Footprinting mRNA–ribosome complexes with chemical probes

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We footprinted the interaction of model mRNAs with 30S ribosomal subunits in the presence or absence of tRNA^{Met} or tRNA^{Phe} using chemical probes directed at the sugar–phosphate backbone or bases of the mRNAs. When bound to the 30S subunits in the presence of tRNA^{Met}, the sugar–phosphate backbones of gene 32 mRNA and 022 mRNA are protected from hydroxyl radical attack within a region of about 54 nucleotides bounded by positions –35 (± 2) and +19, extending to position +22 when tRNA^{Phe} is used. In 70S ribosomes, protection is extended in the 5' direction to about position –39 (± 2). In the absence of tRNA, the 30S subunit protects only nucleotides –35 (± 2) to +5. Introduction of a stable tetraloop hairpin between positions +10 and +11 of gene 32 mRNA does not interfere with tRNA^{Met}-dependent binding of the mRNA to 30S subunits, but results in loss of protection of the sugar–phosphate backbone of the mRNA downstream of position +5. Using base-specific probes, we find that the Shine–Dalgarno sequence (A–12, A–11, G–10 and G–9) and the initiation codon (A+1, U+2 and G+3) of gene 32 mRNA are strongly protected by 30S subunits in the presence of initiator tRNA. In the presence of tRNA^{Phe}, the same Shine–Dalgarno bases are protected, as are U+4, U+5 and U+6 of the phenylalanine codon. Interestingly, A–1, immediately preceding the initiation codon, is protected in the complex with 30S subunits and initiator tRNA, while U+2 and G+3 are protected in the complex with tRNA^{Phe} in the absence of initiator tRNA. Additionally, specific bases upstream from the Shine–Dalgarno region (U–33, G–32 and U–22) as well as 3' to the initiation codon (G+11) are protected by 30S subunits in the presence of either tRNA. These results imply that the mRNA binding site of the 30S subunit covers about 54–57 nucleotides and are consistent with the possibility that the ribosome interacts with mRNA along its sugar–phosphate backbone.

Key words: chemical probing/hydroxyl radical/tetraloop/30S subunit

Introduction

One of the most biologically important macromolecular interactions is that between mRNA and ribosomes. Yet,

apart from the well-known Shine–Dalgarno interaction (Shine and Dalgarno, 1974), little is understood about the molecular mechanisms that govern this process. Early nuclease protection studies showed that the ribosome protects about 30 nucleotides of the mRNA chain, centered on the initiation codon, in 70S initiation complexes. (Steitz, 1969; Platt and Yanofsky, 1975). Base-specific chemical probing experiments (Murukawa and Nierlich, 1989) have provided further clues about which features of mRNA might be recognized by the ribosome. More recently, Gold and co-workers have used reverse transcriptase to 'toeprint' the position of mRNA with respect to the physical frame of the ribosome (Hartz *et al.*, 1988). In addition, information about the proximity of specific positions within mRNA with respect to landmarks in the ribosome has been obtained using modified mRNAs that can be cross-linked to rRNA or ribosomal proteins (Wollenzien *et al.*, 1991; Bhangu and Wollenzien, 1992; Dontsova *et al.*, 1992; Rinke-Appel *et al.*, 1993).

Because the coding sequences of mRNA can have virtually any possible nucleotide sequence, it would seem unlikely that interaction of mRNA with ribosomes takes place through its bases, except for special cases such as during translational initiation. Indeed, searches of *Escherichia coli* mRNAs for conserved sequence elements that could serve as recognition elements for the ribosome has revealed very little sequence conservation, apart from the Shine–Dalgarno sequence and initiator codon (Stormo *et al.*, 1982). Moreover, these few conserved elements are found only in the context of the translational initiation site. Yet it is hard to imagine how the ribosome could accurately maintain the translational reading frame, among other things, without some regular, periodic contact with the mRNA chain. Trifonov (1987) has reported a statistically significant periodicity in mRNA sequences from different organisms and has suggested that this could reflect base pairing interactions with 16S rRNA.

An alternative possibility is that contacts with the ribosome may involve the sugar–phosphate backbone of mRNA, which could provide a sequence-independent structural basis for mRNA–ribosome interaction. We have addressed this possibility by probing mRNA–ribosome complexes with hydroxyl radicals, which attack the RNA backbone at its ribose C1' and C4' positions (Wu *et al.*, 1983). When bound to 70S ribosomes or 30S subunits, a well-defined region of mRNA is protected from hydroxyl radicals. The extent of gene 32 mRNA protected by 30S subunits ranges from about 40 nucleotides in the absence of tRNA to 54 in complexes containing initiator tRNA and 57 in complexes containing the first elongator tRNA (tRNA^{Phe}). The 5' boundary of the protected region, around position –35, is unaffected by the presence of tRNA, while the 3' boundary depends on whether or not tRNA is present and differs according to whether initi-

ator tRNA or tRNA^{Phe} is bound. When a stable tetraloop hairpin is incorporated into the mRNA structure between positions +10 and +11, protection of the mRNA downstream from position +5 is disrupted. Certain bases of mRNA are also protected from base-specific chemical probes; these include the Shine–Dalgarno sequence, the codon corresponding to the bound tRNA and several other discrete positions within the hydroxyl radical-protected region. These results imply that the mRNA binding site of the 30S subunit covers about 54–57 nucleotides and are consistent with the possibility that the ribosome interacts with mRNA along its sugar–phosphate backbone.

Results

T7 transcription of mRNAs and formation of mRNA–ribosome complexes

Three different mRNAs were used in these studies: gene 32 mRNA (Hartz *et al.*, 1988), 022 mRNA (La Teana *et al.*, 1993) and a derivative of gene 32 mRNA which contains a tetraloop hairpin (gene 32-TL). All mRNAs were transcribed by T7 RNA polymerase from DNA templates derived by PCR amplification from the respective cloned genes (see Materials and methods). To test for binding to either 30S subunits or 70S ribosomes, 5' ³²P-end-labeled mRNAs were incubated with either 30S subunits and tRNA_f^{Met} (Figure 1A) or 30S subunits and tRNA_f^{Met} followed by addition of 50S subunits (Figure 1B) and analyzed by sucrose gradient sedimentation. Figure 1A shows that 5' ³²P-end-labeled gene 32 mRNA co-sediments with 30S ribosomal subunits; upon addition of 50S subunits, the labeled mRNA co-sediments with the 70S ribosome peak (Figure 1B).

Toeprint analysis of mRNA–ribosome complexes

We chose gene 32 mRNA as a focus for these studies because its interaction with 30S ribosomes has been characterized extensively by Gold and co-workers using both genetic and biochemical approaches, including toeprint analysis (Hartz *et al.*, 1988). In the latter method, ribosome-bound mRNA is used as a template for reverse transcriptase, which terminates cDNA synthesis when its progress is blocked by collision with the ribosome (Figure

2; Hartz *et al.*, 1988). The premature reverse transcriptase stop, called a toeprint, is identified by gel electrophoresis. Hartz *et al.* (1988) showed that binding tRNAs in response to codons at different positions of the mRNA, including the AUG start codon, gave rise to different characteristic toeprint signals indicative of the tRNA-dependent positioning of mRNA on the ribosome.

Gene 32 mRNA contains an AUG initiation codon, followed by a UUU (phenylalanine) codon. To test for specific binding of gene 32 mRNA to 30S subunits, a toeprint assay was performed in the presence of tRNA_f^{Met} or tRNA^{Phe}. As expected, either tRNA_f^{Met} or tRNA^{Phe} were able to participate in formation of a ternary complex with gene 32 mRNA and 30S ribosomal subunits, as shown by their respective toeprint signals (Figure 2). Each of the tRNAs is presumed to bind to the ribosomal P site of the 30S subunit in the complex (Hartz *et al.*, 1988); indeed, the main toeprint obtained in the presence of tRNA_f^{Met} differs by three nucleotides from that obtained with tRNA^{Phe} (Figure 2, lanes 2 and 3). No toeprint signal is observed when gene 32 mRNA is reverse transcribed in the absence of tRNA or in the absence of 30S subunits (Figure 2, lanes 1 and 4), in agreement with the results of Hartz *et al.* (1988).

Hydroxyl radical footprints of gene 32 mRNA and 022 mRNA by 30S ribosomal subunits

To examine protection of the sugar–phosphate backbone of mRNA by the 30S ribosomal subunit, 5' ³²P-end-labeled gene 32 mRNA was bound to 30S subunits in the presence of tRNA_f^{Met} and the resulting complex was purified on a Sepharose 6-B spin column and probed with hydroxyl radicals generated by Fe²⁺–EDTA (see Materials and methods). As shown in Figure 3A, 30S ribosomal subunits strongly protect about 54 nucleotides of gene 32 mRNA from hydroxyl radical attack in a region surrounding the Shine–Dalgarno and initiation codon sequences, extending from position –35 (±2) to +19 (Figure 3A, lane 2). In the complex formed in the presence of tRNA^{Phe}, a similar region is protected, except that the 3' border of the footprint is extended to position +22 (Figure 3A, lane 3). To test the generality of these results, we probed a different mRNA, 022 mRNA (La

Fig. 1. Isolation of mRNA–ribosome complexes by sucrose gradient centrifugation. 5' ³²P-end-labeled gene 32 mRNA was bound to (A) 30S ribosomal subunits in the presence of tRNA_f^{Met} or (B) to 70S ribosomes in the presence of tRNA_f^{Met}.

Fig. 2. Toeprint analysis of gene 32 mRNA bound to 30S ribosomal subunits. A, G, dideoxy sequencing lanes; lane 1, mRNA only; lane 2, complex containing 30S subunits and tRNA^{Met}; lane 3, complex containing 30S subunits and tRNA^{Phe}; lane 4, 30S subunits alone. Arrows indicate stops ('toeprints') of AMV reverse transcriptase due to collision with the 30S subunit (Hartz *et al.*, 1988).

Fig. 3. Hydroxyl radical footprints of 5' ³²P-end-labeled mRNA bound to 30S subunits. (A) Gene 32 mRNA. T, partial RNase T₁ digest; C, untreated mRNA; lanes 1–4, hydroxyl radical probing of: lane 1, mRNA only; lane 2, gene 32 mRNA, 30S subunits and tRNA^{Met}; lane 3, gene 32 mRNA, 30S subunits and tRNA^{Phe}; lane 4, gene 32 mRNA and 50S subunits; lanes 5–6, unmodified: lane 5, gene 32 mRNA, 30S subunits and tRNA^{Met}; lane 6, gene 32 mRNA, 30S subunits and tRNA^{Phe}. (B) 022 mRNA. T, partial RNase T₁ digest; lanes 1–4, hydroxyl radical probing of: lanes 1 and 3, 022 mRNA only; lane 2, 022 mRNA, 30S subunits and tRNA^{Met}; lane 4, 022 mRNA, 30S subunits and tRNA^{Met}, unmodified. (C) Gene 32 mRNA bound in the absence of tRNA. T, partial RNase T₁ digest; C, untreated mRNA; lanes 1–4, hydroxyl radical probing of: lane 1, mRNA only; lane 2, gene 32 mRNA, 30S subunits and tRNA^{Met}; lane 3, gene 32 mRNA and 30S subunits; lane 4, gene 32 mRNA only. The positions of the Shine–Dalgarno sequence (SD) and AUG initiation codon are indicated. Arrows show end points of digestion of mRNA by an exonuclease activity associated with 30S subunits.

Teana *et al.*, 1993), bound to 30S subunits in the presence of tRNA_f^{Met} (Figure 3B). A similar region of about 54 (± 2) nucleotides surrounding the initiation codon and Shine-Dalgarno sequence was protected from hydroxyl radical cleavage (Figure 3B, lane 2), however, protection of the upstream region of 022 mRNA was less pronounced than that observed for gene 32 mRNA.

We then probed gene 32 mRNA bound to 30S subunits in the absence of tRNA. Affinity of the mRNA for 30S subunits in the absence of tRNA was found to be reduced compared with binding in the presence of tRNAs, as judged by the reduced amount of end-labeled mRNA comigrating with 30S subunits on Sepharose 6-B spin columns (data not shown). In the absence of tRNA, the protected region is reduced to about 40 nucleotides, extending from positions -35 to +5 (Figure 3C, lane 3). Thus, the presence of tRNA in the complex appears to be important for establishing protection of the 3' end of the mRNA from position +6 to position +19 (or +22). Nevertheless, the tRNA-independent footprint has the same 5' border as that observed in the presence of tRNA, despite the absence of a detectable toeprint signal.

3'-Exonuclease activity associated with 30S subunits

In both the probed (Figure 3A, lanes 2 and 3) and non-probed (Figure 3A, lanes 5 and 6) lanes, prominent bands can be seen corresponding to gene 32 mRNA fragments extending from its 5' end to positions +20 (lanes 2 and 5) or +23 (lanes 3 and 6). Several lines of evidence suggest that these bands mark the end-points of digestion by a 3'-exonuclease activity associated with 30S ribosomal subunits.

First, these bands are observed in the presence but not in the absence of 30S subunits (Figure 3A, compare lanes C and 5). Second, cleavage products are seen with 5'-labeled but not with 3'-labeled mRNAs (data not shown). Third, these bands are not observed in primer extension analysis (Figure 2 and see below). Fourth, analysis of the products resulting from incubation of gene 32 message with 30S subunits, in which all U residues contained a 5'-[³²P]phosphate, revealed 5'-[³²P]UMP as the sole product (unpublished results). We infer that these bands are the result of a 3'-exonuclease activity, most probably RNase II (Donovan and Kushner, 1986), associated with our 30S ribosomal subunit preparations.

The 3' limits of these putative exonuclease effects differ by three nucleotides, depending upon whether tRNA_f^{Met} or tRNA_f^{Phe} is bound (Figure 3). This result suggests that the bands at positions +20 and +23 are the result of an exonuclease toeprint. The positions of these toeprints are thus five nucleotides downstream of those created by reverse transcriptase. This difference is probably due to differences between the structures of these different enzymes and how they match up to the structure of the 30S ribosomal subunit. An interesting question is whether this exonuclease activity acts as a bound integral part of the 30S subunit. This could have an important bearing on the mechanism of mRNA degradation in *E. coli*.

Hydroxyl radical probing of gene 32 mRNA bound to 70S ribosomes

To study the effects of the presence of 50S subunits, gene 32 mRNA was probed with hydroxyl radicals in a complex

Fig. 4. Hydroxyl radical footprint of 5' ³²P-end-labeled gene 32 mRNA by 70S ribosomes. T, partial RNase T₁ digest; lanes 1–5, hydroxyl radical probing of: lanes 1, 3 and 5, gene 32 mRNA only; lane 2, gene 32 mRNA, 30S subunits and tRNA_f^{Met}; lane 4, gene 32 mRNA, 70S ribosomes and tRNA_f^{Met}. The positions of the Shine-Dalgarno sequence (SD) and AUG initiation codon are indicated. Arrow shows end point of digestion of mRNA by a 3'-exonuclease activity associated with 30S subunits or 70S ribosomes.

with 70S ribosomes and tRNA_f^{Met} (Figure 4). Prior to probing, complexes were analyzed by sucrose gradient sedimentation, to confirm binding of mRNA to 70S ribosomes (Figure 1B). In the 70S complex, a similar region of the mRNA is protected from hydroxyl radical cleavage, except that the 5' border of the footprint is extended by about four nucleotides compared with that observed for 30S subunits (Figure 4, compare lanes 2 and 4). This could indicate interaction of the 5' region of the mRNA with 50S subunits or that 50S subunits influence the mode of interaction of mRNA with 30S subunits. This same footprint was observed whether the 70S complex was constructed from 30S and 50S subunits or from 70S tight couple ribosomes (data not shown). We note that the same exonuclease toeprint is observed at position +20

Fig. 5. Hydroxyl radical footprint of 5' 32 P-end-labeled gene 32-tetraloop mRNA (gene 32-TL) bound to 30S ribosomal subunits. T, partial RNase T_1 digest; lanes 1–5, hydroxyl radical probing of: lanes 1, 3 and 5, gene 32-TL mRNA alone; lane 2, gene 32-TL mRNA, 30S subunits and tRNA^{Met}; lane 4, gene 32-TL mRNA, 30S subunits and tRNA^{Phe}; lanes 6 and 7, unmodified: lane 6, gene 32-TL mRNA, 30S subunits and tRNA^{Met}; lane 7, gene 32-TL mRNA, 30S subunits and tRNA^{Phe}. The positions of the Shine–Dalgarno sequence (SD), the tetraloop hairpin and AUG initiation codon are indicated. Arrow shows end-point of digestion by a 3'-exonuclease activity associated with 30S ribosomal subunits.

near the 3' border of the footprint in both 30S and 70S ribosomes (Figure 4).

A stable tetraloop hairpin locally disrupts interaction of gene 32-TL mRNA with 30S subunits

Our current understanding of mRNA–ribosome interaction suggests that unstructured, easily deformable mRNA chains are most readily accommodated by the ribosome. To explore the effects of stable mRNA structure on mRNA–ribosome interaction, we introduced a tetraloop

Fig. 6. Extent of protection of mRNA from hydroxyl radical attack by 30S ribosomal subunits. Protected nucleotides are underlined; dotted lines indicate weaker protection. The sequence of the tetraloop hairpin which was introduced into gene 32 mRNA is boxed. The Shine–Dalgarno sequences (SD) and P and A site codons are indicated.

hairpin structure between positions +10 and +11 of gene 32 mRNA to create gene 32-TL mRNA (Materials and methods; see Figure 6E). RNA tetraloops, hairpin stems that typically contain UUCG or GNRA loops, have been shown to be unusually stable structures (Uhlenbeck, 1990). Kethoxal probing of the free gene 32-TL mRNA confirms the formation of a stable stem-loop between positions +9 and +22 (Figure 6E, data not shown). The G residues in the predicted stem of gene 32-TL mRNA are resistant even to digestion with RNase T_1 in the presence of 7 M urea at 50°C, providing additional evidence for formation of the predicted stem-loop structure (Figure 5, lane T).

Prior to probing, complexes containing 30S subunits, tRNA^{Met} or tRNA^{Phe} and 5' 32 P-end-labeled gene 32-TL mRNA were purified on a Sepharose-6B spin column. Co-elution of gene 32-TL mRNA with 30S subunits shows that this structurally modified mRNA retains its ability to bind to 30S subunits. Binding was further confirmed by chemical probing of the complex with kethoxal (KE), which showed protection of the guanines within the Shine–Dalgarno sequence (G–10 and G–9) and in the initiation codon (G+3) of gene 32-TL mRNA (data not shown).

Hydroxyl radical probing of the complexes shows that introduction of the tetraloop causes a dramatic loss of protection downstream of the initiation codon, rendering the mRNA accessible to hydroxyl radical attack in the normally protected region beyond position +5 (Figure 5, lanes 2 and 4). The exposed region includes the tetraloop hairpin itself, as well as several nucleotides in the surrounding region of the mRNA. In the case of gene 32-TL mRNA, a strong band is observed around position +22 for RNA isolated from unprobed 30S complexes. This result suggests stalling of the 3'-exonuclease activity at

the base of the tetraloop hairpin structure, consistent with the known resistance of stable RNA secondary structure to digestion by RNase II (McLaren *et al.*, 1991). The results of hydroxyl radical probing are summarized in Figure 6.

Base-specific probing of gene 32 mRNA bound to 30S subunits

We next asked whether the mRNA bases are protected when gene 32 mRNA is bound to 30S subunits. The chemical probes 1-cyclohexyl-2-morpholino-carbo-diimide-*p*-toluene sulfonate (CMCT), kethoxal (KE) and dimethyl sulfate (DMS) and KE, in conjunction with primer extension analysis (Stern *et al.*, 1988), were used to study interactions between the Watson–Crick pairing positions of all four bases and 30S subunits. Gene 32 mRNA was bound in complexes with 30S subunits and tRNA^{Met} or tRNA^{Phe} and binding was confirmed by a toeprint assay as described above. Figure 6 shows the results of probing gene 32 mRNA bound to 30S subunits in the presence of tRNA^{Met} (lanes 2) or tRNA^{Phe} (lanes 3) with CMCT, KE or DMS.

Bases A–12, A–11, G–10 and G–9 of the Shine–Dalgarno sequence are strongly protected in either the tRNA^{Met} or tRNA^{Phe} complexes (Figure 7B and C, lanes 2 and 3) or in the absence of tRNA (data not shown). In the complex with tRNA^{Met}, bases A+1, U+2 and G+3 of the initiation codon are protected from chemical modification, directly demonstrating their Watson–Crick pairing with the anticodon of tRNA^{Met} (Figure 7, lanes 2). Unexpectedly, A–1 is also protected from DMS attack (Figure 7C, lane 2).

In the complex containing tRNA^{Phe}, bases U+4, U+5 and U+6 of the phenylalanine codon immediately following the AUG initiation codon are protected (Figure 7A, lane 3). Note that spontaneous (CMCT-independent) reverse transcriptase stops occur at positions +1 through +6 (Figure 7A, lane K), partially masking the CMCT-dependent signal (Figure 7A, lane 1). Consequently, full protection against CMCT attack can at best result in a reduction in the band intensity to the level of lane K, which is what is observed (Figure 7A, lane 3). Interestingly, U+2 and G+3 of the AUG initiation codon preceding the phenylalanine codon are strongly protected from chemical modification, even though tRNA^{Met} is absent (Figure 7A and

Fig. 7. Chemical probing of gene 32 mRNA. Gene 32 mRNA or mRNA–30S complexes were probed with (A) 1-cyclohexyl-2-morpholino-carbodiimide-metho-*p*-toluene sulfonate (CMCT), (B) kethoxal (KE) or (C) dimethyl sulfate (DMS) and analyzed by primer extension (Stern *et al.*, 1988). A, G, dideoxy sequencing lanes; K, primer extension of unmodified gene 32 mRNA; lanes 1–3, chemical probing of: lane 1, gene 32 mRNA alone; lane 2, gene 32 mRNA, 30S subunits and tRNA^{Met}; lane 3, gene 32 mRNA, 30S subunits and tRNA^{Phe}. The positions of bases that were reproducibly protected in several experiments are indicated.

B, lanes 3). In the region upstream of the initiation codon, U-5 is protected in the presence of tRNA^{Phe} , but not in the presence of tRNA^{Met} (Figure 7A, lanes 2 and 3). In addition, several other bases, U-33, G-32, U-22 and G+11, 5' of the Shine-Dalgarno sequence and 3' of the initiation codon are less strongly, but reproducibly, protected from chemical modification in the 30S complexes.

Discussion

A region of 54 (± 2) nucleotides in gene 32 mRNA or 022 mRNA was found to be protected from hydroxyl radical attack by 30S ribosomal subunits. The protected region spans positions -35 (± 2) to position +19 of the mRNA in the presence of initiator tRNA and extends in the 3' direction to position +22 in the presence of the first elongator tRNA (tRNA^{Phe}). The Shine-Dalgarno sequence and the initiation codon are thus centrally located within the footprinted region. The length of the protected region is in agreement with the results of Kang and Cantor (1985), who found about 55 nucleotides of poly(U) mRNA protected from RNase cleavage by 30S subunits or 70S ribosomes. However, other studies using natural mRNAs have typically found a smaller region of about 30-40 nucleotides protected by the ribosome in translational initiation complexes (Steitz, 1969; Platt and Yanofsky, 1975; Murukawa and Nierlich, 1989).

The 3' ends of the protected regions in these earlier studies were localized between positions +16 and +21, in good agreement with our findings. However, the 5' boundaries varied from around position -14 to -46, depending on RNase concentrations, temperature and other experimental conditions. In some of our experiments, the region upstream of the Shine-Dalgarno sequence was less strongly protected from hydroxyl radical attack. Thus, observed differences in the 5' limit of the ribosome-protected region of mRNA, but not in the position of the 3' boundary, might be explained by weaker interaction between the ribosome and positions -17 to -35.

Protection by 70S ribosomes extends to the same downstream boundary as for 30S subunits, in agreement

with the toeprinting results of Hartz *et al.* (1988), however, about four additional nucleotides are weakly protected at the 5' end by 70S ribosomes. This additional protection could alternatively be explained by interaction of nucleotides in the region around -36 to -39 of mRNA with the 50S subunit, influence of the 50S subunit on interaction of mRNA with the 30S subunit or by enclosure of the 5' region of mRNA within the 30S-50S subunit interface.

In the absence of tRNA, protection of mRNA downstream from position +5 was lost, indicating that shielding of the mRNA backbone between positions +6 and +19 (or +22) is dependent on binding of tRNA, even though the codon recognized by the bound tRNA is not located within the latter region. This suggests that codon-anticodon interaction influences the pathway of the downstream region of the mRNA through the ribosome (Figure 9). This finding is in agreement with the results of Ringquist *et al.* (1993), who came to similar conclusions based on toeprinting experiments.

The two well-known canonical Watson-Crick interactions involving mRNA are base pairing of the Shine-Dalgarno sequence with the 3' end of 16S rRNA and the initiation codon-anticodon pairing. Although there is extensive evidence for these interactions, to our knowledge they have not yet been demonstrated directly at nucleotide resolution by chemical probing. Binding of gene 32 mRNA to 30S subunits in the presence of tRNA^{Met} or tRNA^{Phe} resulted, as expected, in protection of the initiator or Phe codons respectively from attack by chemical probes that are specific for the Watson-Crick pairing positions of their bases, providing direct evidence for the base pairing of these two codons with the anticodons of their respective tRNAs (Figures 7 and 8).

We also observe strong protection of bases A-12, A-11, G-10 and G-9 of the Shine-Dalgarno sequence from these same probes when gene 32 mRNA is bound to 30S subunits in the presence or absence of tRNA. It is noteworthy that the Shine-Dalgarno interaction is maintained in the presence of bound tRNA^{Met} , even

Fig. 8. Bases and riboses protected in gene 32 mRNA by 30S subunits and 70S ribosomes. (A) 30S or 70S complex containing gene 32 mRNA and tRNA^{Met} . (B) 30S complex containing gene 32 mRNA and tRNA^{Phe} . Base protections: ●, protection from CMCT; ■, protection from kethoxal; ▲, protection from DMS. Protection of the mRNA backbone from hydroxyl radical attack is shown by underlining; weaker protection is indicated by dotted lines. Additional protection observed in 70S ribosomes is shown by the extended underlining at the 5' end. The fragment of R17 mRNA protected from RNase A (Steitz, 1969) is shown for comparison. The toeprint signals and end-points of digestion of gene 32 mRNA by a 3'-exonuclease activity are indicated by arrows.

Fig. 9. Interaction of gene 32 mRNA with 30S subunits in the presence or absence of tRNA^{Met} .

though this interaction is presumed to play no further role once the initiator tRNA is correctly bound and paired with its start codon. Binding of gene 32 mRNA in the presence of tRNA^{Phe} is believed to result in formation of a complex in which the mRNA is translocated by three nucleotides relative to a complex formed with tRNA^{Met} (Hartz *et al.*, 1988; see Figure 2), thereby mimicking the first step of elongation. Yet protection of the Shine–Dalgarno sequence persists in the tRNA^{Phe} complex. This raises the questions as to at what point during translation the Shine–Dalgarno interaction is disrupted and whether the interaction plays a functional role beyond translational initiation. One possibility is that pairing with the Shine–Dalgarno sequence stabilizes mRNA–ribosome interaction during the first rounds of translational elongation and is disrupted at some later, as yet unknown, step.

Interestingly, discrete bases outside the Shine–Dalgarno sequence were also protected in complexes containing gene 32 mRNA, tRNA and 30S subunits. In the presence of tRNA^{Met}, base A–1 was protected from modification with DMS and in the presence of tRNA^{Phe}, bases U+2 and G+3 were protected from modification with KE or CMCT respectively (Figures 7 and 8). Assuming, as is most likely, that the respective tRNAs bind to the 30S P site under these conditions, the latter protections correspond formally to immediate upstream or E site codons. In the case of initiator tRNA, there can, of course, be no physiologically meaningful E site codon. Indeed, there is disagreement over whether E site tRNA is involved in codon–anticodon interactions at all (Lill and Wintermeyer, 1987; Rheinberger *et al.*, 1990). Most intriguing is the protection of the U and G bases of the AUG initiation codon when mRNA is bound in a complex with tRNA^{Phe}. Protection of U+2 and G+3 in 30S complexes with tRNA^{Phe} raises the possibility that the 30S subunit may help to recognize the initiation triplet, even in the absence of initiator tRNA. Such a mechanism could account for the observation that wobble occurs in the first position, rather than the third position, of initiation codons. On the other hand, protection of A–1 in the 30S complex formed with initiator tRNA suggests that bases one or two positions upstream from an occupied P site codon may more generally be protected in 30S complexes. In this case, such interactions could play a role in preventing or destabilizing codon–anticodon interactions in the E site.

In addition, several other bases were reproducibly protected in the 30S complexes. U–33, G–32, U–22 and G+11 were all protected in both the tRNA^{Met} and tRNA^{Phe} complexes, while U–5 was only protected in the tRNA^{Phe} complex. These additional protections may indicate periodic contact points with the 30S subunit along the mRNA chain. Whether or not it is a coincidence that these protections are spaced at intervals of ~11 nucleotides, the periodicity of an RNA A-form helix, is an intriguing question. Lagunez-Otero and Trifonov (1992) have suggested that mRNA sequences carry a cryptic consensus (GCU)_n repeating motif that might interact with rRNA. However, only one of the protected bases (U–22) falls in the reading frame predicted from the cryptic consensus motif.

Sprengart *et al.* (1990) recently proposed base pairing of sequences downstream of the initiation codon of an mRNA with bases 1471–1482 of 16S rRNA, in addition

to the Shine–Dalgarno interaction. In their study a downstream sequence in gene 32 mRNA (positions +21 to +36) was suggested to base pair with the latter region of 16S rRNA during translation initiation due to base complementarity. We fail to observe protection of any bases in the +21 to +36 region of gene 32 mRNA; in fact, a moderate enhancement of reactivity of bases towards chemical probes is observed here (Figure 7).

The structure of mRNA has an important bearing on its interaction with the ribosome. For example, a number of special coding events, such as ribosomal frameshifting in retroviruses and suppression of a UGA stop codon, have been shown to be influenced by mRNA structure (for a review see Gesteland *et al.*, 1992). In these studies we have introduced a stable tetraloop hairpin (Cheong *et al.*, 1990) between positions +10 and +11 of gene 32 mRNA. The presence of the tetraloop does not inhibit binding of the mRNA to 30S subunits, in fact, we observe the same protection of the mRNA backbone from hydroxyl radical attack between positions –35 and +5 as for mRNA lacking the tetraloop (Figures 5 and 6). However, the tetraloop abolishes interaction of the mRNA with the 30S subunit downstream from position +5. Disruption of interactions involving positions +6 through +8, even though they are not part of the hairpin structure itself, shows that this stable structure can exert its effect for at least a short distance along the unstructured part of the mRNA chain. This suggests that the structure of the 30S subunit may restrict the entry of all but unstructured RNA chains to its mRNA binding site. Balakin *et al.* (1990) have reported that binding of initiator tRNA promotes opening up of a hairpin structure in *cro* mRNA containing the initiation codon. In our experiments, neither the Shine–Dalgarno nor initiator codons are contained within the tetraloop hairpin structure; furthermore, we first isolated mRNA–30S complexes prior to probing, to eliminate signal coming from unbound mRNA. Thus the apparent contradiction between the effects of mRNA structure in the two studies can be explained by the inability of ribosomes to bind *cro* mRNA until the AUG codon is bound by initiator tRNA. It should be noted that relatively little is understood about how ribosomes melt mRNA structure, but it is likely that ribosomes deal with mRNA structure during translational initiation differently from the melting of structure that is observed during the elongation phase.

Materials and methods

mRNAs

mRNAs were transcribed from DNA templates containing a T7 promoter (Milligan and Uhlenbeck, 1989) followed by a section of the mRNA gene which included the translation initiation region. DNA templates were generated by PCR amplification of plasmid DNA essentially as described by Saiki *et al.* (1988). PCR reactions were performed in a reaction volume of 100 µl in the presence of 0.2 µM primers and 1 µg plasmid DNA. Plasmids used for PCR amplification were: pRS170 for gene 32 mRNA and gene 32-TL mRNA, generously provided by L. Gold (Hartz *et al.*, 1989), and pXR022 for 022 mRNA, generously provided by C. Pon and C. Gualerzi (La Teana *et al.*, 1993). DNA oligonucleotide primers used in PCR amplification reactions (Table I) were synthesized on a Milligen/Bioscience 8600 automated synthesizer and purified by preparative gel electrophoresis on 20% polyacrylamide–7 M urea gels. Upstream primers contained the T7 RNA polymerase promoter sequence (Milligan and Uhlenbeck, 1989) in addition to a sequence complementary to the respective plasmid DNA (Table I). Introduction of a tetraloop

Table 1. Plasmids and primers used for PCR amplification of templates for transcription of mRNA

mRNA	Plasmid	Primer	Position
Gene 32	pRS170	T7/-40: 5'-TAATACGACTCACTATAGGTAAGTGTCATAGC-3' A12: 5'-CTTTATCTTCAGAAGAAAAACC-3'	-57 to -50 +64 to +85
022	pXR022	022/T7: 5'-TAATACGACTCACTATAGGCAACGTTAAAGGGAATTCAAAAAT-3' 022/B23-13G1: 5'-GCCTGCAGACGCATTAAGTGAAG-3'	-39 to -26 +23 to +46
Gene 32-TL	pRS170	T7/-40: 5'-TAATACGACTCACTATAGGTAAGTGTCATAGC-3' TLO-14L1: 5'-GCGAGTTCAGCAGTAGATTACCGCCGAAGCGGTTTAAACATTTT TATTTC-3'	-57 to -40 -10 to +10 and +11 to +32

sequence into gene 32 mRNA (gene 32-TL mRNA) was performed by PCR amplification of pRS170 plasmid DNA, as described above, using a downstream primer containing the sequence complementary to the tetraloop hairpin located between sequences complementary to gene 32 (between positions -10 to +10 and +11 to +32; Table I).

PCR reaction products were phenol- and chloroform-extracted and DNA fragments were further purified by centrifugation on 1 ml Sephadex G-50 spin columns (Sigma) for 5 min at 3000 r.p.m. prior to transcription reactions. Transcription using T7 RNA polymerase was performed directly from PCR-amplified DNA fragments (about 2–5 µg template) in a reaction volume of 100 µl (Milligan and Uhlenbeck, 1989). RNA transcripts were phenol-extracted, purified on Sephadex G-50 spin columns and ethanol-precipitated. Subsequently, mRNAs were dissolved in 20 µl CIP buffer (50 mM Tris, pH 9.0, 1 mM MgCl₂, 0.1 mM ZnCl₂, 1 mM spermidine) treated with 2 U calf intestinal alkaline phosphatase (Sigma) for 30 min at 37°C (Maniatis *et al.*, 1987), extracted with phenol and chloroform and ethanol-precipitated. Dephosphorylated transcripts were then purified by electrophoresis on 8% (w/v) polyacrylamide–7 M urea gels. Full-length transcripts were identified by UV shadowing, excised and passively eluted into 0.3 M sodium acetate (pH 5.2), 0.1% SDS at 4°C for 12 h. After phenol–chloroform extraction, mRNAs were precipitated, redissolved in 10 µl kinase buffer (50 mM Tris, pH 7.6, 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA) and 5' end-labeled using 20 U T4 polynucleotide kinase (New England Biolabs) and [γ -³²P]ATP (5000 Ci/mmol; Amersham) for 30 min at 37°C (Maniatis *et al.*, 1987). Labeled mRNAs were purified on 8% (w/v) polyacrylamide–7 M urea gels, located by autoradiography and passively eluted and ethanol-precipitated as described above. They were then dissolved in H₂O at a concentration of 1 µg/µl and stored at -20°C.

tRNAs

tRNA^{Phe} (*E.coli*), tRNA^{Met} (*E.coli*) and tRNA^{Phe} (yeast) (Subriden) were dissolved in 10 mM Tris, pH 7.2, 10 mM MgCl₂ at a concentration of 10 mg/ml and used as described below.

Preparation of ribosomes and subunits

Escherichia coli 600 MRE 0.5 M salt-washed 70S ribosomes were prepared as described by Moazed and Noller (1989) and 30S and 50S subunits were obtained as described by Moazed *et al.* (1986). 30S subunits were activated by heating in reaction buffer A (10 mM MgCl₂, 140 mM NH₄Cl, 80 mM potassium cacodylate, pH 7.2) at 42°C for 20 min before being used for tRNA binding (Zamir *et al.*, 1971).

Binding of mRNAs

Binding of 5' ³²P-end-labeled mRNAs (used in hydroxyl radical footprinting or sucrose gradient analysis) or unlabeled mRNAs (used for chemical probing, see below) was performed by incubating 50 pmol 30S ribosomal subunits or an equimolar mixture of 30S and 50S subunits with 10 pmol mRNA (when labeled, ~1.5×10⁶ c.p.m.) and 250 pmol tRNA (molar ratio tRNA:ribosomes:mRNA 25:5:1) in 50 µl reaction buffer A at 37°C for 10 min and then for 20 min on ice. mRNA–tRNA–ribosome complexes were then purified by loading 50 µl complex onto 1 ml Sepharose 6-B spin columns (Sigma) and repeated centrifugations (1 min, 3000 r.p.m.), essentially as described in Langer *et al.* (1984). After each centrifugation step, 50 µl reaction buffer A were loaded onto the spin column. Fractions containing the ternary complex (usually fractions 2–6) were pooled and distributed into 50 µl aliquots.

Sucrose gradient analysis

mRNA–ribosome complexes were loaded onto 10–40% sucrose gradients in buffer A and centrifuged for 11 h in a SW41 rotor (Beckman)

at 35 000 r.p.m. at 4°C. Fractions (200 µl) were collected and the absorbance (A₂₆₀) or Cerenkov radioactivity of each fraction was determined.

Toeprinting assays

Extension inhibition (toeprinting) of gene 32 mRNA was performed as described by Hartz *et al.* (1988) using primer A12 (Table I), 5' ³²P-end-labeled according to Maniatis *et al.* (1987), which was complementary to positions +64 to +85 of gene 32 mRNA.

Hydroxyl radical footprinting

Hydroxyl radical footprinting was performed essentially according to Tullius and Dombrowski (1985), modified according to Hüttenhofer and Noller (1992), by adding 1 µl 50 mM Fe(NH₄)₂(SO₄)₂·6H₂O, 1 µl 100 mM EDTA, 1 µl 2.5% (v/v) H₂O₂ and 1 µl 250 mM DTT to 50 µl mRNA–ribosome complex (see above) or free mRNA, followed by incubation at 4°C for 10 min. Reactions were stopped by precipitation with 0.1 volumes 3 M sodium acetate (pH 5.2) and 3 volumes ethanol in the presence of 10 µg oyster glycogen (Sigma). RNA was redissolved in 200 µl 0.3 M sodium acetate (pH 5.2), extracted three times with phenol and twice with chloroform and precipitated with 3 volumes ethanol. Sequencing markers were generated by partial cleavage of mRNAs with T₁ ribonuclease (Sankyo) as described by Donis-Keller *et al.* (1977). Samples [2 µl in 7 M urea, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue] were applied to 10% (w/v) polyacrylamide–7 M urea gels (60×20×0.02 cm) and electrophoresed at 2000 V for 2 h.

Chemical probing and primer extension

Chemical probing (Stern *et al.*, 1988) of mRNA–ribosome complexes (see above) or free mRNAs was performed by addition of 2 µl DMS (1:10 dilution in 95% ethanol), 2 µl KE (19 mg/ml in H₂O) or 10 µl CMCT (84 mg/ml in H₂O) to 50 µl reaction mixture (see above), followed by incubation at 30°C for 10 min. All modification reactions were stopped by addition of 150 µl 95% ethanol followed by rapid mixing. For DMS reactions, 25 µl DMS stop solution (1 M Tris–HCl, pH 7.5, 1 M 2-mercaptoethanol, 0.1 M EDTA) were added prior to addition of ethanol. KE-modified samples were adjusted to 25 mM potassium borate (pH 7.0). The pellets were resuspended in 200 µl 0.3 M sodium acetate (pH 5.2) and extracted three times with phenol and twice with chloroform in the presence of 0.5% SDS and resuspended in 10 µl H₂O (for DMS and CMCT samples) or in 10 µl 25 mM potassium borate (KE-modified samples). Primer extension reactions were performed as described by Stern *et al.* (1988), using 5' ³²P-end-labeled primer A12 (Table I).

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