Identification of the Single-Strand Regions in *Escherichia coli* 5S RNA, Native and A Forms, by the Binding of Oligonucleotides†

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ABSTRACT: The binding of oligonucleotides to *Escherichia coli* 5S RNA has been interpreted on the basis of a previous study of the binding of oligonucleotides to synthetic polynucleotides. Those oligonucleotides whose binding constants certified that the entire sequence of the oligomer was binding to the RNA were classed as binding. Those oligonucleotides that were definitely not binding to sites complementary to the entire oligomer sequence were classed nonbinding, while those with intermediate binding constants were interpreted as binding to sites with unfavorable conformations or to sites present on a small fraction of the molecules. Binding and intermediate oligonucleotides whose complementary sequences occurred more than once in the 5S RNA sequence were assigned sites overlapping those of oligonucleotides whose complementary sequences occurred only once. Five single-strand regions were identified in the native conformation of 5S RNA: positions 10–14, 28–31, 39–49, 60–62, and 78–82. If very strong oligonucleotide–5S RNA interactions are assumed capable of interrupting a few weak intramolecular pairings, the results support the model of Fox and Woese (Fox, G. E., and Woese, C. R. (1975), *Nature (London) 256,* 505). These same regions and an additional one at 95–98 are identified in the closely related A form of the molecule. Furthermore, the regions 10–14 and 60–62 show greater availability in the A form, whereas the region 39–49 is less available than in the native form.

The secondary structure of *Escherichia coli* 5S RNA has been studied by a variety of physical and chemical methods (for a list of references, see Siddiqui, 1973; Kearns and Wong, 1974). At the time these experiments were done, no uniquely plausible secondary structure, analogous to the tRNA cloverleaf, existed to guide experiments, and no unique structure seemed to satisfy all experimental evidence. However, the determination of the sequences of 5S RNAs from a large variety of organisms has provided additional constraints on structural models. Fox and Woese (1975) have proposed a secondary structure for 5S RNA consisting only of helical regions common to all known 5S RNA sequences. Physical and chemical studies of the molecule can now be compared against this a priori plausible model. In particular, one approach that has been used to identify the exposed regions of an RNA molecule has been the specific binding of oligonucleotides to the single-strand sequences in the molecule. This method has been used to study tRNA (Uhlenbeck et al., 1970; Uhlenbeck, 1972), and many of the assumptions upon which it rests have been tested by studying the binding of oligonucleotides to synthetic polynucleotides (Lewis et al., 1975). An earlier report (Lewis and Doty, 1970) showed that specific sequences in *E. coli* 5S RNA could be identified as single stranded by the binding of oligonucleotides. Our subsequent studies have shown that the 5S RNA used in this first study was a mixture of native and A form 5S RNA. The A form has been shown to be closely related to the native form and very different in structure from the denatured B form, but nevertheless only partially able to compete with the native form upon reconstitution of ribosomal subunits (Aubert et al., 1968). Here the binding of oligonucleotides to 5S RNA is characterized in greater detail, and the bindings to the native and the A forms have been separately studied. The differences in oligonucleotide binding observed indicate substantial differences in tertiary structure for these two molecules.

Experimental Procedure

Preparation of 5S RNA. 5S RNA was prepared from *E. coli* B by the procedure of Comb and Zehavi-Willner (1967), modified so that either 1 M NaCl or 0.01 M $MgCl_2$ was always present. Final purification was by Sephadex G-100 gel filtration chromatography to yield 7–14 mg of 5S RNA per lb of cells. Rechromatography in 1 M NaCl of the 5S RNA peak (Figure 1a) shows the product to be free of large ribosomal RNA, tRNA, and also of denatured B form 5S RNA, which would aggregate in 1 M NaCl and elute in the exclusion volume of the column. This material was precipitated with ethanol and used as native 5S RNA.

Denatured 5S RNA was prepared by heating at 60 °C in the absence of magnesium (Aubert et al., 1968). The A and B forms were separated by gel filtration chromatography (Figure 1b). The A form eluted from this column was precipitated with ethanol and used for these experiments. The B form was not tested because of its tendency to aggregate in the high salt buffers used to assay oligonucleotide binding.

Synthesis of Oligonucleotides. Tritium-labeled oligonucleotides were synthesized using primer-dependent polynucleotide phosphorylase as previously described (Uhlenbeck et al., 1970; Lewis, 1971).

Measurement of Oligonucleotide Binding. The binding of oligonucleotides to 5S RNA was measured by equilibrium dialysis, as previously described (Uhlenbeck et al., 1970), except that the equilibration buffer used in these experiments was 1 M NaCl, 5 mM EDTA, and 10 mM cacodylate (pH 7.2). The $MgCl_2$ previously used (Uhlenbeck et al., 1970; Lewis and Doty, 1970) was omitted because a few oligomers rich in G residues attain equilibrium very slowly in the presence of magnesium. In the absence of magnesium, most oligonucleotides were completely equilibrated within 3 days, although a very few took as long as 6 days.

Calculation of the Binding Constant K. In these experiments

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the total concentration of 5S RNA ranged from 30 to 50 μM, while that of the oligomers ranged from 0.1 to 2.0 μM. Assuming one binding site per 5S RNA molecule, the equilibrium binding constant (K) is related to the experimentally measured ratio (R) of the counts inside the dialysis membrane to counts outside by the equation:

$$K = [(R - 1)830\,000]/A_{260}$$

where A_{260} is the absorbance (inside the membrane) due to 5S RNA. The extinction coefficient is from Scott et al. (1968).

The extent of binding ($R - 1$) increases linearly with increased 5S RNA concentration over the range of RNA concentrations used in these experiments.

A determination of the number of binding sites per molecule can be made by measuring the binding over a large range of ratios of RNA to oligomer concentration, and plotting the results in the Scatchard convention (Edsall and Wyman, 1958). Unfortunately, only a few of the oligomers found to bind to 5S RNA can be synthesized by our methods in sufficient quantity to permit this analysis. In addition, oligomers rich in G residues tend to aggregate at high concentrations (Jaskunas et al., 1968; Lewis et al., 1975). Thus, except where otherwise indicated, all of the K values reported here have been measured at only one ratio of RNA to oligomer concentration and calculated assuming one site per molecule. This assumption is not unreasonable since 5S RNA contains only one or a few copies of the Watson–Crick antisequences of the tri- and tetranucleotides used. However, some complications might be introduced by the presence in *E. coli* strain B of sequence variability at positions 3, 12, 13, and 116 (Pace and Pace, 1971).

The Classification of Oligomer Binding. To decide if an oligomer is binding to its Watson–Crick complementary sequence on the 5S RNA, or is involved in some weaker, less specific interaction, the binding constant (K) of the oligomer is compared to the K values of other oligomers binding to 5S RNA. A classification of "binding", "not binding", or "intermediate" is then made according to the rules previously suggested (Lewis et al., 1975). A classification of binding means that the Watson–Crick complement of the entire oligomer sequence is available for binding. A classification of nonbinding means that no more than some part of the entire complement is available. A classification of intermediate could mean that the particular oligomer is not binding to its entire sequence but is one of the few oligomers in which a nonbinding base makes a larger than usual contribution to K. Alternatively, an intermediate classification could mean that the entire sequence is binding but that the binding site is sterically unfavorable for binding the entire sequence. A final possible cause of intermediate binding is that, contrary to our assumption, the binding is only to a small fraction of the 5S RNA molecules, indicating the presence of different conformations or sequence variants.

Results

The Binding of Oligomers to Native 5S RNA. Binding constants were measured for 46 trimers, 7 of which contain the sequence G-G and will be considered separately in the following section. Each oligomer sequence is complementary to some part of the 5S RNA sequence. Any trimer containing neither a G nor a C was not included, since it would not be expected to exhibit detectable binding, even if its entire Watson–Crick antisequence were available for binding. A trimer containing only one G or C was classified as binding if any binding greater than experimental error was detected. However, the converse is not true. A trimer of this class not exhibiting binding greater than experimental error was not classified as not binding. Several of these oligomers bound so weakly to polynucleotides that it is unlikely that any possible binding to 5S RNA could be detected in these experiments. A trimer with a G + C content of 2 was classed as nonbinding only if it had a K less than 1000. All trimers with a G + C content of 2 and with a K between 1000 and 2000 were arbitrarily classed as intermediate, and as binding with a K greater than 2000. Similarly, with the trimers with a G + C content of 3, the limits were arbitrarily set at a K of greater than 1000 but less than 4000 for intermediate, and greater than 4000 for binding.

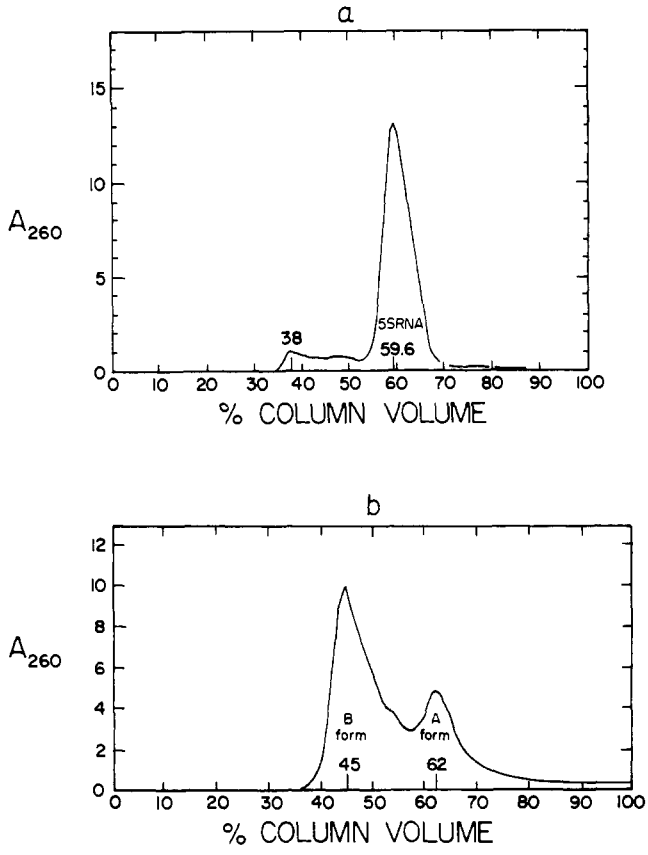

FIGURE 1: Chromatographic and electrophoric properties of 5S RNA. (a) Gel filtration chromatography of a sample of native 5S RNA already purified on a similar column (not shown). The column was Sephadex G-100, eluted with 1.0 M NaCl–0.01 M $MgCl_2$–0.01 M KOAc. (b) Chromatographic separation of form A and form B denatured 5S RNA. The conditions used were the same as in a.

The binding constants of trimers to 5S RNA are given in Table I. The complementary sequences of three of these trimers are contained only once in the 5S RNA sequence. Six trimers definitely do bind and eight definitely do not. Eleven trimers are classed intermediate. In 14 cases no binding was detected, but the K values of such sequences might be too low to detect.

Binding constants were measured for 57 tetramers, 10 of which contain the sequence G-G and will be considered separately. Each oligomer sequence is complementary to some part of the 5S RNA sequence. Those 29 tetramers whose K values are too low to be binding the entire sequence are listed in Table II. The 15 tetramers listed in Table III displayed intermediate binding. The binding constant of one pentamer was also measured, and was found to be intermediate. The three tetramers in Table IV have antisequences in 5S RNA that are definitely available for binding.

The Binding of Oligomers Containing G-G. Because G-G was the only dinucleotide whose binding was large enough to measure, this class of oligomers is considered separately.

TABLE I: Binding of Trinucleotides to Native 5S RNA.[a]

Oligomer	$A_{260}{}^{B}$ [b]	$A_{260}{}^{F}$ [b]	R	K
	A. Binding			
*A-C-A	37.2	5.8	1.06	1600
	27.8	1.4	1.08	2500
A-C-G	25.8	0.8	1.12	4300
	33.6	2.2	1.18	4500
A-C-U	27.4	0.8	1.05	1600
G-A-G	29.4	1.6	1.11	3300
G-C-G	41.8	3.0	1.32	6800
	34.7	0.6	1.36	8800
*G-U-G	41.2	1.4	1.20	4200
	35.0	0.6	1.22	5300
	B. Intermediate			
A-C-C	43.8	3.2	1.05	1000
C-A-G	(29.9	1.8)	1.06	1900
C-C-C	(29.9	1.8)	1.06	1900
C-C-G	(29.9	1.8)	1.10	3000
C-G-C	36.0	1.4	1.05	1200
C-U-G	(33.0	1.8)	1.05	1400
G-C-A	(30.0	2.4)	1.05	1600
G-C-C	(30.0	2.4)	1.12	3700
G-C-U	(30.0	2.4)	1.06	1800
*G-U-C	(30.0	2.4)	1.06	1800
U-C-G	25.4	1.4	1.04	1300
	C. Nonbinding			
C-A-C	29.7	1.2	1.00	0
C-C-A	30.2	2.5	1.01	200
C-C-U	32.2	1.8	1.03	800
C-G-U	(33.0	1.8)	1.03	800
C-U-C	33.7	1.8	1.03	700
G-A-C	(30.0	2.4)	1.02	700
U-C-C	(26.8	1.1)	1.00	0
U-G-C	28.2	0.8	1.02	600
	D. Not Classified			
A-A-G	25.4	1.0	1.02	800
A-G-A	28.6	1.4	1.01	400
A-G-U	28.6	1.4	1.02	700
A-U-C	26.4	0.5	0.99	0
A-U-G	28.2	1.1	1.00	0
C-A-U	(29.9	1.8)	1.00	0
C-U-A	(33.0	1.8)	1.01	200
C-U-U	(30.0	2.4)	1.00	0
G-U-U	(30.0	2.4)	1.03	1000
U-A-C	(30.0	2.4)	1.00	0
U-C-A	(30.0	2.4)	1.01	300
U-G-A	(26.8	1.1)	1.01	400
U-C-U	(26.8	1.1)	0.98	0
U-U-C	(26.8	1.1)	1.01	400

[a] In all experiments, a K of less than 600 is indistinguishable from no binding. The asterisk indicates an oligomer whose complementary sequence is contained only once in the 5S RNA sequence. [b] The absorbance at 260 nm of the solution in the side of the chamber with ($A_{260}{}^{B}$) and without ($A_{260}{}^{F}$) 5S RNA. The absorbance was in each case calculated from the absorbance of a 5-μL aliquot diluted to 1.00 mL. In a few cases, the absorbance was not measured for each experiment, but rather the average of the absorbances of other experiments in the same set, using the same sample of 5S RNA, was used. Such A_{260} values are indicated by parentheses.

TABLE II: Tetranucleotides Whose Binding to Native 5S RNA Was Not Significant.

A. Tetramers Classified as Nonbinding because of a K under the Minimum Limit (K = 1000 for 25% G + C, K = 3000 for 50% G + C)

Oligomer	$A_{260}{}^{B}$	$A_{260}{}^{F}$	R	K
A-C-C-A	27.6	1.6	1.08	2400
A-C-C-C	(28.1	1.6)	1.09	2800
A-C-U-A	(28.1	1.6)	1.02	600
A-C-U-C	28.6	1.6	1.06	2000
A-C-U-U	28.6	2.4	1.03	800
A-U-G-C	(28.7	2.2)	1.04	1300
C-A-C-A	33.4	2.1	1.03	800
C-A-C-C	34.0	5.2	1.07	1900
C-A-C-U	28.8	2.0	1.04	1100
C-A-G-U	(34.0	5.2)	1.03	800
C-A-U-C	(34.0	5.2)	1.04	1200
C-A-U-G	36.0	5.0	1.06	1600
C-U-A-A	33.6	2.0	1.01	200
C-U-A-C	34.6	2.0	1.02	400
C-U-C-U	35.8	2.8	1.09	2200
C-U-G-A	34.6	1.2	1.10	2400
C-U-U-C	32.6	2.6	1.12	3000
U-A-C-U	35.2	3.5	1.00	0
U-C-A-G	32.0	4.8	1.05	1500
U-C-U-C	34.7	3.1	1.08	2000
U-C-U-G	35.4	3.2	1.07	1900
U-U-C-C	33.2	1.4	1.04	1100
U-U-C-G	33.4	1.6	1.11	2800
U-U-U-C	33.4	1.6	1.03	700

B. Tetramers Classified as Nonbinding because of a K Not Significantly Greater Than the Sum of the K Values of Their Two Constituent Trimers

Oligomer	$A_{260}{}^{B}$	$A_{260}{}^{F}$	R	K	K_{tri}
A-G-U-U	(28.7	2.2)	1.07	2300	1600
C-C-U-G	37.8	3.4	1.15	3600	2200
U-A-A-G	33.0	2.8	1.04	1200	600[a]
U-A-C-G	32.2	3.0	1.15	4300	4400
U-G-C-C	33.7	3.2	1.13	3600	4300

[a] This is only the K for AAG. The binding of UAA was not measured.

Those trimers containing the sequence G-G can be classified by comparing their K values to the K of G-G, especially if the third base of the trimer is A or U, since the second dimer would then be expected to have a very low K (Lewis et al., 1975). The K values for these oligomers are listed in Table V. It is clear that G-G-U and G-G-C are binding to their Watson–Crick antisequences since they have K values 8 and 12 times, respectively, the K for the binding of G-G. Thus, there must be at least two sites for G-G. The other trimers are binding at the intermediate level or only as the dimer G-G. These results bring to eight the total number of trimers identified as binding.

The tetramers containing the sequence G-G are to be classified by comparison with their constituent trimers. The K values measured for these tetramers are listed in Table VI. The first four tetramers are controls whose entire complementary sequences are not present in 5S RNA so that they can only bind as trimers. As expected, their K values are not larger than the sum of the K values of their constituent trimers. Similarly, the tetramers A-G-G-U, A-U-G-G, and A-A-G-G are not binding as tetramers even though their entire complementary sequences are present. C-A-G-G binds better than its trimers, but by less than twofold, so it is still classed as nonbinding.

The remaining tetramers bind two to four times as well as do their constituent trimers and are thus classed as intermediate by our general criteria, bringing to 21 the total number of intermediate tetramers. One possible cause of an intermediate classification is that the tetramer is only binding as a trimer, but that the nonbinding base makes, in some way, a contribution to K. Since the addition of nonbinding bases to oligomers containing the sequence G-G has been studied with synthetic polynucleotides (Lewis et al., 1975), this possibility can be evaluated for these tetramers.

G-G-C-C and U-C-G-G each involve adding a pyrimidine to the more strongly binding trimer adjacent to another py-

TABLE III: Tetranucleotides Displaying Intermediate Binding to Native 5S RNA.

Oligomer	$A_{260}{}^B$	$A_{260}{}^F$	R	K	K_{tri}
*A-U-C-G[a]	(28.7	2.2)	1.14	4 400	1300
*C-C-C-C	34.0	2.0	1.44	11 400	3800
C-C-G-C	(34.0	5.2)	1.48	13 700	4200
*C-G-C-A	34.6	2.0	1.35	8 900	2800
	31.6	1.2	1.51	13 900	
*C-G-C-C	34.0	5.2	1.42	12 200	4900
C-G-C-U	39.4	7.1	1.52	13 400	3000
*C-U-C-G	33.4	2.0	1.25	6 600	2000
G-A-C-C	32.8	2.2	1.27	7 300	1700
*G-A-G-A	34.8	2.2	1.51	13 000	3700
*G-C-C-U	32.6	2.0	1.67	18 200	4500
*G-C-G-U	24.4	0.6	1.94	32 800	8600
	33.7	2.7	2.65	44 200	
*G-C-G-U-U	(30.0	2.4)	5.70	141 000	
*G-U-C-A	33.5	2.2	1.34	9 000	2100
*G-U-U-U	34.2	1.3	1.09	2 300	1000
*U-C-C-C	30.9	1.9	1.19	5 400	1900
*U-C-G-C	33.4	1.9	1.34	9 100	2500

[a] The asterisk indicates an oligomer whose complementary sequence is contained only once in the 5S RNA sequence.

TABLE IV: Tetranucleotide Binding to Native 5S RNA.

Oligomer	$A_{260}{}^B$	$A_{26}{}^F$	R	K	ΣK_{tri}
*G-A-G-U[a]	34.2	3.8	2.40	38 200	4000
	22.9	2.2	2.12	45 000	
G-C-A-U	34.0	5.2	1.42	12 100	1600
G-U-U-C	34.0	1.8	1.74	20 000	1400

[a] The asterisk indicates an oligomer whose complementary sequence is contained only once in the 5S RNA sequence.

TABLE V: Binding to 5S RNA of G-G and of All Trinucleotides Containing the Sequence G-G.

Oligomer	Potential sites[a]	K	
G-G		4 300	4 500
A-G-G	3, 11 (U), 30	5 000	5 800
C-G-G	11 (G), 42, 62, 70	7 000	10 000
G-G-A	25, 89	7 700	
G-G-C	2, 10, 41, 61, 69, 112	48 900	56 700
G-G-G	26, 35, 36, 90, 91	16 300	19 200
G-G-U	29, 34	35 200	35 600
U-G-G	27, 37, 92, 113	3 100	3 600

[a] The numbers identify the first residue of all sites on 5S RNA (see Figure 6) complementary to the given trimer. A U or G in parentheses behind a number indicates that this position is a potential site only on the sequence variant with the specified base at position 13.

rimidine. It has been shown that in such cases the nonbinding base does not increase the K of the trimer. A-C-G-G and A-G-G-C involve adding an A residue to the more strongly binding trimer. In no case with the experiments binding oligomers to polynucleotides did adding one nonbinding A residue to an oligomer increase the K. Thus the intermediate values of K shown by these four tetramers cannot be attributed to a larger than usual effect upon K of a nonbinding base. Reasonable explanations for these intermediate K values would include steric hindrance to the binding of the entire tetramer and the binding of these oligomers to other than one site per molecule of 5S RNA.

In contrast, U-G-G-C and C-G-G-C involve adding a pyrimidine adjacent to a G, a situation that often involves a two- or threefold increase in K even if the added base is nonbinding. Furthermore, in the case of U-G-G-C, if the U residue were binding, only a two- or threefold increase in K compared to G-G-C would be expected, since the UG double-strand stacking interaction seems to be quite weak. Thus, we cannot rule out the possibility of a nonbinding base raising the trimer K enough to cause an intermediate classification. The fivefold increase seen for C-G-G-C is, however, significantly greater than the effect of nonbinding C residue seen in the polynucleotide studies.

Scatchard Analysis. To investigate for two examples the possibility that the intermediate values of K observed might be due to binding to other than one site per RNA molecule, the binding of G-G-C and of A-G-G-C to 5S RNA was measured at several oligomer concentrations. There are six complementary sequences to G-G-C and 1.2 to A-G-G-C (because of the sequence variation at position 13). The intermediate value of K for A-G-G-C could be explained if G-G-C was binding to more sites than was A-G-G-C. The results are plotted in the Scatchard convention in Figure 2.

The plot for G-G-C shows considerable curvature, indicating more than one type of binding site. A quantitative interpretation is precluded by the limited range of oligomer concentration experimentally accessible and by the scatter of the data. It does seem apparent, however, that there is a small class of sites, contained only on a few molecules of 5S RNA, to which G-G-C binds very well, and a larger class of sites to which the oligomer binds less well. The parameter values given in Figure 2 provide a reasonable, but not unique, fit to the data.

The plot for A-G-G-C is much less curved. The best straight line through these points gives 0.6 site per molecule, with a K = 200 000. Alternatively, the curved line drawn in Figure 2 corresponds to two types of binding. The first is binding of A-G-G-C as the trimer G-G-C to the small class of sites to which G-G-C binds well. The K of A-G-G-C is very similar to

TABLE VI: Binding to 5S RNA of Tetranucleotides Containing the Sequence G-G.

Oligomer	K	K of the 5′ constituent trimer	K of the 3′ constituent trimer	Classification
A-G-G-A	11 400	5 000	7 700	
	12 600	5 800		
G-G-A-A	10 300	7 700	Sequence not present	
G-G-U-U	26 900	35 200	1 000	
	30 400	35 600		
U-G-G-U	42 400	3 100	35 200	
		3 600	35 600	
A-G-G-U	25 000	5 000	35 200	
	31 500	5 800	35 600	
A-U-G-G	4 200	0	3 100	
			3 600	
A-A-G-G	5 200	800	5 000	
	5 700		5 800	
	6 200			
C-A-G-G	11 000	1 900	5 000	
			5 800	
G-G-C-C	105 000	48 900	3 700	I
	115 000	56 700		
U-C-G-G	21 200	1 300	7 000	I
	26 400		10 000	
U-G-G-C	104 000	3 100	48 900	I
		3 600	56 700	
A-C-C-G	37 200	4 300	7 000	I
		4 500	10 000	
A-G-G-C	129 000	5 000	48 900	I
	129 000	5 800	56 700	
C-G-G-C	239 000	7 000	48 900	I
	264 000	10 000	56 700	

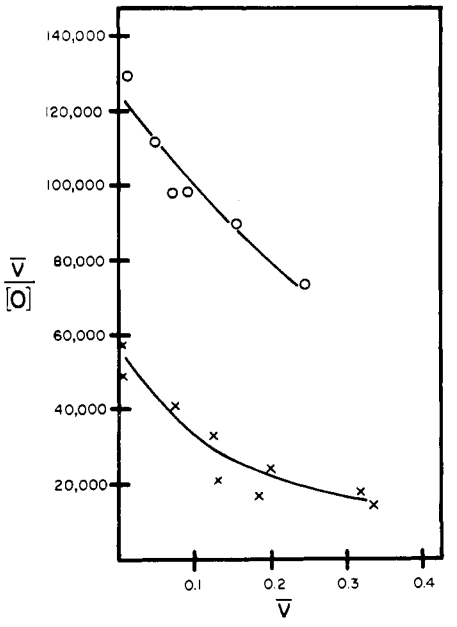

FIGURE 2: Binding of G-G-C (×) and A-G-G-C (○) to 5S RNA. $\bar{v}$ is the fraction of 5S RNA with a bound oligomer, and [O] is the concentration of the free oligomer. The lines correspond to $n_1 = 0.1$, $K_1 = 400\,000$, $n_2 = 1.0$, and $K_2 = 15\,000$ for G-G-C, and $n_1 = 0.1$, $K_1 = 500\,000$, $n_2 = 0.6$, and $K_2 = 125\,000$ for A-G-G-C.

the K of G-G-C for this set of sites. The second is binding of A-G-G-C as a tetramer to more numerous sites. The K for this binding is much larger than the K of G-G-C to these sites. The nonintegral value of n_2 (the number of binding sites of the second class per molecule of RNA) and also the nonintegral value of n if a straight line is drawn suggest that the binding site is position 10–13, although the ratio of molecules with U at position 13 to molecules with G at position 13 would be somewhat higher than given by Pace and Pace (1971). The only other possible site for A-G-G-C is position 2–5, which is base paired (Jordan, 1971). Such an assignment would also explain the intermediate K value of C-G-G-C, which would be the complement to those RNA molecules having a G residue at position 13. Similarly the molar K values of C-G-G and A-C-G-C would be 2.5 times the values listed above since there would be only 0.4 site per molecule. If C-G-G and A-C-G-G bind to position 11–14, then A-G-G and A-A-G-G should also bind. The K of A-G-G is not inconsistent with binding, and although A-A-G-G does not bind, neither does it bind well to poly(U,C) (Lewis, 1971).

Thermal Stability of Oligomer–5S RNA Complexes. It can be shown that at least several of the oligomer–RNA associations measured here exhibit the thermal stability expected for the association of complementary nucleic acid sequences. The 5S RNA is in great molar excess so that the dissociation of the complex must be measured in terms of the oligomer. It follows that the fraction (f) of the oligomer bound to 5S RNA at any temperature is given by the equation: $1 - f = 1/R$.

To study the entire thermal dissociation curve at temperatures accessible in aqueous solutions, only the dissociations of very strong complexes were examined. Accordingly, the dependence of f upon temperature for the binding of A-G-G-C and C-G-G-C to 5S RNA, each of four RNA concentrations, is shown in Figure 3. A portion of the binding of these tetramers is possibly due to the binding of the G-G-C sequence to a site on a small fraction of the 5S RNA molecules. Thermodynamic values measured here will thus represent the average of this interaction and the expected binding of the tetramers to their antisequences. These results cannot be used to determine precise thermodynamic parameters for the A:U and G:C bonds, but they can show the stability of these complexes to be in the range expected.

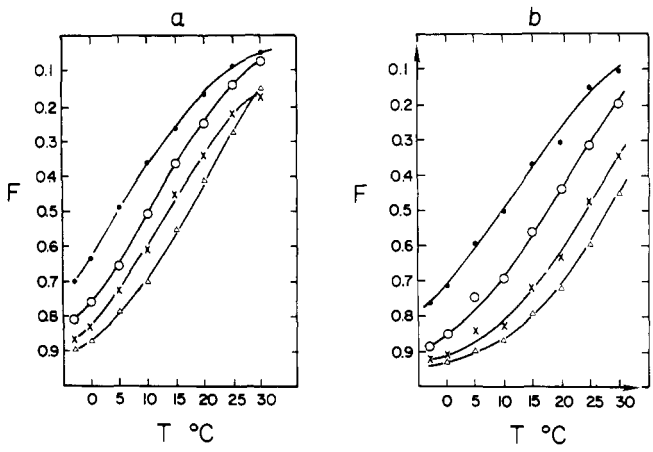

FIGURE 3: The melting of oligomer binding to 5S RNA. All experiments were done in 1.0 M NaCl, 5 mM EDTA, and 10 mM cacodylate (pH 7.2). Three days were allowed for equilibration at each temperature. (a) A-G-G-C binding to 5S RNA at RNA concentrations of 10 μM (•), 20 μM (○), 32 μM (×), and 53 μM (△). (b) C-G-G-C binding to 5S RNA at RNA concentrations of 7 μM (•), 15 μM (○), 34 μM (×), and 52 μM (△).

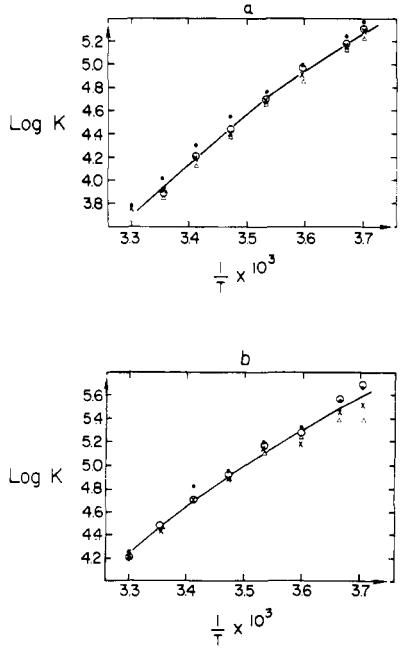

FIGURE 4: van't Hoff plots of melting curves from Figure 3. Symbols refer to same RNA concentrations as in Figure 3: (a) A-G-G-C; (b) C-G-G-C.

Besides showing the T_m values of these complexes, the data in Figure 3 allow the calculation of ΔH in three ways. The simplest way to obtain ΔH is from the van't Hoff equation:

$$\frac{d \log K}{d(1/T)} = \frac{-\Delta H}{2.3R} \tag{1}$$

van't Hoff plots of the melting curves of A-G-G-C and C-G-G-C are shown in Figure 4. Log K values are consistently slightly higher at lower RNA concentrations, indicating a slight association of the 5S RNA. The calculation of ΔH would be unaffected since the slope is identical at all RNA concentrations. However, this slope is not constant, but rather ΔH is about −4 kcal/mol greater at the higher temperatures. This result can be rationalized in terms of the contribution to ΔH of single-strand stacking. At higher temperatures both oligomer and site would be less stacked as single strands, increasing the enthalpy change for forming a complex. Such an effect has been postulated to explain the enthalpies for melting self-complementary oligomers of the series A_nU_n (Martin et al., 1971).

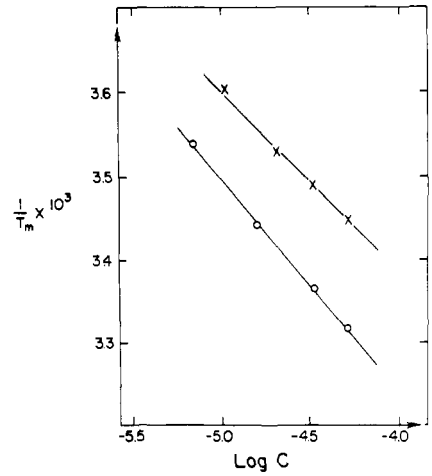

FIGURE 5: Concentration dependence of the melting of A-G-G-C (×) and C-G-G-C (○) from 5S RNA.

TABLE VII: ΔH for the Binding of A-G-G-C and C-G-G-C to 5S RNA.

Method of calculation	ΔH for C-G-G-C (kcal/mol)	ΔH for A-G-G-C (kcal/mol)
Eq 1		
Slope at low T	−16.3	−14.9
Slope at high T	−20.0	−18.8
Eq 2		
20 μM 5S RNA	−18.3	−17.0
53 μM 5S RNA	−19.2	−18.5
Eq 3	−21.9	−18.7

Applequist and Damle (1965) have provided an analysis that allows calculation of ΔH directly from the melting profile. Their result was calculated for the condition that both components are present in equal concentration. If their equations are rewritten for the case in which one component is present in great excess so that its concentration as an uncomplexed species is invariant, the result is:

$$\left(\frac{df}{dT}\right)_m = \frac{\Delta H}{4RT_m^2} \tag{2}$$

where m signifies the slope of the melting curve near T_m.

The same model can also be used to predict that ΔH can be obtained from the concentration dependence of T_m:

$$\frac{1}{T_{m'}} = \frac{1}{T_m} + \frac{2.3R}{\Delta H} \log \frac{C'}{C} \tag{3}$$

where C is the total molar concentration of strands, which is essentially equal to the concentration of 5S RNA in this case. Note that the number of binding sites per molecule is irrelevant to the calculation of ΔH. The dependence of T_m on concentration for the melting of A-G-G-C and C-G-G-C is shown in Figure 5 and is linear as expected.

The compilation in Table VII shows the ΔH values calculated from all three equations to be in close agreement.

The Binding of Oligomers to A Form 5S RNA. Because the B form of denatured 5S RNA aggregates at the high ionic strengths used in equilibrium dialysis experiments, only oli-

TABLE VIII: Binding to A Form 5S RNA of Those Oligomers That Identify No Differences between the Two Conformations.

Oligomer	R	K	Oligomer	R	K
A-C-A	1.03	1 100	C-U-G-A	1.08	2 600
A-G-A	1.04	600	C-U-U-C	1.06	2 000
A-C-C	1.05	1 000	G-A-C-C	1.14	5 500
A-C-G	1.12	5 000	G-C-A-U	1.34	13 800
A-C-U	1.05	1 100	G-C-C-U	1.42	13 200
G-G-U	2.21	49 700	G-C-G-U-U	5.34	178 000
G-U-G	1.08	3 200	G-G-C-C	4.70	152 000
U-G-G	1.06	2 600	G-U-U-C	1.68	27 800
A-C-C-C	1.05	2 100	U-A-A-G	1.02	600
A-C-U-U	1.02	1 000	U-A-C-U	1.03	900
A-G-U-U	1.08	3 400			
A-U-G-C	1.02	700	U-C-A-G	1.06	2 400
C-A-C-A	1.03	1 200	U-C-C-C	1.27	8 300
C-A-C-U	1.04	1 900	U-C-G-G	1.86	35 500
C-A-G-G	1.33	15 000	U-C-U-G	1.05	1 500
C-A-G-U	0.99	0	U-G-C-C	1.06	1 700
C-A-U-C	1.02	600	U-U-C-C	1.05	1 400
C-A-U-G	1.02	600	U-U-U-C	1.01	300
C-G-C-C	1.27	8 600	U-G-G-C	3.44	77 400
C-U-A-A	1.01	400			

gomer binding to the A form of denatured 5S RNA was investigated. Binding was measured for 1 dimer, 15 trimers, 51 tetramers, and 1 pentamer. The binding of an oligomer to the A form was compared with its binding to the native form. A substantial difference for the binding of a particular oligomer identified a sequence at which the two conformations differed. Table VIII lists those 38 oligomers whose K values are not substantially different (less than a 50% increase or a 30% decrease) for the two forms. Table IX lists those 30 oligomers that do bind differently to the A form. All but four of these bind better to the A form than to native 5S RNA.

TABLE IX: Oligomers That Bind to A Form 5S RNA Differently Than to Native 5S RNA.

Oligomer	R	K	$K_{\text{A form}}/K_{\text{native}}$
C-C-C-C	1.10	3 900	0.34
G-A-G-U	1.24	9 800	0.24
G-U-C-A	1.15	4 900	0.54
G-U-U-U	1.05	1 400	0.61
G-G	1.19	7 800	1.8
A-A-G	1.06	1 300	2.2
A-G-G	1.30	12 400	2.3
A-G-U	1.07	1 400	2.0
C-G-C	1.12	4 800	4.0
C-G-G	1.42	17 000	2.0
G-C-G	1.36	14 600	1.9
G-G-A	1.41	16 900	2.2
A-A-G-G	1.25	9 800	1.7
A-C-C-A	1.16	6 000	2.5
A-C-U-A	1.04	1 700	2.8
A-C-U-C	1.09	3 900	2.0
A-C-G-C	4.62	163 000	4.4
A-G-G-C	6.00	220 000	2.2
A-G-G-U	3.48	102 000	3.6
A-U-C-G	1.22	10 000	2.3
A-U-G-G	1.23	8 900	2.1
C-C-U-G	1.24	9 200	2.6
C-G-C-A	2.44	59 200	5.2
C-G-C-U	1.64	24 800	1.8
C-G-G-C	12.06	454 000	1.8
C-U-C-U	1.16	5 100	2.3
G-A-G-A	1.60	23 000	1.8
G-C-G-U	4.14	129 000	3.4
U-C-G-C	1.56	17 500	1.9
U-C-U-C	1.18	5 600	2.0

Discussion

General Considerations about Oligomer Binding to 5S RNA. What these experiments can say about the structure of 5S RNA is limited by the lack of Scatchard plots to certify that the oligomer binding observed is to one site per RNA molecule. Indeed the only two Scatchard experiments done gave puzzling results. To be sure, the tetramers studied in this experiment were classed as intermediates so that one could expect that oligomers classed as binding might give more clear-cut results, had it been possible to synthesize sufficient quantities of these for the experiment. Nevertheless, one cannot assert that the exposed sequences identified are present on every molecule of 5S RNA. However, the limited number of such sequences identified hints strongly that most of these sequences are exposed on most of the RNA molecules in the population.

These oligomer–RNA complexes have the stability expected for the association of complementary nucleic acid sequences. Studies with self-pairing oligonucleotides indicate a ΔH of -5.5 kcal/mol for an A:U pair (Martin et al., 1971) and several kcal/mol higher for a G:C pair (Uhlenbeck et al., 1971). Thus the values of ΔH in Table VII are in the right range. Uhlenbeck (1972) has measured ΔH for the binding of U-A-C-A and A-U-G-A to the anticodon loops of *E. coli* $tRNA^{Tyr}$ and $tRNA^{Met}_f$, and found them to be -33.4 and -28.1 kcal/mol, respectively. In view of their lower G + C composition, it is surprising that they have higher enthalpies of binding than do A-G-G-C and C-G-G-C, but this may be due to an especially favorable conformation of the anticodon loop.

The T_m values of these complexes may be compared with the T_m values of complexes between tetramers and tRNA anticodon loops. At a 5S RNA concentration equal to the tRNA concentration used by Uhlenbeck (1972), A-G-G-C and C-G-G-C would have T_m values of 20 and 32 °C, respectively, while A-U-G-A and U-A-C-A binding to tRNA exhibit T_m values of 0 and 15 °C. If A-G-G-C and C-G-G-C are binding to 0.6 and 0.4 sites per 5S RNA molecule, then their T_m values at equivalent RNA concentrations would be 24 and 42 °C. To compare the 5S RNA and tRNA results requires an estimate of the dependence of T_m upon G + C content. Studies with self-pairing oligomers yield a value of 0.9 °C increase in T_m

for a 1% increase in G + C content (Uhlenbeck et al., 1971). Although these T_m values are much higher than those of A-U-G-A and U-A-C-A, the difference is not as large as would be expected from the large difference in G + C content. This discrepancy probably reflects an especially favorable conformation of tRNA anticodon loops.

Mapping the Secondary Structure of 5S RNA. We now use these results to determine which parts of the 5S RNA sequence are available for binding to oligonucleotides. If an oligomer is classed as nonbinding, then at least one residue of its antisequence is unavailable for binding. To show that a region is unavailable requires that the oligomers whose complementary sequences overlap that region do not bind. The first step in mapping the secondary structure of the 5S RNA is thus to underline the sequences complementary to the oligomers classed as nonbinding. If the complementary sequence of an oligomer is contained several times in the RNA sequence, each site may be underlined since binding to any one of them would have been detected.

The sequences complementary to those oligomers that do not bind have been underlined with dotted lines in Figure 6. Most of the regions so underlined have been underlined several times. Such regions could not harbor many residues that are available for binding. Single-strand residues one or two bases long could not be identified by our technique, however. Thus, these regions are largely base paired, although it is also possible that the tertiary structure of the RNA may impose upon some single-strand regions a conformation incompatible with double helix formation.

If an oligomer is classed as binding, then its entire complementary sequence is available for binding. To show that a region is available, it is only necessary to underline it once (with a heavy solid line, denoting binding). However, if the sequence complementary to an oligomer is contained several times in the 5S RNA sequence, any one of these sites would account for the binding of the oligomer. The binding sites of some oligomers are easily identified since the oligomer antisequence is contained once in the 5S RNA sequence. Such oligomers (binding and intermediate) are identified by an asterisk (*) in Tables I, III, and IV and in Figure 6. These include 2 binding and 1 intermediate trimer; 1 binding and 14 intermediate tetramers; and 1 pentamer, classed intermediate.

Only tentative assignments can be made for the binding sites of the remaining oligomers, both binding and intermediate, by grouping these oligomers around the previously identified sites to provide the minimum number of single-strand regions consistent with the data. This procedure may not yield the most likely binding site in all cases. Independent evidence (see below) indicates that position 39–42 is the probable binding site for G-C-A-U, even though a site at 94–97 would overlap three unique intermediate tetramers. The assignment of A-G-G-C and C-G-G-C to position 10–13 was rationalized above. However, C-G-G-C is probably also binding to position 41–44 since there are binding tetramers overlapping that position on both sides. C-G-C-U and G-C-U, both classed intermediate, are not shown in Figure 6 since they could be assigned equally well to position 15–18 or 66–69.

Many of the intermediate oligomers can be assigned sites overlapping the sites for binding oligomers. The single-strand regions apparently extend one or several residues beyond the sequences that bind oligomers, but these residues have conformations unfavorable for oligomer binding. Many of the remaining intermediate oligomers are tetramers capable of forming three or four G:C bonds and would thus be expected to exhibit very high K values. The small K values of these oligomers might signify competition for binding sites with intramolecularly paired regions. Such a hypothesis has been put forward to explain the weak binding of several oligomers to the variable region in *E. coli* $tRNA^{Tyr}$ (Uhlenbeck, 1972).

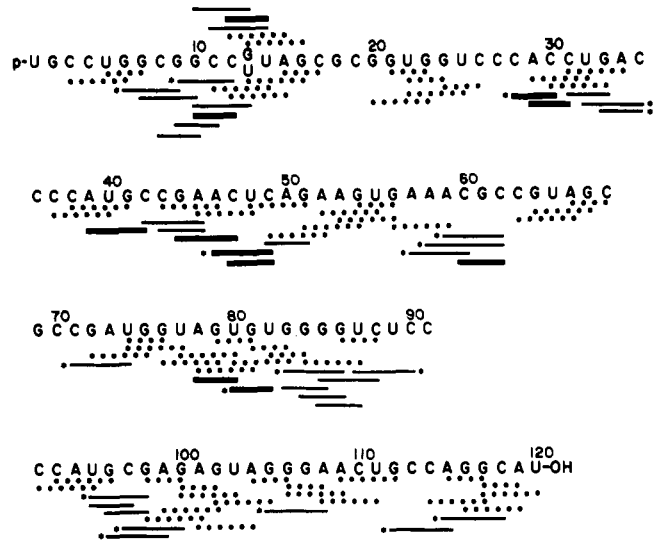

FIGURE 6: Oligomer binding to native 5S RNA. Those sequences underlined with dotted lines are those whose antisequences did not bind. Those sequences underlined with a thin solid line or a heavy solid line are the unique or most probable sites for those oligomers that were classed as intermediate or binding, respectively. The lines marked with an asterisk are the sites that are uniquely determined because they occur only once in the RNA sequence. Lines above the RNA sequence represent oligomers complementary to the variant of 5S RNA with G in position 13.

Figure 6 does contain several apparent contradictions. In three cases (45–48, 78–81, 79–81) a nonbinding oligomer is completely overlapped by binding oligomers. The structure of these two single-strand sequences must be such that the two ends of the sequence are available for base pairing while the center of the sequence is somehow constrained.

Five sequences available for oligomer binding have been identified: positions 10–14, 28–31, 39–49, 60–62, and 78–82. These results are slightly different from our previous study in which the available sequences were identified as 9–13, 25–32, 59–65, and 95–98.

In our earlier study, the sequences 25–27 and 63–65 were considered single strand because they provided binding sites for oligonucleotides containing the sequence G-G that exhibited very large K values. Applying criteria for classifying oligomer binding derived from our subsequent studies with synthetic polynucleotides, several of these oligonucleotides were reclassified as intermediate. For reasons explained above, the most likely binding sites for these molecules are in the region from residue 10 to 14. The region 78–82 was missed in the earlier studies because the trimers A-C-A and A-C-U were not included in the earlier work. The major difference between these results and our earlier results is that the region 39–49 was not originally included as a single-strand region, while the region 95–98 no longer appears single strand.

The 5S RNA used in the earlier experiments had been stored lyophilized after having been dialyzed against distilled water. Such treatment had not been shown to denature RNA. Moreover, these samples were free of any B form 5S RNA upon gel filtration chromatography. However, successive dialysis against water led to changes in K for the binding of several oligomers. It seems probable that this treatment leads to the formation of some conformation resembling A form 5S RNA.

The greatest difference in conformation between the native and A conformation of 5S RNA is the definite existence in the

A form of a single-strand region for 95–98. The K of C-G-C-A increases by a factor of five, warranting a classification of binding to the A form. The K values of G-A-G-A, A-U-G-G, U-C-G-C, U-C-U-C, C-U-C-U, and C-G-C also increase. Thus, the total region of increased availability in the A form compared to the native form stretches from 87 to 103, although only the central portion from 95 to 98 is completely exposed for oligonucleotides to bind. Because this region of native RNA binds intermediate oligomers, it may be that the native population contains a few molecules with this region single strand, while the A form population has many molecules with this region single strand.

The other large changes in K involve oligomer binding to the same regions identified as single strand in the native form. Each of the oligomers binding to position 10–14 did so to the A form about twice as well as to the native, except A-C-G-G, which binds four times as well to the A form and is classed "binding" to the A form, and A-C-G, which is also binding, but no better to the A form than to native 5S RNA.

There is a decrease in availability at position 49 since the K of G-A-G-U decreases by a factor of four. The A form shows greater availability toward the 3′ end of region around residue 60 and lesser availability toward the 5′ end. The K values of G-C-G and G-C-G-U increase twofold and threefold, respectively. However, K for G-C-G-U-U is only slightly higher than for the native form, while K for G-U-U-U is lower in the A form. Thus, the degree of availability of several of the single-strand regions already present in the native form seems to have been changed in the A form. This type of change may represent changes in the constraints placed upon the single-strand regions by the tertiary structure of the molecule.

The sites for the remaining oligonucleotides that show increased binding to the A form are scattered along the 5S RNA molecule, and show no evidence of major change in any one region. These oligomers are all classed as "intermediate". Possibly the small degree of binding observed is due to binding to other conformations that constitute a small fraction of the population of A form 5S RNA molecules.

Comparison of These Results with Other Work. Oxidation with monoperphthalic acid has identified ten unpaired A residues at unknown positions (Cramer and Erdmann, 1968). The single-strand regions identified here contain five A residues. If these regions are extended to include those positions complementary to intermediate tetramers that overlap binding oligomers, then ten A residues are included.

Carbodiimide was used to detect two unpaired U residues: one which reacts very readily and is either at position 14, 65, 77, or 103, and one which reacts more slowly and is at position 40 (Lee and Ingram, 1969). These are accounted for by the first and third single-strand regions identified here. The single-strand regions identified here also contain U residues at positions 48, 80, and 82 that did not react with carbodiimide. Apparently access to carbodiimide can be hindered in such a way that oligomer binding is not prevented.

Completely native 5S RNA is first cleaved by ribonuclease T1 after position 41 or 44 with subsequent cleavages after 13, 56, and 59 (Jordan, 1971; Vigne and Jordan, 1971). Pancreatic ribonuclease is less selective, cleaving after 14, 19, 22, 47, 48, 49, 65, and 70. Likewise, ribonuclease IV preferentially attacks 5S RNA between positions 41 and 45 while sheep kidney nuclease makes a preferred cut after residue 42 or 43 (Bellemare et al., 1972). An exposed region around position 40 is a common feature of several 5S RNAs from diverse organisms (Vigne, et al., 1973). Different results with ribonuclease T1 have been obtained by Mirzabekov and Griffin (1972), who find the first break after nucleotide 61 and other breaks after 16, 18, 56, 101, and 102.

The binding of oligonucleotides to 39–49 is in accord with the great sensitivity of this region to nucleases. The cleavages after residues 13, 14, and 61 are also in regions that bind oligonucleotides. The other cleavages are mostly in both of the two possible sites for the intermediate oligomers C-G-C-U and G-C-U. It is possible some cleavages are in exposed regions too small to bind trimers and tetramers well. Furthermore, many of these cleavages seem to occur only after the most preferred cleavages so that these regions may not be exposed in the intact molecule.

In view of the different susceptibilities of 5S RNA to ribonuclease T1 found by Vigne and Jordan (1971) and by Mirzabekov and Griffin (1972), it is interesting to note that oligonucleotide binding shows increased availability of the A form compared to the native form in region 59–62 and decreased availability at position 49. There may also be decreased availability of 39 to 44 in the A form, but this cannot be shown by these experiments. G-C-A-U (which binds to 39 to 42 in the native form) has the same K binding to the A form, but it may not be binding to the same site as in the native form. An alternate site for G-C-A-U is 94 to 97, which is in a region more exposed in the A form than in the native form. Thus, it appears likely that 5S RNA can exist in two conformations in aqueous solution, the "native" form and the "A" form, which forms easily from the "native" form, even without exposure to denaturing conditions. The conformation studied in our earlier work and the work of Mirzabekov and Griffin (1972) is probably the A form, whereas the "native" conformation of this report appears to be similar to that of Vigne and Jordan (1971). It is not impossible that the A form is even more stable in aqueous solution than is the native form. Indeed, Jordan et al. (1974) have found that the "native" conformation of *Chlorella* 5S RNA does not contain the highly exposed region near position 40 characteristic of the "native" forms of other 5S RNA species. The conformation of *E. coli* 5S RNA studied by Kearns and Wong (1974) was most probably the A form, since their 5S RNA was exposed to 58 °C during isolation.

The information furnished by oligonucleotide binding studies is not in itself sufficient to deduce a secondary structure of 5S RNA. We cannot estimate what fraction of the single-strand regions of the molecule might be protected from oligonucleotide association by the tertiary structure of the molecule. Further, one suspects that in those areas which are accessible to oligonucleotide binding, an oligonucleotide with a very high K (high G + C content) might disrupt weak intramolecular pairings. Examples of both phenomena can be seen by comparing these results with the model of Fox and Woese (1975), which is based on those helical regions common to 5S RNA sequences from many organisms. All of the accessible regions identified here are in single-strand regions of the Fox and Woese model, except for 60–62 (Figure 7). However, in each case the accessible region includes one (or in the case of 39–49, two) base pair of an adjacent helix, suggesting a competition between intramolecular and intermolecular base pairing. Oligonucleotide binding at 60–62 presumably represents the disruption of a weak intramolecular association (two G-C and one G-U pair) by a stronger intermolecular pairing (three G-C pairs). This competition is suggested by the fact that although the binding of G-C-G (which we map as binding at 60–62) is high enough to merit a binding classification, using the somewhat arbitrary rules for trimer binding, it is substantially weaker than that of other binding trinucleotides of comparable or even lower G + C composition (K_{GCG} = 7800 vs. K_{GGC} = 52 800 and K_{GGU} = 35 400).

It must also be noted that the single-strand region is only

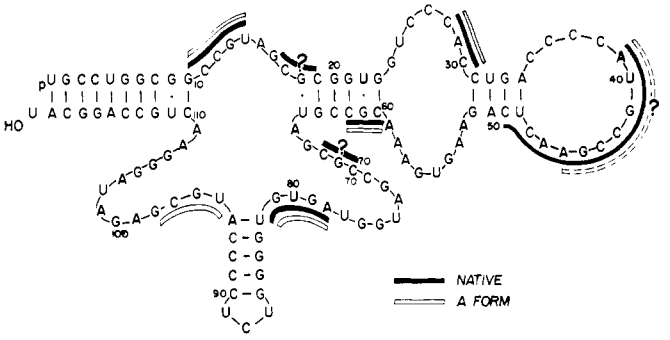

FIGURE 7: Different accessibilities of native and A form 5S RNA to oligonucleotide binding. The secondary structure of *E. coli* 5S RNA is represented according to the model of Fox and Woese (1975), illustrating the sequences of the native molecule and of the denatured A form which are accessible to oligonucleotide binding. As discussed in the text, the binding site for G-C-G could alternatively be placed at 17–19 or 68–70 (indicated by solid bar with a question mark) as well as at 60–62. Whether the region 39–45 (indicated by dashed line and a question mark) is available for oligonucleotide binding in the A form is unknown since the oligonucleotide G-C-A-U, which binds to both native and A form 5S RNA, has in the A form an additional possible binding site partially overlapping the region 95–98, which is available in the A form but not in the native conformation.

partially accessible to oligonucleotide binding. Accessible regions range from 25% of the single-strand region between the "prokaryotic loop" and the "tuned helix" to 70% of the loop connecting the "common arm base" helix. The single-strand region between the "molecular stalk" and the "prokaryotic loop" is not accessible to oligonucleotides in the native form (only intermediate oligonucleotides map there).

The relative accessibilities of different conformations of 5S RNA to oligonucleotide binding are a sensitive probe of changes in secondary structure. The A form shows substantially greater binding at positions 10–14, 59–62, and 95–98, and less binding at 47–50. Since Gray et al. (1973) have shown that 1–11 and 69–120 are involved in binding to protein within the 50S subunit, it is tempting to speculate that these regions might be relatively inaccessible to oligonucleotide binding because they are part of a hydrophobic region of the native tertiary structure. Since the tertiary structure of the A form would not be constrained by binding to ribosomal proteins, it could be free to assume a conformation in which the protein binding and adjacent sequences would be more accessible to oligonucleotide binding.

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