USE OF DIETHYL PYROCARBONATE REACTIVITY AS A PROBE FOR THE TOPOGRAPHY OF 5.8 S rRNA IN YEAST RIBOSOMES

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1. Introduction

Although 5.8 S rRNA was identified as an integral eukaryotic ribosomal component over a decade ago [1–3], little is known about the molecule's role in ribosome structure and function. This RNA, which is ~160 nucleotides long, is hydrogen bonded [1–3] to the high molecular RNA component of the large sub unit (25 S rRNA in yeast). It also appears to interact with ribosomal proteins [4–6], although the nature of the interactions remains to be defined and there is some controversy over the number and type of proteins involved. The nucleotide sequence of many 5.8 S rRNA species have been determined and a variety of studies on the secondary structure of free 5.8 S RNA suggest that the sequence is highly conserved and that a universal secondary structure ('burp gun' model) may be retained (see [7]). Nevertheless, it remains unclear if this structure is retained in the ribosome, if any of the sequences play an active role in protein synthesis, or which nucleotides in the sequence interact with proteins or RNA. Sequences in both the 5′- and 3′-termini may be involved in the 5.8 S–25 S to 28 S rRNA interaction [8,9] and a tRNA-binding function has been postulated [10,11]. Both of these suggestions are tentative and further direct evidence is essential.

Partial ribonuclease digestions have been extensively used to elucidate the secondary structure of RNA molecules including free 5.8 S rRNA (see [7]). This approach, however, usually cannot be used with ribosome-associated RNAs because fragments from the 5.8 S molecule, for example, cannot readily be distinguished from those of the other RNA components. In *Escherichia coli* ribosomes, the topography of RNA components has been explored using various chemical modification techniques [12] or tritium exchange [13]. Significant structural information has been obtained but both approaches require ribonuclease digestion and extensive oligonucleotide analyses to determine the modified residues. Here we have explored the possibility of using a modified version of the rapid chemical degradation and gel sequencing techniques [14], to determine exposed regions in ribosome-associated 5.8 S rRNA. Diethyl pyrocarbonate-reactive sites were determined under buffer and temperature conditions which permit protein synthesis in vitro; the results suggest that portions of the two highly conserved 'GC-rich' and 'AU-rich' stem structures are exposed on the surface of the yeast ribosome or large subunit.

2. Materials and methods

2.1. *Isolation and purification of yeast ribosomes and the 5.8 S rRNA*

Saccharomyces cerevisiae, strain S288C, grown aerobically at 28°C in 0.3% yeast extract, 0.5% Bactopeptone, and 2% glucose were harvested in late log phase. Ribosomes and ribosomal subunits were prepared essentially as in [14]. Briefly, 10 g cells were suspended in 10 ml 10 mM KCl, 5 mM Mg-acetate, 10 mM Tris–HCl (pH 7.5) and 10 mM β-mercaptoethanol. Glass beads (50 g) were added and the cells were disrupted for three 30 s periods in a Braun homogenizer. The cell debris were removed by centrifugation at 27 000 × *g* for 20 min and the ribosomes were then pelleted by centrifugation at 105 000 × *g* for 2 h. Ribosomal subunits were prepared by suspending the ribosomal pellet in 0.8 M KCl, 12 mM Mg-acetate, 50 mM Tris–HCl (pH 7.5) and 10 mM β-mercaptoeth-

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anol (100 A_{260} units/ml) and purified by centrifugation on a 10–30% sucrose gradient in the same buffer. The 60 S subunits were collected by centrifugation at 250 000 × g for 18 h.

RNA was prepared from 60 S subunits by suspending the pellets in 10 vol. 0.3% (w/v) sodium dodecyl sulfate (SDS), 0.14 M NaCl, 0.05 M sodium acetate (pH 5.1) and extracting with an equal volume of phenol solution at room temperature. The 5.8 S rRNA was subsequently purified on an 8% polyacrylamide slab gel [15].

2.2. *Determination of diethyl pyrocarbonate-reactive sites*

Free 5.8 S rRNA, whole ribosomes or 60 S subunits were dissolved in 0.2–12.5 ml (2.0 A_{260} units/ml) of cacodylate buffer containing diethyl pyrocarbonate (50 μl/ml) and incubated at 25°C for 30 min. The reaction was terminated with 1.5 M sodium acetate (0.25 ml/ml reaction mixture), the ribosomal components were precipitated with 2 vol. ethanol, and the RNA was extracted with SDS-phenol.

The extracted RNA was dissolved in 50% formamide, heated for 3 min at 65°C and fractionated on an 8% polyacrylamide slab gel [15]. The purified 5.8 S rRNA (10–20 μg) was labelled at the 3′-end with cytidine 3′,5′-[5′-^{32}P]bisphosphate (Amersham) using RNA ligase (P-L Biochemicals) and repurified on a 12% polyacrylamide sequencing gel as in [16]. The diethyl pyrocarbonate modified residues were chemically degraded using aniline [16]; the samples were dissolved in 10 μl formamide containing 0.03% bromophenol blue and 0.03% xylene cyanol, heated for 3 min at 90°C and loaded on a 12% polyacrylamide sequencing gel [16]. A standard diethyl pyrocarbonate-induced (1 μl diethyl pyrocarbonate for 10 min at 90°C) aniline degraded 5.8 S rRNA sample was loaded on the gel as a chain marker. Half of each sample was loaded initially and the other half 12 h later [7].

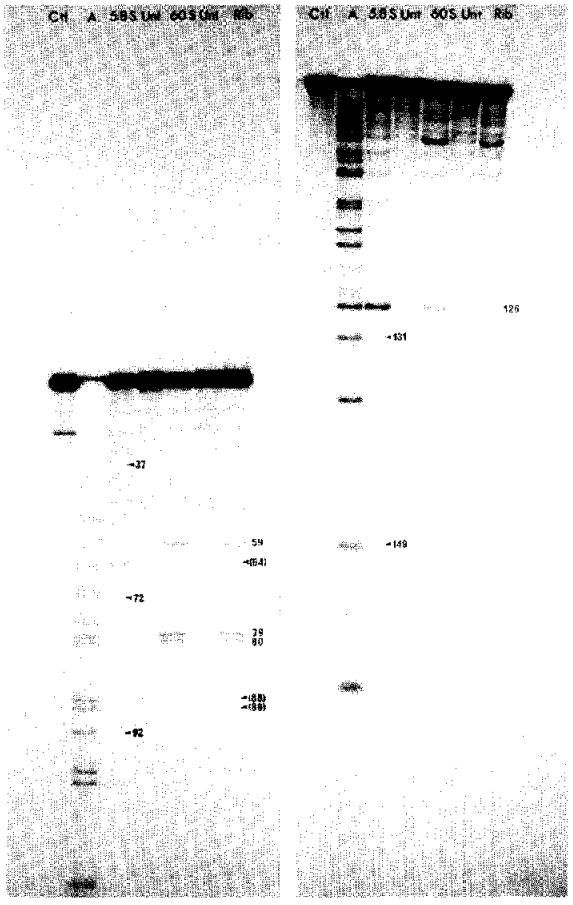

Fig.1. Autoradiograph of 3′-end-labelled yeast free, 60 S subunit and whole ribosome-associated 5.8 S rRNA partially degraded with diethyl pyrocarbonate-aniline and separated on a 12% polyacrylamide gel. Electrophoresis was carried out at 1050 V for 16 h (left) and 4 h (right). Fragments for a standard diethyl pyrocarbonate-induced aniline degradation are included as markers for residue determination; specific cleavage sites are identified by the residue numbers in the margin. Ctl, aniline-treated free 5.8 S rRNA; A, RNA chemically degraded at adenosine residues; 5.8 S, diethyl pyrocarbonate-induced partial cleavages in free 5.8 S rRNA; Unt, 60 S subunit or ribosome-associated 5.8 S rRNA incubated in the absence of diethyl pyrocarbonate and treated with aniline; 60 S, diethyl pyrocarbonate-induced partial cleavages in 60 S subunit-associated 5.8 S RNA; Rib, diethyl pyrocarbonate-induced partial cleavages in ribosome-associated 5.8 S RNA.

3. Results and discussion

The chemical degradation procedures developed [16] for RNA sequencing are carried out as two step reactions; for adenylate specific cleavages, for example, the purified and labelled RNA is first partially modified with diethyl pyrocarbonate and subsequently cleaved with aniline. We adapted this procedure to examine the topography of 5.8 S rRNA in yeast ribosomes by making two changes;

(i) The chemical reactivity of diethyl pyrocarbonate was initially used as a probe under 'physiological-like' conditions similar to those in [17] rather than the original denaturing conditions [16];

(ii) The modified 5.8 S rRNA was subsequently extracted, purified and labelled before being cleaved with aniline. These changes avoided the difficulty in specifically identifying the 5.8 S fragments when partial ribonuclease digestion is used and permitted a much more rapid gel analysis of modified residues.

As shown in fig.1, under our reaction conditions (50 μl pyrocarbonate/ml for 30 min at 25°C) the free 5.8 S RNA molecule was readily susceptible to diethyl pyrocarbonate modification while in ribosomes or subunits, the cleavages were essentially restricted to only two regions of the molecule. In 60 S subunits (60 S) only 4 sites (residues 59, 79, 80, 126) were modified to a significant extent when bands in the diethyl pyrocarbonate untreated control (Unt) are taken into consideration; slight cleavages were also observed at three adenylate residues (residues 64, 88, 89) in some gels. The results with whole ribosomes (Rib) were essentially identical except for residue 126 where modification was now largely eliminated.

In general the untreated controls (i.e., in the absence of diethyl pyrocarbonate) show somewhat higher than expected background levels (fig.1) and must be taken into consideration carefully when the data are interpreted. The problem is significantly less important under standard sequencing conditions because usually the degree of modification and the bands resulting from subsequent aniline cleavage are considerably more intense. Further repurification of the labelled RNA failed to eliminate this background but since it could affect the detection of slightly modified residues we are continuing to search for the cause. Nevertheless, as shown in fig.1, when the untreated controls were considered the accessible sites were easily distinguishable.

Under the reaction conditions used in fig.1, free 5.8 S rRNA (5.8 S) was found to be extensively modified; in fact, under these conditions only 5 adenylate nucleotides (residues 37, 72, 92, 131, 149) remained largely or entirely uncleaved. As would be expected, these are located within the strongly base-paired regions of the 'burp gun' model for 5.8 S rRNA (fig. 2). In contrast, the greatly restricted modification

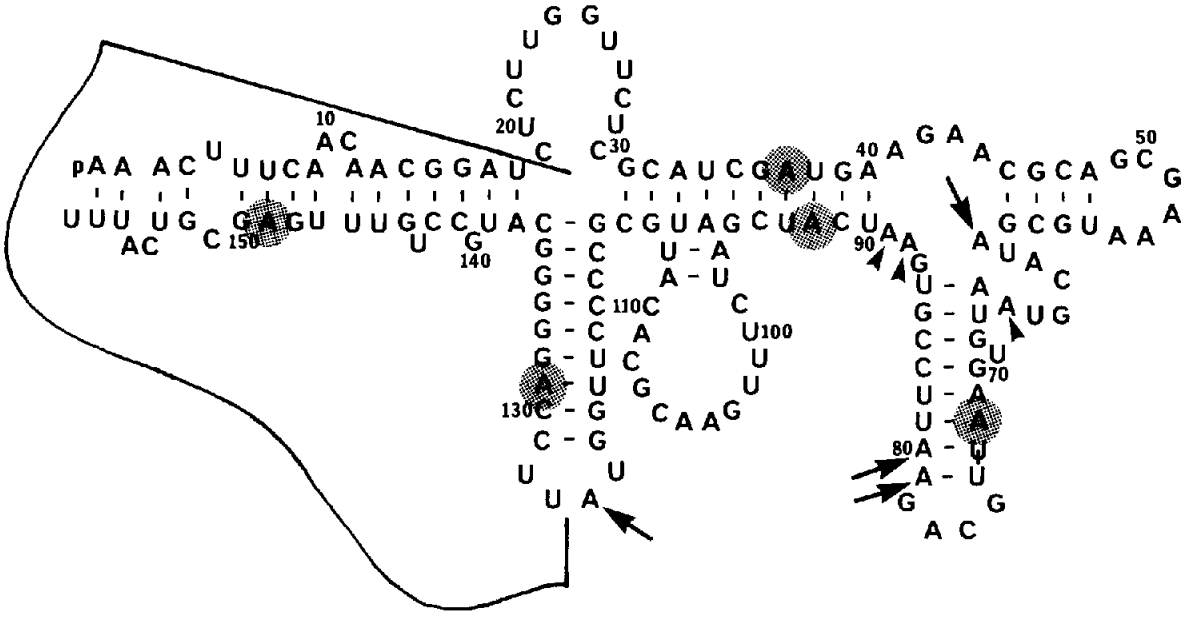

Fig.2. Diethyl pyrocarbonate-reactive sites in ribosome associated yeast 5.8 S rRNA. Free 5.8 S rRNA, 60 S subunits and whole ribosomes were treated with diethyl pyrocarbonate as in section 2; reactive sites were detected by subsequent aniline degradation and analyses on 12% polyacrylamide gels as shown in fig.1. The nucleotide sequence was determined in [18] and the secondary structure is based on the 'burp gun' model [19]. Large arrows and small arrowheads indicate strongly or weakly reactive sites, respectively, in 60 S subunits and whole ribosomes; the shaded areas indicate residues in free 5.8 S RNA which were essentially unreactive. The solid line indicates regions of the 5'- and 3'-termini which are believed to contain sequences that interact with the 25 S rRNA [8,9].

of ribosome-associated 5.8 S RNA strongly supports suggestions that the molecule interacts with ribosomal proteins [4–6] as well as with the large RNA component. Furthermore, the fact that the reactivity of one of the exposed residues in 60 S subunits (A_{126}) is significantly reduced in whole ribosomes suggests that part of this RNA may be localized in the ribosomal interface. Studies on the affinity of rat liver ribosomal proteins for immobilized 5.8 S rRNA have suggested an interaction with two proteins (S9, S13) from the small subunit [6]; the reduced reactivity at residue A_{126} is consistent with this idea.

As indicated in fig.2, the reactive residues in bound 5.8 S RNA are located in two specific regions of the molecule; all but one are in or around the 'AU-rich' stem structure (residues 66–86) and one (A_{126}) is in the looped portion of the 'GC-rich' stem structure (residues 116–137). It is possible that these sites have a function in protein synthesis such as the binding of RNA or translation factors and should be carefully examined in further studies on the role of 5.8 S RNA in ribosome function. In contrast, the two –G–A–A–C– sequences (residues 42–45 and 103–106), which would be putative tRNA binding sites if the suggestions [10,11] for 5.8 S RNA function are correct, appear not to be accessible to diethylpyrocarbonate modification. It seems that this role for 5.8 S RNA must be more stringently questioned.

These studies do not distinguish specific regions of 5.8 S rRNA which interact with the 25 S rRNA or with ribosomal proteins. It is possible that with further studies on free RNA, the 5.8 S–25 S RNA complex, ribosomal subunits and whole ribosomes under a variety of kinetic conditions, specific regions of interaction can be delineated and such studies have been initiated. Nevertheless, these data clearly show that modified applications of recently developed RNA sequencing techniques are potentially useful, not only to probe the secondary structure of free RNA [17], but also for studies on the topography of protein- or ribosome-associated RNA.

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