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Affinity Labeling of the P Site of *Drosophila* Ribosomes: A Comparison of Results from (Bromoacetyl)phenylalanyl-tRNA and Mercurated Fragment Affinity Reactions†

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ABSTRACT: The binding site of the peptidyl group of peptidyl-tRNA in the P site of *Drosophila* ribosomes was probed with (bromoacetyl)phenylalanyl-tRNA (BrAcPhe-tRNA). This affinity label binds specifically to the P site by virtue of its ability to participate in peptide bond formation with puromycin following its attachment to ribosomes. As many as nine ribosomal proteins may be labeled under these conditions; however, the majority of the labeling is associated with three large-subunit proteins and two small-subunit proteins. Two of the large-subunit proteins, L4 and L27, are electrophoretically very similar to the proteins labeled by the same reagent in *Escherichia coli* ribosomes L2 and L27. Reexamination by a different two-dimensional gel system of the ribosomal components labeled by a second P site reagent, the 3′ pentanucleotide fragment of *N*-acetylleucyl-tRNA which is derivatized to contain mercury atoms at the C-5 position of all three cytosine residues, shows two major and three minor labeled proteins. These proteins, L10/L11, L26, S1/S4, S13, and S20, are likely present in the binding site of the 3′ end of peptidyl-tRNA, a site that appears to span both subunits. These results have allowed us to construct a model for the protein positions in and near the peptidyl-tRNA binding site of *Drosophila* ribosomes.

Affinity labeling has proved to be an important and useful technique for establishing structure–function relationships in the ribosome. In particular, the bromoacetyl derivative of aminoacyl-tRNA has been used by several investigators to obtain information on the P site components of bacterial and eukaryotic ribosomes [reviewed by Cooperman (1980)]. The α-bromocarbonyl group is potentially reactive toward nucleophiles present in either the protein or RNA molecules in the ribosomal site. We have used this reagent to probe the binding neighborhood of the peptidyl moiety of peptidyl-tRNA located in the P site of *Drosophila* ribosomes.

We also reexamined the protein components labeled by a second P site reagent, a mercurated analogue of the 3′-terminal pentanucleotide fragment of *N*-acetylaminoacyl-tRNA. In this reagent three mercury atoms are substituted at the C-5 position of the three cytosine residues of the fragment. The mercury atoms are capable of reaction with sulfhydryl-containing protein side chains in the P site. When we first used this reagent to probe the P site of *Drosophila* ribosomes (Fabijanski & Pellegrini, 1979), we identified one ribosomal protein as the major affinity-labeled product. Subsequently, we applied the reagent to a study of rat liver ribosomes. In this system we compared two different two-dimensional (2-D)[1] gel systems for the analysis of the affinity-labeled proteins (Fabijanski & Pellegrini, 1981). The acidic first dimension, SDS second dimension gel system allowed us to identify four additional labeled proteins. Therefore, we have reexamined the *Drosophila* proteins labeled by the mercurated fragment reagent in this gel system. We now can identify two heavily labeled and three less heavily labeled proteins in that region of the P site that binds the 3′ terminus of tRNA.

Our results from the use of these two different P site reagents can be readily correlated with data on other proteins in the peptidyltransferase center. We, therefore, present a model of the arrangement of proteins present in or near this active site in *Drosophila* ribosomes.

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[1] Abbreviations: rRNA, ribosomal RNA; DTT, dithiothreitol; TCA, trichloroacetic acid; 1-D, one dimensional; 2-D, two dimensional; SDS, sodium dodecyl sulfate; BrAcPhe-tRNA, (bromoacetyl)phenylalanyl-tRNA; C(Hg)-A-C(Hg)-C(Hg)-A(Ac[^{3}H]Leu) or mercurated fragment, the 3′-terminal pentanucleotide fragment of *N*-acetylleucyl-tRNA containing a mercury atom at the three cytosine residues; RNase, ribonuclease; Tris, tris(hydroxymethyl)aminomethane.

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Materials and Methods

Materials. Phenylalanine-specific tRNA from yeast (approximately 1200 pmol/A_{260}), yeast aminoacyl-tRNA synthetase, polyuridine, phosphocreatine, phosphocreatine kinase, and RNase A were purchased from Sigma. RNase inhibitor (RNasin) was from Promega Biotech. [^{3}H]-Phenylalanine was obtained from New England Nuclear, and [^{3}H]puromycin was from Amersham. Autofluor was from National Diagnostics.

Preparation of (Bromoacetyl)phenylalanyl-tRNA. tRNA was charged with [^{3}H]phenylalanine essentially as described by White and Tenner (1973) except that the ratio of phenylalanine to tRNA^{Phe} was reduced to 2:1. Reactions were carried out for 40 min at 23 °C and contained 50 mM Tris, pH 7.5, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 4 mM ATP, 0.65 μM phenylalanine-specific tRNA, and 1.3 μM [^{3}H]phenylalanine (specific activity 60.0 Ci/mmol). To initiate the reaction, a combination of RNasin and aminoacyl-tRNA synthetase was added, bringing the final mixture to 1000 units/mL RNasin and 80 units/mL synthetase. Reactions were stopped by extracting 3 times with an equal volume of phenol followed by one extraction with $CHCl_3$/isoamyl alcohol (24:1). The charged tRNAs were precipitated with 2.5 volumes of 95% ethanol and stored at −20 °C. The extent of charging ranged from 30% to 60% as assayed by the amount of radioactivity that was TCA insoluble.

N-(Bromoacetyl)succinimide ester was prepared and reacted with aminoacylated tRNA as described by Pellegrini et al. (1972). The reaction between *N*-(bromoacetyl)succinimide ester and the charged tRNA was found to go to completion in less than 20 min at 0 °C. The product was precipitated with 2.5 volumes of 95% ethanol at −20 °C. The reaction yielded approximately 70% of the desired (bromoacetyl)-phenylalanyl-tRNA (BrAcPhe-tRNA) as shown by its insensitivity to hydrolysis in the presence of Cu^{2+} (Schofield & Zamecnik, 1968).

Mercurated fragment [C(Hg)-A-C(Hg)-C(Hg)-A(Ac-[^{3}H]Leu)] was prepared exactly as described previously (Fabijanski & Pellegrini, 1979).

Affinity Labeling Reactions. Affinity labeling was performed under essentially the same conditions that Nirenberg and Leder (1964) used to show the poly(uridylic acid) directed binding of phenylalanine-specific tRNA to *Escherichia coli* ribosomes. Typical 1-mL reactions contained 500 pmol of ribosomes, 3600 pmol of poly(uridylic acid), and 2700 pmol of BrAcPhe-tRNA in 100 mM Tris, pH 7.2, 50 mM KCl, and 20 mM $Mg(OAc)_2$. In the direct labeling reactions [^{3}H]-BrAcPhe-tRNA was used. In reactions requiring peptidyltransferase activity, cold BrAcPhe-tRNA was used and 15 nmol of [^{3}H]puromycin was added to each 1-mL reaction. The large molar excess of puromycin was necessary both because of its lower specific activity and to bring it to a physiologically active concentration.

In other cases mercurated fragment radiolabeled with ^{203}Hg was added to *Drosophila* ribosomes under fragment assay conditions at 0 °C for 45 min (Munro, 1971; Fabijanski & Pellegrini, 1979). Ribosomes (80S) were isolated from *Drosophila melanogaster* 0–20-h embryos as previously described (Fabijanski & Pellegrini 1979) except that 2-mercaptoethanol and DTT were excluded.

Isolation and Characterization of Ribosomal Components. Following the labeling reactions, any remaining charged tRNAs were removed by adding 100 μg of RNAse A to each milliliter of reaction and incubating 15 min at 37 °C. Ribosomal proteins were dissociated and dissolved by using the Mg^{2+}/acetic acid procedure of Sherton and Wool (1974). To do that, the reaction mixtures were chilled on ice and stirred with 100 μL of 1 M $Mg(OAc)_2$ and 2.3 mL of acetic acid for 30 min. The insoluble material was removed by pelleting in an SS-34 rotor, 10K rpm, 4 °C, for 20 min. The soluble proteins were either dialyzed extensively against 10% acetic acid or passed over a desalting column (Bio-Gel P-6DG, exclusion limit 6000 daltons) equilibated in 1% acetic acid. In either case, the proteins were quick frozen and lyphilized.

To extract rRNA, aliquots of the reaction mixtures were brought to pH 8 with KOH and incubated 20 min at 37 °C to discharge any remaining aminoacyl-tRNAs. The aliquots were then extracted 3 times with phenol and once with $CHCl_3$/isoamyl alcohol (24:1). The remaining aqueous layer was precipitated with 2.5 volumes of ethanol.

Ribosomal proteins were resolved on 2-D polyacrylamide gels using the system of Mets and Bogorad (1974) as modified by Warner and Gorenstein (1977). The first dimension separated proteins on the basis of their charge at pH 5, and the second was a 15% acrylamide SDS sizing gel. Visualization of proteins was by Coomassie blue staining. The gels were then impregnated with Autofluor, dried, and exposed to X-ray film at −70 °C. The ribosomal proteins that appeared on the gels were numbered according to the system of Chooi et al. (1980).

One-dimensional SDS gels were prepared and run in a manner identical with the second dimension of the 2-D gels except that wells were cast in the stacking gel. Five hundred microgram protein samples were dissolved in SDS loading buffer (Laemmli, 1970) and loaded into the wells.

Results

Phenylalanine-Specific Yeast tRNA Promotes Polyphenylalanine Synthesis in a Drosophila Translation System. As large quantities of *Drosophila* tRNA^{Phe} were difficult to prepare, we examined the possibility of substituting commercially available (Sigma) tRNA^{Phe} from yeast for use in the *Drosophila* system. Scott et al. (1979) showed that the addition of a mixture of yeast tRNAs increased activity in the in vitro translation system they derived from *Drosophila* embryos; but they did not show that any specific individual tRNAs were interchangeable in the heterologous system. To show that phenylalanine-specific tRNA from yeast can be used for protein synthesis by *Drosophila* ribosomes, [^{3}H]phenylalanyl yeast tRNA was added to a *Drosophila* in vitro translation system. We observe that charged yeast tRNA^{Phe} produced polyphenylalanine (1.8 pmol/A_{260} 80S ribosome/30 minutes) at the direction of poly(uridylic acid) and that the addition of puromycin to the system caused the expected reduction of polyphenylalanine synthesis. Since yeast tRNA^{Phe} was active in the *Drosophila* system, was readily available, and could be labeled to high specific activities, it was used as the source of phenylalanine-specific tRNA in our experiments.

Affinity Labeling of Drosophila Ribosomal Proteins with BrAcPhe-tRNA Is Site Specific. Panels A and B of Figure 1 show that the poly(uridylic acid) directed binding of both [^{3}H]Phe-tRNA and BrAc[^{3}H]Phe-tRNA occurs under the conditions of the Nirenberg and Leder (1964) filter binding assay. To show that the binding reactions involved complete 80S ribosomes, sucrose gradients were run on the products of the binding reactions. The gradients confirmed that the binding was taking place with either complete 80S ribosomes, polysomes, or both.

Two types of affinity labeling experiments resulted in the radioactive labeling of a few specific ribosomal proteins. In the first type of reaction, which will be referred to as direct

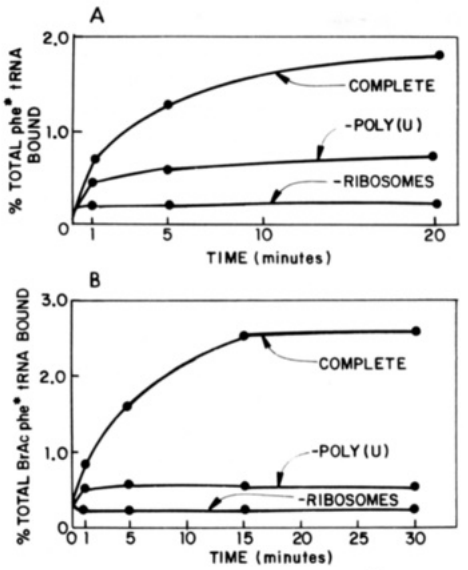

FIGURE 1: Poly(uridylic acid) directed binding of [^{3}H]Phe-tRNA and BrAc[^{3}H]Phe-tRNA to *Drosophila* ribosomes under the conditions of the Nirenberg and Leder (1964) filter binding assay. The graphs show time courses of binding of [^{3}H]Phe-tRNA and BrAc[^{3}H]Phe-tRNA to ribosomes at the direction of poly(uridylic acid) and subsequent retention of that complex on nitrocellulose filters. Each of the points plotted represents the percentage of total counts in the aliquot removed at that time point which bound to the nitrocellulose filter. In panel A, [^{3}H]Phe-tRNA binding was examined. In the complete reaction (upper curve), ribosomes, poly(uridylic acid), and [^{3}H]Phe-tRNA were combined. The curve marked −poly-U is the complete system without poly(uridylic acid). The lowest curve (−ribosomes) is the complete system without ribosomes. Panel B is identical with panel A except BrAc[^{3}H]Phe-tRNA was substituted for [^{3}H]Phe-tRNA and time points were taken over a 30-min period.

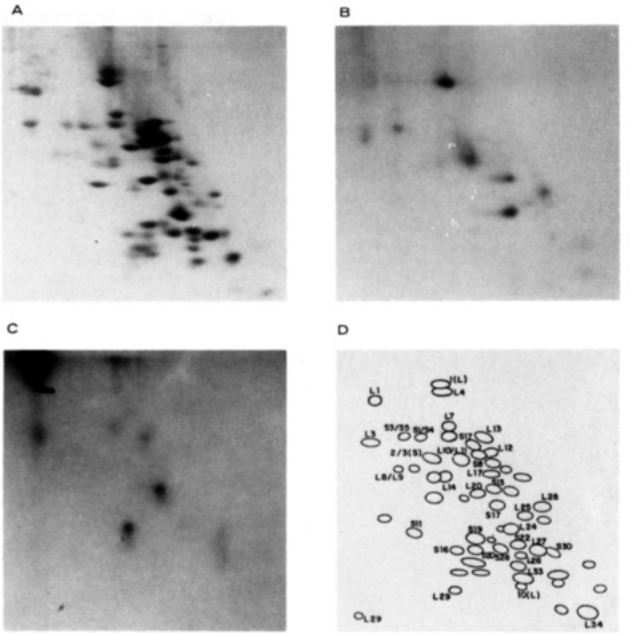

FIGURE 2: Comparison of 2-D gels of ribosomal proteins labeled by the two P site specific affinity labels BrAc[^{3}H]Phe-tRNA and mercurated fragment. Panel A shows the pattern that results when *Drosophila* ribosomal proteins are dissociated, electrophoresed on 2-D acid/SDS gels, and stained with Coomassie blue. Panel B is an autoradiograph of ribosomal proteins affinity labeled by BrAc-[^{3}H]Phe-tRNA and separated on the same gel system. Panel C is an autoradiograph of ribosomal proteins labeled by the mercurated fragment reaction and separated on the acid/SDS gel system. Panel D is a drawing of the Coomassie blue staining pattern generated by *Drosophila* ribosomal proteins separated on the 2-D acid/SDS gel. The numbering system is that of Chooi et al. (1980).

labeling, ribosomal proteins were reacted directly with the affinity label BrAc[^{3}H]Phe-tRNA. In the second type of reaction, referred to here as peptidyltransferase labeling, the action of peptidyltransferase was required to mediate the labeling of ribosomal proteins in the P site. In those reactions cold BrAcPhe-tRNA was preincubated with ribosomes and poly(uridylic acid) before the addition of [^{3}H]puromycin. Ribosomal proteins could only be labeled if BrAcPhe-tRNA bound a ribosomal protein and that complex was subsequently joined to [^{3}H]puromycin by the action of peptidyltransferase. In the direct labeling reactions up to 2% of the available ribosomes were labeled, and in the reaction utilizing [^{3}H]puromycin 3.6% of the ribosomes were labeled.

Identification of the specific proteins that were labeled was made possible by autoradiography of two-dimensional gels. The first dimension gel separated proteins on the basis of charge at pH 5 while the second dimension (SDS) separated proteins on the basis of molecular weight. The gels were Coomassie stained, impregnated with scintillation fluor, dried, and autoradiographed. Ribosomal proteins labeled directly by BrAc[^{3}H]Phe-tRNA could be seen after 2–5 months of autoradiography by using this technique. Autoradiography of the entire 2-D gel in this manner ensured that all areas of the gel were examined for radioactivity. This is important because it is possible that the proteins bound by the affinity label could show altered mobilities on the 2-D gel system. However, as all of the spots on the autoradiograph coincide very closely with stained spots on the dried gel, it seems clear that the small amount of charge and/or molecular weight added to the proteins by the label did not cause a significant change in migration in either dimension. The characteristic pattern of labeling by the reaction is shown in Figure 2.

Substantial losses of radioactivity have been reported for ribosomal proteins run on 2-D gels (Reboud et al., 1981; Grant et al., 1979). Similarly, we noted that approximately 10% of the loaded radioactivity was recovered on the 2-D acid/SDS gels and 3–7-month exposure times were necessary for autoradiography. Because of this and the lower specific activity of the [^{3}H]puromycin it was not practical to identify ribosomal proteins labeled by the peptidyltransferase-catalyzed joining of BrAcPhe-tRNA and [^{3}H]puromycin in the same way. Examination of the 2-D gels obtained from the direct labeling reaction shows that most of the labeled proteins are relatively evenly distributed in the second, SDS, dimension. Because of this it is possible to identify labeled ribosomal proteins solely by their position on a 1-D SDS gel. To correlate proteins in the 2-D gel system with those in the 1-D system, proteins of known molecular weight were run in a slot prepared in the second dimension of a 2-D gel of directly labeled proteins. The same molecular weight standards were also run for size comparison with a directly labeled protein sample on a 1-D SDS gel. By aligning the molecular weight markers on the two types of gels, the identification of the corresponding labeled proteins was possible. Elimination of the acid/urea first dimension increased the recovery of labeled protein and allowed a more rapid assay of ribosomal proteins labeled via peptidyltransferase to [^{3}H]puromycin.

The 1-D SDS gels were subsequently subjected to densitometry (Figure 3). The tracing in Figure 3B shows the intensity of labeling of different ribosomal proteins labeled by the action of peptidyltransferase on BrAcPhe-tRNA and [^{3}H]puromycin. The proteins that are most heavily labeled are L10/L11, L4, S13, and the L3–S3/S5 pair. Another three proteins, L28, S20, and L27, are lightly labeled by both the direct labeling reaction (Figure 3A) and the peptidyltransferase reaction. Although light, the labeling is proportional in both

reactions. Both L24 and L25 are substantially less labeled in the peptidyltransferase reaction than they are by direct labeling with BrAc[^{3}H]Phe-tRNA. Therefore, although they may be near a second site that has an affinity for tRNAs, they are probably not near the P site.

Reexamination of the P Site Proteins Labeled by a Mercurated Fragment of tRNA. We found that the use of a basic first dimension, acidic second dimension 2-D gel system did not allow the complete identification of all protein products affinity labeled by the mercurated fragment in the rat liver ribosome system (Fabijanski & Pellegrini, 1981). Therefore, we reexamined the labeling of the *Drosophila* ribosomal proteins previously separated in the basic, acidic 2-D gel system in an alternate 2-D gel system that we had shown to more completely resolve the labeled products (Fabijanski & Pellegrini, 1981). This acidic first dimension, SDS second dimension gel analysis shows that five rather than one (or possibly two) *Drosophila* ribosomal proteins are labeled by this reagent. In the numbering system of Chooi et al. (1980), these proteins are S13 and S20 (most heavily labeled) and S1/S4, L10/L11, and L26 (less heavily labeled). One other protein, 2/3(S), may also be labeled but to a much lesser degree. Figure 3D is an autoradiograph showing ribosomal proteins labeled by the mercurated fragment.

There Is Little Labeling of Drosophila rRNA by BrAc-[^{3}H]Phe-tRNA. Although the mercurated fragment likely reacts only with sulfhydryls, α-halo carbonyls are reactive toward amines and should have the ability to label rRNA that is located near the P site. Under the labeling conditions used here, the labeling of RNA was 100-fold less than the labeling of proteins. The labeled rRNAs were denatured with glyoxal and run on 1% agarose gels. However, even after 7-month exposures, autoradiographs showed no labeled bands.

Discussion

Drosophila Ribosomal Proteins Were Effectively Labeled Both Directly by BrAc[^{3}H]Phe-tRNA and Indirectly via the Peptidyltransferase Joining of Bound BrAcPhe-tRNA to [^{3}H]Puromycin. The labeling of ribosomal proteins via the peptidyltransferase reaction presents very strong evidence that the labeling is P site specific. For labeling to proceed by this reaction two events must occur: (1) the BrAcPhe moiety of cold BrAcPhe-tRNA must covalently bind a ribosomal protein, and (2) cold BrAcPhe-tRNA must occupy the P site and be catalytically joined to [^{3}H]puromycin in the A site (events referred to as labeling by the peptidyltransferase reaction). Although theoretically these events could occur in any order, BrAcPhe-tRNA was added to the reactions 30 min prior to the addition of [^{3}H]puromycin to allow covalent binding to ribosomal proteins to occur. If the reaction were to go in the reverse order, then BrAcPhe–[^{3}H]puromycin could still react at the P site but it could also be released from the ribosome. In that case, site-specific labeling could no longer be assured; however , it should be noted that neither ([^{3}H]bromoacetyl)trichodermin nor BrAc[^{3}H]Phe-tRNA shows a significant amount of random labeling, indicating that α-halo carbonyls that do not have a specific affinity for ribosomes are not very reactive toward them.

The peptidyltransferase reaction labels five large-subunit and three small-subunit proteins. This is similar to the number of proteins labeled by other α-halo carbonyl affinity labels. Gilly et al. (1985) labeled six *Drosophila* ribosomal proteins with (bromo[^{14}C]acetyl)trichodermin, and in a more analogous experiment, Perez-Gosalbez et al. (1978) labeled nine rat ribosomal proteins with (iodoacetyl)phenylalanyl-tRNA.

Labeling of ribosomal proteins by the addition of BrAc-[^{3}H]Phe-tRNA (referred to as direct labeling) is less specific than peptidyltransferase labeling. As expected, it labels all of the proteins that are labeled in peptidyltransferase reactions plus two additional proteins, L24 and L25. Two other proteins, L3 and S3/S5, are labeled by the peptidyltransferase reaction, but they are labeled with greater intensity by the direct labeling reactions. Of the approximately sixty-five proteins that can be distinguished on 2-D gels, only an additional two are labeled in direct labeling reactions. This indicates that although L24 and L25 are not in the P site, they may be in another site that has an affinity for BrAcPhe-tRNA.

Reexamination of Proteins Labeled by Mercurated Fragment. Drosophila ribosomal proteins can be labeled by a P site specific fragment consisting of the mercurated 3′-C-A-C-C-A end of tRNA^{Leu} charged with the peptide analogue *N*-acetylleucine (Fabijanski & Pellegrini, 1979). We have reexamined the proteins labeled by this rection using a different gel system (acid first dimension, SDS second dimension). This was done because the acid/SDS gel system had improved the resolution of rat liver ribosomal proteins labeled by the same affinity reagent (Fabijanski & Pellegrini, 1981). The revised system allowed five labeled *Drosophila* ribosomal proteins to be resolved rather than the single protein that was found on the basic/acid 2-D gels used previously. In addition, use of the acid/SDS system allowed a direct comparison of proteins labeled by the mercurated fragment to the BrAcPhe-tRNA-labeled proteins presented here and to other affinity labels recently used to probe *Drosophila* ribosomes (Gilly & Pellegrini, 1985; Gilly et al., 1985).

A Comparison of the Proteins Labeled by the Two P Site Affinity Labels—BrAcPhe-tRNA and Mercurated Fragment. Labeling under the conditions of either the peptidyltransferase reaction or the mercurated fragment reaction limits the labeling strictly to the P site. Of the eight proteins labeled by the peptidyltransferase reaction, three proteins (L10/L11, S13, and S20) are also labeled by the mercurated fragment. Since large subunits of ribosomes contain the peptidyltransferase activity, it is reasonable that the three proteins most heavily labeled by the BrAcPhe-tRNA and [^{3}H]puromycin peptidyltransferase reaction are large-subunit proteins. The mercurated fragment reacts with proteins by the attachment of one or more of the mercurated cytosines to the cysteine residues of proteins. These reactive moieties extend down the tRNA arm, away from the catalytic site, toward the site of codon/anticodon alignment on the small subunit. The three proteins most heavily labeled by the mercurated fragment are small-subunit proteins. The ribosomal proteins labeled by both of these P site analogues are likely to be at or near the 60S/40S interface.

Model for the Peptidyl-tRNA Binding Site on Drosophila Ribosomes. The densitometry of Figure 3B shows three ribosomal proteins to be most heavily labeled by BrAcPhe-tRNA when it is constrained to the P site by the conditions of the peptidyltransferase reaction with [^{3}H]puromycin. In our model of the *Drosophila* peptidyltransferase region those three protens, L4, L10/L11, and S13, are placed nearest the reactive, bromoacetyl portion of the label. As L10/L11 and S13 were also labeled by the fragment reaction, they were positioned near the 60S/40S interface. L3 and S3/S5 were labeled by direct labeling with BrAc[^{3}H]Phe-tRNA, and one or both were less labeled by the reaction requiring peptidyltransferase to join the reagent to [^{3}H]puromycin. Because these two proteins had previously been affinity labeled by (bromoacetyl)trichodermin (Gilly & Pellegrini, 1985) and trichodermin inhibits the binding of both A and P site ana-

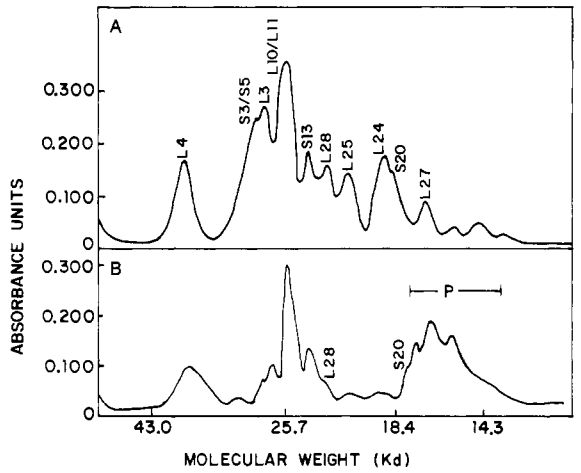

FIGURE 3: Densitometry of the autoradiographs of affinity-labeled ribosomal proteins separated on 1-D SDS gels. Proteins labeled directly by the reaction of BrAc[^{3}H]Phe-tRNA (panel A) and proteins labeled by the action of peptidyltransferase on BrAcPhe-tRNA and puromycin (panel B) were separated on 1-D SDS gels, autoradiographed, and evaluated by densitiometry. Peaks were identified by correlating labeled proteins on the 1-D gels to direct labeled proteins on a 2-D gel (see text). Panel B contains a broad low molecular weight peak labeled P on the densitometry tracing. The peak probably results from the puromycin-induced termination of nascent peptides which were retained by the sucrose-washed ribosomes.

logues, the proteins are placed between the A and P site. Although the densitometry of the autoradiograph of the gel resulting from the labeling reaction utilizing BrAcPhe-tRNA and [^{3}H]puromycin (the peptidyltransferase reaction) indicates that other identifiable ribosomal proteins may be labeled, the amount of labeling was slight. Therefore, except for S20 which was labeled heavily by the fragment reaction, those proteins do not appear on the model.

The reexamination of the proteins labeled by the 3′-terminal pentanucleotide mercurated fragment of peptidyl-tRNA shows that two small-subunit proteins are the most heavily labeled, S13 and S20. We therefore place these proteins in the region that most overlaps two of the three cytosine residues in the fragment, since it is from the C-5 position of these bases that the attached mercury atoms cross-link to the ribosomal proteins. The less heavily labeled proteins L10/L11, L26, and S1/S4 are then placed as overlapping only one of the cytosine residues. It is clear from these data that although the peptidyltransferase catalytic site is known to reside in the large subunit, the binding site for peptidyl-tRNA extends onto the small subunit.

In addition, we have previously shown that a small-subunit protein 2/3(S) is labeled by an affinity analogue of the ribosome-binding antibiotic trichodermin (Gilly et al., 1985). This protein is also labeled, though only to a very small extent, by the mercurated fragment. Therefore, we have placed the trichodermin binding site proteins in the model in Figure 4. The small overlap of the trichodermin binding site and the P site is expected since trichodermin is known to partially inhibit the binding of P site substrates [reveiwed by Gale et al. (1981)].

Finally, it is of interest to note that the overall pattern of labeled proteins in the P site of *Drosophila* ribosomes, particularly with respect to their electrophoretic characteristics, is remarkably similar to that found in the *E. coli* ribosome (Whittman, 1983). As cited above, the most striking correspondence is between the proteins labeled by BrAcPhe-tRNA in *E. coli* ribosomes L2 and L27 (Oen et al., 1973) and in *Drosophila* ribosomes L4 and L23. The exact nature and

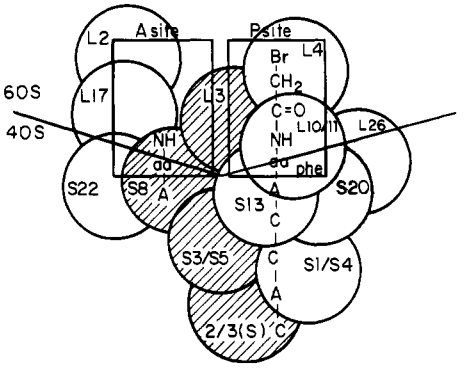

FIGURE 4: Model for the placement of ribosomal proteins in the *Drosophila* P site. Proteins are numbered according to Chooi et al. (1980). P site proteins were labeled by either BrAcPhe-tRNA or mercurated fragment or both. A site proteins were labeled by photoincorporated puromycin (Gilly & Pellegrini, 1985). Each protein was placed according to which label(s) bound to it, the intensity of the labeling, and the origin of the protein (60S or 40S).

reason for the apparent conservation of charge to mass ratio in these P site proteins has yet to be explored.

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Registry No. Ribosome peptidyltransferase, 9059-29-4.

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Reductive Activation of Mitomycin C†

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ABSTRACT: Mitomycin C, an antitumor antibiotic, is known to require reductive activation in order to function as an alkylating agent. In this work reduction has been carried out by using radiolytically produced formate radicals that reduce mitomycin C to its semiquinone in a clean rapid one-electron reaction. The ultimate products of the reduction are *cis*- and *trans*-2,7-diamino-1-hydroxymitosene (B_1 and B_2) and 2,7-diaminomitosene (C). The yields of these compounds were found to be the same when the rate of reduction was varied by 11 orders of magnitude. At pH 7, one mitosene molecule is formed for every two formate radicals, while at pH 9.1, about eight mitosene molecules are formed per formate radical. The ratio of (B_1 + B_2)/C is <0.4 at pH 5.7, 1.0 at pH 7, and >3.5 at pH 9.1. Observations have been made of changes in optical absorption due to the formation of the semiquinone and hydroquinone of both mitomycin C itself and 2,7-diamino-1-hydroxymitosene (B). The direct conversion of the semiquinone form of mitomycin C into the semiquinone of B proceeds slowly, if at all. The semiquinone form of B will rapidly reduce mitomycin C ($k = 7.2 \times 10^8$ M^{-1} s^{-1}). The hydroquinone of mitomycin C undergoes changes resulting in the formation of B and C. The yields of B and C depend on pH. The kinetic data fit a scheme in which the mitomycin C hydroquinone has a pK_a of 5.1, with its protonated form changing to C by a concerted rearrangement with $k = 1.2$ s^{-1}, its deprotonated form changing to the hydroquinone of B with $k = 0.015$ s^{-1}, and the hydroquinone of B reducing mitomycin C with $k = 5$ M^{-1} s^{-1}. The 2,7-diamino-1-hydroxymitosenes are thus formed in a chain reaction with the formation of 2,7-diaminomitosene as a chain termination step. It is suggested that increased cell toxicity at lower pH could be due to the precursor of 2,7-diaminomitosene being an effective alkylating agent.

Mitomycin C, the clinically important antibiotic antitumor drug isolated from *Streptomyces caespitous*, is believed to function by inhibiting DNA replication. This inhibition is brought about by covalent binding to cellular DNA, followed by cross-linking between the complementary strands, thus preventing replication (Tomasz et al., 1985; Iyer & Szybalski, 1963, 1964). It is generally accepted that mitomycin C must be activated by reduction before binding can occur. Activation can be brought about in a variety of systems, e.g., in rat liver microsomes (Tomasz & Lipman, 1981), in hypoxic tumor cells in vivo and in vitro (Keyes et al., 1985), by purified reducing enzymes (Pan et al., 1984; Peterson & Fisher, 1986), and by chemical reduction (Tomasz et al., 1986; Tomasz & Lipman, 1981; Lown et al., 1976).

Recently, the structure of the mitomycin C–nucleotide adduct has been proposed to be formed by covalent linkages at the N^2, N^7, or O^6 positions of guanine and also the N^6 of adenine (Tomasz et al., 1985, 1986, 1987; Hashimoto et al., 1984; Zein & Kohn, 1987; Pan et al., 1986). Some of these adducts has been isolated and characterized. The most recent work (Tomasz et al., 1987) used proton magnetic resonance, differential Fourier transform infrared spectroscopy, circular dichroism, mass spectrometry, and computer-constructed molecular modeling to show conclusively that the major covalent link occurs at the N^2 position of guanine. Activation results in both mono- and bifunctional adducts, i.e., N^2-guanosyl-1-mitosene and N^2,$N^{2'}$-bisguanosyl-1,10-mitosene. Reduction by sodium dithionite leads to almost exclusive bifunctional alkylation, whereas use of the reducing enzyme systems or H_2/PtO_2 in vitro results in >90% monofunctionality. The same bisadduct was formed when DNA was isolated from the liver of rats injected with mitomycin C. Computer simulations have shown that the drug molecule fits neatly into the minor groove for B-DNA without appreciable disruption of the DNA structure.

The increasing clinical importance of the drug and its analogues has brought about a recent upsurge in the study of the mechanisms of bioactivation of mitomycin C as well as other antitumor quinone drugs [e.g., Pan et al. (1984), Peterson and Fisher (1986), Bachur et al. (1978), Land et al. (1985), and Butler and Hoey (1987)]. It was originally believed that the activation consists of a two-electron reduction of mitomycin C to the hydroquinone that results in the molecular rearrangements required to produce reactive centers at carbon 1 and carbon 10. Using reduced nicotinamide adenine dinucleotide phosphate (NADPH) and Old Yellow enzyme to initiate the reduction of mitomycin C, a recent study (Peterson & Fisher, 1986) has also put forward a mechanism of activation involving the hydroquinone route. However, another

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