Reconstitution of biologically active 50S ribosomal subunits with artificial 5S RNA molecules carrying disturbances in the base pairing within the molecular stalk

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ABSTRACT

Bacillus stearothermophilus 50S ribosomal subunits were reconstituted *in vitro* using artificial 5S RNA molecules constructed by combining parts of major and minor type (Raué *et al.* (1976) Europ.J.Biochem. 68, 169-176) *B. licheniformis* 5S RNA. The artificial 5S RNA molecules carry defined disturbances (A.C juxtapositions and extra G.U pairs) in the base pairing between the 5'- and 3'-terminal sequences of the molecule (the molecular stalk region). The biological activity of the reconstituted subunits was determined in an *E. coli* cell-free system programmed with poly-U. The results show that conservation of the base pairing within the molecular stalk is not required for biological activity of 5S RNA. Disturbances of the base pairing within this region do reduce the rate of reconstitution, however. Normal base pairing in the molecular stalk is thus required to ensure efficient ribosome assembly.

INTRODUCTION

In vitro reconstitution experiments on 50S subunits have left no doubt that 5S RNA is vital for biological activity of prokaryotic ribosomes (see Refs. 1 and 2 for reviews). The same most likely is true for their eukaryotic counterparts although this has not been tested. Despite intensive effort, however, the precise role of 5S RNA still remains elusive. The debate centers on the question whether 5S RNA only serves as a scaffold ensuring correct anchoring of a well-defined set of ribosomal proteins (3,4) or whether it also takes part directly in the biological processes carried out by the ribosome, for example in the binding of elongator tRNAs (1).

In vitro reconstitution using modified 5S RNA constitutes a promising approach towards resolving the question of 5S RNA function. So far the main body of experiments of this type consists of studies in which 5S RNA from various sources was incorporated into 50S subunits of either *B. stearothermophilus* or *E. coli* (reviewed in Refs. 1 and 2). The main conclusion from these experiments is that the protein binding sites as well

as the possible functional parts of the 5S RNA molecule are largely conserved in all prokaryotes.

One rigorously conserved feature in prokaryotic 5S RNA is the possibility to form a relatively long uninterrupted double helix by normal base pairing (including G.U pairs) between the 5'- and 3'-terminal sequences of the molecule (5,6). Biochemical (7-9) as well as physical chemical (10,11) studies have demonstrated the actual existence of this double helical region, named the molecular stalk (5), at least in 5S RNA in solution. We have tested the importance of this structural feature for 5S RNA function by preparing artificial 5S RNA molecules in which the base pairing within the molecular stalk was disrupted in a known way. The efficiency of these molecules in *in vitro* reconstitution of biologically active *B. stearothermophilus* 50S subunits was then determined.

MATERIALS AND METHODS

a. *Isolation of B. licheniformis 5S RNA*

5S RNA was prepared by phenol extraction of ribosomes isolated from late-log phase *B. licheniformis* cells (strain S244) according to the procedure described previously (11). The 5S RNA was purified by chromatography on Sephacryl S200 and finally renatured by heating at 65°C for 10 min in 0.05 M Tris-HCl (pH 7.5), 0.02 M $MgCl_2$, 0.2 M NaCl (12) and cooling slowly to room temperature.

b. *Preparation of artificial 5S RNA molecules*

The major and minor type of 5S RNA were separated by preparative gel electrophoresis of total *B. licheniformis* 5S RNA on 15% polyacrylamide slab gels (20x40x0.4 cm) containing 0.04 M Tris-Ac (pH 8.0) and 7 M urea (13). About 10 mg of RNA was run on a single gel. The bands were located by UV-shadowing (14) and the RNA was eluted by shaking the crushed gel overnight at 40°C in 1.0 M NaCl containing 0.1% SDS. Purified major and minor type 5S RNA were treated with RNAase T1 under conditions where the molecule is cut principally next to residue G39 (15). The resulting fragments, called 5'-half (residues 1-39) and 3'-half (residues 40-116), were isolated by preparative gel electrophoresis under denaturing conditions. Artificial 5S RNA molecules were constructed by annealing the appropriate 5'- and 3'-halves for 10 min at 65°C in 0.05 M Tris-HCl (pH 7.5), 0.02 M $MgCl_2$, 0.2 M NaCl and cooling slowly to room temperature. The reannealed material was purified by Sephacryl S200 chromatography in 0.01 M Tris-HCl (pH 7.0), 0.01 M $MgCl_2$, 0.2 M NaCl.

c. *Preparation of B. stearothermophilus 50S ribosomal subunits*

B. stearothermophilus strain 795 was grown as previously described (16) and the 50S ribosomal subunits isolated as reported (17).

d. *Reconstitution of 50S subunits and assay for biological activity*

B. stearothermophilus 50S ribosomal subunits were reconstituted for 4 hrs at 60°C under conditions previously described (16,18). The biological activities were determined in a poly (U)-dependent polyphenylalanine synthesizing system (17,18), in which control(undissociated)50S subunits were able to polymerize 45-50 phenylalanines per 50S subunit per 30 min.

RESULTS AND DISCUSSION

B. licheniformis cells contain two types of 5S RNA in a ratio of about 5:1 which differ in sequence at eight positions (19). Six of these differences are located in the molecular stalk, the double-helical region formed by base pairing between the 5'- and 3'-ends of the molecule. Base pairing is conserved, however, since in all cases both members of the affected base pair have undergone transition (Fig. 1).

The two types of 5S RNA, called major and minor respectively (19), can be separated by electrophoresis on gels containing urea (13). Both major and minor 5S RNA can be cut preferentially at residue G39 by RNAase T1 (19). The resulting "halves" can be purified by gel electrophoresis under denaturing conditions. Reannealing of the 5'-half (residue 1-39) of the major type to the 3'-half (residue 40-116) of the minor type or vice-versa produces artificial 5S RNA molecules carrying well-defined disturbances of the normal base pairing within the molecular stalk (Fig. 1). Base pairing according to the Fox and Woese model (5) elsewhere in the constructs is not affected.

Fig. 2 shows the results of an experiment in which the artificially constructed 5S RNAs, as well as intact B. stearothermophilus and B. licheniformis 5S RNA, were used in in vitro reconstitution of B. stearothermophilus 50S subunits. It is clear that intact B. licheniformis 5S RNA (panel B) supports reconstitution just as efficiently as does homologous B. stearothermophilus 5S RNA (panel A). The same is true for the major 5'/major 3' construct (panel C) which, apart from a cut between residues 39 and 40 is identical to intact major B. licheniformis 5S RNA. Obviously, this discontinuity in the phosphodiester backbone is not detrimental to the biological function of the molecule. This finding is not entirely surprising in view of the observation that the 5S RNA maturation enzyme RNAase M5 recognizes the major 5'/major 3' construct just as efficiently as the intact 5S RNA molecule (20). The conformations of the two molecules must thus closely resemble each other. Furthermore, E. coli 5S RNA kethoxalated at G41, which is equivalent to G39 in Bacillus 5S RNA, functions

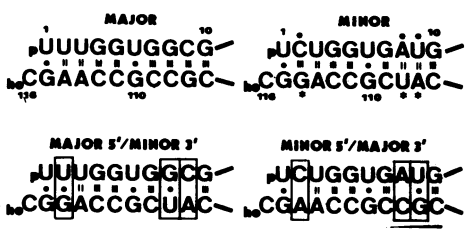

Fig. 1. Structure of the molecular stalk region of major and minor type B. licheniformis 5S RNA and the two heterologous constructs. Asterisks identify sequence differences between major and minor 5S RNA Abnormal juxtapositions in the constructs are boxed.

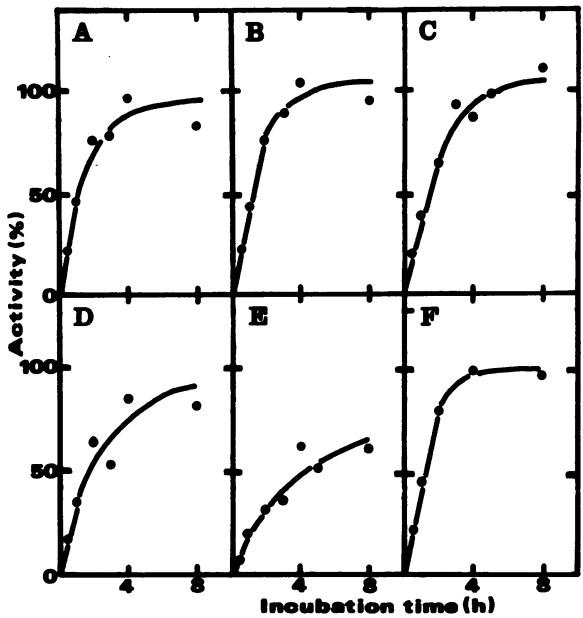

Fig. 2. Time course of reconstitution using intact B. stearothermophilus (panel A) or B. licheniformis (panel B) 5S RNA; the major 5'/major 3' (panel C), major 5'/minor 3' (panel D) or minor 5'/major 3' (panel E) constructs and an unannealed mixture of the major 5' and major 3' "halves" in a molar ratio of one to one (panel F). In all cases the molar ratio of "5S RNA" to 23S RNA was one. The value of 100% (7 phenylalanines polymerized per 50S subunit per 30 min) corresponds to the activity of the ribosomal subunits reconstituted with B. stearothermophilus 5S RNA. Each point is the mean of three independent determinations.

normally in in vitro reconstitution of 50S subunits (21). The two "half molecules" of major 5S RNA may even be added to the in vitro reconstitution mixture without prior reannealing (Fig. 2, panel F). This alters neither the time course of the reconstitution nor the final activity reached. When only one of the "halves" is included in the mixture, however, no biologically active subunits are formed (data not shown).

Panels D and E in Fig. 2 depict the behavior of the two constructs in which normal base pairing in the molecular stalk is disrupted. Clearly the presence of an A.C juxtaposition at positions 9/107 and extra G.U pairs at positions 2/114 and 8/108, as occurring in the major 5'/minor 3' construct (Fig. 1), has very little effect on the ability of the molecule to support the formation of biologically active subunits (Fig. 2, panel D). The final

activity reached is almost identical to that obtained in the controls (see also Table I). This shows the construct to be as active as normal intact 5S RNA once it is incorporated. Reconstitution proceeds at a slightly lower rate though, which can be plausibly explained by a somewhat decreased affinity of the construct for the 5S RNA binding proteins. Previous studies have led to the conclusion that the molecular stalk constitutes part of the binding site for at least one of these proteins (7,22,23). Alternatively the rate of assembly of the 5S RNA·protein complex into the 50S subunit might be reduced, for instance because of an altered conformation of this complex. The minor 5'/major 3' construct contains two A·C juxtapositions as well as an extra G·U pair in the molecular stalk (Fig. 1). Although the resulting destabilization of the base pairing has a significant effect on reconstitution (Fig. 2, panel E), still a biological activity of about 70% of that obtained in the controls is reached after eight hours incubation. Since even at that time the reconstitution has not reached a plateau the biological activity of the minor 5'/major 3' construct once it is incorporated is likely to be close to that of normal 5S RNA. The experiment shown in Table I demonstrates that such is indeed the case. By increasing the amount of the construct added to the reconstitution mixture the total biological activity can be raised to a value equal to the maximum obtained with intact 5S RNA. Consequently the minor 5'/major 3' construct once it is incorporated into the ribosomal subunit must function normally. The reduced activity observed in the experiment in Fig. 2 (panel E) has to be due to a reduction in the rate of reconstitution which can be overcome by increasing the concentration of the construct.
The reason for the decrease in reconstitution observed upon increasing the concentration of intact 5S RNA or the other two constructs (Table I) is not immediately clear. One possible explanation is that the excess RNA causes the

Table I. Effect of the concentration of 5S RNA on the extent of reconstitution of biologically active 50S subunits. Activity is expressed as in Fig. 2. Each value is the mean of two independent determinations.

	% Activity			
5S/23S mol.ratio	B. stearo 5S	major 5'/major 3'	major 5'/minor 3'	minor 5'/major 3'
1	100	110	98	59
3	91	102	76	90
6	70	83	86	116

formation of incomplete, tight, 5S RNA-protein complexes. A lower affinity of the minor 5'/major 3' construct for the 5S RNA binding proteins would still allow exchange of protein between incomplete complexes.

In conclusion we would like to state that the strong conservation of base pairing in the molecular stalk of prokaryotic 5S RNA is not required for the biological function of the molecule once it is incorporated into the 50S ribosomal subunit. It should be kept in mind, however, that biological activity was assayed in the relatively unsophisticated poly-U system. The effect(s) of helix destabilization in the molecular stalk may be more subtle than can be detected with this system, necessitating more detailed studies to bring them to light. In this respect one can think of analysing processes such as initiation and termination of protein synthesis, tRNA binding and fidelity of translation.

Although, with the proviso mentioned above, a disturbed molecular stalk has no effect on ribosome function, it clearly does affect the rate of reconstitution of 50S subunits (Fig. 2). The remaining single sequence alteration present in each of the constructs (position 95 in the major 5'/minor 3' and position 13 in the minor 5'/major 3') can not be responsible for the effects observed. Firstly these residues do not participate in base pairing in the Fox and Woese scheme. Secondly, the specific nucleotides located at these positions in the constructs are present in identical combination in naturally occurring 5S RNA species: the major 5'/minor 3' construct contains A13 and C95 as does B. stearothermophilus 5S RNA at equivalent positions (6); the minor 5'/major 3' construct (G13 + U95) corresponds in this respect to the minor type of 5S RNA from B. subtilis and Bacillus Q (13). Thus, normal base-pairing in the molecular stalk is required to ensure efficient ribosome assembly. This by itself would be sufficient to explain the strong conservation of this structural feature.

The absence of any measurable effect of the 5S RNA constructs on ribosome function in the poly-U system does not necessarily mean that in this system the conformation of the molecular stalk is irrelevant. Interaction of the constructs with the 5S RNA binding proteins may well impose a helical character on this region even though A.C juxtapositions are present. Comparative studies on the structure of prokaryotic 16S RNA (24) strongly suggest the frequent occurrence of non-Watson-Crick juxtapositions (in this case G.A and A.G) in helical regions.

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