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From stand-by to decoding site. Adjustment of the mRNA on the 30S ribosomal subunit under the influence of the initiation factors

ANNA LA TEANA,[1,2] CLAUDIO O. GUALERZI,[1] AND RICHARD BRIMACOMBE[3]

[1] Laboratory of Genetics, Department of Biology, University of Camerino, 62032 Camerino (MC), Italy
[2] Faculty of Science, University of Ancona, via Brecce Bianche, 60131 Ancona, Italy
[3] Max-Planck-Institut für Molekulare Genetik, Ihnestrasse 73, D-14195 Berlin, Germany

ABSTRACT

The hypothesis of an adjustment of the mRNA in its ribosomal channel under the influence of the initiation factors has been tested by site-directed crosslinking experiments. Complexes containing 30S subunits with bound mRNA having 4-thio-uracil at specific positions were prepared in the presence or absence of initiation factors and/or fMet-tRNA and subjected to UV irradiation to obtain specific crosslinks of the radioactively labeled mRNA with bases of the 16S rRNA and with ribosomal proteins. The subsequent identification of the specific sites of both mRNA and rRNA and individual ribosomal proteins involved in the crosslinking, obtained under different conditions of complex formation, provide direct evidence for the occurrence of a partial relocation of the mRNA on the 30S ribosomal subunits under the influence of the factors. The nature of this mRNA relocation is compatible with our previous proposal of a shift of the template from an initial ribosomal "stand-by site" to a second site closer to that occupied when the initiation triplet of the mRNA is decoded in the P-site.

Keywords: mRNA channel; mRNA shift; ribosomes; site-directed crosslinking; translation initiation

INTRODUCTION

One of the first events in translation is the recognition of the translation initiation region (TIR) of the mRNA by ribosomes. The main elements of the TIR include the initiation triplet (in most cases AUG), the purine-rich Shine–Dalgarno (SD) sequence complementary to the 3′-end region of 16S rRNA, and a spacer, of variable length, separating SD and initiation triplet (Gualerzi & Pon, 1990; Ringquist et al., 1992; McCarthy & Brimacombe, 1994).

The interaction between 30S ribosomal subunits and mRNA, and the influence of the initiation factors on this process, had been studied using MS2 RNA, polyribonucleotides, and model mRNAs. In all cases, the results indicated that the translation initiation factors (IFs) affect neither the SD–anti-SD interaction, nor the affinity of the 30S for the mRNA (Calogero et al., 1988; Canonaco et al., 1989). Competition experiments between different mRNAs and an SD deoxy-octanucleotide showed, however, that, in the presence of IFs, the SD octamer had a decreased ability to compete with mRNAs for binding to 30S ribosomal subunits. These results indicated that the relative positions of the two ribosomal ligands were modified by the presence of IFs and suggested that the mRNA is distributed between two ribosomal binding sites. In the absence of IFs, the mRNA would preferentially occupy a "stand-by" site corresponding to the region where the SD interaction takes place, whereas, in the presence of factors, it would be shifted toward another site, presumably closer to the decoding region (Canonaco et al., 1989). In support of this hypothesis, diepoxybutane and UV-induced crosslinking of model mRNAs to 30S subunits yielded a different distribution of the crosslinked mRNA among ribosomal proteins (r-proteins) in the presence and absence of IFs and initiator tRNA (Brandt & Gualerzi, 1991). However, the overall extent of the observed modifications in these experiments was only modest and, furthermore, no information was available concerning either the crosslinked positions on the mRNA or the sites of crosslinking to the rRNA. In consequence, the actual existence of the postulated shift of the mRNA induced by the factors on the ribosome and its molecular nature has remained elusive until

Reprint requests to: A. La Teana, Laboratory of Genetics, Department of Biology, University of Camerino, 62032 Camerino (MC), Italy; e-mail: GENETICA@CAMVAX.CINECA.IT.

now. A significant improvement in the definition of the mRNA path through the 30S ribosomal subunit, with the identification of the ribosomal components in close contact with the mRNA, both 5′ and 3′ to the decoding site, has been made possible in recent years by the introduction of the "site-directed crosslinking" technique (for recent review see Brimacombe, 1995). This technique makes use of mRNA analogues carrying 4-thio-uridine (4-thio-U) residues at selected positions, which, upon UV irradiation, can form specific and identifiable crosslinks with rRNA and r-proteins within the mRNA–ribosome complexes.

In the present paper, we have applied site-directed crosslinking to study mRNA localization in different types of initiation complexes. The results allowed us to demonstrate the existence of the mRNA shift during the early stages of translation initiation and to obtain a better characterization of the two mRNA-binding sites on the 30S ribosomal subunit occupied by the mRNA in the absence and presence of IFs.

RESULTS

Several model mRNAs were prepared and used in this study; they all had an AUG initiation codon, an SD sequence (AAGGAGG) and a spacer 4 or 8 nt long. Furthermore, the mRNAs had three U's in their nucleotide sequence, two at a fixed position (i.e., +2, corresponding to the central base of the initiation triplet, and +11, the central base of the fourth codon), and the third occupying a variable position within the spacer region. These mRNAs were named according to the length of the spacer and the position of the U within the spacer. Most of the data presented in this paper was obtained with 4N/−3 mRNA (i.e., an mRNA with a 4-nt spacer, and a U residue at position −3) whose sequence is shown in Figure 1.

The mRNA analogues were transcribed in the presence of an appropriate mixture of ^{32}P-UTP and nonradioactive 4-thio-UTP (Stade et al., 1989), purified, and bound to 30S ribosomal subunits under the following conditions: (1) in the absence of other components; (2) in the presence of IF1, IF2, and IF3; (3) in the presence of fMet-tRNA; (4) in the presence of IFs and fMet-tRNA. Some additional experiments were also conducted in which individual factors were omitted.

The different complexes were subjected to UV irradiation at wavelengths above 300 nm (Tate et al., 1990), and the crosslinked products were isolated by two successive sucrose gradient centrifugations essentially as described by Dontsova et al. (1991). The crosslinks between mRNA and 16S rRNA were analyzed by the RNase H method (Stade et al., 1989) using oligodeoxyribonucleotides (10- or 17-mers) complementary to selected regions of the 16S rRNA sequence. The r-proteins crosslinked to the mRNA were identified by electrophoresis followed by immunoprecipitation (Gulle et al., 1988).

Titration of 30S ribosomal subunits with increasing amounts of ^{32}P-labeled 4N/−3 mRNA showed, in agreement with previous results (Calogero et al., 1988; Canonaco et al., 1989), that the presence of IFs did not influence the binding (Fig. 2). As seen below, however, the IFs caused the appearance of a different crosslinking pattern between the mRNAs and various components of the 30S subunits.

The products of RNase H digestion of 16S rRNA crosslinked to 4N/−3 mRNA were analyzed by polyacrylamide gel electrophoresis. Each panel of Figure 3 presents the analysis of the crosslinking results obtained with a different type of complex. The individual slots of each panel correspond to different combinations of oligodeoxyribonucleotides used in the reaction covering the 3′-end region (i.e., nt 1300–1542) of 16S rRNA; the corresponding results concerning the 530 loop (i.e., nt 451–553) are presented in Figure 4. The results can be interpreted by reference to the diagrams presented below the gels.

Concerning the 3′-end of the 16S rRNA, in agreement with the data reported by Dontsova et al. (1991), the 30S-mRNA complex yields single radioactive bands (140, 150, and 45 nt) with the three combinations of oligonucleotides (Fig. 3A, lanes 1–3). These three bands correspond to a single crosslink of the mRNA to the 3′-end of the 16S rRNA and, more precisely, to a position close to 1530 (Rinke-Appel et al., 1994). In the complex formed in the presence of the three IFs (Fig. 3B), two additional bands of 80 nt (lane 2) and 110 nt

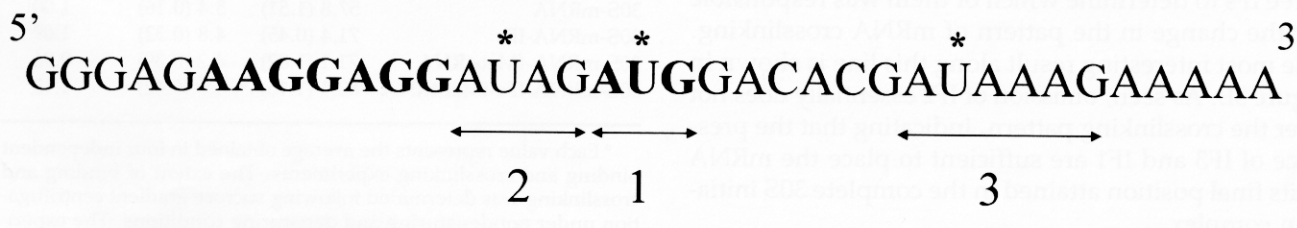

FIGURE 1. Sequence of the 4N/−3 model mRNA. Asterisks mark the positions in which ^{32}P-UTP or 4-thio-UTP were incorporated in the transcripts by the T7 RNA polymerase. The SD sequence and the initiation triplets are in bold letters. Arrows underline the mRNA fragments produced by ribonuclease T_1 digestion that are relevant for the identification of the sites of crosslinking (cf. Fig. 5).

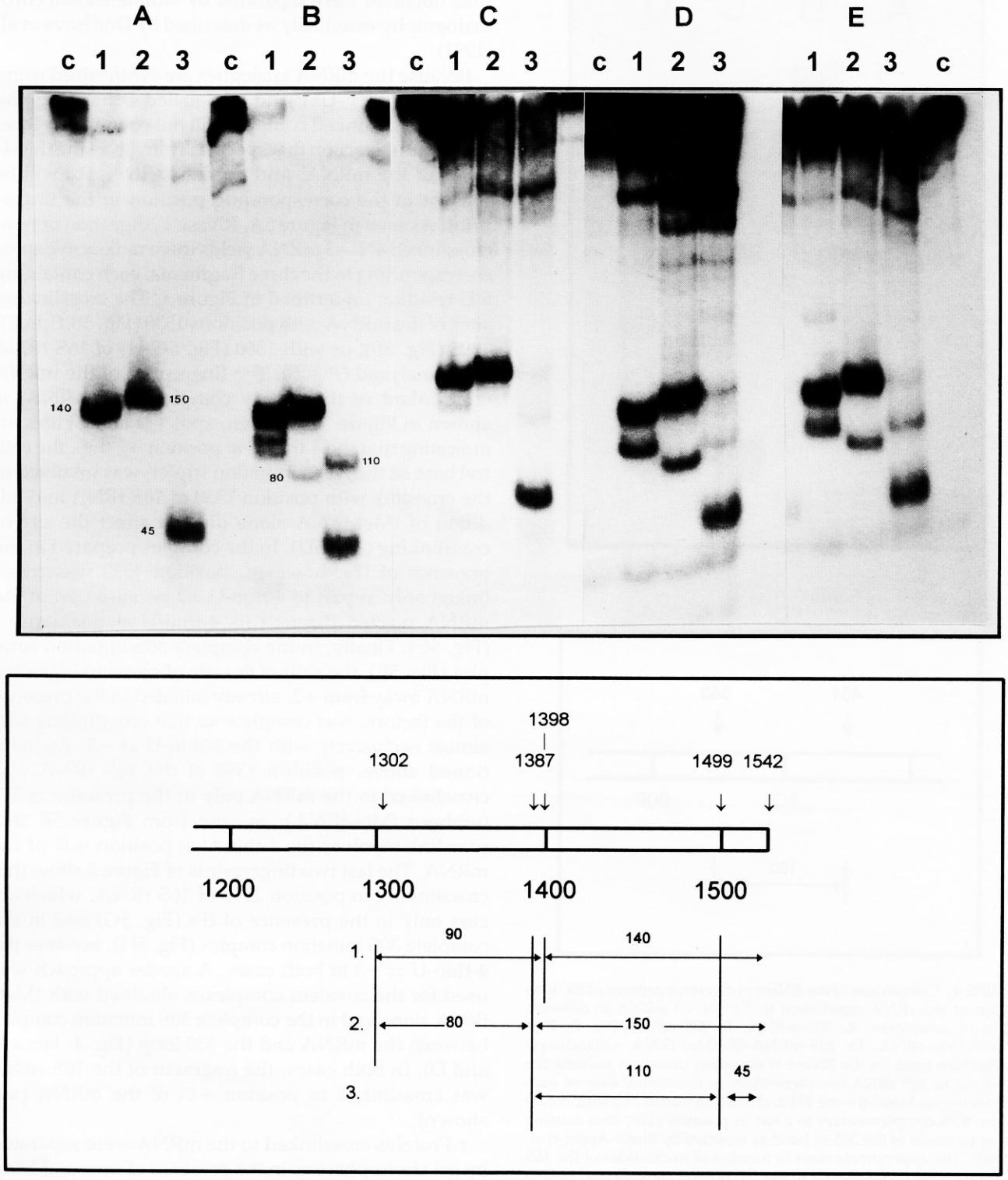

FIGURE 3. Comparison of the RNase H digestion patterns of 3′-end region of 16S rRNA crosslinked to ^{32}P-4N/−3 mRNA in different types of complexes. **A:** 30S-mRNA. **B:** 30S-mRNA-IFs. **C:** 30S-mRNA-fMet-tRNA. **D:** 30S-mRNA-IFs-fMet-tRNA. **E:** 30S-mRNA-IF1-IF3-fMet-tRNA. The oligodeoxynucleotides used for the RNase H digestion were the following: lanes c, none (undigested 16S rRNA); lanes 1, 1302, 1398; lanes 2, 1302, 1387; lanes 3, 1387, 1499. These numbers indicate the positions in 16S rRNA complementary to the central base of each oligodeoxynucleotide. The approximate sizes of the 16S rRNA fragments crosslinked to the ^{32}P-mRNA are indicated (in number of nucleotides) next to the relevant bands. The locations of these fragments within the 16S sequence are shown in the diagram.

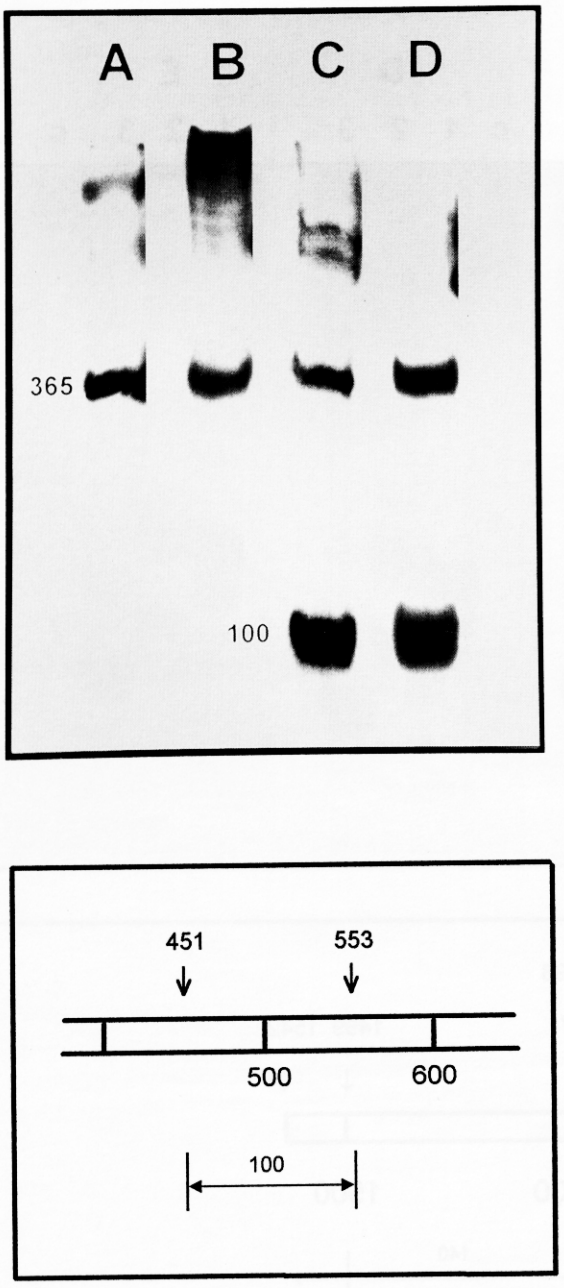

FIGURE 4. Comparison of the RNase H digestion patterns of 530 loop region of 16S rRNA crosslinked to ^{32}P-4N/−3 mRNA in different types of complexes. **A:** 30S-mRNA. **B:** 30S-mRNA-IFs. **C:** 30S-mRNA-fMet-tRNA. **D:** 30S-mRNA-IFs-fMet-tRNA. Oligodeoxynucleotides used for the RNase H digestion (numbers indicate the positions in 16S rRNA complementary to the central base of each oligodeoxynucleotide) were 451 and 553 (the former oligonucleotide is also 80% complementary to a site at position 1178, thus causing the appearance of the 365-nt band as reported by Rinke-Appel et al. [1994]). The approximate sizes in number of nucleotides of the 16S rRNA fragments crosslinked to the ^{32}P-mRNA are indicated next to the relevant bands. The locations of these fragments within the 16S sequence are shown in the diagram.

To determine which of the three 4-thio-U bases of the mRNA was involved in each crosslinked product, the ^{32}P-mRNA-16S rRNA fragments obtained after RNase H digestion and electrophoresis were extracted from the gel and digested with RNase T_1. RNA fragments thus obtained were separated by bidimensional chromatography essentially as described by Dontsova et al. (1991).

Because the mRNA analogues are synthesized using a mixture of ^{32}P-UTP and nonlabeled 4-thio-UTP, the isolated crosslinked complex will not contain any label in the site of reaction that specifically involves the thio-U bases of the mRNA, and no radioactive spot will be present in the corresponding position in the fingerprint. As seen in Figure 5A, RNase T_1 digestion of non-crosslinked 4N/−3 mRNA yields three radioactive spots corresponding to the three fragments, each containing a U residue, underlined in Figure 1. The crosslinking sites of the mRNA with positions 1530 (Fig. 5B,C,D,E), 1395 (Fig. 5F), or with 1360 (Fig. 5G,H) of 16S rRNA were analyzed (Fig. 5). The fingerprint of the mRNA crosslinked in the binary complex (30S-mRNA) is shown in Figure 5B; as seen, spot 1 is almost absent, indicating that the 4-thio-U at position +2 (i.e., the central base of the AUG initiation triplet) was involved in the crosslink with position 1530 of 16S rRNA and addition of fMet-tRNA alone did not affect the site of crosslinking (Fig. 5D). In the complex prepared in the presence of IFs, however, position 1530 was crosslinked only in part to 4-thio-U +2 because part of the mRNA reacted through its 4-thio-U at position −3 (Fig. 5C). Finally, in the complete 30S initiation complex (Fig. 5E), the shift of the site of crosslinking of the mRNA away from +2, already initiated in the presence of the factors, was complete so that crosslinking was almost exclusively with the 4-thio-U at −3. As mentioned above, position 1395 of the 16S rRNA was crosslinked to the mRNA only in the presence of IFs (without fMet-tRNA); as seen from Figure 5F, this crosslink involves the 4-thio-U at position +11 of the mRNA. The last two fingerprints of Figure 5 show that crosslinking to position 1360 of 16S rRNA, which occurs only in the presence of IFs (Fig. 5G) and in the complete 30S initiation complex (Fig. 5H), involves the 4-thio-U at −3 in both cases. A similar approach was used for the covalent complexes obtained with fMet-tRNA alone and in the complete 30S initiation complex between the mRNA and the 530 loop (Fig. 4, lanes C and D). In both cases, the fragment of the 16S rRNA was crosslinked to position +11 of the mRNA (not shown).

r-Proteins crosslinked to the mRNA were separated by gel electrophoresis in the presence of urea and SDS, and the radioactive protein–mRNA complexes, localized by autoradiography (Fig. 6), were extracted from the gel. Proteins present in the complexes were identified using the antibody agarose affinity chromatography method (Gulle et al., 1988). The four lanes (A–D) seen in Figure 6 correspond to the r-proteins crosslinked to mRNA in the four different types of complexes. Three r-proteins (i.e., S1, S18, and S21, the

TABLE 2. Semi-quantitative determination of the extent of mRNA crosslinking to the individual 16S rRNA sites.[a]

Type of complex	Sites of crosslinking in 16S rRNA				
	1530	1360	665	1395	532
30S-mRNA	97.9 (1.0)	0	2.0 (1.0)	0	0
30S-mRNA-IFs	64.7 (6.5)	14.5 (5.5)	2.5 (0.9)	18.1 (1.4)	0
30S-mRNA-fMet-tRNA	24.1 (4.2)	3.4 (1.0)	10.1 (2.9)	1.7 (0.9)	60.9 (1.1)
30S-mRNA-IFs-fMet-tRNA	17.2 (1.0)	15.1 (3.2)	3.2 (0.8)	0.5 (0.2)	62.7 (1.9)

[a] Values represent the average obtained in four independent crosslinking reactions. The mRNA radioactivity covalently linked to each position was determined densitometrically. The values are expressed as percentage of crosslinked mRNA associated with each site with respect to the yield of crosslinking. Standard error is indicated in parenthesis.

latter two always identified together in the same band) were the main targets of mRNA crosslinking in the 30S-mRNA binary complex (lane A). In addition to these proteins, some other very faint radioactive bands were also present. These corresponded to r-proteins S2 (giving rise to a double band) and S7. In the complex prepared in the presence of the three IFs (Fig. 6, lane B), in addition to S1, S18, and S21, strong crosslinking was found also with S7 (giving rise to a split band) and S9 and S11, present together in the same band. The crosslinking pattern obtained in the presence of fMet-tRNA (Fig. 6C) was almost identical to that seen in the binary complex (cf. Fig. 6A,C), whereas that obtained in the complete 30S initiation complex (Fig. 6D) did not substantially change with respect to that seen in the presence of the IFs, but for the almost complete lack of S9 and S11.

As with the 16S rRNA, also the sites of the mRNA crosslinked to individual r-proteins were identified. The relative intensities of the three radioactive spots corresponding to positions −3, +2, and +11, obtained by RNase T_1 fingerprinting, were measured by densitometry to determine the approximate contribution of each site to the crosslinking reaction. As seen from Table 3, the four types of complexes not only gave a different r-proteins crosslinking pattern, but also involved the reaction of different bases of the mRNA.

The main conclusions drawn from the analysis of the mRNA sites crosslinked to r-proteins can be summarized as follows. S1 crosslinking seemed to depend primarily on the UV irradiation per se, rather than on the reaction with 4-thio-U. Concerning the latter, S1 was crosslinked with −3 slightly more than with +2; this bias seemed to increase somewhat going from binary complex to the complex with IFs to the complete 30S initiation complex. Protein S7 was crosslinked very weakly in the binary complex and in the complex containing fMet-tRNA but no IFs; strong crosslinking,

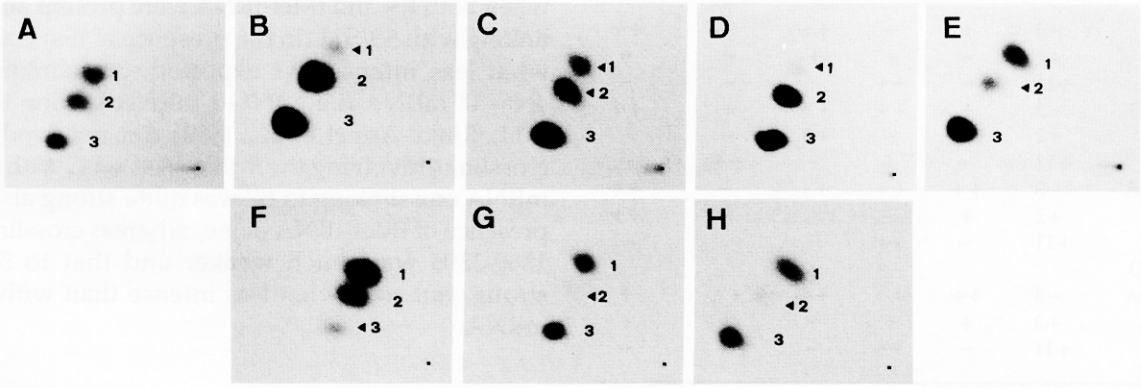

FIGURE 5. Determination of the mRNA sites involved in crosslinking by RNase T_1 fingerprinting. The crosslinked complexes electrophoretically separated after RNase H digestion were extracted and subjected to fingerprinting as described in the Materials and methods. **A:** Control (noncrosslinked) 4N/−3 mRNA. Fingerprints of mRNA crosslinked to the 3′ end of 16S (around position 1530) are shown in panels B–E for the following complexes. **B:** 30S-mRNA. **C:** 30S-mRNA-IFs. **D:** 30S-mRNA-fMet-tRNA. **E:** 30S-mRNA-IFs-fMet-tRNA. **F:** Fingerprint of the mRNA crosslinked to position 1395 in the complete 30S initiation complex. **G,H:** Fingerprints of mRNA crosslinked to position 1360 in 30S-mRNA-IFs complex (G) and 30S-mRNA-IFs-fMet-tRNA complex (H). The sample loading point is marked with a dot. First and second dimension of chromatography were from right to left and from bottom to top, respectively. As shown in Figure 1, the radioactive fragments marked 1, 2, and 3 correspond to oligonucleotides with thio-U at positions +2, −3, and +11, respectively. Arrows indicate spots missing or reduced in intensity.

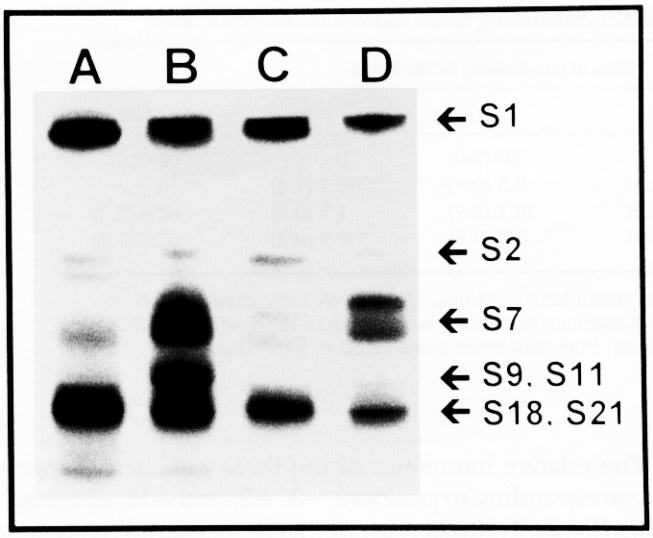

FIGURE 6. Identification of the r-proteins crosslinked to 4N/−3 mRNA in different types of complexes. The figure shows an autoradiogram of an SDS-urea-PAGE of r-proteins crosslinked in: lane A, 30S-mRNA; lane B, 30S-mRNA-IFs; lane C, 30S-mRNA-fMet-tRNA; lane D, 30S-mRNA-IFs-fMet-tRNA.

however, occurred in the presence of IFs, with or without fMet-tRNA. Concomitant with these drastic changes in the extent of crosslinking, the site of reaction of the mRNA was shifted from almost exclusively −3 (in the binary complex), to −3 and +2, the latter site being proportionally more reactive in the complex containing IFs. S18 and S21 are strongly crosslinked in all complexes, particularly in the binary complex and in the complex with IFs. The main target of crosslinking is position −3 of the mRNA in the 30S-mRNA binary complex, in the complex with IFs and in the complex with fMet-tRNA, in which, however, extensive crosslinking with +2 was also observed. Finally, positions −3 and +2 were almost equally reactive within the 30S initiation complex, although the overall efficiency of crosslinking was somewhat reduced.

Proteins S9/S11 were crosslinked very strongly only in the complex containing IFs primarily via the 4-thio-U at +11, with some minor reaction at −3. In consideration of the location of these proteins on the 30S subunit (Capel et al., 1988; Stöffler-Meilicke & Stöffler, 1990), we assume that +11 is crosslinked to S9 and −3 to S11. The weak crosslinking of S9/S11 found in the complete 30S initiation complex was found to be to −3 and +2.

Finally, weak crosslinking of S2 was observed with all complexes, but the site of crosslinking was not the same in all cases; the main sites were +11 in the 30S-mRNA complex and −3 in the presence of IFs, whereas in the presence of fMet-tRNA and in the 30S initiation complex, −3 and +11 contributed almost equally to the reaction.

All data presented so far were obtained with 4N/−3 mRNA (Fig. 1). Other model mRNAs were also used, however, and the crosslinking results obtained with them are briefly outlined below. Crosslinking patterns very similar to those obtained with 4N/−3 mRNA were obtained with 8N/−7 mRNA and 4N/−1 mRNA. With 8N/−7 mRNA (Rinke-Appel et al., 1994), however, crosslinking to 1360/1395 was somewhat weaker. With 4N/−1 mRNA, crosslinking to 1360 appeared only when both IFs and fMet-tRNA were present and crosslinking with S9/S11 (in the presence of IFs) was somewhat less intense. As expected, crosslinking with 4N/−1* mRNA (i.e., 4N/−1 mRNA lacking the U at +11, Rinke-Appel et al., 1994) did not produce the crosslinks involving the 4-thio-U at +11. With 4N/−4, mRNA crosslinking to S7 was quite strong also in the presence of fMet-tRNA alone, whereas crosslinking to 1360/1395 was much weaker and that to 532 was strong, but somewhat less intense than with 4N/−3 mRNA.

TABLE 3. Sites of 4N/−3 mRNA crosslinking to individual r-proteins in different complexes.[a]

Addition to 30S-mRNA complex	mRNA site	Crosslinked ribosomal proteins				
		S1	S2	S7	S9/S11	S18/S21
None	−3	+	−	++	−	++
	+2	−	+	+	−	+
	+11	−	++	−	−	−
IF1/IF2/IF3	−3	+	++	++	+	++
	+2	+	−	++	−	−
	+11	−	+	−	++	−
fMet-tRNA	−3	++	++	++	−	++
	+2	+	−	+	−	++
	+11	−	++	−	−	−
IF1/IF2/IF3 fMet-tRNA	−3	++	++	++	++	+
	+2	+	−	+	+	+
	+11	−	++	−	−	−

[a] The electrophoretically resolved mRNA-r-protein crosslinked complexes (Fig. 6) were extracted from the gels. Part of the material was used for the immunological identification of the proteins (Gulle et al., 1988; Rinke-Appel et al., 1991), and the rest was treated with proteinase K and digested with RNase T1 to yield fingerprints similar to those shown in Figure 5. The extent of reaction of each mRNA site was estimated by densitometry. Bold and nonbold symbols are attributed to r-proteins giving strong and weak crosslinking respectively. The relative extent to which each site of mRNA is involved in crosslinking is indicated by: high (++ or ++); medium (+ or +); low or none (− or −).

DISCUSSION

In the present paper, the position of the mRNA and its possible adjustment on the 30S ribosomal subunit under the influence of the initiation factors, of fMet-tRNA, and in the complete 30S initiation complex were analyzed by site-directed crosslinking. The sites of the mRNA covalently linked to specific positions of 16S rRNA and to individual r-proteins were determined

and the extent of their crosslinking was analyzed semiquantitatively. Identification of the crosslinked sites was aided a great deal by previous results, essentially confirmed by the present work, on the topographical localization of the same or similar model mRNAs (for a review, see Brimacombe, 1995). In addition, the present results demonstrated that, depending upon the nature of the complex, a redistribution of the crosslinking sites on 16S rRNA, r-proteins, and mRNA takes place. Most of the results presented here were obtained with the model mRNA named 4N/−3, whose sequence is presented in Figure 1. Essentially similar results were obtained, however, with other model mRNAs, and the few differences observed can be easily rationalized taking into account the nature of the differences among the various model mRNAs used. Thus, these results support and confirm the conclusions and the model (see below) drawn from the data obtained with 4N/−3 mRNA. These findings also provide evidence that, under the experimental conditions used, crosslinking was selective enough to detect small differences in the relative positions of the reactive sites, thus attesting to the relevance of the modifications in the crosslinking patterns observed in the various types of complexes and, more particularly, under the influence of the IFs. Furthermore, neither the amount of mRNA bound nor the total mRNA crosslinked were significantly different in the four types of complexes. Thus, taken together, the data lead to the main conclusion that the position of the mRNA on the ribosome is readjusted during 30S initiation complex formation.

The main crosslinking result that seems to be difficult to interpret in our experiments is that to r-protein S1. This was probably the only case, however, in which the crosslinking reaction was not entirely "site-specific," depending not so much on a reaction with 4-thio-U, but more on the direct UV irradiation. This is not surprising, however, because a high efficiency of mRNA crosslinking to S1 by UV irradiation is well known and has already been reported, for instance, for a 30S initiation complex (Brandt & Gualerzi, 1992).

To illustrate the idea of a shift of the mRNA position on the ribosome during formation of the 30S initiation complex, most of the results obtained are summarized in Figure 7 (to simplify the picture, the data obtained with 30S-mRNA-fMet-tRNA complex have been omitted from the figure); this picture schematically depicts the occupancy of the 30S mRNA channel by 4N/−3 mRNA in the 30S-mRNA complex without Ifs (Fig. 7A), with IFs (Fig. 7B), and in the complete 30S initiation complex (Fig. 7C). Approximate positions of the relevant crosslinking sites on the 30S subunit (r-proteins and 16S rRNA nucleotides), or, at least, their relative positions, are based on the most up-to-date topographical models of the subunit (Brimacombe, 1995; cf. Brimacombe et al., 1988; Stern et al., 1988; Malhotra & Harvey, 1994).

Compared to the simplest case of the 30S-mRNA complex, in which the mRNA is crosslinked almost exclusively to 1530 of 16S rRNA through its +2 position and to r-proteins S18 and S21 through its −3 position, the crosslinking pattern becomes much more complex in the presence of IFs. New, strong crosslinks appeared between S7 and both +2 and −3, between S9 and +11, 1395 and +11, between 1360 and −3, whereas crosslinking of 1530 was partially shifted from +2 to −3. All these changes suggest "a leftward" shift of the mRNA with respect to the 30S subunit, as drawn in Figure 7. It is noteworthy that most of the changes in the crosslinking pattern obtained in the presence of the three IFs are also seen in the absence of IF2, indicating that the effects are primarily due to IF1 and IF3. Because the overall crosslinking pattern obtained in the presence of IFs is intermediate between those obtained with the 30S-mRNA complex and with the 30S initiation complex, the factors seem to be responsible, to a large extent, for the adjustment of the mRNA that occurs in preparation for the decoding of the initiation triplet in the ribosomal P site by the initiator tRNA. Furthermore, the crosslinking pattern obtained with the 30S subunits in the presence of the IFs resembles that observed by Rinke-Appel et al. (1994) in a 70S-mRNA complex formed in the absence of IFs with or without uncharged $tRNA_f^{Met}$, suggesting that the 50S subunit mimics the presence of the IFs.

A further rearrangement of the mRNA on the 30S subunit took place in the complete 30S initiation complex: position +11 of the mRNA was no longer crosslinked to S9 and only marginally to 1395, but moved further "leftward" to crosslink position 532 of 16S rRNA. Also, position +2 moved away from S7 and 1530 because crosslinking to these two elements was reduced, whereas +2 and −3 approached S9, with which a weak yet significant crosslinking was established. It should be noted that, in the course of the mRNA shift, the central base of the initiation triplet moves away from 1530 toward 1395, i.e., toward the P-site of the subunit.

In addition to IFs, fMet-tRNA also contributes to the placement of the mRNA in its final position in the 30S initiation complex and the most relevant new crosslinking, contributed, also by the initiator tRNA alone, is the formation of crosslinking between position 532 of 16S rRNA and +11 of the mRNA. On the other hand, the initiator tRNA causes very little changes in the crosslinking pattern in the 5′-end of the mRNA. However, because the fMet-tRNA bound under these conditions to the 30S subunits is not puromycin reactive upon addition of 50S subunits (A. La Teana, C.L. Pon & C.O. Gualerzi, in prep), it can be inferred that the crosslinking pattern obtained with fMet-RNA in the absence of IFs does not entirely reflect P-site binding and that the relocation of the 5′-side of the mRNA with respect to the 30S subunit, contributed primarily by the IFs, is

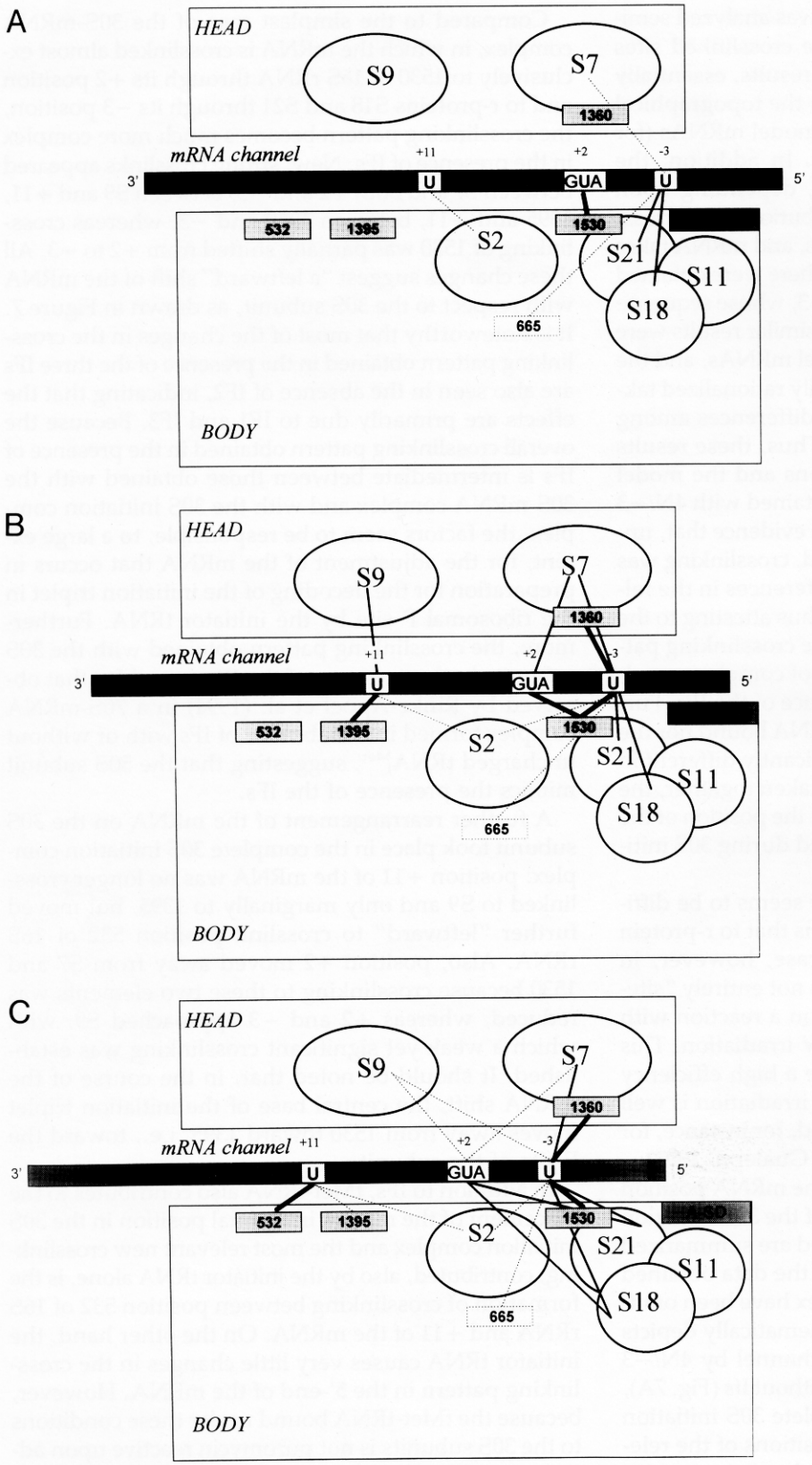

FIGURE 7. Scheme illustrating the crosslinking pattern of 4N/−3 mRNA in its binary complex with the 30S ribosomal subunit (**A**), in the presence of IF1, IF2, and IF3 (**B**), and in the presence of IFs and fMet-tRNA (**C**). The approximate relative positions of the relevant ribosomal components in the head or body of the 30S ribosomal subunit are shown. In the mRNA (black bar) the SD sequence, the initiation triplet, and the positions of the potential crosslinking sites are indicated. Major RNA–RNA crosslinks (thick lines); major RNA–protein crosslinks (thin lines); quantitatively minor crosslinks of either type (dotted lines). The enclosure of 665 within a dotted box indicates the minor nature of this crosslink.

necessary for the proper placement of the initiator tRNA on the ribosome. Finally, it should be noted that the adjustment of the mRNA with respect to the ribosome (which we defined "leftward" by reference to the scheme in Fig. 7) occurs in a direction contrary to that expected (i.e., "rightward") during translation. This "backward" movement of the mRNA during 30S initiation complex formation, which precedes the "for-

ward" movement necessary for elongation, fits well with the current model of initiation site selection that entails an initial (not necessarily specific) mRNA-ribosome interaction in a stand-by position, followed by a kinetic selection of the correct initiation site occurring during decoding of the initiator tRNA in the P-site under the influence of the IFs (Gualerzi & Pon, 1990).

In conclusion, the findings of this paper support the earlier conclusion that the position of the mRNA on the ribosomes is shifted under the influence of the IFs, obtained by a completely different approach, i.e., the competition for ribosomal binding between mRNA and oligonucleotides (Canonaco et al., 1989); they also provide a more precise physical basis for the existence and a topographical description of the two postulated mRNA binding sites (i.e., the stand-by site and P decoding site) preferentially occupied by the mRNA in the absence and presence of the IFs (Canonaco et al., 1989).

MATERIALS AND METHODS

Buffers

Buffer A: 20 mM Tris-HCl, pH 7.8, 7 mM $MgCl_2$, 100 mM NH_4Cl, 0.5 mM GTP, 2 mM 2-mercaptoethanol. Buffer B: 20 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 200 mM NH_4Cl, 2 mM 2-mercaptoethanol.

General preparations

Escherichia coli MRE600 30S and 50S ribosomal subunits, fMet-$tRNA_f^{Met}$ (70% aminoacylated and 96% formylated), and purified initiation factors IF1, IF2, and IF3 were prepared essentially as described (Ohsawa & Gualerzi, 1983; Gualerzi et al., 1991).

Model mRNAs

^{32}P-labeled mRNA analogues containing 4-thio-U in different positions (see Results) were transcribed from synthetic DNA templates using T7 RNA polymerase as described (Stade et al., 1989).

Preparation of mRNA-30S complexes and crosslinking reaction

A typical binding reaction mixture contained, in 0.4 mL of Buffer A, 10 A_{260} units of 30S ribosomal subunits (preincubated for 15′ at 42 °C in Buffer B), and 0.2 molar equivalents of mRNA. When appropriate, the reaction mixture also contained purified initiation factors IF1, IF2, and IF3 (in stoichiometric equivalent amounts to the 30S subunits) and/or fMet-tRNA in a two-fold molar excess over ribosomes. After incubation at 37 °C for 15 min, the complexes were subjected to 3 min at 4 °C UV (>300 nm) irradiation as described (Tate et al., 1990) and the crosslinked products were separated by two successive sucrose gradient centrifugations (Dontsova et al., 1991).

Analysis of the crosslinking sites

Identification of the sites of crosslinking on the 16S RNA and of the crosslinked r-proteins was carried out essentially as described (Stade et al., 1989). The position of the 4-thio-U residues of the mRNAs involved in the crosslinking reaction were identified by ribonuclease T_1 finger-printing as described elsewhere (Dontsova et al., 1991, 1992).

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