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**Fluorescence modification of Escherichia coli 5S RNA**

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ABSTRACT

Reaction of 5S RNA with chloroacetaldehyde leads to the conversion of unpaired adenines to the fluorescent 1,N<sup>6</sup>-etheno-adenine derivatives. Up to 16 of the 23 adenines in free 5S RNA can be modified, the fastest reacting are A<sub>29</sub>, A<sub>34</sub>, A<sub>39</sub> and A<sub>57-59</sub>. Partial modification of adenines in this area results in a 20% reduction in the efficiency of 5S RNA incorporation into 50S subunits during reconstitution and a 15% reduction in the activity of these subunits in peptide synthesis. Fluorescence from 1,N<sup>6</sup>-etheno-adenine is quenched in free 5S RNA and is not detectably further influenced by the binding of proteins E-L5, E-L18 and E-L25, nor by the first stage of the two step *E. coli* 50S subunit reconstitution procedure. However, the fluorescence is further reduced to near zero after the second step of the reconstitution. Thus, 5S RNA free in solution contains 16 unpaired adenines, those in the region between A<sub>29</sub> and A<sub>59</sub> being particularly accessible to modification by chloroacetaldehyde. This portion of the 5S RNA molecule appears to undergo either a conformational change or interacts with other ribosomal components in the last stage of subunit reassembly.

INTRODUCTION

In the course of our study of 5S RNA structure and function (1,2) we have become interested in applying fluorescent probes to answer specific questions, particularly in order to measure distances between 5S RNA and other components in the ribosome using energy transfer measurements.

As a first attempt in this field we have chosen to study the fluorescent adenine derivatives produced by reaction of chloroacetaldehyde with unpaired adenines (3-5). The derivative etheno-adenine shows a fluorescence emission at 415nm when excited at 300nm and thus represents a potentially useful donor in energy transfer experiments to suitable chromophores attached to other sites in the ribosome or to components required for protein synthesis. For example, tRNA<sup>Phe</sup> in which 1-aminoanthracene

replaces the Y-base and which has excitation and emission maxima of 395 and 486nm respectively (6).

Additionally, chemical modification of RNA has proved to be a useful technique for studying structure and function (7-11) so that determining the sites of modification and their effect on function could be expected to yield valuable information.

In this paper we describe conditions for modification of 5S RNA with chloroacetaldehyde, details of the effects of modification on function, the results of preliminary sequence analyses and the first fluorescence measurements.

#### MATERIALS AND METHODS

Unless otherwise indicated, all chemicals were from Merck, Darmstadt, biochemical reagents, nucleotides, tRNA and enzymes were from Boehringer, except T1 ribonuclease which was from Sankyo, Japan. Chloroacetaldehyde, prepared according to Secrist *et al* (4), was a kind gift from Dr. H. Wombacher (Freie Universität Berlin).

#### Ribosomes and 5S RNA

E. coli A 19 and Bacillus stearothermophilus 799 cells were grown (12), 70S ribosomes and 50S and 30S subunits were isolated as described (13).

Ribosomal proteins from E. coli and Bacillus stearothermophilus were extracted as published (13). Specific 5S RNA binding proteins were isolated by affinity chromatography essentially as reported (14). 5S RNA was prepared by phenol extraction or urea/LiCl treatment of ribosomes followed by Sephadex G-100 gel filtration to separate 5S RNA from the larger RNA molecules (15). For the sequencing experiments, the RNA was further purified by preparative gel electrophoresis on 20% polyacrylamide slab gels in 7M urea. After localization of the 5S RNA band through UV-shadowing, the gel was excised, crushed into buffer (10mM Tris-HCl, pH 7.8, 1% SDS) and incubated with an equal volume of phenol for 4 hours at 4°C. The aqueous phase was recovered by centrifugation and the 5S RNA precipitated with ethanol at -30°C for several hours. After repeated ethanol washes, the 5S RNA pellet was resuspended in water, remaining ethanol removed by vacuum and the 5S RNA stored at -80°C.

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### Modification with chloroacetaldehyde

5S RNA was added to solutions of chloroacetaldehyde adjusted to pH 5.0 with 2% Na-bicarbonate solution or with 20mM Na-acetate buffer, pH 5.0.

Concentrations of chloroacetaldehyde ranged from 40mM to 5M, 5S RNA concentration remained constant at 4.0 $\mu$ M. Incubation was carried out at 25°C. After incubation, chloroacetaldehyde was removed by dialysis (3 x 1:1500 at 4°C), by gel filtration on Sephadex G50 or by repeated precipitation of the RNA with ethanol.

After removal of chloroacetaldehyde the 5S RNA was examined on 10% polyacrylamide gels in 7M urea and found to be intact. The extent of the modification was established by comparison of fluorescence intensity with a standard solution of etheno-AMP after digestion of the modified 5S RNA with phosphodiesterase for 24 hours at a ratio of 1:100, enzyme:substrate. Fluorescence was measured in an Aminco-Bowman spectrofluorimeter at room temperature with the excitation monochromator set at 300nm and sweeping the emission wavelength from 325nm to 600nm, 2mm slits were used. The absorption of the samples at 260nm was measured to establish the concentration of RNA (19.18 A<sub>260</sub>/mg, MW 40.778 (16)). Comparison of the concentration of etheno-adenine determined by fluorescence with the concentration of the 5S RNA yields the statistical ratio of modification.

Reconstitution of Bacillus stearothermophilus and E. coli 50S subunits was carried out as previously described (12,13,17). The activity of the subunits in poly(U)-dependent synthesis of poly-phenylalanine was assayed as described (13,18). The incorporation efficiency of modified 5S RNA into 50S ribosomal subunits was determined using uniformly labelled 5S RNA prepared as described (19), Uniformly <sup>32</sup>P-labelled 5S RNA (specific activity: 12.5 $\mu$ Ci/ $\mu$ mole) was modified as above and samples with increasing numbers of modified adenines per molecule were reconstituted into 20 A<sub>260</sub> units of 50S subunits. The reconstituted subunits were separated from excess protein and RNA by centrifugation on 10%-30% sucrose gradients (Spinco, SW 27). The gradients were fractionated and the absorbance at 260nm and the radioactivity of each fraction measured (13). The ability of the subunits to support the

synthesis of polyphenylalanine was measured in the poly(U)-directed system, as above.

### RNA Sequencing

E. coli 5S RNA was modified to various extents with chloroacetaldehyde and digested with T1 ribonuclease at an enzyme to substrate ratio of 1:10 at 37°C. 5µg of the fragments were labelled at the 5' end with <sup>32</sup>P-phosphate. The reaction mixture of 10µl contained 10µCi (8-<sup>32</sup>P)-ATP at 2000 Ci/nmole, 1-2 units T4-poly nucleotide kinase, 70mM Tris-HCl pH 7.6, 10mM MgCl<sub>2</sub>, 5mM DTE. After incubation for 30 mins at 37°C, glucose to 2mM and 2m units yeast hexokinase were added, incubation was continued for 15 minutes followed by two incubations each with the addition of 3nmoles of ATP, each for 15 minutes. After this last reaction to remove excess (<sup>32</sup>P)-ATP, an equal volume of 0.3% xylene cyanole blue was added and then aliquots of 1-3µl loaded into PEI-cellulose plates (Machrey-Nagel Polygram CEL 300 PEI). The fragments were resolved by chromatography in two dimensions as described (20). The fragments were located by autoradiography on Kodak XR-5 film at room temperature for 1-2 hours. The fragment locations were then marked onto the plates, cut out and the radioactivity measured in a Beckman LS-8000 scintillation counter.

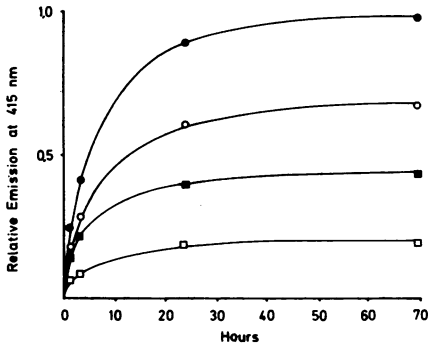
### Fluorescence measurements

Fluorescence measurements at the various stages of the two-step E. coli 50S reconstitution procedure (17) were made with the steady state photon-counting fluorimeter described by Odom *et al.* (6). This instrument uses a xenon light source with a double monochromator (SLM Instruments, Urbana, IL). The effective intensity of this light source is low at 300 nm. For this reason samples were excited at 330nm. Emission was measured at 430nm. Four 1-minute measurements of fluorescence were made for each sample; these were averaged. Appropriate blanks containing all components except etheno-adenine 5S RNA were subtracted in each case.

## RESULTS

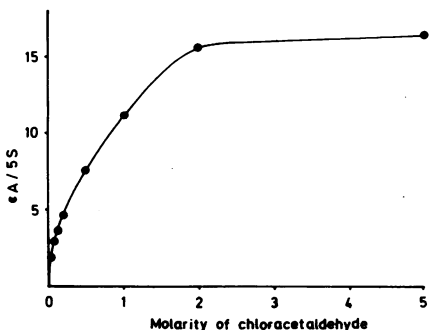
### Modification of 5S RNA

The extent of modification was determined by comparison of the fluorescence intensity at 415nm with a standard solution of etheno-AMP. The fluorescence of etheno-adenine in 5S RNA is so



**Figure 1.** Increase in fluorescence during phosphodiesterase digestion of *E. coli* 5S RNA containing 14 (●), 9 (○), 6 (■) and 3 (□) modified adenines per molecule.

severely quenched that it was necessary to digest the sample with phosphodiesterase before the fluorescence was measured. Figure 1 shows the increase in fluorescence upon incubation with the enzyme. Since fluorescence and concentration are linearly proportional in the range under consideration (data not shown) and the concentration of RNA could be determined by absorbance at 260nm, the number of modified adenines per molecule 5S RNA could be determined. The effect of reagent concentration and incubation time on the extent of 5S RNA modification was examined. Figure 2 shows the results of incubating 5S RNA with increasing concentrations of reagent. A plateau is reached at 16 modified adenines per molecule. This observation agrees well with a previous chemical modification study in which 16 adenines could be modified with monoperphthalic acid (7). The time dependence of modification of  $4.0\mu\text{M}$  5S RNA with 40mM chloroacetaldehyde was found to be linear for 24 hours (data not shown). One modified adenine per molecule



**Figure 2.** Number of modified adenines in 5S RNA as a function of chloroacetaldehyde concentration. Incubation was at 25°C (24 hrs) with a constant 5S RNA concentration ( $4.0\mu\text{M}$ ).

is achieved after 12.5 hours under these conditions.

#### Reconstitution experiments with modified 5S RNA

5S RNA uniformly labelled with  $^{32}\text{P}$ -phosphate was modified to various levels with chloroacetaldehyde, and the recovery of counts in reconstituted *Bacillus stearothermophilus* 50S subunits used to establish the extent of incorporation after 90 minutes incubation at  $60^\circ\text{C}$ . The reconstituted 50S subunits were also tested in the poly(U) assay. From the results summarized in Figure 3 it is apparent that one and two modified adenines per 5S RNA lead to a reduced incorporation of the RNA into the ribosomal subunit and therefore to a corresponding reduction in biological activity. Further modification of the 5S RNA does not influence its incorporation into the ribosomal subunit but its biological activity.

Figure 4 shows a fingerprint autoradiogram of 5' labelled T1 fragments of *E. coli* 5S RNA, the oligonucleotides were given Brownlee assignments (21) after identification of their 5' nucleotides by PEI-thin-layer-chromatography of complete T2 digests. Since the efficiency of labelling with  $^{32}\text{P}$ -phosphate through T4 polynucleotide kinase is dependent upon the length of an oligonucleotide, and the nature of its 5' terminal nucleotide, the recovered counts are not always proportional to the amount of fragment expected. Table 1 shows the molar yields for control 5S RNA T1 fragments. As can be seen, most fragments are found in the amounts expected, except for the longer oligonucleotides, which are less efficiently labelled. However, as the standard deviations show, these relative yields remain constant over many fingerprints, so that comparisons within a series may be made in order to detect reductions in the levels of fragments from, for example, 5S RNA molecules modified to increasing levels with chloroacetaldehyde.

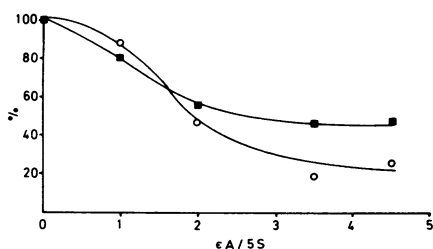
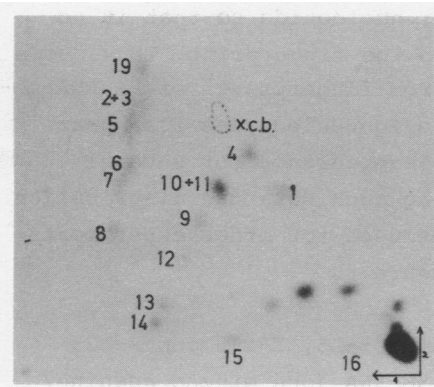


Figure 3. Level of incorporation into reconstituted 50S subunits (■) and the activity in protein synthesis (○) of modified 5S RNAs. 100 % activity represents the performance of untreated 5S RNA and corresponds to a polymerization of 4.8 mol Phe/mol reconstituted ribosome. Native ribosomes polymerize 16.5 mol phenylalanine under otherwise identical conditions.



**Figure 4.** PEI-thin layer fingerprint of  $^{32}\text{P}$ -5' labelled T1 RNase fragments of *E. coli* 5S RNA. The spots are numbered according to Brownlee (21). X.C.B. is the xylene cyanole blue dye, glucose-6- $^{32}\text{P}$  remains at the start point in the lower right hand corner.

Since etheno-adenine has different mobility properties compared to adenine itself (3), and since the fingerprint method used is based upon the differential mobilities of nucleotides, it may be reasonably assumed that decreasing yields of an oligonucleotide re-

**TABLE 1**

Yield of 5'-labelled T1 RNase Oligonucleotides from *E.coli* 5S RNA

Spot No.	Sequence	Expected relative yield	Observed relative yield
1	G	11	11.4
2+3	CG,AG	7	6*
4	UG	4	4.2
5	CCG	3	3.1
6	AAG	1	1.4
7	CCAG	1	1.1
8	AAACG	1	1.3
9	CCUG	1	1.0§
10+11	UAG,AUG	4	4
12	AACUG	1	0.7
13	AACUCAG	1	0.5
14	ACCCCAUG	1	0.6
15	UCCACCUG	1	0.7
16	UCUCCCAUG	1	0.8
17	-	-&	-
18	pUG	1	0#
19	CAU <sub>OH</sub>	1	4.1•

\* Std. deviation 14% over 14 fingerprints.

§ T9 taken as 1.0 for the calculation of relative yields.

& T17 is not present in 5S RNA from *E. coli* A19.

# 5S RNA used was not dephosphorylated, so that the 5' terminal fragment could not be labelled.

• 3' end deviation was 11% over 14 fingerprints.

flect increasing modification of that nucleotide so that it no longer runs to its normal position in the fingerprint. Thus, some oligonucleotides disappear rapidly from fingerprints of 5S RNA modified to increasing extents, other oligonucleotides disappear slowly or not at all. The rates of disappearance for several fragments are shown in Figure 5. Using such data the distribution of the modification in several probes used for other experiments may be determined and is shown in Figure 6.

Fluorescence studies

The specific 5S RNA binding proteins E-L5, E-L18 and E-L25 from *E. coli* were added to modified 5S RNA (one etheno-adenine/RNA molecule) without significant change in fluorescent intensity (Table 2). Similarly, the fluorescent 5S RNA was incorporated into *E. coli* 50S subunits as described, and the fluorescence monitored. Table 2 shows the results of these experiments in which the second step of the reconstitution leads to a threefold reduction in 5S RNA fluorescence. Addition of all components essential for poly(U)-dependent polyphenylalanine synthesis did not bring about a change of the fluorescence from the reconstituted ribosomal subunits (data not shown).

DISCUSSION

We have shown that the chloroacetaldehyde modification reaction is applicable to RNA. Limited modification led to relatively little disturbance of the 5S RNA structure as shown by reconsti-

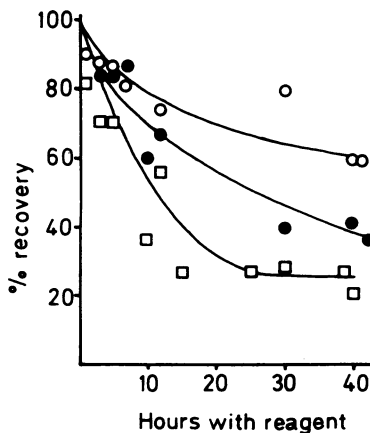
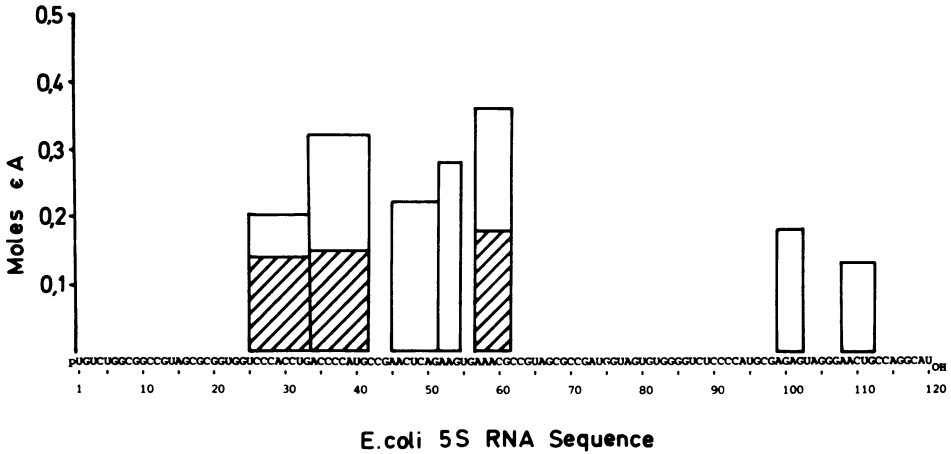


Figure 5. The recovery of three T1 oligonucleotides from fingerprints after increasing modification with chloroacetaldehyde. o-o T12 (AACUG), ●-● T13 (AACUCAG), □-□ T8 (AAACG).





**Figure 6.** The distribution of modification in two samples of chloroacetaldehyde treated 5S RNAs as deduced from the disappearance of T1 oligonucleotides. Shaded areas indicate the oligonucleotides affected by modification to produce one modified adenine per molecule 5S RNA. Unshaded areas are oligonucleotides affected by modification of two etheno-adenines per molecule 5S RNA.

tution experiments. Further modification results in a reduction in 5S RNA incorporation efficiency and in activity in protein synthesis. In the first probe tested (statistically one modified adenine per 5S molecule) incorporation and activity are reduced to the same extent, suggesting that the modification is more likely to disturb the structure and/or ability to interact with other ribosomal components than the function of 5S RNA. When the modification is taken further there is an even greater reduction in activity than in incorporation, suggesting that interactions with other ribosomal components, important for biological function, are impaired. The nature of these interactions is not known.

The fact that a maximum number of 16 adenines per 5S RNA are modifyable by chloroacetaldehyde agrees well with earlier *E. coli* 5S RNA modification studies in which 16 adenines could be modified by monoperphthalic acid (7). Both modification studies agree well with a recently proposed *E. coli* 5S RNA structural model (Pieler and Erdmann, manuscript in preparation, and ref. 22).

Sequencing studies indicate, so far, that the fastest reacting adenines are A<sub>29</sub>, A<sub>34</sub>, A<sub>39</sub> and A<sub>57-59</sub>, this area would therefore appear to be a relatively well accessible and at least partially

**TABLE 2**  
**EFFECT OF RECONSTITUTION ON 5S RNA FLUORESCENCE AND ACTIVITY FOR POLYPHENYLALANINE SYNTHESIS**

	1. Step Free 5S RNA* $\xrightarrow{\text{E-L5, E-L18, E-L25}}$ 5S RNA*-Protein Complex $\xrightarrow{\text{23S RNA and remaining 50S Proteins}}$ Inactive E. coli 50S Subunits $\xrightarrow{\text{Heat activation}}$ Active E. coli 50S Subunits			
Relative Fluorescence: (photon counts/min x 10 <sup>3</sup> )	1.4	1.3	1.6	0.5
Biological Activity of modified 5S RNA: ( <sup>14</sup> C Phe cpm)	0	0	150	960
Biological Activity of unmodified 5S RNA: ( <sup>14</sup> C Phe cpm)	0	0	308	1 877

\* E. coli 5S RNA was modified with chloroacetaldehyde to one etheno-adenine per 5S RNA molecule.

single stranded region. This is also found to be the case from the S1 digestion studies of Pieler and Erdmann cited above. This area between A<sub>29</sub> and A<sub>59</sub> includes the highly conserved CGAA sequence which has been proposed to interact with tRNA (1). Direct evidence for this type of interaction has so far not been presented. Nevertheless, the fluorescence measurements suggest that this region undergoes a final conformational change and/or participates in interaction with other ribosomal components when the ribosome is activated (Table 2).

Since the fluorescence is so highly quenched in the RNA molecule and disappears completely in the active subunit, it is not possible to carry out the sort of energy transfer experiments wished. However, as an agent of chemical modification chloroacetaldehyde would seem to be very interesting. We now intend to examine the modification of 5S RNA in complex with its specific ribosomal binding proteins and in situ in the 50S subunit where it can be expected that certain areas will be shielded and therefore are not available for modification. Similarly, the examination of the modification patterns of other 5S RNA species may yield valuable information on evolution of this molecule.

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