Complementary Oligodeoxynucleotide Probes of RNA Conformation within the *Escherichia coli* Small Ribosomal Subunit†

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ABSTRACT: The large RNA molecule within each ribosomal subunit is folded in a specific and compact form. The availability of specific 16S RNA sequences on the surface of the small ribosomal subunit has been probed by using complementary oligodeoxynucleotides. The hybridization of 8–15-nucleotide-long oligomers to their RNA complements within the subunit was quantitated by using a nitrocellulose membrane filter binding assay. The probes have been grouped into classes on the basis of sequence-specific binding ability under different conditions of ionic environment, incubation temperature, and subunit activation state [as defined by the ability to bind phenylalanyl-tRNA in response to a poly(uridylic acid) message]. Oligodeoxynucleotides complementary to nucleotides flanking 7-methylguanosine residue 527 and to the 3′-terminal sequence bound 30S subunits regardless of the activation state. Oligodeoxynucleotides that complement 16S ribosomal RNA residues 1–16, 60–70, 685–696, and 1330–1339 and the sequence adjacent to the colicin E3 cleavage site at residue 1502 all bound efficiently only to subunits in an inactivated conformation. Probes complementary to residues 1–11 and 446–455 bound only inactivated subunits, and then with low efficiency. Sequences complementary to nucleotides 6–16, 99–109, 1273–1281, and 1373–1383 bound 30S subunits poorly regardless of the activation state. With one exception, each probe was bound by native or heat-denatured 16S ribosomal RNA (as determined by size-exclusion chromatography). We conclude that complementary oligodeoxynucleotide binding efficiency is a sensitive measure of the availability of specific RNA sequences under easily definable conditions.

The *Escherichia coli* ribosome includes within its 2 subunits over 50 different proteins as well as 3 RNA molecules. About two-thirds of the mass of each subunit is contributed by the RNA, which also defines the overall shape and size of each subunit (Noller, 1984; Vasiliev et al., 1978, 1980). The RNA is more than a simple scaffold for ribosomal proteins; e.g., it functions directly in the placement of mRNA (Shine & Dalgarno, 1974) and tRNA (Ofengand et al., 1986; Moazed & Noller, 1986) during protein biosynthesis. Specific RNA segments are involved in the interactions between ribosomal subunits (Santer & Shane, 1977; Herr et al., 1979; Tapprich & Hill, 1986), and the small (5S) molecule is perhaps a peptidyltransferase component (Erdmann et al., 1986).

Information regarding the three-dimensional structure of the RNA within the ribosome remains limited. Immune electron microscopy has allowed localization of several chemically unique sites including the RNA termini and the modified nucleotides N^6,N^6-dimethyladenosine and N^7-methylguanosine [summarized in Stöffler and Stöffler-Meilicke (1984) and Noller et al. (1986)]. Two-dimensional stem–loop structures have been proposed, based on observations of chemical and enzymatic reactivity and sequence comparisons (Noller et al., 1986; Brimacombe et al., 1986). Folding of these two-dimensional structures into the compact three-dimensional conformations characteristic of each subunit has been proposed in very general form [e.g., see Noller and Lake (1984), Noller (1984), Expert-Besançon and Wollenzein (1985), Brimacombe et al. (1986), and Noller et al. (1986)], but details of the real structure remain obscure.

One way to identify surface RNA sequences within the ribosomal subunit is to quantitate their ability to interact with complementary oligodeoxynucleotides (Backendorf et al., 1980, 1981; Glitz et al., 1986; Oakes et al., 1986; Tapprich & Hill, 1986). This approach should also permit examination of the degree of availability of a specific ribosomal RNA (rRNA)[1] sequence segment under conditions in which the ribosomal subunit assumes an active or reversibly inactivated conformation (Zamir et al., 1971, 1974). In this paper, we examine the ability of several complementary oligodeoxynucleotides to bind both active and inactivated 30S ribosomal subunits. The results are used to define sequence availability, to help to select between alternative potential secondary structures, and to identify oligodeoxynucleotides of potential value for sequence placement by immune electron microscopy.

Experimental Procedures

Probe Selection and Design. Putative single-stranded segments of *E. coli* 16S rRNA were identified from the secondary structure of Noller et al. (1986); oligodeoxynucleotides 8–15 residues in length and complementary to 14 of these sequences were selected for study. Potential secondary binding sites for each oligodeoxynucleotide were evaluated through a computerized search for partially complementary sequences within the 16S (and 23S) sequence; the subsequence homology search of Pustell et al. (1984) was used to identify RNA sequences with more than 50% complementarity to the probe. Free energies of binding (ΔG) were calculated according to Tinoco

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[1] Abbreviations: rRNA, ribosomal RNA; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; cDNA, oligodeoxynucleotide complementary to rRNA; Phe-tRNA^{Phe}, charged phenylalanyl transfer RNA.

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et al. (1983) and evaluated for binding in those instances in which the secondary structure of Noller et al. (1986) indicated a potential for significant interaction.

Oligodeoxynucleotide Synthesis and Characterization. A Vega Biotechnologies/Du Pont Coder 300 automated DNA synthesizer was used to prepare oligodeoxynucleotides by the phosphoramidite method (Matteuci & Caruthers, 1981) but using β-cyanoethyl nucleoside phosphoramidites (Sinha et al., 1984). Reagents were obtained from Du Pont, Glen Research, or ABN (American Bionetics). After being deblocked (20 h at 55 °C in concentrated NH_3 solution), each oligodeoxynucleotide was purified by ion-exchange HPLC with a Bio-Rad TSK DEAE 5PW column (7.5 × 75 mm) on a Waters chromatograph at a flow rate of 0.8 mL/min. The usual program was the following: 20 mM Tris-HCl, pH 7.2 (buffer A), 5 min; linear gradient to 15% buffer B (buffer A plus 1 M NaCl) over 5 min; parabolic gradient to 70% buffer B (Waters curve 7) over 60 min; linear gradient to 100% buffer B over 5 min; 10 min at 100% buffer B; 5-min linear gradient to 100% buffer A. Purified oligomers were desalted by dialysis (Spectrapor 1 tubing) against distilled water.

The composition of each oligodeoxynucleotide was verified by analysis of enzymatic hydrolysates: 0.1–0.2 A_{260} unit (5–10 μg) of oligodeoxynucleotide was treated with a mixture of 6 μg of DNase I (Pharmacia), 30 μg of snake venom phosphodiesterase (Worthington), and 0.3 unit of bacterial alkaline phosphatase (Pharmacia) in 100 μL of 0.1 M Tris-HCl/0.015 M $MgCl_2$, pH 9, at 37 °C overnight. Analysis by reversed-phase HPLC with a Supelco 5-μm C18 column was essentially as described by Buck et al. (1983); a Perkin-Elmer Series 400 chromatograph and a Shimadzu CR3A integrator were used.

Terminal labeling of oligodeoxynucleotides with T4 polynucleotide kinase (Pharmacia) was based on the procedure of Donis-Keller et al. (1977). The 5′-phosphorylated oligomers [(1–2) × 10^3 cpm/pmol] were then usually passed through 0.9-mL Exocellulose GF5 desalting gel (Pierce Chemical Co.) spun columns that had been equilibrated with 10 mM Tris-HCl, pH 7.5. Final purification was by ion-exchange HPLC using a modification of the program above in which a very shallow gradient was used in the region of oligodeoxynucleotide elution. Oligodeoxynucleotides were desalted by dialysis vs water and dried by vacuum evaporation.

Ribosomes and Subunits. E. coli strain Q13 or MRE600 cells were grown in the UCLA fermentation facility on Difco antibiotic medium 3. Ribosomes, ribosomal subunits, and rRNA were prepared as described (Traub et al., 1971; Trempe et al., 1982).

Ribosomal 30S subunits were inactivated by dialysis overnight in the cold against 10 mM Tris-HCl, pH 7.5, 150 mM NH_4Cl, 1 mM magnesium acetate, and 6 mM 2-mercaptoethanol; reactivation was accomplished by adjusting the magnesium ion concentration to 10 mM and incubating the subunits at 37 °C for 10 min (Zamir et al., 1971). Subunit activity was assayed essentially as described by Zamir et al. (1971). [^{14}C]Phe-tRNA^{Phe} for the assay was prepared just before use by using 1 nmol of tRNA^{Phe} (Sigma), 2.5 nmol of [^{14}C]Phe (405 mCi/mmol), and ca. 20 μL of enzyme preparation (10^5g supernatant; Vogel et al., 1968) in 400 μL of buffer (25 mM Tris-HCl, pH 7.5, 15 mM NH_4Cl, 15 mM $MgCl_2$, and 1 mM ATP). The reaction was incubated at 37 °C for 20 min and the charged tRNA isolated by phenol extraction followed by ethanol precipitation. Assay mixtures containing 5 μg of poly(U), 15 pmol of 30S subunits, and ca. 10 pmol of [^{14}C]Phe-tRNA^{Phe} in 50 μL of buffer (50 mM Tris-HCl, pH 7.2, 30 mM magnesium acetate, and 150 mM NH_4Cl) were incubated for 60 min on ice and then passed through nitrocellulose filters (which retain subunits and complexes but not free tRNA).

Subunit–Oligodeoxynucleotide Interactions. A nitrocellulose membrane filter binding assay was used to quantitate subunit–oligodeoxynucleotide binding (Glitz et al., 1986). The standard reaction mixture included 30 pmol of radiolabeled probe (heated for 3 min in a boiling water bath and then quick-chilled on ice immediately before use if self-aggregating) and 15 pmol of 30S subunits in 50 μL of 10 mM Tris-HCl, pH 7.5, 150 mM NH_4Cl, and 1 or 10 mM $MgCl_2$. Duplicate samples were incubated for 15 min at 37 or 0 °C and then kept overnight on ice. Each mixture was then diluted to 1 mL in the same buffer and rapidly poured through a prewet Millipore HA 0.45-μm filter. Each filter was washed with three 2-mL portions of cold buffer; subunits and bound oligomer are retained by the filters, while unbound oligodeoxynucleotide passes through. Filter-bound ^{32}P was measured by liquid scintillation counting; values were corrected for background retention of oligodeoxynucleotides measured in the absence of ribosomal subunits. Nonspecific binding of oligomers was estimated by using *E. coli* 50S ribosomal subunits under identical conditions.

16S rRNA–Oligodeoxynucleotide Interactions. The binding of each oligodeoxynucleotide to 16S rRNA was evaluated by using size-exclusion HPLC. *E. coli* 16S rRNA was isolated from purified 30S subunits by extraction at room temperature with phenol that had been equilibrated with 0.1 M Tris-HCl, pH 7.5 (Traub et al., 1971). The RNA was precipitated with ice-cold ethanol and dissolved in cold 10 mM Tris-HCl, pH 7.5. A portion of the RNA was denatured by placing it in a boiling water bath for 3 min, followed by quick-chilling in an ice slurry.

Reaction mixtures included 15 pmol of rRNA and 30 pmol of ^{32}P-labeled oligodeoxynucleotide in 50 μL of 10 mM Tris-HCl, pH 7.5, 150 mM NH_4Cl, and 10 mM $MgCl_2$. The samples were incubated overnight on ice and then chromatographed on a Beckman TSK-3000 column (7.5 × 300 mm); both buffer (identical with that used in the reaction) and column were packed in ice. At a flow rate of 1 mL/min, RNA (with bound oligodeoxynucleotide) eluted at 6–8 min while free oligomer eluted at about 15 min. As a control, oligodeoxynucleotides were allowed to react with inactive 30S subunits and chromatographed as above (except in three cases in which the $MgCl_2$ level was reduced to 1 mM in the hope of maximizing binding of cDNA species that react poorly with 30 S subunits).

Results

Oligodeoxynucleotide Selection, Purification, and Characterization. The 16S rRNA secondary structure proposed by Noller et al. (1986) was used to design 14 different oligodeoxynucleotide probes that complement putative single-stranded segments of *E. coli* small subunit RNA. These probes are illustrated in Figure 1; each was synthesized by the phosphoramidite approach, purified by ion-exchange HPLC, radiolabeled at the 5′ end using polynucleotide kinase, and repurified by ion-exchange HPLC. Each oligodeoxynucleotide was chromatographically homogeneous and gave the expected products upon nucleoside analysis. The sequence of each oligodeoxynucleotide and indications of the probable availability of its rRNA complement are summarized in Table I.

Analysis of Oligodeoxynucleotide Binding to 30S Subunits. A nitrocellulose membrane filter binding assay was used to quantitate the interaction between radiolabeled probe and ribosomal subunits. Oligodeoxynucleotide retention by filters

Table I: Synthetic Oligodeoxynucleotides Used To Probe 30S Ribosomal Subunits

cDNA sequence	16S RNA segment	sequence conservation[a]	no. of reactive nucleotides			additional evidence of availability
			RNA[b,c]	active 30S[c,d]	inactive 30S[d]	
CTCTTCAATTT	1–11	+	5	3	3	microscopy[e]
CAAACTCTTCAATTT	1–15	+	6	4	4	cDNA binding[f]
TCAAACTCTTC	6–16	++	3	1	1	
ACCGTTCGACT	60–70	+	4	2	2	
TCCGCCACTCG	99–109	+	1	0	0	
CCCTTCCTCC	446–455	+	4	3	3	
ACCGCGGCTGCT	520–531	++++	8	5	5	microscopy,[g] tRNA binding[h]
TTTCACCGCTAC	685–696	+++	9	3	3	
GTCCGCTTG	1273–1281	+	4	3	decreased	
TCCGACTTCA	1330–1339	+++	7	4	4	
GGAACGTATTC	1373–1383	+++	4	1	1	
CCTTGTTACGA	1495–1505	++++	8	2	increased	nuclease sensitive[j]
TAAGGAGGTGAT	1531–1542	++	NA[i]	NA	decreased	microscopy,[k] functional
AAGGAGGT	1534–1541	++	NA	NA	decreased	role,[l] cDNA binding[m]

[a] Gutell et al. (1985). One (+) indicates slight conservation, while four (+) signify rich in universally conserved residues. [b] Van Stolk & Noller (1984). [c] Moazed et al. (1986a). [d] Moazed et al. (1986b). [e] Mochalova et al. (1982). [f] Skripkin et al. (1979). [g] Trempe et al. (1982). [h] Lake (1980). [i] NA, not available. [j] Senior & Holland (1971). [k] Olson & Glitz (1979). [l] Shine & Dalgarno (1975). [m] Backendorf et al. (1980, 1981).

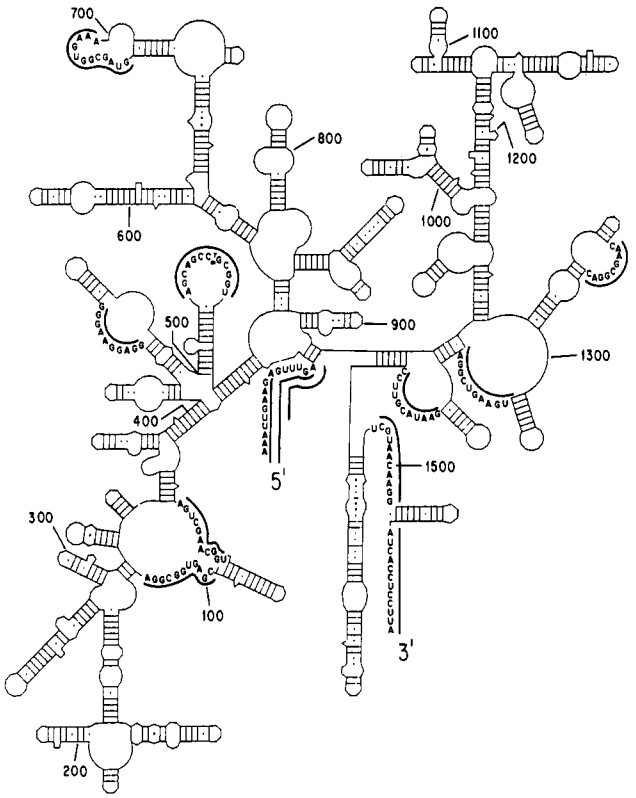

FIGURE 1: Oligodeoxynucleotide probes of 16S RNA structure. The secondary structure of Noller et al. (1986) is shown; the particular base sequences probed are indicated, and cDNA probes are identified by solid lines.

in the absence of subunits was usually below 1% of the total (and was subtracted from specific binding data). Nonspecific binding of oligonucleotides was estimated by using 50S subunits in place of 30S subunits; the values measured were usually 0–0.02 mol of oligodeoxynucleotide/mol of 50S subunit.

The stoichiometry of binding of probe was measured by using varying amounts of oligodeoxynucleotide with a constant quantity (15 pmol) of subunits. Oligodeoxynucleotide binding in each instance approached a maximum at a 1 to 1.5 molar ratio of probe to subunit regardless of subunit conformation, oligodeoxynucleotide chain length, or composition, and under all incubation conditions examined. Figure 2 illustrates typical results; in this experiment, inactivated 30S subunits and oligodeoxynucleotide were allowed to interact only at 0 °C. Panel

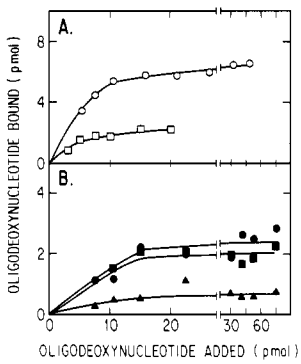

FIGURE 2: Saturation of oligodeoxynucleotide binding. Reaction mixtures included 15 pmol of 30S subunits and varying amounts of oligomer in 10 mM Tris-HCl, pH 7.5, 150 mM NH_4Cl, and 10 mM $MgCl_2$ and were incubated overnight on ice. (A) Oligodeoxynucleotides complementary to the 3′ terminus: (○) cDNA 1531–1542; (□) cDNA 1534–1541. (B) Oligodeoxynucleotides complementary to the 5′ terminus: (●) cDNA 1–11; (■) cDNA 1–15; (▲) cDNA 6–16.

A compares two oligodeoxynucleotides complementary to the 3′-terminal sequence of 16S RNA: binding of cDNA 1531–1542 levels off at about 0.36 mol of probe/mol of subunit, while the shorter cDNA 1534–1541 saturates at ca. 0.15 mol/mol. This level of oligodeoxynucleotide binding is comparable to the extent of poly(U)-stimulated binding of [^{14}C]Phe-tRNA^{Phe} to activated 30S subunits (typically 0.1–0.2 mol/mol) as measured in simultaneous assays or reported elsewhere (Zamir et al., 1971; Politz & Glitz, 1980). Figure 2, panel B, compares three oligomers that complement the 5′-terminal sequence of 16S RNA. These results indicate that the extent of binding depends on more than chain length. cDNA 1–11 shows saturation at a level of ca. 0.13 mol/mol of 30S subunit, while the overlapping cDNA 6–16 binds at a level of no more than 0.05 mol/mol and the longer cDNA 1–15 is essentially indistinguishable from cDNA 1–11.

Effect of Incubation Conditions on Oligodeoxynucleotide Binding and Subunit Activation. The subunit environment, and particularly the concentration of Mg^{2+} in the medium, is known to strongly affect the conformation and functional activity of the ribosome (Zamir et al., 1971, 1974). Preliminary experiments with cDNA 1534–1541 indicated that the levels of Mg^{2+} affected oligodeoxynucleotide binding but that the results were essentially identical whether the NH_4Cl concentration was 60, 150, or 500 mM. We therefore examined the ability of subunits to bind each oligodeoxynucleotide as the concentration of $MgCl_2$ was varied from 1 mM

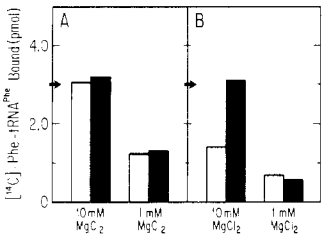

FIGURE 3: Effect of oligodeoxynucleotide binding conditions on poly(U)-dependent binding of phenylalanyl-tRNA^{Phe} to 30S subunits. Reaction mixtures contained 15 pmol of 30S subunits activated by a 10-min incubation in 10 mM $MgCl_2$ at 37 °C (panel A) or inactivated by dialysis overnight in 1 mM $MgCl_2$ in the cold (4 °C) (panel B). Reactions were done under cDNA probe binding conditions with either 10 mM $MgCl_2$ or 1 mM $MgCl_2$ buffer. Incubation for 15 min at either 0 °C (open bars) or 37 °C (shaded bars) was followed by overnight incubation on ice. Subsequently, buffer was adjusted to conditions for the tRNA binding assay (Zamir et al., 1971). The arrow indicates the activity of control-activated 30S subunits.

(characteristic of an inactivated conformation of 30S subunits) to 10 mM (characteristic of an activated conformation) in the 150 mM NH_4Cl buffer that we prefer for immune electron microscopy. The data allowed classification of the cDNA probes into four groups: (1) oligodeoxynucleotides that showed binding at both low and high magnesium ion concentrations; (2) probes that bound minimally in high $MgCl_2$ concentration and to a much greater extent in low Mg^{2+} concentration; (3) oligomers that only bound when the magnesium ion concentration was low; and (4) those probes that did not show any significant binding regardless of the conditions used.

In order to relate the results above to a quantitative measure of subunit conformation, oligodeoxynucleotide binding was determined by using 30S subunits of defined activation state. Ribosomal subunits were activated by incubation with 10 mM $MgCl_2$ at 37 °C for 10 min, inactivated by overnight dialysis against 1 mM $MgCl_2$, and reactivated by restoring the magnesium ion concentration to 10 mM and incubating at 37 °C for 10 min. The activation state was defined by the ability of a subunit preparation to bind [^{14}C]Phe-tRNA^{Phe} in response to a poly(U) message. Activated 30S subunits bound 0.2 mol of tRNA/mol, while inactivated 30S subunits showed 0–15% of this level; reactivation restored the initial binding capacity. Since the ionic environment and incubation conditions used in cDNA binding reactions could further affect the subunit activation state, reaction mixtures without cDNA were first subjected to cDNA assay conditions and then immediately assayed for poly(U)-dependent Phe-tRNA^{Phe} binding. Figure 3 illustrates the results. Overnight incubation of activated subunits (panel A) resulted in no loss of activity in 10 mM $MgCl_2$, while incubation in 1 mM $MgCl_2$ led to a ca. 60% reduction in tRNA binding (whether or not a 15-min incubation at 37 °C was included). Incubation of inactivated subunits (panel B) in 10 mM Mg^{2+} resulted in partial (0 °C) or complete (37 °C) reactivation; in 1 mM $MgCl_2$, tRNA binding activity remained minimal. Consequently, during the course of the oligodeoxynucleotide binding reaction, the incubation conditions and buffers can cause partial or complete subunit activation or inactivation.

Relationship of Oligodeoxynucleotide Binding to Subunit Conformation. Subunit binding of each cDNA probe was quantitated and directly related to the tRNA binding capacity of subunits that had been identically treated. In these experiments, four sets of reaction conditions were used; the concentration of $MgCl_2$ was either 1 or 10 mM, and incubation of the assay mixtures at 37 °C for 15 min was either included or specifically omitted from the protocol. After overnight incubation on ice, levels of cDNA binding were measured in the filter binding assay, while duplicate reaction mixtures (identically treated but lacking cDNA) were assayed for poly(U)-stimulated Phe-tRNA^{Phe} binding. In all instances, fully reactivated subunits were indistinguishable from activated 30S particles with respect to both cDNA and tRNA binding.

Table II: Nitrocellulose Membrane Filter Assays of Probe–30S Subunit Complexes

cDNA	chain length	ΔG (kcal)[a]	% G + C	extent of cDNA binding (mol/mol of subunit) active 30S	inactive 30S
1–11	11	−18	27	0.03 ± 0.01	0.09 ± 0.03
1–15	15	−24	27	0.05 ± 0.01	0.24 ± 0.05
6–16	11	−19	27	0.01 ± 0.01	0.04 ± 0.01
60–70	11	−26	55	0.03 ± 0.01	0.14 ± 0.06
99–109	11	−32	73	0.03 ± 0.01	0.03 ± 0.02
446–455	10	−30	70	0.03 ± 0.02	0.08 ± 0.05
520–531	12	−40	75	0.14 ± 0.03	0.39 ± 0.04
685–696	12	−28	50	0.05 ± 0.01	0.36 ± 0.10
1273–1281	9	−23	67	0.04 ± 0.02	0.05 ± 0.02
1330–1339	10	−23	50	0.02 ± 0.02	0.15 ± 0.06
1373–1383	11	−23	45	0.02 ± 0.01	0.06 ± 0.01
1495–1505	11	−23	45	0.05 ± 0.02	0.30 ± 0.12
1531–1542	12	−28	42	0.25 ± 0.02	0.34 ± 0.03
[^{14}C]Phe-tRNA^{Phe}				0.20[b]	0.02[c]

[a] Calculated according to Tinoco et al. (1973). [b] Minimum value used to define active conformation. [c] Maximum value used to define inactive conformation.

The results summarized in Table II are interpreted in terms of the usual two-state model of the 30S subunit in which subunit conformation is defined by poly(U)-dependent Phe-tRNA^{Phe} binding capacity (Zamir et al., 1971; Noller et al., 1986). In our experiments, subunits that bound at least 0.2 mol of tRNA/mol of 30S subunit were categorized as active, while those that bound 0.02 mol or less of tRNA/mol of subunit were categorized as inactive. Any reaction conditions that resulted in an intermediate level of tRNA binding were considered to have produced a mixture of active and inactive subunits; hence, these results were excluded from the data presented in Table II. The values shown for the binding of each cDNA under reaction conditions that maintained (or generated) an active 30S conformation represent the average of three measurements; little variation was seen, and the standard deviation in each case was ca. ±0.02. This variation is equivalent to the level of nonspecific binding (measured with 50S subunits). Values for the binding of each cDNA under conditions that maintained (or generated) an inactive subunit conformation represent the average of at least six determinations (except for cDNA 520–531 in which case only two sets of conditions resulted in inactivated subunits as defined above). The standard deviations shown in Table II reflect in some instances (cDNAs 1–15, 60–70, 446–455, 685–696, and 1405–1505) a tendency for these probes to show a considerable increase in cDNA binding as the subunit tRNA binding capacity approached zero (but within the range defined as inactive: 0–0.02 mol/mol). In these instances, the two-state model of subunit conformation (Zamir et al., 1971) may be in part an oversimplification.

The results presented in Table II allow categorization of the cDNA probes into four groups, on the basis of their interaction with active or inactive 30S subunits. Two oligodeoxynucleotides (cDNA 520–531 and cDNA 1531–1542) bind efficiently to subunits in either conformation. Several probes bind activated 30S subunits but are much more efficient in binding to inactivated subunits (e.g., cDNAs 1–15, 685–696, and 1495–1505). Others bind only inactivated subunits, and

Table III: Gel Filtration of Probe–16S RNA Complexes

cDNA	extent of cDNA binding (mol/mol)[a]		
	native 16S	heated 16S	30S subunit
1–11	0.04	0.26	0.10
1–15	0.56	1.21	0.19
6–16	0.05	0.36	0.04
60–70	0.41	0.73	0.02[b]
99–109	0.06	1.09	0.04[b]
446–455	1.11	1.75	0.01
520–531	0.49	1.24	0.92
685–696	1.05	1.35	0.24
1273–1281	0.23	0.62	0.01
1330–1339	1.03	0.70	0.04[b]
1373–1383	0.10	0.03	0.01
1495–1505	0.39	0.12	0.10
1531–1542	0.85	0.52	0.30

[a] Calculated from materials recovered in the 6–8-min peak. [b] Assay done in 1 mM $MgCl_2$ to promote binding.

then with low efficiency (e.g., cDNAs 1–11 and 446–455). A final group of oligodeoxynucleotides bind 30S subunits poorly,regardless of the subunit activation state (e.g., cDNAs 6–16 and 99–109).

Results of cDNA binding under conditions in which subunits occurred in both active and inactive conformations were also valuable. In general, as the percentage of inactivated subunits in a preparation was increased, oligodeoxynucleotide binding also increased. In one instance, cDNA 1531–1542, binding was slightly lower in partially inactivated 30S populations (but never less than 0.2 mol/mol of 30S subunits). Binding of this probe increased if the 30S population was extensively inactivated, e.g., by freezing in the activation buffer. (Usually such inactivation was not fully reversible.) A relatively sharp increase in binding to these inactivated subunits was also observed with cDNAs 60–70, 685–696, and 1495–1505. The efficiency of oligodeoxynucleotide binding in these three instances was also more variable from preparation to preparation of inactivated 30S subunits; the values presented in Table II represent numerical averages of seven such determinations, while the standard deviations reflect this variability.

Binding of Oligodeoxynucleotide Probes to Ribosomal RNA. The potential interaction between a complementary oligodeoxynucleotide and the RNA in the ribosome could be decreased by involvement of the RNA segment in secondary structure, by its location in the subunit interior, or by blockage of the RNA sequence with a ribosomal protein. To delineate such effects, we have investigated the ability of oligodeoxynucleotides to bind to native or to heat-denatured 16S RNA. Since naked RNA is not adsorbed by nitrocellulose filters, size-exclusion HPLC was used to separate rRNA–probe complexes from uncomplexed oligodeoxynucleotide probes. As a control for the assay procedure, similar measurements were made using inactive 30S subunits under conditions used for rRNA binding (with three exceptions). Results are summarized in Table III.

Native 16S rRNA bound a significant quantity of each probe that was shown to bind active and/or inactivated 30S subunits (Table II), except that cDNA 1–11 was only bound by denatured 16S RNA. Several other probes that were not found to bind subunits did bind native 16S RNA (cDNAs 60–70, 446–455, 1273–1281, and 1330–1339); these RNA sequences are therefore most likely hidden by ribosomal proteins (or by an RNA conformation induced by ribosomal proteins). Other oligodeoxynucleotides bound only to denatured RNA (cDNAs 6–16 and 99–109), suggesting that these sequences either are internal or are involved in stable secondary or tertiary interactions in both subunit and native RNA. One probe, cDNA 1373–1383, was bound poorly to either native or denatured RNA; this may indicate the involvement of the RNA segment in a very stable secondary structure. Only one oligodeoxynucleotide (cDNA 446–455) was bound at a level significantly greater than 1 mol/mol of RNA.

The quantitative aspects of the RNA binding data of Table III are substantiated by the comparison of oligodeoxynucleotide binding to 30S subunits in these experiments with the binding to inactive 30S particles shown in Table II. In all but four instances, the size-exclusion HPLC values fall within the ranges shown in the filter binding assay results. Three of the exceptions (cDNAs 1–15, 685–696, and 1495–1515) show binding that is intermediate between that of active and inactive subunits in Table II; we attribute this to partial activation of the subunits under the conditions of the chromatographic assay (10 mM $MgCl_2$ buffers, but all steps were carried out in the cold). In one instance, cDNA 520–531, the HPLC assay showed binding to 30S subunits that was significantly greater than that for inactive subunits in the filter assay.

Specificity of Oligodeoxynucleotide–Subunit Interactions. The data of Tables II and III are meaningful only if they represent binding (or lack of binding) of a cDNA to its target rRNA sequence. Three types of evidence suggest that binding is highly specific. First, no cDNA probe was bound to a significant level by 50S ribosomal subunits under any set of conditions used in these experiments. Moreover, a variety of treatments used in preliminary experiments (e.g., higher or lower ionic strength buffers, $MgCl_2$ levels up to 15 mM, or intermediate incubation temperatures) never led to binding of these cDNAs to 50S subunits. Second, the stoichiometry of cDNA binding to 30S subunits is consistent with single specific binding sites; saturation of subunit binding is seen, and RNA binding significantly exceeds 1 mol/mol of 16S RNA in only one case, cDNA 446–455 (see below). Finally, computerized subsequence homology searches of both 16S and 23S RNA sequences were done; RNA sequences that were partially complementary to each oligodeoxynucleotide were identified, and the probability of probe binding was evaluated from calculations of the free energy of the interaction and the availability of the partially homologous sequence in the secondary structures of rRNA of Noller et al. (1986). The rRNA sequences with the greatest potential as target sites are shown in Table IV.

The data of Table IV generally indicate very little likelihood of nontarget binding. In most cases, the alternative targets in 16S RNA include mismatches that interupt (and destabilize) any potential base-paired sequences. Moreover, several of the alternative target sequences are at least partially located in stable stem structures (Noller et al., 1986). An equivalent or more plausible alternative target sequence occurs in 23S rRNA (with one exception); control experiments consistently show no binding of any probe to 50S particles, indicating that binding of probes to secondary targets in 30S subunits is, at best, improbable. The sole exception is cDNA 446–455. Here, the alternative sequence is almost equivalent to the target sequence, and the RNA binding data of Table III clearly show a 2/1 oligonucleotide stoichiometry that is compatible with interaction at two sequences. However, binding of the cDNA to active 30S ribosomes is insignificant, and binding to inactivated subunits is very slight, suggesting that both the target and the alternative sequences are largely unavailable in the subunit.

Discussion

Given the great conservation of probable elements of sec-

Table IV: Specificity of Probe–Subunit Interactions

cDNA probe sequence	target 16S RNA segment	ΔG (kcal)[a]	potential nontarget sequences: 16S sequence,[b] 3′ → 5′	16S RNA segment	ΔG (kcal)[a]	% C[c]	23S sequence,[b] 3′ → 5′	23S RNA segment	ΔG (kcal)[a]	% C[c]
CTCTTCAATTT	1–11	−18	aAGAAGUaugu	405–415	−10	54	cucAAGUUAAA	2013–2023	−11	72
CAAACTCTT-CAATTT	1–15	−24	ccggGgGcAGUUAAg	917–931	−7	53	GUcgcucAAGUUAAA	2017–2027	−11	66
TCAAACTCTTC	6–16	−19	AGUagGAGAgu	296–306	−9	63	uacUUGAGAAc	2467–2477	−11	63
ACCGTTCGACT	60–70	−26	gGGCAgGCgGu	103–113	−12	63	aGGguAGCUGA	1373–1383	−15	72
TCCGCCACTCG	99–109	−32	AaGCGGUGgcC	719–729	−16	72	AGGCGuUGgGC	2215–2225	−15	81
CCCTTCCTCC	446–455	−30	uGGAAGGAGG	1174–1183	−18	90	GGGAAccAGa	1302–1311	−12	70
ACCGCGGCTGCT	520–531	−40	UGGCGauGugGA	681–692	−15	66	UGGCcCCGAaGc	1887–1898	−12	75
			UuGCGCaauCGA	860–871	−17	66				
TTTCACCGCTAC	685–696	−28	AuAaUGGCGccG	524–535	−15	66	cgAGcGGCGAUG	242–253	−20	75
GTCCGCTTG	1273–1281	−23	CAGGCGgug	102–110	−18	66	CAGGCGuug	2218–2226	−18	66
TCCGACTTCA	1330–1339	−23	cGGCgGAAGc	726–735	−12	70	AGGCUGgucc	2300–2309	−10	66
							gGGCUucAaU	1688–1697	−10	66
GGAACGTATTC	1373–1383	−23	guggcCAUAAG	714–724	−10	54	CgUgGCAUcAc	1531–1541	−7	63
CCTTGTTACGA	1495–1505	−23	GGAAggAgGgg	444–454	−8	54	GGggCAAUGua	1129–1139	−10	63
TAAGGAGGTGAT	1531–1542	−28	AgUguUuCACcA	1465–1476	−4	58	gUagCUCCACgg	2494–2505	−12	58

[a] Calculated according to Tinoco et al. (1973). [b] Complementary residues shown in capitals; underlined residues in single-stranded segments (Noller et al., 1986). [c] Percent complementarity

ondary structure in ribosomal RNA (Noller, 1984; Noller et al., 1986) and the major role of RNA in defining ribosome shape and size, it is logical to assume that the RNA of each subunit has a specific and defined three-dimensional structure. There is considerable information to support this assumption: immune electron microscopy places RNA features such as chain termini and modified nucleosides at specific sites, unique elements of the rRNA are involved in site-specific functional interactions, and other specific segments of RNA interact with ribosomal proteins that are themselves uniquely localized on the subunit surface [see, e.g., Noller et al. (1986), Brimacombe et al. (1986), and Noller and Lake (1984)].

Our long-term interest is to better describe the tertiary structure of the rRNA species using defined oligodeoxynucleotides as probes in immune electron microscopy. Our aim in this work was to determine the factors that influence the extent of specific binding of potential probes and to use this information to extend our knowledge of the surface elements of rRNA within the 30S ribosomal subunit. The probes selected included complements to sequences known to be on the ribosomal surface (e.g., the 3′ and 5′ termini, the 7-methylguanosine residue, and the colicin E3 cleavage site), sequences likely to be available because they are reactive to chemical or enzymatic attack in the subunit (near residues 448, 691–693, 1278, and 1338), apparently buried or nonreactive sequences (e.g., residues 60–70 and 99–109), and sequences for which more than one secondary structure has been proposed (e.g., the 5′ terminus).

A major concern in these experiments must be that the cDNA probes are indeed specific for the targeted sequences and that the binding we observe reflects their interaction with these sequences. Binding saturation [see also Hill et al. (1986)], lack of interaction with 50S subunits, and the subsequence homology search results of Table IV all indicate that targeting specificity is unique. The major exception, cDNA 446–455, has two almost equivalent targets since residues 1174–1182 are also complementary, and in this instance, heat-denatured 16S RNA binds 2 mol of cDNA (Table III). Since this cDNA binds very poorly to 30S subunits, regardless of their conformation, it is likely that both rRNA sequences are unavailable in the subunit.

Three probes, cDNAs 99–109, 685–696, and 1273–1281, have partially complementary 16S rRNA sequences whose potential binding energies (Table IV) or approximate T_m values [calculated according to Maniatis et al. (1982); results not shown] suggest that duplex structures could exist at 0 °C. Two of these sequences (cDNAs 99–109 and 1273–1281) fail to bind 30S subunits of either conformation, and so a possible secondary interaction is not relevant. The third sequence (cDNA 685–696) partially complements residues 524–535 in 16S RNA; this is part of another target sequence known to be available in active 30S subunits from this work (Table II) and earlier results from this laboratory (Trempe et al., 1981) and others (Noller et al., 1986). Since the binding of cDNA 685–696 to active 30S subunits is barely measurable, it appears likely that the interaction with five consecutive available nucleotides (one of which is methylated) is insufficient to generate a stable complex in this instance. Finally, cDNA 520–531 is partially complementary to two additional 16S sequences (residues 681–692 and 860–871). Our data (Table II) indicate the first sequence to be unavailable in active subunits, and Noller et al. (1986) find the second chemically unreactive in 30S particles, but both sequences show increased chemical reactivity in isolated 16S RNA. This suggests that our probe is likely to correctly recognize the highly available 520–531 sequence (Trempe et al., 1982; Noller et al., 1986) in 30S particles but that the more than stoichiometric binding to heat-denatured 16S RNA (Table III) is likely to include interaction with the highly reactive pentanucleotide complement at positions 688–692 or the near-match at positions 866–871.

An additional concern arises regarding cDNA 520–531. Probe binding to subunit populations with mixed conformations (hence, data not included in Table II) showed extreme variability, and the results of the membrane filter assay in Table II show a much lower level of binding than the size-exclusion assay of Table III. Several factors offer possible reasons for these observations. Foremost, this cDNA includes a short self-complementary sequence of six G-C base pairs; it required heat denaturation immediately before use. In our experiments, any metastable bimolecular complexes that formed could have been dissociated under the extreme dilution inherent in washing immobilized subunits on a filter, while the lesser dilution of the HPLC separation could be less perturbing. It is also possible that weak interactions between cDNA 520–531 and the complementary pentanucleotide of residues 688–692 could survive gel filtration but not washing on a filter. Secondarily, any interactions between this probe and the 30S subunit might be particularly sensitive to disruption upon subunit interaction

with the filter, or might alter the quantitative interaction of subunits to filters.

Three other probes (cDNAs 1–15, 685–696, and 1495–1505) show less binding in the HPLC assay. In these assays, the HPLC buffer contained 10 mM $MgCl_2$, and although a 37 °C incubation step was avoided, a partial activation of subunits should have occurred (Figure 3). Hence, these data represent assays of a mixture of active and inactive 30S particles, with binding levels that reflect this fact. Far more important for these experiments, binding of oligodeoxynucleotide to 30S subunits shows that the procedure is able to detect complexes that are formed (with subunits or with RNA).

Oligodeoxynucleotide binding to 30S subunits in our nitrocellulose filtration assay ranges from 0.4 mol/mol to zero. There is only a marginal relationship between the extent of binding and values of ΔG [calculated according to Tinoco et al. (1973)] for the interaction with ribosomes, but both a better correlation of ΔG and more efficient binding in general to isolated 16S RNA are observed (Tables II and III). It is therefore clear that the ribosome imposes constraints on the conformation of its RNA that prevent formation of simple A-form helical complexes. Instead, we must be observing a point in an equilibrium that itself involves partial or distorted base pairing, tertiary interactions, and perhaps distortion of the normal ribosomal RNA conformation [see also Hill et al. (1986)]. At lower concentrations of Mg^{2+} or with free RNA, conformational constraints are reduced, and the equilibrium is often shifted toward complex formation. Nevertheless, it is possible that conformational restrictions imposed by very stable interactions perclude effective cDNA binding even to heat-denatured 16S RNA (e.g., cDNA 1373–1383).

The 3′-terminal sequence of *E. coli* 16S ribosomal RNA functions in positioning messenger RNA during the initiation of protein biosynthesis (Shine & Dalgarno, 1974), and it is known to be available for oligodeoxynucleotide binding (Backendorf et al., 1980, 1981; Glitz et al., 1986). As expected, our probes of this region (cDNAs 1531–1542 and 1534–1541) confirm its availability on the 30S subunit surface and help to substantiate the methodology of these experiments. Binding of cDNA 1531–1542 is only slightly affected by changes in the magnesium ion concentration or the activation state of the 30S subunit; this result contrasts with the observations of Backendorf et al. (1981) in which an octadeoxynucleotide complement to rRNA nucleotides 1534–1541 was bound by active subunits, but not by inactivated 30S or by 16S RNA. Their results may differ from ours primarily because we used a longer oligodeoxynucleotide which, as shown in Figure 1, is better able to form a stable complex with the ribosome. Moazed et al. (1986b) show that subunit inactivation leads to lessened chemical reactivity of several nucleotides that complement the Backendorf et al. (1981) octamer but that the additional nucleotides complementing cDNA 1531–1542 either are unaffected or become more reactive (i.e., available) upon subunit inactivation. Moreover, cDNA 1531–1542 binds somewhat less effectively to subunit populations with mixed conformations; binding increases only when subunits are fully inactive.

The 5′ terminus of 16S RNA has been derivatized and localized in reconstituted subunits by immune electron microscopy (Mochalova et al., 1982). This region of the RNA has also been proposed to bind an octadeoxynucleotide complementary to positions 8–15 (Skripkin et al., 1979), although the efficiency of binding shown was less than 1%. Our data show that cDNA 1–11 is bound effectively only by inactivated 30S subunits. The longer cDNA 1–15 is bound to some extent by active 30S subunits, but subunit inactivation considerably enhances probe binding; this must be due to interactions involving nucleotides 12, 13, 14, and/or 15. This added stabilization is subtle, since cDNA 6–16 does not bind to active 30S subunits and the stimulation of binding by subunit inactivation is slight. We conclude that the primary stabilization of cDNA 1–11 or cDNA 1–15 is through interactions that involve positions 1–5 of the rRNA, with secondary stabilization in inactivated subunits occurring through complementation of rRNA nucleotides 12–15. This interpretation fits well with results of chemical modification (Moazed et al., 1986b; Noller et al., 1986); nucleotides 1, 2, 4, 5, and 7 of 16S rRNA are somewhat reactive (i.e., available) in 30S subunits, as are residues 12–15. It is possible that subunit activation involves a change in the conformation of the RNA, making some of residues 11–15 more available to our probe. However, inactivation does not cause all of rRNA sequence 1–15 to become available and single stranded, as proposed in earlier models [e.g., see Noller (1984)] of the 16S secondary structure, since cDNA 6–16 is not bound whatever the subunit conformation.

Colicin E3 is a ribonuclease that cleaves 16S RNA in the ribosome at position 1493 (Senior & Holland and, 1971; Bowman et al., 1971). The complement to residues 1495–1505 probes the adjacent segment that Moazed et al. (1986b) find largely available in free RNA but strongly protected in 30S subunits. The transition to an inactive subunit conformation results in increased reactivity at ring nitrogen positions 1 and 3, suggesting an increased potential to participate in base-pairing interactions with our cDNA at 6 of the 11 nucleotides (residues 1495, 1497, 1499, 1500, 1502, and 1505). Our results thus fit the chemical modification data well. The oligodeoxynucleotide binds active 30S subunits (although not as effectively as cDNA 1531–1542), but subunit inactivation increases binding of the probe, and the cDNA binds free RNA.

The single 7-methylguanosine residue of 16S RNA at position 527 has been localized by immune electron microscopy (Trempe et al., 1982) and is known to be on the subunit surface. Our cDNA 520–531 binds well to subunits regardless of the state of activation, although the sequence appears more available in inactivated subunits. The last result would not have been predicted from the chemical reactivity data of Moazed et al., (1986b), who see no major changes in this segment upon subunit inactivation. The cDNA may be the more effective probe; it is G-C rich, and its binding to RNA is thermodynamically favorable. At low concentrations of Mg^{2+}, the probe may be able to disrupt or distort elements of RNA secondary structure. However, as also noted by Hill et al. (1986), potential self-complementarity in this probe, a potential (but questionable) secondary interaction at residues 688–692, and our observation of highly variable binding to subunit populations of mixed conformation all lead us to be less confident of the significance of the quantitative increase in binding of cDNA 520–531 upon subunit inactivation.

The cDNA 685–696 probe complements four nucleotides that are highly reactive in isolated RNA (Van Stolk & Noller, 1984) but much less so in 30S subunits regardless of the activation state (Moazed et al., 1986a,b). Our binding data suggest greater availability, particularly in inactivated 30S particles. Two additional oligodeoxynucleotides, cDNAs 60–70 and 1330–1339, show small but significant levels of binding to inactivated subunits and marginal binding to active 30S subunits. Both sequences show chemically reactive nucleotides in RNA, and the latter has one highly reactive site in 30S subunits, with no changes upon subunit inactivation (Noller

et al., 1986). In each of these cases, quantitation of oligodeoxynucleotide binding appears to allow a very sensitive measure of RNA sequence availability in a given subunit conformation.

Four additional sequences, cDNAs 99–109, 446–455, 1273–1281, and 1373–1383, bound very poorly, if at all, to ribosomal subunits. All except the first of these sequences include at least one chemically reactive nucleotide (positions 447, 1278, and 1381; Moazed et al., 1986a) and might be predicted to be at least partially available. Our results indicate that such a level of availability is insufficient to permit formation of a stable cDNA–subunit complex.

A preliminary model of 16S RNA folding has been proposed by Expert-Bezançon and Wollenzein (1985). Our results suggest that residues included within nucleotides 1–6, 520–531, 685–696, 1495–1505, and 1531–1542 are easily available at the subunit surface; residues 1, 527, and 1542 have already been localized by immune electron microscopy. The model would predict that residues 685–696 might be found on the subunit platform surface and residues 1495–1505 should be localized in the cleft and near the site of the 3′ terminus. Our data also suggest partial availability of residues 60–70 and 1330–1339. The former sequence would be predicted to lie at the base of the subunit, while the latter would be in the cleft. Finally, residues 99–109, 446–455, 1273–1281, and 1373–1383 all are unavailable to our probes. The model predicts that residues 99–109 should be available at the subunit base and residues 1373–1383 might be on the subunit surface near the platform; neither prediction is supported by our data. However, the model also predicts that residues 446–455 could be inside the particle and hidden by protein S20 and residues 1273–1281 could be in the particle head, hidden by protein S7; our results are consistent with such placements. The oligomer binding results can thus contribute to model building and testing and can help identify sequences that are good candidates for localization by immune electron microscopy.

References

Backendorf, C., Overbeek, G. P., van Boom, J. H., Van Der Marel, G., Veeneman, G., & Van Duin, J. (1980) *Eur. J. Biochem. 110*, 599–604.

Backendorf, C., Ravensbergen, C. J. C., Van, der Plas, J., van Boom, J., Veeneman, G., & Van Duin, J. (1981) *Nucleic Acids Res. 9*, 1425–1444.

Bowman, C., Dahlberg, J., Ikemura, T., Konisky, J., & Nomura, M. (1971 *Proc. Natl. Acad. Sci. U.S.A. 68*, 964–968.

Brimacombe, R., Atmadja, J., Kyriatsoulis, A., & Stiege, W. (1986) in *Structure, Function and Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.) pp 184–202, Springer-Verlag, New York.

Buck, M., Connick, M., & Ames, B. N. (1983) *Anal. Biochem. 129*, 1–13.

Donis-Keller, H., Maxam, A. M., & Gilbert, W. (1977) *Nucleic Acids Res. 4*, 2527–2537.

Erdmann, V. A., Pieler, T., Wolters, J., Digweek, M., Vogel, D., & Hartmann, R. (1986) in *Structure, Function and Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.) pp 164–183, Springer-Verlag, New York.

Expert-Bezançon, A., & Wollenzein, P. (1985) *J. Mol. Biol. 184*, 53–66.

Glitz, D. G., Olson, H. M., & Lasater, L. S. (1986) *Biophys. J. 49*, 11–13.

Gutell, R. R., Weiser, B., Woese, C., & Noller, H. F. (1985) *Prog. Nucleic Acid Res. Mol. Biol. 32*, 155–216.

Herr, W., Chapman, N., & Noller, H. F. (1979) *J. Mol. Biol. 130*, 433–449.

Hill, W. E., Tapprich, W. E., & Tassanakajohn, A. (1986) in *Structure, Function and Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.) pp 233–252, Springer-Verlag, New York.

Lake, J. A. (1980) in *Ribosomes: Structure, Function, and Genetics* (Chambliss, G., Craven, G., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 207–236, University Park Press, Baltimore, MD.

Maniatis, T., Fritch, E. F., & Sambrook, J. (1982) *Molecular Cloning*, pp 388–389, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Matteuci, M., & Caruthers, M. H. (1981) *J. Am. Chem. Soc. 103*, 3185–3191.

Moazed, D., & Noller, H. F. (1986) *Cell (Cambridge, Mass.) 47*, 985–994.

Moazed, D., Stern, S., & Noller, H. F. (1986a) *J. Mol. Biol. 187*, 399–416.

Moazed, D., Van Stolk, B., Douthwaite, S., & Noller, H. F. (1986b) *J. Mol. Biol. 191*, 483–493.

Mochalova, L. U., Shatsky, I. N., Bogdanov, A. A., & Vasiliev, V. D. (1982) *J. Mol. Biol. 159*, 637–650.

Noller, H. F. (1984) *Annu. Rev. Biochem. 53*, 119–162.

Noller, H. F., & Lake, J. A. (1984) *Membr. Struct. Funct. 6*, 217–297.

Noller, H. F., Asire, M., Barta, A., Douthwaite, S., Goldstein, T., Gutell, R., Moazed, D., Normanly, J., Prince, J. B., Stern, S., Triman, K., Turner, S., Van Stolk, B., Wheaton, V., Weiser, B., & Woese, C. R. (1986) in *Structure, Function and Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.) pp 143–163, Springer-Verlag, New York.

Oakes, M. I., Clark, M. W., Henderson, E., & Lake, J. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 275–279.

Ofengand, J., Ciesiolka, J., Denman, R., & Nurse, K. (1986) in *Structure, Function and Genetics of Ribosomes* (Hardesty, B., & Kramer, G., Eds.) pp 473–494, Springer-Verlag, New York.

Olson, H. M., & Glitz, D. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3769–3773.

Politz, S. M., & Glitz, D. G. (1980) *Biochemistry 19*, 3786–3791.

Pustell, J., & Kafatos, F. C. (1984) *Nucleic Acids Res. 12*, 643–655.

Santer, M., & Shane, S. (1977) *J. Bacteriol. 130*, 900–910.

Senior, B. W., & Holland, J. B. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 959–963.

Shine, J., & Dalgarno, L. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1342–1346.

Sinha, N. D., Biernet, J., McManus, J., & Koster, H. (1984) *Nucleic Acids Res. 12*, 4539–4557.

Skripkin, E. A., Kopylov, A. M., Bogdanov, A. A., Vinogradov, S. V., & Berlin, Yu. A. (1979) *Mol. Biol. Rep. 5*, 221–224.

Stöffler, G., & Stöffler-Meilicke, M. (1984) *Annu. Rev. Biophys. Bioeng. 13*, 303–330.

Tapprich, W. E., & Hill, W. E. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 556–560.

Tinoco, I., Borer, P., Dengler, B., Levine, M., Uhlenbeck, O., Crothers, D., & Gralla, J. (1973) *Nature (London), New Biol. 246*, 40–41.

Traub, P., Mizushima, S., Lowry, C. V., & Nomura, M. (1971) *Methods Enzymol. 20*, 391–407.

Trempe, M. R., Oghi, K., & Glitz, D. G. (1982) *J. Biol. Chem. 257*, 9822–9829.

Van Stolk, B., & Noller, H. F. (1984) *J. Mol. Biol. 180*, 151–178.
Vasiliev, V. D., & Zalite, O. M. (1980) *FEBS Lett. 121*, 101–104.
Vasiliev, V. D., Selivanova, O. M., & Koteliansky, U. E. (1978) *FEBS Lett. 95*, 273–276.
Vogel, Z., Zamir, A., & Elson, D. (1968) *Proc. Natl. Acad. Sci. U.S.A. 61*, 701–707.
Zamir, A., Miskin, R., & Elson, D. (1971) *J. Mol. Biol. 60*, 347–364.
Zamir, A., Miskin, R., Vogel, Z., & Elson, D. (1974) *Methods Enzymol. 30*, 406–426.

Hydroxyl Radical Induced Cross-Linking between Phenylalanine and 2-Deoxyribose†

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ABSTRACT: Hydroxy radicals induce cross-linking between phenylalanine (Phe) and 2-deoxyribose (dR) via formation of corresponding free radical intermediates. The cross-linked products were separated and identified by capillary gas chromatography–mass spectrometry. When phenylalanine and 2-deoxyribose radicals were generated in a 1:1 ratio, the predominant interaction was between Phe and dR radicals while the Phe–Phe and dR–dR cross-links were less abundant. The newly discovered cross-link between 2-deoxyribose and phenylalanine may serve as a model for radiation or free radical induced cross-linking between DNA and proteins and in general between sugar moieties and amino acids.

The least studied and understood type of DNA damage in cells is the cross-linking between DNA and proteins (Oleinick et al., 1986). Radiation-induced cross-links were shown to occur in chromatin, in vivo and in vitro (Mee & Adelstein, 1981), and in mouse leukemia cells (Bowden et al., 1982). These cross-links have been suggested to result from free radicals of DNA and protein components generated in close proximity (Simic & Dizdaroglu, 1985). The most likely components of proteins to be involved in cross-linking would be aromatic and positively charged amino acids since they interact most intimately with DNA in the DNA–protein complex (Takeda, 1983).

Cross-linking mechanisms between thymine (T) and either phenylalanine (Phe) (Dizdaroglu & Simic, 1985) or tyrosine (Tyr) (Simic & Dizdaroglu, 1985) have been suggested on the basis of the observed T–Phe and T–Tyr cross-linked products. Participation of 2-deoxyribose (dR) in the DNA–protein cross-linking has not been considered so far mainly because of the inability to observe any radiation-induced sugar–sugar cross-links in model systems (Schulte-Frohlinde & von Sonntag, 1972; von Sonntag, 1987). In the present work, we find that hydroxy radical induced cross-links between phenylalanine and 2-deoxyribose can take place in model aqueous systems. It is suggested that this type of sugar–amino acid cross-link may be more relevant than base–amino acid cross-links in radiation-induced cross-linking between DNA and protein because of steric considerations associated with the DNA–protein interactions.

Materials and Methods

Materials.[1] Phenylalanine (ultrapure) was purchased from Vega Biochemicals and 2-deoxy-D-ribose (ultrapure) from Sigma. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine (ultrapure) were from Pierce. Water purified through a Millipore reverse osmosis system was used for all solutions.

Irradiations. Aqueous solutions of the mixture of Phe (0.5×10^{-3} M, pH 6.3) and dR (0.92×10^{-3} M) were saturated with oxygen-free N_2O (Matheson) for 30 min and irradiated in a ^{60}Co γ source (Woolf & Burke, 1984) (dose range 110–440 Gy, dose rate 110 Gy/min). Dose rate of the source was determined by using a Fricke dosimeter [$G(Fe^{3+}) = 15.6$; (Fricke & Hart, 1966)].

Trimethylsilylation. Samples (about 10 mg) dried with a rotary evaporator were trimethylsilylated (TMS) in Teflon-capped Hypovials (Pierce) with 0.1 mL each of BSTFA and pyridine (1:1) by heating for 30 min at 140 °C.

Gas Chromatography–Mass Spectrometry (GC–MS). A Hewlett-Packard Model 5880A microprocessor-controlled gas chromatograph interfaced to a Hewlett-Packard Model 5970A mass selective detector was used. The injection port and GC–MS interface were both maintained at 250 °C. Separations were carried out by using a fused-silica capillary column (12 m long × 0.2 mm i.d.) coated with cross-linked SE-54 (5% phenyl methyl silicon gum). Helium was used as the carrier gas at an inlet pressure of 100 kPa (split ratio 4:1, ion source temperature ca. 200 °C, electron energy 70 eV).

High-Performance Liquid Chromatography (HPLC). A Water Model 600 microprocessor-controlled liquid chromatograph equipped with a WISP Model 512 autoinjector and a Model 990 diode-array spectrophotometric detector was used. Separations were carried out on a 25 × 1 cm semiprep Supelcosil column SPLC-8-DB (particle size, 5 μm; Supelco) (eluent A, 0.1% trifluoroacetic acid (TFA) in water; eluent

† Taken from the Ph.D. dissertation of Mahnaz Farahani, Chemistry Department, The American University, Washington, DC 20016.

[1] The mention of commercial products is to provide technical information and is not intended as an endorsement.