Heckler, T. G., Chang, L.-H., Zama, Y., Naka, T., & Hecht, S. M. (1984b) *Tetrahedron 40*, 87-94.
Heckler, T. G., Roesser, J. R., Xu, C., Chang, P. I., & Hecht, S. M. (1988) *Biochemistry 27*, 7254-7262.
Heinrikson, R. L., Mora, R., & Maraganore, J. M. (1987) in *Modern Methods in Protein Chemistry* (L'Italien, J., Ed.) Pergamon Press, New York.
Hilvert, D., Hatanaka, Y., & Kaiser, E. T. (1988) *J. Am. Chem. Soc. 110*, 682-689.
Hudson, D. (1988) *J. Org. Chem. 53*, 617-624.
Khan, A. S., & Roe, B. A. (1988) *Science 241*, 74-79.
Konarska, M. M., Padgett, R. A., & Sharp, P. A. (1984) *Cell 38*, 731-736.
Kozak, M. (1984) *Nature 308*, 241-246.
Leinfelder, W., Zehelein, E., Mandrand-Berthelot, M.-A., & Bock, A. (1988) *Nature 331*, 723-725.
Milligan, J. F., Groebe, D. R., Witherell, G. W., & Uhlenbeck, O. C. (1987) *Nucleic Acids Res. 15*, 8783-8798.
Noren, C. J., Anthony-Cahill, S. J., Griffith, M. C., & Schultz, P. G. (1989) *Science 244*, 182-188.
Noren, C. J., Anthony-Cahill, S. J., Suich, D. J., Noren, K. A., Griffith, M. C., & Schultz, P. G. (1990) *Nucleic Acids Res. 18*, 83-88.
Payne, R. C., Nichols, B. P., & Hecht, S. M. (1987) *Biochemistry 26*, 3197-3205.
Pezzuto, J. M., & Hecht, S. M. (1980) *J. Biol. Chem. 255*, 865-869.
Roberts, J. W., & Carbon, J. (1975) *J. Biol. Chem. 250*, 5530-5541.
Roesser, J. R., Chorghade, M. S., & Hecht, S. M. (1986) *Biochemistry 25*, 6361-6365.
Roesser, J. R., Xu, C., Payne, R. C., Surratt, C. K., & Hecht, S. M. (1989) *Biochemistry 28*, 5185-5195.
Sampson, J. R., & Uhlenbeck, O. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1033-1037.
Scalfi-Happ, C., Happ, E., Ghag, S., & Chládek, S. (1987) *Biochemistry 26*, 4682-4688.
Scherberg, N. H., Seo, H., & Hynes, R. (1978) *J. Biol. Chem. 253*, 1773-1779.
Schimmel, P. R. (1989) *Acc. Chem. Res. 22*, 232-233.
Schimmel, P. (1990) *Biochemistry 29*, 9495-9502.
Schimmel, P. R., & Söll, D. (1979) *Annu. Rev. Biochem. 48*, 601-648.
Shih, L. B., & Bayley, H. (1985) *Anal. Biochem. 144*, 132-141.
Snopek, T. J., Sugino, A., Agarwal, K. L., & Cozzarelli, N. R. (1976) *Biochem. Biophys. Res. Commun. 68*, 417-424.
Söll, D. (1988) *Nature 331*, 662-661.
Sugino, A., Snopek, T. J., & Cozzarelli, N. R. (1977) *J. Biol. Chem. 252*, 1732-1738.
Tanimura, H., Fukazawa, T., Sekine, M., Hata, T., Efcavitch, J., & Zon, J. (1988) *Tetrahedron Lett. 29*, 577-578.
Usman, N., Ogilvie, K. K., Jiang, M.-Y., & Cedergren, R. J. (1987) *J. Am. Chem. Soc. 109*, 7845-7854.

A Photolabile Oligodeoxyribonucleotide Probe of the Peptidyltransferase Center: Identification of Neighboring Ribosomal Components†

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ABSTRACT: In this work we report the synthesis of a radioactive, photolabile oligodeoxyribonucleotide probe and its exploitation in identifying 50S ribosomal subunit components neighboring its target site in 23S rRNA. The probe is complementary to 23S rRNA nucleotides 2497-2505, a single-stranded sequence that has been shown to fall within the peptidyltransferase center of *Escherichia coli* ribosomes [Cooperman, B. S., Weitzmann, C. J., & Fernandez, C. L. (1990) in *The Ribosome: Structure, Function, & Evolution* (Hill, W. E., Dahlberg, A., Garrett, R. A., Moore, P. B., Schlesinger, D., & Warner, J. R., Eds.) pp 491-501, American Society of Microbiology, Washington]. On photolysis in the presence of 50S ribosomes, it site-specifically incorporates into protein L3 (identified by both SDS-PAGE and immunological methods) and into three separate 23S rRNA regions: specifically, nucleotides 2454; 2501, 2502, 2505, 2506; and 2583, 2584. These results provide clear evidence that G-2505 in 23S rRNA is within 24 Å (the distance between G-2505 and the photogenerated nitrene) of protein L3 and of each of the nucleotides mentioned above and are of obvious importance in the construction of detailed three-dimensional models of ribosomal structure. The approach we present is general and can be applied to determining ribosomal components neighboring regions of rRNA that are susceptible to binding by complementary oligodeoxyribonucleotides, both in intact 30S and 50S subunits and in subunits at various stages of reconstitution.

Bogdanov and his co-workers (Skripkin et al., 1979; Mankin et al., 1981) first introduced the notion of using oligodeoxyribonucleotides that are complementary to rRNA sequences to probe the structure of rRNA, both of the native molecule and within ribosomal subunits. More recently, Hill and his co-workers (1990) have demonstrated that single-stranded regions of rRNA can form stable complexes with their complementary oligodeoxyribonucleotides, as evidenced both by filter-binding assays using a ^{32}P-labeled probe and by the demonstration that treatment of the complex with RNase H cleaves rRNA at the appropriate position. They have exploited

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this approach in determining the functional importance of a targeted rRNA sequence. Of particular note for the work reported here are the results of Marconi and Hill (1988, 1989) and Marconi et al. (1990) demonstrating that the oligodeoxyribonucleotide 5′-CATCGAGGT-3′ that is complementary to nucleotides 2505–2497 within the central loop of domain V of 23S rRNA competes with the binding of deacylated tRNA to the ribosome, thus providing further evidence for the involvement of this rRNA region at the peptidyltransferase center (Cooperman et al., 1990). A further application has been to attach electron microscopic markers to complementary oligodeoxyribonucleotides and to visualize complexes of such oligodeoxyribonucleotides bound to ribosome subunits, thus obtaining a three-dimensional localization of the target rRNA sequence [Olson et al., 1988; Lasater et al., 1989, 1990; Oakes & Lake, 1990; McWilliams & Glitz, 1991].

In this work we convert the oligodeoxyribonucleotide 5′-CATCGAGGT-3′ into a radioactive photoaffinity labeling reagent, *O*-[*N*-(*p*-azidobenzoyl)-6-aminohexyl]-pCATCGAGGTA* (ABAH2505–2497A*: the asterisk indicates the presence of radioactivity)[1] and use this reagent to identify ribosomal components in the vicinity of the 23S rRNA sequence 2497–2505.

Experimental Procedures

Materials

Buffers. TKM0.3: 40 mM Tris-HCl (pH 7.4), 60 mM KCl, 0.3 mM $MgCl_2$. TKM1: 10 mM Tris-HCl (pH 7.4), 100 mM KCl, 1 mM $MgCl_2$. TKM1.5: 10 mM Tris-HCl (pH 7.4), 100 mM KCl, 1.5 mM $MgCl_2$. TKM10: 10 mM Tris-HCl (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$. TNM: 50 mM Tris-HCl (pH 7.4), 30 mM NH_4Cl, 1 mM $MgCl_2$. TBE: 89 mM Tris–borate (pH 8.3), 8 mM EDTA. TE: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. TND: 20 mM Tris-HCl (pH 7.9), 50 mM NaCl, 1 mM dithiothreitol.

RNase H was isolated from *Escherichia coli* Q13 according to the method of Darlix (1975). TP30 was produced by acetic acid extraction of 30S subunits and acetone precipitation as described (Kerlavage & Cooperman, 1986). The following materials were purchased and used without further purification: Terminal deoxynucleotidyltransferase and a calibrated RNA ladder (Bethesda Research Labs), avian myeloblastosis virus reverse transcriptase (AMV-RT), (Molecular Genetic Resources, Tampa, FL), *N*-hydroxysuccinimidyl 4-azidobenzoate (HSAB) (Pierce), bacteriophage T4 polynucleotide kinase (New England Biolabs), chemicals used in the synthesis of oligodeoxynucleotides (Glenn Research, Sterling, VA), Sep-pak C-18 cartridges (Waters), [γ-^{32}P]ATP (3000 Ci/mmol; Du Pont/New England Nuclear), [α-^{32}P]ddATP (5000 Ci/mmol; Amersham).

Preparation of 50S Subunits. All operations were performed at 0–4 °C. Ribosomes (70S) were prepared from *E. coli* Q13 bacteria harvested at midlog phase. Frozen cells (25g) were ground with 50 g of alumina and extracted with TKM10 buffer. Following sedimentation for 30 min at 30000*g*, the upper three-fourths of the supernatant was sedimented at 45 000 rpm for 3.5 h in a Beckman Ti70 rotor. The ribosomal pellet was dissolved in TKM10 buffer containing 0.5 M NaCl, left on ice for 2 h, and sedimented as above to pellet ribosomes. The ribosomal pellet was dissolved in TNM buffer at a concentration of 1000 A_{260}/mL, and subunits were separated by zonal centrifugation as described by Sypherd and Wireman (1974). The 50S and 30S fractions were pelleted separately by centrifugation at 45 000 rpm in a Beckman Ti50 rotor for 16 h. The 50S subunits were further purified by sucrose gradient centrifugation (15–30% in TKM1 buffer, 50 000 rpm, 1.5 h in a Beckman VTi50 rotor; Goldman et al., 1983), pelleted as before, and dialyzed against 2 × 2 L of TKM1.5 buffer over 24 h. Aliquots of 100 μL were stored at −80 °C.

Synthesis and Purification of Oligodeoxyribonucleotides. All cDNA probes were synthesized by use of phosphoramidite chemistry on a Milligen Biosearch Cyclone automated DNA synthesizer and deblocked according to the manufacturer's protocol. They were purified both before and after removal of the 5′-dimethoxytrityl (DMT) blocking group by RP-HPLC. A Synchropack RP-P column (C18, 250 × 4.6 mm i.d.) was used. Column conditions: linear gradient, 5–40% acetonitrile in 0.1 M triethanolamine acetate, pH 7.2; flow rate, 0.7 mL/min, 30 min.

cDNA 2505–2497, having the sequence 5′-CATCGAGGT-3′ and the corresponding mismatched oligonucleotide 5′-CATATAGGT-3′ (MM-cDNA 2505–2497) were synthesized as described above. cDNA 2505–2497 labeled at its 5′ end, p*2505–2497, was made by incubation of cDNA 2505–2497 with [γ-^{32}P]ATP and polynucleotide kinase and purified by use of Sep-pak (C-18) cartridges, according to Sambrook et al. (1989).

The photolabile, radioactive cDNA ABAH2505–2497A* was synthesized by suitable modification of the synthesis of cDNA 2505–2497 described above. Prior to deblocking, the 5′ terminus of cDNA 2505–2497 was coupled with *O*-[*N*-(4-monomethoxytrityl)-6-aminohexyl] *O*′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite), in which the amine is protected with the 4-monomethoxytrityl group according to the protocol and with reagents supplied by Glen Research (Sterling, VA). Deblocking and RP-HPLC purification was carried out as above. A total of 12 A_{260} units of this 5′-amino-derivatized cDNA 2505–2497 in 250 μL of 0.05 M Na_2CO_3 buffer (pH 10.0) was mixed with 250 μL of a DMF solution of HSAB (10 mg/mL), and the resulting solution was incubated in the dark for 16 h at room temperature. The resulting photolabile cDNA, ABAH2505–2497, was purified by passage through a small Sephadex G-25 column and RP-HPLC, as described earlier. ABAH2505–2497 eluted at 19.5 min, 4.5 min later than cDNA 2505–2497. It was labeled at the 3′ end by use of [α-^{32}P]ddATP and terminal deoxynucleotidyl transferase following the procedure of Yousaf et al. (1984). The final product, ABAH2505–2497A*, was purified by Sep-pak as described earlier.

Methods

Binding of Oligonucleotides to 50S Subunits. Noncovalent binding of p*2505–2497 to 50S subunits was performed as described by Marconi et al. (1990) with some modifications. The 50S subunits (12.5 pmol) were incubated with varying amounts of p*2505–2497 (250–500 cpm/pmol) in 25 μL of TKM0.3 at 37 °C for 5 min and left on ice for 15 min, after which time the $MgCl_2$ concentration was raised to 10 mM and the incubation on ice was continued for an additional 2 h. The reaction mixtures were then diluted with 0.5 mL of cold TKM0.3 containing 10 mM $MgCl_2$ and filtered through HWAP 0.45-μM nitrocellulose filters (Millipore), followed by three 1-mL washes of the filter with binding buffer. The

[1] Abbreviations: ABAH-, *O*-[*N*-(*p*-azidobenzoyl)-6-aminohexyl]-; AMV-RT, avian myeloblastosis virus reverse transcriptase; DMT, dimethoxytrityl; HSAB, *N*-hydroxysuccinimidyl 4-azidobenzoate; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reversed-phase high-performance liquid chromatography; TP30, total protein from 30S subunits; TP50, total protein from 50S subunits.

amount of filter-bound oligonucleotide was determined by liquid scintillation counting of the dried filters.

RNase H Digestion. The binding procedure was followed as above through the addition of $MgCl_2$ to a final concentration of 10 mM, except that 50S subunits (50 pmol) were first incubated with either cDNA 2505–2497 or ABAH2505–2497A* (50 pmol) in 25 μL of TKM0.3. RNaseH (5 International units) was added and incubation proceeded at 4 °C for 4–18 h. Reaction mixtures were extracted three times with phenol and two times with chloroform. The rRNA was precipitated with 3 volumes of 95% ethanol at −20 °C for 1 h, pelleted by centrifugation, dissolved in loading solution containing 7 M urea/0.01% xylene cyanol/0.01% bromophenol blue, incubated at 60 °C for 5 min, and analyzed by electrophoresis on a 4% polyacrylamide/7 M urea (20:1) gel made up in TBE buffer. The RNA bands were visualised by staining with methylene blue (0.2% in 0.2 M sodium acetate, pH 4.7).

Photoincorporation of ABAH2505–2497A or ABAH2505–2497 into 50S Subunits.* In a typical experiment, 150 pmol of 50S subunits was incubated with 50 pmol of ABAH2505–2497A* (30000 cpm/pmol) in 150 μL of TKM0.3 at 37 °C for 5 min and left on ice for 15 min. The $MgCl_2$ concentration was then increased to 10 mM and incubation was continued for an additional 16–18 h at 4 °C. The incubation was carried out in a quartz EPR tube. Photolysis was carried out for 5 min in a Rayonet RPR 100 reactor equipped with RPR 3000-Å lamps at 4 °C as described (Jaynes et al., 1978). Following photolysis, 2-mercaptoethanol was added to a final concentration of 20 mM to quench any light-independent reaction (Goldman et al., 1983). One volume of 9:1 ethanol/2-mercaptoethanol was added, the mixture was allowed to stand at 0 °C for 30 min, and 50S subunits were collected by centrifugation for 15 min in an Eppendorf microcentrifuge.

Identification of Labeled Proteins. Proteins were prepared from labeled 50S subunits by acetic acid extraction and acetone precipitation in the usual fashion (Kerlavage & Cooperman, 1986). Prior to PAGE analysis, protein samples were dissolved in 8 M urea/10 mM 2-mercaptoethanol (~75 μg of TP50/100 μL) and heated to 65 °C for 10 min in the presence of 0.1% SDS. SDS-PAGE analysis was performed following Thomas and Kornberg (1975), using a 1-mm-thick separating gel prepared with 12% acrylamide/0.06% bisacrylamide in 0.75 M Tris-HCl (pH 8.8)/0.1% SDS, which was run for 2–3 h at 150 V. The electrode buffer was 0.05 M Tris/0.38 M glycine (pH 8.3)/0.1% SDS. Labeled protein bands were detected by overnight exposure to an X-ray film (Kodak XAR-5). Labeled proteins were extracted from the gel with 66% acetic acid (Bernabeu et al., 1978), concentrated by acetone precipitation in the presence of TP30 (50 μg) as carrier, and identified immunologically by use of the agarose method of Gulle et al. (1988).

Measurement of Photoincorporation into 23S rRNA. Following Cooperman et al. (1977), labeled 50S subunits (8 A_{260} units) were dissolved in 200 μL of TKM10, SDS was added to a final concentration of 1%, and the mixture was heated at 37 °C for 5 min and layered on a 5–20% sucrose gradient containing 0.05 M sodium acetate (pH 5.8), 0.01 M EDTA, 0.5 M LiCl, 3 M urea, 0.1% 2-mercaptoethanol, and 0.1% SDS. Centrifugation was carried out at 4 °C in a Beckman VTi80 rotor for 75 min at 50000 rpm. Fractions containing 23S rRNA were pooled, and the RNA was precipitated with 2.5 volumes of ethanol, redissolved in 200 μL of TE buffer, extracted once with phenol and once with $CHCl_3$, precipitated with ethanol, and redissolved in 1 mM EDTA (pH 8.0). The radioactivity of the solution was determined by liquid scintillation counting with use of Ecolite (+) cocktail (ICN Chemicals), and the RNA concentration was determined by A_{260} (1 A_{260} unit equals 36 pmol).

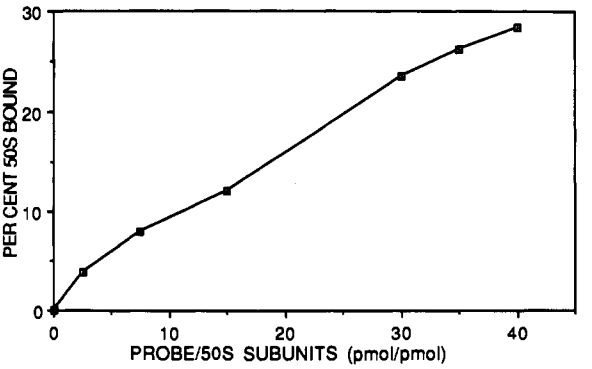

FIGURE 1: Binding of p*2505–2497 to 50S subunits. Conditions were as described in Experimental Procedures.

Localization of Photoincorporation Sites within 23S rRNA. Partial localization of sites of photoincorporation into 23S rRNA was carried out following Brimacombe et al. (1990a) with some modifications. Typically, labeled rRNA (16 pmol), purified by SDS–urea sucrose density gradient centrifugation as described above, was incubated with a cDNA probe (16 pmol) in 10 μL of TND buffer for 5 min at 55 °C and left at 32 °C for 15 min. The $MgCl_2$ concentration was raised to 10 mM, RNase H (2 units) was added, and incubation was continued for 30 min. Urea–PAGE analysis of RNA and autoradiography were carried out as described above. Precise photoincorporation sites were identified by primer extension using AMV-RT, as described by Muralikrishna and Wickstrom (1989). Four synthetic oligodeoxynucleotides, complementary to 23S rRNA nucleotides 2904–2888, 2797–2781, 2697–2681, and 2576–2560 were used as primers.

Results

Noncovalent Binding of Deoxyoligonucleotides. The noncovalent binding of cDNA p*2505–2497 to 50S subunits was determined by use of a Millipore assay, reaching a value of 28% of 50S subunits at a probe:50S subunit molar ratio of 40:1. This result is similar to that reported earlier by Marconi and Hill (1988), who found binding of the same probe to 32% of 50S subunits at a probe:50S molar ratio of 30:1. Although most of this binding is nonspecific (see Discussion), some of it occurs specifically to the target complementary region in 23S rRNA, as evidenced by the 400-nt RNA fragment that is released following RNase H treatment of the complex (Figure 2, lane 2). Marconi and Hill (1988, 1989) obtained similar results. The same fragment is obtained on RNase H digestion of the complex formed between 50S subunits and the photolabile probe ABAH2505–2497A* (Figure 2, lane 1), but is not formed in the absence of either RNase H (lane 3) or of cDNA probe (lane 4).

In addition to the 400-nt fragment, three faint oligodeoxyribonucleotide-specific fragments, corresponding to 670, 500, and 360 nt, are also visible. These fragments do not correspond to RNase H cleavage of intact 23S RNA at secondary sites of oligodeoxyribonucleotide binding (there are 5-nt complementarities with bases 47–51 and 1375–1380 and a 4-nt complementarity with bases 1727–1731) but may arise from partial RNA fragmentation during isolation and incubation.

Protein L3 Is Specifically Labeled on Photolysis of the 50S/ABAH2505–2497A Complex.* In a typical photoincorporation experiment, 50S subunits (150 pmol) were com-

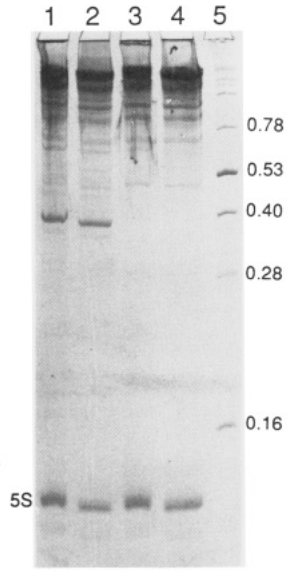

FIGURE 2: RNase H digestions of 50S subunits with cDNA 2505–2497 and with ABAH2505–2497A*. RNase H digestion assays and PAGE were performed as described in Experimental Procedures: lane 1, RNase H with ABAH2505–2497A*; lane 2, RNase H with cDNA 2505–2497; lane 3, cDNA 2505–2497 without RNase H; lane 4, RNase H without added oligodeoxyribonucleotide; lane 5, RNA size markers (in kilobases).

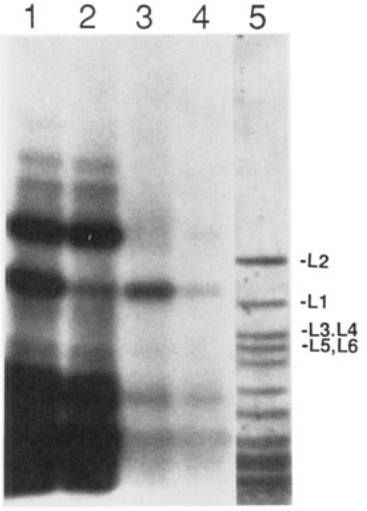

FIGURE 3: Autoradiogram of a PAGE analysis of proteins photoaffinity labeled with ABAH2505–2497A*. Conditions were as described in Experimental Procedures. The reaction mixture (total volume 75 μL) subjected to photolysis contained 50S subunits (150 pmol) and ABAH2505–2497A* (5 pmol) in the absence or presence (90 pmol) of cDNA probe 2505–2497: lane 1, 67% acetic acid soluble fraction from 50S subunits photolyzed in the absence of cDNA 2505–2497; lane 2, same as lane 1 except that photolysis was carried out in the presence of cDNA 2505–2497; lanes 3 and 4 are the acetic acid insoluble fractions corresponding to lanes 1 and 2, respectively; lane 5, TP50. Proteins in lane 5 were visualized by Coomassie Blue staining.

bined with ABAH2505–2497A* (5 pmol) in the presence or absence of cDNA 2505–2497 (90 pmol) or of MM-cDNA 2505–2497 (90 pmol), the reaction mixture (75 μL) was photolyzed, the 50S subunits were extracted with acetic acid, and the acetic acid soluble material (essentially pure 50S protein) as well as the acetic acid insoluble material (50S rRNA with a trace of protein) was analyzed by SDS–PAGE and autoradiography. The results are quite striking. Considering the acetic acid soluble fractions first (Figure 3, lanes 1 and 2), it is clear that ABAH2505–2497A photoincorporates into many 50S proteins. However, we are able to detect substantial reduction in the labeling of only one protein band,

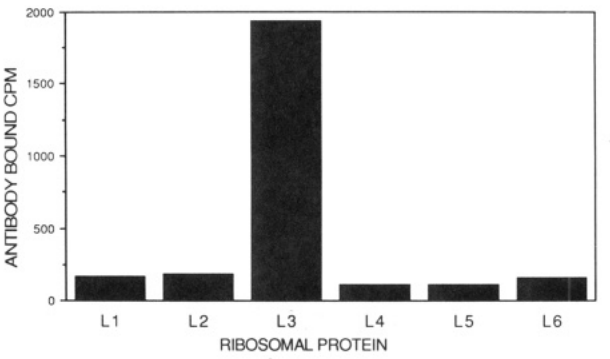

FIGURE 4: Immunological identification of 50S ribosomal proteins labeled with ABAH2505–2497A*. Bars denote counts per minute bound to antibody to each of the indicated ribosomal proteins, performed as described by Gulle et al. (1988).

migrating just above L1 and with an apparent molecular mass of 26 kDa, when photoincorporation is carried out in the presence of cDNA 2505–2497 [in contrast, addition of MM-cDNA 2505–2497 had no effect on photoincorporation into the 26-kDa band or any of the other bands (data not shown)]. Moreover, it is precisely this band that shows greatly increased relative photoincorporation of radioactivity in the acetic acid insoluble fraction of the 50S subunits labeled in the absence of cDNA 2505–2497 (Figure 3, lane 3); this enhanced relative labeling is abolished when photoincorporation is carried out in the presence of cDNA 2505–2497 (Figure 3, lane 4).

Acetic acid extraction is generally a very efficient way of removing proteins from 50S subunits (Hardy et al., 1969; Jaynes et al., 1978). From the results in Figure 3, this appears to be true even for proteins into which ABAH2505–2497A* has photoincorporated. The one exception is the specifically labeled protein, an appreciable fraction of which is left behind in the pellet after acetic acid extraction (Figure 3, lane 3). We speculate that this partitioning reflects an incomplete denaturation of the specifically formed heteroduplex between ABAH2505–2497A* and 23S rRNA.

The specifically labeled protein was extracted from the electrophoresis gel and identified by testing its binding to antibodies to 50S proteins having electrophoretic mobilities close to that of the labeled protein. The results (Figure 4) unambiguously show the protein to be L3. This identification is consistent with the mobility of the labeled protein on SDS–PAGE analysis, since its apparent molecular mass of 26 kDa is equal to the sum of the molecular masses of L3 (22.2 kDa) and ABAH2505–2497A* (3.5 kDa). In the experiment analyzed in Figure 3, lane 1, approximately 17% of the total radioactivity incorporated into protein was found in the band now identified definitively as labeled L3. Under photolysis conditions in which a higher ratio of ABAH2505–2497A* to 50S subunits was employed than above (ABAH2505–2497A*, 100 pmol; 50S, 300 pmol; 150 μL), approximately 0.8% of L3 was labeled.

Photoincorporation of ABAH2505–2497A into 23S rRNA.* To measure ABAH2505–2497A* photoincorporation into 23S rRNA, labeled 50S ribosomes were subjected to sucrose gradient centrifugation in an SDS–urea buffer (Figure 5). Under these conditions, noncovalent binding is virtually abolished (i.e., the specific heteroduplex formed between ABAH2505–2497A* and the complementary region of 23S rRNA is denatured). The results presented in Figure 5 show that incorporation of ABAH2505–2497A* into 23S rRNA is light-dependent, is almost completely abolished on addition of cDNA 2505–2497, and is unaffected by the addition of MM-cDNA 2505–2497. Thus, when ABAH2505–2497A*

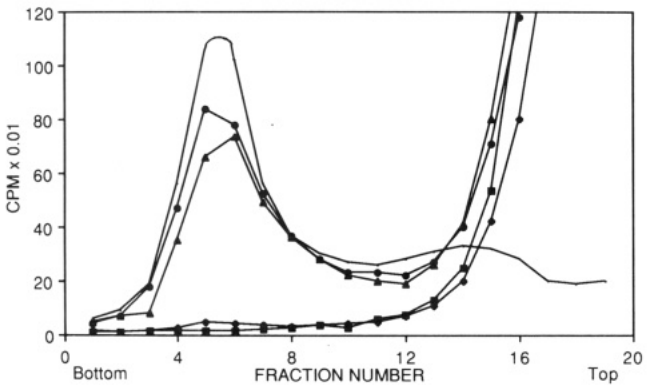

FIGURE 5: Photoincorporation of ABAH 2505–2497A* into 23S rRNA. Sucrose gradient in the presence of urea and SDS was done as described in Experimental Procedures. Aliquots of gradient fractions were counted by liquid scintillation to monitor the probe. A total of 150 pmol of 50S was incubated with 10 pmol of ABAH2505–2497A* in a total volume of 75 μL. Key: (■) without photolysis; (●) with photolysis; (◆) with photolysis in the presence of cDNA 2505–2497 (150 pmol); (▲) with photolysis in the presence of MM-cDNA 2505–2497 (150 pmol). The solid line is a trace of absorbance at 260 nm.

(20 pmol) was photolyzed in the presence of 300 pmol of 50S subunits (total volume, 0.15 mL), 1.8 pmol of reagent was photoincorporated into 23S rRNA. This number decreased to 0.08 pmol of ABAH2505–2497A* when photolysis was repeated in the presence of added cDNA 2505–2497 (300 pmol) but remained essentially unchanged (1.7 pmol) when photolysis was carried out in the presence of added MM-cDNA 2505–2497. The background incorporation level in the absence of photolysis was 0.03 pmol.

Partial Localization of ABAH2505–2497A Photoincorporation Sites in 23S rRNA.* In the Brimacombe et al. (1990a) approach to identifying sites of rRNA labeling, pairs of oligoDNAs complementary to different rRNA sequences are simultaneously hybridized to a labeled rRNA, the resulting complex is digested with RNase H, and the radioactivity in the excised piece of RNA is determined following PAGE separation. For ease of discussion in the presentation that follows, we will generally assume that RNase H treatment removes all of the nucleotides complementary to the added oligodeoxyribonucleotide. The apparent sizes of labeled fragments [as estimated from a semilog plot of the number of nucleotides vs migration distance for a set of RNA size markers (Figure 6, lane 1)] are typically larger (10–20 nt) than would be expected for the corresponding unlabeled fragments generated with complete RNase H digestion. This is so for two reasons. First, the size of the photoincorporated probe itself is 10 nucleotides. Second, "frayed" ends sometimes result from incomplete RNase H digestion.

Autoradiographic analysis of a PAGE separation of the reaction mixture resulting from RNase H digestion of ABAH2505–2497A*-labeled 23S rRNA, taken up in a nondenaturing buffer, reveals the presence of a ^{32}P-labeled 400-nt fragment (Figure 6, lane 3). This result indicates that the specific heteroduplex between photolyzed, covalently incorporated ABAH2505–2497A* and 23S rRNA is reformed under the conditions used for RNase H digestion and provides a site for RNase H cleavage. It also shows that photoincorporation takes place into 23S rRNA between nucleotides 2506 and 2904. This same analysis—consisting of RNase H cleavage, PAGE separation, and autoradiography—was also performed on ABAH2505–2497A*-labeled 23S rRNA that had been hybridized with cDNA probes added either one at a time (2310–2301, 2377–2368, 2576–2560, and 2740–2731; Figure 6, lanes 4, 5, 6, and 7, respectively) or two at a time (2377–2365 and 2447–2438, Figure 6, lane 8).

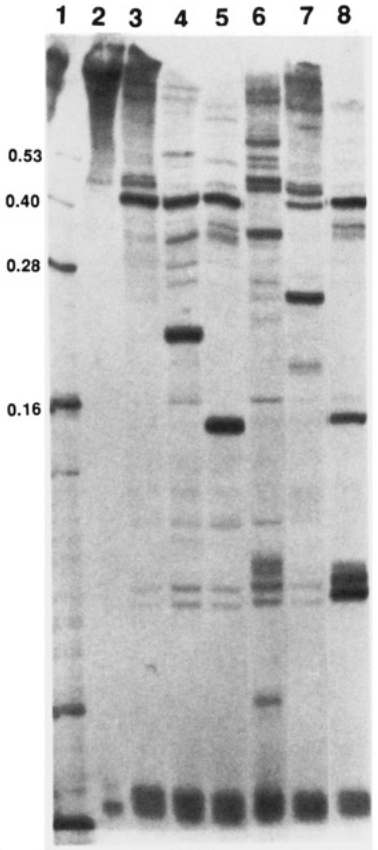

FIGURE 6: Autoradiogram of a PAGE analysis of 23S rRNA photoaffinity labeled with ABAH2505–2497A* and treated with cDNA probes and RNase H: lane 1, RNA size markers (in kilobases); lane 2, labeled 23S rRNA in the absence of RNase H; lane 3, labeled 23S rRNA digested with RNase H; lane 4, labeled 23S rRNA hybridized with cDNA probe 2310–2301 and digested with RNase H; lane 5, labeled 23S rRNA hybridized with cDNA probe 2377–2368 and digested with RNase H; lane 6, labeled 23S rRNA hybridized with cDNA probe 2576–2560 and digested with RNase H; lane 7, labeled 23S rRNA hybridized with cDNA probe 2740–2731 and digested with RNase H; lane 8, labeled 23S rRNA hybridized with cDNA probes 2377–2368 and 2447–2438 and digested with RNase H.

The results obtained in Figure 6 strongly indicate the presence of at least three major sites of ABAH2505–2497A* photoincorporation into 23S rRNA. The labeled fragments seen in lane 6 provide clear evidence for two sites of photoincorporation between nucleotides 2506 and 2904, one giving rise to a fragment of approximately 340 nt and corresponding to a site between nucleotides 2577 and 2904 and one giving rise to the cluster of fragments of approximate sizes 55–70 nt and corresponding to a site between nucleotides 2506 and 2559. (The heterogeneity reflects incomplete RNase H digestion, so the site of incorporation could fall, at the outside, between nucleotides 2497 and 2576. Such heterogeneities are presumably also present for fragments of larger sizes but are not apparent because single-base differences in larger fragments are not well resolved.) The first of these sites is further localized between nucleotides 2578 and 2730 by the approximately 240-nt fragment seen in lane 7, corresponding to the fragment 2506–2730. RNase H digestion in the presence of cDNA 2310–2301 (lane 4) leads to the appearance of a new labeled fragment (approximately 210 nt) that is clearly not derived from the 400-nt fragment already discussed. This result indicates the presence of a third site of ABAH2505–2497A* photoincorporation, falling between nucleotides 2311 and 2496. This new fragment is presumably derived from the

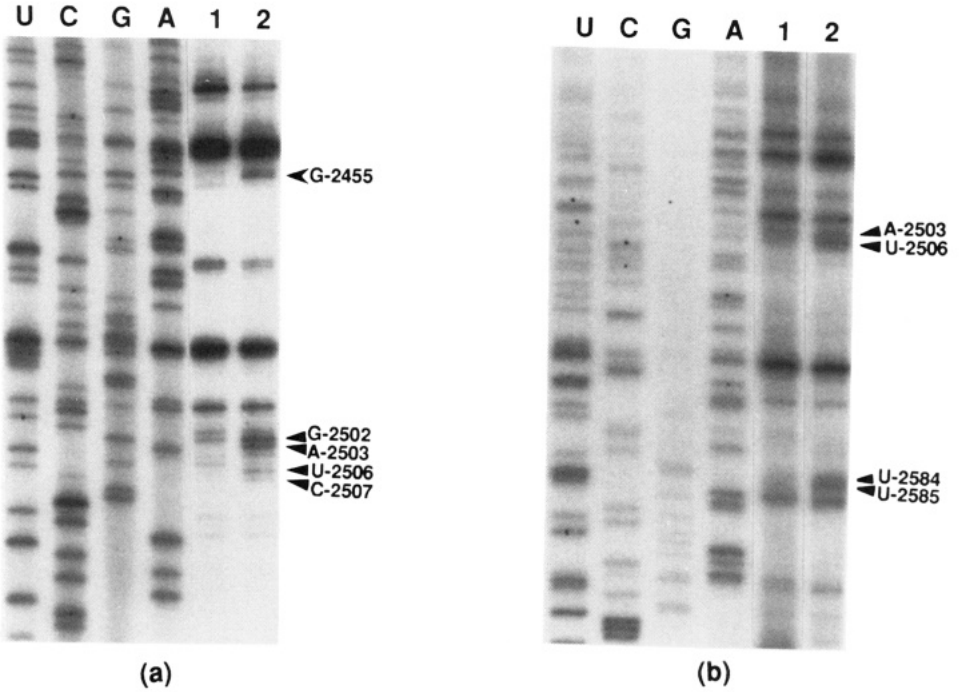

FIGURE 7: Autoradiograms showing reverse transcription of RNA photolabeled with ABAH2505–2497. Photoincorporation was carried out in a reaction mixture containing 150 pmol of 50S subunits and 4.5 nmol of ABAH2505–2497 in a total volume of 75 μL (see Experimental Procedures): lane 1, primer extension on rRNA extracted with 50S subunits photolyzed in the absence of ABAH2505–2497; lane 2, primer extension on rRNA extracted from 50S subunits photolyzed in the presence of ABAH2505–2497. Lanes U, C, G, and A are sequencing products generated in presence of ddATP, ddGTP, ddCTP, and ddTTP, respectively. Nucleotides at which pauses or stops are observed are indicated. Primers used as 2576–2560 in part a and 2697–2681 in part b.

1–2496 fragment formed on digestion of ABAH2505–2497A*-labeled 23S rRNA (lane 3, top). The approximately 130-nt fragment found in lane 5 restricts this labeling site to between nucleotides 2377 and 2496, and the cluster of fragments of 60–70 nt found in lane 8 further restricts the site to between nucleotides 2447 and 2496.

Similar experiments were also performed by adding 23S rRNA cDNA probes 360–351, 750–741, 1050–1041, 1400–1391, 1750–1741, and 2116–2100 to ABAH2505–2497A*-labeled 23S rRNA one at a time or in pairs, in an effort to determine whether there were other major sites of photoincorporation between 23S rRNA nucleotides 1–2300. No such sites were found.

Identification of Specific Nucleotides in 23S rRNA into Which ABAH2505–2497A Photoincorporates.* Specific sites of photoincorporation within the three nucleotide regions (2447–2497, 2506–2559, 2578–2730) described above were identified by use of the primer extension approach (Hall et al., 1988; Muralikrishna & Wickstrom, 1989) on 23S rRNA extracted from 50S subunits photolabeled in the presence of ABAH2505–2497. Both ABAH2505–2497-photolabeled 23S rRNA and control 23S rRNA prepared from 50S subunits photolyzed in the absence of probe were hybridized to single-stranded oligodeoxynucleotide primers complementary to 23S rRNA. The resulting heteroduplexes were examined as substrates for AMV-RT by PAGE and autoradiographic analysis, the underlying premise being that nucleotide modification resulting from photoincorporation of the probe should result in a detectable halt or pause at the position preceeding the site of photoincorporation (Youvan & Hearst, 1979, 1981).

The results of such analyses are shown in Figure 7. Part a of this Figure shows clear pauses or stops in analysis of the photoincorporation experiment (lane 2), not seen in the analysis of the control (lane 1), at positions G-2455, G-2502, A-2503, U-2506 and C-2507, implicating nucleotides G-2454, C-2501, G-2502, G-2505, and U-2506 as sites of photoincorporation. Similarly, part b of Figure 7 implicates nucleotides G-2583 and U-2584 as sites of photoincorporation and provides confirmatory evidence for photoincorporation into G-2502 and G-2505. Thus, ABAH2505-2497 photoincorporates not only into the rRNA single-stranded region to which it is complementary but also into two other sites as well, separated by some 130 nucleotides within the 23S rRNA primary structure.

Discussion

A great deal of effort is currently being devoted toward constructing detailed topographical and functional maps of both the 30S and 50S subunits of *E. coli* ribosomes (Brimacombe et al., 1990b; Noller et al., 1990). Photoaffinity labeling studies have provided a significant fraction of the information used in constructing these maps (Cooperman, 1987, 1988; Cooperman et al., 1989, 1990). Until now the photoaffinity labeling approach has been confined to traditional ribosomal ligands: i.e., tRNAs, mRNAs, antibiotics, protein factors. Here we extend this approach to cDNAs complementary to rRNA sequences known to be exposed in intact subunits.

As in any photoaffinity labeling study, interpretation of the results depends on the demonstration that photoincorporation occurs from the target site, in this case the single-stranded 2497–2505 sequence in 23S rRNA. With respect to protein labeling, our results indicate that protein L3 is labeled by ABAH2505-2497A* specifically binding to its complementary sequence in 23S rRNA. That the labeling of protein L3 is site-specific is shown by the results in Figure 3 (lanes 1 and 2), since cDNA probe 2505–2497 should, by competition, reduce photoincorporation arising from site-specific binding but should have no effect on photoincorporation arising either via nonspecific binding of ABAH2505–2497A* or from photoactivated ABAH2505–2497A* in solution. The experiment with MM-cDNA 2505–2497 was carried out to test whether the specific site from which labeling occurs is, in fact, the target site. We reasoned that, with two mismatches in the

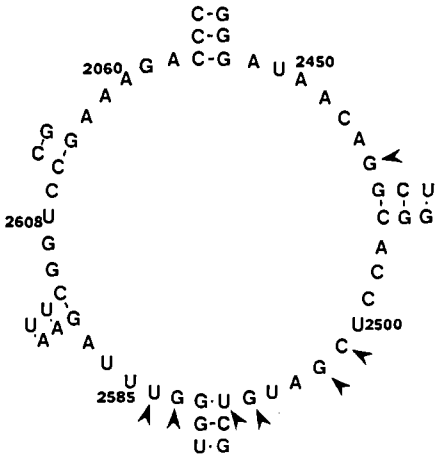

FIGURE 8: Secondary structure of the central loop of domain V of 23S rRNA [from Noller (1984)]. The arrows indicate positions of ABAH2505–2497A* photoincorporation.

middle of its sequence, MM-cDNA 2505–2497 would compete only very poorly with ABAH2505–2497A* binding to the target sequence but, with seven of the nine other residues conserved, could compete with the binding of ABAH2505–2497A* to other sites in the 50S subunit, if such binding occurred. The contrasting effects on the photoincorporation of ABAH2505–2497A* into protein L3 of addition of cDNA 2505–2497 and of MM-cDNA 2505–2497 is strong evidence that L3 is labeled from the target site. That neither cDNA 2505–2497 nor MM-cDNA 2505–2497 has any apparent effect on photoincorporation into any other proteins (at least at the concentrations employed) indicates that photoincorporation into such proteins arises either from nonspecific (or less specific) binding of ABAH2505–2497A* or from photoactivated ABAH2505–2497A* in solution.

In contrast to photoincorporation of ABAH2505–2497A* into 50S protein (but in parallel with the photoincorporation of ABAH2505–2497A* into protein L3), the results presented in Figure 5 provide strong evidence that essentially all photoincorporation of ABAH2505–2497A* into rRNA arises from ABAH2505–2497A* specifically bound to the complementary sequence in 23S rRNA. This is shown by the large decrease of photoincorporation observed for photolyses carried out in the presence of cDNA 2505–2497, as contrasted with the absence of effect on photoincorporation of MM-cDNA 2505–2497.

We infer from the effects of cDNA 2505–2497 on ABAH2505–2497A* labeling of L3 and rRNA that most of the noncovalent binding of cDNA p*2505–2497 seen at high stoichiometric ratios of oligodeoxyribonucleotide to 50S subunit (Figure 1) is not at the target site. This inference is based on the finding that almost all of the specific photoincorporation of ABAH2505–2497A* from the target site is abolished at cDNA 2505–2497 to 50S ratios of 0.6–1.0 (Figures 3 and 5), implying that binding of cDNA 2505–2497 to the target site is largely saturated at these values and that the remainder of the noncovalent binding observed at higher ratios must take place elsewhere. This would account for the large amount of apparently nonspecific protein labeling seen in Figure 3. The results of Marconi and Hill (1988, 1989) also indicate that only a portion of the noncovalent binding measured by Millipore filtration is specific for the target site.

In specifically bound and photoactivated ABAH2505–2497A*, the photogenerated nitrene is a maximum of 24 Å from G-2505 in 23S rRNA, and this distance represents an upper limit of the distance between this base and the ribosomal components into which photoincorporation occurs. The important new information from our results, therefore, is that protein L3 and nucleotides G-2454, G-2583, and U-2584 lie within 24 Å of G-2505. A considerable body of evidence (as reviewed in Cooperman et al., 1990) suggests that the central loop of domain V of 23S rRNA (Figure 8) is part of the peptidyltransferase center, and that each of three single-stranded sequences within this loop (nucleotides 2448–2454, 2497–2505, and 2583–2587) form part of this center. Our RNA labeling results clearly provide direct evidence for the proximity of these sequences within the 50S subunit. Interesting extensions of this work will be to examine the effects on the photoincorporation products both of varying the size of the linker between the reactive nitrene and the complementary oligodeoxyribonucleotide and of attaching the aryl azide to the opposite end (3′ rather than 5′) of the complementary oligodeoxyribonucleotide.

Our results also represent the first report of the labeling of L3 by a reagent bound at the peptidyl transferase center. In this connection it is important to note that protein L3 is one of only three 50S proteins (the others are L2 and L4) that might be essential for peptidyl transferase activity (Tate et al., 1987). Further, the chemical and enzymatic footprinting work of Leffers et al. (1988) has shown that L3 binds in region VIA of 23S rRNA, with the strongest protection effects seen on nucleotides 2634–2638 and 2770–2785, thus placing these 23S rRNA sequences within some proximity of nucleotides 2505–2497. All of this information is directly relevant for the efforts currently underway to construct a three-dimensional model of the 50S subunit (Brimacombe et al., 1990b).

We targeted nucleotides 2497–2505 in this first study using photolabile complementary cDNA probes because of their importance for ribosomal function and as a followup to a previous study by this laboratory showing incorporation of a photolabile derivative of the peptidyltransferase substrate puromycin into nucleotides G-2502 and U-2504 (Hall et al., 1988). However, it is clear that this is a general approach for exploring the neighborhoods of other rRNA regions in ribosomal subunits to which oligodeoxynucleotides can bind [e.g., Olson et al. (1988), Hill et al. (1990), and Oakes et al. (1990)] as well as in subparticles that have been only partially reconstituted. Such studies are underway.

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Registry No. Guanine, 73-40-5; peptidyl transferase, 9059-29-4; *O*-[*N*-(4-monomethoxytrityl)-6-aminohexyl[*O'*-(2-cyanoethyl *N,N*-diisopropylphosphoramidite, 114616-27-2.

REFERENCES

Bernabeu, C., Conde, F. P., & Vazquez, D. (1978) *Anal. Biochem. 84*, 97–102.

Brimacombe, R., Greuer, B., Gulle, H., Kosack, M., Mitchell, P., Osswald, M., Stade, K., & Stiege, W. (1990a) in *Ribosomes and Protein Synthesis, A Practical Approach* (Spedding, G., Ed.) pp 131–159, Oxford University Press, Oxford.

Brimacombe, R., Greuer, B., Mitchell, P., Osswald, M., Rinke-Appel, J., Schuüler, D., & Stade, K. (1990b) in *The Ribosome: Structure, Function, & Evolution* (Hill, W. E., Dahlberg, A., Garrett, R. A., Moore, P. B., Schlesinger, D.,

& Warner, J. R., Eds.) pp 93–106, American Society of Microbiology, Washington.
Cooperman, B. S. (1987) *Pharmacol. Ther. 34*, 271–302.
Cooperman, B. S. (1988) *Methods Enzymol. 164*, 341–361.
Cooperman, B. S., Dondon, J., Finelli, J. Grunberg-Manago, M., & Michelson, A. M. (1977) *FEBS Lett. 76*, 59–63.
Cooperman, B. S., Buck, M. A., Fernandez, C. L., Weitzmann, C. J., & Ghrist, B. F. D. (1989) in *Photochemical Probes in Biochemistry* (Nielsen, P. E., Ed.) NATO ASI Series C, Vol. 272, pp 123–139, Kluwer Academic, Boston.
Cooperman, B. S., Weitzmann, C. J., & Fernandez, C. L. (1990) in *The Ribosome: Structure, Function, & Evolution* (Hill, W. E., Dahlberg, A., Garrett, R. A., Moore, P. B., Schlesinger, D., & Warner, J. R., Eds.) pp 491–501, American Society of Microbiology, Washington.
Darlix, J. L. (1975) *Eur. J. Biochem. 51*, 369–376.
Goldman, R. A., Hasan, T., Hall, C. C., Strycharz, W. A., & Cooperman, B. S. (1983) *Biochemistry 22*, 359–368.
Gulle, H., Hoppe, E., Osswald, M., Greuer, B., Brimacombe, R., & Stoffler, G. (1988) *Nucleic Acids Res. 16*, 815–832.
Hall, C. C., Johnson, D., & Cooperman, B. S. (1988) *Biochemistry 27*, 3983–3990.
Hardy, S. J. S., Kurland, C. G., Voynow, P., & Mora, G. (1969) *Biochemistry 8*, 2897–2905.
Hill, W. E., Weller, J., Gluick, T., Merryman, C., Marconi, R. T., Tassanakajohn, A., & Tapprich, W. E. (1990) in *The Ribosome: Structure, Function, & Evolution* (Hill, W. E., Dahlberg, A., Garrett, R. A., Moore, P. B., Schlesinger, D., & Warner, J. R., Eds.) pp 93–106, American Society of Microbiology, Washington.
Jaynes, E. N., Jr., Grant, P. G., Giangrande, G., Wieder, R., & Cooperman, B. S. (1978) *Biochemistry 17*, 561–569.
Kerlavage, A. R., & Cooperman, B. S. (1986) *Biochemistry 25*, 8002–8010.
Lasater, L. S., Cann, P. A., & Glitz, D. G. (1989) *J. Biol. Chem. 264*, 21798–21805.
Lasater, L. S., Montesano-Roditis, L., Cann, P. A., & Glitz, D. G. (1990) *Nucleic Acids Res. 18*, 477–485.
Leffers, H., Egebjerg, J., Andersen, A., Christensen, T., & Garrett, R. A. (1988) *J. Mol. Biol. 204*, 507–522.
Mankin, A. S., Skripkin, E. A., Chichkova, N. V., Kopylov, A. M., & Bogdanov, A. A. (1981) *FEBS Lett. 131*, 253–256.
Marconi, R. T., & Hill, W. E. (1988) *Nucleic Acids Res. 16*, 1603–1615.
Marconi, R. T., & Hill, W. E. (1989) *Biochemistry 28*, 893–899.
Marconi, R. T., Lodmell, J. S., & Hill, W. E. (1990) *J. Biol. Chem. 265*, 7894–7899.
McWilliams, R. A., & Glitz, D. G. (1991) *Biochimie* (in press).
Muralikrishna, P., & Wickstrom, E. (1989) *Biochemistry 28*, 7505–7510.
Noller, H. (1984) *Annu. Rev. Biochem. 53*, 119–162.
Noller, H., Moazed, D., Stern, S., Powers, T., Allen, P. N., Robertson, J. M., Weiser, B., & Triman, K. (1990) in *The Ribosome: Structure, Function, & Evolution* (Hill, W. E., Dahlberg, A., Garrett, R. A., Moore, P. B., Schlesinger, D., & Warner, J. R., Eds.) pp 93–106, American Society of Microbiology, Washington.
Oakes, M. I., & Lake, J. A. (1990) *J. Mol. Biol. 211*, 897–906.
Olson, H. M., Lasater, L. S., Cann, P. A., & Glitz, D. G. (1988) *J. Biol. Chem. 263*, 15196–15204.
Sambrook, J., Maniatis, T., & Fritsch, E. F. (1989) in *Molecular Cloning: A Laboratory Manual*, 2nd ed., pp 11–39, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Skripkin, E. A., Kopylov, A. M., Bogdanov, A. A., Vinogradov, S. V., & Berlin, Yu, A. (1979) *Mol. Biol. Rep. 5*, 221–224.
Sypherd, P. S., & Wireman, J. W. (1974) *Methods Enzymol. 30*, 349–354.
Tate, W. P., Sumpter, V. G., Trotman, C. N. A., Herold, M., & Nierhaus, K. H. (1987) *Eur. J. Biochem. 165*, 403–408.
Thomas, J. O., & Kornberg, R. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2626–2630.
Youvan, D. C., & Hearst, J. E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3751–3754.
Youvan, D. C., & Hearst, J. E. (1981) *Nucleic Acids Res. 9*, 1723–1741.
Yousaf, S. I., Carroll, A. R., & Clarke, B. E. (1984) *Gene 27*, 309–313.