Ivanov, V. I., & Minyat, E. E. (1981) *Nucleic Acids Res. 9*, 4793-4798.
Jovin, T. M., van de Sande, J. H., Zarling, D. A., Arndt-Jovin, D. J., & Eckstein, F., et al. (1982) *Cold Spring Harbor Symp. Quant. Biol. 47*, 143-154.
Jovin, T. M., McIntosh, L. P., Arndt-Jovin, D. J., Zarling, D. A., Robert-Nicoud, M., & van de Sande, J. H., et al. (1983) *J. Biomol. Struct. Dyn. 1*, 21-56.
Kleppe, K., van de Sande, J. H., & Khorana, H. G. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 68-72.
Lilley, D. M. J. (1981) *Nucleic Acids Res. 9*, 1271-1289.
Marky, K. A., Blumenfeld, K. S., Kozlowski, S., & Breslauer, K. J. (1983) *Biopolymers 22*, 1247-1257.
Mizuuchi, K., Mizuuchi, M., & Gellert, M. J. (1982) *J. Mol. Biol. 256*, 229-243.
Nordheim, A., Lafer, E. M., Peck, L. J., Wang, J. C., Stollar, B. D., & Rich, A. (1982) *Cell (Cambridge, Mass.) 31*, 309-318.
Panayotatos, N., & Wells, R. D. (1981) *Nature (London) 289*, 466-470.
Patel, D. J., Canuel, L. L., & Pohl, F. M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2508-2511.
Patel, D. J., Kozlowski, S. A., Ikuta, S., Itakura, K., Batt, R., & Hare, D. R. (1982) *Cold Spring Harbor Symp. Quant. Biol. 47*, 197-206.
Pohl, F. M. (1976) *Nature (London) 260*, 365-366.
Pohl, F. M. (1982) *Cold Spring Harbor Symp. Quant. Biol. 47*, 113-117.
Pohl, F. M., & Jovin, T. M. (1972) *J. Mol. Biol. 67*, 375-396.
Quadrifoglio, F., Manzini, G., Vasser, M., Dinkelspiel, K., & Crea, R. (1981) *Nucleic Acids Res. 9*, 2195-2206.
Singleton, C. K., Klysik, J., Stirdivant, S. M., & Wells, R. D. (1982) *Nature (London) 299*, 312-316.
Tinoco, I., Uhlenbeck, O. C., & Levine, M. D. (1971) *Nature (London) 230*, 362-367.
van de Sande, J. H., & Jovin, T. M. (1982) *EMBO J. 1*, 115-120.
van de Sande, J. H., McIntosh, L. P., & Jovin, T. M. (1982) *EMBO J. 1*, 777-786.
Wang, A. H.-J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., van Boom, J. H., van der Marel, G., & Rich, A. (1979) *Nature (London) 282*, 680-686.

Articles

Mapping Labeled Sites in *Escherichia coli* Ribosomal RNA: Distribution of Methyl Groups and Identification of a Photoaffinity-Labeled RNA Region Putatively at the Peptidyltransferase Center†

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ABSTRACT: We have developed a method for the rapid localization of sites of ribosomal RNA labeling to limited regions (~200 bases). The method is based on the formation and polyacrylamide gel electrophoretic separation of hybrids between restriction fragments of *rrn*B DNA and isotopically labeled rRNA and the subsequent determination of radioactivity across the gel. Using [^{3}H]adenine-labeled rRNA as a control sample, we optimized experimental conditions with respect to a number of variables, including rRNA:DNA stoichiometric ratio, temperature of the annealing step, and levels of nucleases. An important result is that different rRNA·DNA hybrid fragments are obtained in different yields. The method was then applied to analyses of C^3H_3-labeled rRNA, giving results in good accord with known and proposed sites of rRNA methylation, and of rRNA that has been photoaffinity-labeled with 5-azido-2-nitrobenzoyl-[^{3}H]Phe-tRNA^{Phe}, a probe directed toward the peptidyltransferase center. The latter study showed a single major site of RNA labeling, falling within bases 2445-2668 of 23S rRNA. The extent of labeling was shown to be dependent on light-induced formation of a reactive intermediate and to be decreased in the absence of poly(uridylic acid) or in the presence of puromycin. The location of this major site of labeling is consistent with recent results obtained with an analogous tRNA photoaffinity label [Barta, A., Steiner, G., Brosius, J., Noller, H. F., & Kuechler, E. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3607-3611] and with related genetic and biochemical studies of antibiotic interaction with ribosomes suggesting that the peptidyltransferase center falls within region V (bases 2043-2625) of 23S rRNA.

In recent years increasing emphasis has been placed on the possible functional roles of rRNA in overall ribosomal function (Noller, 1984). An important step for the development of appropriate models is the placement of specific functional sites within the rRNA structure. Although affinity labeling is a potentially powerful way of identifying such sites and several affinity labels of ribosomal ligands have been known for some time to incorporate covalently into rRNA (Cooperman, 1980), localization of such incorporation to either specific bases or limited rRNA regions has proven to be quite difficult. As a

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result, few such localizations have been reported, and up until quite recently (Barta et al., 1984), those that were reported depended on experimental approaches that were not applicable generally (Wagner et al., 1976; Prince et al., 1982).

In this paper we first develop a general method for the rapid localization of sites of labeling to limited (~200 bases) rRNA regions that is based on the formation and polyacrylamide gel electrophoretic (PAGE)[1] separation of hybrids between restriction fragments of *rrn*B DNA and rRNA. We then apply the method in examining the distribution of methyl groups within mature, posttranscriptionally modified rRNA and in localizing the site of photoincorporation of an aryl azide derivative of Phe-tRNA^{Phe} designed as a probe of the peptidyltransferase center. In the latter study, we exploit the capability of the method for quantitatively measuring even very low levels of labeling to determine the effects of several experimental variables on the level of labeling, thereby probing the significance of the labeling result.

Materials and Methods

The following materials were purchased from the commercial sources indicated: [^{3}H]adenine, [*methyl*-^{3}H]-methionine, and [^{3}H]phenylalanine, Amersham-Searle; RNase A, bentonite, and ethidium bromide, Sigma Chemical Co.; S1 nuclease, tRNA^{Phe}, and *Hpa*II restriction endonuclease, Boehringer-Mannheim; restriction enzymes *Hin*fI, *Hae*III, and *Taq*I, Bethesda Research Laboratories; RNase T1, Calbiochem; technical-grade 5-amino-2-nitrobenzoic acid, Fluka; all other chemical reagents, Aldrich. All plasticware was treated with 0.2% diethyl pyrocarbonate and autoclaved. All buffers were autoclaved. All glassware was heated to 200 °C before use.

Ribosomes and Subunits. Washed 70S ribosomes, and ribosomal subunits, were prepared from *Escherichia coli* Q13 as previously described (Traub et al., 1971; Jaynes et al., 1978).

[^{3}H]Adenine-Labeled rRNA. E. coli Q13 cells were grown at 37 °C to an A_{550nm} of 1.3 in minimal medium (Zimmermann, 1979) containing 2 mCi/L of [2-^{3}H]adenine or [8-^{3}H]adenine. The harvested cells were resuspended and sonicated in buffer A [10 mM Tris-HCl (pH 7.6), 15 mM $MgCl_2$, 30 mM KCl, 6 mM β-mercaptoethanol] made up in 30% sucrose. After removal of cell debris by centrifugation at 30000*g* (30 min), ribosomes were sedimented by ultracentrifugation (200000*g*, 3 h) and resuspended at a final concentration of 40 A_{260nm}/mL in SET buffer [15 mM Tris-HCl (pH 7.6), 150 mM NaCl, 10 mM Na_2EDTA]. Following addition of 0.1 volume of 10% SDS and 0.05 volume of 2% bentonite, the resulting mixture was extracted a total of 4 times with equal volumes of SET buffer–equilibrated phenol. The rRNA was precipitated by addition of 2 volumes of ethanol to the final aqueous layer and overnight incubation at −20 °C and was pelleted by centrifugation (8000*g*, 20 min). For use in hybridization experiments, the rRNA was resuspended in hybridization buffer (Berk & Sharp, 1977) [80% formamide containing at a final concentration 40 mM PIPES (pH 6.4), 0.4 M NaCl, and 1 mM Na_2EDTA]. The specific radioactivity was 2×10^5 cpm/A_{260nm}. Ribosomal RNA was stable toward storage in hybridization buffer at −80 °C.

C^{3}H$_3$-Labeled rRNA. E. coli cells [strain JC355, a methionine auxotroph (Flaks et al., 1966; Chang et al., 1974)], obtained from Prof. Joel Flaks, were labeled by a 10-min pulse of L-[*methyl*-^{3}H]methionine (sp act. 8.8 Ci/mmol, 0.1 mCi/g of cells) in M9 medium (Anderson, 1946; Davis et al., 1980). Ribosomal RNA was extracted from labeled cells as described for [^{3}H]adenine-labeled rRNA. The specific radioactivity of the rRNA was approximately 3×10^4 cpm/A_{260nm}.

Restriction Fragments of pKK3535. E. coli HB101 cells containing pKK3535 were obtained from Dr. H. Noller (Brosius et al., 1981a). Cells were grown, and the plasmid was isolated according to Lillis et al. (1982) except that LB medium (Davis et al., 1980) was used. Restriction enzyme digests of pKK3535 were performed on plasmid solutions (0.14–0.25 mg/mL of DNA) with manufacturer-described protocols. For use in hybridization experiments, they were deproteinized by extraction with a one-third volume of SET buffer–equilibrated phenol, precipitated from the aqueous layer by addition of 2 volumes of ethanol and overnight incubation at −20 °C, collected by centrifugation (20000*g*, 1 h), and resuspended in hybridization buffer at a final concentration of 0.5–2 mg/mL.

5-Azido-2-nitrobenzoyl-[^{3}H]Phe-tRNA^{Phe} (ANB-[^{3}H]Phe-tRNA^{Phe}). [^{3}H]Phe-tRNA^{Phe} was prepared by enzymatic charging of tRNA^{Phe} with [^{3}H]phenylalanine (38.6 Ci/mmol), yielding 340–500 pmol of [^{3}H]Phe/A_{260nm} of tRNA^{Phe}. 5-Amino-2-nitrobenzoic acid was converted to 5-azido-2-nitrobenzoic acid and then condensed with *N*-hydroxysuccinimide essentially as described by Seela & Hansske (1976). The resulting *N*-succinimidyl-5-azido-2-nitrobenzoate was dissolved in Me_2SO (1.5 mg/mL), and 1.5 mL of this solution was added to 0.3 mL of 0.05 M sodium phosphate (pH 6.8) containing 8 A_{260nm} of [^{3}H]Phe-tRNA^{Phe}. Following incubation for 3 h at 37 °C, the reaction was quenched by addition of 75 μL of 1 M HCl. tRNA was precipitated by addition of 3.75 mL of ethanol and brief chilling in dry ice–ethanol and collected by centrifugation. The pellet was redissolved in 450 μL of 0.05 M sodium phosphate (pH 4.5), and tRNA was again ethanol precipitated and collected by centrifugation. The washed pellet was resuspended in 450 μL of the same buffer and stored at −80 °C prior to use. This preparation of ANB-[^{3}H]Phe-tRNA^{Phe} is a modification of an analogous procedure of Hoffman et al. (1969) for the preparation of a spin-labeled tRNA. The extent of NH_2 derivatization of [^{3}H]Phe-tRNA^{Phe} was typically 70–90%, as measured by the method of Schofield & Zamecnik (1968). All synthetic steps were carried out in reduced light to minimize photolysis of the azide group.

Photoincorporation of ANB-[^{3}H]Phe-tRNA^{Phe} into E. coli Ribosomes. Photolysis reaction mixtures contained 70S ribosomes, poly(U), ANB-[^{3}H]Phe-tRNA^{Phe}, and/or various other components, as indicated in the text, were made up in photolysis buffer [10 mM HEPES (pH 7.2), 100 mM KCl 20 mM $MgCl_2$, 2 mM β-mercaptoethanol] and preincubated at 37 °C for 20 min. In a typical experiment, an aliquot was withdrawn to measure noncovalent binding of ANB-[^{3}H]-Phe-tRNA^{Phe} to ribosomes (see Table IV). The remaining solution was placed in a quartz tube and photolyzed for 3 min in a Rayonet RPR 100 reactor equipped with RPR 3500-Å lamps at 4 °C, as described previously (Jaynes et al., 1978) (the $t_{1/2}$ for photolysis of the azide group in our apparatus is 28 s). Following photolysis, β-mercaptoethanol was added to a final concentration of 20 mM to quench the light-independent reaction, the photolysis mixture was centrifuged (20000*g*, 90 min) to remove polymeric material formed on azide photolysis (Nicholson et al., 1982), and ribosomes and tRNA were collected by addition of 2 volumes of 9:1 ethanol–β-mercaptoethanol and centrifugation. The RNA from

[1] Abbreviations: ANB, 5-azido-2-nitrobenzoyl; PAGE, polyacrylamide gel electrophoresis; poly(U), poly(uridylic acid); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Na_2EDTA, disodium ethylenediaminetetraacetate; PIPES, 1,4-piperazinediethanesulfonic acid.

this pellet was isolated from the ribosomes as described for [^{3}H]adenine-labeled rRNA and dissolved in hybridization buffer prior to further analysis.

Polyacrylamide Gel Electrophoresis of ^{3}H-Labeled rRNAs. The specific radioactivities of 23S and 16S rRNAs prepared from [^{3}H]adenine-labeled, C^{3}H$_3$-labeled, and ANB-[^{3}H]-Phe-tRNA^{Phe}-labeled ribosomes were determined by PAGE analysis. Samples of these materials were each added to 1 volume of 8 M urea, 1 mM Na_2EDTA, and 0.1% bromophenol blue made up in 40% sucrose, and the resulting solutions were analyzed by PAGE. The gel contained 2.85% acrylamide–0.15% bis(acrylamide) made up in TBE gel buffer (Peacock & Dingman, 1968) [89 mM Tris–borate (pH 8.3), 2.5 mM Na_2EDTA] containing 8 M urea. The RNA bands were detected by ethidium bromide staining. Radioactivity across the gel was determined by gel slicing and peroxide solubilization (Jaynes et al., 1978).

Standard Procedure for Hybridization of ^{3}H-Labeled rRNAs to pKK3535 Restriction Fragments and Analysis of the Resulting Hybrids. Hybridizations were performed as described below except as indicated in the text. A solution of pKK3535 restriction fragments (final concentration 0.32 mg/mL) and ^{3}H-labeled rRNA (final concentration 0.18 mg/mL) made up in hybridization buffer was heated at 80 °C for 15 min and then incubated for 1 h at 55 °C. This solution was then rapidly cooled by dilution with 9 volumes of 0 °C S1 buffer [30 mM sodium acetate (pH 4.6), 0.25 M NaCl, 1 mM $ZnSO_4$] (Berk & Sharp, 1977). S1 nuclease [6.4 units (as defined by Boehringer-Mannheim)/μg of nucleic acids] was then added. Digestion was allowed to proceed at 45 °C for 20 min and was terminated by addition of 0.1 volume of 0.6 M Tris-HCl (pH 8.3). The resulting mixture was extracted with 0.4 volume of SET buffer–equilibrated phenol, and nucleic acid was precipitated by addition of 2 volumes of ethanol and overnight incubation at −20 °C. The pellet obtained by centrifugation was rinsed with ethanol to remove traces of phenol, dried under a stream of N_2, dissolved in T1 buffer (Padgett et al., 1983) [10 mM HEPES (pH 7.6), 0.2 M NaCl, 1 mM Na_2EDTA], 0.265 mL/mg of total original nucleic acid, and digested with RNase T1 [0.146 unit (as defined by Calbiochem)/μg of original rRNA] for 30 min at 25 °C. The resulting mixture was extracted with 1.0 volume of SET buffer–equilibrated phenol, and the aqueous layer was dialyzed overnight (Spectrapor membrane tubing I, Spectrum Medical Industries) against SET buffer. The dialyzed sample was added directly to a 0.5 volume of 6.5% Ficoll 400, 250 mM NaCl, and 0.1% bromophenol blue in TBE gel buffer and applied to a 4.75% acrylamide −0.25% bis(acrylamide) gel made up in TBE gel buffer. Visualization of bands and determination of radioactivity across the gel were performed as described above. In an earlier version of this procedure, digestion with RNase A [0.0032 Kunitz (1946) units/μg of original rRNA] for 15 min at 0 °C in 2 × SSC buffer [0.3M NaCl, 30 mM sodium citrate (pH 7.0)] replaced digestion with RNase T1.

Results

Outline of the Method. The method described in this paper for localizing sites of radioactive labeling of rRNA is based upon the hybridization of defined fragments of *rrn*B DNA, generated from plasmid pKK3535 by restriction enzyme digestion, to a stoichiometric excess of labeled rRNA, under conditions modeled on those described by Casey & Davidson (1977) and Berk & Sharp (1977). Digestion of the resulting reaction mixture by S1 nuclease (to remove denatured single-stranded DNA and single-stranded RNA) and RNase T1 (to remove residual RNA) leaves only the RNA·DNA hybrids intact. The hybrids, in the amount of tens to hundreds of micrograms, are then separated by PAGE essentially according to size, and the radioactivity in each hybrid is determined by gel slicing and counting. A simplified schematic of this method is presented in Figure 1.

Below we describe the application of this method to determining the labeling patterns of three different kinds of radioactively labeled rRNA: (1) RNA isolated from cells grown on [^{3}H]adenine, which provides a useful control for the optimization of a variety of experimental conditions; (2) RNA isolated from cells grown on C^{3}H$_3$-labeled methionine, which allows quantitative comparison of our results with predictions based on published studies identifying (with varying degrees of certainty) sites of methylation in rRNA; (3) RNA isolated from purified ribosomes that have been photoaffinity labeled with ANB-[^{3}H]Phe-tRNA^{Phe}, which allows the identification of a region of rRNA at or near the peptidyltransferase center.

Analysis of [^{3}H]Adenine-Labeled rRNA. The result of a typical PAGE analysis of the rRNA·rDNA hybrids formed when a *Hin*fI digest of pKK3535 is hybridized to [^{3}H]-adenine-labeled rRNA and then nuclease digested, as described under Materials and Methods, is shown in Figure 2 (lane 3). A total of 11 major bands (corresponding to duplexes of 120 base pairs or more) are visible. That these bands correspond to RNA·DNA hybrids is demonstrated by the large reduction in ethidium-staining material seen if RNA is omitted from the hybridization procedure (Figure 2, lane 6) and the total absence of ethidium-staining material seen if *Hin*fI fragments are omitted from the hybridization procedure (Figure 2, lane 7). The low-intensity bands seen in lane 6 are due to renatured DNA. The amount of such material increases if the temperature is reduced below 50 °C or if the amount of S1 nuclease employed is reduced. The numbered bands correspond to the rRNA sequences shown in Table I. The assignments are made on the basis of the sequence length of the hybrids, with *Hin*fI and *Hae*III restriction fragments of pKK3535 [whose sizes may be predicted with confidence since the plasmid sequence is known (Brosius et al., 1981a)] to calibrate the gel from a plot of log (sequence length) vs. migration (Figure 3). Although the observed migrations of RNA·DNA hybrids generally correspond to those predicted on the basis of DNA·DNA duplex migrations, slight deviations are sometimes observed. A check of the assignments is provided by repeating the experiments and replacing total ribosomal RNA either with a combination of 23S rRNA and 5S RNA extracted from 50S subunits (Figure 2, lane 4) or with 16S rRNA extracted from 30S subunits (Figure 2, lane 5).

Determination of the radioactivity in the RNA·DNA hybrids such as those shown in Figure 2, lane 3 (Figure 4), demonstrates the quantitative validity of our method, since the radioactivity in a band clearly increases as a function of size (i.e., adenine content). There are, however, two problems that have the potential for limiting the precision of the method. First, as is obvious from Figure 4, there is a region of radioactivity (which is subject to considerable variation from experiment to experiment) that migrates more slowly than the largest predicted RNA·DNA hybrid. No radioactivity is observed in this region in a control experiment lacking DNA restriction fragments, and we believe the slow-moving radioactivity represents hybrids containing incompletely digested RNA and/or DNA. Second, although the overall yield of RNA·DNA hybrids (determined from the radioactivity comigrating with hybrid bands that is recovered from the gel compared to that applied, and corrected for the stoichiometric

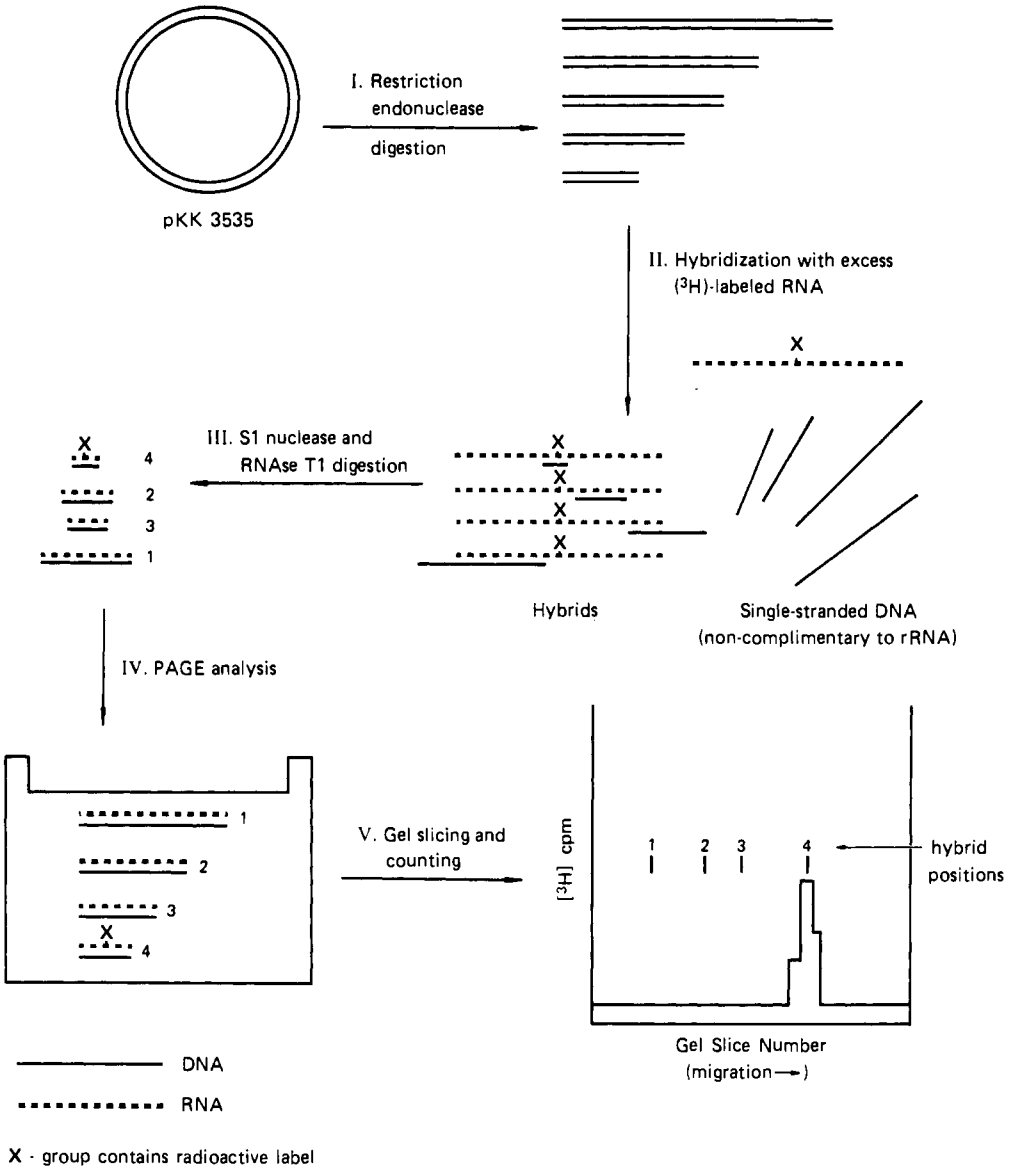

FIGURE 1: Schematic of method for localizing sites of labeling within ribosomal RNA.

Table I: rRNA·DNA Hybrids:[a] Sequences and Predicted Methylation[b] Stoichiometries

	restriction enzyme								
	*Hinf*I			*Hpa*II			*Taq*I		
hybrid	rRNA source	sequence	no. of Me	rRNA source	sequence	no. of Me	rRNA source	sequence	no. of Me
1	23 S	2008–2904	7	16 S	1–505	0	16 S	65–824	1
2	23 S	1345–2007	3	23 S	924–1299	0	23 S	368–1110	2
3	16 S	658–1313	3	23 S	1501–1839	1	23 S	1754–2481[c]	7
4	23 S	640–1113	2	23 S	1897–2216	4	23 S	2503–2904	1
5	23 S	281–639	0	23 S	186–488	0	23 S	1378–1753	1
6	16 S	1–340	0	16 S	859–1138	2	16 S	964–1323	3
7	16 S	341–657	1	23 S	2445–2668	3	23 S	1111–1346	0
8	23 S	1114–1344	0	23 S	489–681	0	23 S	142–367	0
9	23 S	1–162	0	23 S	2217–2396	2	16 S	1324–1542	9
10	16 S	1342–1478	3	16 S	1143–1304	1	23 S	1–141	0
11	5 S	1–120	0	16 S	1386–1542	9	5 S	1–120	0
12	23 S	163–235	0	23 S	1300–1448	0	16 S	878–963	0
13	16 S	1479–1542	6	16 S	722–858	0	16 S	1–64	0
14	23 S	236–280	0	23 S	2719–2842	0	16 S	825–877	0
15	16 S	1327–1341	0	5 S	1–120	0	23 S	1347–1377	0
16	16 S	1314–1326	0	23 S	682–797[d]	2	23 S	2482–2502	1

[a] Arranged in order of decreasing size. [b] According to Noller (1984) and references cited therein. [c] The *Taq*I site at position 1884 of 23S rRNA is methylated in HB101 and is not cleaved (Brosius et al., 1981b). [d] Hybrids smaller than 116 base pairs are not listed.

excess of RNA over DNA) is generally reproducible (typically 35 ± 10%), there is considerable variation in the yields of the individual hybrids. This variation is clear from the results presented in Table II, which show that, on average, we recover more than twice as much of the highest yield hybrids (hybrids 8 and 9) than of the hybrids obtained in lowest yield (hybrids

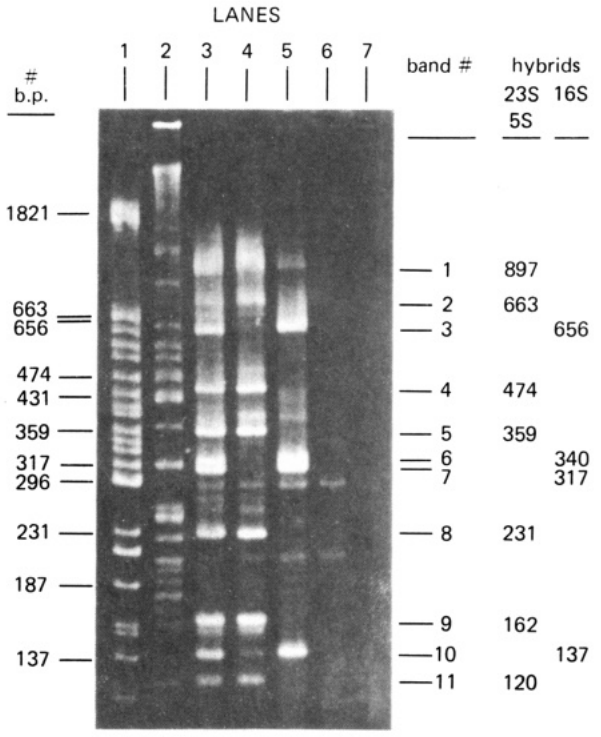

FIGURE 2: PAGE analysis of rRNA·*rrn*B hybrids. The following samples were electrophoresed on a 4.4% polyacrylamide gel: (lane 1) *Hin*fI digest of pKK3535 (pKK3535/*Hin*fI); (lane 2) *Hae*III digest of pKK3535 (this is included only to aid in calibrating the gel); (lane 3) 32 μg of pKK3535/*Hin*fI hybridized to 33 μg of rRNA extracted from 70S ribosomes (5-fold molar excess), in 124 μL. (lane 4) 32 μg of pKK3535/*Hin*fI hybridized to 30 μg of rRNA extracted from 50S ribosomal subunits (7-fold molar excess), in 124 μL. (lane 5) 32 μg of pKK3535/*Hin*fI hybridized to 13 μg of rRNA extracted from 30S ribosomes (6-fold molar excess), in 124 μL. (lane 6) minus RNA control, 16 μg of pKK3535/*Hin*fI in 60 μL; (lane 7) minus DNA control, 16 μg of rRNA (from 70S) in 65 μL. Unhybridized nucleic acid was degraded with S1 nuclease, followed by limited RNase A digestion. The columns to the right of the gel show the positions and sizes of hybrids derived from the DNA restriction fragments.

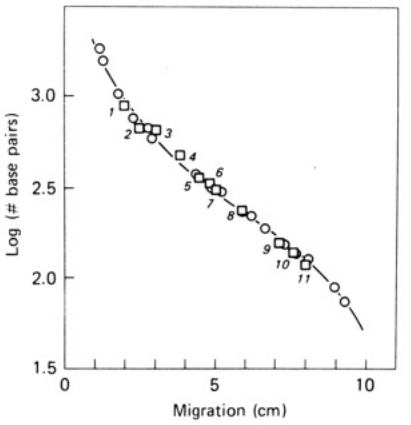

FIGURE 3: Size dependence of electrophoretic mobility of pKK3535 restriction fragments and rRNA:DNA hybrids: *Hin*fI and *Hae*III fragments (○); rRNA·DNA hybrids (□). Based on results in Figure 2.

3 and 4). Such variation has clear significance for the interpretation of PAGE analyses of specific labeling experiments, a point we return to below.

Several experimental parameters affect hybrid yield. An important one is the RNA:DNA stoichiometric ratio. We found that at a ratio of 1.0 or less the only hybrids found in reasonable yield were those corresponding in size to 23S, 16S, and 5S rRNA. Small amounts of *Hin*fI hybrid bands 9 and

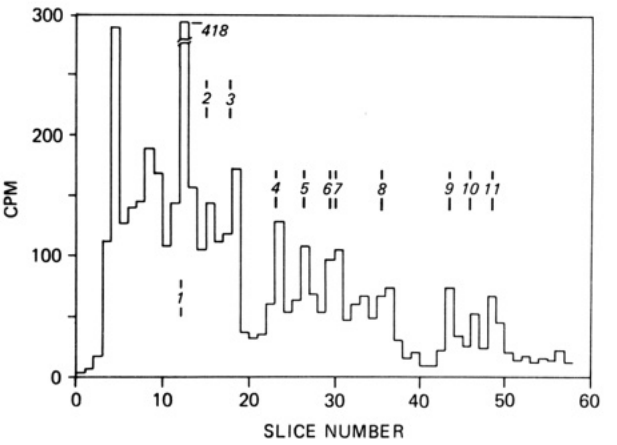

FIGURE 4: Radioactivity profile of PAGE analysis of [^{3}H]adenine-labeled rRNA hybridized to *Hin*fI-digested pKK3535. Hybridization conditions were 36 μg of DNA and 20 μg of rRNA in 123 μL of hybridization buffer, incubated for 60 min at 50 °C, followed by S1 nuclease and RNase A digestions. The resulting hybrids were electrophoresed on a 4.4% polyacrylamide gel. The positions of ethidium bromide stained hybrid bands, numbered as in Table I, are indicated. The gel was sliced and radioactivity determined as described under Materials and Methods.

Table II: Yields of *Hin*fI Hybrids of [^{3}H]Adenine-Labeled rRNA

hybrid	no. of base pairs	relative [^{3}H]adenine yield[a]
1	897	1.37 ± 0.23(7)
2	663	0.93 ± 0.18 (7)
3	645	0.80 ± 0.30 (7)
4	474	0.74 ± 0.16 (7)
5	359	1.24 ± 0.30 (7)
6, 7[b]	340 (6) 317 (7)	0.84 ± 0.18 (7)
8	231	2.15 ± 1.21 (7)
9	162	1.42 ± 0.54 (6)
10	137	1.76 ± 1.17 (3)
11	120	2.03 ± 0.21 (3)

[a] Calculated as the ratio of the fraction of total recovered cpm in the hybrid band (or bands) divided by the fraction expected on the basis of the known primary structure of rRNA. For example, in one gel analysis, a total of 1520 cpm was recovered in all of the hybrid bands. There are 213 adenines in the rRNA in hybrid 1, which is 18.1% of the total of 1177 adenines in rRNA. The expected radioactivity in hybrid 1 is 1520 × 0.181 or 275 cpm. The observed radioactivity of 434 cpm corresponds to a relative yield of 1.6. Note that if all hybrids were obtained in the same yield, the relative yield for each hybrid would be equal to 1.0. The number of independent determinations is given in parentheses. [b] Unresolved.

10 (Figure 2, lane 3) were also visible, but the remainder of the hybrids were absent. We infer from this result that, under our conditions, S1 nuclease does not digest between DNA fragments lined up in an adjacent manner along the RNA. Similar resistance to S1 cleavage at small single-stranded sites has been previously reported (Dodgson & Wells, 1977). The obvious solution to this problem is to use a stoichiometric excess of RNA to ensure gaps between the DNA fragments. However, the magnitude of this excess will often be limited by the quantity of labeled RNA available. The RNA:DNA ratio of 2.5–3.0 used routinely in this work represents a reasonable compromise between those two considerations.

A second important parameter is the temperature of the annealing step leading to preferential formation of the RNA·DNA hybrids. We performed hybridizations over the temperature range 40–75 °C. At temperatures of 60 °C or greater, some hybrids do not form, and by 75 °C virtually no hybrids can be seen on PAGE analysis. On the other hand, at the lower end of the range, bands due to double-stranded DNA persist at temperatures below 50 °C. These results have

Table III: Quantitation of Methyl Groups in *Taq*I Hybrids

hybrids[a]	no. of bases	predicted no. of CH_3 groups[b]	relative [^{3}H]adenine yield[c]	fraction of CH_3 groups[d]	no. of CH_3 groups, corrected[e]
1, 2, 3 (unresolved)	760 (1) 742 (2) 728 (3)	10	1.18 ± 0.11	0.475 ± 0.054	8.7 ± 1.3
4, 5 (unresolved)	404 (4) 376 (5)	2	1.23 ± 0.25	0.175 ± 0.025	3.1 ± 0.9
6	360	3	0.72 ± 0.05	0.108 ± 0.013	3.3 ± 0.5
7, 8, 9 (unresolved)	236 (7) 226 (8) 221 (9)	9	0.60 ± 0.11	0.246 ± 0.067	8.9 ± 2.8

[a] Hybrids are not fully resolved (see Figure 5). Numbers are the average of four to five experiments. [b] According to Dubin & Gunalp (1967), Fellner (1969), and Noller (1984). [c] See footnote *a*, Table II. [d] cpm in hybrid band(s) divided by total cpm in all hybrid bands 1–9. [e] Calculated by dividing the fraction of methyl groups by the relative [^{3}H]adenine yield and normalizing the total to 24, the total known number of CH_3 groups in hybrids 1–9.

dictated the use of 55 °C as the standard temperature of choice. Although this temperature may be subject to some variation for the hybrids formed with other restriction fragments, it is unlikely to ever lie outside the 50–60 °C range.

Our method involves the use of RNase T1 in addition to S1 nuclease. Use of S1 nuclease alone results in a large, broad peak of radioactivity in the region of the gel corresponding to ≤100 base pairs that interferes with quantitation of the radioactivity in the smaller hybrid bands. Addition of RNase T1 decreases the size of this broad peak, which we assume corresponds to a variety of RNA fragments that resist S1 digestion since it is seen on PAGE analyis of control experiments containing only rRNA and lacking plasmid fragments. S1-resistant fragments of rRNA up to 100 nucleotides in length have been reported previously (Ross & Brimacombe, 1979).

Analysis of C^3H_3-Labeled rRNA. There are a total of 13 methyl groups within mature, posttranscriptionally modified 16S rRNA, and their positions within the primary sequence are known (Noller, 1984). Studies of methylation within 23S rRNA are less definitive. Locations within the 23S rRNA sequence have been proposed for 12 methyl groups (Fellner, 1969; Noller et al., 1981). Reported levels of total 16S and 23S rRNA methylation (Fellner, 1969; Hayashi, 1966; Dubin & Gunalp, 1967), as well as our own experiments, suggest the presence of one to six additional sites not yet identified within the 23S rRNA sequence. Thus, our method can be applied to both confirm and extend our knowledge of the sites of methylation within rRNA. An example of typical results is provided by Figure 5. Two points of procedure are worth noting. First, the restriction enzyme employed, *Taq*I, was selected because it yields a DNA fragment overlapping with a relatively small piece (hybrid fragment 9 containing 224 bases, see Table I) of RNA at the 3′ end of 16S RNA that contains eight methyl groups. Second, an experiment with [^{3}H]methyl-labeled rRNA was run in parallel with an experiment with [^{3}H]adenine-labeled rRNA. This was to allow the observed yields for the hybrids in the latter experiment to be applied as correction factors for the [^{3}H]methyl-labeled hybrids. The qualitative results of this experiment are clearly in accord with predictions. Most notably, hybrid 9 accounts for only a very small fraction of the [^{3}H]adenine labeling (Figure 5A) but is a major site of [^{3}H]methyl labeling (Figure 5B). The levels of radioactivity found in each of the hybrid bands in both labeling experiments are summarized in Table III. As the results make clear, we are at present capable of semiquantitative precision through application of this method. Hybrids 4 and 5 show a particularly large deviation from the labeling expected on the basis of the known methylation sites,

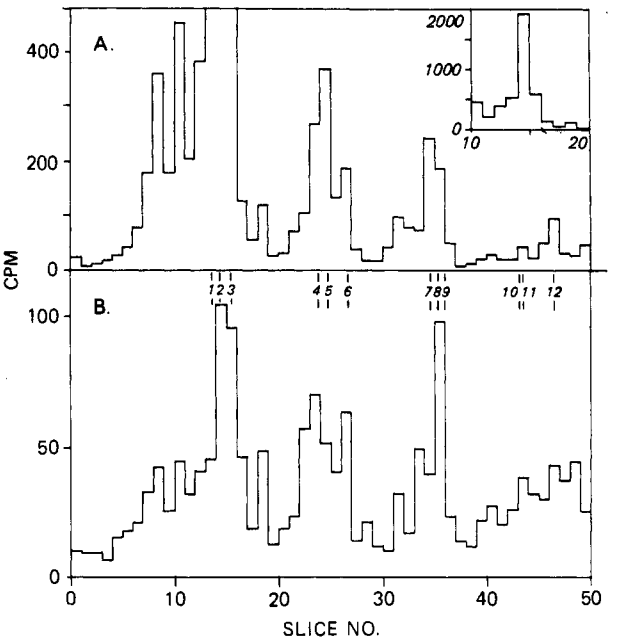

FIGURE 5: Radioactivity profiles of PAGE analysis of [^{3}H]-adenine-labeled rRNA and C^3H_3-labeled rRNA hybridized to *Taq*I-digested pKK3535. (A) [^{3}H]Adenine-labeled rRNA. Hybridization conditions were 25 μg of DNA and 13 μg of rRNA in 60 μL of hybridization buffer. The inset shows gel slices 11–20 on an expanded scale. (B) C^3H_3-labeled rRNA. Hybridization conditions were as in part A, except that 40 μg of DNA and 21 μg of rRNA were hybridized in 120 μL of hybridization buffer. The positions of ethidium bromide stained hybrid bands (numbered as in Table I) for both panels are marked along the top of the lower panel.

leading us to suspect that there may be as yet unidentified sites of methylation within 23S RNA region 2503–2904 or 1378–1753. In any event, these and similar results lead us to conclude that we can estimate the extent of labeling in a hybrid band within no worse than a factor of 1.5. Further experiments are under way to localize sites of 23S rRNA methylation with greater precision.

Analysis of rRNA Photoaffinity Labeled with ANB-[^{3}H]Phe-tRNA^{Phe}. The results presented in Table IV show that ANB-[^{3}H]Phe-tRNA^{Phe} binds noncovalently to 70S ribosomes in a partially poly(U)-dependent manner, which is typical for *N*-acyl-Phe-tRNA^{Phe} derivatives at the high Mg^{2+} levels used in the present work (Odinzov & Kirillov, 1978). Photolysis of the ANB-[^{3}H]Phe-tRNA^{Phe}–ribosome complex, for a time corresponding to 6.4 half-lives for destruction of the aryl azide moiety, leads to photoincorporation of approximately 1.5×10^{-3} mol of label/mol of ribosomal RNA, corresponding to 0.45% of noncovalently bound label. The

great majority of this labeling takes place, as expected, in 23S RNA, as shown by the PAGE analysis presented in Figure 6A.

In early attempts to use the hybridization method to extend the localization of the site of photoincorporation of ANB-[^{3}H]Phe-tRNA^{Phe} to a more limited region of 23S RNA, we found that the yield of radioactivity from the photoaffinity label recovered in hybrids decreased as S1 nuclease concentration was increased above some minimal level. This contrasts with our experiments with [^{3}H]adenine-labeled RNA, for which the yield of hybrids is relatively insensitive to S1 nuclease levels over a wide range. The level of S1 nuclease used in the standard procedure reflects the low concentration required for good recovery of radioactivity from photoaffinity-labeled rRNA in the hybrids. A relatively high level of RNase T1 ensures fairly complete hydrolysis of RNA not involved in hybrid formation, including most or all of the tRNA portion of the photoaffinity label.

Hybridization of ANB-[^{3}H]Phe-tRNA^{Phe}-labeled rRNA with *Hin*fI fragments of pKK3535, followed by PAGE analysis (Figure 6B), shows that virtually all of the radioactivity is found in hybrid 1, corresponding to bases 2008–2904 (Table I). The major site of labeling is further localized to bases 2445–2668 by PAGE analysis of the hybrids formed between labeled ribosomal RNA and *Hpa*II fragments of pKK3535 (Figure 6C), in which hybrid 7 (see Table I) clearly contains the most radioactivity. A check on the assignment of this band is provided by repeating the hybridization procedure on photoaffinity-labeled RNA in the presence of a 3-fold stoichiometric excess of either unlabeled 23S RNA or unlabeled 16S RNA. As expected, addition of the unlabeled 23S RNA lowers the amount of radioactivity recovered in hybrid 7 by approximately 3-fold, presumably by competition with labeled 23S RNA for the *Hpa*II fragment, whereas addition of excess 16S RNA has little effect (Table IV, experiments 5 and 6).

A series of controls were conducted to test the significance of the photolabeling within *Hpa*II hybrid 7, the results of which are summarized in Table IV. As is clear from these results, there is a clear parallelism between effects on photoincorporation of ANB-[^{3}H]Phe-tRNA^{Phe} measured at the level of either 23S RNA or *Hpa*II hybrid 7. Thus, incorporation clearly depends on the photogeneration of a highly reactive intermediate, since separate photolysis of the photoaffinity label followed by dark incubation with 70S ribosomes (experiment 4) leads to little or no incorporation. In addition, both omission of poly(U) (experiment 2) and addition of puromycin (experiment 3) lead to reproducible decreases in the extent of photoincorporation measured at both levels, although in the latter case the decrease appears more marked at the level of incorporation into hybrid 7.

Discussion

One characteristic of photoaffinity labeling as a technique for identifying receptor sites is the large number of control experiments that are required to assess the significance of a labeling result. Typically, one is required to determine the dependence of labeling on variables such as light fluence, photoaffinity-label concentration, and the presence or absence of other ligands (cooperative, competitive, or allosteric). For a receptor the size and complexity of the *E. coli* ribosome, it is not uncommon for labeling to be widespread over a large number of components, even if major amounts of labeling are limited to one or a limited number of components. As a consequence, it is in general necessary to evaluate the results of control experiments at the level of individual ribosomal components, rather than at the overall ribosomal level. This

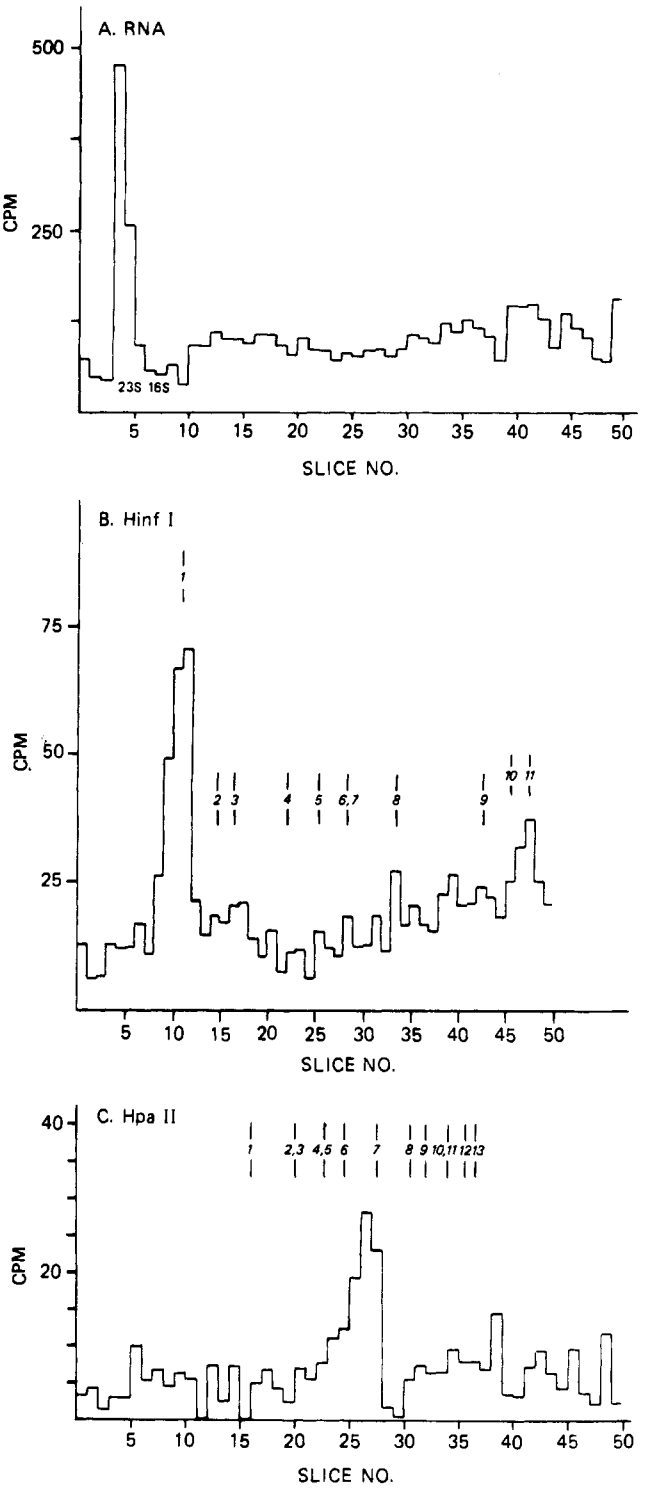

FIGURE 6: Radioactivity profiles of PAGE analysis of ANB-[^{3}H]-Phe-tRNA^{Phe} photoaffinity-labeled rRNA and rRNA–restriction fragment hybrids. (A) The 70S ribosomes (100 A_{260nm}/mL) were photolyzed in the presence of ANB-[^{3}H]Phe-tRNA^{Phe} (1.0 μM) and poly(U) (0.5 mg/mL) in photolysis buffer, and the extracted rRNA was subjected to PAGE analysis, as indicated under Materials and Methods. (B) Hybrids with *Hin*fI fragments. Hybridization conditions were 1.04 mg of DNA and 0.59 mg of rRNA in 3.1 mL of hybridization buffer. The standard procedure was used. (C) Hybrids with *Hpa*II fragments. Hybridization conditions were 0.60 mg of DNA and 0.34 mg of rRNA in 1.8 mL of hybridization buffer. The standard procedure was used. The positions of ethidium bromide stained rRNA (a) or hybrid bands (b and c) (numbered as in Table I) are indicated.

is quite straightforward for ribosomal proteins, since they may be easily separated by one- or two-dimensional PAGE (Jaynes et al., 1978) or, as we have more recently demonstrated, by

Table IV: ANB-[^{3}H]Phe-tRNA^{Phe} Binding and Photoincorporation into Ribosomes

expt no.	conditions	noncovalent binding to 70S ribosomes (% mol/mol)[a]	relative covalent incorporation into 23S rRNA[b]	relative covalent incorporation into *Hpa*II hybrid 7[c]
1	standard[d]	32 ± 11	1.00	1.00
	changes prior to photolysis			
2	−poly(U)	14 ± 4	0.78 ± 0.05	0.68
3	+puromycin (1.0 mM)		0.75 ± 0.26	0.52
4	prephotolysis of ANB-[^{3}H]Phe-tRNA^{Phe}[e]		0.024 ± 0.026	0.06
	additions to hybridization mixture			
5	unlabeled 23S rRNA[f]			0.31
6	unlabeled 16S rRNA[f]			0.88

[a] Measured by millipore filter binding assay (Jaynes et al., 1978). [b] Corresponds to 0.142 ± 0.053% mol/mol. Corrected for gel yield (~78%) based on [^{3}H]adenine-labeled rRNA recovery. [c] Corresponds to 0.048 ± 0.015% mol/mol. Corrected for gel yield (~56%) based on recovery of [^{3}H]adenine-labeled rRNA·DNA hybrid 7. [d] The standard reaction mixture contained 70 S ribosomes (100 A_{260nm}/mL), poly(U) (0.5 mg/mL), and ANB-[^{3}H]Phe-tRNA^{Phe} (1.0 μM) made up in photolysis buffer. In some experiments, the Mg^{2+} concentration was 10 mM. [e] A solution of ANB-[^{3}H]Phe-tRNA^{Phe} was separately prephotolyzed to destroy the azide group and then incubated in the dark with ribosomes for 3 h at 0 °C. The remainder of the experiment exactly paralleled experiment 1. [f] In 3-fold stoichiometric excess over photoaffinity-labeled rRNA.

high-performance liquid chromatography (Kerlavage et al., 1983a,b). For ribosomal RNA, on the other hand, the problem is more difficult, since the localization of labeling to a limited region (or, in the limit, to a single base) has usually involved a tedious, multistep procedure of uncertain quantitative accuracy. As a result, only a few localizations of the sites of RNA photoaffinity labeling have been reported (Wagner et al., 1976; Prince et al., 1982; Barta et al., 1984), and in no case has an extensive set of controls been carried out for labeling within a limited RNA region.

The method presented in this paper offers one solution to this problem. It is simple to perform, requiring only total rRNA and a plasmid, pKK3535, encoding the entire *rrn*B DNA (i.e., there is no need to separately purify components of these materials), it is suitable for the detection and quantitation of even very low stoichiometric levels of labeling anywhere in rRNA, as the results with ANB-[^{3}H]Phe-tRNA^{Phe} (Table IV) demonstrate, and it allows, in a process requiring only a single separation step, direct localization of sites of labeling to limited regions of rRNA (~200 bases for the data presented in Figure 7, but easily extendable to more limited regions through use of restriction enzymes having cleavage sites within the pKK3535 sequence corresponding to *Hpa*II hybrid 7). The method provides a convenient way of screening rRNA labeling reactions at a level suitable for the evaluation of control experiments, so that the considerable effort required to further localize the site of labeling to the single-base level need only be expended on sites of labeling shown to be significant. A very similar approach has recently been described in studies on chemical modification of 16S rRNA (Van Stolk & Noller, 1984).

Several aspects of the method merit further comment. (1) Phenol extractions follow both restriction enzyme nuclease digestion of the plasmid and each of the nuclease digestions (S1 and RNase T1) of the hybrids. Each is necessary since the presence of restriction enzymes can interfere with proper hybrid formation and nuclease digestion, whereas the presence of the nuclease proteins can interfere with PAGE analysis. The importance of multiple phenol extractions only became obvious to us in the later phases of this work. Some of our earlier results (particularly those summarized in Tables II and III) were obtained omitting one or more of these extractions, which we believe accounts, at least in part, for their modest precision. The localization of the site of ANB-[^{3}H]Phe-tRNA^{Phe} photoincorporation was carried out largely with the improved methodology and has yielded results of considerably higher reproducibility. (2) As already pointed out, the yields of different hybrids vary over a considerable range (Table II), and this variation has to be considered in making quantitative comparisons of the extents of labeling of different regions of rRNA. We have been unsuccessful in attempting to correlate hybrid yield with variables such as G-C content, hybrid length, or proposed RNA secondary structure (Noller, 1984) and at present lack a rationale for the variation we observe. (3) Although the yield of radioactivity from ANB-[^{3}H]Phe-tRNA^{Phe} recovered with hybrids is strongly decreased as S1 nuclease is increased above some minimal level, the overall yield of hybrids (estimated by the intensity of ethidium staining) in the same experiment is insensitive to the level of S1 nuclease. We interpret this result as suggesting that the incorporation of the photoaffinity label into rRNA, which proceeds to only a minor stoichiometric extent (Table IV), disrupts the local double-stranded structure within the RNA·DNA hybrid and that this disrupted region is excised by high levels of S1 nuclease.

Electrophilic (Bochkareva et al., 1971; Breitmeyer & Noller, 1976; Yukioka et al., 1975) and photolabile (Bispink & Matthei, 1973; Girshovich et al., 1974; Barta et al., 1975; Sonenberg et al., 1975, 1977; Leitner et al., 1982) derivatives of Phe-tRNA^{Phe}, similar in structure to ANB-Phe-tRNA^{Phe}, have been shown to mimic *N*-acetyl-Phe-tRNA^{Phe} as a peptidyl-tRNA analogue and to affinity label *E. coli* ribosomes, predominantly in 23S rRNA. Up until 1983 the site or sites of such labeling were further localized, if at all, only to very extensive rRNA regions (≥1000 bases (Leitner et al., 1982; Barta & Kuechler, 1983)]. However, quite recently, while the work reported in this paper was nearing completion, Barta et al. (1984) demonstrated that 3-(4′-benzoylphenyl)-propionyl-[^{3}H]Phe-tRNA^{Phe}, a photoaffinity label they had first described in 1975 (Barta et al., 1975), photoincorporates into uridine-2584 and uridine-2585 of 23S rRNA. Barta et al. used a two-part method to identify these sites. In the first, which has significant similarity to the method we describe in this paper, they hybridized affinity-labeled rRNA to a nitrocellulose filter blot of a PAGE separation of DNA restriction fragments generated from a portion of the rrnB operon corresponding to bases 846–2904 of 23S RNA and detected the major site of incorporation by autoradiography. In agreement with our own results, they localized the site of labeling to the *Hpa*II site corresponding to bases 2445–2668 and, through use of *Cfo*I, narrowed this region slightly further to 2445–2625. Second, using this affinity-labeled fragment as a template and base pairs 2719–2842 as a primer, they took advantage of interruptions in reverse transcriptase activity at the sites of labeling to identify the two bases. Though this approach has considerable appeal, the first part is clearly more time consuming vis-a-vis the more general approach described in this paper. Moreover, it is unclear how suitable it will be

for quantitative studies. No information on yields was provided, although the problem of a lower efficiency of transfer of smaller fragments (≤300 bp) to nitrocellulose was noted. Nevertheless, it is gratifying that both Barta et al. and ourselves, using different photolabile Phe-tRNA^{Phe} derivatives and somewhat different analytical procedures, find the same region of 23S rRNA to include the major sites of photoincorporation. Given the difference in the nature of the photogenerated species leading to photoincorporation and the fact that the nitrene generated from the ANB moiety is about 3 Å closer to the α-amino nitrogen of phenylalanine than is the aromatic ketone in the Barta derivative, it will be of interest to see how close the base (or bases) labeled by ANB-Phe-tRNA^{Phe} is (are) to U-2584 and U-2585.

The results of both Barta et al. (1984) and ourselves strongly suggest that bases within 2445–2668 falling within domain V (bases 2043–2625) of the 23S rRNA structure, are at or near the peptidyltransferase center. This suggestion is supported by the strong evolutionary conservation of domain V between procaryotes, mitochondria, and eucaryotes (Noller, 1984), as well as by genetic and biochemical studies of antibiotic interaction with 23S rRNA. Thus, domain V of mitochondrial rRNA contains sites of mutation leading to resistance to chloramphenicol (Dujon, 1980) and erythromycin (Sor & Fukuhara, 1982), antibiotics that either directly, or indirectly, inhibit peptidyl transferase, respectively. In addition, an electrophilic derivative of puromycin, an antibiotic that is an A-site substrate for peptidyltransferase, covalently incorporates into a 23S rRNA pentanucleotide identified as either GUCCG or GUUCG (Eckermann & Symons, 1978). Of the four such sites in 23S rRNA, two occur within bases 2445–2668 (Simund etal., 1984). Puromycin itself photoincorporates into protein L23 (Jaynes et al., 1978; Grant et al., 1979), a protein that, when bound to 23S rRNA, protects several oligonucleotides in domain V from RNase T1 digestion (Vester & Garrett, 1984).

Lastly, our result that preincubation of the ANB-Phe-tRNA^{Phe}–ribosome complex with puromycin decreases total incorporation into bases 2445–2668 by only 48% (Table IV) strongly suggests that photoincorporation proceeds from more than one site. In fact, despite the preference of *N*-acylated Phe-tRNA^{Phe} derivatives for binding to the P site, we would expect partial binding of ANB-Phe-tRNA^{Phe} to both the A and P sites, since our reaction mixtures contain a fair amount of deacylated tRNA, which also has a higher preference for P vs. A site binding (Nierhaus, 1984). Thus, as we both localize the incorporation site(s) still further and examine the labeling pattern as a function of additional experimental variables (e.g., Mg^{2+} and uncharged tRNA concentrations), we may well be able to obtain clear evidence for assigning RNA regions within bases 2445–2668 as being at or near portions of the A and P sites associated with the 3′-terminal ends of the bound tRNAs.

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Registry No. Peptidyltransferase, 9059-29-4.

REFERENCES

Anderson, E. H. (1946) *Proc. Natl. Acad. Sci. U.S.A. 32*, 120–128.

Barta, A., & Kuechler, E. (1983) *FEBS Lett. 163*, 319–323.

Barta, A., Kuechler, E., Branlant, C., Sriwidada, J., Krol, A., & Ebel, J. P. (1975) *FEBS Lett. 56*, 170–174.

Barta, A., Steiner, G., Brosius, J., Noller, H. F., & Kuechler, E. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3607–3611.

Berk, A. J., & Sharp, P. A. (1977) *Cell (Cambridge, Mass.) 12*, 721–732.

Bispink, L., & Matthei, H. (1973) *FEBS Lett. 37*, 291–294.

Bochkareva, E. S., Budker, V. G., Girshovich, A. S., Knorre, D. G., & Teplova, N. M. (1971) *FEBS Lett. 19*, 121–124.

Breitmeyer, J. B., & Noller, H. F. (1976) *J. Mol. Biol. 101*, 297–306.

Brosius, J., Dull, T. J., Sleeter, D. D., & Noller, H. (1981a) *J. Mol. Biol. 148*, 107–127.

Brosius, J., Ullrich, A., Raker, M. A., Gray, A., Dull, T. J., Gutell, R. R., & Noller, H. F. (1981b) *Plasmid 6*, 112–118.

Casey, J., & Davidson, N. (1977) *Nucleic Acids Res. 5*, 1539–1552.

Chang, F. N., Chang, C. N., & Piak, W. K. (1974) *J. Bacteriol. 120*, 651–656.

Cooperman, B. S. (1980) in *Ribosomes* (Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds.) pp 531–554, University Park Press, Baltimore.

Davis, R. W., Botstein, D., & Roth, J. R. (1980) *Advanced Bacterial Genetics: A Manual for Genetic Engineering*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Dodgson, J. B., & Wells, R. D. (1977) *Biochemistry 16*, 2374–2379.

Dubin, D. T., & Gunalp, A. (1967) *Biochim. Biophys. Acta 134*, 106–123.

Dujon, B. (1980) *Cell (Cambridge, Mass.) 20*, 185–197.

Eckermann, D. J., & Symons, R. H. (1978) *Eur. J. Biochem. 82*, 225–234.

Fellner, P. (1969) *Eur. J. Biochem. 11*, 12–27.

Flaks, J. G., Leboy, P. S., Birge, E. A., & Kurland, C. G. (1966) *Cold Spring Harbor Symp. Quant. Biol. 31*, 623–631.

Girshovich, A. S., Bochkareva, E. S., Kramarov, V. M., & Ovchinnikov, Yu. A. (1974) *FEBS Lett. 45*, 213–217.

Grant, P. G., Strycharz, W. A., Jaynes, E. N., Jr., & Cooperman, B. S. (1979) *Biochemistry 18*, 2149–2154.

Hayashi, Y., Osawa, S., & Miura, K. (1966) *Biochim. Biophys. Acta 129*, 519–531.

Hoffman, B. M., Schofield, P., & Rich, A. (1969) *Proc. Natl. Acad. Sci. U.S.A. 62*, 1195–1202.

Jaynes, E. N., Jr., Grant, P. G., Giangrande, G., Wieder, R., & Cooperman, B. S. (1978) *Biochemistry 17*, 561–569.

Kerlavage, A. R., Hasan, T., & Cooperman, B. S. (1983a) *J. Biol. Chem. 258*, 6313–6318.

Kerlavage, A. R., Weitzmann, C., Hasan, T., & Cooperman, B. S. (1983b) *J. Chromatogr. 266*, 225–237.

Kunitz, M. (1946) *J. Biol. Chem. 164*, 563–568.

Leitner, M., Wilchek, M., & Zamir, A. (1982) *Eur. J. Biochem. 125*, 49–55.

Lillis, M., Nick, H., Lu, P., Sadler, J., & Caruthers, M. H. (1982) *Anal. Biochem. 120*, 52–58.

Nicholson, A. W., Hall, C. C., Strycharz, W. A., & Cooperman, B. S. (1982) *Biochemistry 21*, 3797–3808.

Nierhaus, K. H. (1984) *Mol. Cell. Biochem. 61*, 63–81.

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

Noller, H. F., Kop, J., Wheaton, V., Brosius, J., Gutell, R. R., Kopylov, A. M., Dohme, F., Herr, W., Stahl, D. A., Gupta, R., & Woese, C. R. (1981) *Nucleic Acids Res. 9*, 6167–6189.

Odinzov, V. B., & Kirillov, S. V. (1978) *Nucleic Acids Res. 5*, 3871–3879.

Padgett, R. A., Hardy, S. F., & Sharp, P. A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5230–5234.

Peacock, A. C., & Dingman, C. W. (1968) *Biochemistry 7*, 668–674.
Peters, M., & Yarus, M. (1979) *J. Mol. Biol. 134*, 471–491.
Prince, J. B., Taylor, B. H., Thurlow, D. L., Ofengand, J., & Zimmermann, R. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5450–5454.
Ross, A., & Brimacombe, R. (1979) *Nature (London) 281*, 271–276.
Schofield, P., & Zamecnik, P. (1968) *Biochim. Biophys. Acta 155*, 410–416.
Seela, F., & Hansske, F. (1976) *Z. Naturforsch., C: Biosci. 31C*, 263–266.
Sigmund, C. D., Ettayebi, M., & Morgan, E. A. (1984) *Nucleic Acids Res. 12*, 4653–4663.
Sonenberg, N., Wilchek, M., & Zamir, A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4332–4336.
Sonnenberg, N., Wilchek, M., & Zamir, A. (1977) *Eur. J. Biochem. 77*, 217–222.
Sor, F., & Fukuhara, H. (1982) *Nucleic Acids Res. 10*, 6571–6577.
Traub, P., Mizushima, S., Lowry, C. V., & Nomura, M. (1971) *Methods Enzymol. 20*, 391–407.
Van Stolk, B. J., & Noller, H. F. (1984) *J. Mol. Biol. 180*, 151–157.
Vester, B., & Garrett, R. A. (1984) *J. Mol. Biol. 179*, 431–452.
Wagner, R., Gassen, H. G., Ehresmann, C., Stiegler, P., & Ebel, J. P. (1976) *FEBS Lett. 67*, 312–315.
Yukioka, M., Hatayama, T., & Morisawa, S. (1975) *Biochim. Biophys. Acta 390*, 192–208.
Zimmermann, R. A. (1979) *Methods Enzymol. 59*, 551–583.

Two $tRNA_1^{Ile}$ Species from an Extreme Thermophile, *Thermus thermophilus* HB8: Effect of 2-Thiolation of Ribothymidine on the Thermostability of tRNA†

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ABSTRACT: From *Thermus thermophilus* HB8 grown at 65 °C, two major $tRNA^{Ile}$ species have been purified by column chromatography and polyacrylamide gel electrophoresis. The nucleotide sequence of one of these two $tRNA_1^{Ile}$ species ($tRNA_{1a}^{Ile}$) has been determined to be pGGGCGAUUAGCUCAGCUGmGUDA-GAGCGCACGCCUGAUt^{6}AAGCGUGAGm^{7}GUCGGUGGs^{2}TΨCAm^{1}AGUCCACCAUCGCCCA-CCA_{OH}. The nucleotide sequence of the other species ($tRNA_{1b}^{Ile}$) is found to be the same as that of $tRNA_{1a}^{Ile}$ except for the modification in position 54; $tRNA_{1a}^{Ile}$ has s^{2}T(54) while $tRNA_{1b}^{Ile}$ has T(54). The melting temperature of $tRNA_{1a}^{Ile}$ is as high as 86.2 °C while that of $tRNA_{1b}^{Ile}$ is 83.3 °C. The single replacement of an oxygen atom (2-carbonyl oxygen) of T(54) by a sulfur atom significantly contributes to the thermostability of the $tRNA_{1a}^{Ile}$ species. In addition, the methylation of G(18) and A(58) possibly contributes to the thermostability of *T. thermophilus* $tRNA_{1a}^{Ile}$ and $tRNA_{1b}^{Ile}$ species.

The organism *Thermus thermophilus* HB8 is an extremely thermophilic bacterium that grows in the temperature range 50–85 °C. Several tRNA species have been purified from *T. thermophilus* HB8 and found to be more thermostable than the corresponding tRNA species from *Escherichia coli* (Oshima et al., 1976; Watanabe et al., 1976a, 1980; Davanloo et al., 1979). Among those tRNA species from *T. thermophilus* HB8, $tRNA_{f1}^{Met}$ and $tRNA_{f2}^{Met}$ have been sequenced and found to include 2-thioribothymidine (s^{2}T)[1] and 1-methyladenosine (m^{1}A) in the TΨC loop and 2′-*O*-methylguanosine (Gm) in the D loop (Watanabe et al., 1979a). These modified nucleosides have also been found in other tRNA species from extreme thermophiles (Watanabe et al., 1980). Among these three modifications, 2-thiolation of ribothymidine (T) in the TΨC loop has been anticipated to be largely responsible for the thermostability of extreme thermophile tRNA species (Oshima et al., 1976; Watanabe et al., 1976a,b, 1979a, 1980, 1983; Davanloo et al., 1979). In order to examine the contribution of the 2-thiolation itself for the thermostability, however, it is necessary to obtain a pair of tRNA species with the same nucleotide sequence except for the modification in position 54 (T or s^{2}T) [the numbering is in accord with Gauss & Sprinzl (1984)].

We have now succeeded in the isolation of such a pair of tRNA species (major $tRNA^{Ile}$ species) from *T. thermophilus* HB8; one species ($tRNA_{1a}^{Ile}$) has s^{2}T(54) while the other species ($tRNA_{1b}^{Ile}$) has T(54). These two tRNA species also

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[1] Abbreviations: acp^{3}U, 3-(3-amino-3-carboxypropyl)uridine; D, dihydrouridine; Gm, 2′-*O*-methylguanosine; m^{1}A, 1-methyladenosine; m^{7}G, 7-methylguanosine; Ψ, pseudouridine; s^{2}T, 2-thioribothymidine; s^{4}U, 4-thiouridine; T, ribothymidine; t^{6}A, *N*^{6}-(threoninocarbonyl)adenosine; HPLC, high-performance liquid chromatography; IleRS, isoleucyl-tRNA synthetase; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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