Ribosome Subunit to Polysome Ratios Affect the Synthesis of rRNA in *Drosophila* Cells†

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ABSTRACT: Many investigations have revealed that ribosome numbers increase in parallel with the growth rate of cells. Here we show that the absolute level of protein synthesis may not be the only factor influencing rRNA synthesis in a nondividing eukaryotic cell. Under conditions of complete (>99%) inhibition of protein synthesis by four different antibiotics, there is a corresponding inhibition of rRNA synthesis. At lower levels of inhibition of protein synthesis (70%), a different effect of individual antibiotics on rRNA synthesis is observed. Cycloheximide and anisomycin, which cause a decrease in the free subunit pool due to a buildup of polysomes, stimulate rRNA synthesis, whereas puromycin and pactamycin, which cause an increase in the free subunit pool, cause a decrease in rRNA synthesis. These effects on rRNA synthesis are not solely due to a low level of completed proteins. Pactamycin treatment allows completed proteins to be made yet lowers rRNA labeling, while anisomycin treatment does not show synthesis of complete proteins yet increases rRNA labeling. The results suggest that eukaryotic cells may regulate ribosome synthesis in response to the number of free versus translating (polysomal) ribosomes as do *Escherichia coli* cells.

Although the biosynthesis of ribosomes usually parallels the growth rate of cells, we had observed that ribosome synthesis can be up- and downregulated in a nondividing tissue of *Drosophila* (Schmidt et al., 1985). We also found a close correspondence between the level of protein and rRNA synthesis in this tissue. To investigate the influence of protein synthesis on rRNA synthesis in this *Drosophila* system, we measured the amount of rRNA which accumulated at different levels of protein synthesis inhibition using a variety of protein synthesis antibiotics. Under conditions of complete protein synthesis inhibition (>99%), we find there is also an inhibition of rRNA transcription, in accordance with the findings reviewed by Sollner-Webb and Tower (1986). However, at lower levels of inhibition (70%) we see a strikingly different effect of specific protein synthesis inhibitors on rRNA synthesis. In all cases these events are taking place in a terminally differentiated nondividing tissue and, therefore, are not directly coupled to a growth response.

Materials and Methods

Drosophila Culture and Isolation and Labeling of Male Accessory Glands. *Drosophila melanogaster* (Oregon R) were raised at 25 °C on a standard medium of yeast, cornmeal, and agar. After eclosion, flies were separated according to sex. From 10- or 11-day-old virgin male flies, male accessory glands were dissected free of testicular and intestinal (gut) tissue and stored in a drop of ice-cold MOPS buffer [10 mM MOPS (4-morpholinepropanesulfonic acid, Sigma), pH 7.0, 80 mM NaCl, 10 mM KCl, 0.2 mM $MgCl_2$, and 0.1 mM $CaCl_2$ (Mitchell et al., 1978)]. The glands from six flies were rinsed and then incubated at room temperature for 1 h with gentle shaking in 1-mL homogenization tubes with 60 μL of MOPS buffer containing variable concentrations of $CaCl_2$, 2 μCi of [^{35}S]methionine [New England Nuclear (1125 Ci/mmol)] or 25 μCi of [^{32}P]orthophosphate [Amersham (1000 Ci/mmol)], and other reagents as indicated.

Protein and RNA Assays. Following a labeling reaction, the glands were homogenized after the addition of 10 μL of 2% Triton X-100 in MOPS buffer. Fifty microliters of a bovine serum albumin (BSA, 50 μg/mL) solution was added as carrier. Proteins were precipitated by the addition of 1 mL of ice-cold 10% trichloroacetic acid (TCA) and maintained on ice for at least 20 min. The precipitate was collected on glass fiber filters and counted in a Beckman LS 7000 liquid scintillation counter. Alternatively, the labeled proteins were assayed by one-dimensional gel electrophoresis in 0.5-mm SDS slab gels of 10% acrylamide, followed by drying and autoradiography.

For analysis of rRNA fractions, male accessory glands were dissected, labeled, and incubated as described above, except that 10 glands per sample were incubated in a total volume of 50 μL of MOPS medium containing the indicated concentration of cycloheximide (Sigma), anisomycin (Sigma), pactamycin (a gift from Upjohn), or puromycin (Sigma). After 1 h, the medium was drawn off, and a mixture of 100 μL of 10 mM Tris-HCl (pH 7.5)–saturated phenol and 100 μL of 2× NETS (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 2 mM EDTA, 0.2% SDS), preheated to 80 °C, was added. The glands were vortexed thoroughly and incubated at 80 °C for 5 min with constant shaking. After being cooled on ice and centrifugation at 15000*g* for 20 s, the phenol phase was extracted once more with 100 μL of 2× NETS. The combined aqueous phases were phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) extracted twice more at room temperature and precipitated with 2.5 volumes of ethanol at −20 °C. The RNA was redissolved in water, denatured by glyoxal (McMaster & Carmichael, 1977), and fractioned on a 1.5% agarose gel (Maniatis et al., 1982) at room temperature, dried, and autoradiographed.

Analysis of Polysomal Profiles. Following dissection, the glands from 30 flies were incubated with antibiotics and homogenized in 1-mL tubes containing 0.1 mL of homogenization buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 25 mM NaCl, 25 mM EGTA, 4 mM dithiothreitol, 1% Triton X-100, 250 mM sucrose, 1 mg/mL heparin). Control glands

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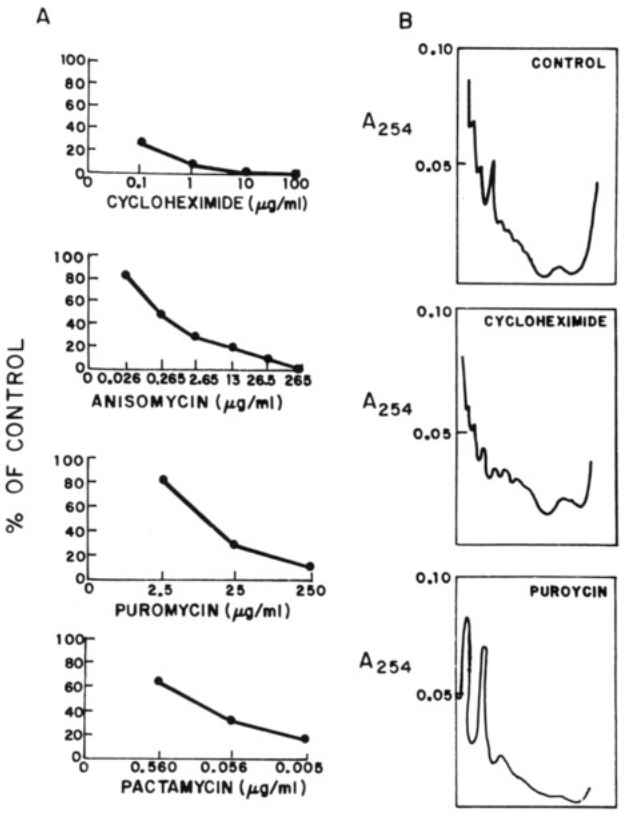

FIGURE 1: (A) Concentration dependence of various antibiotics on inhibition of protein synthesis. The male accessory glands from five virgin male flies were incubated in MOPS medium with the indicated concentration of either cycloheximide, anisomycin, puromycin, or pactamycin and 2 μCi of [^{35}S]methionine for 1 h at room temperature. Proteins were precipitated as described under Materials and Methods. Control values were derived from glands incubated in the absence of any antibiotic. The graphs show the averages of duplicate samples. (B) Absorbance profiles of ribosomal material from glands treated with a concentration of antibiotic which reduced protein synthesis by 70%.

were incubated in MOPS buffer alone. The homogenate was centrifuged at 15000*g* for 15 min at 4 °C. For polysomal analysis, the supernatant was layered over a 5-mL linear 7–50% sucrose gradient. The sucrose was prepared in gradient buffer containing 100 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris-HCl, pH 7.5. Gradients were centrifuged at 50000*g* for 2 h at 4 °C. Gradients were analyzed with an ISCO UV-5 absorbance detector with continuous recording at 254 nm.

Results

Effects on rRNA Synthesis of Protein Synthesis Inhibition Using Different Antibiotics. We had previously found that many of the reagents that had an effect on the level of protein synthesis in *Drosophila* cells had similar effects on the level of rRNA synthesis (Yamamoto et al., 1988). Here we report a further investigation of the nature of the relationship of these events. The level of male accessory gland rRNA synthesis was examined after exposure to various protein synthesis inhibitors, and at different levels of inhibition. An equal number of glands were incubated in MOPS medium containing varying concentrations of cycloheximide, anisomycin, pactamycin, or puromycin. The level of protein synthesis inhibition was determined as a percent decreases in TCA-precipitable material relative to an equal number of control glands that were incubated with no inhibitor. Figure 1A shows the resulting dose–response curves.

The effect of cycloheximide and puromycin on the polysome profiles in the glands (at 70% inhibition of protein synthesis)

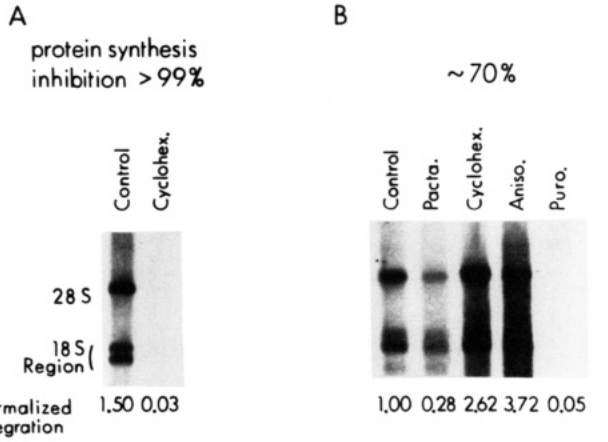

FIGURE 2: Autoradiographs of [^{32}P]phosphate-labeled total cellular RNAs. Glands were dissected and incubated in MOPS medium containing cycloheximide (Cyclohex), anisomycin (Aniso), pactamycin (Pacta), or puromycin (Puro) and 25 μCi of [^{32}P]phosphate for 1 h. Control glands were incubated in the absence of any inhibitor. Total RNA was extracted, glyoxylated, and size fractionated on a 1.5% agarose gel as described under Materials and Methods. Each lane represents RNA from the glands of 10 flies. Scanning densitometry of band intensities was performed, and the normalized values are presented under each lane. (A) Effect of complete inhibition (>99%) of protein synthesis on the level of rRNA synthesis. (B) Effect of various protein synthesis inhibitors on the level of rRNA synthesis at incomplete levels of protein synthesis inhibition (70% inhibition).

was measured as shown in Figure 1B. The profile for anisomycin was very similar to that for cycloheximide, and the profile for pactamycin was similar to that for puromycin (data not shown). Cycloheximide and anisomycin are both inhibitors of translational elongation that do not elicit premature chain termination but stabilize polysomes, although through different mechanisms of action. Anisomycin is thought to inhibit the peptidyl transferase reaction directly, whereas cycloheximide, which does not inhibit this reaction, is thought to inhibit translocation by inhibiting the EF-2 (elongation factor) GTPase reaction [reviewed in Gale et al. (1981)]. The polysomes of cells treated with either cycloheximide (Figure 1B) or anisomycin are increased in proportion to control cells. Puromycin is a tRNA analogue that participates in the peptidyl transferase reaction which results in premature chain termination and thus destabilizes polysomes. Pactamycin acts on protein synthesis by inhibiting the formation of complete initiation complexes. Treatment with either puromycin or pactamycin results in a decrease of polysomes and a buildup of subunits as seen in Figure 1B. Therefore, these antibiotics affect the polysome to subunit ratios in the glands as expected from their proposed mechanisms of action.

To examine the effects of the protein synthesis inhibitors on rRNA synthesis, male accessory glands from an equal number of adult virgin males were incubated in MOPS medium containing [^{32}P]phosphate and varying concentrations of either cycloheximide, anisomycin, pactamycin, or puromycin. Total RNA was extracted, denatured, and separated as described under Materials and Methods. Sample autoradiograms are shown in Figure 2. As can be seen in Figure 2A, when protein synthesis is completely inhibited (>99%) by cycloheximide (10 μg/mL for these *Drosophila* cells), there is also a complete inhibition of rRNA synthesis. The same inhibition of rRNA synthesis can be seen after complete inhibition of protein synthesis by each of the other antibiotics (data not shown). Substantially different results are seen when protein synthesis is inhibited to a lesser degree. Figure 2B shows that when the glands are incubated in concentrations of cycloheximide, anisomycin, pactamycin, or puromycin that

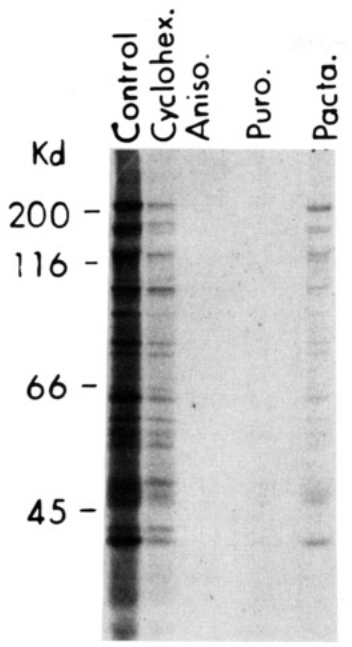

FIGURE 3: Proteins synthesized in glands treated with protein synthesis antibiotics. Labeled proteins from glands treated with antibiotics to reduce total TCA-precipitable peptides to 30% of that in control glands were run on polyacrylamide gels and autoradiographed. The protein patterns observed for cells treated with cycloheximide and pactamycin are the same as those from control gland cells in vitro (Yamamoto et al., 1988).

cause only a 70% inhibition of protein synthesis, rRNA synthesis is not always inhibited. When cycloheximide or anisomycin (which increase polysomes) are used at concentrations that result in a 70% reduction in protein synthesis activity, rRNA accumulation is not inhibited and appears to be stimulated. With puromycin and pactamycin (which increase subunits), however, at the same 70% reduction in protein synthesis activity, rRNA synthesis is inhibited as is the case during complete inhibition of protein synthesis. A high or low ratio of subunits (40S and 60S) to polysomes (80S and larger) correlates directly with the level of labeled rRNA we observe in parallel rRNA labeling and polysome analysis experiments. The subunit to polysome ratio parallels though does not precisely predict the level of accumulation of rRNA. This may indicate that other factors can also influence rRNA accumulation as is the case for *Escherichia coli*.

Effect of Different Antibiotics on the Synthesis of Complete Proteins. Both the chemical structures and mechanisms of action of the antibiotics used in this study are significantly different from each other [reviewed in Gale et al. (1981)]. In addition, at 70% protein synthesis inhibition, the level of synthesis of completed proteins that each antibiotic permits was examined by one-dimensional gel analysis. As shown in Figure 3, treatment with anisomycin and puromycin allows very low levels of completed proteins to be made as detected after a 1-h labeling with [^{35}S]methionine. On the other hand, exposure of the glands to levels of cycloheximide or pactamycin that lower overall polymerization of amino acids by 70% from control values allows relatively greater amounts of proteins to be visible on a gel. Then, treatment of cells with either pactamycin or cycloheximide allows gel-detectable levels of complete proteins to be synthesized, yet of these two antibiotics only cycloheximide treatment stimulates rRNA synthesis. Anisomycin and puromycin both inhibit completion of proteins while only anisomycin treatment stimulates rRNA synthesis. There is no correlation between the low levels of completed proteins and the changes in rRNA levels observed in treated cells. It is interesting that cycloheximide and anisomycin each inhibits protein synthesis elongation yet cycloheximide does not allow detectable levels of completed proteins. This may be accounted for by the fact that anisomycin directly binds to and affects the peptide bond forming site of the ribosome, allowing few if any proteins to be completed. Cycloheximide affects elongation through inhibition of the recycling of an elongation factor which would stall a protein chain but could allow functional elongation factors to affect completion of a few protein copies.

Discussion

Lack of Coordination of Protein Synthesis and rRNA Synthesis in Drosophila Cells. As we have shown previously in the male accessory gland system (Schmidt et al., 1985; Yamamoto et al., 1988; Weber and Pellegrini, unpublished results), many reagents that suppress protein synthesis similarly inhibit rRNA synthesis, whereas reagents or events that stimulate protein synthesis can also stimulate rRNA synthesis. However, these results showing the effects of protein synthesis inhibitors on rRNA synthesis indicate that the level of protein synthesis may not be the only factor influencing rRNA synthesis. Under conditions of complete (>99%) protein synthesis inhibition, we see a corresponding inhibition of rRNA synthesis (Figure 2A). This is in accordance with the work of others [reviewed in Sollner-Webb and Tower (1986)]. Under conditions of incomplete inhibition of rRNA synthesis (70%) we see either a stimulation or an inhibition of rRNA synthesis (Figure 2B). At this lower level of protein synthesis, inhibitors that stabilize polysomes (cycloheximide and anisomycin) and cause a relative decrease in the free ribosomal subunit pool stimulate rRNA synthesis, whereas puromycin and pactamycin, which cause a relative increase in the free ribosomal subunit pool, inhibit rRNA synthesis. Since the different responses of rRNA synthesis were obtained under conditions where protein synthesis was lowered to the same level by these various and differently acting antibiotics, these results indicate that the absolute level of protein synthesis, or polymerization of amino acids, may not be the only factor influencing rRNA synthesis in *Drosophila* cells.

In prokaryotic systems it has been suggested that the signal for the stimulation of ribosome biosynthesis may be due to the detection of a lowered pool of free nontranslating ribosomes and that the rate of synthesis of new ribosomes may be feedback inhibited by free, nontranslating ribosomes (Nomura et al., 1984; Yamagishi & Nomura, 1988). This would account for the rapid induction of ribosome synthesis in nutritional upshift experiments where the corresponding stimulation of total protein synthesis would cause a mobilization of free ribosomes, and thus lower the free nontranslating ribosome pool. Nomura and colleagues (Cole et al., 1987) also demonstrated that an initiation event must be able to occur for the extra subunit accumulation to have an effect on rRNA synthesis.

Our results also suggest that the ratio of free subunits to polysomes influences rRNA synthesis. In male accessory glands, when there is also only a minimal level of ongoing protein synthesis due to low levels of an antibiotic present, we see that rRNA synthesis is inhibited under conditions that increase the relative free subunit pool by disrupting polysomes. However, rRNA synthesis continues and appears to be stimulated when the relative free subunit pool is decreased due to the stabilization of polysomes. These results indicate that the level of the free ribosomal subunit pool relative to the polysome pool may influence rRNA synthesis and that this influence requires a minimal amount of protein synthesis. Our previous

results are consistent with this interpretation. Conditions that stimulate overall protein synthesis (treatment with juvenile hormone III or higher levels of calcium) also stimulate rRNA synthesis (Schmidt et al., 1985; Yamamoto et al., 1988; Weber and Pellegrini, unpublished results). It is possible that this is also a response to the lowering of the ribosomal subunit pool relative to the polysomal pool caused by a recruitment of free nontranslating ribosomes during the stimulation of protein synthesis.

Other Examples of the Effects of Protein Synthesis on rRNA Synthesis. In *Tetrahymena* nutritional downshift experiments, it has been shown that rRNA synthesis is reduced (Eckert & Kaffenberger, 1980). Interestingly, this inhibition of rRNA transcription can be prevented by a pretreatment with low levels of cycloheximide. Puromycin and pactamycin in concentrations exhibiting a similar inhibitory effect on protein synthesis (70%) do not prevent an inhibition of rRNA transcription but instead produced an additional reduction of rRNA accumulation. Again, this indicates that a factor not depending on the absolute level of protein synthesis per se can be responsible for the negative regulation of rRNA synthesis.

Under heat-shock conditions, *Drosophila* and plant tissue culture cells still continue to transcribe rRNA (Lengyel et al., 1980; Nover et al., 1986; Bell et al., 1988). Under conditions of heat shock, the cells cease to synthesize ribosomal proteins and most other 25 °C cellular proteins, but there is not a total inhibition of protein synthesis, as the heat-shock proteins mRNAs are translated to a high degree and low ratios of subunits to polysomes are maintained.

In summary, our evidence indicates that nondividing eukaryotic cells may regulate rRNA synthesis levels in response to a signal or signals that convey the relative ratio of those ribosomal subunits engaged in protein synthesis (free subunit to polysomes). This is clearly the case in the prokaryote *E. coli*. It is possible that this is a widespread mechanism by which cells, whether growing or not, can modulate ribosome synthesis in response to the increased need for protein synthesis.

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