Affinity Labeling the Ribosome with Eukaryotic-Specific Antibiotics: (Bromoacetyl)trichodermin†

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ABSTRACT: Trichodermin, a eukaryotic-specific antibiotic, inhibits protein synthesis in *Drosophila* cells. We have synthesized a ^{14}C-labeled bromoacetyl derivative of trichodermin that binds to *Drosophila* 80S ribosomes and once bound reacts covalently with ribosomal proteins. It does not react with rRNA. Three large-subunit proteins (L1, L3, and L24) and three small-subunit proteins (S3/S5, 2/3S, and S8) are labeled by [^{14}C](bromoacetyl)trichodermin. Reaction with each of these proteins can be competed by an excess of unmodified trichodermin, indicating that the labeling has occurred from the native binding site of the parent drug. One of the (bromoacetyl)trichodermin-labeled proteins (S8) is also labeled by photoactivated puromycin in the A site. A second protein (S3/S5) is found to be labeled by a P-site affinity reagent. The results suggest that the trichodermin binding site spans both the small and large subunits and portions of both the A and P sites. These data combined with previous studies on the A and P sites of *Drosophila* ribosomes have allowed us to construct a model of the protein locations in this important active site.

Antibiotics that inhibit protein synthesis have often and effectively been used to investigate the details of this essential biological process. The great majority of antibiotics that interfere with protein synthesis do so by inhibiting the function of ribosomes. Many antibiotics show selective toxicity in that they are active against bacteria but not against eukaryotes or vice versa. Frequently, the particular step or steps in protein synthesis effected by an antibiotic have been identified, but the nature of the molecular interaction of antibiotic and ribosome is rarely understood [reviewed by Gale et al. (1981)]. Furthermore, although a small number of specific ribosomal components involved in antibiotic binding have been characterized in bacterial and organelle ribosomes, even fewer have been identified in eukaryotic ribosomes (Gale et al., 1981). The only cytoplasmic ribosomal components characterized as responsible for eukaryotic-specific antibiotic function have been ribosomal proteins from antibiotic-resistant mutants. In yeast and mammalian tissue culture cells, electrophoretically variant ribosomal proteins have been observed in the mutant cells [for example, Boersma et al. (1979) and Stocklein & Piepersberg (1980)]. Clearly, such proteins are responsible for maintaining the ribosomal antibiotic binding site. However, it has not been determined whether the altered proteins are at the binding site or affect it allosterically.

In order to identify the ribosomal components actually present at the binding site of a eukaryotic-specific antibiotic, we have utilized the principles of antibiotic affinity labeling. Trichodermin is an antibiotic selectively toxic to eukaryotic ribosomes. Its action is at the peptidyltransferase step of protein synthesis (Carrasco et al., 1973). Since trichodermin partially blocks both A- and P-site substrates, its binding site is not well defined nor is its precise mode of action [reviewed by Gale et al. (1981)]. Therefore, we prepared a chemically modified trichodermin that should be able to covalently react with nucleophilic ribosomal components. Here we present evidence that this modified trichodermin, (bromoacetyl)trichodermin, reacts covalently and site specifically with a small number of ribosomal proteins in *Drosophila* ribosomes. Since trichodermin is implicated as acting at or near the peptidyltransferase center, these affinity-labeled proteins may be implicated as being part of the active site for that function. A comparison with other active site proteins and RNAs can provide a more detailed picture of the molecular architecture of both ribosomal active sites and antibiotic binding sites.

Materials and Methods

Trichodermin. Trichodermin was obtained from W. O. Godfredsen (Godfredsen & Vangedal, 1965): mp 45–46 °C; ^{13}C NMR ($CDCl_3$) δ 0.5.79 (q), 15.98 (q), 21.13 (q), 23.24 (q), 24.45 (t), 27.97 (t), 36.65 (t), 40.41 (s), 47.84 (t), 48.91 (s), 65.50 (s), 70.49 (d), 75.06 (d), 79.11 (d), 118.59 (d), 140.17 (s), and 170.92 (s); mass spectrum, m/z 292.

Trichodermol. Ethyl acetate and pyridine were distilled under nitrogen from BaO. All reaction vessels were dried in an oven, and all reactions were performed under a dry nitrogen atmosphere. A 5-mL round-bottom flask was equipped with a magnetic stir bar and reflux condenser to which 1 mL of ethanol, 3 mL of 2 M aqueous NaOH, and 100 mg (0.34 mmol) of trichodermin were added. The solution was heated to reflux and allowed to stir for 1 h. This was allowed to cool to room temperature and concentrated under reduced pressure and then taken up in 5 mL of diethyl ether and extracted with 3 mL of water. The organic layer was removed and dried over anhydrous $MgSO_4$, and the aqueous layer was extracted 3 times with 5-mL portions of ether. The organic extracts were combined and concentrated, affording a white powder. The powder was recrystallized from ether by layering pentane or hexane over the ether solution and placing it at 4 °C overnight. This procedure produced long colorless needles that melted at 117.5–118 °C (70% yield). This material, used in subsequent experiments, was dried under vacuum and stored in a dessicator under nitrogen. ^{13}C NMR ($CDCl_3$) showed the following: δ 6.2, 15.79, 23.22, 24.39, 27.97, 39.76, 40.1, 47.54, 49.09, 65.72, 70.23, 73.97, 78.71, 118.68, and 140.11.

(Bromoacetyl)trichodermin. To a dry 5-mL round-bottom flask equipped with stir bar was added 165 mg (0.80 mmol)

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of dicyclohexylcarbodiimide, 203 mg (1.5 mmol) of bromoacetic acid, and 4 mL of dry ethyl acetate under a dry nitrogen atmosphere. A white precipitate was formed, and the solution was allowed to stir for 1 h at room temperature under nitrogen. To this solution of bromoacetic anhydride was added 50 mg (0.20 mmol) of trichodermol and 70 μL of dry pyridine. The yellow solution was allowed to stir at 25 °C for 24 h. The reaction mixture was taken up in 5 mL of ether, and the solution was filtered. The filtrate was washed 5 times with 8-mL portions of water and then 5 times with 8-mL portions of 1% aqueous $NaHCO_3$. The ether solution was dried ($MgSO_4$) and concentrated. The resulting oil sometimes contains solids that must be filtered out. The oil was dried under reduced pressure and stored neat at −20 °C (45% yield): ^{13}C NMR ($CDCl_3$) δ 5.82, 15.99, 23.24, 24.36, 26.01, 27.93, 36.44, 40.45, 47.9, 49.3, 65.39, 70.51, 76.83, 77.28, 78.96, 118.41, and 140.35; mass spectrum, m/z 369.9, 371.9.

[^{14}C](*Bromoacetyl*)*trichodermin*. The supplier's flask (Amersham) containing the labeled acid was used as a reaction vessel. Three vials containing 250 μCi (51.2 mCi/mmol) of bromoacetic acid were broken open and washed consecutively with the same 50-μL portion of dry ethyl acetate. This was repeated 3 times, and all organics were combined and pooled in the final flask equipped with a micro stir bar. To this was added 31 μL of a dry solution of dicyclohexylcarbodiimide in ethyl acetate (59 mg/mL), and the solution was allowed to stir 1 h. The anhydride precipitate formed. After 1 h, 23 μL of a trichodermol solution in ethyl acetate (22.3 mg/mL) was added, and the solution was stirred overnight at 25 °C. The solution was filtered after the addition of 1 mL of ether and extracted 5 times with 5-mL portions of water followed by 5 washings with 1% aqueous $NaHCO_3$. The ether solution was dried ($MgSO_4$) and concentrated. The yield based on recovered radiolabel was 10%.

Protein Synthesis in the Presence of Trichodermin. Exponentially growing Schneider's line 2 cells were aliquoted into tubes (1×10^6 cells/tube) and incubated with 0.5 mL of fresh modified Schneider's media for 30 min. Then both trichodermin (0–50 μM) and 20 μL of [^{35}S]methionine (1000 Ci/mmol, 1 mCi/mL, New England Nuclear) were added simultaneously. At various times, aliquots of the reactions were stopped by the addition of 200 μL of cold Hanks buffered saline, and the cells were centrifuged for 5 s in a microfuge. The cell pellets were resuspended in 200 μL of water and mixed with 200 μL of 100% trichloroacetic acid (TCA).[1] The solution was heated to 95 °C for 15 min to hydrolyze charged tRNAs. The precipitates were collected on glass-fiber filters and counted for radioactivity.

Ribosome Isolation and Labeling. Ribosomes were isolated from dechorionated 0–24-h *Drosophila melanogaster* embryos as described elsewhere (Gilly & Pellegrini, 1985). The affinity-labeling conditions are identical with those used for puromycin affinity labeling (Gilly & Pellegrini, 1985) except that the trichodermin or (bromoacetyl)trichodermin (5 μM) was added to the ribosomes as a solution in dimethyl sulfoxide (Me_2SO). In every case, the final reaction mixture was 10% Me_2SO. Ribosomes were again added last. Unless otherwise specified, the reaction was allowed to proceed for 1 h at 23 °C, which is an optimal temperature for *D. melanogaster* growth. Following the affinity-labeling reaction, ribosomes were ethanol precipitated, washed to remove unbound label, and then treated with Mg^{2+} and acetic acid to separate the

[1] Abbreviations: rRNA, ribosomal RNA; Me_2SO, dimethyl sulfoxide; TCA, trichloroacetic acid; 2D, two dimensional; SDS, sodium dodecyl sulfate.

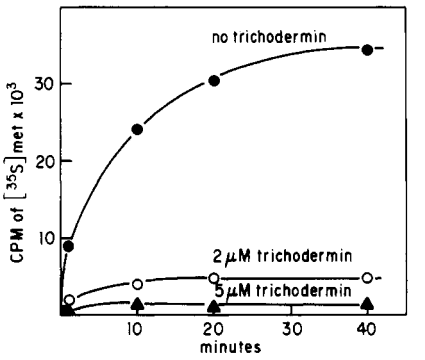

FIGURE 1: Sensitivity of protein synthesis to trichodermin in *Drosophila* cells. Schneider's line 2 cells were incubated with either 5 (△), 2 (○), or 0 μM (●) trichodermin for the times indicated. Total TCA-precipitable cpm from [^{35}S]methionine incorporation into protein was measured.

soluble protein and insoluble RNA components (Sherton & Wool, 1974). After extensive dialysis against 10% acetic acid/1 mM dithiothreitol, both the protein and redissolved RNA fractions were assayed for remaining radioactivity (Gilly & Pellegrini, 1985).

Gel Electrophoresis and Analysis of Labeled Proteins. Ribosomal proteins were resolved by two-dimensional (2D) gel electrophoresis in the system of Mets & Bogorad (1974) as modified by Warner & Gorenstein (1977). Separation in the first dimension is based on charge at pH 5, and the second dimension is an SDS sizing gel. Coomassie Blue stained gels were prepared for autoradiography by impregnation with Autofluor (National Diagnostic Products) according to the manufacturer's instructions. Dried gels were exposed to X-ray film at −70 °C. The protein numbering system is according to Chooi et al. (1980); see Gilly & Pellegrini (1985) for details.

RESULTS

Design and Synthesis of a Trichodermin Affinity Label. In order to determine the feasibility of constructing a modified trichodermin to use as an affinity label for *Drosophila* ribosomes, it was first necessary to test the response of these ribosomes to the unmodified antibiotic. Treatment of *Drosophila* tissue culture cells (Schneider's line 2) with even 2 μM trichodermin inhibited the uptake of [^{35}S]methionine into acid-precipitable protein molecules by 85%. This occurred less than 15 min after addition of the drug to the cell culture medium. Complete inhibition of protein synthesis was observed at a concentration of 5 μM trichodermin as shown in Figure 1. Therefore, this antibiotic acts to decrease protein synthesis in *Drosophila* as it does in numerous other eukaryotes [reviewed by Gale et al. (1981)].

Two facts led us to synthesize the bromoacetyl derivative of trichodermin in an attempt to use this molecule as a ribosome affinity label. First, the site of action of trichodermin has been shown to be on the ribosome itself rather than on one or more of the various protein factors involved in protein synthesis (Carrasco et al., 1973; Grant et al., 1976). Second, trichodermin contains a C-4 acetyl group that could be modified to an α-halo carbonyl. Such compounds have been successfully used to affinity label a number of ribosome active sites [reviewed by Cooperman (1980)]. This is probably due to the nucleophilic nature of a number of amino acid and nucleic acid moieties, particularly the sulfhydryls of cysteine. Therefore, we synthesized [^{14}C](bromoacetyl)trichodermin from [^{14}C]bromoacetic acid and trichodermol via a condensation reaction with dicyclohexylcarbodiimide. The synthesis of [^{14}C](bromoacetyl)trichodermin is diagrammed in Figure 2.

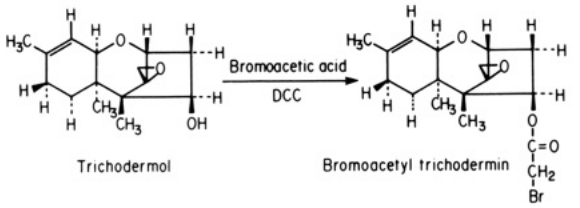

FIGURE 2: Synthesis of (bromoacetyl)trichodermin. Trichodermol was reacted with [^{14}C]bromoacetic acid in the presence of dicyclohexylcarbodiimide (DCC) to give [^{14}C](bromoacetyl)trichodermin.

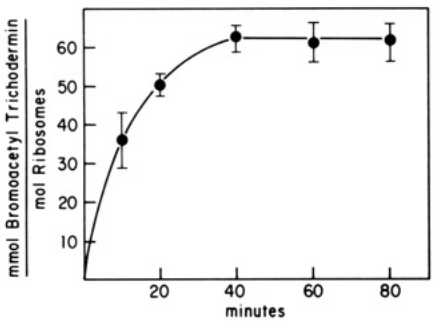

FIGURE 3: Time course of the reaction of (bromoacetyl)trichodermin with 80S ribosomes. [^{14}C](Bromoacetyl)trichodermin (5 μM) was incubated with *Drosophila* 80S ribosomes for the times indicated. The total radioactivity associated with isolated ribosomal proteins was then measured. The error bars represent uncertainty in the average of three experiments.

(Bromoacetyl)trichodermin Binds Covalently to Drosophila Ribosomes. In order to determine whether [^{14}C](bromoacetyl)trichodermin would covalently bind to any ribosomal components, we added this molecule to *Drosophila* ribosomes and incubated the mixture at 23 °C for up to 80 min. This temperature was chosen since it is the same as that at which our *Drosophila* stocks are kept and is nearly optimal for the maintenance of these insects. Following incubation with 5 μM [^{14}C](bromoacetyl)trichodermin, the ribosomes were ethanol precipitated and separated into acetic acid soluble (protein) and insoluble (RNA) fractions. Radioactivity was found exclusively in the protein fraction. The presence of radioactivity still bound to either RNA or protein following this treatment (see Materials and Methods) is a strong indication for covalent binding. Separation of ^{14}C-labeled proteins on SDS gels, as described below, is further proof of a covalent linkage with the affinity label. The time course of the (bromoacetyl)trichodermin reaction is shown in Figure 3. It is complete at 60 min, and all future reactions were run to this extent. The efficiency of labeling is over 6% (Figure 3). No labeling of ribosomal components was observed with the nonbrominated derivative [^{14}C]acetyltrichodermin under identical conditions.

Attachment of [^{14}C](Bromoacetyl)trichodermin to Ribosomal Proteins Is Site Specific. In order to identify which of the ribosomal proteins had become covalently attached to the modified trichodermin affinity label, we separated these proteins by 2D gel electrophoresis. As shown in Figure 4, three large-subunit proteins (L1, L3, and L24) and three small-subunit proteins (S3/S5, 2/3S, and S8) show labeling. In addition, three proteins found in crude, i.e., non salt washed, ribosome preparations were also labeled (C2, C5, and C6).

The fact that only a few of the 75 ribosomal proteins were labeled by (bromoacetyl)trichodermin is evidence for the specificity of this reaction. However, in order to demonstrate that the reaction with these proteins occurs from the trichodermin binding site, we incubated ribosomes with 5 μM affinity label and increasing amounts of nonradioactive trichodermin.

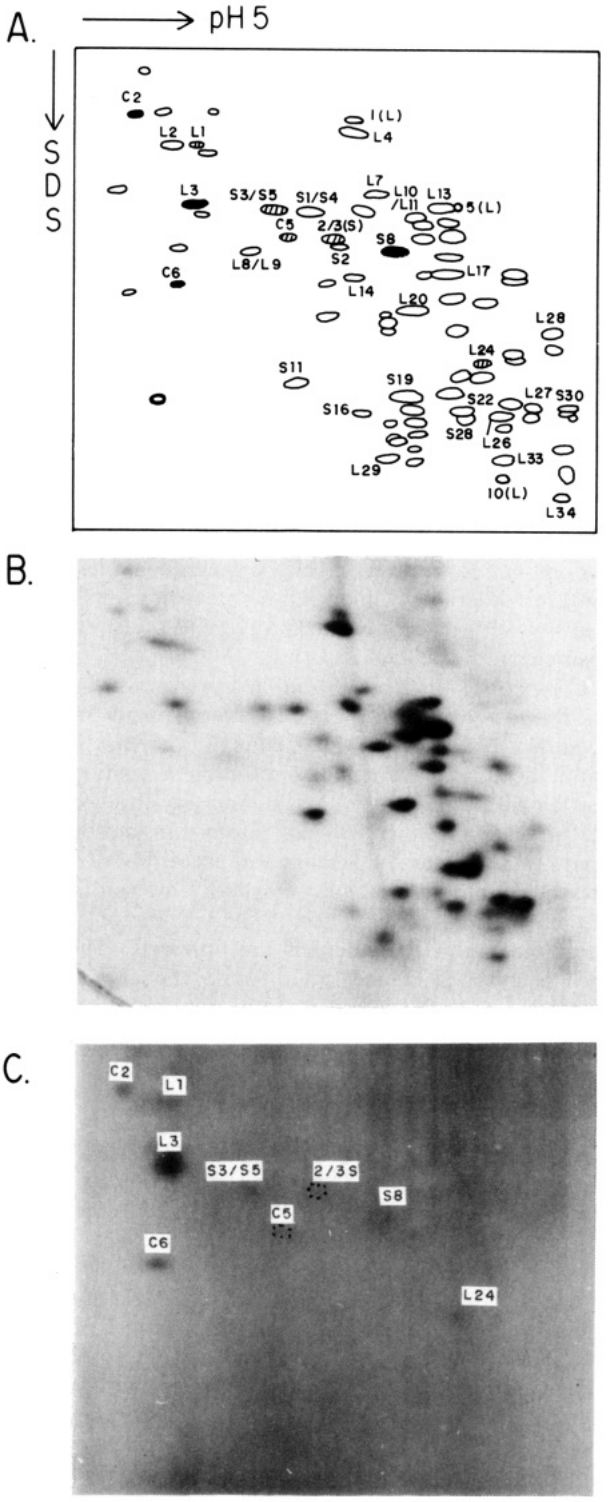

FIGURE 4: Labeling pattern of (bromoacetyl)trichodermin-reacted ribosomal proteins. (A) Diagram of a 2D gel of 80S-labeled proteins. Filled-in spots were the most intensely labeled proteins in three experiments. Cross-hatched spots represent less intensely labeled proteins. The proteins are designated as either small- (S) or large- (L) subunit proteins or from crude, non salt washed ribosomes (C). (B) Coomassie Blue stained 2D gel of *Drosophila* 80S proteins labeled with [^{14}C](bromoacetyl)trichodermin. (C) Autoradiograph of [^{14}C]-(bromoacetyl)trichodermin-labeled 80S proteins.

As seen in Figure 5, trichodermin is a competitive inhibitor of the labeling of total ribosomal proteins by (bromoacetyl)-trichodermin. This suggests that the affinity-labeling reaction is taking place at or near the trichodermin binding site.

The competitive inhibition by trichodermin of (bromoacetyl)trichodermin labeling of ribosomal proteins was also

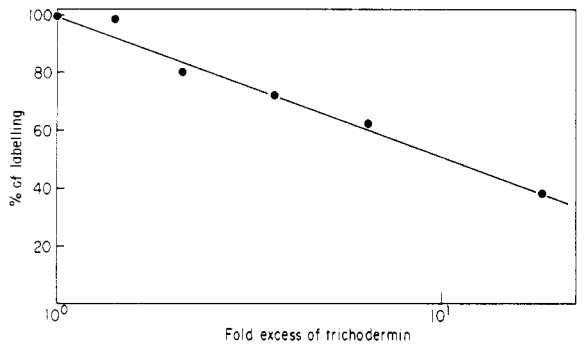

FIGURE 5: Competition of the affinity-labeling reaction with trichodermin. [^{14}C](Bromoacetyl)trichodermin (5 μM) was incubated with 80S ribosomes in the presence of increasing amounts of unmodified trichodermin. The total radioactivity remaining associated with the isolated ribosomal protein fraction was measured.

observed by 2D gel analysis. In the presence of either 50 or 500 μM trichodermin and 5 μM [^{14}C](bromoacetyl)trichodermin, no radioactively labeled proteins could be observed by autoradiography while control autoradiograms showed a clearly visible pattern of labeled proteins under similar exposure conditions.

Competition of Other Peptidyltransferase Antibiotics for the Trichodermin Binding Site. In order to obtain information about the effects that other peptidyltransferase inhibitory antibiotics may have on the trichodermin binding site, we reacted ribosomes with 5 μM (bromoacetyl)trichodermin in the presence of either 500 μM puromycin, anisomycin, or emetine. The pattern of labeling was not visibly altered in the presence of puromycin or anisomycin. However, in the case of emetine, an intensification of the labeling of one of the autoradiographic protein spots was observed. This protein 2/3S is shown in Figure 4. Otherwise, the labeling pattern was the same as the control performed in the absence of any competing drug.

Discussion

(Bromoacetyl)trichodermin Reacts with Ribosomes in a Covalent Site Specific Manner. In the presence of dicyclohexylcarbodiimide, [^{14}C]bromoacetic acid reacts with the alcohol, trichodermol, to yield a C-4-substituted acetate, [^{14}C](bromoacetyl)trichodermin (Figure 2). This molecule, which contains an α-halo carbonyl, should be available for nucleophilic substitution reactions with a number of ribosomal components. When bound to 80S *D. melanogaster* ribosomes [^{14}C](bromoacetyl)trichodermin but not [^{14}C]trichodermin (the nonbrominated compound) coisolates with ribosomal proteins. Following both 67% acetic acid separation of ribosomal proteins and rRNA and SDS gel electrophoresis of the proteins, the ^{14}C label remains bound to these proteins. This is evidence for a covalent reaction of (bromoacetyl)trichodermin with ribosomal proteins.

Only a small subset of the 75 proteins reacts with this affinity label (Figure 4). These results suggest a site-specific reaction for (bromoacetyl)trichodermin. In order to prove this more directly, we performed the affinity-labeling reaction in the presence of increasing amounts of nonradioactive trichodermin. As shown in Figure 5, trichodermin successfully competes for the same binding site as (bromoacetyl)trichodermin, thereby eliminating the affinity label's ability to react with ribosomal proteins. Two-dimensional gel analysis of the ribosomal proteins labeled by (bromoacetyl)trichodermin in the presence of a 10-fold excess of trichodermin shows that the reaction with all of the proteins is reduced. That is, none of the proteins escapes labeling inhibition by unmodified trichodermin, indicating that the affinity labeling of each of the reactive proteins by (bromoacetyl)trichodermin is taking place from the native binding site of the parent antibiotic.

Trichodermin is known to bind to the separated 60S subunit as well as 80S ribosomes of yeast (Barbacid & Vazquez, 1974), and trichodermin-resistant yeast strains have a mutant 60S protein (Grant et al., 1976). Yet, we labeled small- as well as large-subunit proteins with (bromoacetyl)trichodermin. These data can easily be reconciled, however, if the proximity of the peptidyltransferase to the subunit interface and the fact that the A and P sites of tRNA binding span both subunits are considered [reviewed by Wittmann (1983)]. Our results then suggest that the trichodermin binding site also occupies a region at the subunit interface.

The fact that six ribosomal proteins react with (bromoacetyl)trichodermin also suggests that the binding site for this antibiotic is relatively large and/or complex. Alternatively, trichodermin may occupy more than one ribosomal site. The α-bromo carbonyl group of the affinity label is a fairly reactive group toward nucleophiles, which could also account for the complex labeling pattern. In addition, three crude ribosomal proteins (removed from 80S ribosomes by a 0.1 M KCl wash) are labeled by (bromoacetyl)trichodermin. Although we do not know the precise nature of these proteins, they may be involved in protein synthesis as auxiliary factors. Since their labeling is competed by excess trichodermin, these crude proteins are certainly implicated as being part of the trichodermin binding site(s).

Finally, it is interesting to note that (bromoacetyl)trichodermin does not react with rRNA, although rRNA has been shown to be labeled by both A- and P-site affinity labels including α-halo carbonyl compounds [reviewed by Cooperman (1980)]. Our own studies show a small amount of labeling of *Drosophila* rRNA with the A-site reagent puromycin (Gilly & Pellegrini, 1985). The data imply that trichodermin does not precisely overlap the active binding sites of A- or P-site substrates.

Interaction of Trichodermin with Other Peptidyltransferase Antibiotics. Several general and eukaryotic-specific antibiotics other than trichodermin have been suggested to act at the peptide bond forming step of protein synthesis. Among these are anisomycin, puromycin, and emetine. It is, therefore, possible that any of these drugs might be expected to perturb the binding of trichodermin. In this study we observed that a 100-fold excess of anisomycin neither significantly reduced nor changed the pattern of labeling of specific ribosomal proteins by (bromoacetyl)trichodermin. However, even a 10-fold excess of trichodermin produced a large reduction in labeling. These data may indicate that (bromoacetyl)trichodermin and trichodermin have largely overlapping binding sites while the (bromoacetyl)trichodermin and anisomycin sites are nonoverlapping. More likely, they suggest that trichodermin and anisomycin have nonidentical ribosomal binding sites.

It is known that anisomycin can inhibit trichodermin binding to 80S ribosomes in human and yeast cells (Barbacid & Vazquez, 1974) and that yeast strains isolated as resistant to trichodermin show an equivalent insensitivity to anisomycin (Jimenez et al., 1975). However, anisomycin, unlike trichodermin, binds normally to the ribosomes from the mutant strains (Jimenez & Vazquez, 1975). These data agree with our conclusion and suggest that these two antibiotics do not bind to identical ribosomal sites.

Puromycin clearly occupies the A site of both prokaryotic and eukaryotic ribosomes since it can participate in peptide bond formation with a P-site substrate. Trichodermin as well

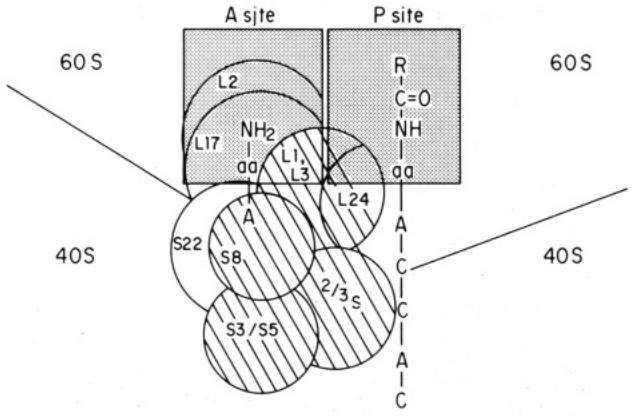

FIGURE 6: Model for protein placement near the *Drosophila* peptidyltransferase center. Proteins are numbered according to Chooi et al. (1980). Proteins at the A site were photoaffinity labeled by puromycin (Gilly & Pellegrini, 1985). The P-site protein 2/3S was affinity labeled via covalent linkage between a mercury atom(s) linked to the three cytosines found in the 3′-pentanucleotide fragment of *N*-acetylleucyl-tRNA (Fabijanski & Pellegrini, 1979; unpublished results) and placed in the P-site neighborhood. As a result of the overlap of the trichodermin binding site with that of the A and P sites, all of the proteins in this site were placed near the peptidyltransferase center as indicated by the hatched area. Where two proteins are indicated for a single circle, it is envisioned that one protein lies above and one below the plane of the diagram.

as other trichothecene toxins can prevent the reaction of puromycin with (acetylleucyl)pentanucleotide fragment. Yet trichodermin inhibits the binding of both A- and P-site oligonucleotide substrates (Carrasco et al., 1973). (Bromoacetyl)trichodermin labeling of ribosomes was not altered by the presence of a 100-fold excess concentration of puromycin. This may be due to the low binding affinity of puromycin although its K_D (200 μM) was exceeded by the concentration used (500 μM). However, it may also suggest that (bromoacetyl)trichodermin and puromycin, like trichodermin and anisomycin, have nonidentical binding sites. It is important to note here that one of the labeled small-subunit proteins (S8) seen in the reaction of (bromoacetyl)trichodermin is also seen to be labeled to a small extent during the photoincorporation of puromycin into *Drosophila* ribosomes (Gilly & Pellegrini, 1985). These data then indicate close if not partially overlapping binding sites for these antibiotics.

Emetine, another eukaryotic-specific protein synthesis inhibitor, was first thought to act at the peptidyltransferase center but now appears to interact with factor EF-2 and thus inhibits peptide bond formation [reviewed by Gale et al. (1981)]. In addition, this drug binds most strongly to the 40S rather than 60S subunit unlike the other antibiotics discussed above. Interestingly, emetine shows a small but significant effect on the labeling of ribosomal proteins by (bromoacetyl)trichodermin. One of the small-subunit proteins (2/3S) exhibits enhanced labeling in the presence of emetine. Since emetine has not been shown to inhibit the binding of trichodermin, it is likely that emetine allosterically effects the trichodermin binding site. In total, these results emphasize the fact that peptidyltransferase-inhibiting antibiotics act at or near the interface of the small and large subunits.

Finally, it is interesting to note that another of the small-subunit proteins labeled by (bromoacetyl)trichodermin (S3/S5) is the same as that labeled by a P site specific affinity reagent, a mercurated 3′-terminal pentanucleotide fragment of *N*-acetylleucyl-tRNA (Fabijanski & Pellegrini, 1979; unpublished results). This again places the trichodermin binding site near the peptidyltransferase center possibly partially overlapping both the A and P sites.

On the basis of these data we have constructed a model to show some of the protein positions within and near the peptidyltransferase center of *Drosophila* ribosomes. It is shown in Figure 6. Puromycin-labeled proteins (L2, L17, S8 and S22) are drawn in the A site. The proteins that were labeled by puromycin from a less active binding mode (L27, L29, S16, and S19) are not included in the model (Gilly & Pellegrini, 1985). P-site proteins were affinity labeled by a mercurated 3′-pentanucleotide fragment of *N*-acetylleucyl-tRNA. They are L10/L11, L26, S13, S16, 2/3S, and S1/S4 (Fabijanski & Pellegrini, 1979; unpublished results). As explained above, one protein from the A site (S8) and one from the P site (2/3S) overlap the trichodermin binding site. We therefore placed the other (bromoacetyl)trichodermin-labeled proteins (S3/S5, L1, L3, and L24) in the near vicinity of the A and P sites. Because of the need to show a three-dimensional molecule in two dimensions, certain protein locations (L1 and L3) are shown as completely overlapping. We envision individual members of these sets of proteins as occupying positions above and below the plane of the diagram. A large number of proteins are necessarily represented as having at least a portion of their sequence near the peptidyltransferase center. This is a situation similar to that seen for *Escherichia coli* and other organism ribosomes [for example, Wittmann (1983)]. Finally, the proteins in this active center of the *Drosophila* ribosome clearly span the domain between the two subunits.

In sum, these data show that we have identified a subset of ribosomal proteins that are at the trichodermin binding site on *Drosophila* ribosomes. This site probably partially overlaps the A and P sites but is not identical with the binding sites of other antibiotics with similar functions. It is clear that the precise nature of the inhibitory actions of these various drugs, including trichodermin, will require more detailed studies to determine the molecular architecture of their ribosomal binding sites.

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Synthesis of [Pro α1(IV)]$_3$ Collagen Molecules by Cultured Embryo-Derived Parietal Yolk Sac Cells†

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ABSTRACT: Electron immunohistochemical studies demonstrate that cultured embryo-derived parietal yolk sac (ED-PYS) carcinoma cells synthesize type IV collagen. This material has been isolated and characterized. The collagen obtained after limited pepsin digestion from the medium in which the cells are grown is composed of homogeneous components with a molecular mass of ~95 000 daltons. When chromatographed on (carboxymethyl)cellulose under denaturing conditions, the chains elute as acidic components slightly before the human α1(I) chain and coincident with the position of elution of the pepsin-derived human α1(IV) chain. This analysis indicates the presence of a single type of collagen chain in the pepsin-derived ED-PYS synthesized material. In addition, the profile of cyanogen bromide (CNBr) cleavage products obtained from the pepsin-derived ED-PYS cell collagen chains is essentially identical with that derived from the human α1(IV) chain. Isolation of the medium collagen in the absence of pepsin digestion reveals the presence of two high molecular weight components equivalent in size to procollagen α chains. However, both high molecular weight products yield CNBr cleavage products that correspond to those obtained from the pepsin-derived α1(IV) chain. The ED-PYS cell-associated collagens obtained with or without the use of pepsin contain components that are essentially identical with those isolated from the culture-medium collagen. These data provide definitive evidence for the existence of type IV collagen molecules composed solely of α1(IV) procollagen chains and further document the usefulness of ED-PYS cells for investigating the biosynthesis of basement membrane components.

Basement membranes are the ubiquitous extracellular matrices found at the boundaries between cells and the connective tissue stroma (Martinez-Hernandez & Amenta, 1983). Significant evidence has accumulated indicating that these structures participate in a variety of physiological processes including filtration, structural support, and cell attachment (Hay, 1981; Kuehn et al., 1982) and that alterations in the functions, composition, and biosynthesis of the components that comprise basement membranes occur in a variety of pathological states (Martinez-Hernandez & Amenta, 1983).

Recent investigations have established that basement membranes are composed of several components with laminin (Timpl et al., 1979), entactin (Carlin et al., 1981), heparan sulfate proteoglycan (Kanwar & Farquhar, 1979), and type IV collagen (Bailey et al., 1979; Bornstein & Sage, 1980; Crouch & Bornstein, 1979; Dehm & Kefalides, 1978; Dixit & Kang, 1979; Gay & Miller, 1979; Glanville et al., 1979; Kresina & Miller, 1978; Mayne & Zethergren, 1980; Miller & Gay, 1982; Qian & Glanville, 1984; Robey & Martin, 1981; Sage et al., 1979; Timpl et al., 1978; Treub et al., 1982) being the best characterized of the constituents. To date, evidence indicates that the collagenous molecules isolated from these structures contain two genetically distinct types of collagen chains, designated α1(IV) and α2(IV) (Bornstein & Sage, 1980; Miller & Gay, 1982). However, the precise organization of these chains in any basement membrane remains unresolved with evidence having been presented for the existence of both homotrimeric (Dehm & Kefalides, 1978; Robey & Martin, 1981) and heterotrimeric (Mayne & Zettergren, 1980; Qian & Glanville, 1984; Treub et al., 1982) molecules. This lack of definition reflects in part both the proteolytic susceptibility of type IV molecules and the lack of suitable in vitro model systems to study the biosynthesis and deposition of the col-

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