Articles

Comparison of Eubacterial and Eukaryotic 5S RNA Structures: A Chemical Modification Study†

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ABSTRACT: The 5S RNAs from *Bacillus stearothermophilus* and *Saccharomyces cerevisiae* were probed by nucleotide-specific reagents, with a view to compare and contrast their higher order structures. The progressive unfolding of the RNAs during heating, in the presence and absence of magnesium, was monitored. Evidence was provided for the double-helical segments which occur in the secondary structural models of both RNAs. The results also placed constraints on the possible structuring of the remainder of the RNA and yielded some insight into ways of folding up the molecule. Together with the data from our earlier studies, employing ribonucleases, these results provide a detailed picture of the structuring and topography of the 5S RNAs. The main structural differences between the eubacterial and eukaryotic RNAs occur throughout the loop D/helix IV/loop E/helix V arm; in particular strong evidence is provided for loop D of the eukaryotic RNA being involved in a tertiary interaction.

The 5S RNA is found in all ribosomes except those from mammalian mitochondria, and together with a group of proteins, it appears to be important for ribosomal function. Although the functional role remains unknown, the complex is proving particularly useful for examining the structural basis of protein–RNA recognition (Garrett et al., 1981). The binding sites of proteins L5, L18, and L25 on *Escherichia coli* 5S RNA (Douthwaite et al., 1979, 1982; Huber et al., 1984) and of protein YL3 on yeast 5S RNA (Nazar & Wildeman, 1983) have been mapped. In addition, evidence has been found for a nucleotide bulged from a helix being involved in protein recognition (Peattie et al., 1981), and recently, a low-resolution X-ray diffraction analysis has been performed on a complex of protein L25 and an RNA fragment from *E. coli* (Abdel-Meguid et al., 1983).

A prerequisite for a better understanding of the protein–RNA interactions is to elucidate further the structure of 5S RNA. After the first minimal model was proposed for the secondary structure of 5S RNA on the basis of sequence comparison evidence (Fox & Woese, 1975), various additions have been made, and there is now general agreement that eubacteria contain four or five double-helical segments and that eukaryotes contain five (Hori et al., 1980; Garrett et al., 1981; Luehrsen & Fox, 1981; Studnicka et al., 1981; De Wachter et al., 1982; Delihas & Andersen, 1982). Numerous experimental approaches have been directed toward characterizing the least structured regions of the *E. coli* RNA which occur at positions 10–13, 40–42, and 87–89 [reviewed by Monier (1974) and Noller & Garrett (1979)] and those nucleotides which are looped out from helices (Peattie et al., 1981; Garrett & Olesen, 1982). Recently, the ribonuclease from *Naja naja oxiana* has also provided a probe for double helices or highly stacked structures, and at least three of the double-helical segments in the models of both eubacterial and eukaryotic RNAs are susceptible to this enzyme (Douthwaite & Garrett, 1981; Garrett & Olesen, 1982). At present, we know little about the tertiary structure, although the formation of a chemical cross-link between G_{41} and G_{72} in the *E. coli* 5S RNA (Hancock & Wagner, 1982) is compatible with three mutually exclusive tertiary interactions (Böhm et al., 1982; Hancock & Wagner, 1982; Pieler & Erdmann, 1982).

In the present work, we have employed a chemical modification approach (Peattie & Gilbert, 1980) to investigate further the higher order structure of 5S RNA and to compare the structures of a eubacterial RNA from *Bacillus stearothermophilus* (the 3′-terminus of *E. coli* 5S RNA is not sufficiently homogeneous for a semiquantitative study of this nature) and a eukaryotic RNA from *Saccharomyces cerevisiae*. This method is ideal for investigating RNA structure since reactions are generally rapid and cause only minimal pertubations in the native structure (Rhodes, 1975; Peattie & Gilbert, 1980; de Bruijn & Klug, 1983). We assumed that at a given magnesium concentration interacting regions would tend to denature, and become reactive, over the same temperature range. Therefore, we examined the reactivities of each adenosine, guanosine, and cytidine at different temperatures in the presence and absence of magnesium. The results are tested against the current secondary and tertiary structural models.

Materials and Methods

The 5S RNA was extracted from the ribosomes of *B. stearothermophilus* strain NCA 1503 and *S. cerevisiae* essentially by the method of Monier & Feunteun (1971). Ten-microgram aliquots of 5S RNA were 3′ end labeled with [^{32}P]pCp (Amersham or New England Nuclear) and RNA ligase (P-L Biochemicals) as described by Bruce & Uhlenbeck (1978). The RNA was renatured by heating in CMK buffer (70 mM sodium cacodylate, 10 mM $MgCl_2$, and 270 mM KCl, pH 7.5) at 65 °C for 10 min with slow cooling (Aubert et al., 1968); the renatured 5S RNA was chemically modified in CMK buffer at 30, 55, 70 and 85 °C. Samples which were

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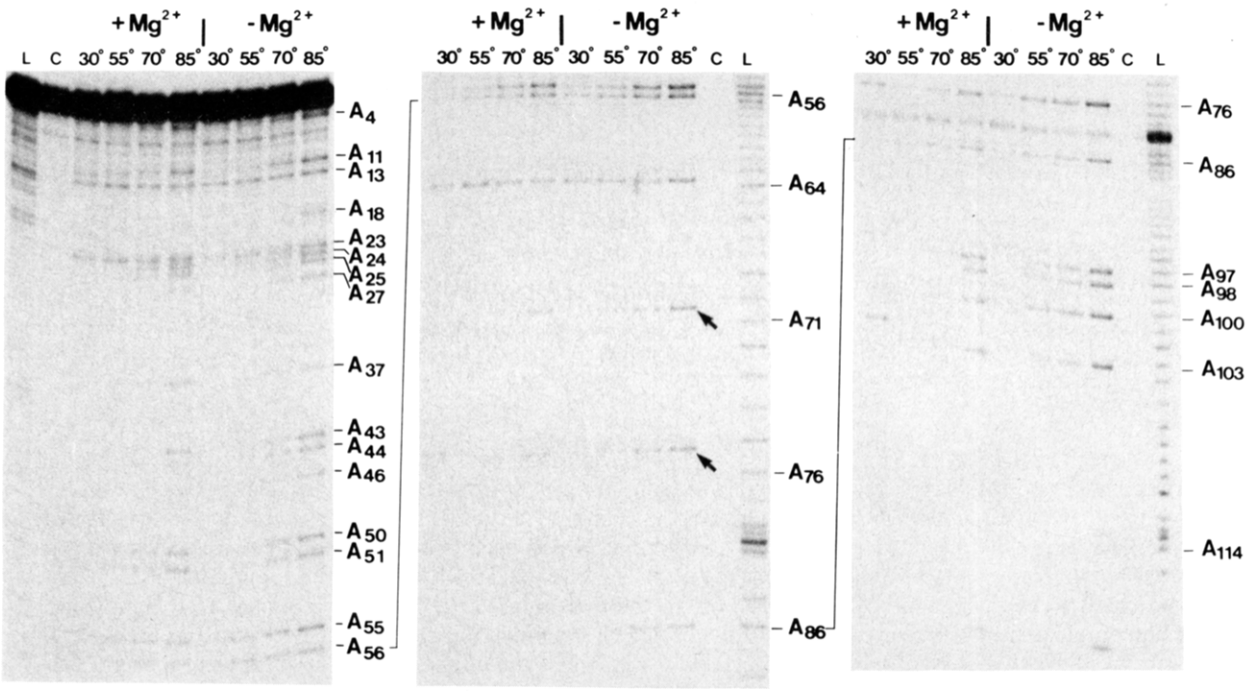


FIGURE 1: Adenosines of the *B. stearothermophilus* 5S RNA were carbethoxylated with diethyl pyrocarbonate at the temperature indicated and in the presence or absence of magnesium. Samples were coelectrophoresed in 12% polyacrylamide sequencing gels. Arrows indicate anomalously migrating bands that are considered in the text. C represents a control RNA sample that was not subjected to chemical treatment, and L denotes a ladder formed by water hydrolysis at 100 °C.

to be modified in the absence of magnesium were renatured in TMK buffer [70 mM tris(hydroxymethyl)aminomethane (Tris), 10 mM $MgCl_2$, and 270 mM KCl, pH 7.5], before precipitating with 2.5 volumes of ethanol. They were redissolved in 70 mM sodium cacodylate, 270 mM KCl, and 5 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5, and modified at the four temperature steps.

Nucleotide-specific modifications of the RNA were performed essentially as described by Peattie & Gilbert (1980). Guanosines were modified with $^1/_2$ μL of fresh dimethyl sulfate (Me_2SO_4) in 300 μL of CMK buffer for 20 min at 30 °C followed by sodium borohydride treatment for 5 min at 20 °C in the presence of 8 M urea to ensure complete denaturation. Adenosines were reacted with 10 μL of fresh diethyl pyrocarbonate (DEP) in 200 μL of CMK buffer for 30 min at 30 °C. Cytidines were treated with $^1/_2$ μL of fresh Me_2SO_4 in 300 μL of CMK buffer for 20 min at 30 °C followed by hydrazine treatment in low salt. At higher temperatures the reaction time was halved for every increase of 10 °C. Prior to the strand scission with aniline/acetic acid, the RNA was purified on a 12% polyacrylamide gel containing 8 M urea, to remove any degraded material. After aniline treatment, samples were heated to 85 °C for 30 s prior to loading on 12% polyacrylamide sequencing gels (40 × 40 × 0.03 cm). Samples subjected to the normal sequencing reactions of Peattie (1979) were coelectrophoresed as markers. The intensities of the bands on the autoradiograms were assessed visually.

RESULTS

Modification Reactions. Adenosines are carbethoxylated at the N-7 position by diethyl pyrocarbonate when they are not stacked in a double helix and when the N-7 position is not involved in a tertiary interaction. Guanosines exhibit similar reactivities toward dimethyl sulfate except that they do react within regular double helices. Cytidines are only methylated at the N-3 position when not involved in base pairing. Adenosines and cytidines have also been probed in various non-Watson–Crick configurations in yeast $tRNA^{Phe}$, including reversed G-C pairings, certain Hoogsteen pairings, and base triples where they are also resistant (Peattie & Gilbert, 1980).

Secondary Structural Models. The generally accepted model for 5S RNA presented here has evolved from a minimal four helix model for eubacterial RNAs which was based on evidence for compensating base pair changes occurring in the putative helices of a group of 5S RNAs (Fox & Woese, 1975); the helical nomenclature also derives from this model. One helical segment, helix V, has been added to the original model; it is regular in eukaryotes (Nishikawa & Takemura, 1974) and contains putative non-Watson–Crick pairings in eubacteria (De Wachter et al., 1982). While direct experimental evidence has been presented for the occurrence of helices I, II, and IV by probing with the cobra venom ribonuclease (Douthwaite & Garrett, 1981; Garrett & Olesen, 1982), support for helices III and V is indirect since they were resistant to all the ribonucleases tested. Minor additions have been made to the model on the basis of both experimental results (Noller & Garrett, 1979; Peattie et al., 1981) and sequence comparison evidence (Hori et al., 1980; Studnicka et al., 1981; Luehrsen & Fox, 1981; De Wachter et al., 1982; Delihas & Andersen, 1982). Below the present data are considered with respect to these models.

Bacillus stearothermophilus. Figure 1 shows the bands resulting from the carbethoxylated adenosines over the range 30–85 °C in the presence and absence of magnesium. Few abrupt changes were observed in the band intensities owing to the dynamic nature of the RNA structure. The relative intensities of the bands were averaged over different experiments, and the results for the adenosine-, cytidine-, and guanosine-specific reactions are summarized in Table I. The lowest temperatures at which nucleotides reacted moderately, in the presence and absence of magnesium, are also indicated on the models of the RNA secondary structure presented in Figure 2.

At 30 °C, in the presence of magnesium, accessibility to the adenosine- and cytidine-specific reagents were restricted to

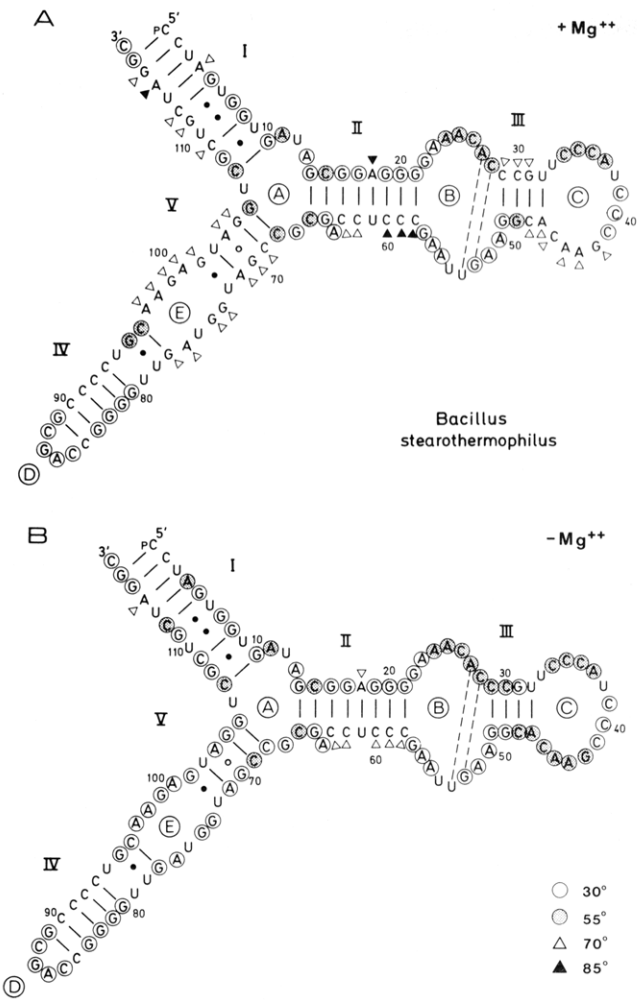


FIGURE 2: Chemical modification results for adenosines, cytidines, and guanosines are superimposed on a secondary structural model of the *B. stearothermophilus* 5S RNA. Dashed lines across loop B depict tentative base pairs. Samples were modified at increasing temperatures (A) in the presence of 10 mM magnesium and (B) in the absence of magnesium. The temperature at which a nucleotide became moderately reactive (+; see Table I) is denoted by circles or triangles as indicated in the figure.

nucleotides A_{13}, A_{23}, C_{39}-C_{41}, A_{50}-A_{51}, A_{55}-A_{56}, A_{64}, C_{85}-C_{88}, and C_{117} which are located in unstructured regions of the model (Figure 2A). Guanosine modifications at the same temperature revealed that helices I, II, and IV were accessible. At 55 °C, increased reactivity was observed in the upper parts of loops B and C, where A_{24}-C_{26} and C_{34}-A_{37} became accessible and the putative base pairing within loop B was disrupted (Figure 2A). Also, the concurrent methylation of C_{15} and C_{66} indicated that helix II melts from the end adjacent to loop A. At 70 °C, helix I was denatured except at the terminal region. Moreover, helix II progressively denatured with C_{62} and C_{63} becoming accessible, and helix III was completely disrupted together with the remainder of loop C; loop E and helix V also melted simultaneously. At 85 °C, helices I and II were completely denatured, whereas the partial resistance of C_{84} and C_{90-94} revealed some residual structuring in helix IV.

Removal of magnesium produced some localized effects (Table I; Figure 2B). The greatest change was in the loop E/helix V region where four potentially juxtapositioned purines are located; the melting temperature decreased by 40 °C in the absence of magnesium. Magnesium depletion also destabilized the G·U pairings in helix I, the segment of helix II adjacent to loop B, and, moreover, helix III and the adjacent sequence G-A-A-C_{45} in loop C (Figure 2B).

Saccharomyces cerevisiae 5S RNA. Bands resulting from cytidines that were methylated at increasing temperatures in the presence and absence of magnesium are depicted in Figure 3. The averaged results from the adenosine-, cytidine-, and guanosine-specific modifications are listed in Table II, and the temperatures at which each nucleotide became moderately reactive, in the presence and absence of magnesium, are indicated on the secondary structural model in Figure 4.

Adenosines and cytidines which were reactive at 30 °C, in the presence of magnesium, occurred at nucleotides C_{10}-A_{11}, C_{39}-C_{40}, A_{51}-A_{56}, A_{63}-A_{64}, C_{73}-C_{74}, A_{84}, and C_{105} within unstructured regions of the model (Figure 4A). These active sites include the extra nucleotides C_{73}-G_{75}/C_{105} in loop E and looped out A_{84}/G_{85} from helix IV that are characteristic of the eukaryotic RNA (Figure 4A). At 55 °C, helix III was denatured and the structuring within loop C, loop D, and the upper part of loop B were disrupted rendering all of the bases exposed: helices I and V were only partly denatured. By 70 °C, the remainder of the secondary structure had melted, and no further reactivity changes were detected at 85 °C.

Magnesium removal resulted in effects that were less localized than in the eubacterial RNA (Table II; Figure 4B). At 30 °C, the structure was destabilized mainly within loops B–D; raising the temperature to 55 °C resulted in a general melting of the whole RNA; only helix III was not markedly destabilized.

Methodological Problems. Bands corresponding to adenosines-71 and -76 of the thermophile RNA migrated more slowly in the diethyl pyrocarbonate track than in the water hydrolysis track (Figure 1), where sequence compression occurs due to the presence of helix IV. The only difference between the two samples is the presence of the 5′-terminal phosphate in the chemically modified sample, and we assume that this helps to destabilize helix IV in the abovementioned bands.

Some artifacts were observed that were attributable to chemical side reactions. For example, guanosine bands were sometimes detected in the cytidine tracks; in the cytidine reaction, 3-methylcytosine is hydrolyzed by hydrazine, but the latter may also emulate the strongly nucleophilic borohydride ion and hydrolyze 7-methylguanosine. Cytidines that were strongly modified during the dimethyl sulfate/hydrazine treatment also tended to appear in the guanosine track. In addition, secondary effects were detected due to methylation of adenosines by dimethyl sulfate; this is compatible with the observed order of reactivities: N-7 in guanosine > N-1 in adenosine > N-3 in cytidine > N-3 in adenosine (Lawley & Brookes, 1966).

There were also limitations in the resolution and quantification of certain bands. For example, the 5′-terminal nucleotides in the G and C tracks were more blurred than in the A track, due to the random methylation of guanosines and cytidines in each of the former; consequently, no results were recorded for C_1 and C_2 in the thermophile RNA. Moreover, a relatively strong background ladder was always observed in the adenosine tracks (Figure 1) because the adenosine reaction proceeded less far than the cytidine and guanosine reactions such that a relatively longer exposure was necessary.

Discussion

The secondary structural models of the eubacterial and eukaryotic 5S RNAs exhibit only minor differences (Figure 5). It is also likely that some facets of the tertiary structure have been conserved since these RNAs have been required for the functioning of ribosomes throughout the evolution of eubacteria and eukaryotes. Below, the two RNA structures are compared and contrasted.

Table I: Adenosine, Guanosine, and Cytidine Modifications in *B. stearothermophilus* 5S RNA at Different Temperatures and Magnesium Concentrations[a]

	10 mM magnesium				no magnesium			
nucleotide	30 °C	55 °C	70 °C	85 °C	30 °C	55 °C	70 °C	85 °C
A_4			++	+++		+	+++	+++
G_5	+++	+++	+++	+++	+++	+++	+++	+++
G_7	+++	+++	+++	+++	+++	+++	+++	+++
G_8	+++	+++	+++	+++	+++	+++	+++	+++
G_{10}	+	+++	+++	+++	+++	+++	+++	+++
A_{11}		+	++	+++		+	+++	+++
A_{13}	+++	+++	+++	+++	+++	+++	+++	+++
G_{14}	+++	+++	+++	+++	+++	+++	+++	+++
C_{15}		+	+++	+++		+	+++	+++
G_{16}	++	++	++	++	++	++	++	++
G_{17}	++	++	++	++	++	++	++	++
A_{18}				+			+	+++
G_{19}[b]	++	++	++	++	++	++	++	++
G_{20}[b]	++	++	++	++	++	++	++	++
G_{21}[b]	++	++	++	+++	++	++	++	++
G_{22}[b]	++	++	++	+++	++	++	++	++
A_{23}	+++	+++	+++	+++	+++	+++	+++	+++
A_{24}		+	++	+++	(+)	++	+++	+++
A_{25}	(+)	+	++	+++	(+)	++	+++	+++
C_{26}		+	+++	+++		+	+++	+++
A_{27}		+	++	+++		+	+++	+++
C_{28}		+	+++	+++		+	+++	+++
C_{29}			++	+++		+	+++	+++
C_{30}			++	+++		+	+++	+++
G_{31}			+	++	+	+	++	+++
C_{34}		+	+++	+++		+++	+++	+++
C_{35}		+	+++	+++		+++	+++	+++
C_{36}		+	+++	+++		+++	+++	+++
A_{37}		+	++	+++		+	++	+++
C_{39}	+++	+++	+++	+++	+++	+++	+++	+++
C_{40}	+++	+++	+++	+++	+++	+++	+++	+++
C_{41}	+++	+++	+++	+++	+++	+++	+++	+++
G_{42}		(+)	+	+++		+	+++	+++
A_{43}		(+)	+	+++		+	++	+++
A_{44}		(+)	+	+++		+	++	+++
C_{45}		(+)	+++	+++		+	+++	+++
A_{46}			+	+++		+	++	+++
C_{47}		(+)	+++	+++		+	+++	+++
G_{48}		+	++	+++	+	++	+++	+++
G_{49}	+	++	++	++	+	++	++	++
A_{50}	+	+	++	+++	+	++	+++	+++
A_{51}	+	++	++	+++	+	++	+++	+++
G_{52}	+++	+++	+++	+++	+++	+++	+++	+++
A_{55}	+	+	++	+++	++	+++	+++	+++
A_{56}	+	+	++	+++	++	+++	+++	+++
G_{57}	++	++	++	+++	++	++	+++	+++
C_{58}			(+)	+++			++	+++
C_{59}			(+)	+++			++	+++
C_{60}			(+)	+++			++	+++
C_{62}		(+)	++	+++		(+)	+++	+++
C_{63}			+	+++			+++	+++
A_{64}	+++	+++	+++	+++	+++	+++	+++	+++
G_{65}	+++	+++	+++	+++	+++	+++	+++	+++
C_{66}		+	++	+++		+	+++	+++
G_{67}	++	++	+++	+++	++	+++	+++	+++
C_{68}		+	++	+++	++	++	+++	+++
C_{69}			++	+++		++	+++	+++
G_{70}			+	+++	+	++	+++	+++
A_{71}			+	+++	+	++	+++	+++
G_{73}			+	+++	+	++	+++	+++
G_{74}			+	++	++	+++	+++	+++
A_{76}			+	+++	+	++	+++	+++
G_{77}			+	++	++	+++	+++	+++
G_{80}[b]	++	++	++	++	++	++	++	++
G_{81}	++	++	++	+++	++	+++	+++	+++
G_{82}[b]	++	++	++	++	++	++	++	++
G_{83}[b]	++	++	++	++	++	++	++	++
C_{84}				(+)				(+)
C_{85}	++	++	++	++	++	++	++	++
A_{86}	+	+	++	+++	+	++	+++	+++
G_{87}	+++	+++	+++	+++	+++	+++	+++	+++
C_{88}	+++	+++	+++	+++	+++	+++	+++	+++
G_{89}	+++	+++	+++	+++	+++	+++	+++	+++
C_{90}				(+)				(+)

Table I (Continued)

nucleotide	10 mM magnesium				no magnesium			
	30 °C	55 °C	70 °C	85 °C	30 °C	55 °C	70 °C	85 °C
C_{91}				(+)				(+)
C_{92}				(+)				(+)
C_{93}				(+)				(+)
G_{95}		+	+	+++	+	++	+++	+++
C_{96}	(+)	+	+++	+++	+	++	+++	+++
A_{97}		(+)	+	+++	+	++	+++	+++
A_{98}			+	+++	+	++	+++	+++
G_{99}		(+)	++	+++	++	+++	+++	+++
A_{100}			+	+++	+	+++	+++	+++
G_{101}	(+)	(+)	+	+++	++	+++	+++	+++
A_{103}			+	+++	+	++	+++	+++
G_{104}			+	+++	+	+++	+++	+++
G_{105}		+	++	+++	++	+++	+++	+++
C_{107}		+	+++	+++		+	+++	+++
G_{108}	++	++	++	+++	+++	+++	+++	+++
C_{109}		(+)	+++	+++	+	++	+++	+++
G_{111}			+	++	+	++	+++	+++
C_{112}		(+)	+++	+++		+	+++	+++
A_{114}			(+)	++			+	+++
G_{115}		(+)	+	+++	+	+++	+++	+++
G_{116}	+++	+++	+++	+++	+++	+++	+++	+++
C_{117}	+++	+++	+++	+++	+++	+++	+++	+++

[a] The quantification system is the following: +++, very strong; ++, strong; +, medium; (+), weak. [b] Quantification less certain due to band compression. Each set of data was averaged from at least three independent experiments.

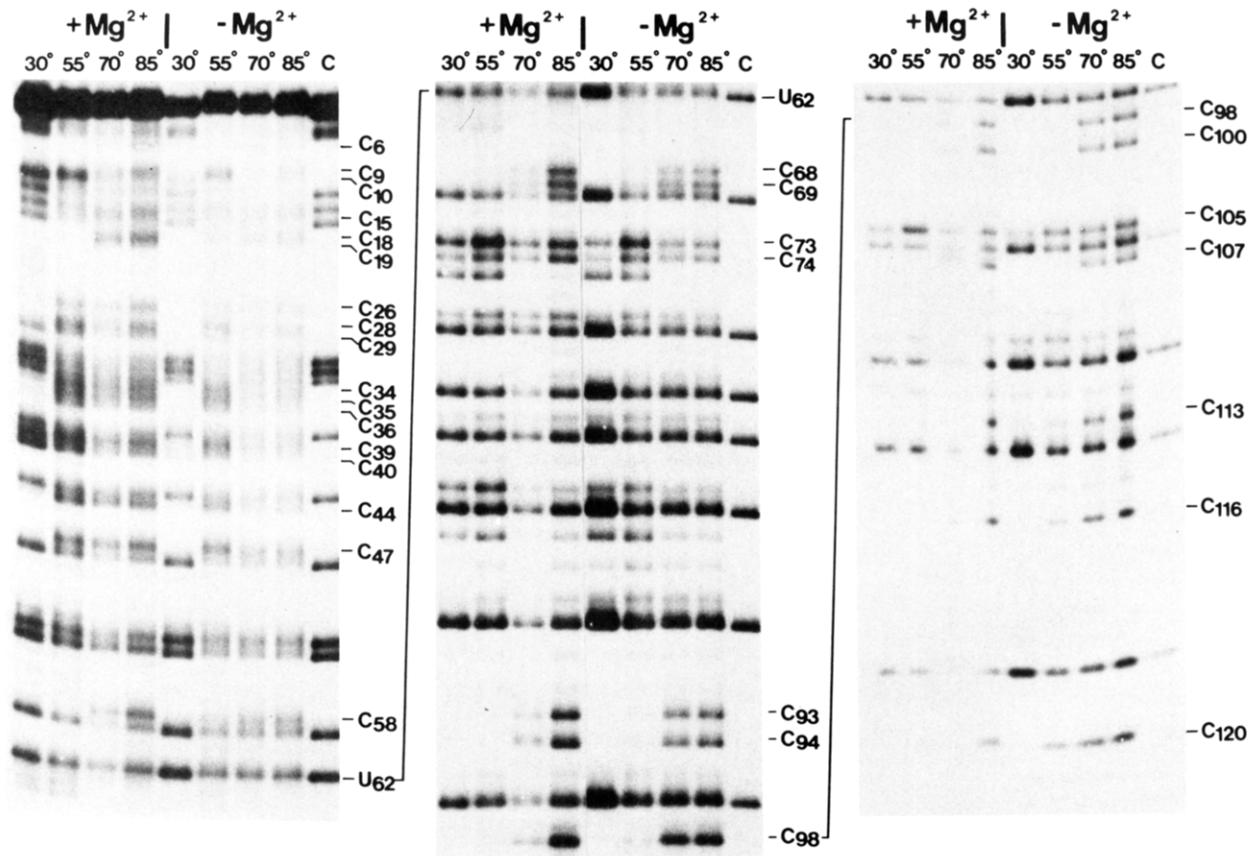


FIGURE 3: Cytidines of the *S. cerevisiae* 5S RNA were methylated with dimethyl sulfate and treated with hydrazine. Samples were modified at the temperatures indicated in the presence and absence of magnesium and run on 12% polyacrylamide sequencing gels. C represents a control sample that was reacted with hydrazine but not with dimethyl sulfate (the U reaction).

Accessible Nucleotides at 30 °C. The adenosines, guanosines, and cytidines that were moderately reactive at 30 °C are summarized on the models of *B. stearothermophilus* and *S. cerevisiae* RNAs in Figure 5. Previously, adenosines accessible at 30 °C in the eukaryotic RNA were located (Lo & Nazar, 1982) and the two sets of data agree well. Superimposed on the models are also the cutting positions of the ribonucleases specific for single strands and double helices, at 0 °C. These data derive from studies in which only primary cutting positions were monitored in essentially intact 5S RNA molecules (Douthwaite & Garrett, 1981; Garrett & Olesen, 1982). The locations of the modified adenosines and cytidines, and the single-strand-specific ribonuclease cuts, correlate well for three accessible regions in loops A, C, and D that were first

Table II: Adenosine, Guanosine, and Cytidine Modifications in *S. cerevisiae* 5S RNA at Different Temperatures and Magnesium Concentrations

	10 mM magnesium				no magnesium			
nucleotide	30 °C	55 °C	70 °C	85 °C	30 °C	55 °C	70 °C	85 °C
G_1[b]	+++	+++	+++	+++	+++	+++	+++	+++
G_2[b]	+++	+++	+++	+++	+++	+++	+++	+++
G_5	+++	+++	+++	+++	+++	+++	+++	+++
C_6			+	+++		+	++	+++
G_7	+++	+++	+++	+++	+++	+++	+++	+++
G_8	+++	+++	+++	+++	+++	+++	+++	+++
C_9			+	+++		+	++	+++
C_{10}	+++	+++	+++	+++	+++	+++	+++	+++
A_{11}	+++	+++	+++	+++	+++	+++	+++	+++
A_{13}		+	+++	+++	+	+++	+++	+++
C_{15}			++	+++		++	+++	+++
A_{17}			++	+++		+	++	+++
C_{18}			++	+++		+	+++	+++
C_{19}			++	+++		+	+++	+++
A_{20}			++	+++		+	++	+++
G_{21}	+++	+++	+++	+++	+++	+++	+++	+++
A_{22}		+	++	+++	+	++	+++	+++
A_{23}		+	++	+++	+	++	+++	+++
A_{24}		+	++	+++		++	+++	+++
G_{25}	+++	+++	+++	+++	+++	+++	+++	+++
C_{26}	(+)	++	+++	+++	(+)	+++	+++	+++
A_{27}		++	+++	+++		++	+++	+++
C_{28}		+++	+++	+++		+++	+++	+++
C_{29}	++	+++	+++	+++	+++	+++	+++	+++
G_{30}	++	+++	+++	+++	+++	+++	+++	+++
C_{34}	+	+++	+++	+++	++	+++	+++	+++
C_{35}		+++	+++	+++	++	+++	+++	+++
C_{36}		+++	+++	+++	+++	+++	+++	+++
G_{37}	++	+++	+++	+++	+++	+++	+++	+++
C_{39}	+++	+++	+++	+++	+++	+++	+++	+++
C_{40}	+++	+++	+++	+++	+++	+++	+++	+++
G_{41}	(+)	+	+++	+++	++	++	+++	+++
A_{42}		+	+++	+++		+	+++	+++
C_{44}	(+)	+++	+++	+++	+++	+++	+++	+++
A_{45}	(+)	+	+++	+++	(+)	+	+++	+++
A_{46}		+	+++	+++		+	+++	+++
C_{47}		++	+++	+++		+++	+++	+++
G_{49}	+++	+++	+++	+++	+++	+++	+++	+++
A_{51}	+++	+++	+++	+++	+++	+++	+++	+++
G_{52}	+++	+++	+++	+++	+++	+++	+++	+++
A_{55}	+	++	+++	+++	+	+++	+++	+++
A_{56}	+++	+++	+++	+++	+++	+++	+++	+++
G_{57}	+++	+++	+++	+++	+++	+++	+++	+++
C_{58}			+	+++		+	+++	+++
G_{60}			++	+++		++	+++	+++
G_{61}			++	+++	++	++	+++	+++
A_{63}	+++	+++	+++	+++	+++	+++	+++	+++
A_{64}	+++	+++	+++	+++	+++	+++	+++	+++
G_{65}	+++	+++	+++	+++	+++	+++	+++	+++
A_{66}		(+)	++	+++	(+)	++	+++	+++
G_{67}	(+)	+	+++	+++	+	+++	+++	+++
C_{68}		(+)	+	+++		+	++	+++
C_{69}		(+)	+	+++		+	++	+++
G_{71}	+	+	+++	+++	+++	+++	+++	+++
A_{72}			+	++		+	++	+++
C_{73}	+++	+++	+++	+++	+++	+++	+++	+++
C_{74}	+++	+++	+++	+++	+++	+++	+++	+++
G_{75}	+++	+++	+++	+++	+++	+++	+++	+++
A_{76}			+	++		+	++	+++
G_{77}	++	+++	+++	+++	++	+++	+++	+++
A_{79}			+	++		+	++	+++
G_{80}			++	+++		++	+++	+++
G_{82}	+	+	++	+++	++	+++	+++	+++
A_{84}	+++	+++	+++	+++	+++	+++	+++	+++
G_{85}	+++	+++	+++	+++	+++	+++	+++	+++
G_{87}	+++	+++	+++	+++	+++	+++	+++	+++
G_{88}	(+)	+	++	++	++	++	++	++
G_{89}	(+)	+	++	++	++	++	++	++
G_{91}	(+)	+	+++	+++	+	+++	+++	+++
A_{92}		+	++	+++	+	++	+++	+++
C_{93}		(+)	+	+++		+	+++	+++
C_{94}		(+)	++	+++		+	+++	+++
A_{95}			+	+++		+	++	+++
A_{97}			+	+++		+	++	+++
C_{98}		(+)	++	+++		+	+++	+++

Table II (Continued)

nucleotide	10 mM magnesium				no magnesium			
	30 °C	55 °C	70 °C	85 °C	30 °C	55 °C	70 °C	85 °C
G_{99}	+	++	+++	+++	++	+++	+++	+++
C_{100}		(+)	++	+++		+	+++	+++
G_{101}	+	+	+++	+++	+	+++	+++	+++
A_{102}			+	+++		+	++	+++
A_{103}			+	+++		+	++	+++
A_{104}			+	+++		+	++	+++
C_{105}	+++	+++	+++	+++	+++	+++	+++	+++
C_{107}		(+)	++	+++		+	+++	+++
A_{108}			+	+++		+	++	+++
G_{109}	+	+	++	+++	++	+++	+++	+++
G_{110}	+++	+++	+++	+++	+++	+++	+++	+++
G_{112}	+++	+++	+++	+++	+++	+++	+++	+++
C_{113}		+	++	+++		+	+++	+++
G_{115}	+++	+++	+++	+++	+++	+++	+++	+++
C_{116}		+	++	+++		++	+++	+++
A_{117}			+	+++		+	++	+++
A_{118}			+	+++		+	++	+++
C_{120}		+	+	+++	(+)	+++	+++	+++

[a] The quantification is the same as for Table I. [b] Quantification uncertain. Each set of data was averaged from at least three independent experiments.

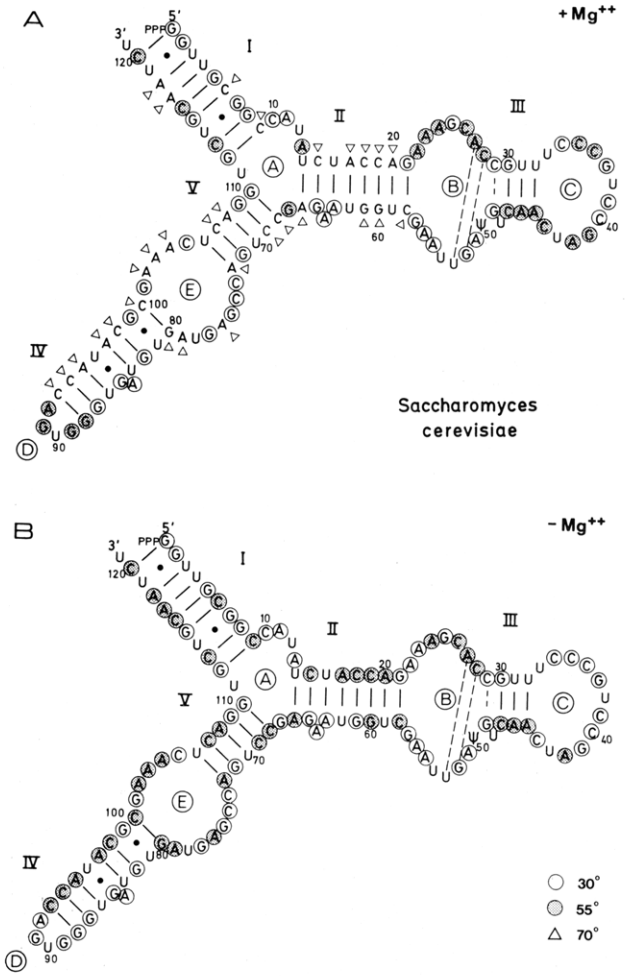


FIGURE 4: Chemically modified adenosines, cytidines, and guanosines are depicted on a secondary structural model of *S. cerevisiae* 5S RNA. Samples were treated at increasing temperatures (A) in the presence of 10 mM magnesium and (B) in the absence of magnesium. The temperature at which a nucleotide became moderately reactive (+; see Table II) is indicated by circles or triangles as shown on the figure.

characterized by Monier and co-workers (Monier, 1974); only loop D was fairly resistant in the yeast RNA (Figure 5). In other regions, however, the correlation was less clear: for example, the adenosine bulged from helix II was only detected by the chemical probes although it was cut by ribonucleases in the *E. coli* RNA (Douthwaite & Garrett, 1981). Structural features special to the eukaryotic RNA were accessible to the different probes; these include the inserts C-C-G_{75} and C_{105} in loop E and A_{84}/G_{85} looped out from helix IV. Moreover, reactivity differences between corresponding regions of the two RNAs were generally found in both sets of data: loop B and the sequence U-C_{34} in loop C were more susceptible by all criteria in the yeast RNA (Tables I and II).

The conserved nucleotides in the 5S RNAs, shown in Figure 5, that were reactive in other eubacterial and eukaryotic 5S RNAs to single-strand-specific reagents such as kethoxal and sodium bisulfite, at 20–30 °C (Noller & Garrett, 1979; Miura et al., 1983; Nishikawa & Takemura, 1978), correlate well with the data summarized in Figure 5, as do the tritium-exchange results for the *E. coli* RNA (Faber & Cantor, 1981).

Double Helices: Location and Accessibility. The chemical criterion for the presence of a double-helical segment is that the reactivities of the adenosines and cytidines on opposite strands of the helix should exhibit the same dependence on temperature and magnesium concentration. Each of the putative double helices in the two RNAs was consistent with this criterion (Figures 2 and 4). In the thermophile RNA, helices III and V denatured in one step whereas the remainder melted in two steps, at both magnesium concentrations. For the yeast RNA each of the helices melted in one step. The overall stability of the helices was higher in the former due to its higher content of G-C pairings. These results reinforce the cobra venom ribonuclease data for helices I, II, and IV (Figure 5) and provide additional support for helices III and V. Cobra venom ribonuclease cuts have been detected in helices III and V in other eukaryotic 5S RNAs (Toots et al., 1981; Troutt et al., 1982), but no precautions were taken to avoid rearrangements in secondary structure during the digestion; when a selection was made for essentially intact molecules, no such cuts were detected (Douthwaite & Garrett, 1981; Garrett & Olesen, 1982).

The degree of accessibility of a helix within the 5S RNA structure should be revealed by its susceptibility to guanosine methylation and to digestion by the cobra venom ribonuclease. Neither criterion is foolproof since stacked guanosines may sometimes exhibit low reactivities (de Bruijn & Klug, 1983) and the ribonuclease may have some special structural requirements. However, when results from the two probes

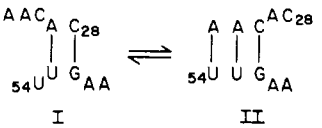
correlate, the data are likely to be reliable. Most of helices I, II, and IV were accessible to both probes, in both RNAs, whereas helix III was only partially accessible. The main difference was found in helix V; it was susceptible in the yeast RNA to dimethyl sulfate but not to the larger ribonuclease whereas it was unreactive to both in the thermophile (Figure 5).

Nucleotides Bulged from Helices II and IV. The bulging of an adenosine from helix II of both RNAs is strongly supported by the carbethoxylation results. In the yeast RNA both adjacent adenosines that can loop out were reactive, suggesting that they are in dynamic equilibrium in the free RNA structure. Chemical modification and ribonuclease digestion results reveal a similar equilibrium for the bulging of A_{84} and G_{85} from helix IV of the yeast RNA (Figure 5B). The nucleotide bulged from helix II is conserved in position and is almost invariably an adenosine in eubacteria and lower eukaryotes, a cytidine in animals, and a uridine in plants (Peattie et al., 1981); it has only been found missing from two archaebacteria (Luehrsen et al., 1981; Stahl et al., 1981). The bulged nucleotide in helix IV is less conserved in position but is generally a purine. Peattie et al. (1981) have provided evidence for the adenosine bulged from helix II of the *E. coli* RNA being involved in protein L18 recognition. The nucleotide bulged from helix IV of the yeast RNA is also protected by protein YL3 (Nazar & Wildeman, 1983), although the latter authors suggest that the role of this nucleotide may be simply to bend helix IV.

Aspects of Tertiary Structure. Insight into the tertiary structure may also be gained by identifying nucleotides in the loop regions that become reactive at the same temperature in the presence and absence of magnesium. Stable structuring occurs in loops B, C, and E in both RNAs, and loop D is structured in the eukaryotic RNA (Figures 2 and 4); some of these structures may participate in interloop interactions.

(*a*) *Structuring within Loop Regions.* The results for loop A are compatible with the formation of the additional base pair A_{11}-U_{106} in the thermophile (Figure 5A) and the A_{13}·G_{67} pairing in the yeast (Figure 5B). Such pairing could facilitate coaxial stacking of either helices I and V in the thermophile or helices II and V in yeast. An exchange between such alternative conformers within a given RNA molecule may be of functional importance (Luehrsen & Fox, 1981; Stahl et al., 1981), and a conformational change detected under physiological conditions could correspond to such a transition (Kao & Crothers, 1980; Rabin et al., 1983).

Additional base pairs have been proposed within loop B [e.g., see Studnicka et al. (1981) and Stahl et al. (1981)] (Figure 5). De Wachter et al. (1982) have shown further that sequence comparison evidence suggests that an exchange between this and an alternative base paired form may occur for the thermophile (A_{25} is converted to a guanosine in yeast).



Our data indicate that the sequence A_{24}-C_{28} is structured whereas A(ψ)-A_{51} and A-A_{56} are accessible. A distinction cannot be made unambiguously between these structures; either is possible as long as the sequences A-A-C_{26} in form I and A-C_{28} in form II are involved in further interactions. Form II is favored for the yeast RNA by the medium RNase T_1 cuts at G_{25} and G_{52} (Figure 5) since both would occur at the G_{25}·U_{53} pairing which is absent from the thermophile RNA. Base pairing within loop B would also be compatible with its low magnesium dependence of melting.

Loop C is also highly structured and may contain as few as three accessible nucleotides. The upper and lower parts of the loop (Figure 5) show different melting characteristics in the presence (thermophile RNA) and absence of magnesium (yeast RNA), which suggests that they are structurally independent and may be involved in interloop interactions. Particularly stable is the conserved sequence G-A-A-C in the eubacteria and G-A-U-C in the eukaryote which have been considered as tRNA binding sites (Pieler et al., 1982). The putative base pairing across loop C between U-C_{33} and G-A_{43} (Thompson et al., 1981) is incompatible with the biochemical data and the sequence comparison evidence.

Loop E in the thermophile and the corresponding region in the yeast RNA exhibit the same stability as the adjacent helix V. Moreover, the common behavior of the juxtapositioned purines in the former and adenosines in the latter loop E provides support for a distorted double helix between helices IV and V as was suggested by Stahl et al. (1981) for *S. caldarius* 5S RNA. Such stable structuring is supported further by the complete resistance of this region to kethoxal (Noller & Garrett, 1979; Nishikawa & Takemura, 1979; Muira et al., 1983) and single-stranded-specific ribonucleases (Douthwaite et al., 1979; Douthwaite & Garrett, 1981). The presence of non-Watson–Crick base pairing is also compatible with proton NMR analyses on the *E. coli* RNA (Kime & Moore, 1983). The only contrary evidence derives from a study of the reactivities of the N-1 position of adenosines in *B. stearothermophilus* RNA with monoperphthalic acid when modifications were detected in loop E (A_{71}, A_{97}, and A_{98}) (Pieler et al., 1984). However, this study was performed in the absence of magnesium, and as we show here, this region is selectively destabilized when magnesium is removed.

(*b*) *Hypothetical Interloop Interactions.* The chemical cross-link induced between G_{41} and G_{72} of the *E. coli* 5S RNA by biphenylglyoxal has provided experimental support for tertiary interactions between partially conserved sequences in loops C and E (Hancock & Wagner, 1982). Base pairing has been proposed for eubacterial RNAs, between C-C-A-U_{38} and A-U-G-G_{74} (Hancock & Wagner, 1982) and between (G)-C-C-G_{44} and U-G-G-$(U)_{77}$ (Pieler & Erdmann, 1982) in the thermophile, but neither meets our criterion for a tertiary interaction. A universal parallel base pairing was also proposed for C-C-A-U_{38}/G-G-U-A_{76} (thermophile) and C-G-U_{38}/G-U-A_{79} (yeast) (Böhm et al., 1982) which could contribute to a triple helix if loop E closed. This suggestion is compatible with our data and also with a study in which the phosphates in this region of loop E were shown to be partially resistant to ethylnitrosourea in three eukaryotic RNAs (McDougall & Nazar, 1983). However, caution is still necessary in interpreting the seminal chemical cross-link since it is difficult to eliminate the possibility that it derives from small amounts of the B conformer of *E. coli* 5S RNA in which G_{41} and G_{72} are juxtapositioned (Jordan, 1971).

The structuring of loop D in the eukaryotic RNA (Figure 4A) reflects a clear difference between the eubacterial and eukaryotic RNAs. This conclusion is compatible with earlier data (Garrett & Olesen, 1982, and references therein) and suggests that part of loop D interacts with another part of the molecule. Vigne et al. (1973) suggested pairing between G-U-C_{39} (loop C) and G-A-C (loop D) for eukaryotic RNAs, but although sequence comparison evidence is favorable for two base pairs, the sequence in loop C is very accessible. Another possibility would be with loop B between G-U_{90} and

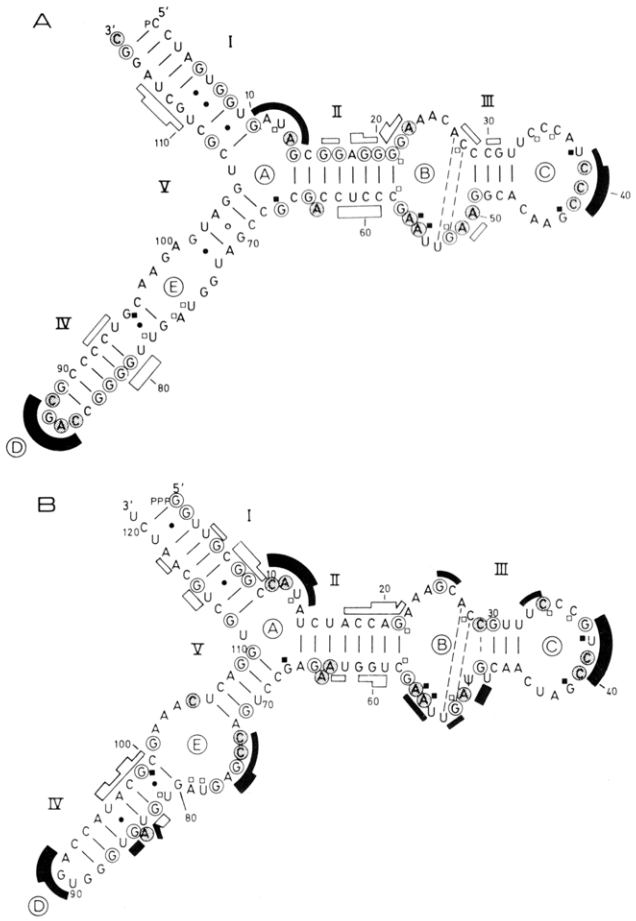


FIGURE 5: Summary of results obtained at low temperatures in the present work and in earlier ribonuclease digestion studies (Douthwaite & Garrett, 1981; Garrett & Olesen, 1982) on the models for *B. stearothermophilus* 5S RNA (A) and *S. cerevisiae* 5S RNA (B). Filled circles represent chemically reactive adenosines and cytidines, and open circles denote reactive guanosines. Filled lines correspond to single-strand-specific ribonuclease cuts, and open lines reflect double-strand-specific cuts with the cobra venom ribonuclease. The thickness of the lines correlates with the degree of ribonuclease cutting. Highly conserved nucleotides in 5S RNAs are also denoted; filled squares correspond to invariant nucleotides, and open squares indicate at least 90% constancy (Delihas et al., 1982).

A-C_{28}; this would favor form II base pairing in loop B (see above) and could be extended to three or four base pairs in many eukaryotic RNAs.

In conclusion, the present data alone are inadequate for establishing tertiary structural interactions in 5S RNA; they give no information about the uridines and yield little or no insight into interactions involving the ribose–phosphate backbone which are found in tRNAs. However, the data do put considerable constraints on the possible interactions involving the adenine, guanine, and cytosine moieties within and between loop regions, and they have, and will, prove valuable for testing tertiary structural models.

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Poly(deoxyadenylic-deoxythymidylic acid) Damage by Radiolytically Activated Neocarzinostatin†

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ABSTRACT: The anaerobic reaction of poly(deoxyadenylic-deoxythymidylic acid) with neocarzinostatin activated by the carboxyl radical CO_2^-, an electron donor generated from γ-ray radiolysis of nitrous oxide saturated formate buffer, has been characterized. DNA damage includes base release and strand breaks. Few strand breaks are formed prior to alkaline treatment; they bear 3′-phosphoryl termini. In contrast, most (66%) of the base release occurs spontaneously. DNA damage is highly (95%) specific for thymidine sites. Neither DNA–drug covalent adduct nor nucleoside 5′-aldehyde, which are major products in the DNA-nicking reaction initiated by mercaptans and oxygen, is formed in this reaction. Data are presented to show that the CO_2^--activated neocarzinostatin intermediate is a short-lived free radical able to abstract hydrogen atoms from the C-1′ and C-5′ positions of deoxyribose. Attack occurs mostly (68%) at the C-1′ position, producing a lesion whose properties are consistent with those of (oxidized) apyrimidinic sites.

Neocarzinostatin (NCS),[1] a protein antibiotic isolated from cultures of a *Streptomyces* species (Ishida et al., 1965), has recently been characterized as a complex in which a non-protein chromophore is bound reversibly to the protein (M_r 10 700) component (Napier et al., 1979; Hensens et al., 1983). The isolated chromophore binds reversibly to DNA by an intercalative mechanism (Povirk et al., 1981) and exhibits the full biological activity of NCS (Kappen et al., 1980). DNA strand breaks constitute the main lesions induced by NCS in vivo (Beerman & Goldberg, 1974, 1977; Ohtsuki & Ishida, 1975; Hatayama & Goldberg, 1979; Moustacchi & Favaudon, 1982). DNA damage is also observed in vitro in the presence of mercaptan and oxygen (Beerman & Goldberg, 1974; Ishida & Takahashi, 1976; Beerman et al., 1977; Kappen & Goldberg, 1978; Burger et al., 1978) or oxygen substitutes such as nitroaromatic compounds (Kappen & Goldberg, 1984). Mercaptan addition to the NCS chromophore triggers the reaction (Napier & Goldberg, 1983) whose products in the presence of O_2 include spontaneous base release, formation of a nucleoside 5′-aldehyde esterified to the 5′ end of the breaks (Kappen et al., 1982), and formation of a covalent DNA–NCS chromophore adduct, presumably on the same oxidized 5′-carbon of the deoxyribose of DNA (Povirk & Goldberg, 1982).

Short-lived radical species that nick DNA have been proposed as intermediates in the mercaptan-induced reaction of NCS (Kappen & Goldberg, 1978; Edo et al., 1980; Sheridan & Gupta, 1981). Although no direct evidence for involvement of a radical form of the NCS chromophore has been presented, we have recently reported that thiol-activated chromophore abstracts a hydrogen atom from the 5′-carbon of deoxyribose in DNA (Charnas & Goldberg, 1984). Furthermore, the incorporation of ^{18}O from molecular oxygen into the 5′-position of nucleoside 5′-aldehyde in DNA (Chin et al., 1984) suggests that, among other possibilities, hydrogen atom abstraction generates a carbon-centered radical at the 5′-carbon to which molecular oxygen can add. It was therefore, of interest to study in detail the DNA-damaging action of a characterizable free radical species of NCS chromophore produced by a

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[1] Abbreviations: $PF_{5.0}$, 20 mM potassium hydrogen phosphate and 100 mM sodium formate buffer, pH 5.0; poly(dA-dT), alternating poly(deoxyadenylic-thymidylic acid) copolymer; NCS, neocarzinostatin; apo-NCS, protein component of NCS; holo-NCS, NCS–chromophore–protein complex; HPLC, high-performance liquid chromatography; DNase II, deoxyribonuclease II; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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