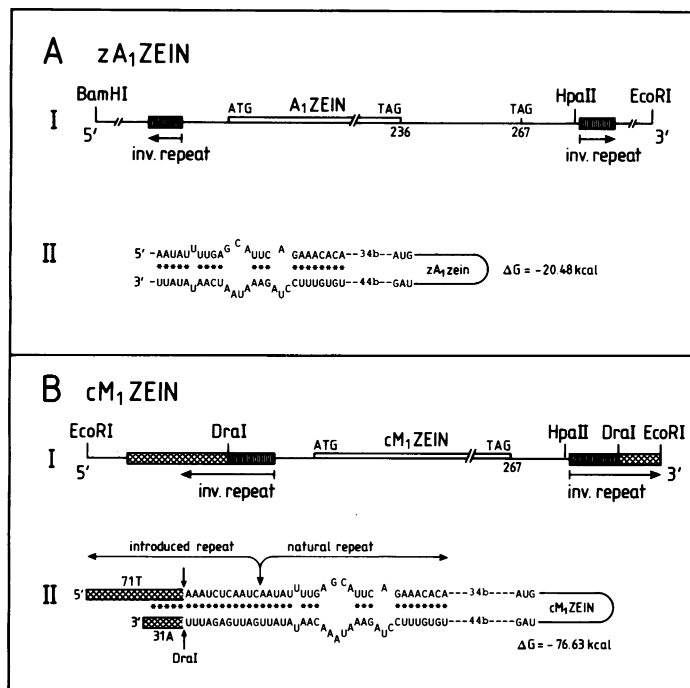

Fig. 1. (A.I) Schematic drawing of the zA_1 zein genomic clone represented as a *Bam*HI-*Eco*RI fragment of ~2.45 kb. The *Eco*RI site flanks its 3′ end while the *Bam*HI site is ~1 kb upstream from the initiation codon. The TAG stop codon at position 236 has shortened its coding capacity to 235 amino acids. The imperfect inverted repeats present in its untranslated regions are indicated. **(A.II)** DNA seuqence of the putative stem structure of the zA_1 clone. The $\triangle G$ value of −20.48 kcal was calculated according to Salser (1977). Asterisks indicate regions of potential base pairing.
(B.I) Schematic drawing of the cM_1 cDNA clone, indicating (i) the zein cM_1 coding sequence of 798 bp (266 amino acids), (ii) the 5′-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 adenosine residues from the poly(A) tail. The flanking *Eco*RI sites, the two *Dra*I sites and the unique *Hpa*II site just preceding the 3′-inverted repeat are also indicated. **(B.II)** DNA sequence and putative stem structure of the cM_1 cDNA clone. The $\triangle G$ value of −76.73 kcal was calculated according to Salser (1977).

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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 A_2 gene

The zein gene in pDS-A_2 is derived from the genomic clone zA_1 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 1 AI and AII. Zein A_2 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-A_2 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 cM_1 full length zein pre-protein (see below). In plasmid pDS A_2Hpa, the 3′-inverted repeat region was deleted by a *Hpa*II 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 A_2 5′-untranslated region on the efficiency of translation, we have selected 5′ deletion derivatives of pDS-A_2. pDS-$A_2$50 has ~50 bases in front of the ATG initiation codon, while pDS-$A_2$8 and pDS-$A_2$2 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

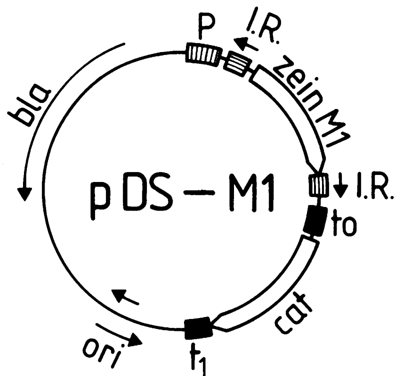

Fig. 2. Schematic representaton of plasmid pDS-M_1 containing zein M_1 cDNA. The T5 promoter is indicated by P, t_0 represents a terminator of phage λ and t_1 is the terminator of the Rrnb operon of *E. coli*. The 5′- and 3′-inverted repeats (I.R.) are indicated. For further details concerning the original vector DS6, see Stueber *et al.* (1984).

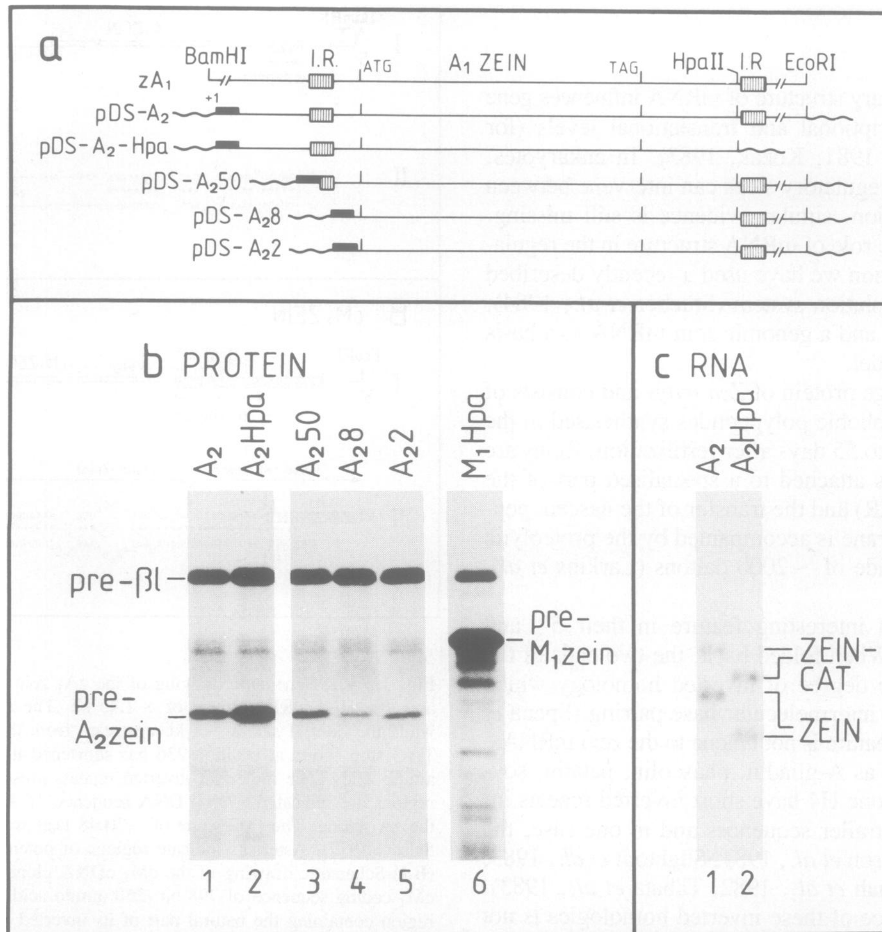

Fig. 3. (a) Schematic drawing of the pDS-A_2 clone and its deletion derivatives. Indicated are, +1: start of transcription; thick black line: 31 nucleotides from pDS6. The imperfect inverted repeats are boxed. The pDS-A_2 clone is a *Bal*31 derivative of pzA_1 starting 143 bases upstream of the AUG initiation codon, while this region in pDS-$A_2$50, pDS-$A_2$8 nd pDS-$A_2$2 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-M_1 zein and of pre-A_2 zein are indicated. (c) Northern blot analysis of mRNA derived from pDS-A_2 and pDS-A_2Hpa.

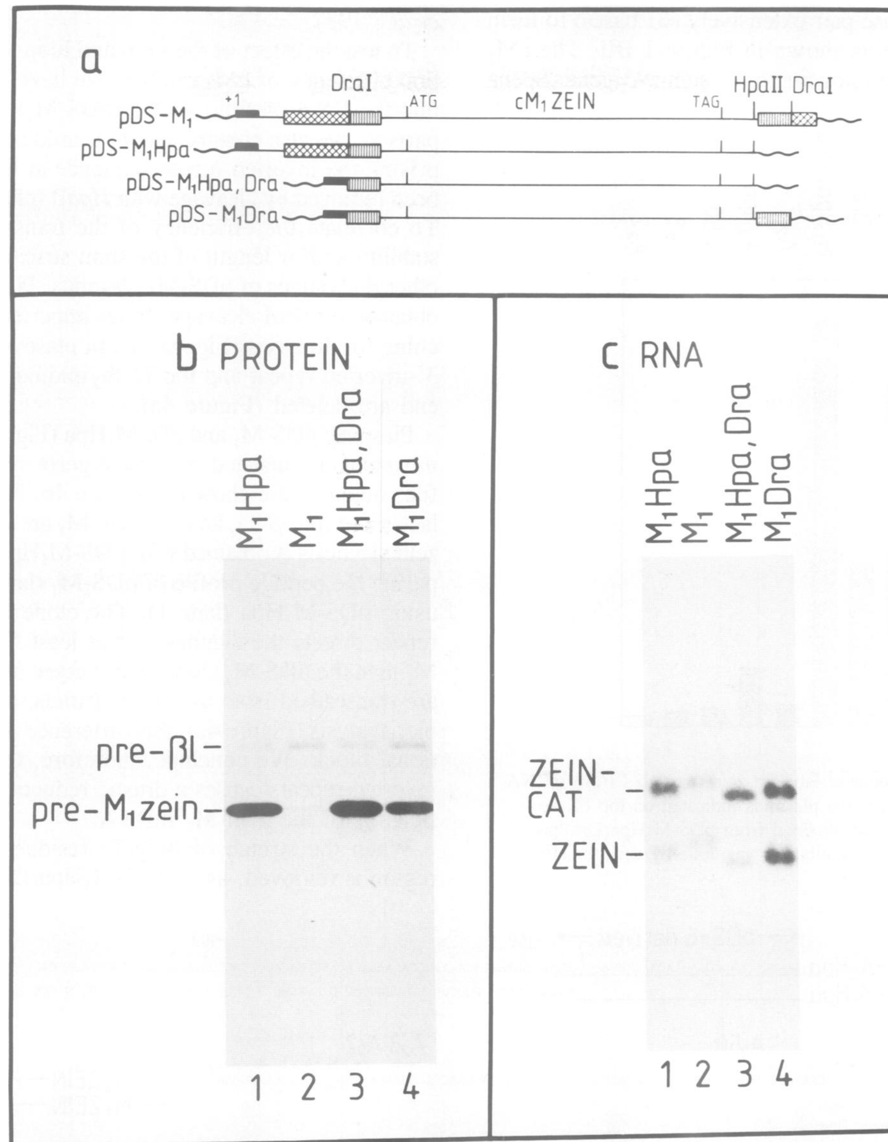

Fig. 4. (**a**) Schematic drawing of inserts in plasmid pDS-M_1 and its deletion derivatives pDS-M_1Hpa, pDS-M_1Dra and pDS-M_1Hpa/Dra. +1 indicates the start of transcription; the thick black line the 31 nucleotides from pDS6, and the boxes, inverted repeats (see Figure 1). (**b**) pDS plasmids were transcribed and capped by *E. coli* RNA polymerase and the resulting mRNA translated in the wheat germ cell-free system. ^{35}S-Labelled proteins were characterized by SDS-PAGE and autoradiography. **Lane 1**: products from pDS-M_1Hpa, **lane 2**: pDS-M_1, **lane 3**: pDS-M_1Hpa/Dra, **lane 4**: pDS-M_1Dra. (**c**) Northern blot analysis. mRNAs transcribed from pDS-M_1Hpa, pDS-M_1 and pDS-M_1Hpa,Dra and pDS-M_1-Dra were run on an agarose/formaldehyde gel, blotted onto nitrocellulose filter and hybridized to a cM_1 *Eco*RI fragment probe. On each lane 10 μl of a transcription mixture were analysed. Two RNA bands can be seen which correspond to zein transcripts terminated at t_0 or to bicistronic zein-CAT transcripts terminated at t_1. Their comparable intensity certifies that zein transcripts are at present at a similar level in the two transcription assays.

plasmids pDS-A_2 and its 3′ deletion derivative pDS-A_2Hpa, 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-A_2 are shown in Figure 3b. mRNA derived from pDS-$A_2$50, contains practically all the inverted repeat and translates with an efficiency similar to pDS-A_2, which contains 143 bp in its 5′-untranslated region. Translation of pDS-A_2.8 and pDS-A_2.2 mRNA is significantly lower than that from pDS-A_2.50. 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 M_1 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 pcM_1 (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 BII. The cM_1 cDNA clone is ~96% homologous to the zein zA_1 gene (Spena et al., 1982).

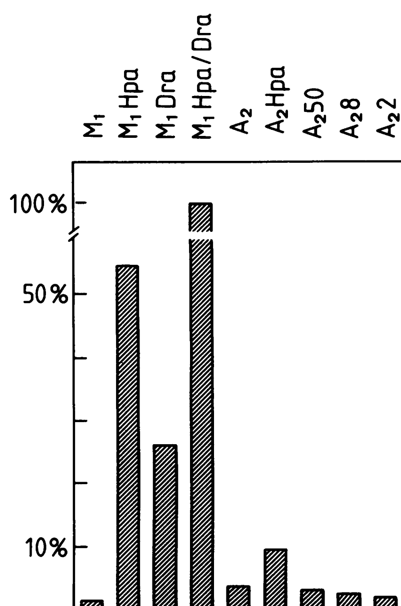

Fig. 5. Quantitation of the amount of labelled zein translated from mRNA derived from 0.5 μg of the respective plasmids indicated on top of the graph. The amount of pre-M_1 zein obtained from pDS-M_1Hpa/Dra has arbitrarily been taken as 100%. Quantitation was done by densitometric analysis of the autoradiographic film.

To test the effect of the stem and loop structure on the translation efficiency of cM_1 mRNA, we have cloned the cM_1 cDNA into the DS-6 plasmid vector (pDS-M_1) (Figure 4a). For comparison, we also constructed a plasmid in which the 72 bp comprising the inverted repeat sequence at the 3′ end of cM_1 have been removed by cleavage with *Hpa*II (pDS-M_1Hpa) (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-M_1 plasmids. Plasmid pDS-M_1 Dra was obtained by *Dra*I cleavage. It has imperfect inverted repeats matching for 30 bases (Figure 4a). In plasmid pDS-M_1Hpa/Dra the 3′-inverted repeat and the 71 thymidine residues at the very 5′ end are deleted (Figure 4a).

Plasmids pDS-M_1 and pDSM_1Hpa (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 M_1 are indicated. Efficient pre-zein synthesis is obtained with pDS-M_1Hpa, as is evident by comparing the peptide profile of pDS-M_1 (lane 2) with that obtained using pDS-M_1Hpa (lane 1). The clone lacking the 3′-inverted repeat directs the synthesis of at least 50 times more pre-zein M_1 than the pDS-M_1 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 M_1 mRNA.

When the stretch of poly(T) residues in the 5′-non-coding region is removed, as in pDS-M_1Hpa/Dra, translation efficien-

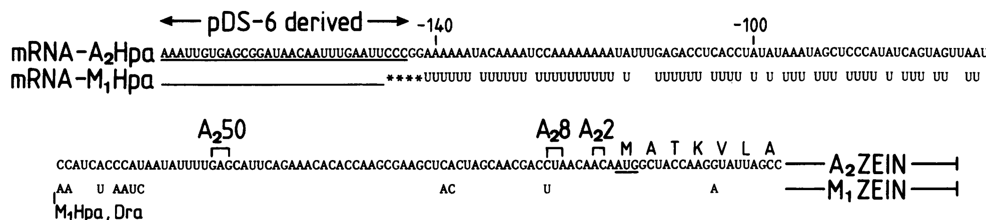

Fig. 6. Alignment of the 5′-untranslated regions of mRNA transcribed from pDS-A_2Hpa and pDS-M_1Hpa. Only those bases in mRNA-M_1Hpa different from mRNA-A_2Hpa are indicated. Base deletions are indicated by an asterisk. Start of the zein A_2 derived leader sequences in mRNA $A_2$50, $A_2$8, $A_2$2, M_1Hpa and Dra. The pDS-6 derived 31 nucleotides of the leader sequence and the AUG translation start site are underlined.

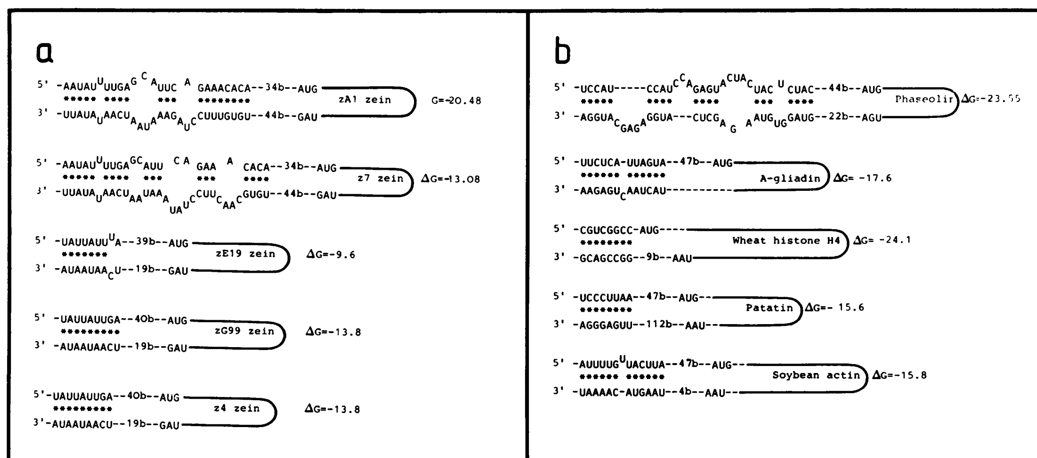

Fig. 7. (a) Putative stem structures between the 5′- and 3′-untranslated regions of five zein genes and their thermodynamic stabilities (Salser, 1977). zA_1, and z4 (Kridl et al., 1984) are members of one zein homology class (opaque-2 dependent). zE19 (Spena et al., 1983), zG99 (Pedersen et al., 1982) 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.

cy is further increased by 50%, as compared with pDS-M_1Hpa (Figures 4b and 5). When, however, the 3'-inverted repeat segment remains as in pDS-M_1 Dra translation efficiency is 4–5 times lower than in pDS-M_1Hpa/Dra (Figures 4b and 5). This would indicate that a stretch of 30 imperfectly matching bases can reduce translation efficiency.

A surprisingly large difference in translation efficiency between mRNA-A_2 Hpa and mRNA-M_1 Hpa was found although the 5'-untranslated regions in these mRNAs are of similar size. They comprise 170 and 174 bases, respectively. The 70 nucleotides upstream from the AUG initiation codon are nearly identical (see Figure 6). Nevertheless, mRNA from pDS-M_1Hpa is translated ~6 times more efficiently than mRNA from pDS-A_2Hpa (Figure 5). The stretch of poly(U) in mRNA M_1Hpa is not responsible for the observed difference in translational efficiency. When this stretch of poly(U) was removed by shortening the 5'-untranslated regions to ~70 nucleotides, no change was observed (data not shown). Most probably the secondary structure of mRNA-A_2Hpa negatively affects translational efficiency.

Discussion

Previous studies of several genes have shown that the degree of evolutionary conservation of the 5'- and part of the 3'-non-coding sequences is higher than that of non-functional DNA (Miyata *et al.*, 1980; Martin *et al.*, 1981). This suggests the existence of evolutionary constraint and implies a functional significance for these regions. Several authors have shown that the tripartite leader is responsible for efficient mRNA translation in adenovirus 5-infected cells (Logan and Shenk, 1984; Thummel *et al.*, 1983; Berkner and Sharp, 1985). An influence of 3'-untranslated regions on gene expression has also been documented in several cases (Kaufman and Sharp, 1982; Zaret and Sherman, 1982; Freytag von Loringhoven *et al.*, 1985).

Other studies have suggested that the secondary structure of eukaryotic mRNAs 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; Gheysen *et al.*, 1982; Tessier *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 zA_1 and cM_1, 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-A_2/Hpa and pDS-M_1/Hpa transcripts in wheat germ extracts suggests the existence of other features in mRNA which affect the translational potential of zein mRNA.

Differences in the translation efficiencies of the various mRNAs described here are not only found when a plant cell-free system is used for the analysis. The same relative translation efficiencies are also found in a rabbit reticulocyte lysate (data not shown).

Anti-sense RNA has been used successfully in interfering with the translation of mRNA. It has been observed that the 5'-untranslated region is a particularly accessible region for anti-sense RNA (see Weintraub *et al.*, 1985). In this *in vitro* study, we have described one example of a *cis*-acting anti-sense mechanism. As few as 20 bases, even matching imperfectly, can have a significant affect on translation.

Materials and methods

E. coli RNA polymerase, and 7mGpppA were from PL-Biochemicals; human placental RNase inhibitor was from BRL; restriction enzymes, nuclease *Bal*31, T4 DNA ligase, DNA *Pol*I and its large fragment (Klenow polymerase) were from Boehringer-Mannheim; [α-^{21}P]GTP and [^{35}S]methionine were from Amersham, UK.

Construction of plasmids and preparation of plasmid DNA

Construction of plasmid DS-M_1 was performed by inserting the zein pcM_1 *Eco*RI fragment of 1059 bp (Spena *et al.*, 1982) into the *Eco*RI site of pDS6 (Stueber *et al.*, 1984). The sense orientation was determined by restriction map analysis. The deletion derivative pDS-M_1Hpa was obtained by *Hpa*II restriction of pcM_1. The *Hpa*II protruding end was filled in by treatment with Klenow polymerase, the plasmid was recut with *Eco*RI and inserted as an *Eco*RI-blunt end fragment into a pDS6 vector cut with *Eco*RI and *Hind*II restriction enzymes. pDS-M_1-Dra was obtained by *Dra*I cleavage of pcM_1, and subcloning into *Sma*I-linearized pDS6. pDS-M_1Hpa/Dra was derived by *Dra*I/*Hind*III cleavage of pDS-M_1Hpa and subcloning into a pDS6 vector cut with *Sma*I and *Hind*III.

The *Bam*HI/*Eco*RI fragment of 2.45 kb, containing the zein zA_1 gene (Spena *et al.*, 1982 and Figure 1A) was subcloned into plasmid pUC8 (Vieira and Messing, 1982). The subclone was linearized with *Bam*HI and digested with *Bal*31 according to the supplier (Boehringer Mannheim). After *Eco*RI digestion, protruding ends were filled in by Klenow polymerase treatment and fragments of ~1.5 kb were subcloned into *Sma*I-linearized pDS6. The length of the 5'-untranslated region of clone pDS-A_2 was precisely mapped to contain 143 bases upstream of the ATG initiation codon. pDS-A_2 Hpa was obtained by *Hpa*II cleavage of plasmid pDS-A_2. The purified fragment was incubated with Klenow polymerase and recloned into pDS6 cut with *Sma*I. The 5'-untranslated regions of pDS-$A_2$50, pDS-$A_2$8 and pDS-$A_2$2 were estimated, by restriction analysis on polyacrylamide gels, to contain ~50, ~8 and ~2 bases, respectively. Plasmid DNA was prepared according to Birnboim and Doly (1979) followed by CsCl/ethidium bromide equilibrium centrifugation (Radloff *et al.*, 1967).

Coupled transcription-translation

The transcription-translation system was previously described (Stueber *et al.*, 1984). Translation in the wheat germ cell-free system, SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Maizel, 1969) and fluorography (ENHANCE, NEN) were done as described.

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 *Taq*I fragment from the zein zA_1 coding region or the *Eco*RI fragment of the cM1 clone were used in the respective hybridizations.

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References

Anderson,O.D., Litts,J.C., Gautier,M. and Green,F. (1984) *Nucleic Acids Res.*, **12**, 8129-8144.
Berkner,L.K. and Sharp,P.A. (1985) *Nucleic Acids Res.*, **13**, 841-857.
Birnboim,H.C. and Doly,J. (1979) *Nucleic Acids Res.*, **7**, 1513-1523.
Freytag von Loringhoven,A., Koch,S., Hofschneider,P.H. and Koshy,R. (1985) *EMBO J.*, **1**, 249-255.
Gheysen,D., Iserentant,D., Derom,C. and Fiers,W. (1982) *Gene*, **17**, 55-63.
Gold,L., Prisbow,D., Schneider,T., Schinedling,S., Swebilius-Singer,B. and Stormo,G. (1981) *Annu. Rev. Microbiol.*, **35**, 365-403.
Gordon,G., Gayda,R.C. and Markovitz,A. (1984) *Mol. Gen. Genet.*, **193**, 414-421.
Gough,N.M., Metcalf,D., Gough,J., Grail,D. and Dunn,A.R. (1985) *EMBO J.*, **4**, 645-653.
Hall,M.N., Gabay,J., Debarbouille,M. and Schwartz,M. (1982) *Nature*, **295**, 616-618.
Horinouchi,S. and Weisblum,B. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 7079-7083.
Hu,N., Pfeifer,M.A., Heidecker,G., Messing,J. and Rubenstein,I. (1982) *EMBO J.*, **1**, 1337-1342.
Hurt,E.C., Pesold-Hurt,B. and Schatz,G. (1984) *EMBO J.*, **3**, 3149-3156.
Kaufman,R.J. and Sharp,P.A. (1982) *Mol. Cell. Biol.*, **2**, 1304-1317.
Kozak,M. (1980) *Cell*, **19**, 79-90.
Kozak,M. (1983) *Microbiol. Rev.*, **47**, 1-45.
Kridl,J.C., Vieira,J., Rubenstein,I. and Messing,J. (1984) *Gene*, **28**, 113-118.
Larkins,B.A., Pedersen,K., Handa,A.K., Hurkman,W.J. and Smith,L.D. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 6448-6452.
Logan,J. and Shenk,T. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 3655-3659.
Maizel,J.V. (1979) in Habel,K. and Salzman,N.P. (eds.), *Fundamental Techniques in Virology*, Academic Press, NY, p. 334.
Maniatis,T., Fritsch,E.F. and Sambrook,J., eds. (1982) *Molecular Cloning, a Laboratory Manual*, published by Cold Spring Harbor Laboratory Press, NY.
Martin,S.L., Zimmer,E.A., Davidson,W.S., Wilson,A. and Kan,Y.W. (1981) *Cell*, **25**, 737-741.
Mignery,G.A., Pikaard,C.S., Hannapel,D.J. and Park,W.D. (1984) *Nucleic Acids Res.*, **12**, 7987-8000.
Miyata,T., Yasunaga,T. and Nishida,T. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 7328-7332.
Pavlakis,G.N., Lockard,R.E., Vamvakopoulos,N., Rieser,L., Rajbhandary,U.L. and Vournakis,J.N. (1980) *Cell*, **19**, 91-102.
Pedersen,K., Devereux,J., Wilson,D.R., Sheldon,W. and Larkins,B.A. (1982) *Cell*, **29**, 1015-1026.
Pelletier,J. and Sonenberg,N. (1985) *Cell*, **40**, 515-526.
Radloff,R., Bauer,W., Vinograd,J. (1967) *Proc. Natl. Acad. Sci. USA*, **57**, 1514-1521.
Rosenberg,M. and Paterson,B.M. (1979) *Nature*, **273**, 636-701.
Salser,W. (1977) *Cold Spring Harbor Symp. Quant. Biol.*, **42**, 985-1002.
Shah,D.M., Hightower,R.C. and Meagher,R.B. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 1022-1026.
Slightom,J.L., Sun,S.M. and Hall,T.C. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1897-1901.
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.
Stueber,D., Ibrahimi,I., Cutler,D., Dobberstein,B. and Bujard,H. (1984) *EMBO J.*, **3**, 3143-3148.
Tabata,T., Sasaki,K. and Iwabuchi,M. (1983) *Nucleic Acids Res.*, **11**, 5865-5875.
Tessier,L., Sondermeyer,P., Faure,T., Dreyer,D., Benavente,A., Villeval,D., Courtney,M. and Lecocq,J. (1984) *Nucleic Acids Res.*, **12**, 7663-7675.
Thummel,C., Tjian,R., Hu,S.L. and Grodzicker,T. (1983) *Cell*, **33**, 455-464.
Vieira,J. and Messing,J. (1982) *Gene*, **19**, 259-268.
Viotti,A., Abildsten,D., Pogan,N. Sala,E. and Pirrotta,V. (1982) *EMBO J.*, **1**, 53-58.
Weintraub,H., Izant,Y.G. and Harland,R.M. (1985) *Trends Genet.*, **1**, 22-25.
Zaret,K.S. and Sherman,F. (1982) *Cell*, **28**, 563-573.

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