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Gene Heterogeneity: A Basis for Alternative 5.8S rRNA Processing†

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ABSTRACT: Two bands of 5.8S rRNA were observed when the total RNA isolated from rat or mouse tissue was separated by electrophoresis on high-resolution polyacrylamide gels under denaturing conditions. The minor form, with a lower mobility, represented 15-35% of the total 5.8S rRNA, depending on the source of the tissue. Sequence analysis and the kinetics of formation showed that this minor form is elongated at the 5′ end and is not a precursor. The sequence of the minor form was found to be p(C)CGAUA[CG-, five or six nucleotides longer than the major form. The minor 5.8S rRNA constituent also formed a more stable junction complex with 28S rRNA than the shorter major sequence. The rat DNA sequence that corresponds to the additional nucleotides at the 5′ end of 5.8S rRNA has been reported to be -CCGTACG- [Subrahmanyam, C. S., Cassidy, B., Busch, H., & Rothblum, L. I. (1982) *Nucleic Acids Res. 10*, 3667-3680], a sequence which does not contain the extra adenylic acid residue at position 4 found in the minor form. This suggests that the rodent rRNA genes are heterogeneous and that the insertion of an A residue in the ribosomal precursor RNA can generate an alternate processing site.

The 5.8S ribosomal RNA (rRNA) in eucaryotic cells is hydrogen bonded to its cognate high molecular weight rRNA (25-28 S) in the large ribosomal subunit (60 S) (Pene et al., 1968; Weinberg & Penman, 1968). The 5.8S rRNA along with the two high molecular weight rRNAs, 18S and 28S rRNA, is processed from the 45S precursor RNA in the nucleolus. The signals which identify the processing sites for specific endo- and exonucleolytic cleavages are probably a combination of specific sequences and secondary and tertiary structure (Perry, 1976) and may also be influenced by ribosomal proteins (Nazar, 1982). For example, the sequence -ACGPuPu- has been postulated to be a nuclease recognition site at the 3′ end of the 18S rRNA region in the precursor (Subrahmanyam et al., 1982) while a secondary structure has been proposed as the recognition site for the processing of 5.8S rRNA in *Xenopus* (Nazar, 1982). Recently, a binary complex between U3 RNA and the 32S precursor rRNA has been postulated to signal the processing at the 3′ end of the 5.8S rRNA (Bachellerie et al., 1983; Crouch et al. 1983). The precise roles of these inter- and intramolecular interactions in processing remain to be elucidated.

In previous studies (Sitz et al., 1978, 1981), we made use of naturally occurring differences in nucleotide sequences of 5.8S rRNA to study the role of specific nucleotides in the intermolecular interactions of this RNA with other 5.8S rRNAs and with 28S rRNA and found that single nucleotide changes had dramatic effects. In the present study, we have characterized an elongated form of 5.8S rRNA, which could be derived from a subpopulation of precursor 45S rRNA which

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contains an additional processing site. On the basis of the rat rDNA sequence (Subrahmanyam et al., 1982), a single nucleotide insertion appears to generate such a site.

Materials and Methods

RNA was isolated from either tissues or tissue culture cells with a phenol–sodium dodecyl sulfate buffer at 55 °C (Nazar et al., 1975). The 5.8S rRNA was purified on a denaturing 15% polyacrylamide slab gel [7 M urea, 1:39 bis(acrylamide):acrylamide, 0.086 M tris(hydroxymethyl)aminomethane (Tris), 0.01 M boric acid, and 0.002 M ethylenediaminetetraacetic acid (EDTA), pH 8.3]. The 5.8S rRNA bands were detected by staining with methylene blue or by autoradiography, eluted (Nazar et al., 1975), and applied to a nondenaturing 15% polyacrylamide gel (no urea) to isolate conformational isomers. For preparations of uniformly labeled [^{32}P]RNA, normal rat kidney (NRK) cells (Duc-Nguyen et al., 1966) were incubated with 1 mCi of [^{32}P]orthophosphate per 75-cm^2 T flask in low-phosphate media (Sitz et al., 1981), and the 5.8S rRNA was purified as described above. The 5.8S rRNA isolated from mouse liver, rat liver, and Ehrlich ascites cells was labeled at the 5′ terminus with [γ-^{32}P]ATP and polynucleotide kinase (Richardson, 1971) or at the 3′ terminus with [5′-^{32}P]pCp and RNA ligase (England & Uhlenbeck, 1978).

The sequences of the postlabeled RNAs were determined by partial nuclease digestion and the rapid-sequencing gel techniques (Donis-Keller et al., 1977) by using nucleases supplied as a kit by P-L Biochemicals, Inc. After the various enzyme digestions, the fragments were separated by electrophoresis on a 12% polyacrylamide slab gel [8.5 M urea, 1:19 bis(acrylamide):acrylamide, 0.043 M Tris, 0.005 M boric acid, and 0.001 M EDTA, pH 8.3]. Partial U2 RNase digestion fragments were produced to obtain homogeneous 5′ termini from the elongated 5.8S rRNA. 5′-Labeled 5.8S rRNA was digested by mixing 1 μL of U2 RNase (0.5 unit/μL) and 3 μL of buffer (33 mM sodium citrate, pH 5, 1.7 mM EDTA, and 1 mg/mL carrier tRNA) with the dried RNA sample and incubating it at 37 °C for 30 min. Labeled RNA fragments 22 and 23 nucleotides long were isolated as described above, and their sequences were determined.

To further characterize the 5.8S rRNA, uniformly labeled RNA was digested with T1 RNase, and the resulting fragments were fractionated by electrophoresis on cellulose acetate at pH 3.5 and then on diethylaminoethyl (DEAE) paper in 7% formic acid (Nazar et al., 1975). The unique oligonucleotide found in the elongated form of 5.8S rRNA was characterized by base composition analysis, and its sequence was deduced after further digestion with pancreatic or U2 RNase.

The stability of the 5.8S–28S rRNA junctions for the different forms of 5.8S rRNA was determined by forming complexes and measuring their T_m's as previously described (Sitz et al., 1981).

Results and Discussion

Two bands of 5.8S rRNA were observed when RNA isolated from rat or mouse tissue was separated by electrophoresis on a high-resolution denaturing, 15% polyacrylamide slab gel (Figure 1, right panel), one faint slower migrating minor component (m) and one intense major form (M). Each of these two RNA fractions migrated as two families of isomers (three bands each) when eluted and applied to a nondenaturing gel. This multiple banding could also be observed if whole-cell RNA was applied directly to a nondenaturing gel (Figure 1, left panel). Three faint bands (labeled a, b, and c) and three intense bands (labeled A, B, and C) were reproducibly observed. The amount of minor form varied from 15% to 35% of the total 5.8S rRNA in rat and mouse tissues and in cultured rodent cells.

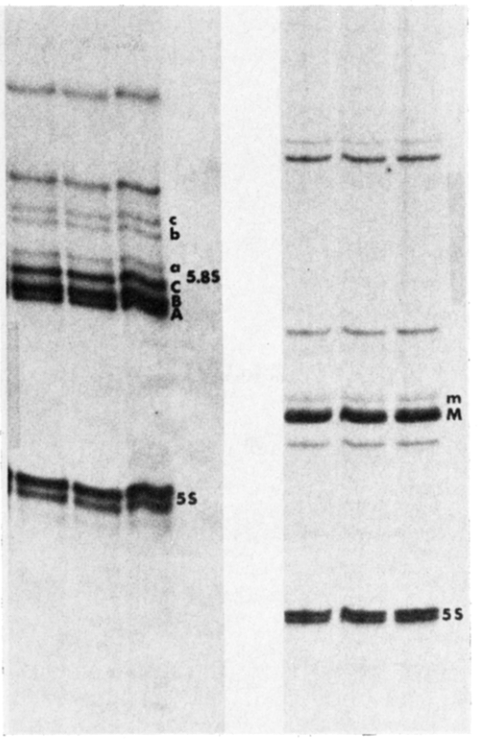

FIGURE 1: Autoradiographs of uniformly labeled NRK cell RNA separated on 15% polyacrylamide slab gels. (Left panel) RNA separated by electrophoresis on a nondenaturing gel. The major conformers of 5.8S rRNA are labeled A, B, and C while the minor forms are labeled a, b, and c. (Right panel) RNA separated on a denaturing, 7 M urea gel. The minor 5.8S rRNA is labeled m while the major 5.8S rRNA is labeled M.

Table I: Methylation Levels in Conformational Isomers of 5.8S rRNA[a]

component	conformational isomers of 5.8S rRNA	% of total 5.8S rRNA	GGUmGGAUp (mol %)
minor	c	3.6	0.58
	b	6.5	0.37
	a	5.0	0.10
major	C	8.0	0.80
	B	38.0	0.87
	A	39.0	0.01

[a] The conformational isomers from NRK cells were isolated on a 15% nondenaturing slab gel (Figure 1), extracted from the gel, and digested with pancreatic ribonuclease. The resultant oligonucleotides were separated by two-dimensional electrophoresis as described under Materials and Methods, and methylation levels were quantitated.

We have previously shown (Nazar et al., 1983) that the conformation of the major isomers is related to the extent of 2′-*O*-methylation of the uridylic acid residue at position 14, i.e., no methylation at residue 14 in the fastest migrating 5.8S rRNA. When the minor 5.8S rRNA isomers were digested with RNase A and the resulting nucleotides mapped, we observed that the methylation levels also varied (Table I). However, while the extent of methylation of "a" was low, it was not completely lacking as was previously observed in the major conformation A (Nazar et al., 1983), suggesting again that the 2′-O-methylation was having an effect on the conformational equilibrium and perhaps the function of this molecule. This fingerprinting procedure also demonstrated that the RNA in bands A, B, C, a, b, and c was 5.8S rRNA.

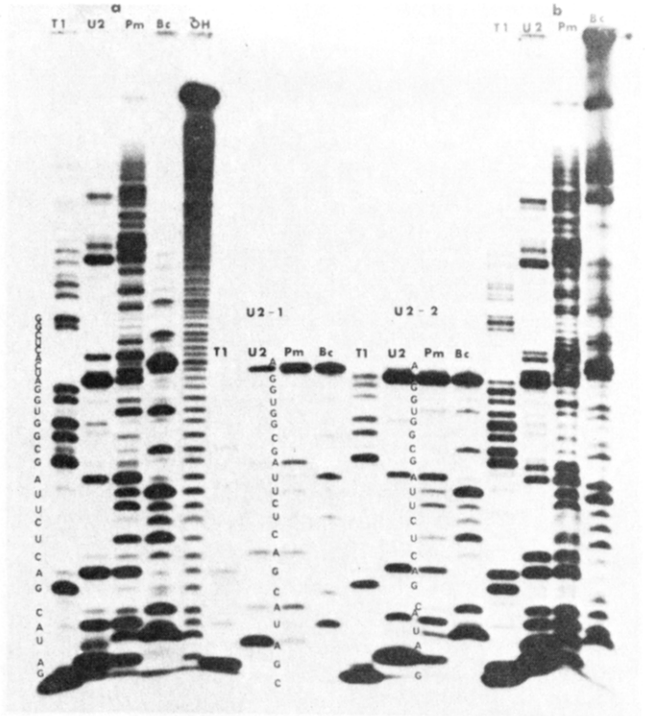

FIGURE 2: Autoradiograph of a rapid-sequencing gel of the 5′-labeled minor conformers a and b (see Figure 1, left panel) and 5.8S rRNA fragments (U2-1 and U2-2) generated by a U2 RNase digest of total minor forms (see Figure 1, right panel).

The minor form of 5.8S rRNA therefore appeared to be an elongated sequence of 5.8S rRNA similar to that which has been found in yeast 5.8S rRNA (Rubin, 1974).

To verify this possibility, major and minor forms of 5.8S rRNA from Ehrlich ascites cells were labeled at the 5′ or 3′ terminus and analyzed by the rapid-sequencing gel method (Doris-Keller et al., 1977). Both forms of 5.8S rRNA produced similar sequencing gels when labeled at the 3′ terminus but different patterns when 5′ labeled (data not shown). Also, the sequencing gels from the 5′-labeled 5.8S rRNAs gave complicated banding patterns characteristic of heterogeneous 5′ termini. Both forms were identical except that the minor form was offset by an additional five or six nucleotides at the 5′ end. This elongated 5.8S rRNA also had two predominant 5′ termini.

We used two approaches to obtain molecules with a homogeneous terminus so we might determine the actual sequence. In the first approach, a partial U2 RNase digestion of minor 5.8S rRNA was used to obtain two fragments (U2-1, 23 nucleotides, and U2-2, 22 nucleotides) which were then subjected to sequence analysis. As shown in Figure 2 (middle), these fragments had homogeneous termini and were 22 and 23 nucleotides long, respectively. The second approach was to separate the minor form into its conformational isomers first (Figure 3), and then sequence each band (Figure 2). Band a had a homogeneous 5′ terminus while bands b and c had both termini present, which produced double enzyme cleavage bands on the sequencing gel (Figure 2, gel not shown for band c). From these gels, we initially deduced the sequence to be p-(N)CGA(U)ACG- with two uncertainties. The *Bacillus cereus* enzyme (labeled Bc in Figure 2) in our experience does not readily cleave a 5′-terminal C residue, consistent with an additional C residue on the 5′ end. Also, the nucleotide identified as U in position 5 sometimes was cleaved as a C residue on some gels (a common problem with this enzyme digestion procedure). To clarify the uncertainty with the 5′ terminus, we digested the three isomers (Figure 3) of the minor

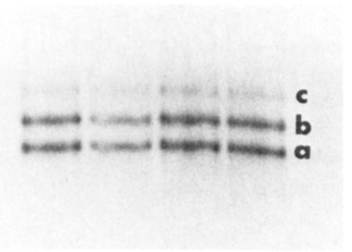

FIGURE 3: Autoradiograph of mouse 5′-terminal labeled minor 5.8S rRNA separated into its conformational isomers on a nondenaturing 15% polyacrylamide slab gel.

FIGURE 4: Autoradiograph of the oligonucleotides resulting from the complete T1 RNase digests of uniformly labeled rat 5.8S rRNA major conformation A and minor conformation a. The first dimension (right to left) was on cellulose acetate at pH 3.5, and the second dimension (top to bottom) was on DEAE paper in 7% formic acid. The arrow indicates the unique minor form fragment, UA_2CGp.

5.8S rRNA with 0.3 N NaOH and separated the resulting terminal nucleoside bisphosphates by paper electrophoresis. All three forms exhibited pCp as their 5′ terminus. In rat 5.8S rRNA, the fastest migrating major and minor forms (labeled A and a in Figure 1) had homogeneous 5′ termini ending in pCG-. The slower migrating conformational isomers of the major and minor 5.8S rRNA (B, C and b, c in Figure 1) had heterogeneous termini, p(C)G- and p(C)CG-, respectively. Therefore, the longer minor form ends in pCCG- while the shorter minor form ends in pCG-. Since a T1 digest of uniformly labeled minor 5.8S rRNA should produce a unique T1 digestion fragment, APyrACGp, we used this approach to finalize the sequence. T1 digestion of RNA bands A and a (Figure 1) produced a unique fragment in the minor form (Figure 4). Thus, the nucleotide composition of this fragment was C, U, A_2, G; the pancreatic RNase digestion products were AU, AC, G, and the U2 RNase digestion products were UA, A, CG, indicating the sequence is AUACGp. Therefore, the overall final sequence of the 5′ end of the minor form of 5.8S rRNA is p(C)CGAUA[CG-, which is six nucleotides longer than the major form and has a heterogeneous 5′ terminus due to the additional cytidylic acid residue in about half of the molecules.

To determine if this elongated form was a precursor to the shorter major 5.8S rRNA, we examined the labeling kinetics of both forms. When NRK cells were labeled for 3, 10, 12 and 24 h with [^{32}P]orthophosphate, the percent of minor form

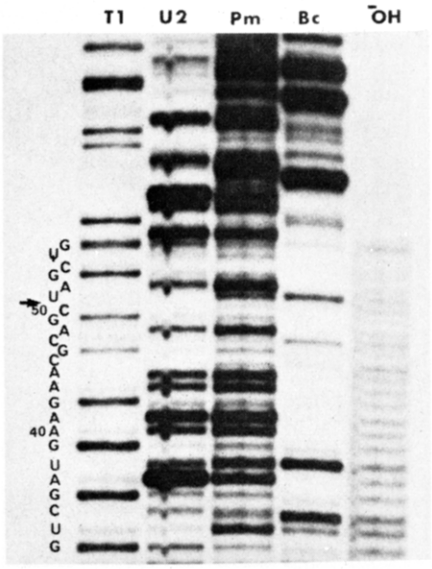

FIGURE 5: Rapid-sequencing gel of the 5.8S rRNA conformational isomer A isolated from rat liver RNA. The arrow indicates the missing -GC- dinucleotide.

was respectively 38%, 30%, 33%, and 24% of the total 5.8S rRNA. From this and other experiments, the percent of the minor 5.8S rRNA was observed to remain relatively constant for 12 h with a slight drop at 24 h. If the minor 5.8S rRNA was an obligatory precursor, it would be expected to have a higher percent of the radioactivity at 3 h when 5.8S rRNA was first detected to any great extent. The minor form of 5.8S rRNA is therefore not a precursor but must result from a variable processing site or gene heterogeneity.

Because the 5′ end of the 5.8S rRNA is involved in binding to the 28S rRNA (Sitz et al., 1981), we examined the effect these added sequences would have on the stability of the 5.8S–28S rRNA complex. The T_m for the complexes of 28S RNA with the major and minor forms of 5.8S rRNA were 54.5 ± 0.2 and 55.7 ± 0.2 °C, respectively. This difference of 1.2 °C was reproducible ($n = 4$ separate preparations) and statistically highly significant ($p < 0.001$) when analyzed by the Student's t test. The greater stability of the minor form of the 5.8S–28S rRNA junction complex indicates some additional base pairing at the 5′ end of the minor 5.8S rRNA with 28S rRNA.

There have been a number of reports (Subrahmanyam et al., 1982; Michot et al., 1982) that a GC dinucleotide reported in our original sequence for the rat 5.8S rRNA was not found in the DNA sequence. Using newer, more definitive gel sequencing techniques, we have therefore reexamined the sequence in this region (position 51 in the sequence). In both the fast migrating major and minor forms (A and a, respectively, Figure 1) of rat 5.8S rRNA, we found the sequence to be -AGCUA- (Figure 5) rather than the originally reported incorrect sequence, -AGCGCUA-. The sequences in this region were easy to read, and there were no signs of compression which was observed in earlier attempts to sequence this region using 3′-labeled RNA. Apparently, when the original primary sequence of the rat 5.8S rRNA (Nazar et al., 1975) was determined by using classical sequencing methods (Brownlee, 1972), extra GCp fragments from minor contaminants resulted in an erroneously high molar yield of this dinucleotide in the oligonucleotide catalogs, resulting in the extra two residues being placed in the sequence. The complete corrected sequence of the major 5.8S rRNA from rat now corresponds to the reported DNA sequence (Subrahmanyam et al., 1982; Michot et al., 1982).

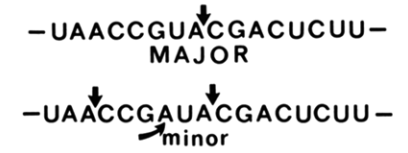

FIGURE 6: Processing of the major and minor forms of 5.8S rRNA from the nucleolar precursor RNA. The curved arrow indicates the adenylic acid insertion while the straight arrows show the processing sites.

The finding of an elongated form of 5.8S rRNA supports the concept that a gene variation with a nucleotide insert results in a change in the cleavage site or an additional processing site at the 5′ end of the 5.8S rRNA. This adenylic acid insert generates a precursor which when cleaved generates a 5.8S rRNA five or six nucleotides longer than the major form (Figure 6). The published sequence for the rDNA from this region cloned from rat DNA (Subrahmanyam et al., 1982) does not have this A insert while the recent published sequence from mouse (Michot et al., 1983) does. Since the rat and mouse major and minor forms of 5.8S rRNA are identical, it is possible that the cloned rat rDNA represents the major sequence (no A insert) while the cloned mouse rDNA represents the minor sequence (an A insert). If this nucleotide insertion generates a new processing site but does not destroy the major site, then two possible processing sites on the 5′ end of the 5.8S rRNA would exist. The cleavage that generates the elongated 5.8S rRNA precludes the additional cleavage at the major site since the minor form is not a precursor of the major. Therefore, the precursor RNA with the adenylic acid insert could represent over 50% of the total precursor pool if the frequency of cleavage was the same for both sites (Figure 6). Then, in the process of cloning an rDNA fragment there might be an equal chance of selecting either DNA sequence (-CCGTACG- or -CCGATACG-). *Saccharomyces cerevisiae* 5.8S rRNA contains 10% of a minor form which is six or seven nucleotides longer (Rubin, 1974) and which corresponds to the published yeast rDNA sequence for this region (Skryabin et al., 1979). The existence of these elongated forms in a primitive eucaryotic organism (yeast) and in mammals may indicate some selective pressure or function that would allow these structures to be maintained in evolution.

The sequence of the cleavage site at the 5′ end of 5.8S rRNA is -ACGA- or -ACCGA- for the major and minor forms, respectively (Figure 6). This is similar to the consensus sequence seen just beyond the 3′ end of 18S rRNA in the internal transcribed spacer (ITS-1) (-ACGPu-) found in rat, *Xenopus* and *Bombyx* (Subrahmanyam et al., 1982). The general location of the processing site could depend on some aspect of secondary or tertiary structure while the specific nucleotide cleavage site depends on the unique sequence within this structure. No obvious unique secondary structure was observed that might help explain this endonuclease recognition region. Although there were no obvious binding sites for U3 RNA, which may be important in the processing of the 3′ end of 5.8S rRNA (Bachellerie et al., 1983; Crouch et al., 1983), this does not preclude the formation of a binary complex, as other small RNAs may function in this regard. While the A insert may generate a new processing site (Figure 6), an alternative explanation may be that this nucleotide insert destroys the cleavage site for the major 5.8S rRNA. After the initial endonuclease cleavage, there must also be some trimming that would generate the sequence heterogeneity found in the major and minor forms [p(C)GACU- and p(C)-CGAUA-]. Thus, this single nucleotide insertion has been useful in gaining additional information about the processing

of 5.8S rRNA, the binding of 5.8S rRNA to 28S rRNA, and the heterogeneity of the rDNA genes.

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Base Pairing in Wheat Germ Ribosomal 5S RNA As Measured by Ultraviolet Absorption, Circular Dichroism, and Fourier-Transform Infrared Spectrometry†

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ABSTRACT: Ultraviolet absorption (UV) and circular dichroism (CD) spectra of wheat germ 5S RNA, when compared to $tRNA^{Phe}$, indicate a largely base-paired and base-stacked helical structure, containing up to 36 base pairs. Fourier-transform infrared (FT-IR) spectra of $tRNA^{Phe}$ and wheat germ ribosomal 5S RNA have been acquired at 30 and 90 °C. From the difference of the FT-IR spectra between 90 and 30 °C, the number of base pairs in both RNAs was determined by modification of a previously published procedure [Burkey, K. O., Marshall, A. G., & Alben, J. O. (1983) *Biochemistry 22*, 4223-4229]. The base-pair composition and total base-pair number from FT-IR data are now consistent for the first time with optical (UV, CD, Raman) and NMR results for ribosomal 5S RNA. Without added Mg^{2+}, $tRNA^{Phe}$ gave 18 ± 2 base pairs [7 A-U and 11 G-C], in good agreement with the number of secondary base pairs from X-ray crystallography [8 A-U, 12 G-C, and 1 G-U]. Within the 10% precision of the FT-IR method, wheat germ 5S RNA exhibits essentially the same number of base pairs [14 A-U, 17 G-C, and 5 G-U; for a total of 36] in the absence of Mg^{2+} as in the presence of Mg^{2+} [14 A-U, 18 G-C, and 3 G-U; for a total of 35], in agreement with the UV hyperchromism estimate of G-C/(A-U + G-C) = 0.58. Addition of Mg^{2+} increases the melting midpoint (T_m) for both A-U and G-C pairs in wheat germ 5S RNA. The average T_m for both types of base pairs increases from 48 (CD) or 54 °C (UV) in the absence of Mg^{2+} to 63 (CD) or 69 °C (UV) in the presence of Mg^{2+}. From FT-IR, addition of Mg^{2+} increases the average base pair melting midpoint by 11 °C for G-C pairs (56 to 67 °C) but only by 4 °C for A-U pairs (54 to 58 °C). The above results are consistent with three generalized eukaryotic 5S RNA secondary base-pairing schemes, of which the cloverleaf model gives the closest match.

5S RNA appears to be present and essential for protein synthesis in virtually all 70-odd species from which the molecule has been isolated and base sequenced (Erdmann, 1976; Singhal & Shaw, 1983). It is therefore widely speculated that a universal secondary structure exists, and several models have been proposed (Fox & Woese, 1975; Nishikawa & Takemura, 1978; Luoma & Marshall, 1978a,b; Hori & Osawa, 1979; Luehrsen & Fox, 1981; Studnicka et al., 1981; Kuntzel et al., 1983; Pieler & Erdmann, 1983; Singhal & Shaw, 1983).

These models necessarily differ in both the *total* number and the *relative* numbers of the three main base-pair types (A-U, G-C, and G-U). Choosing between the existing models or devising a new model therefore requires techniques that can discriminate between the four bases with respect to base

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