Higher Order Structures of the 5'-Proximal Region Decrease the Efficiency of Translation of the Porcine Pro-opiomelanocortin mRNA*

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The SP6 polymerase/promoter system was used to synthesize porcine pro-opiomelanocortin mRNAs with nucleotide sequence deletions in the 5'- as well as 3'-untranslated and coding regions. The translational efficiency of the mutant mRNAs was evaluated by cell-free translation or by monitoring the rate and extent of ribosome binding in the presence of sparsomycin. The results of these experiments indicate that specific nucleotide sequences in the 5'-untranslated and coding regions of the pro-opiomelanocortin mRNA decrease its rate of translation. Structure mapping of the mRNA with double-strand and single-strand specific nucleases suggests that these sequences can form stable secondary structures.

Initiation of protein synthesis in eukaryotic cells is a complex event that involves the molecular interaction of many components (for reviews, see Moldave, 1985; Pain, 1986). In an early step, mRNA molecules in the cellular pool associate with a 43 S preinitiation complex (formed by the 40 S ribosomal subunit, eukaryotic initiation factor-2, -3, and -4C, GTP, and the initiator methionine-tRNA) (Jagus et al., 1981). The binding of mRNAs to the 43 S preinitiation complexes is strongly dependent on the presence of the cap structure, m7GpppN, located at the 5'-end of most eukaryotic mRNAs (Shatkin, 1976). Several polypeptides which appear to specifically interact with the cap structure have been identified (Sonenberg et al., 1978; Tahara et al., 1981; Grifo et al., 1983; Edery et al., 1983). These proteins referred to as cap binding proteins stimulate mRNA binding to ribosomes. The binding of mRNAs to the 43 S complexes requires also the hydrolysis of ATP (Marcus, 1970; Trachsel et al., 1977). The precise role of the cap binding proteins and of ATP hydrolysis is not established, but several lines of evidence suggest that they melt secondary structures in the 5'-proximal region of mRNAs (Sonenberg et al., 1981; Lee et al., 1983; Ray et al., 1985). It was postulated that after binding at or near the 5'-end of mRNAs, the complex moves along the RNA, scanning the sequence for a suitable AUG initiation codon (Kozak, 1983).

Two structural features of mRNAs are believed to modulate the rate at which they are translated. First, Kozak (1984) has shown that the nucleotide sequence around the initiation codon AUG plays an important role in the translational efficiency of the proinsulin mRNA. Second, several studies have associated secondary structures located in the 5'-untranslated region of mRNAs with translational efficiency. By insertion mutagenesis, Polliette and Sonenberg (1985a) and Kozak (1986) have shown that extensive secondary structures in the 5'-untranslated sequence can block the translation of mRNAs. Furthermore, it was suggested by Saito et al. (1983) and Spena et al. (1985) that regions of secondary structure reduce the rate of translation of the c-myc and zen mRNA, respectively.

In this paper we have studied the influence of the structure of the 5'-untranslated region on the translation of the porcine pro-opiomelanocortin (POMC) mRNA. Using exonuclease Bal31 and restriction endonucleases we have generated several mutant POMC mRNAs with deletions in the 5'- and 3'-untranslated portions of the molecule as well as in the coding region. The translation efficiency of these mutant mRNAs was analyzed in a cell-free rabbit reticulocyte lysate and/or by a ribosome binding assay in the presence of sparsomycin. The results indicate that in vitro the translational efficiency of the porcine POMC mRNA can be increased when deletions are introduced that either eliminate a hairpin structure (∆G° = -15.8 kcal/mol) in the 5'-untranslated region or make the 5'-proximal region more accessible to ribosomes.

**MATERIALS AND METHODS**

Construction of 5'-Deletion Mutants—Plasmid pSRT contains a porcine POMC cDNA (Boileau et al., 1983a) cloned between the restriction sites Poul and PstI of pBR327. The Poul site is located at the end of the cDNA corresponding to the 5'-end of the POMC mRNA and will be referred to as the 5'-end throughout the paper. A set of mutant porcine POMC cDNAs with random deletions in the sequences corresponding to the mRNA 5'-untranslated region was constructed by digestion of plasmid pSRT with exonuclease Bal31 as follows. The plasmid containing the cDNA was linearized with the restriction endonuclease Poul and digested with exonuclease Bal31 according to the recommendations of the supplier (Bethesda Research Laboratories). After incubations of 0.5, 1, or 2 min with the nuclease, the plasmid ends were filled in with the Klenow fragment of DNA polymerase I (Pharmacia LKB Biotechnology Inc.) in the presence of 250 μM of each dNTP, and XbaI linkers were added with T4 DNA ligase (Pharmacia LKB Biotechnology Inc.). The plasmids were re-circularized by ligation with T4 DNA ligase and used to transform Escherichia coli HB101 competent cells. Tetracycline-resistant colonies were isolated.

1. The abbreviations used are: POMC, pro-opiomelanocortin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
nies were picked for each incubation time, and the length of the 5'-
untranslated region of the pSRT mutant cDNAs was precisely deter-
mmined by analysis of restriction fragments on polyacrylamide gels and by nucleotide sequencing (Maxam and Gilbert, 1980).

Five mutants termed pSRA clones were selected, and their truncated cDNAs were cloned downstream of the SP6 promoter in plasmid pSFP6 (Meltzer et al., 1984). This cloning was achieved by digesting the pSRA DNA with endonuclease Xbal and filling in the cohesive ends with the Klenow fragment of DNA polymerase I. The cDNA was then released from the plasmid by digestion with endonuclease PstI. Plasmid pSFP6 was restricted with endonuclease HindIII, and the cohesive ends were filled in as described above and then digested with restriction endonuclease PstI. The DNAs from both plasmids were mixed, ligated with T4 DNA ligase, and used to transform Escherichia coli HB101 competent cells. Ampicillin-resistant colonies were screened with restriction endonucleases for the presence of mutant cDNAs, and the positive clones were amplified and characterized by nucleotide sequencing as described above. Five mutants corresponding to the deletions mentioned above in the 5'-untranslated region of the POMC cDNA were thus obtained and named pSPP-24, pSPP-47, pSPP-73, pSPP-95, and pSPP-129. The porcine POMC cDNA contained in pSRT was also inserted in pSFP6 in a manner similar to that described above, except that the HindIII cohesive end of pSFP6 and PstI cohesive ends of PstI were digested with nuclease S1 before treatment with the Klenow fragment of DNA polymerase I. This plasmid was named pSPP-21.

Construction of 3'-Deletion Mutants—The 3'-deletion mutants pSPP-21 RE were obtained by linearization of pSPP-21 with different restriction endonucleases in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl2, 0.5 mg/ml RNAsin (Boehringer Mannheim Canada), 500 pM of each ribonuclease triphosphate (Bethesda Research Laboratories), 500 units/ml tRNA, and at least 5000 cpm of labeled RNA in a final volume of 10 ml.

In Vivo Transcription.—The POMC mRNAs were synthesized in vitro by transcription of the linearized plasmids essentially as described by Melton et al. (1984). Linearization of the plasmids was accomplished by digestion of the DNA with the restriction endonucleases indicated in the legends to the figures. A typical transcription reaction contained 2 pg of linearized plasmid, 40 mM Tris-HCl, pH 7.5, 6 mM MgCl2, 10 mM dithiothreitol, 500 units/ml RNasin (Boehringer Mannheim Canada), 50 units/ml SP6 polymerase (Du Pont-New England Nuclear) in a final volume of 80 ml.

As a tracer, 1 pCi of [α-32P]GTP (Du Pont-New England Nuclear, specific activity 3000 Ci/mmole) was added, and the reaction was incubated at 37°C for 1 h. After treatment with 20 μg/ml RNase-free DNase (Bethesda Research Laboratories) in the presence of 10 mM vanadyl ribonucleosides (Bethesda Research Laboratories), 500 μM of each ribonuclease triphosphate (Bethorcher Mannheim Canada) and 250 units/ml SP6 polymerase (Du Pont-New England Nuclear) at 37°C for 90 min.

In Vitro Capping of RNA—For 5'-end labeling, RNAs (25 μg/ml) produced in the SP6 system were capped with 50 μM each of 20 amino acids, 2 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 5 mM creatine phosphate, 25 μg/ml of ribosyltransferase, 1.5 mM Mg(OAc)2, and 70 mM 5'-MgCl2, for 4 min at 37°C (Lockard and Kumar, 1981). The reaction was stopped as described above. The RNA fragments produced by the nuclease digestions or partial hydrolysis were analyzed on an 85-cm-long, 20-cm-wide, and 0.4-mm-thick polyacrylamide gel containing 7 M urea before nuclease digestion (Pavlakis et al., 1984). Digestions with nuclease S1 (Boehringer Mannheim Canada) were performed in 40 mM sodium acetate, pH 4.5, 200 mM NaCl, and 10 mM ZnSO4, for 5 min at 37°C (Wurst et al., 1978). Digestions with RNases T1 and U2 (Pharmacia LKB Biotechnology Inc.) were done in 25 mM Tris-HCl, pH 7.2, 250 mM NaCl, and 10 mM MgCl2, for 4 min at 37°C (Lockard and Kumar, 1981). The enzyme to substrate ratio for each digestion are included in the figure legends. In all instances, the RNA was precipitated before adding the enzyme, and the reaction mixture contained 1 μg of rRNA and at least 5000 cpm of labeled RNA in a final volume of 10 μl.

Reactions were stopped by the addition of EDTA (pH 7.0) to a final concentration of 25 mM and ethanol precipitation of the RNA. RNA fragments were collected by centrifugation, washed with 70% ethanol at -20°C, and resuspended in 90% formamide containing 50 mM Tris-borate, pH 8.3, 1 mM EDTA, and 0.2% xylene cyanol and bromophenol blue.

The nucleotide ladder was obtained either by partial alkaline hydrolysis of 5'-32P-labeled RNA in 50 mM NaHCO3/NaOH, pH 9.0, 1 mM EDTA at 90°C for 10 min (Trowell et al., 1987) or by heating the RNA at 100°C for 30 min in deionized formamide containing 1 mM EDTA. Positions of the guanine residues in the ladder were determined by digesting 5'-32P-labeled RNA with T1 in the presence of 7 M urea (Donis-Keller et al., 1984). The reaction was stopped as described above.

The RNA fragments produced by the nuclease digestions or partial hydrolysis were analyzed on an 85-cm-long, 20-cm-wide, and 0.4-mm-thick polyacrylamide gel containing 7 M urea (Maxam and Gilbert, 1980). The positions of the fragments were detected by exposing the gel to Fuji X-ray films.

Ribosome Binding Assay.—5'-32P-labeled POMC mRNA was incubated for various periods of time (specified in the legend to Fig. 4) at 25°C in 50 μl of an S23 wheat germ extract containing 20 mM Hapes (pH 7.4), 50 μM each of 20 amino acids, 2 mM dithiothreitol, 1 mM ATP, 0.2 mM GTP, 5 mM creatine phosphate, 25 μg/ml of ribosomal protein L10, 1.5 mM Mg(OAc)2, and 70 mM 5'-MgCl2. At a concentration of 0.2 mM was added to inhibit elongation of protein synthesis. Initiation complex formation was determined by analysis on gel at gradients as described by Sonenberg and Stark (1977). Centrifugation was for 3 h at 90,000 rpm and 4°C in a Beckman SW 41 rotor. Fractions were collected, and creatine was used to determine radioactivity.

RESULTS

Translation of Porcine POMC mRNAs—Porcine POMC mRNAs were synthesized in vitro from plasmid pSPP-21 linearized with PstI, which contains the full-
length cDNA indicated that six nucleotides from the 5'-end of the POMC mRNA were lost and replaced by eight nucleotides from vector pSP64 and from a PvuI linker during the cloning procedure (data not shown). This mRNA is referred to as the full-length POMC mRNA in the present work.

The pSPP-Δ series of plasmids directed the synthesis of mRNAs with deletions in the 5'-untranslated region (see Fig. 1, panel A). Nucleotide sequence analysis of the plasmids suggested that the mRNAs transcribed from these templates are fusion mRNAs with an extension of 17 nucleotides at their 5'-end. These nucleotides (5' GAATACAAGCTCTAG 3') originate from vector pSP64 and from the XbaI linker used in the cloning procedure (data not shown). The pSPP-21 RE series of plasmid directed the synthesis of mRNAs with identical 5'-untranslated regions but with 3'-untranslated and coding regions harboring large deletions (see Fig. 3, panel A). The 5'-untranslated region of these mutant mRNAs is identical to that of the full-length pSPP-21 mRNA.

![Diagram of POMC mRNA Translation](image)

**Fig. 1. Structure and translation of the pSPP-Δ POMC mRNAs.** A, schematic representation of pSPP-21 and pSPP-Δ mRNAs. The black box at the beginning of each mRNA represents the common 17 nucleotides transcribed from the plasmid. The thin black line corresponds to the POMC sequences. The numbers under the black boxes indicate the number of the nucleotide where exonuclease Bal31 stopped. Some unique restriction sites present in the cDNA are also indicated. B, polyacrylamide gel electrophoresis of the 35S-labeled cell-free translation product of pSPP-21 and pSPP-Δ mRNAs. All cell-free translations were primed with 0.05 μg of in vitro synthesized mRNA as evaluated from the incorporated radioactivity. Lane 1, no mRNA added; lanes 2-7, pSPP-21, pSPP-Δ24, pSPP-Δ47, pSPP-Δ73, pSPP-Δ95, and pSPP-Δ129 mRNAs, respectively. C, densitometric analysis of the POMC protein. Quantification of the POMC protein synthesized in each translation assay was done by soft laser densitometry (LKB) analysis of the autoradiogram. The amount of protein synthesized by the assay primed with the pSPP-Δ73 mRNA was arbitrarily set at 100%. The amount of protein produced from the other mRNAs is expressed as a percent of pSPP-Δ73.
Translation of the pSPP-Δ mRNAs—The porcine POMC mRNA has a 5'-untranslated region of 129 nucleotides (Boileau et al., 1983a). To determine the effect of this 5'-untranslated region on the efficiency of translation, we have selected mRNA has a 5"untranslated region of 129 nucleotides with nucleases T1 and U2 which cut 3' to guanine and adenine when translated in a rabbit reticulocyte lysate these mRNAs directed the synthesis of a polypeptide of apparent M, of 37,500 (Fig. 1B) consistent with the previously characterized porcine prepro-opiomelanocortin protein (Boileau et al., 1983b). Scanning of the autoradiogram by soft laser densitometry showed that this polypeptide represents more than 75% of the total radioactivity detected (result not shown). In addition to the major 37,500-dalton species, a minor polypeptide of apparent M, of 34,000 is also synthesized in response to the addition of exogenous POMC mRNA. This polypeptide most probably corresponds to initiation of translation at the second in-frame AUG codon.

The translational efficiency of the different truncated mRNAs was assessed by the amount of protein synthesized by the reticulocyte lysate. As can be seen in Fig. 1B deletions of noncoding sequences in several mutants influence the level of synthesis of the POMC precursor. The densitometric analysis of the autoradiogram (Fig. 1B) is represented in Fig. 1C. This analysis reveals that the mutant pSPP-Δ73 mRNA (lane 5) promotes the synthesis of at least twice as much POMC protein as the control pSPP-21 mRNA (lane 2). We also observed an increase in translational efficiency for the pSPP-Δ55 (lane 6) and pSPP-Δ129 (lane 7) mutant mRNAs as compared to the control pSPP-21 mRNA. In contrast, translation of the mutants pSPP-Δ24 (lane 3) and pSPP-Δ47 (lane 4) was similar to that of the control pSPP-21 mRNA (lane 2).

These results indicate that translational efficiency is not directly correlated with the length of the 5'-untranslated region of the POMC mRNA and strongly suggest that specific features confined to discrete regions of the mRNA structure can affect its translational rate.

Structure Mapping of the POMC mRNA—Stable secondary structure is one feature of mRNAs that has been shown to greatly reduce translational efficiency (Pelletier and Sonenberg, 1985a; Kozak, 1986). To examine the possibility that the increased translation of the pSPP-Δ73 mRNA is a consequence of deletion of secondary structures, we mapped the same region of the 5'-proximal region of the POMC mRNA by partial digestions under non-denaturing conditions of 5'-32P-end-labeled RNA with T1, S1, U2, and V1 nucleases followed by separation of the fragments by electrophoresis on polyacrylamide gels containing 7 M urea. The radioactive fragments were detected by autoradiography, and the nucleotides susceptible to nuclease attack were located by comparing the mobility of the fragments produced by digestion of the same RNA with T1 nuclease under denaturing conditions or by partial hydrolysis. Fig. 2 shows the structure mapping analysis of the pSPP-21 mRNA on a 15% polyacrylamide gel containing 7 M urea.

An examination of Fig. 2 shows regions of enhanced cleavage by the single strand-specific nucleases S1, T1, and U2 as well as regions of inaccessibility. These results suggest a complex secondary structure for the porcine POMC mRNA. S1 nuclease treatment of the 5'-32P-end-labeled RNA revealed three regions of strong cleavage in the 5'-segment of the molecule. These regions are located between nucleotides 31 and 47, 57 and 60, and around nucleotide 135. These results were substantiated when we digested the 5'-end-labeled RNA with nucleases T1 and U2 which cut 3' to guanine and adenine residues, respectively. The major difference found is between nucleotides 17 and 24 where nucleases T1 and U2 detected a region of accessibility whereas nuclease S1 did not cleave. The very high content of purine in this sequence is possibly responsible for the failure of nuclease S1 to digest this region. This is consistent with the appearance of a band of medium intensity in the S1 lane corresponding to a cleavage 3' to the cytosine residue in position 21. It is noteworthy that the AUG initiation codon (nucleotides 130-132) of the porcine POMC mRNA is located in a very accessible region of the molecule.

Digestion of 5'-end-labeled RNA with the double-strand specific V1 nuclease detected regions of secondary structure around nucleotides 38, 53, 67, 84, 118, and 133. These findings are consistent with the results of single-strand specific nuclease digestions presented above except for the region around nucleotide 38 which has been shown to be partially accessible to nucleases S1, T1, and U2. A possible explanation for this observation is the formation of weak alternative secondary interactions in that area.

As noticed in Fig. 1, a deletion of the nucleotide sequence between positions 47 and 73 caused an increase in the translation of the mRNA. Examination of this region in Fig. 2 suggests that it forms a hairpin loop with nucleotides around position 60 very accessible to nuclease S1 (lanes 2 and 3) and strong V1 cuts on either side (lanes 11 and 12). Computer analysis (Zuker and Stiegler, 1981) of that sequence shows that a hairpin stem can be formed by pairing nucleotides 50–56 with nucleotides 65–71. Nucleotides 57–64 would then form the loop. The predicted stability of that structure would be −15.8 kcal/mol (Tinoco et al., 1979). Such a structure is perfectly consistent with the structure mapping analysis except that the guanine nucleotides at positions 51 and 71 are slightly accessible to nuclease T1 (lanes 6 and 7). Their position near the beginning of the stem may explain this discrepancy. Since this structure was detected at 37 °C by both the single- and double-strand specific nucleases, it is likely to exist under the cell-free translation conditions. Therefore, the results suggest that the increased rate of translation observed for the pSPP-Δ73 mutant mRNA is brought about by the deletion of the hairpin structure located between nucleotides 50 and 71 of the POMC mRNA sequence.

The hairpin structure located between nucleotides 50 and 71 is not the only secondary structure present in the 5'-region of the POMC mRNA. As mentioned above, examination of Fig. 2 reveals other areas protected from nuclease digestion. However, computer analysis (Zuker and Stiegler, 1981) showed that the stability of these structures is lower than the stability of hairpin structure 50–71. The deletion of these other potential secondary structures did not result in a significant increase in the translational rate of the POMC mRNA (Fig. 1).

Pelletier and Sonenberg (1985b) and Lawson et al. (1986) have shown that 5'-proximal hybrid structures can reduce the rate of translation of mRNAs by restricting access to the 5'-cap structure. To examine the possibility that the nucleotide sequence between positions 47 and 73 of the porcine POMC mRNA reduces translational efficiency by a similar mechanism, we synthesized 5'-32P-end-labeled pSPP-21, pSPP-Δ47, pSPP-Δ73, and pSPP-Δ129 mRNAs and assessed the accessibility of the cap structure of each mRNA to tobacco acid pyrophosphatase in the conditions described by Godefroy-Colburn et al. (1985). The results of these experiments (not shown) indicated no significant difference between the mRNAs studied suggesting that nucleotides 47–73 decrease translational efficiency through another mechanism.

Translation of the pSPP-21 RE mRNAs—The pSPP-21 RE
mRNAs are a series of mutant RNAs with large deletions in the 3′-untranslated and coding regions created by linearization of plasmid pSPP-21 with different restriction enzymes (Fig. 3A). The translational efficiency of these mRNAs was assessed as described for the pSPP-Δ mRNAs. Fig. 3B shows the sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the translation products of pSPP-21 PstI (lane 1), pSPP-21 EcoRV (lane 2), and pSPP-21 XhoI mRNAs (lane 3). The translation product of the shorter pSPP-21 BglII mRNA could not be detected by this technique. As expected, both pSPP-21 PstI and EcoRV mRNAs directed the synthesis of a polypeptide of apparent Mr of 37,500 corresponding to the full-length POMC precursor. The pSPP-21 XhoI mRNA directed the synthesis of a shorter polypeptide of apparent Mr of 23,000, consistent with the Mr of the protein expected due to the deletion of approximately 100 amino acids. Fig. 3C shows the densitometric scanning of the autoradiogram presented in B. The value obtained from the scanning of lane 3 (pSPP-21 XhoI mRNA) was corrected for the loss of two of the five pre-POMC methionine residues. For comparison purposes, the pSPP-21 PstI mRNA which is the full-length unmodified mRNA was adjusted to the same level as in Fig. 1C. As can be seen in Fig. 3C no significant variations in the rate of translation of the three mRNAs can be observed. These results suggest that in the rabbit reticulocyte lysate the poly(A) tail, the 3′-untranslated region, and the termination codon are not necessary features for maximum translation of the POMC mRNA (the POMC cDNA used in these studies...
has a poly(A) tail of approximately 75 nucleotides).

Ribosome Binding to POMC mRNAs—Since the translation product of the pSPP-21 BglII mRNA could not be detected in the previous experiment, the ribosome binding efficiency of each mRNA was assessed in the presence of sparsomycin. Initiation complex formation was measured by centrifugation through glycerol gradients, and the amount of radioactivity found associated with the initiation complexes was expressed as the percent of total recovered radioactivity. When the ribosome binding efficiency of the pSPP-21 RE mRNAs (Fig. 3, panel A) was assessed, only the BglII mRNA showed a significant increase (factor of 3) over the full-length PstI mRNA (results not presented). The results are in agreement with those presented in the previous section, suggesting that removing the poly(A) tail as well as the entire 3’-untranslated region and part of the coding sequences (downstream from the XhoI site) does not affect the ribosome binding efficiency of the POMC mRNA. On the contrary, the removal of sequences located between the BglII and the XhoI restriction sites favors binding of the mRNA to the ribosomes. Ribosome binding of all mRNAs could be totally inhibited by the addition of 100 μM of the cap analog m’GpppG (results not presented) showing that it is specific.

To further characterize the binding of the POMC mRNAs to the ribosomes, we have determined the kinetics of ribosome binding to the BglII and PstI mRNAs. Fig. 4 shows the formation of 80 S initiation complexes and of a faster moving disosome peak when 32P-labeled PstI mRNA (panel A) or BglII mRNA (panel B) is incubated in a wheat germ extract in the presence of sparsomycin and analyzed on glycerol gradients. It can be seen that the rate of ribosome binding on the shorter BglII mRNA is much faster, since after only 0.25 min of incubation maximum formation of 80 S initiation complexes has been obtained; with the PstI mRNA, binding continues over a period of 10 min. However, the rate of formation of disomes is not increased to the same extent. Formation of a disosome peak is observed because the 5’-untranslated region of the POMC mRNA is long enough (129 nucleotides) to accommodate more than one ribosome (Filipowicz and Haenni, 1979).

The absolute amount of radioactivity found associated with ribosomes is higher for the PstI mRNA than for the BglII mRNA, although the percentage of bound mRNA out of total input is higher for BglII mRNA (maximum binding of 39%).
than for PstI mRNA (maximum binding of 9.3%). This is due
to the fact that equimolar amounts of mRNAs were added in
the incubation mixture. Since the mRNAs are evenly labeled
with \(^{32}\)P-GTP throughout the chain and since the PstI
mRNA is roughly four times larger than the BglII mRNA, the
amount of radioactivity added in the incubation mixture is
four times higher for the PstI mRNA than for the BglII
mRNA.

Sensitivity of the BglII and PstI mRNA 5'-Untranslated Sequences to T1 Nuclease Digestion—A possible explanation
for the results of the ribosome binding assay would be that
removal of the sequences between the BglII and XhoI restriction
sites has somehow disrupted the secondary or tertiary structure of the 5' untranslated region of the mRNA making
it more available for ribosome binding. To verify this hypothesis, 5'-\(^{32}\)P-end-labeled BglII and PstI mRNAs were
digested with T1 ribonuclease, and the digestion fragments were sep-

ared on a 15% polyacrylamide gel containing 7 M urea. Only T1 ribonuclease was used in this experiment because of the
high guanine content of the 5'-proximal region of the porcine
POMC mRNA (Boileau et al., 1983a). As can be seen in Fig.
5, several regions of the BglII mRNA are more sensitive to
T1 ribonuclease attack than in the PstI mRNA. Specifically
it can be noticed that the guanine residues from position 9 to
18 have an increased sensitivity to T1 nuclease digestion.
When 5'-\(^{32}\)P-end-labeled BglII and PstI mRNAs were treated with tobacco acid pyrophosphatase under the conditions
described by Godfrey-Colburn et al. (1985), both the BglII and
PstI mRNAs showed similar accessibility to the 5'-cap structure (result not shown). These results support the idea that the
increase in ribosome binding observed for the BglII mRNA is
brought about by an increase in accessibility of the 5'-untranslated region of the mRNA and further suggest that in
the mutant POMC mRNAs, 5'-proximal structures decrease
translational efficiency by a mechanism different than re-
stricting access to 5'-cap structure.

It is difficult to assess whether this increased accessibility
is caused by disruption of secondary or tertiary structure.
However, a computer search for complementary sequences to
region 9-18 revealed a sequence located between nucleotides
636 and 646 possessing 10 out of 11 nucleotides complemen-
tary to position 8-18. Furthermore, in the XhoI mRNA the
guanine residues from position 9-18 do not show the increased
sensitivity to ribonuclease T1 observed in the BglII mRNA
(data not shown) (XhoI endonuclease cuts plasmid pSPP-21
at nucleotide 643 of the POMC sequence).

DISCUSSION

We have presented evidence that the translational effi-
ciency of \textit{in vitro} synthesized mutant porcine POMC mRNAs
is decreased by the presence of 5'-noncoding sequences. This
conclusion was reached after analysis of two types of mutant
mRNAs. The first mutants (plasmid pSPP-\(\Delta\)) were obtained
by exonuclease Bal31 treatment and correspond to successive
deletions of nucleotide sequences in the 5'-noncoding region
of the porcine POMC mRNA. Removal of nucleotides 47-73
(mutant pSPP-\(\Delta73\) ) increased the translational rate of mRNA
by a factor of about 2. Structure mapping of the \textit{in vitro}
synthesized mRNA showed that this sequence can form a
hairpin structure with a Gibbs energy change of \(-15.8\) kcal/
mol. Recently, Pelletier and Sonenberg (1985b) and Lawson
et al. (1986) have shown that hybrid structures within the
first 18 nucleotides of mRNAs restrict the accessibility of the
cap structure to the cap binding proteins, thus reducing trans-
lational efficiency. Hybrid structures located downstream
from this region had no apparent effect. Since all the pSPP-
\(\Delta\) mRNAs have the same 17-nucleotide sequence at their 5'-'end (see "Results") and since the hairpin structure formed by
nucleotides 59-71 starts 67, 43, and 20 nucleotides from the
5'-cap structure in the control mRNA, pSPP-\(\Delta24\), and pSPP-
\(\Delta47\) mutants, respectively, such a mechanism cannot be in-
voked to explain their reduced rate of translation. This assertion
was supported by cap accessibility studies. Rather it is
possible that the hairpin structure decreases the translational
efficiency of the porcine POMC mRNA by slowing down the
migration of the 43 S initiation complex along the mRNA.
Further deletions of sequences (mutants pSPP-\(\Delta95\) and
\(-\Delta129\) ) produced a decline in the synthesis of the POMC
proteins. The reason for this behavior is not clear, but one
possibility is that the close proximity of the AUG initiation
endonucleases before in vitro transcription. All these mutant mRNAs have the same 5'-untranslated segment but differ by large deletions in the 3'-untranslated and coding regions. Deletion of the poly(A) tail (mutant pSPP-21 EcoRV) or of all the 3'-untranslated region and part of the coding segment (mutant pSPP-21 XhoI) did not affect the rate of translation nor the affinity for ribosomes of the mutant mRNAs. This is in agreement with in vitro and in vivo experiments where the 3'-noncoding sequences from β-globin (Kronenberg et al., 1979) and human γ and β interferon mRNAs (Soreq et al., 1981) were deleted without affecting their translational rate and further suggests that the translational termination codon is not an essential feature of the mRNA for efficient termination of protein synthesis. However, when we removed sequences from the coding region that led to greater accessibility of the 5'-untranslated region to T1 nuclease, we noticed an increased affinity of the mutant mRNA (pSPP-21 BglII) for ribosomes.

The results presented here indicate that secondary and possibly tertiary structures located in the 5'-untranslated region of the porcine POMC mRNA decrease its rate of translation. Our experimental data suggest that these structures act by decreasing the accessibility of the 5'-proximal region of the mRNA to the ribosome and also possibly by interfering with its migration toward the initiation codon.

In conclusion, we have presented evidence that sequences proximal to the 5'-end of the porcine POMC mRNA reduce its in vitro translational efficiency by promoting the formation of secondary structures. Our studies were performed with in vitro transcribed mRNA and thus ignored the role of the RNA-bound proteins in translation. For this reason, we are presently assessing the in vivo translational efficiency of some of our mutants.

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