and lactose are added to the system.

Although the binding protein is composed of multiple subunits, a single transition was observed upon thermal denaturation for all forms of the lectin, suggesting that the binding protein is devoid of vastly different independent structural domains, such as those observed in a number of other proteins, e.g., prothrombin (Ploplis et al., 1981), fibrinogen (Donovan & Mihalyi, 1974), and plasminogen (Castellino et al., 1981). It should be pointed out, however, that the large $\Delta H_{cal}/\Delta H_{vH}$ ratios may be consistent with the existence of separate domains undergoing more or less independent denaturation at similar temperatures. These results show that the A and B subunits do not differ markedly in their denaturation parameters, implying that they are structurally similar. This is in accord with their similar amino acid compositions (Kawasaki & Ashwell, 1976). The minor transition, at approximately 46 °C, may well be contributed by dissociated subunits, but this remains to be established.

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Esterification of an Endogenously Synthesized Lipoxygenase Product into Granulocyte Cellular Lipids†

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ABSTRACT: The human promyelocytic leukemia cell line HL60 can be induced to differentiate into mature granulocytes by exposure to Me_2SO. [1-^{14}C]Arachidonic acid incubated overnight with these cells was incorporated mainly into membrane phospholipids. Stimulation of these cells with the calcium ionophore, A_{23187}, resulted in a rapid release of esterified arachidonic acid from phosphatidylethanolamine and phosphatidylcholine. The released arachidonic acid was metabolized via both the cyclooxygenase and lipoxygenase pathways into three major hydroxylated products, 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT), 5(*S*)-hydroxy-6,8,11,14-icosatetraenoic acid (5-HETE), and 5(*S*),12(*R*)-dihydroxy-6,8,10,14-icosatetraenoic acid (leukotriene B). Arachidonic acid was also incorporated into triacylglycerols and phosphatidylinositol. The lipoxygenase product, 5-HETE, was rapidly esterified into cellular lipids. Thirty minutes after ionophore stimulation, 55% of the total 5-HETE synthesized was esterified into phopholipids and 35% incorporated into acylglycerols. In contrast, the other hydroxylated derivatives of arachidonic acid (HHT and leukotriene B) were not incorporated into acylglycerols or phospholipids. Esterification of hydroxylated metabolites of arachidonic acid into membrane phospholipids may serve to regulate a number of granulocyte functions.

Arachidonic acid metabolism in granulocytes has been the subject of extensive research recently. An activatable phospholipase, which releases arachidonic acid from membrane phospholipids, has been described in neutrophils (Stenson & Parker, 1979a; Waite et al., 1979), and these cells are known to metabolize free arachidonic acid via the cyclooxygenase and lipoxygenase pathways (Goldstein et al., 1978; Borgeat & Samuelsson, 1979a; Goetzl & Sun, 1979; Siegel et al., 1980). The lipoxygenase products have been shown to be chemotactic for neutrophils (Tainer et al., 1975; Turner et al., 1976; Goetzl et al., 1977) and to induce degranulation of specific granules (Stenson & Parker, 1980). Furthermore, arachidonic acid and its metabolites have been implicated in a number of other physiological functions of the neutrophil. These include aggregation and degranulation (Ford-Hutchinson et al, 1979; O'Flaherty et al., 1979; Naccache et al., 1979), the chemotactic process (Hirata et al., 1979), changes in the permeability of the plasma membrane to calcium (Volpi et al., 1980),

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stimulation of the hexose monophosphate shunt oxidation of glucose, and regulation of superoxide generation (Bokoch & Reed, 1979).

The precise role of arachidonic acid metabolism in neutrophil function remains unclear. Stenson & Parker (1979a) reported that human neutrophils generated 5(*S*)-hydroxy-6,8,11,14-icosatetraenoic acid (5-HETE)[1] during calcium ionophore stimulation. 5-HETE was shown to be released into the medium, taken back up by the cells, and further metabolized. Futhermore, exogenously supplied 5-HETE was shown to be readily incorporated into triacylglycerols and phospholipids, strongly suggesting that endogenously synthesized 5-HETE might be esterified into cellular lipids. It has been suggested that the reesterification of these hydroxy fatty acids into membrane lipids may be one mechanism for controlling such functions as degranulation and chemotaxis (Stenson & Parker, 1979a,b, 1980).

Recently, we reported the appearance of two arachidonic acid metabolizing enzymes in the human promyelocytic leukemic cell line HL60 during dimethyl sulfoxide (Me_2SO) induced differentiation (Bonser et al., 1981). This study describes in detail endogenous arachidonic acid metabolism in HL60 cells which have been induced to differentiate into mature granulocytes by exposure to dimethyl sulfoxide. Data are presented which directly demonstrate the rapid esterification of endogenously synthesized 5-HETE into granulocyte cellular lipids.

Materials and Methods

Cells. The HL60 cells (a generous gift from Dr. R. C. Gallo, National Institutes of Health, Bethesda, MD) were cultured as previously described (Bonser et al., 1981). Differentiation was induced by adding dimethyl sulfoxide to a final concentration of 1.3%. Cell cultures, which had been exposed to Me_2SO for 5 days, were incubated overnight with [1-^{14}C]arachidonic acid (Amersham, 55 μCi/μmol) at a concentration of 2 μM (0.1 μCi/mL). The labeled cells were harvested by centrifugation and washed twice in RPMI 1640 medium, without Phenol Red (B and B Research Laboratory, Baltimore, MD). The washed cells were resuspended to a final concentration of 4×10^7 cells/mL in the same medium, which had been adjusted to 1 mM with respect to calcium. The cell suspensions (1 mL) were incubated at 37 °C with 10 μM calcium ionophore A_{23187} (Calbiochem).

Extraction of Lipid. After the reaction was stopped by the addition of 2.4 mL of chloroform/methanol (1:1 v/v), containing 0.001% butylated hydroxyanisole and butylated hydroxytoluene, and 0.1 mL of 2% formic acid, the suspension was vortexed, cooled in ice, and centrifuged. The organic layer was withdrawn and evaporated to dryness under N_2. The dry extract was redissolved in a small volume of chloroform/methanol (1:1 v/v) and spotted on silica thin-layer plates (Sil G25, without gypsum, Brinkmann). Chromatograms were developed in an ascending fashion by using the following solvent systems: I, ligroine/ether/acetic acid (40:60:1 v/v/v); II, the organic phase of ethyl acetate/isooctane/acetic acid/water (90:50:20:100 v/v/v/v). Labeled products were located by autoradiography, and the appropriate regions of the plates were scraped and counted in a liquid scintillation counter. Products were identified by cochromatography with authentic standards on thin-layer and high-performance liquid chromatographic (HPLC) systems (Skipski et al., 1965; Porter et al., 1979a,b). The putative 5-HETE and leukotriene B chromatographed on HPLC systems with retention times similar to those reported by others (Borgeat & Samuelsson, 1979a) and had the characteristic ultraviolet spectra (Borgeat & Samuelsson, 1979b). Furthermore, the production of HHT was inhibited by 10 μM indomethacin, and the synthesis of leukotriene B and 5-HETE as well as HHT was inhibited by 50 μM BW 755C, a cyclooxygenase and lipoxygenase inhibitor (Higgs et al., 1979).

Lipid Hydrolysis. The dry total lipid extract was redissolved in 1 mL of 80% methanol containing 0.2 N NaOH and heated at 45 °C overnight, under N_2. Chloroform (1.2 mL), methanol (0.4 mL), and water (0.8 mL) were added to the hydrolysate, and the pH was adjusted to 4 with formic acid. The mixture was vortexed, cooled on ice, and centrifuged. The organic layer was withdrawn and evaporated to dryness under N_2. Hydrolysis products were separated by thin-layer chromatography using the procedure described above.

Separation of Lipid Classes. Neutral lipids were separated from phospholipids on a silicic acid column by using the procedure described by Borgeat & Samuelsson (1979b). The total lipid extract was applied to the column in a small volume of hexane/ether (8:2 v/v). Neutral lipids were eluted with 20 mL of hexane/ether (8:2 v/v); phospholipids were eluted from the column with 20 mL of methanol. The methanol fraction was evaporated to dryness under N_2, and the different phospholipid classes were separated by high-performance liquid chromatograhy using the procedure described by Geurts Van Kessel et al. (1977). The identity of each phospholipid class was confirmed by cochromatography with authentic standards on HPLC and also on silica gel H (Applied Science, State College, PA) using the solvent system chloroform/methanol/acetic acid/water (75:45:12:2 v/v/v/v). The phospholipid fractions recovered after HPLC were evaporated to dryness under N_2 and hydrolyzed, and the products were separated by thin-layer chromatography, using the procedure described earlier. The sources of the chromatographic standards were as follows: phospholipid and acylglycerol standards were purchased from P-L Biochemicals, and chemically synthesized 5-HETE was a gift from Dr. N. A. Porter, Duke University, Durham, NC.

Recovery of Lipids. The recovery of radiolabel from Me_2SO-differentiated HL60 cells prelabeled overnight with [1-^{14}C]arachidonic acid, using the extraction procedure described above, was 96.94 ± 0.25% (mean ± SE of 10 determination). Alkaline hydrolysis (see above) released 99.49 ± 0.04% (mean ± SE of 10 determinations) of the incorporated radiolabel as free arachidonic acid.

Experiments were also done to establish the recovery of phospholipids after silicic acid column chromatography and high-performance liquid chromatography (see above). Recoveries were monitored by using [^{3}H]dipalmitoylphosphatidylcholine (a generous gift from Dr. D. Ahern, New England Nuclear). After silicic acid column chromatography, 92.02 ± 1.32% (mean ± SE of five determinations) of the radiolabeled phosphatidylcholine was recovered. The recovery following high-performance liquid chromatography was 70.62 ± 2.18% (mean ± SE of five determinations).

Results

Me_2SO-Induced Differentiation. Several striking morphological changes occur in HL60 cells following 6 days of

[1] Abbreviations used: TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; HHT, 12-L-hydroxy-5,8,10-heptadecatrienoic acid; 5-HETE, 5(*S*)-hydroxy-6,8,11,14-icosatetraenoic acid; leukotriene B, 5(*S*),12(*R*)-dihydroxy-6,8,10,14-icosatetraenoic acid; AA, arachidonic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; cpm, counts per minute.

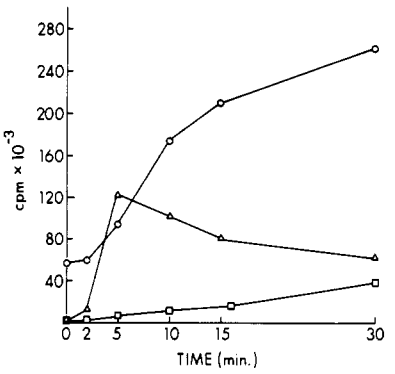

FIGURE 1: Distribution of [1-^{14}C]arachidonic acid among the neutral lipid fractions of Me_2SO-differentiated HL60 cells during calcium ionophore stimulation. Prelabeled cells (4×10^7/mL) were incubated at 37 °C in RPMI 1640 medium with 10 μM A_{23187} for varying periods of time. The cell suspensions were extracted and the different lipid fractions separated by TLC (see Materials and Methods). Results are the mean of duplicate experiments. All values are within ±10% of the mean. (○) Triacylglycerols; (△) arachidonic acid; (□) diacylglycerols.

exposure to Me_2SO. Differentiated cells exhibit the following changes: smaller size, decreased nuclear/cytoplasmic ratio, marked reduction or complete disappearance of nucleoli, and distinct indentation, convolution, and segmentation of the nuclei. All of these observed changes are characteristic of the differentiation of promyelocytes of mature granulocytes (Collins et al., 1978).

Incorporation of Arachidonic Acid. Differentiated HL60 cells incorporate over 95% of the [1-^{14}C]arachidonic acid added to the culture medium into their cellular lipids after overnight labeling. More than 97% of the label taken up is esterified into phospholipids, the remainder being incorporated into triacylglycerols. The cells contain only trace amounts of free arachidonic acid. Alkaline hydrolysis of the total lipids releases virtually all of the radiolabel as free arachidonic acid, demonstrating that the fatty acid is not significantly metabolized by the cells before being esterified.

Ionophore-Stimulated Arachidonic Acid Metabolism. The calcium ionophore, A_{23187}, stimulates the release of free arachidonic acid in Me_2SO-differentiated HL60 cells. Arachidonic acid accumulates rapidly, reaching a peak 5 min after stimulation. The level of free arachidonate then falls slowly (Figure 1). There is a dramatic incorporation of radiolabel into triacylglycerols during ionophore stimulation, and this incorporation continues throughout the time course of the experiment (Figure 1). Radiolabel also accumulates within the diacylglycerol fraction (Figure 1). Me_2SO-differentiated HL60 cells metabolize endogenous arachidonic acid via both cyclooxygenase and lipoxygenase. 12-L-Hydroxy-5,8,10-heptadecatrienoic acid (HHT), a cyclooxygenase product (Hamberg & Samuelsson, 1974), accumulates rapidly during ionophore stimulation (Figure 2). These cells also synthesize thromboxane A_2 from endogenous arachidonic acid (Bonser ett al., 1981). 5(*S*-Hydroxy-6,8,11,14-icosatetraenoic acid (5-HETE) and leukotriene B [5(*S*),12(*R*)-dihydroxy-6,8,10,14-icosatetraenoic acid], both lipoxygenase products (Borgeat & Samuelsson, 1979a; Borgeat et al., 1976), are also rapidly synthesized from endogenous arachidonic acid during ionophore stimulation (Figures 2 and 3).

The level of free 5-HETE increases rapidly during the first 5 min of stimulation but then falls sharply (Figure 3). To test the hypothesis that the loss of radiolabel from the free 5-HETE fraction might be due to the esterification of this arachidonic acid metabolite into cellular lipids, we subjected the total lipid extracts to alkaline hydrolysis (see Materials and Methods).

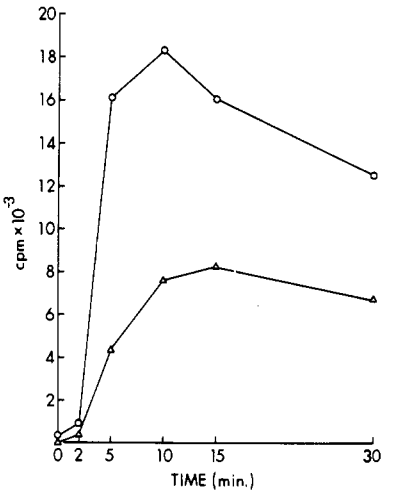

FIGURE 2: Time course for production of leukotriene B and HHT from endogenous arachidonic acid in Me_2SO-differentiated HL60 cells stimulated with A_{23187}. Prelabeled cells (4×10^7/mL) were incubated at 37 °C for varying periods of time with 10 μM A_{23187}. Cell suspensions were extracted and products separated by TLC (see Materials and Methods). Results are the mean of duplicate experiments. All values are within ±10% of the mean. (○) leukotriene B; (△) HHT.

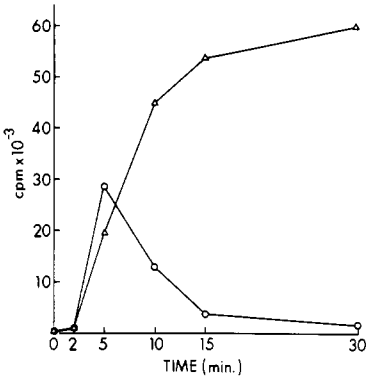

FIGURE 3: Time course of 5-HETE production and esterification from endogenous arachidonic acid in Me_2SO-differentiated HL60 cells stimulated with A_{23187}. Prelabeled cells (4×10^7/mL) were incubated for varying periods at 37 °C with 10 μM A_{23187}. Cell suspensions were extracted and half of the total lipid extracts subjected to alkaline hydrolysis. 5-HETE present in the hydrolyzed and unhydrolyzed fractions was isolated by TLC (see Materials and Methods). Esterified 5-HETE is expressed as total 5-HETE (hydrolyzed fraction) − free 5-HETE (unhydrolyzed fraction). Results are the mean of duplicate experiments. All values are within ±10% of the mean. (○) Free 5-HETE; (△) esterified 5-HETE.

All of the radiolabel which is lost from the free 5-HETE fraction is recovered after hydrolysis, indicating that free 5-HETE had been esterified into cellular lipids (Figure 3). Alkaline hydrolysis does not release any HHT or leukotriene B, demonstrating that these arachidonic acid metabolites are not incorporated into cellular lipids during ionophore stimulation.

The total lipid extract was fractionated into its different lipid classes (see Materials and Methods) to determine into which particular lipid(s) the free 5-HETE had been esterified. The distribution of [1-^{14}C]AA among the different lipid classes of unstimulated cells is shown in Table I. The results of alkaline hydrolysis reveal that of the total AA esterified, 65% is in PE, 28% is in PC, 2% is in PI and PS, 2.5% is in the neutral lipid fraction (which is composed almost exclusively of triacylglycerols), and 0.25% is in PA. Alkaline hydroylsis releases only trace amounts of 5-HETE from the lipids of unstimulated cells (Table I).

Table I: Distribution of AA and 5-HETE among the Different Lipid Fractions of Me_2SO-Differentiated HL60 Cells before and after Ionophore Stimulation[a]

	cpm			
lipid fraction	AA control	AA + A_{23187}	5-HETE control	5-HETE + A_{23187}
free	3 972	75 877	37	18 068
neutral lipid	231 196	1 149 477	304	67 164
PA	25 178	38 398	70	23 404
PE	6 090 151	4 554 604	1 057	16 951
PI	191 093	585 554	107	5 001
PS	196 849	140 062	147	1 032
PC	2 602 289	2 177 585	446	56 614

[a] Prelabeled cells (2×10^8) were incubated at 37 °C for 30 min in RPMI 1640 medium with and without 10 μM A_{23187}. The total lipid extracts were fractionated into their individual lipid classes and hydrolyzed, and the products of hydrolysis were separated by TLC (see Materials and Methods). The results shown above are representative of three separate experiments.

The distribution of [1-^{14}C]AA within the various phospholipids was studied following calcium ionophore stimulation (Table I). Exposure to the ionophore for 30 min results in a loss of AA from PE and PC. In contrast, there is a dramatic increase in the AA esterified in the neutral lipid (tri- and diacylglycerols) and PI fractions. Alkaline hydrolysis of the lipids from ionophore-stimulated cells release large amounts of 5-HETE (Table I and Figure 3). Approximately 35% of the total 5-HETE synthesized is esterified into neutral lipids; 30% is incorporated into PC, 12.4% into PA, 9% into PE, 2.6% into PI, and 0.5% into PS; the remaining 10% is recovered as free 5-HETE. The incorporation of 5-HETE into the phospholipid classes did not significantly affect their retention times on HPLC.

Discussion

The human promyelocytic leukemia cell line HL60 can be induced to differentiate into mature granulocytes by exposure to dimethyl sulfoxide (Collins et al., 1978; Newberger et al., 1979). Differentiated HL60 cells display many of the functions characteristic of polymorphonuclear leukocytes (Newberger et al., 1979). This study clearly demonstrates that in the presence of the calcium ionophore, A_{23187}, Me_2SO-differentiated HL60 cells metabolize endogenous arachidonate via both the cyclooxygenase and lipoxygenase pathways. Furthermore, during ionophore stimulation the lipoxygenase product, 5-HETE, is very rapidly esterified into neutral lipids and phospholipids. In contrast, other products of arachidonate metabolism (HHT and leukotriene B) are not similarly esterified into cellular lipids. The esterification of exogenously supplied 5-HETE by human peripheral blood neutrophils into triacylglycerols and phospholipids has been described recently by Stenson & Parker (1979a). However, we believe that this is the first direct demonstration of the esterification of an endogenously synthesized hydroxyarachidonic acid metabolite.

Monohydroxyicosatetraenoic acids are chemotactic for neutrophils and induce degranulation of specific granules (Tainer et al., 1975; Turner et al., 1976; Goetzl et al., 1977; Stenson & Parker, 1980). However, the precise role of these arachidonic acid metabolites in neutrophil function remains unclear. It has been suggested that esterification of hydroxy fatty acids into membrane phospholipids may be a more generalized mechanism for controlling membrane characteristics and may explain the degranulating and chemoattractant properties of these arachidonic acid metabolites (Stenson & Parker, 1980). For example, the introduction of hydroxy fatty acids into granulocyte membrane lipids may increase permeability to cations (Volpi et al., 1980) and change the fluidity of local sites on membranes, permitting fusion and degranulation (Stenson & Parker, 1980).

Inhibitors of neutrophil arachidonate metabolism block degranulation induced by a variety of stimuli (O'Flaherty et al., 1979; Naccache et al., 1979; Northover, 1977). While the relative contributions of the cyclooxygenase and lipoxygenase pathways to the degranulation process have not yet been ascertained, it has been reported that aspirin, a potent inhibitor of cyclooxygenase, does not block neutrophil degranulation (O'Flaherty et al., 1979). This suggests that lipoxygenase but not cyclooxygenase products are important in this process. Bass et al. (1980) have demonstrated that the stimulation of hexose transport in neutrophils by chemoattractants is blocked by inhibitors of arachidonate metabolism. Futhermore, they have suggested that this process is also dependent upon lipoxygenase, rather than cyclooxygenase, product formation. The evidence presented in this study further strengthens the theory put forward by Stenson & Parker (1979a,b, 1980) that the esterification of lipoxygenase products into membrane lipids may be involved in a number of functions of the neutrophil. The observation that Me_2SO-differentiated HL60 cells metabolize endogenous arachidonate in a manner very similar to that of peripheral blood neutrophils (Stenson & Parker, 1979a) suggests that these cells may be a useful model system for investigating the precise role of arachidonic acid metabolism in neutrophil function.

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Position of Transfer Ribonucleic Acid on *Escherichia coli* Ribosomes. Distance from the 3′ End of 16S Ribonucleic Acid to Three Points on Phenylalanine-Accepting Transfer Ribonucleic Acid in the Donor Site of 70S Ribosomes†

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ABSTRACT: *Escherichia coli* 16S RNA from 30S ribosomal subunits was isolated, oxidized at the 3′ end, and labeled with the thiosemicarbazide derivatives of fluorescein or eosin. Labeled 16S RNA was reconstituted into 30S subunits. They were almost fully active compared to 30S subunits reconstituted from unlabeled 16S RNA by using a poly(uridylic acid)-directed polyphenylalanine synthesis assay. Fluorophores were placed at three different positions of $tRNA^{Phe}$. *E. coli* and yeast $tRNA^{Phe}$ were oxidized at the 3′ end and labeled with the thiosemicarbazide derivative of fluorescein or with the hydrazide of *N*-methylanthranilic acid. The Y base in the anticodon loop of yeast $tRNA^{Phe}$ was replaced by proflavin or 1-aminoanthracene. Also, *E. coli* $tRNA^{Phe}$ was photochemically cross-linked between 4-thiouridine at position 8 and cytidine at position 13. After reduction, this site was used as a fluorescent probe. The labeled tRNAs were bound into the peptidyl site of 70S ribosomes, and then the distances from the fluorophore in the modified tRNA to the fluorophore at the 3′ end of 16S RNA were measured by nonradiative energy transfer. Calculations were based on measurements of fluorescence lifetimes. The distances to the 3′ end of 16S RNA were found to be as follows: 3′ end of tRNA, 67–74 Å; cross-linked tRNA, 53–60 Å; anticodon loop of tRNA, >61 Å.

Very little is known about the relative location of tRNA[1] bound to the peptidyl site of 70S ribosomes and the 3′ end of 16S RNA in the small ribosomal subunit. The 3′ end of 16S RNA has been found to be particularly sensitive to nucleases, chemical modification, and antibodies (Zimmermann, 1980) and has been implicated in the process of subunit association. When in 30S subunits, 16S RNA was found to react at 22 sites with the guanine-specific reagent kethoxal, most of them in the 3′ half of the molecule (Noller, 1974). Reactive sites in the 5′-end 600 nucleotides are equally reactive in either 30S subunits or 70S ribosomes (Chapman & Noller, 1977), while the middle and 3′ regions were protected in 70S ribosomes, leading to the conclusion that these portions of the 16S RNA are near the 30S–50S interface.

The 3′ region of 16S RNA also has been implicated in the recognition of a complementary nucleotide sequence in messenger RNA (Shine & Dalgarno, 1974; Steitz & Jakes, 1975;

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[1] Abbreviations used: RNA, ribonucleic acid; tRNA, transfer RNA; $tRNA^{Phe}$, phenylalanine-accepting tRNA; Ac-Phe-tRNA, *N*-acetyl-Phe-tRNA; $tRNA^{Phe}_{XL}$, $tRNA^{Phe}$ that has been photochemically cross-linked from 4-thiouridine in position 8 to cytidine in position 13; $tRNA^{Phe}_{XLR}$, $tRNA^{Phe}_{XL}$ where cross-links have been reduced with $NaBH_4$; Srd_8, 4-thiouridine at position 8 in *E. coli* $tRNA^{Phe}$; Cyd_{13}, cytidine at position 13 in *E. coli* $tRNA^{Phe}$; $tRNA^{Phe}_{Pf}$, $tRNA^{Phe}$ in which the Y base has been replaced by proflavin; DNP, dinitrophenyl moiety; FTSC, fluorescein thiosemicarbazide; ETSC, eosin thiosemicarbazide; NMA, *N*-methylanthranilic moiety; DEAE, diethylaminoethyl; BD-cellulose, benzoylated DEAE-cellulose; poly(U), poly(uridylic acid); Me_2SO, dimethyl sulfoxide; EF-G, elongation factor G; EFTu, elongation factor Tu; Tris, tris(hydroxymethyl)aminomethane; Bicine, *N,N*-bis(2-hydroxyethyl)glycine; CP, creatine phosphate; CPK, creatine phosphokinase; ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate.

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