New RNA-protein crosslinks in domains 1 and 2 of *E.coli* 30S ribosomal subunits obtained by means of an intrinsic photoaffinity probe

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ABSTRACT

Functionally active 70S ribosomes containing 4-thiouridine (s^4U) in place of uridine were prepared by a formerly described *in vivo* substitution method. Proteins were crosslinked to RNA by 366nm photoactivation of s^4U . We observe the systematic and characteristic formation of 30S dimers; they were eliminated for analysis of RNA-protein crosslinks. M13 probes containing rDNA inserts complementary to domains 1 and 2 of 16S RNA from the 5' end up to nucleotide 868 were used to select contiguous or overlapping RNA sections. The proteins covalently crosslinked to each RNA section were identified as S3, S4, S5, S7, S9, S18, S20 and S21. Several crosslinks are compatible with previously published sites for proteins S5, S18, S20 and S21; others for proteins S3, S4, S7, S9, S18 correspond necessarily to new sites.

INTRODUCTION

Precise localization of covalent RNA-protein crosslinking points in the sequence of the RNA of large ribonucleoprotein particles is one of the methods of choice to obtain structural information concerning their architecture. With the notable exception of UV light (254nm), most of the crosslinking agents used so far yield only neighbourhood information (1-6). In order to obtain more precise complementary structural data we have developed new reagents able to introduce direct crosslinks and thus permit analysis of close contacts (from van der Waals contacts to 5Å) between RNA and protein. Thus EDC, a soluble carbodiimide activates free carboxyl groups in proteins triggering crosslinking reactions (7). More recently we have used an intrinsic photoaffinity probe: 4-thiouridine (s^4U) (8), which occurs naturally in tRNA of *E.coli* cells (9) where it plays an antiphotomutagenic role against solar radiation (10). As an analogue of uridine it can be incorporated *in vivo* into RNAs. Active thiolated 70S ribosomes can be obtained and their 365nm photoactivation lead to RNA-protein crosslinks. The advantages of s^4U as an intrinsic probe have previously been discussed (8,11,12). The limits of this type of approach are set not only by the crosslinking procedure used but also by the methods employed to analyse

covalent crosslinked complexes. As a general and systematic approach to obtain low resolution mapping of crosslinked 30S proteins on 16S RNA. We have used single stranded M13 DNA probes (ssDNA) containing rDNA inserts complementary to 16S RNA sections. These probes were hybridized to 16S RNA-protein complexes and RNase T1 hydrolysis was used to eliminate the non selected regions. The proteins covalently linked to the RNA sections protected by the complementary rDNA were identified by two-dimensional gel analysis. This method was previously described in details for domains 3 and 4 (12-14).

We report here the use of 8 other M13 ssDNA probes to identify proteins crosslinked to sections between nucleotides 1 and 869, covering domains 1 and 2. We have found several RNA-protein crosslinks which are compatible with previous results and a few others that are necessarily new sites.

MATERIAL AND METHODS.

Material and Enzymes.

4-thiouridine was from Sigma. (^{125}I) and the T4 DNA ligase were from Amersham (England). Carrier free ($\text{H}_3^{32}\text{PO}_4$) was from CEA (France). The Klenow fragment of DNA polymerase I was from Boehringer and RNase T1, RNase T2 from Sankyo.

Buffers.

Buffer A: 10 mM triethanolamine / HCl pH 7.5, 1 mM Mg AC_2 , 50 mM KCl

Buffer B: 10mM triethanolamine / HCl pH 7.5; 0.1% SDS, 100 mM LiCl.

Buffer SCE1X: 15mM sodium citrate pH 7, 150mM NaCl, 10mM Na_2EDTA .

Buffer TBE1X: 90mM Tris, 90mM borate, 2.5mM Na_2EDTA pH 8.3.

Cell strains and media used for ribosome preparation.

(^{32}P) labelled MRE 600 cells were grown in low phosphate medium (15). AB1157 sfia pyrD was used for 4-thiouridine incorporation. The conditions were improved so that incorporation now occurs in exponentially growing cells (Bezerra, R. & Favre, A. in preparation). *E. coli* JM103 was employed for growth of recombinant phage in 2 x TY medium (16).

Preparation of 30S ribosomal subunits, carrier 30S proteins and 16S RNA.

Thiolated 70S ribosomes were separated from unassociated particles and separated into 30S and 50S subunits as previously described (8). Two different preparations with respectively 2% and 7.5% $\text{s}^4\text{U/U}$ substitution level in 30S subunits were used in this work : the former being the preparation used in previous work (13). Thiolated 30S particles were stored at -70°C in buffer A supplemented with 1 mM dithiothreitol and samples were thawed only once. Uniformly labelled (^{32}P)30S subunits and 16S RNA were prepared from MRE 600

cells as in (17). Carrier 30S proteins were extracted from 30S subunits by the acetic acid procedure and stored in 8M urea (18).

Illumination and analysis of dimer formation.

The illumination system and fluence conditions were as in (11). For analytical purposes, thiolated 30S subunits were adjusted to the desired ionic conditions before illumination by dialysis (Spectrapor tubing MW Cut off 12000) for 1.5h. Samples ($1.5A_{260}$ units, 200 μ l) were either illuminated in a 1ml cuvette (1cm pathlength) in conditions previously described (8), or kept in the dark (control) and then analysed by centrifugation on a 5-20% sucrose gradient in buffer A (SW41 rotor, 41 000 rpm for 4h at 4°C). For preparative purposes, 40 A_{260} units of thiolated 30S subunits were illuminated in Buffer A in a 5ml cylindrical cuvette (2.5cm pathlength) (13), and the suspension was then layered on a 5-20% sucrose gradient made in Buffer A which was centrifuged at 19 000 rpm for 15.5h at 4°C (SW28 rotor) for further purification.

Purification and labelling of crosslinked RNA-protein complexes from illuminated 30S subunits.

Elimination of non crosslinked proteins was performed by sedimentation on LiCl-SDS sucrose gradients (11). Crosslinked proteins were labelled with 125 I (usually 1mCi for 200 μ g of 16S RNA) in 4M urea as described in (19). RNA complexes were then purified from free iodine on a G25 column, and from aggregates on a 5-20% sucrose gradient in Buffer B (SW 60 Ti rotor at 10°C, 60 000 rpm for 100 min).

Obtention of M13 single stranded DNA probes.

The general methods used for cloning and the different strains were as described in (20-21). The size of cloned inserts was determined in 2 ways : (i) uniformly (32 P) labelled 16S RNA was hybridized to each M13 ssDNA probe, hybrids were incubated with RNase T1 and the size of the protected RNA fragment was measured on 7M urea polyacrylamide gels. (ii) M13 DNA probes were hybridized to the universal primer and the complementary DNA was synthesized by the Klenow fragment of DNA polymerase I in the presence of (α^{32} P)dATP. The double stranded DNA corresponding to the insert was excised by BamHI and PstI and its size controlled on 7M urea polyacrylamide gels.

Recombinant M13 phage particles released from JM103 cells were concentrated by polyethylene-glycol precipitation and DNA was prepared by phenol-chloroform extractions followed by incubation in 0.4N NaOH for 1h at 37°C to degrade RNA contaminants.

Hybridization of 16S RNA with M13 ssDNA probes.

Typically 20 μ g of purified RNA-(125 I)protein complex or of (32 P)16S RNA was

hybridized with 400µg of M13 DNA probe (a 4 molar excess of DNA with respect to 16S RNA) in 50% formamide 0.5 x SCE Buffer (250µl) for 3h at 35°C. Nucleic acids were recovered by ethanol precipitation. The efficiency of hybridization of (³²P)16S RNA and (¹²⁵I) protein-16S RNA complexes to each probe was checked before each experiment as follows : 1µl of each hybridization mixture was mixed with 5µl glycerol 10%, SDS 0.1%, xylene cyanol blue 0.03%, bromophenol blue 0.03% and loaded onto a 0.7% agarose mini gel made in TBE 1X, SDS 0.1%. After electrophoresis, gels were dried and autoradiographed. Radioactive bands were cut out and counted in a α counter (¹²⁵I) or a β counter (³²P Cherenkov emission).

RNase T1 hydrolysis and gradient purification of the RNase T1 digested hybrid.

To avoid aggregate formation hybrid pellets were gently rehydrated in 10µl water, 90µl SCE1X was then added and samples were incubated at 40°C for 10 min to ensure complete resuspension (if necessary they were centrifuged to eliminate aggregates). RNase T1 hydrolysis and gradient purification conditions are identical to (13). Before collecting the gradient from the bottom of the tube, the upper four fractions were removed to eliminate over 80% of RNase T1-released radioactivity.

Preparation of crosslinked proteins from T1- hybrids.

T1-hybrids recovered from gradients were hydrolysed by NaOH, neutralized and proteins were separated from DNA and from mononucleotides as previously described (13). They were then analysed on two-dimensional gels : the first dimension was in Urea at pH4.5, acrylamid 10%, bisacrylamid 0.15% (22) followed by a second dimension in acrylamid 15%, SDS 0.1%, Urea 2M (23). This gel system was previously used to identified proteins crosslinked to 16S RNA domains 3 and 4 (13)

RESULTS

The 30S ribosomal subunits used in this study were obtained by dissociation of 70S ribosomes extracted from *E.coli* AB1157 sf1A pyrD grown in the presence of uridine and 4-thiouridine (s⁴U) (8). Two preparations were used ; the first (2% thiolation) was obtained as described previously (10-12), while for the second culture conditions were improved yielding a substitution level of 7.5% (Bezerra,R. and Favre,A. in preparation). Thiolated 30S subunits were illuminated at 366nm under nitrogen to avoid RNA breakage (10), crosslinked 16S RNA-protein complexes were separated from free (non crosslinked proteins) by two successive sedimentations through SDS-containing sucrose gradients and proteins covalently bound to RNA were subsequently labelled with ¹²⁵I. The

Fig. 1 : Schematic derivatization of the M13 ssDNA probes from plasmid pKK3535
 The restriction fragments Aha3-MluI (1515-2382) and MluI-MluI (2383-3127) (pKK3535 numbering), were blunt ended, subcloned into pUC8. They cover respectively 16S RNA nucleotides 1 to 869 and 866 to 1542. These two large sections were cloned into M13 and M13 phages, whose DNA is complementary to 16S RNA, were selected.
 pUC8(1-869) insert was further restricted into smaller fragments. Enzymes used in this process are indicated ; the position of the restriction sites is numbered according to their corresponding position on the 16S RNA. These smaller fragments were cloned into M13.

identification of 30S ribosomal proteins bound to selected portions of 16S RNA domains 1 and 2 was performed by the strategy previously described in the study of domains 3 and 4 (12).

M13 DNA probes.

Eight probes were used in this study. For convenience, they are named by the positions of the RNA section to which they hybridize. Preparation of the different M13 ssDNA probes derived from pKK3535 is described schematically in Fig.1. The different probes hybridize to contiguous sections of domains 1 and 2 and cover the 5'-half of 16S RNA. The sizes of the cloned rDNA sections vary (in length) from 84 to 235 nucleotides. This last probe was useful to control the results obtained using 2 smaller adjacent probes 88 nucleotides and 112 nucleotides long which it covers. Two additional probes M13(1-869) and M13(866-1542) respectively complementary to domains 1+2 and domains 3+4 were used as

Fig. 2: Yield of 30S dimer after illumination.

The yield of 30S dimers after illumination is expressed as percent of input thiolated subunits as a function of Mg^{++} concentration at 50mM KCl (■) and 150mM KCl (●).

references for the hybridization efficiency of the smaller probes and for evaluation of the percentage of crosslinked (^{125}I) labelled proteins remaining in the T1-hybrid. These two points will be discussed in detail below.

Dimer formation during illumination

The illumination of thiolated 30S particles led to non negligible formation of complexes with a sedimentation coefficient of approximately 50S on sucrose gradients, they will be referred to as dimers. When thiolated 50S particles are illuminated under the same conditions no dimerization is observed (data not shown). Since contamination by dimers may lead to errors in the interpretation of crosslinking data we have examined the ionic conditions under which illumination was carried out in order to minimize their formation. The results presented in Fig. 2 show that Mg^{++} and K^+ have opposite effects: increasing Mg^{++} concentrations favour dimer formation whereas the formation of dimers is lower with 50mM KCl than with 150mM KCl in the presence of 1mM Mg^{++} . Therefore, thiolated 30S subunits have been illuminated at low concentration, 8A₂₆₀ units/ml (i.e. 0.6 μ M), in 1mM magnesium acetate, 50mM KCl, 10mM TEA/HCl pH7.5. Under these conditions only 3% of input 30S subunits are converted to dimers. They were carefully removed from the 30S monomers by an additional sucrose gradient

Hybridization efficiency

The problem of RNA-DNA hybridization efficiency is of importance because it

Table 1 : Hybridization efficiency of (125 I) labelled protein-RNA complexes to M13 ssDNA probes.

Probe	1-84	81-213	214-417	418-505	504-615	418-652
RNA size (N)	84	133	204	88	112	235
Hybridization efficiency (%)	63	65	52	67	67	72
Probe	649-734	735-869	1-869	866-1542		
RNA size (N)	86	135	869	677		
Hybridization efficiency (%)	67	50	100	100		

Line 1 : the DNA probes are numbered from the section of the 16S RNA they protect in the RNA-DNA hybrid. Line 2 : length of the protected RNA section.

can limit the usefulness of the method. Thus if the hybridization of the 16S RNA-protein complexes to a given DNA probe is not quantitative preferential hybridization of the probe with some RNA-protein complexes and elimination of others may occur and this will result in loss of information. Hybridization efficiency was checked as follows. After hybridization of 16S RNA or 16S RNA-protein complexes with a given probe, an aliquot was removed and analysed by electrophoresis on SDS-agarose gel. After electrophoresis, autoradiography of the dried gel reveals only one band at the position of M13 DNA when RNA or RNA-protein complexes are fully hybridized whereas an additional band at the position of 16S RNA is detected when hybridization is incomplete. The hybridization efficiency is determined by counting of the radioactive bands. Hybridization of uniformly (32 P) labelled 16S RNA to a complementary M13 DNA probe is quantitative whatever the probe and the insert size used. In contrast with (125 I) labelled proteins crosslinked 16S RNA only a fraction of RNA molecules hybridize to DNA. The size of this fraction depends on the probe used (as shown in Table 1) and varies from 50% to 72% for probes smaller than 235 nucleotides long. Varying the experimental conditions indicates that for a given DNA probe there is a maximum level in crosslinked RNA hybridization (data not shown). We have finally adopted the conditions giving maximum hybridization at 35°C. Although the reason for the observed selectivity cannot be clearly determined from our present results several arguments lead to a plausible explanation. At a 7.5% thiolation level, an RNA section 100 nucleotides long contains less than 2 s⁴U residues in place of uridines which means that at most an average of 2 proteins can be simultaneously crosslinked to this section. A

Fig. 3 : Purification of "T1-hybrids" on SDS-sucrose gradients.

Panel A : "T1-hybrids" (735-869) (●), (214-417) (■), (81-213) (▲), panel B : "T1-hybrids" (504-615) (●), (418-505) (■), (1-84) (▲), panel C : "T1-hybrids" (418-652) (●), (649-754) (■), panel D : second purification gradient of T1-hybrids (418-652) (●), (649-734) (■).

crosslinked protein is expected to prevent efficient hybridization over a region only about 10 bases long (roughly one helix turn) and the remaining 90 bases should be largely sufficient to hybridize the DNA probe efficiently. EDC which introduces crosslinks at sites of protein-RNA contact with a comparable efficiency allows quantitative hybridization except for the short probe M13(214-247) (14). However there are two differences between the behaviour of EDC and s^4U (i) formation of the transiently activated EDC carboxylate excludes some sites due to steric constraints (ii) s^4U leads to RNA-RNA crosslinks with an efficiency comparable to that of the formation of RNA-protein bridges, thus possibly stabilizing hairpins. Hence non quantitative hybridization with thiolated RNA could be due either to the intramolecular formation of hairpins within the explored regions and/or to the fact that s^4U induced RNA-protein crosslinks may lead to the stabilization of the two dimensional structure of the RNA.

RNase T1 hydrolysis of hybrids.

As already mentioned (13), the identification of crosslinked proteins to the different 16S RNA sections is based upon protection of a given RNA section against RNase T1 hydrolysis by a complementary DNA probe. The T1-treated

Table 2 : Summary of the 30S ribosomal proteins found crosslinked to the different RNA sections.

16S RNA position	α %	β %	S3	S4	S5	S7	S9	S12	S14	S18	S19 S20	S20 S21
1-84	0.5(*)	0.8	€	€	.	€	€	.	.	.	€	€
81-213	0.2(*)	0.3	€	.	.	€	€	.	€	.	.	.
214-417	3.2(*)	6.1	.	.	.	.	.	.	.	.	++	.
418-652	4.5(*)	6.2	+++	+++	+	+	++	+	.	.	.	.
649-734	4.0(*)	6.0	.	.	.	.	.	.	.	+	.	+
735-869	7.2(*)	14.4	.	.	.	.	.	.	.	++	.	.
418-505	0.5(*)	0.7	€	€	.	€	€	.	.	.	.	.
504-615	3.6(*)	5.3	+++	++	+	+	+++	+	.	.	.	.
1-869	35.6(**)	35.6	not determined									
866-1542	63.4(**)	63.4	not determined									

Column 1 : for the smaller probes the section protected in the RNA-DNA hybrids is indicated by a vertical bar the length of which is proportional to the number of protected nucleotides. The position of the nucleotides at the boundaries are indicated for each section. Section (418-651) was also analysed with smaller probes protecting sections (418-505) and (503-614). Column 2 : α (%) is the fraction of (125 I) labelled crosslinked proteins found in the different RNA sections, α = (cpm T1-hybrid/total cpm recovered on gradient) x 0.01. Column 3 : β (%) = (α) x (M13 probe hybridization efficiency).

(*) analysis done with 7.5% thiolated 16S RNA and (**) with 2% thiolated 16S RNA. Results are based on visual inspection of different exposures of several gels, for this reason only qualitative results are presented here. € indicates traces of radioactivity not taken into account for further analysis. Significantly crosslinked proteins are indicated by (+); (++); (+++).

hybrids (T1-hybrid) are separated from non protected oligonucleotides and proteins on SDS-LiCl sucrose gradients (Fig.3(A,B,C)). Central fractions corresponding to the T1-hybrid are pooled and when necessary purified again on a second identical gradient. This was necessary for T1-hybrids (1-84), (81-213),

A

214 - 417

504 - 615

418 - 652

Fig. 4: Two dimensional PAGE analysis of proteins crosslinked by s^4U to domains 1 and 2.

Analysis of sections (214-417); (504-615); (418-652) and (649-734); (735-869) are presented respectively on panel A and B. Left side ; reference proteins , right side; (^{125}I) autoradiography. Arrow 1 : direction of the first dimension, arrow 2 : direction of the second dimension.

(418-505) where the ratio of signal to background is low. T1-hybrids (418-652) and (649-734) were also purified a second time in order to control the purification efficiency of the first gradient (Fig. 3D) which was respectively 93% and 82%.

The values presented in Table 2 are (i) α : the percentages of (^{125}I) radioactivity associated with the T1-hybrids; the sum of the α values of all contiguous probes is 19.6%. (ii) β is the percentage of radioactivity associated to each T1-hybrid normalized to their corresponding hybridization efficiency (Table 1); the sum of the β values for contiguous probes is 34% which is comparable to the figure of 35.8% found for probe (1-869). Hence our data are consistent and we conclude that there is no notable selective loss of crosslinked proteins with the smaller probes as compared to the larger probes (above 80 nucleotides long). The same conclusion is obtained if one compares the values obtained with probes (418-505) and (504-615) and the overlapping probe M13(418-652). Table 2 shows that for three T1-hybrids, the value β is inferior to 1% and that for five T1-hybrids this value is higher than 5%. We show below that no significant protein crosslinks could be attributed to the sections where β is lower than 1%, which sets the limits of the method.

Identification of crosslinked proteins.

The proteins crosslinked to the different RNA sections were identified by 2D gel electrophoresis after NaOH hydrolysis of the purified T1-hybrids. For T1-hybrids (1-84), (81-213) and (418-505) for which the value of β is lower than 1% (Table 2), almost all the 30S proteins are detected after an extended gel exposition and are considered as the experimental background (results not shown). Autoradiographs of 2D gels of proteins significantly crosslinked to the other sections are presented in Fig.4(A, B) and the results are summarized in Table 2. Efficiently crosslinked proteins are few and in contrast to domains 3 and 4 (13) they do not exhibit multiple crosslinking sites. Since data shown in Table 2 are based on visual inspection of different exposures of several gels, only qualitative results are presented.

In section (214-417) a single radioactive spot is detected (Fig.4A). Although centered on S19 it could be identified as S19 or S20 (see below discussion).

In section (649-734) two radioactive spots are detected (Fig.4B): one corresponds to S18: it is situated to the left of the reference protein S18. The second spot centered on reference S20 with its center on the right of reference protein S20 may be identified as S20 or S21 (see discussion below).

In section (735-869) only one spot is detectable and corresponds clearly to S18 (Fig.4B).

Section (504-615) and (418-652) which overlap widely show the same distribution of spots (Fig.4A) showing that no proteins are crosslinked in detectable amounts to sections (418-505) and (615-652). The major spots can be attributed with no ambiguity to S3, S4 and S9 and the minor spots to S5, S7 and

Fig. 5 : Summary of the protein-RNA crosslinks in domains 1 and 2 of 16S RNA
 Upper part : protein^a schematizes the sites obtained by Brimacombe et al. (6) for proteins S4, S5, S18 and S21. The RNA section for protein S20 interaction is indicated S20^b (28). Middle part : numbers indicate the distance in nucleotide residues from the 5'-terminus of the 16S RNA molecule. The RNA sections covered by the 8 ssDNA probes used in this study are indicated by heavy bars. Lower part : proteins crosslinked to RNA by s⁴U photoactivation and associated to the different RNA sections.

S12. Other fainter spots can also be detected ; they probably belong to a general background.

DISCUSSION

As already mentioned the DNA-RNA hybridization efficiency in the case of s⁴U crosslinking reaches 100% only for large probes. This effect can potentially lead to loss of information ; but it can be precisely quantitated and obviated in some cases, if necessary, by the use of long DNA probes. When compared to other methods which rely on limited RNase digestion for selection of small RNA sections, the DNA-RNA hybrid method is clearly more satisfactory.

The identification of the crosslinked proteins by the 2D gel technique poses a problem in some cases. However for all the proteins which are well resolved in 2D gels (S2, S3, S4, S5, S7, S9, S10, S12, S13, S18) the radioactive spot of iodinated crosslinked protein is uniformly displaced about one diameter in the second dimension and generally less in the first dimension relative to the reference spot. We have no reason to suspect exceptions to this rule for the other 10 proteins. The only group of proteins which cannot be separated contains S15, S16 and S17. These considerations allow us to choose S20 rather than S19 and S20 rather than S21 as the proteins crosslinked to, respectively, sections

(214-417) and (649-734). To do this we make the reasonable assumption that the link between the photocrosslinked s⁴U 2'-3' monophosphate and the aminoacid residue is stable during the whole purification process and identification step.

It is remarkable that a very low efficiency of crosslinking is obtained from nucleotides 1 to 505, the major part of domain 1 (1 to 563) with only one protein S20, significantly crosslinked (Fig.5). With the same DNA probes and methodology but with another contact crosslinker (EDC) protein S20 was not crosslinked and the only significant crosslinked proteins were located in the 5'-terminal section (1-84), where S2, S4, S5 were tentatively identified (24). Although EDC and s⁴U are efficient contact crosslinkers, they do not necessarily crosslink all the 30S ribosomal proteins to a significant extent (as we can conclude after (125I) labelling of proteins). However, the few proteins which are not crosslinked to 16S RNA cannot account for the remarkably low reactivity of most of domain 1. These results agree with the observations of others showing that the largest part of domain 1 is devoid of interacting proteins (25-26).

The highly significant crosslinking of proteins S7 and S9 to section (504-615) is quite puzzling since they are well known to be associated with domain 3. The same result was obtained with the overlapping probe (418-652) and no proteins were found crosslinked to section (418-505), thus limiting their interaction to section (504-615). All available data concerning the structure of the overall 30S particle is compatible with a model in which it is divided into three substructures each containing a specific domain of 16S RNA ; namely the head containing domain 3 (nucleotides 923 to 1393) with which proteins S7 and S9 are associated ; the platform containing essentially domain 2 (nucleotides 564 to 922) and the body containing domain 1 (nucleotides 1 to 563). According to this model, section (504-615) clearly belongs partly to domain 1 (nucleotides 504 to 563) and partly to domain 2 (nucleotides 564 to 615) and thus should not interact with proteins S7 and S9. Our results indicating unexpected contacts between proteins S7, S9 and domain 2 (Fig.5) could be reconciled with the current model of 30S structure by considering only the part of section (504-615) located in domain 2 (nucleotides 564-615) and proposing a possibility of movement of the part of domain 2 located in the platform which could bring nucleotides (564-615) close to the head of the 30S particle. Finally, in spite of stringent controls : careful elimination of 30S dimers ; use of overlapping M13 probes and two step purification of T1-hybrids, these crosslinks clearly have to be confirmed by determination of sequences of crosslinked RNA segments.

In this report we observe that proteins S3, S4 and S5 which are clearly known from different reports to belong to domain 1 close to the junction with

domain 2 (23-25), interact after nucleotide 504 i.e probably between position 504 and the end of domain 1 (A563) (Fig.5). This correlates well with the results of protein-protein crosslinking and neutron diffraction which show that these proteins are very close together (27). Protein S12 is crosslinked to the same (504-615) section by s^4U and by EDC ; it was shown to be close to S4 and S5 by neutron diffraction (27). Several sites of crosslinking of two of these proteins have already been identified : S5 was crosslinked to position 559-561 and to the 5'-terminus by APAI (6) and protein S4 to position 413 by nitrogen mustard (6) ; the S4-crosslinking site detected with s^4U and EDC situated in section (504-615) are therefore different sites.

In domain 2, the crosslink of S18 in sections (649-734) and (735-869) is also not surprising for S18 has clearly been shown to belong to domain 2. The S18 crosslinking sites we detect are not necessarily far from each other because in the secondary structure these two sections form a discontinuous double helix between (A655-U672) and (G734-U751). One site obtained by nitrogen mustard crosslinking has been identified by Brimacombe and al (6) at position 845-851 i.e. in section (735-869), the site we detect in section (649-734) is therefore a new site. A site was also obtained in this section with EDC (24).

Protein S21 is crosslinked to section (649-734). This is compatible with two sites which were localized on 693-697 by nitrogen mustard and on 723-724 by iminothiolane (6).

Protein S20 is crosslinked to a large section (214-417) after UV crosslinking and RNase digestion experiments, its site of interaction has been limited to a small region (221-296) probably corresponding to the helix (G240-C286) (28), but to our knowledge has up to now, not been localized.

To summarize the overall results from preliminary screening of 16S RNA by use of contact crosslinking reagents and use of ssDNA probes : most 30S proteins can be crosslinked to 16S RNA by 366nm photoactivation of s^4U , our preliminary localization clearly shows that by this technique we detect sites which were not previously detected by others (here S7 and S9, S4, S18 for example) we do not know if this is due to the specificity of our intrinsic photolabelling probe or to the technique of isolation.

Several proteins (S7, S9, S13) were shown to have multiple crosslinking sites in domains 3 and 4 by s^4U (and EDC) (14). Here we detect also crosslinking of S7 and S9 to an RNA section which spans a portion of domain 2. As previously discussed this multiplicity of sites could reflect multiple conformational states in the 30S particle.

Protein S3 also has multiple sites : it has previously been crosslinked to sections

(868-971) ; (972-1068) and (1134-1281) (13) and here it is crosslinked to section (503-614). These multiple sites are easily explained by recalling that part of sections (503-614) and (868-971) are close together in the 16S RNA secondary structure and also that large parts of section (972-1068) and (1134-1281) (in domain 3) are base paired. Taken together these results predict that a part of domain 3 common to the two latter sections must fold to approach the junction of domain 1 and 2 formed by the two former sections.

The low resolution mapping reported here allows us to make predictions and to direct further investigations to specific protein-RNA complexes likely a priori to yield new information bearing on 30S subunit tertiary structure. For the precise localization of the RNA-protein crosslinking points we use the following method (i) the separation of the RNA and RNA-protein complexes from the DNA probe (ii) the purification of the different complexes from uncrosslinked RNA (iii) the primer extension by reverse transcriptase on purified RNA-protein complexes and visualisation of the pauses which are specific of the presence of the protein. Precise localization of a number of RNA-protein crosslinks is in progress and should help to refine the three dimensional structure of the 30S particle.

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