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Decoding the translational termination signal: The polypeptide chain release factor in *Escherichia coli* crosslinks to the base following the stop codon

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ABSTRACT

Protein release factors act like tRNA analogues in decoding translational stop signals. Statistical analysis of the sequences at translational stop sites and functional studies with particular signals indicate this mimicry involves an increase in the length of the signal in the mRNA. The base following the stop codon (+4 base) is of particular interest because it has a strong influence on the competitiveness of the stop signal at recoding sites, suggesting it might form part of the release factor recognition element. Site-directed crosslinking from the +4 base showed that it is in close proximity to the *Escherichia coli* release factor-2 in a termination complex, a prerequisite for the +4 base being part of the recognition element. Fingerprinting analysis indicates that crosslinking to the release factor occurred from both +1 and +4 positions of the stop signal in the same RNA molecule. This provides more evidence that the +4 base may be an integral part of the decoding signature in the mRNA during the termination phase of protein biosynthesis.

Keywords: molecular mimicry; release factor-2; site-directed crosslinking; termination complex

INTRODUCTION

The mechanism by which translational stop signals are decoded is unresolved, but it has been clear since the 1960s that this process does not utilize tRNA, as for the sense codons, but rather involves proteins, the polypeptide chain release factors (RFs) (Tate et al., 1996b). Moffat and Tate (1994) proposed a "tRNA analogue" model for how RFs function, providing evidence that, like a tRNA, the proteins spanned the decoding site of the small subunit of the ribosome and the peptidyltransferase center of the large subunit. The term "molecular mimicry" was introduced for this kind of phenomenon following the resolution of the structures of various functional states of the elongation factors (Czworkowski et al., 1994; Nissen et al., 1995; Al-Karadaghi et al., 1996; Nyborg et al., 1996). Nakamura and colleagues refined this idea in their tRNA-protein mimicry hypothesis by proposing that there is a domain of the RF mimicking a tRNA, specifically the tRNA anticodon (Ito et al., 1996; Nakamura et al., 1996; Uno et al., 1996). Although these ideas await further testing, they have given an exciting impetus to achieve a better understanding of how a protein can decode the translational stop signal in the mRNA. An important aspect is a definitive description of what comprises the translational stop signal.

Several studies of suppression of nonsense codons suggested that termination of protein synthesis might involve a larger signal than simply three nucleotides (Salser, 1969; Salser et al., 1969; Fluck et al., 1977; Bossi & Roth, 1980; Bossi, 1983; Miller & Albertini, 1983; Smith & Yarus, 1989; Buckingham et al., 1990; Pedersen & Curran, 1991; Kopelowitz et al., 1992; Björnsson & Isaksson, 1993; Mottagui-Tabar et al., 1994; Björnsson et al., 1996; Zhang et al., 1996). For example, the nucleotides immediately 3′ to stop codons influence UGA suppression (Buckingham et al., 1990; Kopelowitz et al., 1992) and selection rates of both suppressor tRNA and RF-1 were shown to vary with the nucleotide following the stop codon (Pedersen & Curran, 1991).

We believed that statistical analysis of the sequences surrounding stop codons at natural termination sites might show particularly important or more subtle features of a larger signal that have been fixed during evolution. For this purpose, a database, TransTerm, of sequences at natural termination sites was established

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(Brown et al., 1993) and developed (Dalphin et al., 1997). Using this database, analysis of sequences around natural stop codons in genes from a wide range of organisms has shown a significant bias in the surrounding codon context, and it is particularly striking for the position following the stop codon (+4 base) (Tate & Mannering, 1996). We tested the influence of this position on termination efficiency in vivo in *Escherichia coli* by altering the +4 base with each stop codon and measured the strength of termination against a frameshifting event at the now classical RF-2 frameshift site (Craigen et al., 1985; Pedersen & Curran, 1991). The termination efficiency of these 12 signals and the rate of RF selection at stop codons were influenced dramatically by the identity of the base in the +4 position (Poole et al., 1995). This influence was paralleled in yeast and mammals (Bonetti et al., 1995; McCaughan et al., 1995). The order of efficiency of the translational stop signals as a function of the +4 base was different between the prokaryotic and eukaryotic examples and correlated with the frequency of their occurrence at natural stop sites within each group. These results implied that an effective stop signal extended at least to the fourth base.

During termination of protein synthesis, the Class I RFs (decoding factors) (Pel et al., 1996) interact with the ribosome to allow release of the completed polypeptide chain. An important question regarding the +4 base influence on termination is whether this effect is mediated by an intimate interaction between the base and the RF, or whether the role of the +4 base is more indirect and influences RF interaction with the stop codon itself. We first addressed this question using small designed mRNAs containing a thio-U residue (Tate et al., 1990) in the first (+1) position of the stop signal (Brown & Tate, 1994). Thio-U residues base pair similarly to U and act as a zero-length crosslinking reagent when photo-activated. This allows a "snapshot" where molecules in close proximity to the thio-U are "fixed." In a termination complex that contained *E. coli* ribosomes, a designed mRNA containing a UGAN signal and RF-2, an RF-dependent crosslink, was formed from the thio-U in the +1 position in relatively high yield and with codon specificity. This implied that the RF is indeed an active decoding molecule and may make direct contact with the stop signal in the ribosomal A-site.

In this study, we have determined that the decoding RF is in close proximity to the +4 base by site-directed crosslinking from this position in designed mRNAs.

RESULTS

Analysis of site-specific crosslinks using ribonuclease T1

For use in a termination complex, two ^{32}P-labeled mRNA analogues were designed and transcribed in vitro from synthetic DNA oligonucleotides. One mRNA analogue contained the zero-length crosslinking reagent thio-U in the +1 and +4 positions of the stop signal (UGAU, Fig. 1). The control mRNA analogue differed only in the +4 position, where C was substituted for thio-U (UGAC). The substitution of thio-U for U in the essential +1 position of the stop codon in mRNA analogues has been demonstrated previously not to affect RF binding in termination complexes (Tate et al., 1990). Termination complexes were formed using *E. coli* ribosomes, with the stop codon of the mRNA positioned in the A-site by a P-site-bound deacylated tRNA^{Ala-2}, in the presence of the factor specific for UGA, RF-2. Control complexes without RF-2 were formed in parallel. UGA-containing mRNAs were used because a higher yield of RF-2.mRNA crosslinked product was produced than with UAA-containing mRNAs (Brown & Tate, 1994). Subsequent irradiation with UV at wavelengths >300 nm specifically activates the thio-U residue to form crosslinks with molecules in the immediate vicinity. By subjecting the mRNA in the crosslinked termination complex to ribonuclease T1 digestion, which specifically cuts to the 3′ side of G nucleotides, the mRNA analogue can be cleaved into fragments of defined lengths. The mRNA crosslinked

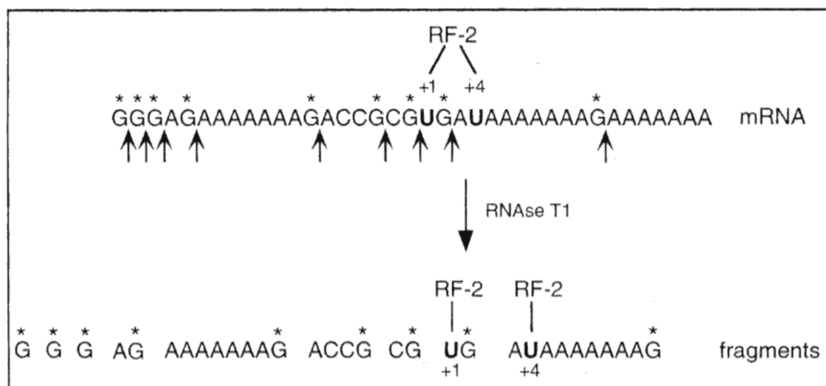

FIGURE 1. Strategy for analysis of crosslinked products generated by ribonuclease (RNAse) T1 digestion of mRNA analogues containing thio-U. Vertical arrows show the sites of RNAse T1 cleavage. Asterisks denote the radiolabeled G. Solid bars represent putative crosslinks to RF-2. The resulting ^{32}P-labeled fragments are shown in the lower part of the diagram. An mRNA with thio-U in the +1 and +4 positions of the stop signal is shown.

to RF-2 is retarded during electrophoresis and the mobility of the RF-2.mRNA fragment will depend on the position in the mRNA from which the crosslink originated. A diagram of the strategy with the potential crosslinked RNA fragments is shown in Figure 1.

Gel separation analysis of RF-2.mRNA crosslinks

After ribonuclease T1 digestion of mRNA, RF complexes containing crosslinks to the mRNA from the +1 and +4 thio-U positions can be separated. A crosslink from the +1 position will result in a dinucleotide fragment attached to RF-2, whereas a crosslink from the +4 position results in the attachment of a decanucleotide fragment (see Fig. 1). Both of these fragments are radiolabeled at the G nucleotide and can be identified by autoradiography. RF crosslinked to a dinucleotide from the +1 position migrates on a polyacrylamide gel close to the position where native RF-2 migrates (46 kDa). In contrast, a crosslink from the +4 base to the RF attaches almost one quarter (10 nt) of the original mRNA and was expected to migrate to a position between the native RF-2 position and the undigested RF-2.mRNA crosslinked species. In this way, a crosslink originating from the +4 position can be identified specifically following autoradiography.

The crosslink patterns were determined for the mRNAs containing the UGAC and UGAU stop signals both in the presence and absence of RF-2, and before and after digestion with ribonuclease T1. The products of these reactions were separated by PAGE and then transferred to nitrocellulose membranes before autoradiography. Figure 2A and B shows the results from a typical experiment. For both the UGAC (Fig. 2A) and UGAU (Fig. 2B) crosslinked complexes, a prominent crosslinked band was seen in complexes containing RF-2 (lane 1, arrows). This band was absent in complexes lacking RF-2 for both mRNAs (lane 3). Following ribonuclease T1 digestion, the crosslinked complex was destroyed and, in the case of the UGAC-containing mRNA, a single band appeared at the position of native RF-2 (Fig. 2A, lane 2). In contrast, an additional radiolabeled band appeared after digestion of the complex containing the UGAU stop signal (Fig. 2B, lane 2). This less-intense RF-specific band appeared between the band migrating at the same position as that seen for the mRNA containing the UGAC stop signal and the position of the undigested RF-2.mRNA complex. Neither of these bands were present in the absence of RF-2 (Fig. 2A,B, lane 4). The higher band was inferred to contain a crosslink from the +4 base to the RF, with the decanucleotide fragment impeding its migration as discussed above. This band should also contain RF molecules with crosslinks from both +1 and +4 positions (a dinucleotide plus a decanucleotide fragment).

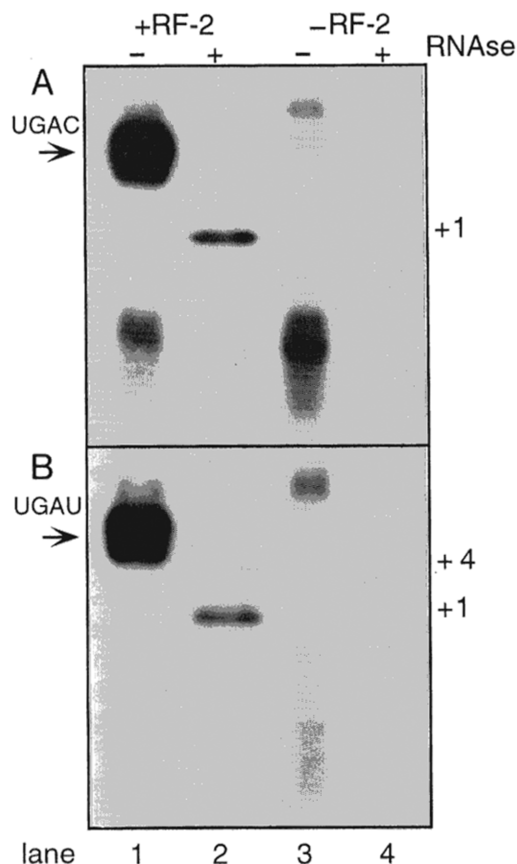

FIGURE 2. Polyacrylamide gel separation of crosslinked complexes. The complexes were detected by autoradiography after transfer to a nitrocellulose membrane. **A:** Analysis of the fragments from mRNA containing UGAC. **B:** Analysis of the fragments from mRNA containing UGAU. Lanes 1 and 2, and lanes 3 and 4 show the crosslinks formed in the presence and absence of RF-2, respectively, both before (lanes 1 and 3) and after (lanes 2 and 4) ribonuclease (RNAse) T1 digestion. The uppermost crosslinked species in lanes 1 and 3 represent probable crosslinks from the mRNA to ribosomal protein S1. Arrows show the position of the RF-2.mRNA crosslinked species prior to RNAse T1 digestion.

The presence of RF-2 in these two radiolabeled products of ribonuclease T1 digestion of the crosslinked complex was confirmed by probing with a polyclonal antibody to the factor. The predominant RF-2 species in the reaction will be those molecules not involved in crosslinking and will migrate to the position of the native molecule, but the antibody should react also with RF-2 species crosslinked to the mRNA. Figure 3A and B shows the antibody pattern for the reactions containing the UGAC and UGAU mRNAs after chemiluminescence detection. As expected, a strong antibody reaction was evident for both signals at the native RF-2 position (arrows) in lanes containing the complete termination complex, both before and after ribonuclease T1 digestion (Fig. 3A,B, lanes 1 and 2). The reaction containing the UGAU mRNA fragments showed an additional band specific for RF-2 that aligned exactly with the putative +4 crosslinked band (Fig. 3B, lane 2). The RF-2 antibody detected a minor product in

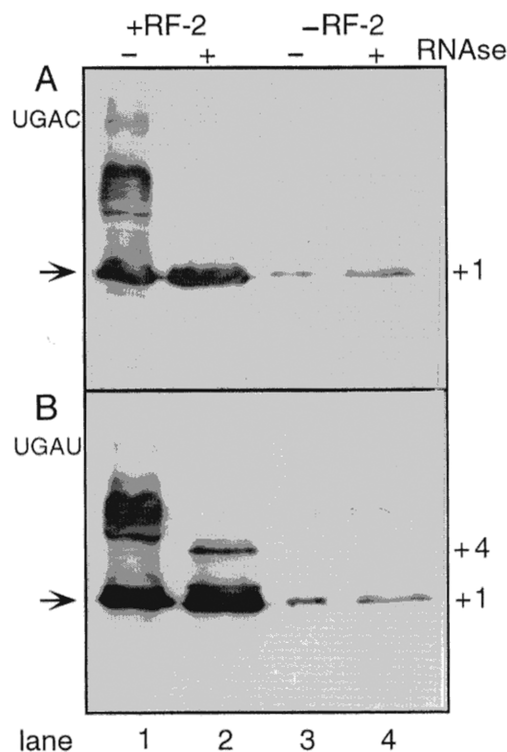

FIGURE 3. Immunological identification of the RF-2.mRNA crosslinked species. Chemiluminescence was used to detect reactivity of an antibody to RF-2 in the crosslinked complexes shown in Figure 2. **A:** RF-2 antibody reactivity to the UGAC crosslinked complexes. **B:** RF-2 antibody reactivity to the UGAU crosslinked complexes. Lanes 1 and 2, and lanes 3 and 4 show the antibody pattern to crosslinks formed in the presence and absence of RF-2, respectively, both before (lanes 1 and 3) and after (lanes 2 and 4) ribonuclease (RNAse) T1 digestion. Arrows show the position of the native RF-2 species.

termination complexes lacking RF-2 at the position of native RF-2 (Fig. 3A,B, lanes 3 and 4). We have shown previously that ribosomal preparations contain a small amount of contaminating RF-2 that can be revealed immunologically (Brown & Tate, 1994), and this is the likely reason for this apparent nonspecific antibody reaction.

Using the same strategy, it was possible to look for RF-2.mRNA crosslinks beyond the +4 base. For these experiments, each designed mRNA was identical to that used in the experiments described here, except that G was the +4 base and the second thio-U in each mRNA substituted for A in positions +5 to +10. Potential crosslinks between the +5 and the +10 position could be analyzed by gel separation in order to define the RF-2 footprint on the mRNA further downstream from the stop codon. Weaker crosslinks than that found at the +4 position were demonstrated from the +5 and +6 bases to RF-2, but were not detected in any of the more distant positions (data not shown). This has renewed our interest in the +5 and +6 positions and their contributions to the translational termination signal, and a more detailed study is now in progress into the role of these bases in the functioning of this signal.

Fingerprint analysis of RF-2.mRNA crosslinks

More definitive evidence that there is a crosslink from the +4 base to RF-2 was obtained by RNA fingerprinting analysis. For this approach, the RF-2.mRNA crosslinked complex (shown in Fig. 2, lane 1) first was separated from the other components of the termination reaction through a polyacrylamide gel. The RF-2.mRNA complex was extracted and then the protein was digested, leaving only crosslinked peptides or amino acids attached to the RNA. In this case, after ribonuclease T1 digestion, the putative +1 and +4 crosslink-containing oligonucleotide fragments each would have an attached peptide or amino acids. These crosslinked fragments were differentiated from free mRNA fragments by their mobility in the two-dimensional fingerprint analysis system of Brimacombe et al. (1990). The extent of the first-dimensional migration from the origin depends firstly on the number of U's in the fragment and then on the size of the oligonucleotide with larger fragments migrating further. The second dimension migration pattern is a property of the number of nucleotides in the fragment, with each additional nucleotide retarding migration incrementally. Any RNA fragment crosslinked to an amino acid(s) remains at the origin, along with undigested fragments from the 5′ end of the oligonucleotide. An absence or a reduction in the intensity of a spot then represents the extent of crosslinking from a base within that particular oligonucleotide. Figure 4A and B shows the results for control UGAC-containing mRNAs. The thio-UG (containing the +1 base of the stop signal) was clearly visible in the sample mRNA not subjected to the crosslinking protocol (spot 5, Fig. 4A). Significantly, this dinucleotide was completely absent after crosslinking (Fig. 4B) and confirmed that all the mRNA in the RF-2.mRNA complex extracted from the gel was crosslinked from the +1 base of the stop signal, as would be predicted.

When the mRNA containing UGAU (+1 and +4 thio-U) was fingerprinted, the two thio-U-containing fragments could be identified (spots 5 and 7, Fig. 5A). In this case, because the oligonucleotide containing the +4 base of the stop signal is a decanucleotide, the fragment barely moves during the second dimension separation and remains well separated from the more mobile dinucleotide fragment (spot 5) containing the +1 base of the stop signal. After crosslinking, fingerprinting showed that not only had the dinucleotide fragment disappeared, but also that the intensity of the larger fragment (+4 oligonucleotide) had been reduced significantly (spot 7, Fig. 5A,B). This confirmed that the +4 base was indeed able to form a crosslink with the RF. There was no change in the relative in-

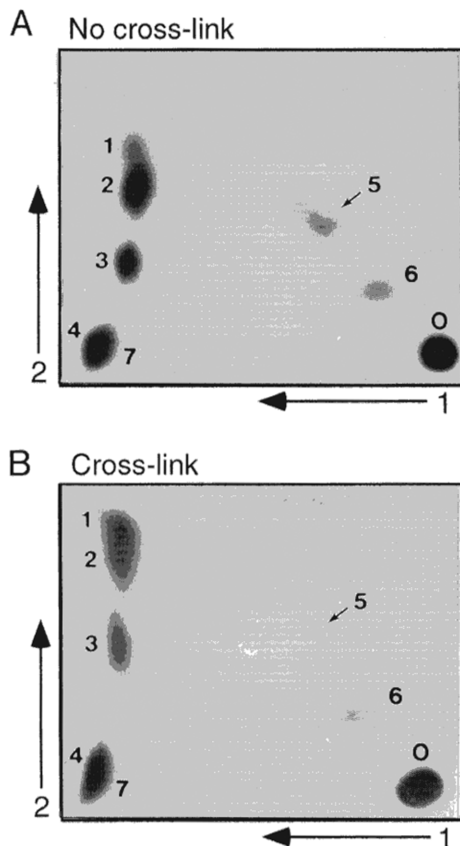

FIGURE 4. Fingerprint analysis of the ^{32}P-labeled UGAC-containing mRNA following ribonuclease T1 digestion. Radioactive oligonucleotide spots are numbered according to the mRNA sequence, namely: 0, pppGp; 1, Gp; 2, NGp; 3, ACCGp; 4, A_7Gp; 5, UGp; 6, pGp (5′-end); 7, ACA_7Gp. **A:** Noncrosslinked mRNA. **B:** Crosslinked mRNA. Direction of the first dimension is from right to left, that of the second from bottom to top, and the application point (0) is at the lower right. The position of the +1 thio-U dinucleotide fragment (5) is marked by an arrow. The faint spot contacting the +1 fragment (5) in A results from residual UDP in the original thio-UDP preparation. The 3′ sequence A_7 is not labeled and is absent from the fingerprint for this reason.

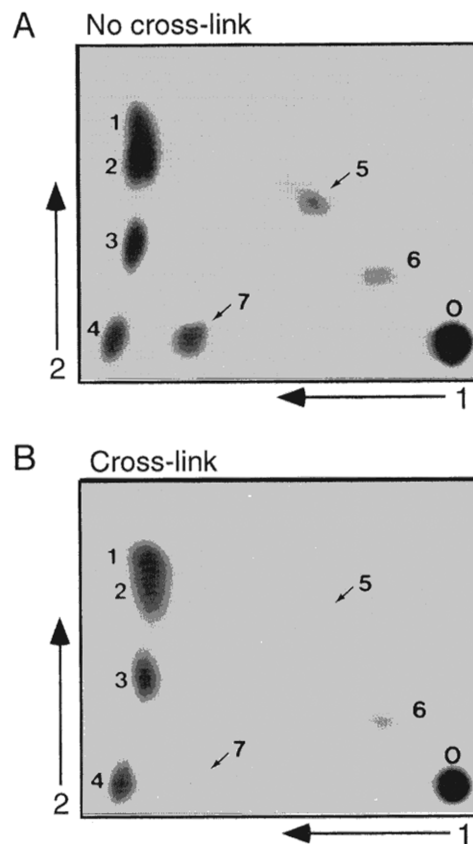

FIGURE 5. Fingerprint analysis of the ^{32}P-labeled UGAU-containing mRNA following ribonuclease T1 digestion. Radioactive oligonucleotides are numered according to the mRNA sequence, namely: 0, pppGp; 1, Gp; 2, NGp; 3, ACCGp; 4, A_7Gp; 5, UGp; 6, pGp (5′-end); 7, AUA_7Gp. **A:** Noncrosslinked mRNA. **B:** Crosslinked mRNA. Direction of the first dimension is from right to left, that of the second from bottom to top, and the application point (0) is at the lower right. Positions of the +1 dinucleotide (5) and +4 decanucleotide (7) thio-U fragments are marked with arrows. The faint spot contacting the +1 fragment (5) in A results from residual UDP in the original thio-UDP preparation. The 3′ sequence A_7 is not labeled and is absent from the fingerprint for this reason.

tensities of the fragments not involved in crosslinking in the two experiments. The spot at the origin (0) in the noncrosslinked samples represents the nucleotide derived from the 5′ end of the mRNA, which has a triphosphate (pppGp). A degradation product of this moiety, pGp (spot 6), appears close to the application point within the chromatograms.

DISCUSSION

Translational stop signal efficiency and the +4 base as part of a recognition element

A comprehensive study of the effect of the +4 base on the efficiency of stop signals in competition with a number of recoding events in vivo, using bacterial, mammalian, and yeast systems (Pedersen & Curran, 1991; Bonetti et al., 1995; McCaughan et al., 1995; Poole et al., 1995), and, where possible, in vitro experiments (McCaughan et al., 1995), has provided clear evidence that the +4 base can influence the efficiency of decoding the stop signal over a wide range. The key question was whether the +4 base changes the conformation of the stop codon and thereby influences recognition of the core triplet through a context effect, or whether it is part of the recognition element itself and has a direct effect on this process. For the latter, the RF and the +4 base must be in close proximity. Previously, we have shown by site-directed crosslinking from the +1 base of the stop codon in a defined mRNA analogue, using the zero-length thio-U residue, that this base is close to the decoding RF (Tate et al., 1990; Brown & Tate, 1994). A schematic "tRNA analogue molecular mimicry" model of RF interaction with the stop signal is shown in Figure 6A.

Two lines of evidence are presented for the RF having close proximity with the +4 base. The mRNA containing a thio-U in both the +1 and +4 positions

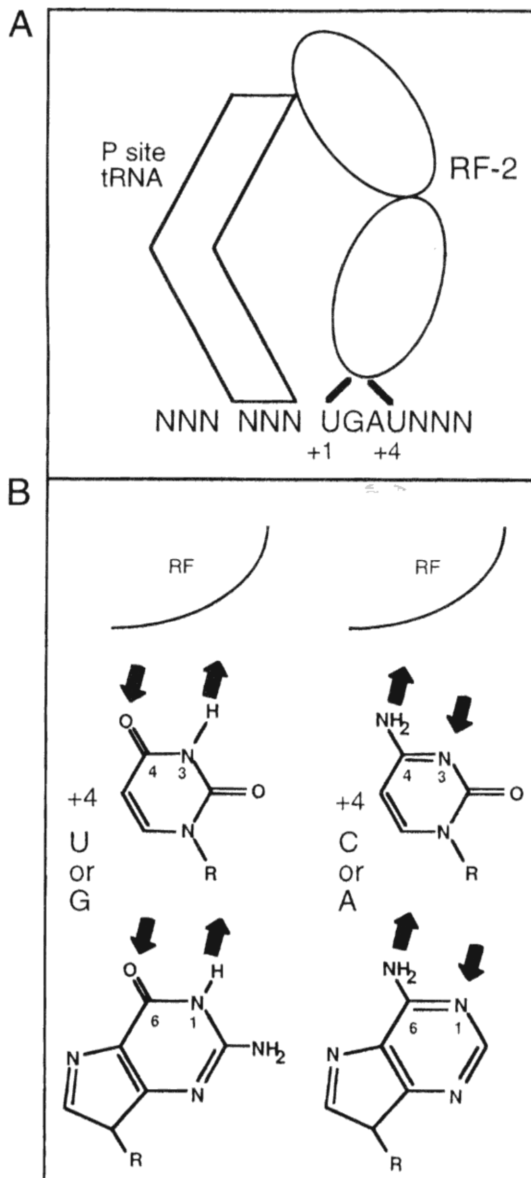

FIGURE 6. Possible interaction between RF and mRNA at the ribosomal decoding site. **A:** Schematic "tRNA analogue" illustration of RF interaction with the +1 base of the stop codon and the following +4 base (bold lines). **B:** Possible hydrogen bonding from the structural moieties of the +4 base to a RF. Arrows indicate the common bonding potential of bases U and G, and of bases C and A.

revealed after electrophoresis an additional retarded crosslinked species that reacted with the RF-2 antibody, compared with the mRNA with a single thio-U in the +1 position. More definitive evidence for the involvement of the +4 base in the crosslinked products was provided by the RNA fingerprinting study. The dinucleotide containing the +1 base was the only fragment missing from the degradation products of the crosslinked RNA with thio-U in the +1 position, whereas both the dinucleotide and decanucleotide fragments were either significantly reduced or absent when a thio-U was present in both the +1 and +4 positions.

Over a range of experiments, the results were consistent, although there were qualitative differences in the relative crosslinks from the two positions. As shown in the experiment in Figure 2B, a crosslink from the +1 position was on the order of 10-fold more efficient than that from the +4 position, although, in the fingerprinting experiment shown in Figure 5B, the difference was somewhat less pronounced. It should be noted, however, that it is inappropriate to conclude anything about the relative strength of contact with the RF from the +1 and +4 positions from the yield of the crosslinks in these data. Indeed, crosslinking from the +1 position from UAA-containing mRNAs is much less efficient than for UGA-containing mRNAs (Brown & Tate, 1994) and suggests that a number of parameters can affect crosslinking efficiency. From the fingerprinting experiment, it seems clear that crosslinking can occur to the RF from both positions in a single mRNA; the +1 dinucleotide fragment was absent completely and the +4 decanucleotide fragment was depleted significantly. The implication of this is that the slower-moving band in Figure 2B should contain some RF molecules crosslinked not only to the decanucleotide fragment from the +4 base, but also to the dinucleotide fragment from the +1 base. Crosslinks must occur with different residues in the RF from the two positions. Our studies cannot eliminate the possibility that it is the result of the RF crosslinking to the +1 base of the stop signal that fixes the decoding factor near the +4 base. Therefore, the crosslink from the +4 base to the RF may not be independent of events happening at the +1 base, but, of course, the same is likely to be true of the codon recognition event in vivo. The RF is apparently also in close proximity to the +5 and +6 bases and analysis of these crosslinks would provide a wider perspective on which residues of the RF contact the mRNA.

The translational stop signal

These data provide a rationale for why the +4 base can have such a profound effect on the competitiveness of a stop codon at a recoding site in vivo or on the efficiency of the codon to direct release of a product from a ribosomal termination complex in vitro (Pedersen & Curran, 1991; Poole et al., 1995; McCaughan et al., 1995). The core termination signal can be redefined as:

URRN (excluding UGGN).

For particular genetic systems, certain bases in the +4 positions are preferred. For example, in mammals, +4 R > Y, whereas, in *E. coli*, +4 U/G > A/C. In prokaryotes, the U preference for the +4 position holds irrespective of the organism and G+C content of the genome, except in genomes with very high G+C con-

tent (≥60%) when G supersede U as the preferred +4 base (Tate et al., 1996a). In genetic codes from some organisms, specificity of the recognition element may have changed. In *Tetrahymena*, UAG and UAA are used for glutamine, and UGA is used for tryptophan in some *Mycoplasma* species (Osawa et al., 1992). This might reflect a real change in the recognition element of the stop signal for the RF, or loss of RF, or simply an overwhelming competition from the new tRNA. For example, the complete sequence of two *Mycoplasma* genomes (Fraser et al., 1995; Himmelreich et al., 1996) has indicated that the gene for RF-2 is not present. However, the recognition of AGG and AGA in bovine and human mitochondria for signaling stop suggests a loosening of the specificity in the normally invariant +1 position.

In the case of *E. coli*, the +4 bases fall into two groups, U or G produce a very competitive strong signal, whereas A or C produce a weaker signal (Poole et al., 1995). How might this be mediated through recognition by the RF? Each of these pairs of bases have unique structures at the same positions of the six-membered ring (3,4 pyrimidines or 1,6 purines) that enable potential hydrogen bonding to occur with amino acid residues of the RF. For example, G and U have a common keto hydrogen bond acceptor and an imino bond donor, which are in similar orientations, whereas A and C have an amino group donor and an acceptor ring nitrogen, as shown in Figure 6B. A tRNA anticodon mimicry domain within the RF as proposed by Nakamura and colleagues (Ito et al., 1996) could be responsible for detecting the difference between these pairs of bases. Although a tRNA has a triplet anticodon to recognize the three-base sense codons, the RFs can provide particular combinations of side chains of amino acid residues from the same or different parts of the protein as recognition elements, and, therefore, it is most likely that the molecular signature in the mRNA will differ from that of translational sense codons.

MATERIALS AND METHODS

Materials

Deoxyoligonucleotides were made using an Applied Biosystems 380B DNA synthesizer. [α-^{32}P] GTP (3,000 Ci/mmol) and the ECL western blotting analysis system were supplied from Amersham. A RiboMAX™ Large Scale RNA Production System (Promega) kit was used for in vitro transcription reactions. tRNA$^{Ala\text{-}2}$ (anticodon VGC) was supplied by Subriden. UV irradiation was performed using either a bank of Philips T129D 16/09N 15-watt lamps with a maximum output of about 350 nm, or a 15-watt National Matsushita black light (FL15 BL) in a Griffin UV light box through a glass filter that was impermeable to light < 300 nm. Ribonuclease T1 and Proteinase K were purchased from Boehringer Mannheim. A Mini-PROTEAN II electrophoresis cell (Bio-Rad) and Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) were used for gel electrophoresis and protein transfer, respectively. The anti-sheep immunoglobulin peroxidase conjugate was purchased from Sigma. POLYGRAM CEL 300 PEI TLC plates were purchased from Macherey-Nagel.

In vitro transcriptions

The 4-thio-UTP was prepared from 4-thio-UDP (Sigma) as described (Stade et al., 1989). Annealing and transcription reactions were performed using reagents from a RiboMAX™ kit, although the protocol used differed from the manufacturers instructions. Briefly, annealing in transcription buffer of the single-stranded DNA templates (~0.2 nmol) to an equimolar amount of T7 polymerase primer took place by heating the mixture to 65 °C for 3 min and then allowing it to cool to room temperature. The annealed primer:templates were then added to transcription reactions containing 7.5 mM ATP and CTP, 0.25 mM GTP, 0.52 mM 4-thio-UTP, 26 μCi [α-^{32}P] GTP, and 3,000 Units T7 RNA polymerase in a 100-μL reaction volume and incubated at 37 °C overnight. The resulting mRNA products were purified on a 15% polyacrylamide [38:2 acrylamide:bis(acrylamide)]/7 M urea/0.1% SDS gel. The bands were located by autoradiography, excised from the gel, extracted with phenol/SDS buffer, and collected by ethanol precipitation. Yields were calculated from Cerenkov radiation of the samples.

Formation of ribosomal complexes and site-directed crosslinking

Ribosomal complexes were formed by incubating 25 pmol 70S ribosomes with 1,000 pmol of [^{32}P]mRNA, 75 pmol tRNA$^{Ala\text{-}2}$ (anticodon VGC), and 140 pmol RF-2 in 50 μL buffer containing 20 mM Tris-HCl, pH 7.4, 100 mM NH_4Cl, 20 mM $MgCl_2$, and 6% (v/v) ethanol. Control reaction mixtures were made in the same manner without RF-2. The complexes were incubated for 30 min at 37 °C, then dispensed onto Parafilm resting on ice and UV irradiated from a distance of 10 cm for 30 min.

Gel separation analysis of crosslinked complexes

Aliquots (10 μL) of crosslinked complexes were digested with 100 Units of ribonuclease T1 at 37 °C for 30 min. Samples were resolved on a 12% polyacrylamide [29:1 acrylamide:bis(acrylamide)] mini gel system prior to transfer to a nitrocellulose membrane, as described (Poole et al., 1995). The crosslinked proteins were visualized initially by autoradiography, then RF-2 protein identified using a primary antibody against RF-2 (1:2,000 dilution) and secondary antibody (1:5,000 dilution), as described (Poole et al., 1995), except that chemiluminescence protein detection was used.

Fingerprint analysis of crosslinked complexes

A total of 200 μL of each crosslinked complex was resolved on a 7% polyacrylamide [38:2 acrylamide:bis(acrylamide)]/7 M urea/0.1% SDS gel. The RF-2.mRNA crosslinked band was located by autoradiography, excised from the gel, extracted with βME/SDS buffer, and collected by ethanol pre-

cipitation. For the fingerprint analysis, aliquots (50 μL) of each crosslinked complex were digested with Proteinase K (37 °C for 30 min), extracted with phenol, followed by ethanol precipitation prior to ribonuclease T1 digestion, as described above. Control mRNA (not crosslinked) was also digested with ribonuclease T1, as described. Digested oligonucleotide fragments were resolved on two-dimensional 10 × 10 cm PEI gels as follows. After the samples were applied and pre-run with water, the first dimension separation was conducted in a buffer containing 5 M urea/7.4 M formic acid. Gels were then rinsed with water and 70% ethanol, dried, then pre-run with water, prior to running the second dimension in a buffer containing 4 M urea/1 M formic acid, pH to 4.3 with pyridine. Following the second-dimension migration, the gels were washed and dried as described, and the separated fragments were located by autoradiography.

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