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Topography of 16S RNA in 30S Ribosomal Subunits. Nucleotide Sequences and Location of Sites of Reaction with Kethoxal†

Harry F. Noller

ABSTRACT: The sites of reaction of kethoxal with 16S RNA in intact 30S ribosomal subunits have been identified. By means of a new "diagonal" electrophoresis method, 53 T1 oligonucleotides from modified sites were isolated, their nucleotide sequences determined, and sites of attachment of kethoxal unambiguously identified. These sequences define 26 sites within the 16S RNA chain, 16 of which can be located in the known sequence. The modified sites appear to fall into clusters, interspersed by regions 200–400 nucleotides long that are known to be involved in binding of ribosomal proteins. In addition to providing a catalog of RNA sequences that includes sites involved in ribosomal function, these data provide new information about the sequence of 16S RNA and its topography within the ribosome.

Indications of the functional participation of ribosomal RNA in protein synthesis have emerged from recent work by several groups (Senior and Holland, 1971; Bowman *et al.,* 1971; Helser *et al.,* 1971, 1972; Noller and Chaires, 1972; Lai *et al.,* 1973a,b; Held *et al.,* 1974). Studies in this laboratory have shown that the guanine-specific reagent kethoxal reacts with 30S ribosomal units causing inactivation of the particle in poly(U) directed *in vitro* protein synthesis (Noller and Chaires, 1972). Inactivation is attributable to loss of tRNA binding ability, and, correspondingly, protection against inactivation is afforded by prior binding of tRNA. Reconstitution experiments indicate that the target of inactivation is RNA. During the course of inactivation about 10 mol of kethoxal become attached/mol of 30S subunits. These modified sites include candidates for possible functional sites in 16S RNA. Thus, it is of great interest to identify these regions in the 16S RNA molecule. In addition, such information will reveal details of the conformation of 16S RNA within the intact ribosome, since kethoxal is known to react only with single-stranded regions of RNA (Litt, 1969).

Identification of modified sites in rRNA and other large RNA molecules is technically difficult due to the large number of fragments obtained from nuclease digests of these molecules. In order to simplify the identification of kethoxal-modified sites, a "diagonal" paper electrophoresis procedure has been devised. By use of this procedure, about 50 oligonucleotides have been isolated and their nucleotide sequences determined. These sequences correspond to about 26 sites within the 16S RNA, 16 of which can be located unambiguously in regions of known or partially known sequence as reported by Ehresmann *et al.* (1974). The modified sites appear to fall into five main clusters, interspersed by regions 200–400 nucleotides long which are known to be involved in binding of ribosomal proteins (Zimmerman *et al.,* 1972; Schaup *et al.,* 1971, 1973; Székely *et al.,* 1973). Some of the kethoxal sites correspond to sites of attack by T1 nuclease on intact 30S ribosomal subunits (Ehresmann *et al.,* 1972; Santer and Santer, 1973).

Materials and Methods

Buffers. Buffer A, 0.1 M NH_4Cl–0.01 M $MgCl_2$–0.02 M Tris (pH 7.5)–0.5 mM EDTA–6 mM β-mercaptoethanol; buffer B, 0.5 M NH_4Cl–0.01 M $MgCl_2$–0.02 M Tris (pH 7.5)–0.5 mM EDTA–6 mM β-mercaptoethanol; TMA II,[1] 0.3 mM $MgCl_2$–30 mM NH_4Cl–10 mM Tris (pH 7.5)–6 mM β-mercaptoethanol; kethoxal reaction buffer, 0.1 M potassium cacodylate (pH 7.0)–20 mM $MgCl_2$; SCE, 0.15 M NaCl–15 mM sodium citrate–10 mM EDTA (pH 7.0).

Enzymes. Ribonuclease T1 (Sankyo) was obtained from Calbiochem, ribonuclease A and snake venom phosphodiesterase from Worthington, and bacterial alkaline phosphatase from Sigma. U2 nuclease was a gift from Dr. Howard

† From Thimann Laboratories, University of California, Santa Cruz, California 95064. *Received July 19, 1974.* Supported by U. S. Public Health Service Grant No. 17129-01 from the National Institute of General Medical Sciences.

[1] Abbreviations used are: TMA II, 0.3 mM $MgCl_2$–30 mM NH_4Cl–10 mM Tris (pH 7.5)–6 mM β-mercaptoethanol; SCE, 0.15 M NaCl–15 mM sodium citrate–10 mM EDTA (pH 7.0); CMCT, *N*-cyclohexyl-*N'*-morpholinocarbodiimide metho-*p*-toluenesulfonate.

Goodman, and silkworm nuclease was a gift from Dr. John Abelson.

Growing and Labeling Bacterial Cells. Escherichia coli, strain MRE 600, was kindly provided by Dr. Paul Sypherd. Cells were grown at 37° on a low phosphate medium (Garen and Levinthal, 1960) supplemented with all 20 amino acids (2 μg/ml). After 30-min growth, 20 mCi of carrier-free [^{32}P]orthophosphate (ICN) was added to 50 ml of medium and the cells were harvested in late-log phase by a brief low-speed centrifugation. Cells were opened by the lysozyme freeze–thaw method of Ron *et al.* (1966) in the presence of buffer A. The crude lysate was centrifuged for 30 min at 16,000 rpm (Servall RC-2B, SS-34 rotor), and the supernatant was layered on 4 ml of buffer B containing 1.1 M sucrose. After centrifugation at 55,000 rpm (IEC B-60 ultracentrifuge, A-321 rotor) for 24 hr at 6°, the 70S ribosome pellet was resuspended by gentle rotary shaking for 1 hr at 0° in 1 ml of TMA II. The resuspended ribosomes were layered on a 35-ml 5–20% sucrose gradient in TMA II and centrifuged for 13.5 hr at 20,000 rpm (Beckman L3-40 centrifuge, SW27 rotor) at 6°. About 20 fractions were collected and the 30S and 50S subunit peaks located by counting Čerenkov radiation for 1-μl aliquots. The fractions containing 30S subunits were pooled, the magnesium ion concentration was raised to 10 mM by the addition of 1 M $MgCl_2$, and 0.65 volume of ethanol (0°) was added. Precipitated subunits were recovered by centrifugation at 15,000 rpm for 45 min (Servall RC-2B, SS-34 rotor) at 0°, and resuspended in 0.5 ml of kethoxal reaction buffer. Subunits were either used immediately or frozen in Dry Ice–acetone and stored at −72° for not more than 48 hr. Ribosomal units prepared in this way were found to have a specific activity of about 10^6 cpm/μg.

Reaction of 30S Subunits with Kethoxal. Ribosomes were treated with kethoxal (Nutritional Biochemicals) as previously described (Noller and Chaires, 1972) for 60 min at 37°, conditions previously shown to produce complete inactivation. Treated ribosomes were precipitated by addition of 0.65 volume of ethanol and centrifuged for 20 min at 6° in a clinical centrifuge. The pellet was resuspended in 0.5 ml of SCE.

Extraction of Modified ^{32}P-Labeled 16S RNA. The modified subunits, in 0.5 ml of SCE containing 1% sodium dodecyl sulfate, were extracted three times with an equal volume of water-saturated phenol by strong agitation on a Vortex mixer for 2–3 min. The phenol phases were reextracted twice with 0.5 ml of SCE and the combined aqueous phases precipitated with 2 volumes of ethanol at −20°. Ethanol-precipitated RNA was recovered by centrifugation in a clinical centrifuge for 20 min at 6°. The precipitate was redissolved in 0.2 ml of 0.2 M NaOAc (pH 5.0) and ethanol precipitation was repeated three to six times, until there was no initial turbidity upon addition of ethanol. The final precipitate was dissolved in 0.05 ml of water and lyophilized.

Diagonal Electrophoresis Procedure. The kethoxal-modified RNA sample was dissolved in 10 μl of a solution containing 0.08 mg/ml of T1 RNase, 1.0 mg/ml of bacterial alkaline phosphatase, 0.01 M Tris (pH 7.8), 0.01 mM $ZnCl_2$, and 0.02 M potassium borate, and incubated in a drawn-out capillary for 1 hr at 37°. The digest was spotted 8 cm from the end of a 58-cm sheet of Whatman DE-81 DEAE-cellulose paper and subjected to electrophoresis in 7% formic acid at 700 V for 12–24 hr, depending on the separation required. After drying the paper, a strip 4 cm × 46 cm containing the separated oligonucleotides was cut out and laid on a strip of Parafilm of slightly larger dimensions. In all manipulations involving the wet DEAE paper, it was conveniently handled by using the Parafilm as a support. The strip was placed in a chromatographic dipping tray and equilibrated with 150 ml of 0.013 M Tris base by gentle agitation. The Tris solution was carefully poured off and the DEAE paper strip rolled up in the Parafilm and incubated in a humid desiccator for 2 hr at 37°, conditions which were found sufficient to remove kethoxal. The DEAE strip was then washed three times with water and dried. T1 nuclease (0.05 mg/ml, in 0.01 M Tris (pH 7.8)) was applied to the strip, and incubation carried out as before. After washing the strip with water to remove residual salts, it was dried thoroughly, and sewn onto a second sheet (46 × 58 cm) of DE-81 paper, 10 cm from the end. The second dimension of the electrophoresis is performed exactly as for the first dimension, but at right angles to the direction of the first dimension. After drying, the oligonucleotides are located by autoradiography, as previously described (Barrell, 1971).

RNA Sequence Methods. Oligonucleotides were eluted from the DE-81 paper and sequenced by the methods of Sanger and coworkers as described by Barrell (1971). In addition, some oligonucleotides were treated with silkworm nuclease (Pinkerton *et al.*, 1973). Oligonucleotides were dissolved in 10 μl of a solution containing 0.6 mg/ml of silkworm nuclease and 0.5 mg/ml of bacterial alkaline phosphatase, 0.5 M Na_2CO_3, 0.1 M NaCl, and 0.005 M $MgCl_2$ (pH 10.5), and incubated for 2 hr at 37°. The digests were spotted onto DEAE paper and electrophoresed at pH 3.5 for 6 hr at 1000 V. The digestion products were eluted and submitted to further degradation by both alkaline hydrolysis and total hydrolysis with snake venom phosphodiesterase.

Digestion with U2 nuclease was in a solution containing 0.5 unit/ml of U2 nuclease, 1 mg/ml of tRNA carrier, 0.1 mg/ml of bovine serum albumin, 0.05 M NaOAc, and 0.002 M EDTA (pH 4.5) at 37° for 16 hr.

Blocking of oligonucleotides with CMCT (*N*-cyclohexyl-*N'*-morpholinocarbodiimide metho-*p*-toluenesulfonate) was carried out in a solution containing 100 mg/ml of CMCT and 0.001 M Tris (pH 7.5) for 16 hr at 37°.

In many cases, oligonucleotides had to be submitted to further purification. Oligonucleotides running faster than the blue marker (xylene cyanol FF) were rerun on DEAE paper at pH 3.5. Nucleotides running slower than the blue marker were repurified by homochromatography on DEAE thin-layer plates (Machery-Nagel MN 300 DEAE) using homomix C (Barrell, 1971).

Results

(*a*) *Isolation of Modified Oligonucleotides by Diagonal Electrophoresis.* Modification of guanine residues by kethoxal renders them resistant to enzymatic cleavage by T1 ribonuclease. Instead, modified guanines will appear in internal positions in the oligonucleotides produced by T1 digestion of kethoxal-modified RNA. After electrophoresis in the first dimension, the kethoxal is removed from the modified nucleotides, allowing T1 cleavage of the bond adjacent to the modified nucleotide. The original kethoxalated T1 oligonucleotide is thus cleaved into two or more smaller fragments, each of which has an electrophoretic mobility which is, in general, different from the original one (Figure 1). By carrying out the second electrophoretic dimension at right angles to the first, a diagonal line is formed, consisting of oligonucleotides not adjacent to modified guanines. Oligo-

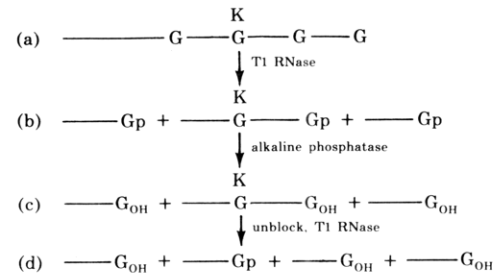

FIGURE 1: A schematic diagram of the basis of the diagonal procedure. (a) 16S RNA containing kethoxal-modified guanine residues is treated with T1 ribonuclease. Cleavage occurs specifically at guanine sites except where blocked by kethoxal (b). Alkaline phosphatase treatment removes all terminal phosphate groups. The first dimension of electrophoresis is carried out on the resulting mixture (c). Removal of kethoxal by treatment with mild base, followed by T1 digestion, gives rise to cleavage only at guanine sites formerly occupied by kethoxal (d). Electrophoresis is then carried out as for the first dimension but at right angles to the original direction. Oligonucleotides adjacent to the reactive guanine are identified by their altered mobility in the second dimension (*cf.* Figure 2).

nucleotides adjacent to sites of attachment of kethoxal lie off the diagonal, and fragments belonging to the same parent kethoxalated T1 oligonucleotide lie on a vertical line with respect to one another (Figure 2). Since the 3′-phosphate group of the parent nucleotide had been removed by the phosphate treatment, any pair of related fragments can be unambiguously ordered, the phosphorylated fragment at the 5′ end, and the dephosphorylated fragment at the 3′ end. Similarly, any 3′-terminal guanosine 3′-phosphate is necessarily a site of kethoxal modification, and any oligonucleotide having a 3′-terminal guanosine must have been directly preceded by a kethoxalated guanine.

Using this technique, 16S RNA isolated from kethoxal-treated ^{32}P-labeled 30S ribosomal subunits was analyzed. The result (Figure 2) shows that about 50 nucleotides lie off the diagonal. These nucleotides were subjected to sequence analysis. Control experiments, in which unmodified RNA was used, gave no off-diagonal spots.

(*b*) *Sequence Determination of RNA Fragments.* After location of oligonucleotides by autoradiography, they were eluted and sequenced, using the methods developed by Sanger and his collaborators (Barrell, 1971). The results of the sequence determination are given in Table I. In most cases, the deduced sequences follow directly from the data given. A few nucleotides require comment, however.

Spots 7 and 8 have the same mobility in the pH 3.5 or 7% formic acid systems on DEAE paper, and yield on alkaline hydrolysis a product with an electrophoretic mobility (pH 3.5, 3 MM paper) identical with that of Ap. Total digestion with snake venom phosphodiesterase gives only guanosine 5′-phosphate, suggesting the sequence AG_{OH}. However, their mobility (R_B) at pH 3.5 on DEAE paper is 0.4, clearly distinguishable from authentic AG_{OH}, which has a mobility of 1.4. Further work is required to clarify the nature of these fragments.

Spots 11a and 12 also probably have identical structures from their identical mobility at pH 3.5 and in 7% formic acid on DEAE paper, and identical pancreatic digestion products. One of the pancreatic products has a mobility identical with AG on DEAE paper at pH 3.5, but quantitative analysis reveals that it is a tetranucleotide. Alkaline hydrolysis yields Cp, Ap, and two products running slightly ahead, and slightly behind, Gp at pH 3.5 on Whatman 3MM paper. The faster running of these two spots has a mobility of 0.82, identical with that of 4-amino-5-(*N*-meth-

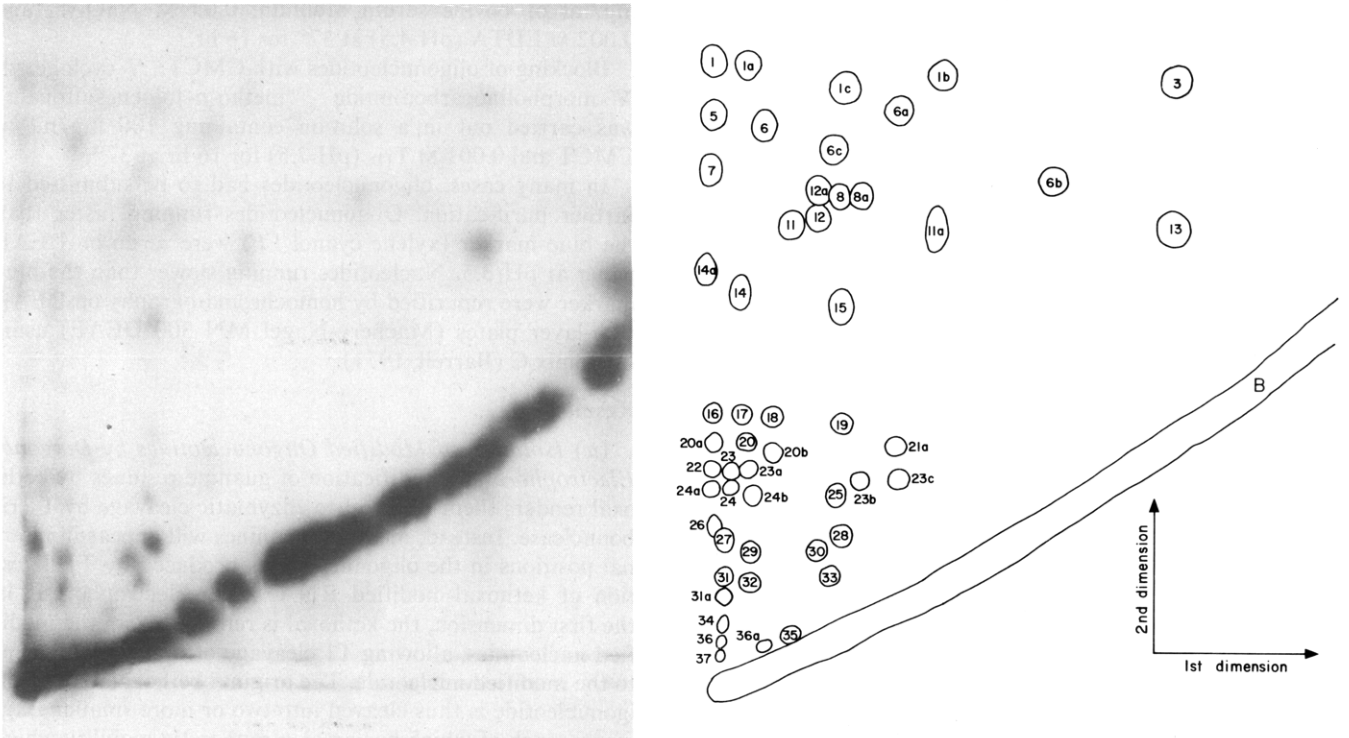

FIGURE 2: Diagonal electrophoresis purification of oligonucleotides from sites of reaction of kethoxal with 16S RNA in intact 30S ribosomal subunits. Preparation of ^{32}P-labeled 30S subunits, kethoxal reaction conditions, and electrophoresis procedures are described in the Materials and Methods section. The origin is in the lower left of the radioautograph. Spots which are reproducibly observed in significant (≥10%) yield are indicated by the numbers in the line drawing at the right. Spots 4 and 9 are observed only in shorter runs, and lie to the right of this autoradiograph. The position of the blue tracking dye (xylene cyanol FF) is indicated by the letter B.

yl)formamidoisocytosine ribonucleotide, the alkaline degradation product of 7-methylguanylic acid (Barrell, 1971). It is therefore possible that the two unidentified components in this sequence are modified guanylic acid residues. The presence of 7-methylguanine is similar to cytosine in its effect on the electrophoretic mobility of an oligonucleotide. This is consistent with the observed mobility of spots 11a and 12. However, it seems unlikely that this sequence contains m^7G, since such a sequence does not appear in methyl-labeled 16S RNA (Fellner and Sanger, 1968; Fellner, 1969). The presence of both modified nucleotides in a pancreatic RNase fragment containing A and C gives the sequence $(A,G^*_2)C$. Silkworm nuclease gives a fragment with the sequence AG*, which, in view of the disappearance of A in the complete snake venom phosphodiesterase digest, extends the sequence to AG*G*C. The complete sequence, AG*G*CCUG, does not correspond to any of the modified nucleotides reported by Fellner (1969). It is possible that this sequence may exist unmodified in some strains, but none of the four CCUG sequences reported by Ehresmann *et al.* (1972) are preceded by the sequence AGG. A second possibility is that the modification does not involve the addition of methyl groups, in which case it would not have been detected in fingerprints of [^{14}C]methylmethionine-labeled RNA. A third possibility is that this sequence is present only in a fraction of the 16S RNA chains.

Nucleotide 30, AUACUCACCG, is isomeric with nucleotide 26 of Fellner *et al.* (1972), Um^{5}CACACCAUG, and gives an identical pancreatic RNase analysis. Its sequence, however, is similar to that of nucleotide 27 (Fellner *et al.*, 1972), AUACUCCG, with the insertion of AC in the latter half of the sequence. It may thus be a variant of nucleotide 27.

Nucleotide 32 is nearly identical with the sequence AUUAAACG given by Fellner *et al.* for their spot 21. However, the products of U2 nuclease digestion, confirmed by alkaline hydrolysis, clearly show the presence of AUA and CUG but not AUUA and CG sequences within this oligonucleotide, and establish the sequence as AUAAACUG.

Nucleotide 37, AUUAG, is not in the catalog of T1 fragments reported by Fellner *et al.* (1972). However, Santer and Santer (1973) have observed a T1 product which gives pancreatic RNase products consistent with this structure.

(*b*) *Molar Yields of Oligonucleotides.* Sequences of the T1 oligonucleotides are listed in Table II, with their relative molar yields and probable location in 16S RNA. There are 34 unique sequences represented in the 53 spots that were analyzed. The relative molar yields were calculated by measuring the amount of ^{32}P in each spot, dividing by the number of phosphorus atoms in the oligonucleotide, and comparing with the specific radioactivity found in spot 3. Absolute molar yields can be calculated from independent measurement of the yield of spot 3 compared with the specific activity of CG. This is conveniently carried out by performing the diagonal electrophoresis without alkaline phosphatase, in which case CG is well isolated on the diagonal. Assuming 27 mol of CG/mol of 16S RNA (Fellner *et al.*, 1972) spot 3 is found in a yield of 0.8–1.0. Absolute molar yields of the oligonucleotides in Table II can thus be estimated by multiplying the relative molar yield by 0.9. The extent of reaction of kethoxal at the identified sites is thus in the range of 10–100%. Some underestimation is possible due to losses during electrophoresis and detachment of kethoxal during the isolation procedures. Overestimates are also possible, due to contaminants which sometimes migrate with the identified nucleotides.

(*c*) *Identification of Kethoxal Attachment Sites.* Sequence analysis of the off-diagonal T1 spots reveals, as expected, two classes of nucleotides: those with 3′-terminal phosphates and those with dephosphorylated 3′ ends. Sites of attachment of kethoxal can be unambiguously identified as any 3′-terminal guanine residue in an oligonucleotide containing a terminal 3′-phosphate. Conversely, any T1 fragment containing a dephosphorylated 3′ terminus must have been linked to a kethoxal-modified guanine residue at its 5′ end (Figure 1). Thus, each kethoxal site gives rise to two fragments which lie off the diagonal in a vertical line parallel to the second electrophoretic dimension. In some cases, there may exist several (n) contiguous kethoxal-reaction T1 oligonucleotides, which will give rise to $n + 1$ fragments. In other instances, where the 3′-terminal oligonucleotide is simply guanosine, it will not be detected by autoradiography, and the number of detectable fragments will be n. The most probable case is that where sites of kethoxal attachment are not adjacent to other reactive oligonucleotides, and so it is expected that the total number of off-diagonal spots will be roughly twice the number of modified sites. This is confirmed by sequence analysis (Table II), which indicates 26 phosphorylated nucleotides and 23 dephosphorylated nucleotides. The number of bound kethoxal molecules was previously estimated to be 10 mol/mol of 30S subunits, using ^{3}H-labeled kethoxal (Noller and Chaires, 1972). The number of moles of kethoxal sites identified by the present method can be estimated from the data in Table II as 10.6, in good agreement with our previous result. In view of the possibility that some oligonucleotides with very low electrophoretic mobilities in this system may not have been detected, this number may be an underestimate.

Identification of adjacent pairs of oligonucleotides is often ambiguous due to the presence of several spots lying on a vertical line. At least one pair, spots 3 and 13, can be readily identified, giving the sequence

$$\begin{matrix} & & & & & & & \mathrm{K} \\ \mathrm{GGAAUCG} & & & & & & & \end{matrix}$$

in agreement with the sequence of Ehresmann *et al.* (1972). Other probable pairs are listed in Table III. Each of the proposed pairs lies on a vertical line in the diagonal fingerprint. Both partners in each pair are found in similar yield, with the possible exception of the pair 19–15. The relative order of the two oligonucleotides is uniquely determined by the presence or lack of a terminal 3′-phosphate, with the exception of the pair 36-3–31a. Furthermore, the proximity of the pairs of nucleotides is supported in many cases by the presence of both nucleotides in subfragments of 16S RNA isolated by Ehresmann *et al.* (1972, 1974) although some of the sequences presented in Table III do not agree with those of the latter authors (see Discussion).

(*d*) *Location of Kethoxal Sites in 16S RNA.* Of the nucleotides listed in Table II, there are 15 sequences that are unique in 16S RNA and that can be located unambiguously within a specific section of the polynucleotide chain (Ehresmann *et al.*, 1974). In addition, the pair 36a–6 defines a unique sequence which can be placed in section K. The distribution of unique kethoxal sequences of known location within the 16S RNA chain is summarized in Figure 3, along with the regions that bind some of the ribosomal proteins (Zimmerman *et al.*, 1972; Székely *et al.*, 1973; Schaup *et al.*, 1971, 1973).

TABLE I: Sequence Determination of T1 Oligonucleotides.[a]

Oligonucleotide No.	Sequencing Results	Oligonucleotide No.	Sequencing Results
1	Alk G	12a-1	Alk A
	Panc G		Panc A-A-G_{OH}
	Sequence: Gp		Sequence: A-A-G_{OH}
1a	Alk G	13	Alk $C_{1.0}$ $A_{1.9}$ $U_{1.0}$
	Panc G		Panc $C_{1.1}$ $AAU_{1.0}$
	Sequence: Gp		Venom pC, pA, pG, pU
1b	Alk $U_{1.0}$ $C_{1.0}$		U2 A, (U,C)-G_{OH}
	Panc $U_{1.0}$ $C_{1.0}$		Sequence: A-A-U-C-G_{OH}
	Venom $pU_{1.0}$ $pG_{0.9}$	14	Panc U, A-A-C, A-G_{OH}
	Sequence: C-U-G_{OH}		Venom pC, pA, pG
1c	Alk G		Sequence: U-A-A-C-A-G_{OH}
	Sequence: Gp	14a	Alk $U_{1.0}$ $A_{3.0}$
3	Alk G		Panc A-A-A-U
	Panc G		Venom $pU_{1.0}$ $pG_{0.9}$ $pA_{2.0}$
	Sequence: Gp		Sequence: A-A-A-U-G_{OH}
4	Alk G	15	Alk $U_{1.1}$ $A_{3.0}$
	Panc G		Panc A-A-A-U
	Sequence: Gp		Venom pA, pG, pU
5	Alk $C_{2.0}$ $G_{0.8}$		U2 A, U-G_{OH}
	Panc $C_{2.0}$ $G_{1.2}$		Sequence: A-A-A-U-G_{OH}
	Sequence: C-C-Gp	16	Alk U, G
6	Alk $C_{3.0}$ $U_{1.1}$		Panc U, G
	Panc $C_{3.0}$ $U_{1.0}$		Sequence: U-Gp
	Venom $pC_{3.0}$ $pG_{0.7}$	17	Alk U, G
	CMCT U-C, C		Panc U, G
	Sequence: U-C-C-C-G_{OH}		Sequence: U-Gp
6a	Alk A, U	18	Alk U, G
	Panc U, A-G_{OH}		Sequence: U-Gp
	Venom A, G	19	Alk U, G
	U2 U-A		Panc U, G
	Sequence: U-A-G_{OH}		Sequence: U-Gp
6b	Alk C, A, U	20a	Alk $C_{1.0}$ $U_{1.1}$ $G_{1.0}$
	Panc C, A-U		Panc $C_{1.0}$ $U_{1.1}$ $G_{1.0}$
	Venom pA, pG, pU		Venom pC, pG, pU, pGp
	Sequence: C-A-U-G_{OH}		Sequence: U-C-Gp
6c	Alk $A_{1.0}$ $U_{1.0}$	20b	Alk C, U, G
	Panc A-U		Panc C, U, G
	Venom $pU_{1.0}$ $pG_{1.0}$		Venom pC, pU, pG, pGp
	Sequence: A-U-G_{OH}		Sequence: C-U-Gp
7[b]	Alk A	22	Alk U, A, G
	Venom pG		Panc U, A-G
8[b]	Alk A		Sequence: U-A-Gp
	Venom pG	23	Alk U, A, G
8a	Alk C, A, U		Panc U, A-G
	Panc $C_{2.0}$, $U_{1.1}$ A-$C_{1.0}$		Venom pA, pGp
	Venom pC, pA, pG		Sequence: U-A-Gp
	U2 (U,C_2)-A, C-G_{OH}	23a	Alk U, A, G
	Sequence: U-C-C-A-C-G_{OH}		Panc U, A-G
9	Alk A		Sequence: U-A-Gp
	Panc A-G_{OH}	23b	Alk U, A, G
	Sequence: A-G_{OH}		Panc U, A-G
11a	Alk $C_{2.0}$ $A_{1.0}$ $U_{1.1}$ $G^*_{\sim 2}$		Sequence: U-A-Gp
	Panc $C_{1.0}$ $U_{1.0}$ (A, G^*_2)-$C_{1.0}$	23c	Alk U, A, G
	Venom C, G, U, "fast C"		Panc U, A-G
	CMCT (A, G^*_2)-C, C-U-G		Sequence: U-A-Gp
	SWP A-G*, C-U-G, C-(C,U)-G	24	Panc U, C, A-U
	Sequence: A-G*-G*-C-C-U-G_{OH}		Venom pC, pG, pU
12c	Panc C, U, (A,G^*_2)-$C_{1.0}$ (*cf.* 11a)		Sequence: A-U-(C,U)-G_{OH}
	Sequence: A-G*-G*-C-C-U-G_{OH}		
24a	Panc U, C, A-U		Alk of "slow A-A-U" C, A, U

TABLE I (*Continued*)

Oligonucleotide No.	Sequencing Results	Oligonucleotide No.	Sequencing Results
	Venom pC, pA, pG, pU		Venom pC, pA, pGp
	Sequence: U-(C, A-U)-G_{OH}		Sequence: U*-A-A-C-A-A-Gp
24b	Panc U, C, A-A-U	31a	Alk $C_{3.3}$ $A_{3.1}$ $G_{0.9}$ $U_{1.0}$
	Venom pC, pA, pU		Panc $U_{0.8}$ $C_{1.0}$ $G_{1.0}$ $AC_{3.0}$
	Sequence: U-(C, A-A-U)-G_{OH}		Venom pA, pC, pGp
25	Alk $U_{1.8}$ $A_{1.1}$ $C_{1.0}$		U2 C-A, U-A, C-C-Gp
	Venom $pU_{1.8}$ $pH_{1.0}$ $pC_{1.0}$		CMCT C, A-C, U̇-A-C, Ġp
	Panc A-$U_{1.1}$ $C_{0.9}$ $U_{1.0}$		Sequence: U-A-C-A-C-A-C-C-Gp
	U2 A, (C,U_2)-G_{OH}	32	Alk $C_{1.0}$ $A_{4.3}$ $U_{1.9}$
	CMCT A-U̇-C		Panc $U_{1.0}$ A-$U_{1.0}$ A-A-A-$C_{1.1}$
	Sequence: A-U-C-U-G_{OH}		Venom pC, pA, pU, pG
26	Alk C, A, U		U2 A, A-A, (C,U)-G_{OH}, U-A
	Panc $C_{1.0}$ A-$U_{1.9}$		CMCT A-U̇-A-A-A-C
	Venom pC, pA, pU, pG		Sequence: A-U-A-A-C-U-G_{OH}
	U2 A, (U,C)-A, U-G_{OH}	33	Alk $C_{1.1}$ $A_{3.4}$ $G_{1.0}$ $U_{1.0}$
	CMCT A-U̇-C, A-U̇-Ġ$_{OH}$		Panc U, G, A-A-A-C
	Sequence: A-U-C-A-U-G_{OH}		Venom pC, pA, pGp
27	Alk $C_{2.5}$ $A_{2.3}$ $U_{2.0}$		U2 A, A-A, U-A, C-G
	Panc $U_{1.1}$ $C_{2.0}$ A-$C_{1.0}$ A-$U_{1.0}$		CMCT U̇-A-A-A-C, Ġp
	Venom pC, pA, pG, pU		Sequence: U-A-A-A-C-Gp
	U2 U-A, (U,C_3)-G_{OH}	34	Alk $U_{2.0}$ $G_{1.0}$
	CMCT C, A-U̇-A-C		Panc $U_{2.0}$ $G_{0.9}$
	Sequence: A-U-A-C-(U,C_2)-G_{OH}		Sequence: U-U-Gp
28	Alk $C_{0.9}$ $A_{2.9}$ $U_{2.0}$	35	Alk $C_{1.0}$ $G_{0.8}$ $U_{2.1}$
	Panc $U_{0.8}$ A-$C_{1.0}$ A-A-$U_{1.0}$		Panc $C_{1.1}$ $G_{0.9}$ $U_{2.0}$
	Venom pC, pA, pG, pU		Venom pU, pGp
	U2 A, U-A, U-A-A, C-G_{OH}		CMCT C, U̇-U̇-Ġ
	CMCT U̇-A-A-U̇-A-C		Sequence: C-U-U-Gp
	Sequence: U-A-A-U-A-C-G_{OH}	36-1	Panc $U_{2.0}$ $G_{0.8}$
29	Alk $C_{2.2}$ $A_{2.7}$ $U_{2.0}$		Sequence: U-U-Gp
	Panc $U_{1.2}$ $C_{2.2}$ A-A-$U_{1.0}$ A-$G_{OH\ 0.8}$	36-2	Alk $C_{1.0}$ $G_{0.6}$ $U_{2.0}$
	Venom pC, pA, pU, pG		Panc $C_{1.0}$ $G_{0.7}$ $U_{2.0}$
	U2 A, A-A, (U_2C_2)-A		Venom pU, pGp
	CMCT C, A-A-U̇-U̇-C, A-G_{OH}		CMCT C, U̇-U̇-Ġ
	Sequence: A-A-U-U-C-C-A-G_{OH}		Sequence: C-U-U-Gp
30	Alk $C_{3.2}$ $A_{2.9}$ $U_{2.0}$	36-3	Alk $C_{2.0}$ $G_{0.8}$ $U_{2.2}$
	Panc $U_{1.1}$ $C_{2.0}$ A-$C_{1.9}$ A-$U_{1.0}$		Panc $C_{2.0}$ $G_{0.9}$ $U_{2.1}$
	U2 A, U-A		Venom pC, pU, pGp
	CMCT C, A-C, A-U̇-A-C		CMCT C, U̇-U̇-Ġp
	SWP U-A, U-A-C, U-C-A, A-(U,A,C)-U		Sequence: C-C-U-U-Gp
	Sequence: A-U-A-C-U-C-A-C-C-G_{OH}	36a	Alk $A_{2.0}$ $G_{0.9}$ $U_{2.5}$
31-1	Alk $U_{1.2}$ $G_{0.8}$ $A_{3.0}$		Panc $U_{2.0}$ A-A-$G_{0.9}$
	Panc $G_{0.9}$ A-A-A-$U_{1.0}$		Sequence: U-U-A-A-Gp
	U2 A, A-A, U-G	37	Alk $A_{2.3}$ $G_{1.0}$ $U_{2.3}$
	Sequence: A-A-A-U-Gp		Panc $U_{1.4}$ A-$U_{1.0}$ A-$G_{0.8}$
31-2	Alk $C_{1.9}$ $A_{4.7}$ $G_{1.0}$ $U_{0.9}$		Venom A, U, pGp
	Panc "slow A-A-$U_{1.0}$" A-A-$G_{1.0}$		Sequence: A-U-U-A-Gp

[a] Throughout this table the following abbreviations are used: alk, alkaline hydrolysis; panc, digestion by pancreatic ribonuclease A; venom, complete hydrolysis by snake venom phosphodiesterase; U2, digestion by U2 ribonuclease; CMCT, digestion with pancreatic ribonuclease after blocking with *N*-cyclohexyl-*N'*-morpholinocarbodiimide metho-*p*-toluenesulfonate; SWP, combined digestion with silkworm nuclease and alkaline phosphatase. Procedures are as given in the Materials and Methods Section. [b] Both spots 7 and 8 gave identical ambiguous results, as discussed in the text. [c] The nucleotides indicated by G* in spot 11a behave like 7-methylguanylic acid residues. Spots 11a and 12 are probably identical.

Discussion

Our earlier observations on modification of 30S ribosomal subunits with kethoxal drew attention to the possibility that certain regions of 16S RNA may play a direct role in binding of tRNA during protein synthesis (Noller and Chaires, 1972). In view of other results which implicate the involvement of a subset of the 30S ribosomal proteins in this function (Randall-Hazelbauer and Kurland, 1972; Rummel

TABLE II: Oligonucleotide Sequences and Molar Yields.

Oligonucleotide No.	Deduced Sequence[e]	Rel Molar Yield[d]	Corresponding Nucleotide in Numbering System of Fellner *et al.* (1972)	Sections in 16S RNA
1	Gp	1.1		
1a	Gp	0.8		
1b	C-U-G_{OH}	0.4	89	
1c	Gp	0.8		
3	Gp	1.0		
4[f]	Gp			
5	C-C-Gp	0.3	109	B,C″,D′,O,K
6	U-C-C-C-G_{OH}	0.7	85	E′?, K
6a	U-A-G_{OH}	0.3	87	
6b	C-A-U-G_{OH}	0.3	79	O′?,E′
6c	A-U-G_{OH}	0.3	82	
8a	U-C-C-A-C-G_{OH}	0.1	76b	D′
9[f]	AG_{OH}		107	
11a	A-G*-G*-C-C-U-G_{OH}	0.1	Not reported	
12	A-G*-G*-C-C-U-G_{OH}	0.1	Not reported	
12a-1	A-A-G_{OH}	0.7	95	
12a-2	N.d.			
13	A-A-U-C-G_{OH}	0.9	70a	E
14	U-A-A-C-A-G_{OH}	0.2	63b	L
14a	A-A-A-U-G_{OH}	0.2	60	K
15	A-A-A-U-G_{OH}	0.6	60	K
16	U-Gp	0.4	91	
17	U-Gp	0.4	91	
18	U-Gp	0.3	91	
19	U-Gp	0.4	91	
20	U-C-Gp	0.4	90	
20a	U-C-Gp	0.3	90	
20b	C-U-Gp	0.2	89	
22	U-A-Gp	0.3	87	
23	U-A-Gp	0.5	87	
23a	U-A-Gp	0.3	87	
23b	U-A-Gp	0.1	87	
23c	U-A-Gp	0.2	87	
24	A-U-(C,U)-G_{OH}	0.2	42a	K′
24a	U-(C,A-U)-G_{OH}	0.3	Not reported	
24b	U-(C,A-A-U)-G_{OH}	0.2	40	E
25	A-U-C-U-G_{OH}	0.4	42a	K′
26	A-U-C-A-U-G_{OH}	0.4	34	L
27	A-U-A-C-(U,C_2)-G_{OH}	0.5	28	D′
28	U-A-A-U-A-C-G_{OH}	0.7	27	C″
29	A-A-U-U-C-C-A-G_{OH}	0.3	24b	C′1
30	A-U-A-C-U-C-A-C-C-G_{OH}	0.2	Not reported	
31-1	A-A-A-U-Gp	(0.3)	60	K
31-2	U*-A-A-C-A-A-Gp		57b	J
31a	U-A-C-A-C-A-C-C-Gp	0.1	56b[a]	A
32	A-U-A-A-A-C-U-G_{OH}	0.5	21[b]	P
33	U-A-A-A-C-Gp	0.4	63a	D′
34	U-U-Gp	0.8	54	
35	C-U-U-Gp	0.8	51	L,C
36-1	U-U-Gp	(0.8)	54	
36-2	C-U-U-Gp		51	L,C
36-3	C-C-U-U-Gp		47a	A
36a	U-U-A-A-Gp	0.8	39a	C,O
37	A-U-U-A-Gp	0.7	Not reported[c]	

[a] Fellner *et al.* (1972) report the sequence of 56b as U(A-C_3,C_2)G. This is probably identical with nucleotide 31a in my numbering system (see text). [b] Fellner *et al.* (1972) report A-U-U-A-A-A-C-G as the sequence of their nucleotide 21, which is similar to, but not identical with, the sequence of my nucleotide 32. [c] An oligonucleotide having a pancreatic RNase composition consistent with this sequence has been identified in a RNase T1 digest of 16S RNA by Santer and Santer (1973). [d] The relative molar yield is based on the average of 3–5 independent determinations, and normalized to nucleotide 3 = 1.0. [e] Sequences are not yet known for nucleotides 8, 11, 12a-2, and 21a. [f] Nucleotides 4 and 9 do not appear in Figure 2, because of their high mobility in the first electrophoretic dimension. They are the only major kethoxal nucleotides which do not appear in Figure 2.

TABLE III: Probable Oligonucleotide Pairs Adjacent to Sites of Kethoxal Attachment.

Oligonucleotide No.	Probable Sequence[a]	Section	Respective Molar Yields (from Table II)
1c–28	K G-G-U-A-A-U-A-C-G K	C''	0.8, 0.7
23a–29	G-U-A-G-A-A-U-U-C-C-A-G K	C'1	0.3, 0.3
33–6c	G-U-A-A-A-C-G-A-U-G K	D'	0.4, 0.3
23b–8a	G-U-A-G-U-C-C-A-C-G K	D'	0.1, 0.1
19–15	G-U-G-A-A-A-U-G K	E'-K	0.4, 0.6
36a–6	G-U-U-A-A-G-U-C-C-C-G K	K	0.8, 0.7
3–13	G-G-A-A-U-C-G K K	E	1.0, 0.9
36-3–31a	G-C-C-U-U-G-U-A-C-A-C-A-C-C-G	A	0.1

[a] K denotes sites of attachment of kethoxal. A G residue is assumed to be at the beginning of each sequence because of the known specificity of T1 ribonuclease.

and Noller, 1973; Lelong *et al.*, 1974), we suggested that the tRNA binding site, and possibly other ribosomal functional sites, may be composite structures made up of both protein and RNA moieties. The present work describes the nature of the sites of reaction of kethoxal with the 30S ribosomal subunit and their location within the 16S RNA chain. Sites which are crucial for ribosomal function are presumably among these nucleotide sequences (Table II) although assignment of possible functional roles awaits further studies.

These results also provide detailed information about the conformation and accessibility of 16S RNA within the 30S ribosomal subunit. Strong RNA–protein or RNA–RNA interactions shield nucleotides from reaction with kethoxal. In particular, it has been demonstrated that base-paired regions of RNA are strongly protected (Litt, 1969). Thus, the kethoxal-reactive guanine sites identified in Table II are not involved in base pairing or in intimate protein–RNA interactions under these reactions conditions.

The accessibility of kethoxal to 16S RNA as revealed by the sequences of fragments 13, 24, 24b, 25, 26, 31-1, 31-2, and 31a are inconsistent with the base-pairing schemes suggested for sections E, K', L, J, and A by Fellner and coworkers (Ehresmann *et al.*, 1974). Thus only about half of the accessible guanine sites are consistent with the secondary structure model proposed by these authors, suggesting that local base pairing of the type found in tRNA molecules may not be sufficient to account for the secondary structure observed in intact ribosomal particles.

Some of the results of partial nuclease digestion of 30S ribosomal subunits are consistent with the present results. For example, oligonucleotide 14 is coincident with the site of partial nuclease cleavage of 16S RNA at the 3' end of section L (Ehresmann *et al.*, 1972) and at the end of band 5' (Santer and Santer, 1972). Oligonucleotide 27 marks the site of partial nuclease cleavage between sections C'2 and D' of Fellner and coworkers (Ehresmann *et al.*, 1972) as well as the 3' end of "band 300" and the 5' end of "band 7" (Santer and Santer, 1973). Similarly fragment 15 lies at the junction of sections E' and K and at the 5' end of "band 475." In addition, RNase T1 digestion of intact 30S subunits results in release of several free oligonucleotides, nearly all of which are from sections A and J (Santer and Santer, 1973). Some of the nucleotides listed in Table II are among those released. Thus, many of the sites of kethoxal attachment (6, 8a, 14, 15, 27, 31-2, 31a, 32, 36-3, 36a and 37) are identical with sites which are accessible to nucleases. However, other sites appear not to be nuclease susceptible (*e.g.*, 13, 25, 26, 28, 29, and 33), although many of them react with kethoxal in high yield. This might be ex-

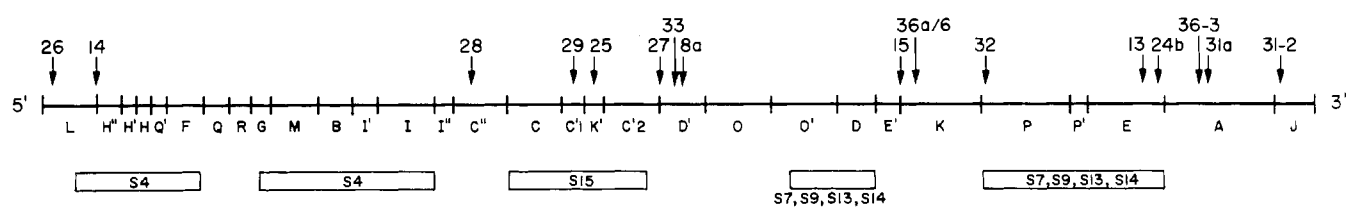

FIGURE 3: Distribution of kethoxal-reactive sites in the 16S RNA chain. Oligonucleotides from Table II which can be unambiguously located in the 16S RNA sequence are located at the positions indicated by the arrows. Arrangement of lettered sections is according to Ehresmann *et al.*, (1974). Sections of RNA previously implicated in the binding of ribosomal proteins S4 and S15 (Zimmerman *et al.*, 1972) and RNA fragments from a subribosomal nucleoprotein particle containing proteins S7, S9, S13, and S14 or S19 (Székely *et al.*, 1973) are indicated by the bars in the lower part of the figure. Ribosomal protein S8 has been reported to bind an RNA fragment corresponding roughly to section C (Schaup *et al.*, 1973), included in the section bound by S15.

plained either by restricted nucleotide conformations or backbone interactions which could render a phosphodiester bond nuclease resistant, or by the existence within the 30S ribosomal subunit of RNA-containing sites too restricted to allow the entry of an enzyme molecule, but large enough for the penetration of kethoxal. Results of partial nuclease cleavage must be interpreted with caution when used as a probe of RNA topography. Any nucleolytic cleavage event gives rise to an interruption of the primary structure of the polynucleotide chain, which in turn may result in major alterations of secondary and tertiary structure. Subsequent cleavages may thus occur at normally buried sites which become exposed as a result of the initial cleavage. Interpretation of kethoxal modification results must also be undertaken with caution, but probably to a more subtle degree, due to the much less drastic effect of kethoxal on ribosome structure (Noller and Chaires, 1972).

Kethoxal-reactive sites are distributed throughout the 16S RNA chain (Figure 3), indicating that folding of the RNA in the 30S subunit leaves exposed single-stranded regions at frequent intervals. Although only 16 of the 26 sites can be uniquely placed in the 16S sequence at present, there appear to be gaps in the distribution of exposed sites. No reactive guanines are found in the 400 nucleotide sequence between sections H″ and C″, which is the region near the 5′ terminus previously found to be protected by ribosomal protein S4 (Zimmerman *et al.*, 1972). There also appear to be protected regions of 200–300 nucleotides in the 3′ half of the chain, between sections D′ and K and K and E, respectively. These are the sections of 16S RNA contained in the nucleoprotein subparticle containing proteins S7, S9, S13, and S14 or S19 which has been isolated after mild nuclease digestion of 30S ribosomal subunits (Morgan and Brimacombe, 1972; Székely *et al.*, 1973). The other reported protein binding region, that of S15 (Zimmerman *et al.*, 1972), contains two exposed sites, indicating that at least part of the region of RNA bound to S15 contains exposed, unpaired nucleotides in the intact 30S particle. This suggests that a possible role for some ribosomal proteins may be to hold critical RNA sites in a specific conformation.

In general, the 3′ half of 16S RNA contains many more exposed sites than the 5′ half. This observation may reflect the involvement of the 5′ half of the chain in early assembly steps, or its more extensive involvement with ribosomal proteins, whereas the 3′ half may contain more of the sites that participate directly in ribosomal function. There are about 400 guanine residues in 16S RNA, and since about 60–70% of the molecule is estimated to be involved in base pairing (Cox, 1965), at least 120 guanines are unpaired. Since only about 26 guanines are available for reaction with kethoxal, it can be concluded that approximately three-fourths of the unpaired guanines must be involved in either RNA–protein interactions or RNA–RNA interactions other than Watson–Crick base pairing.

The wide range of values observed for molar yields of oligonucleotides (Table II) is of some interest. It might be attributable to variable rates of reaction of various sites due to conformational restraints or differing degrees of accessibility, or it may reflect structural heterogeneity. It is unlikely that all differences can be explained on the basis of reaction rates alone, since the molar yields of some fragments appear to level off at fractional values which do not increase appreciably with prolonged incubation (H. F. Noller, unpublished results). Structural heterogeneity may be of two kinds. There may be sequence heterogeneity at a given site in 16S RNA, or there may be variable accessibility to a given RNA sequence. Evidence for sequence heterogeneity has been reported (Ehresmann *et al.*, 1974), but is not sufficient to account for the data found for the sequences in Table II. Accessibility to a site might become altered in several ways. A conformational change in the 30S particle during isolation, such as the one described by Zamir *et al.* (1969), or radiolytic damage, could lead to changes in the accessibility of regions of the RNA chain. Alternatively, loss of a ribosomal protein from the particle could expose a polynucleotide region that is normally protected. Loss of a protein from a fraction of the ribosome population, as has been reported to be the case *in vitro* (Voynow and Kurland, 1971), would thus expose a fraction of the 16S RNA molecules at a given site. It is conceivable that such fractional accessibility of rRNA could be of functional significance. Further work is required to clarify the structural and functional basis of partially reactive sites. It is worthy of note that the two kethoxal-reactive sites in 5S RNA are both fully accessible in the intact 50S subunit (Noller and Herr, 1974).

The diagonal electrophoresis technique described here, in conjunction with kethoxal modification, is a convenient method for isolation and identification of accessible guanine residues in large RNA molecules. It should also prove to be of use in RNA sequence determination. Partial nuclease digestion is usually employed for establishing the order of oligonucleotides within a long sequence, but it is often difficult to avoid cleaving at particularly susceptible sites. Such sites are likely to be readily modified by kethoxal, and use of the diagonal method can provide the nucleotide sequence around susceptible sites, thus providing overlaps between large fragments obtained by partial digestion.

Use of this technique to provide overlap information is illustrated by the data in Table III. The sequences defined by the pairs 1c–28, 23a–29, 3–13, and 36-3–31a confirm the sequences proposed by Fellner and coworkers (Ehresmann *et al.*, 1974). The pair 19–15 provides an overlap between sections E′ and K, and indicate a missing GUG sequence linked to the 5′ end of section K. Three other pairs indicate transpositions of the order of oligonucleotides within the sequence reported by the latter authors. Pairs 33–6c and 23b–8a give the sequences GUAAACGAUG and GUAGUCCACG, respectively, whereas Fellner and coworkers have reported the sequence GUAGUAAACGUCCACGAUG in their section D′. Both sequences are consistent with the T1 and RNase A digestion products obtained, but only the sequences in Table III are consistent with the results of the kethoxal diagonal analysis, in which the dephosphorylated oligonucleotide in a pair must be linked to the 3′ end of the oligonucleotide bearing a 3′-phosphate. The pair 36a–6, GUUAAGUCCCG, similarily corresponds to the sequence GUCCCGUUAAG reported by the latter authors for section K, where it seems likely that two T1 oligonucleotides have been transposed. Use of this method with fragments of 16S RNA might provide a means of clarifying the order of oligonucleotides in uncertain regions of the polynucleotide chain.

Use of the approach presented here with ribosomes in various functional states should shed light on the possible role of rRNA in protein synthesis. For example, protection of some of these sites from reaction with kethoxal by bound mRNA or tRNA would provide evidence for the possible interaction of these ligands with specific RNA sequences. Studies of this kind are in progress.

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