THE REACTION OF 5S RNA in 70S RIBOSOMES WITH KETHOXAL

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1. Introduction

The reaction of 5S RNA in 70S ribsomes with kethoxal (β-ethoxy-α-ketobutyraldehyde), which is specific for single stranded guanine residues [1], and the functional capacity of reconstituted ribosomes containing modified 5S RNA has been studied. Two nucleotides react: G_{41} and G_{13}. Residues G_{44} and G_{107} that are in 5S RNA sequences complementary to TΨC of tRNAs are not reactive. Reconstituted ribosomes containing 5S RNA modified at residues G_{41} and G_{13} are fully active in poly U directed polyphenylalanine synthesis. These results are discussed in terms of the possible requirement for a protein synthesis initiation complex to expose the 5S RNA sequence complementary to TΨC.

2. Materials and methods

Kethoxal was a gift from Dr Gerald Underwood of the Upjohn Company. [^{3}H] kethoxal of specific activity of 8.9 mCi/mmole was from New England Nuclear Co. Solutions for ribosome isolation were as follows: Solution 1 (10 mM Tris–HCl, pH 7.8, 10 mM $MgCl_2$, 30 mM NH_4Cl, 6 mM β-mercaptoethanol). For ribosome reactions with kethoxal and subsequent procedures, Solution 1A was 10 mM Tris–borate, pH 7.8, 10 mM $Mg(OAc)_2$, 30 mM NH_4Cl. ^{32}P-labeled ribosomes were prepared from *E. coli* strain MRE 600 Str^R RNAse I- (from Dr Julian Davies). Cells were grown in low phosphate medium and labeled for 4 generations with $H_3\,^{32}PO_4$ (100 μCi/ml. 70S ribosomes were prepared according to Traub et al. [2] from 0.1 g of ^{32}P labeled cells after adding 2 g of unlabeled MRE 600 cells (from Grain Processing Corp.). rRNAs were purified by extraction with phenol equilibrated with Solution 1A and precipitated twice with ethanol. Prior to phenol extraction, reacted ribosomes were centrifuged through 30% sucrose in Solution 1A to remove unbound kethoxal.

For nucleotide sequence studies, rRNAs were resuspended in 0.01 M Tris–borate, pH 7.5, 0.05 M potassium acetate followed by the addition of solid potassium acetate to a final concentration of 3 M. After 24-48 hr at 0°C, high mol. wt RNAs were removed by centrifugation at 10 000 rev/min for 15 min; the supernatant RNA, containing mostly 5S RNA, was precipitated with ethanol. Final purification of ^{32}P labeled 5S RNA was by electrophoresis on a 10% acrylamide slab gel [3].

For reconstruction studies, 5S RNA was purified by Sephadex G-100 chromatography. Electrophoretic separations of ribosomes was by a modification of the vertical method of Dahlberg et al. [4] using cylindrical gels of 2% acrylamide plus 0.5% agarose prepared according to Peacock and Dingman [5]. Fingerprinting was essentially according to Brownlee et al. [6]. Individual spots were identified by comparison with published patterns and by their pancreatic RNase digestion products which were separated by electrophoresis on DE-81 paper at pH 3.5 [7]. Reconstitution of 50S *B. stearothermophilus* subunits with 5S RNA and poly U directed polyphenylalanine synthesis were according to previously published reports [8].

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3. Results

70S ribosomes were reacted with kethoxal and analyzed by composite agarose-acrylamide gel electrophoresis (fig.1). No significant dissociation to subunits was observed and no significant aggregation occurred with 50 μmol/ml kethoxal (fig.1B). However, with higher concertrations of kethoxal (500 μmol/ml) aggregates are formed with approximately 66% of the ribosomes (fig.1C). These results were confirmed by sedimentation analysis of reacted ribosomes in the analytical ultracentrifuge.

For nucleotide sequence analysis, 5S RNAs from reacted (using 500 μmol/ml kethoxal), and unreacted ribosomes were isolated, digested to completion with T_1 RNase and the resulting oligonucleotides separated by the Brownlee and Sanger two-dimentional technique (fig.2). Of the 19 major T_1 RNase derived spots observed in fingerprints of unreacted 5S RNA [6], all were present in the fingerprints of reacted material

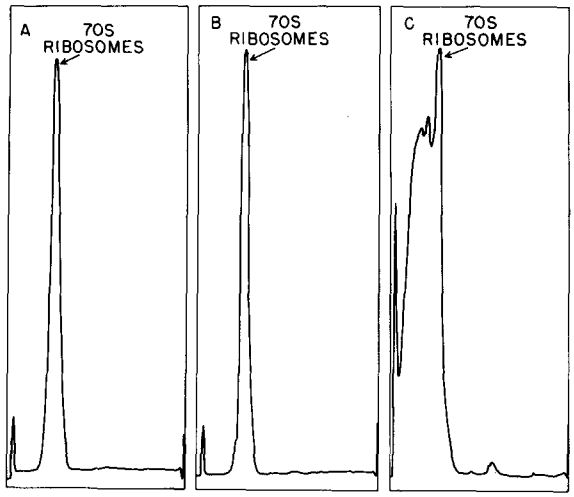

Fig.1. Composite agarose-acrylamide gel electrophoresis of unreacted and kethoxal reacted ribosomes. Electrophoresis was with Solution 1A. Electrophoresis was from left to right. (A) Unreacted 70S ribosomes; (B) 70S ribosomes reacted with 50 μmol/ml kethoxal; (C) 70S ribosomes reacted with 500 μmol/ml kethoxal.

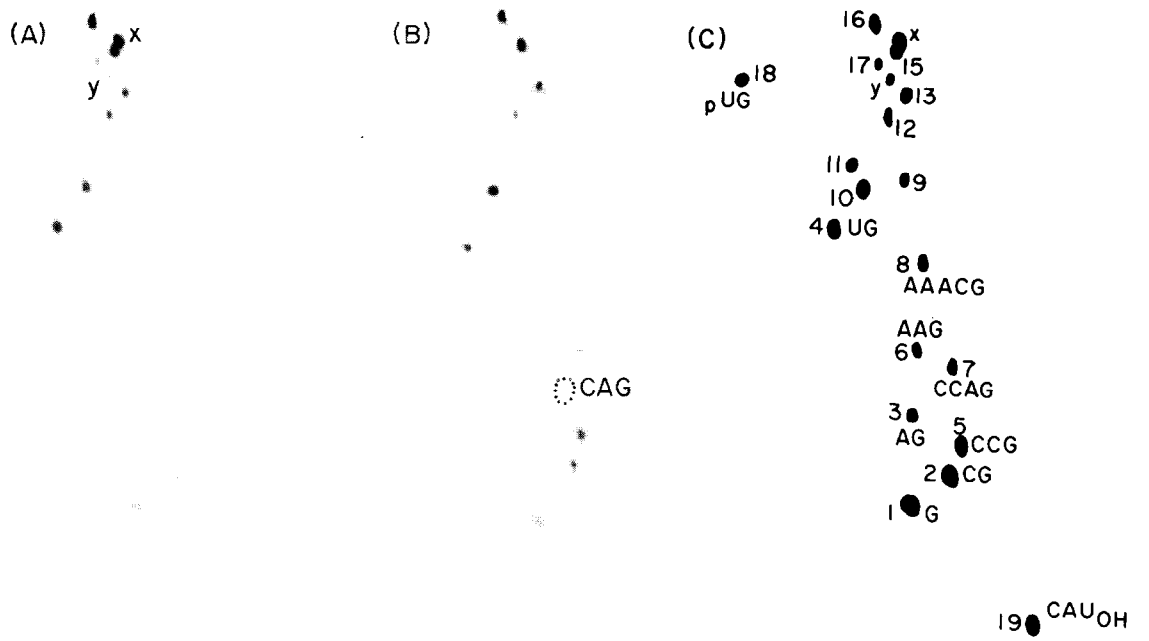

Fig.2. Fingerprints of 5S RNA. (A) 5S RNA was from 70S reacted ribosomes. The reaction volume in solution 1A was 0.23 ml and contained 50 A_{260} ribosomes, [^{3}H]kethoxal (10 μmol), unlabeled kethoxal (94 μmol). [^{3}H]kethoxal was added at the initial time of reaction and after 1 h unlabeled kethoxal was added; total reaction time was 2 h at 37°C. 5S RNA was isolated, hydrolyzed with T_1 RNase and the resulting oligonucleotides were separated by two-dimentsional electrophoresis [18]; (B) oligonucleotide separation of 5S RNA fragments from unreacted 70S ribosomes; (C) map of the fragment spots from kethoxal reacted 5S RNA. Spot identification and numbering system according to Brownlee et al. [18].

except spot 14, which was completely absent. Two new spots (X and Y) were observed in patterns from reacted material. Similar patterns were obtained using 50 μmol/ml kethoxal. Spots X and Y, as well as all 19 spots from the unreacted fingerprint were eluted with 30% triethylammonium carbonate pH 9.5 and lyophilyzed to dryness from H_2O. During elution, modified G residues are unblocked and become sensitive to further digestion with T_1 [9]. Each eluted spot was resuspended in 10 μl of 0.01 M Tris–HCl, pH 7.5; 0.001 M EDTA containing 2 mg/ml carrier RNA and 0.1 mg/ml T_1. After incubation for 30 min at 37°C, the digests were spotted on DE-81 paper and then electrophoresed in one dimension in 7% formic acid. As expected, each of the unreacted 5S RNA spots gave only the original spot on further incubation with T_1. Spots X and Y each gave rise to two additional spots plus some residual blocked material. Both X and Y yielded CCG (number 5), while X also gave ACCCCAUG (number 14) and Y also gave UAG (number 10).

From the sequence of 5S RNA [6,7], spot X could arise only from modification of G_{41} to yield ACCCCAUG^{k}CCG, while modification of either G_{13} or G_{64} could yield spot Y (CCG^{k}UAG). In the fingerprint of unreacted 5S RNA (Fig.2B) there is a minor amount of CAG present, corresponding to a change of C to A at position 12 in a small fraction of the population [10]. This minor spot was absent in the fingerprints of reacted material indicating that it is G_{13} and not G_{64} that was modified.

Spots 12 and 13 in reacted 5S RNA were found in the expected molar yield, (related to unreacted 5S RNA) and no new spots except X and Y were detected. Reaction of G_{44} would produce a decrease in spot 13 while reaction of G_{107} would decrease the yield of spot 12. Thus, our data indicate no significant reaction of 5S RNA nucleotides G_{44} or G_{107} in 70S ribosomes.

Having found that G_{41} of 5S RNA had been modified in 70S ribosomes, it was of interest to determine whether or not this modification would influence the biological activity in reconstituted 50S ribosomal subunits. Therefore, reconstitutions were carried out for 30, 60, and 90 min with *B. stearothermophilus* 5S RNA, *E. coli* 5S RNA, and *E. coli* 5S RNA which had reacted with 1.3 mol kethoxal per mole 5S RNA (in 70 ribosomes) and analyzed as follows: (1) in a poly U directed polyphenylalanine synthesizing system; (2)

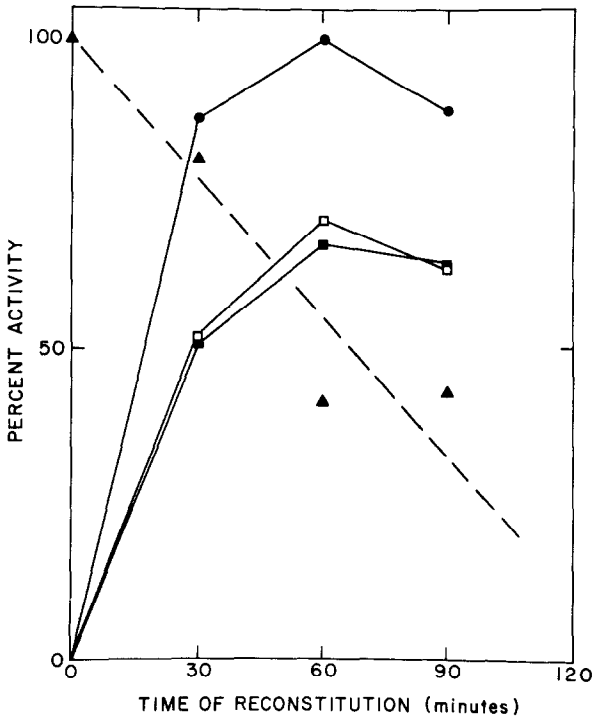

Fig.3. Poly U directed polyphenylalanine synthesis of *B. stearothermophilus* 50S ribosomal subunits reconstituted with *B. stearothermophilus* 5S RNA (●——●) *E. coli* 5S RNA (□——□) and kethoxal reacted *E. coli* 5S RNA (■——■). Activity (100%) corresponds to 3000 cpm [^{14}C]phenylalanine. In the poly U assay 1.0 A_{260} units 50S ribosomes were incubated with 0.6 A_{260} units 30S subunits. Native ribosomes yielded 9400 cpm. Reconstitution of *B* stearothermophilus 50S ribosomal subunits with various 5S RNAs and the poly U assay were carried out as previously described [20]. To determine the activity of [^{3}H] kethoxal remaining bound to the 5S RNA during reconstitution (▲——▲] at 60°C, 1.0 A_{260} unit reconstituted 50S ribosomes (150 μl) were precipitated with 1 ml ice cold 10% TCA and filtered on Whatman glass fiber filters. Activity (100%) corresponds to 195 cpm ^{3}H label retained on the filter.

by TCA precipitation to determine the amount of kethoxal remaining covalently attached to the 5S RNA during reconstitution. Fig.3 shows that the half life of the 5S RNA bound kethoxal is approx. 60 min under these conditions. However, the activity of the modified 5S RNA is similar to a nonmodified control and does not change relative to the controls with increased time of incubation, suggesting that G_{41} is not crucial for biological activity.

4. Discussion

With high concentrations of kethoxal, aggregates of 70S ribosomes form, but we think it unlikely that aggregation alters the 5S RNA reaction sites. At a lower kethoxal concentration, a minor fraction of 70S particles aggregate, and a similar oligonucleotide fingerprints pattern was obtained compared to patterns at higher concentrations.

Previous studies showed that 5S RNA in 70S ribosome has 1–2 exposed guanine residues [11]. The present studies show that neither G_{44} or G_{107} are available for chemical modification in 70S particles but that two residues, G_{41} and G_{13}, do react. Free 5S RNA and 5S RNA in 50S subunits also have G_{41} and G_{13} exposed [9,12]. It is of considerable interest that with expected 5S RNA conformational changes to occur when this RNA is packaged into a completed ribosome, and ribosomal structural changes that occur with the joining of the 50S and 30S subunits to form the 70S ribosome [13], that the identical base residues react with kethoxal.

Assuming chemical modification measures all exposed guanines, two interpretations of these results can be made. 5S RNA is not part of the common binding site for tRNAs, but 23S RNA fulfills that function; or, there are structural changes in ribosomes during protein synthesis which expose G_{44}. In view of the evidence supporting 5S RNA as the binding site for TΨC [14–16], we think it likely that the latter interpretation is correct. The region about G_{44} is the most probable region for tRNA binding. G_{41} is highly reactive and only a small change in structure should be necessary to expose G_{44}. Since other bacterial 5S RNAs do not have a similar AAG sequence around postition 110 [17], but are nevertheless active in reconstituted *B. stearothermophilus* 50S ribosomal subunits [18], it would appear that only the common sequence around position 44 could possibly be important for ribosomal 5S RNA–TΨC interaction. Also, nucleotides 70–110 are involved in specific protein recognition sites for ribosomal proteins that bind directly to 5S RNA [19].

Ribosomes reconstituted with modified 5S RNA isolated from reacted 70S ribosomes were as active as control samples in polyphenylalanine synthesis. This is an indication that G_{41} is not especially crucial to the functioning of ribosomes in the in vitro poly U directed reaction, which is in agreement with unpublished data cited in [9]. At the most, kethoxylated G_{41} may produce some mild structural changes in the region close to $A_{46}AG_{44}$ which apparently are not of any great significance to the functioning of ribosomes.

From chemical modification studies, it appears that TΨC is not exposed in tRNAs [20]. Schwarz et al. [21] have presented a model for the exposure of TΨC with the codon–anticodon interaction of tRNA and messenger RNA. Similarly, we suggest that the 5S RNA sequence AAG_{44} is exposed in ribosomes during the elongation process in protein synthesis. It is possible that AAG_{44} is exposed only after the initiation complex is formed with the initiation codon of messenger RNA, f-met-tRNA and 30S subunits, and that the conformational change in 5S exposing AAG_{44} is produced after the binding of 50S subunits to this complex. More generally, we feel that the AAG_{44} sequence of 5S RNA becomes exposed when the ribosomal P site is occupied in the 70S ribosome. Work is in progress in an attempt to determine whether G_{44} is exposed in 5S RNA with ribosomes complexed in protein synthesis and in the initiation process.

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