Mechanisms of Action of Aminoglycoside Antibiotics in Eucaryotic Protein Synthesis

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Tetrahymena thermophila is a eucaryotic organism that is highly susceptible to growth inhibition by aminoglycoside antibiotics. Concentrations of paromomycin, gentamicin G418, and hygromycin B at 22, 10, and 17 μ M, respectively, inhibited growth by 50%. A combination of in vitro and in vivo methods was used to determine the mechanisms of action of these aminoglycoside antibiotics on protein synthesis in *T. thermophila*. Analysis of polysome profiles from paromomycin- and gentamicin G418-treated cells showed clear, progressive depletions of polysomes concomitant with an inhibition of in vivo [14 C]lysine incorporation. In vitro, paromomycin and gentamicin G418, which are disubstituted 2-deoxystreptamine-containing molecules, were not very effective inhibitors of either the translocation of peptidyl-tRNA or the elongation of nascent polypeptide chains on polysomes. In contrast, we found that the translocation of phe-tRNA on polyuridylylated programmed ribosomes was susceptible to inhibition by paromomycin. We conclude that the primary inhibitory action of paromomycin and gentamicin G418 was at (i) an early stage of elongation after initiation, (ii) the initiation stage of translation, or (iii) a stage of translation before initiation. Hygromycin B, which is a monosubstituted 2-deoxystreptamine-containing aminoglycoside, potentially inhibited the elongation of nascent chains during the translation of polysomes. In addition, the in vitro translation of polysomes from two hygromycin B-resistant mutants was resistant to the inhibition of elongation caused by hygromycin B.

Aminoglycoside antibiotics are well-established inhibitors of growth, stimulators of misreading, and inhibitors of protein synthesis in procaryotes and eucaryotes (18). Vazquez and co-workers have studied the in vitro activities of many aminoglycoside antibiotics in eucaryotic model systems derived from yeasts and mammalian cells (20 and references therein). Of the aminoglycoside antibiotics tested, those active in inhibiting protein synthesis were usually found to inhibit the polypeptide chain elongation phase (20). Model systems to study the interaction of antibiotics with the ribosome at separate stages of the elongation cycle have proven to be powerful tools in the elucidation of antibiotic action on ribosomes. Furthermore, these studies have greatly enhanced the understanding of the structure-function relationships of ribosomes (8, 15).

Eucaryotic protein synthesis has not, however, received as much attention as have procaryotic systems, and the majority of current concepts about the structure of eucaryotic ribosomes have been derived by analogy with the extensive information available from *Escherichia coli* ribosomes. Model systems derived from different eucaryotic sources do not have uniform responses to a particular aminoglycoside, as is exemplified by the failure of paromomycin (PM) to inhibit the cytoplasmic protein synthesis of yeasts (14), mammalian cells (21), and wheat germ (22). In contrast, cytoplasmic protein synthesis in the lower eucaryote *Tetrahymena thermophila* is effectively inhibited by PM, as is growth (13). *Tetrahymena* spp. have a high susceptibility to a wide range of antibiotics, suggesting that these organisms may be an ideal model system to study antibiotic action in a eucaryotic organism (6, 13). Mutants of *Tetrahymena* spp. have also been obtained which display high levels of resistance to PM or hygromycin B (HM) or both (S. M. Skelly, D. C. Eustice, and J. W. Wilhelm, Abstr. Annu. Meet. Am.

Soc. Microbiol. 1983, I112, p. 158), as well as to cycloheximide (17). These mutants have altered cytoplasmic (80S) ribosomes. To further understand the reactions of eucaryotic protein synthesis and the mechanism of action of translation inhibitors in eucaryotes, we have developed homologous systems from *T. thermophila* for each of the partial reactions of elongation and studied the interaction of antibiotics with ribosomes. Optimal conditions were established and antibiotics were tested. The results from analyses of partial reactions are compared with in vitro translation of endogenous mRNA on polysomes, in vivo changes in polysome profiles, in vivo inhibition of protein synthesis, and in vivo growth inhibition. In addition, we compare our results to those of previous reports of aminoglycoside activity in *T. thermophila* with respect to codon-misreading potential and the inhibition of in vitro translation on polyuridylylated [poly(U)] templates. In vitro, we also compared polysomes from the wild-type strain to polysomes from two aminoglycoside-resistant mutants of *T. thermophila*.

(A preliminary account of portions of this work was given previously [D. C. Eustice, S. Skelly, and J. M. Wilhelm, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, I111, p. 158].)

MATERIALS AND METHODS

Materials. HM, PM, and gentamicin G418 (G418) (potency of monosulfate, 460 μ g/mg) were generous gifts from Eli Lilly & Co.; Parke, Davis & Co.; and Schering Corp., respectively. All other antibiotics were purchased from Sigma Chemical Co.

Strains. *T. thermophila* B1868 was grown as described previously (13), except the growth medium contained 0.003% chelated iron. The mutant strains, designated HYR3 and HYR16, were obtained from a collection isolated by one of us (J.M.W.); results of the genetic analysis of this collection will be detailed elsewhere. Strains HYR3 and HYR16 are respectively homozygous in the germline nucle-

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us (micronucleus) for alleles designated *Hmr-3* and *Hmr-16* and express these alleles uniformly in the somatic nucleus (macronucleus). Special aspects of the genetics of *T. thermophila* have been described by Orias and Bruns (12).

In vivo treatment with antibiotics. Cultures (250 ml of medium per 1-liter flask) were grown to mid-log phase ($\sim 1.4 \times 10^5$ cells per ml). Antibiotics (see Fig. 1 and 2 for concentrations) were added directly to the medium, and incubation was continued with shaking at 30°C. At the indicated times, 25 ml of cell suspension was removed and cells were pelleted at $300 \times g$ for 2 min, washed with 10 mM Tris-hydrochloride (pH 7.6), and repelleted at $300 \times g$.

Analysis of polysome profiles. Cell pellets were lysed by the method of Calzone et al. (3), except the aurin tricarboxylic acid was omitted from the lysing buffer. Approximately 4 to 5 absorbance at 260 nm (A_{260}) units of supernatant were layered onto 5.0-ml sucrose gradients (15 to 50% sucrose) prepared in 10 mM Tris-hydrochloride (pH 7.6)–100 mM KCl–5 mM $MgCl_2$. Centrifugation was carried out for 70 min at $150,000 \times g$ in an SW50.1 rotor. Gradients were analyzed at 260 nm with a flow cell.

Incorporation of [^{14}C]lysine by whole cells. Concomitant with the time points for the extraction of polysomes during drug treatments, duplicate 100- μ l portions of cell suspension were removed from each flask and mixed with 0.2 μ Ci of [^{14}C]lysine (specific activity, 342 mCi/mmol). The labeling mixture was incubated for 10 min at 30°C, 50 μ l of 1.0 N KOH was then added, and 100 μ l of the mixture was pipetted onto Whatman 3MM filter paper disks (2.3 cm in diameter). Incorporation of label into protein was determined as described previously (13). Results are expressed as counts incorporated per minute per 10^5 cells.

Preparation of polysomes for in vitro translation. Cells from approximately 1 liter ($\sim 2 \times 10^5$ cells per ml) were harvested by centrifugation and washed as described above. Cells were homogenized in an equal volume of homogenization buffer as described previously (13), except in some preparations, 2% spermidine was included in the homogenization buffer. The lysed cell mixtures were centrifuged at $30,000 \times g$ for 15 min, and the resulting supernatant (S-30 supernatant) was layered over 34-ml sucrose gradients prepared as described above. Gradients were centrifuged at $65,000 \times g$ for 4.5 h. Gradients were fractionated and monitored with a flow cell. Polysomes containing more than two ribosomes were collected and pelleted by centrifugation at $160,000 \times g$ for 6 to 10 h. Polysomes were resuspended in 18 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-KOH (pH 7.6)–3.6 mM magnesium acetate–90 mM KCl–3.6 mM 2-mercaptoethanol–10% glycerol and stored in small portions at -70°C .

Preparation of S-100 supernatants for polysome translation. Supernatants were prepared from the S-30 fraction (13) by centrifugation at $100,000 \times g$ for 90 min (S-100 supernatants). Typical S-100 supernatants were about 3 to 5 A_{260} units per ml and had an A_{260}/A_{280} ratio of 1.1.

In vitro polysome translation assay. Reaction mixtures (50 μ l) contained 20 mM HEPES-KOH (pH 7.6), 63 mM KCl (90 mM K^+), 3.5 mM magnesium acetate, 2.0 mM dithiothreitol, 5.0 mM K_2HPO_4 , 1.0 mM ATP, 0.5 mM GTP, 6.0 mM phosphoenolpyruvate, 10 μ g of pyruvate kinase per ml, 1.0 mM spermidine, 1.0 mM putrescine, 100 μ M each of 19 amino acids (minus leucine), 10 μ M [^{14}C]leucine (specific activity, 339 mCi/mmol), 1.0 A_{260} units per ml of *T. thermophila* tRNA (6), and polysomes and S-100 supernatant as indicated in Results. Reaction mixtures were incubated at 30°C for 20 min, and 40 μ l was pipetted onto filter paper

disks. Incorporation of label into protein (radioactivity insoluble in hot trichloroacetic acid) was determined as described previously (13).

Peptide bond formation. Peptide bond formation between [3H]puromycin and peptidyl-tRNA was performed as described previously (2). Reaction mixtures (50 μ l) contained 20 mM HEPES-KOH (pH 7.6), 3.1 mM magnesium acetate, 80 mM KCl, 2.0 mM dithiothreitol, 40 μ M [3H]puromycin (specific activity, 400 mCi/mmol), and polysomes (see Fig. 4 and Table 4). Reaction mixtures were incubated for 10 min at 30°C, and 40 μ l was pipetted onto filter paper disks which had been pretreated with 100 μ l of 10-mg/ml ADP. Pretreatment of the disks with ADP helped reduce nonspecific adsorption of [3H]puromycin. Disks were dried, and [3H]puromycin-peptides were precipitated with ice-cold 10% trichloroacetic acid for 1 h. Disks were extensively washed with several changes of 5% trichloroacetic acid over the course of 2 h and then washed with 95% ethanol. Counting efficiencies were compensated for by the method of Albright et al. (1), where 1.0 pmol of [3H]puromycin-peptide was equal to 232 cpm.

Assay of peptidyl-tRNA translocation. To study the enzymatic translocation of peptidyl-tRNA, we supplemented reaction mixtures identical to those described for peptide bond formation assay with 200 μ M GTP and S-100 supernatant (concentrations as indicated in Results). Incubation was carried out for 10 min at 30°C, and products were processed as described for the peptide bond formation assay. Values for enzymatic translocation were corrected for the amount of (puromycin-reactive) P site-bound peptidyl-tRNA in the absence of GTP and S-100 supernatant.

Purification of elongation factors. *T. thermophila* elongation factor 1 (EF1) and EF2 were resolved by chromatography on DEAE-Bio-Gel (16) or hydroxylapatite (6). One unit of EF1 was defined as the amount that bound 1 pmol of phe-tRNA to poly(U)-programmed ribosomes.

Assay for aminoacyl-tRNA translocation. To measure the translocation of [^{14}C]phe-tRNA on poly(U)-programmed ribosomes, we used a coupled binding-translocation assay at pH 7.6 (6). Products were analyzed as described previously (5).

RESULTS

Effects of antibiotics on *T. thermophila* growth and in vivo protein synthesis. To determine which aminoglycoside antibiotics were the most effective inhibitors of growth, we tested antibiotics for effects on long-term growth (overnight cultures); concentrations which prevented growth completely or reduced the number of doublings by 50% are shown in Table 1. PM, G418, and HM were all inhibitors of growth between 10 and 22 μ M. In contrast, neomycin (NM) and streptomycin (SM) were very ineffective inhibitors even at millimolar concentrations.

To determine whether PM, G418, and HM had effects on protein synthesis in vivo, we tested various concentrations of the antibiotics at various exposure times for their effects on growth rate and the incorporation of [^{14}C]lysine into protein. The short-term exposure of log-phase cells (15 to 30 min) to PM concentrations between 10 and 100 μ M was not effective in inhibiting either growth or [^{14}C]lysine incorporation. At a PM concentration of 100 μ M and an exposure time of 60 min, a significant inhibition of [^{14}C]lysine incorporation was observed (Table 2). Similarly, HM and G418 inhibition of [^{14}C]lysine incorporation was also not observed until about 60 min of incubation with 100 μ M of either drug (Table 2). Higher concentrations of PM (300 μ M), however,

TABLE 1. Effect of antibiotics on growth of *T. thermophila* strains B1868, HMR16, and HMR3

Strain	Antibiotic	Concn (μ M) required for following % inhibition of growth ^a :	
		50	100
B1868 (wild type)	PM	22	70
	G418	10	30
	HM	17	85
	NM	$\geq 5,000^b$	ND ^c
	SM	5,000	ND
HYR16	PM	140	560
	G418	170	520
	HM	180	540
HYR3	PM	280	1,100
	G418	28	100
	HM	1,400	>2,700

^a Determined as described previously (6).^b This was the highest concentration tested, and it had no effect at all on growth.^c ND, Not determined.

caused a response at 30 min. These results suggest that the uptake of these antibiotics was a slow process. Furthermore, we deduced that, for the determination of the primary effect of these drugs in vivo, a reasonable exposure time was 60 or 90 min. Table 2 also shows the effect of other antibiotics on the in vivo incorporation of [¹⁴C]lysine into protein after 60 min of exposure.

Effect of antibiotics on polysome profiles. PM at a concentration of 300 μ M caused a clear, progressive loss of polysome content (Fig. 1). In addition, the losses in poly-

TABLE 2. Effect of various antibiotics on in vivo incorporation of [¹⁴C]lysine into protein after 1 h of exposure to the drugs

Antibiotic and concn (μ M) ^a	% Inhibition
Expt 1	
None (0)	0
Emetine (300)	88
Cycloheximide (1)	49
Pactamycin (100)	46
Expt 2	
None (0)	0
PM (100)	35
HM (100)	30
G418	
100	55
50	33

^a The control incorporation values were 5.5×10^4 and 7.2×10^4 cpm per 10^5 cells in experiments 1 and 2, respectively.

some content produced by PM or G418 correlated well with the in vivo inhibitions of [¹⁴C]lysine incorporation (Table 2). Higher concentrations of PM (or G418) caused a greater shift of ribosomes into the monosome region of the gradient (cf. Fig. 1 with Fig. 2). Treatment with HM, however, did not result in the same shift of ribosomes into the monosome region of the gradient (Fig. 2). HM-induced alterations in polysome patterns were subtle and complex as compared with control patterns but were clearly different from the changes induced by PM and G418. We made reproducible observations of some broadening of the polysome region of the gradient during HM treatment (Fig. 2). This may be similar to the superloading effect caused by cycloheximide

FIG. 1. Effect of paromomycin in vivo on polysome profiles. Cells were grown, treated with 300 μ M of paromomycin, and analyzed for [¹⁴C]lysine incorporation, and polysomes were extracted as described in Materials and Methods. (A) Incubation (15 min) in which no inhibition of [¹⁴C]lysine incorporation occurred; (B) 30 min of incubation with PM in which 42% inhibition of [¹⁴C]lysine incorporation occurred; (C) 60 min of incubation with PM in which 68% inhibition of [¹⁴C]lysine incorporation occurred.

FIG. 2. Effect of aminoglycoside antibiotics on polysome profiles. Cells were treated with antibiotics for 90 min and analyzed for [^{14}C]lysine incorporation, and polysomes were extracted as described in Materials and Methods. (A) Control; (B) 100 μM PM and 57% inhibition of [^{14}C]lysine incorporation; (C) 100 μM HM and 43% inhibition of [^{14}C]lysine incorporation; (D) 50 μM G418 and 55% inhibition of [^{14}C]lysine incorporation.

on *T. thermophila* polysomes in vivo (3). To determine in more detail the effects of these antibiotics on translation, we tested them for activity during in vitro polysome translation.

In vitro translation with polysomes. Reaction mixtures for the translation of polysomes were optimized for Mg^{2+} (3 to 4 mM) and K^+ (~90 mM) concentrations. The addition of 1.0 A_{260} unit of *T. thermophila* tRNA or increasing the amino acid concentration from 10 to 100 μM stimulated translation by 76 and 30%, respectively, suggesting that *T. thermophila* extracts were limiting for either tRNA or tRNA synthetases or both. Maximal rates of translation and the highest levels of incorporation were obtained when the ratio of polysomes to S-100 supernatant was the same as that found in S-30 supernatants. A typical reaction was greater than 90% complete after 20 min of incubation at 30°C and synthesized proteins ranging in molecular weight from 40,000 to 110,000 (as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography).

Effect of antibiotics on translation with polysomes containing endogenous mRNA. Emetine and cycloheximide were effective inhibitors of *T. thermophila* translation and inhibited protein synthesis by 50% at about 2.0 μM (Table 3). Inhibitions of 91 or 81% could be obtained at 25 μM emetine or cycloheximide, respectively. The most effective inhibitor was HM, which inhibited translation by 50% at concentrations between 0.1 and 0.2 μM and by 97% at a concentration of 2.0 μM . NM and PM inhibited translation by 50% at about 19 and 54 μM , respectively, whereas SM and G418 were relatively ineffective in this reaction. To determine whether the inhibition of nascent-chain elongation caused by NM and PM was the primary effect on translation, we compared polysomes from two aminoglycoside-resistant mutants to

wild-type polysomes for inhibition during in vitro translation.

Analysis of aminoglycoside-resistant mutants. Two classes of mutants were obtained which showed altered responses to aminoglycoside antibiotics (Table 1). When compared with wild-type susceptibility levels, mutant strain HYR3 was resistant to high levels of HM and moderate levels of PM. Strain HYR16 was resistant to low levels of HM and PM. Translation of polysomes from strains HYR3 and HYR16 with several concentrations of PM and HM is shown in Fig. 3. The results in Fig. 3A clearly show that the resistance of these polysomes to HM inhibition was an accurate reflection of the drug resistance phenotype displayed in vivo. In contrast to the HM resistance demonstrated in vivo, the effect of PM on the polysomes from mutants was not much different from that on wild-type polysomes (Fig. 3B). This result suggested that the inhibition of chain elongation obtained with PM was not the primary action of PM and that PM affects some aspect of translation other than polypeptide chain elongation.

Partial reactions of protein synthesis in the presence of antibiotics. To determine which phase of elongation was sensitive to each antibiotic, we performed an analysis of peptide bond formation and enzymatic translocation with isolated polysomes. The inset in Fig. 4 shows the progression of translocation and peptide bond formation when measured by the reaction of [^3H]puromycin with peptidyl-tRNA. The effect of S-100 supernatant on the translocation of peptidyl-tRNA suggest that EF2 acted stoichiometrically in this reaction. Since the inhibitory action of many drugs on translocation can be overcome by the addition of excess EF2 (19), we tested the effect of supernatant fraction on the level

TABLE 3. Effect of antibiotics on in vitro translation of polysomes

Antibiotic and concn (μ M)	Incorporation ^a		% of control	Estimated concn (μ M) for 50% inhibition
	Net pmol	Leucines per 80S		
Expt 1				
None	21.4	4.41	100	
PM				
0.2	23.6	4.85	110	54.0
2.0	19.5	4.02	91	
10.0	17.1	3.51	80	
25.0	14.7	3.02	69	
50.0	11.5	2.37	54	
HM				
0.2	5.2	1.07	24	0.15
2.0	0.6	0.12	3	
10.0	0.4	0.09	2	
25.0	0.3	0.06	1	
NM				
2.0	21.7	4.47	101	19.0
10.0	20.1	4.13	94	
25.0	5.3	1.09	25	
50.0	0.5	0.08	2	
Expt 2				
None	18.7	3.84	100	
Cycloheximide				
2.0	8.1	1.66	43	1.7
10.0	5.2	1.07	28	
25.0	3.5	0.71	19	
Emetine				
2.0	9.9	1.87	53	2.0
10.0	3.1	0.64	17	
25.0	1.8	0.36	10	
Expt 3				
None	26.3	5.42	100	
G418				
0.1	27.1	5.59	103	ND ^b
1.0	19.5	4.02	74	
10.0	18.5	3.81	70	
25.0	18.4	3.78	70	
SM				
1.0	24.6	5.08	94	ND
10.0	24.1	4.96	92	
25.0	27.0	5.56	103	

^a Reaction mixtures (50 μ l) contained 6.06 pmol of polysomes, and 40 μ l of the reaction mixtures were processed for hot trichloroacetic acid insoluble radioactivity after 20 min at 30°C.

^b ND, Not determined.

of inhibition obtainable. Figure 4 shows the stimulatory pattern by supernatant fraction when antibiotics were present at 10 μ M. It is clear that emetine inhibition was easily overcome by additional supernatant fraction. This result was consistent with the activity of this antibiotic in yeast translation (19). HM, emetine, and cycloheximide did not inhibit peptide bond formation (Table 4). The levels of inhibition of peptidyl-tRNA translocation by PM and cycloheximide were

FIG. 3. Translation of polysomes from aminoglycoside-resistant mutants of *T. thermophila* with HM (A) and PM (B). All conditions were as described in Materials and Methods. Reaction mixtures contained 3.02 A₂₆₀ units per ml of polysomes (66.3 nM of 80S ribosomes), 0.754 A₂₈₀ units per ml of S-100 supernatant (291 μ g/ml of protein), and antibiotics as indicated. All values were corrected for incorporation in the absence of polysomes. Uninhibited incorporation values were: 5.47 leucines per ribosome for wild type (●), 3.15 leucines per ribosome for HYR3 (■), and 3.77 leucines per ribosome for HYR16 (▲).

TABLE 4. Effect of various antibiotics on peptide bond formation and translocation of peptidyl-tRNA bound to polysomes^a

Antibiotic ^b	Peptide bond formation		Enzymatic translocation ^c	
	pmol formed	% Inhibition	pmol formed	% Inhibition
None	1.33		2.57	
PM	1.30	3	1.92	26
G418	1.31	1	2.30	11
HM	1.52	0	1.62	37
NM	1.20	10	2.52	2
SM	1.41	0	2.72	0
Emetine	1.44	0	2.41	7
Cycloheximide	1.31	1	1.57	40

^a Reaction mixtures contained 11.74 pmol of polysomes, and peptide bond formation was determined as described in Materials and Methods. The values shown have been corrected for those obtained in the absence of polysomes (0.58 pmol in the absence of supernatant and 1.17 pmol when S-100 supernatant was present).

^b All antibiotics were present at a concentration of 10 μ M.

^c These values are the amounts (in picomoles) of peptidyl-tRNA translocated to the P site by 65.3 μ g of S-100 supernatant and 200 μ M GTP. These values were corrected for the amount of puromycin-reactive peptides formed in the absence of S-100 supernatant and GTP (peptide bond formation).

FIG. 4. Effect of S-100 supernatant concentration on peptidyl-tRNA translocation with antibiotics. Reaction mixtures (50 μ l) contained 294 pmol of polysomes, 1,306 μ g of S-100 supernatant per ml and 200 μ M GTP, and 40 μ l was processed for trichloroacetic acid-insoluble radioactivity. All other conditions were as described in Materials and Methods. All values shown were corrected for [3 H]puromycin-reactive peptides in the absence of S-100 supernatant and GTP (1.43 pmol per 40 μ l). Symbols: ●, no antibiotic; □, 10 μ M emetine; ■, 10 μ M HM. Inset, Time course of the reaction progression with 322 pmol of polysomes per ml in uninhibited controls. Symbols: △, no S-100 supernatant and no GTP added (peptide bond formation); ▲, 1,430 μ g of S-100 supernatant per ml and 200 μ M of GTP added (peptide bond formation plus translocation together).

comparable to those found during polysome translation at 10 μ M (Tables 3 and 4). In contrast, the inhibition of peptidyl-tRNA translocation by 10 μ M HM was markedly less than that of polysome translation. At 10 μ M HM, translation was inhibited by 98%, whereas peptidyl-tRNA translocation was inhibited by only 37%. Clearly, the levels of inhibition by HM and emetine during a single round of translocation was not adequate to explain the potent inhibitions observed with polysome translation. Preincubation of polysomes with HM did not increase the level of inhibition observed during peptidyl-tRNA translocation (data not shown). It is possible that the presence of peptidyl-tRNA bound at the A site of the ribosome posed a steric hindrance to the binding of HM. Precedents for steric hindrance of drug binding are well established in *E. coli* (4; for a review, see reference 20).

Since PM was a relatively poor inhibitor of translation in vitro, a poor inhibitor of peptidyl-tRNA translocation in vitro, and an inducer of polysome breakdown in vivo, we examined the possibility that PM inhibits an early stage of translation. At early stages of translation, when peptidyl groups are very small, PM may be a more effective inhibitor of translocation. Therefore, we examined the translocation of aminoacyl-tRNA as a model reaction for this stage of translation.

Effect of antibiotics on translocation of aminoacyl-tRNA. The translocation of aminoacyl-tRNA was measured with a coupled binding and translocation system. In the coupled system, a residual EF2-like activity translocated significant amounts of phe-tRNA to the P site (Table 5), although insufficient EF2 was present to support polypeptide synthesis (6). This may be analogous to the phenomenon of

spontaneous translocation recently reported by Wormbach and Neirhaus (23) with *E. coli* ribosomes in the absence of elongation factor G. The inhibition of phe-tRNA translocation by antibiotics with a coupled assay is shown in Table 5. Residual translocation activity was susceptible to inhibition not only by cycloheximide and emetine, but also by PM. The addition of 2.0 μ g of EF2 partially overcame the inhibition by emetine or PM. Figure 5 shows the inhibition of aminoacyl-tRNA translocation by PM when EF2 was present at two concentrations. Clearly, PM was an effective inhibitor of this reaction with 0.113 μ g of EF2. When EF2 was increased to 0.57 μ g, PM inhibition could be effectively prevented.

TABLE 5. Effect of various antibiotics on the translocation of phe-tRNA in the presence or absence of 2 μ g of EF2^a

Antibiotic	Concn (μ M)	% Inhibition	
		Control ^b	With 2.0 μ g of EF2 ^c
None		0	0
PM	2	93	15
Cycloheximide	100	100	56
Emetine	10	100	5
Emetine	100	100	45

^a Reaction mixtures contained 2.0 pmol of ribosomes, 3.0 pmol of [14 C]phe-tRNA, and 7.5 U of EF1.

^b A total of 0.64 pmol of phe-tRNA was bound to the ribosome; 0.32 pmol of phe-tRNA reacted with puromycin.

^c A total of 1.11 pmol of phe-tRNA was bound to the ribosome; 0.61 pmol of phe-tRNA reacted with puromycin.

FIG. 5. Inhibition of aminoacyl-tRNA translocation by PM in the presence of different concentrations of EF2. A coupled assay system was used as described in Materials and Methods. Reaction mixtures (50 μ l) contained 8.0 U of EF1, 4.0 pmol of 80S ribosomes, 4.0 pmol of [14 C]phe-tRNA, and PM as indicated. Symbols: ■, 0.113 μ g of EF2; ●, 0.57 μ g of EF2. Parallel reaction mixtures tested for total binding demonstrated that binding in the presence of 0.113 and 0.57 μ g of EF2 was 0.301 and 0.345 pmol of [14 C]phe-tRNA, respectively. The levels of P site-bound [14 C]phe-tRNA showed that virtually all bound products were translocated to the P site with either concentration of EF2 when no PM was present.

DISCUSSION

With a combination of in vitro and in vivo methods to analyze the effects of aminoglycoside antibiotics on protein synthesis, it was possible to discriminate between primary and secondary effects for closely related aminoglycoside antibiotics. NM, which is a disubstituted 2-deoxystreptamine-containing molecule with a 4 substituent of 2',6'-diamino sugar, had no effect on the growth of *T. thermophila* at concentrations up to 5 mM. In contrast, PM and G418, which are disubstituted 2-deoxystreptamine-containing molecules with a 4 substituent of 2' monoamino sugar, were potent inhibitors of growth and in vivo protein synthesis. The common difference between NM and the two inhibitors of growth is the presence of an amino versus a hydroxyl group in the 6' position. We do not know whether the nature of the 6' hydroxyl group caused differences in transport into cells or whether it resulted in different properties with respect to translation. When tested in vitro on poly(U) translation, PM at concentrations much lower than 10 μ M is a potent inhibitor of translation, whereas NM is not an effective inhibitor until concentrations are greater than 10 μ M (13).

We report here that PM and NM were very similar in the inhibition of in vitro polypeptide elongation when tested on polysomes bearing natural mRNA. This inhibition was significant only when the aminoglycoside concentration exceeded 10 μ M. We suggest that the difference found in PM susceptibility of poly(U) translation systems when compared with polysome translation systems are due to PM interference with either the formation of pseudo-initiation complexes in the poly(U) system or inhibition of the first rounds of

elongation when the peptidyl-tRNA is very small. In addition, we suggest that the inhibitory effect produced by PM during in vitro translation of *T. thermophila* polysomes represents a secondary effect which is not a reflection of its mechanism of action in vivo. In vivo treatments with PM or G418, which inhibited the incorporation of [14 C]lysine into protein, also caused shifts in polysome patterns: polysomes were depleted and monosome content rose with drug treatment. Coupling these in vivo results with the relative inability of PM and G418 to inhibit the translation of polysomes in vitro clearly showed that an inhibition of nascent-chain elongation was not responsible for the inhibition of protein synthesis and that the loss of polysome content observed during in vivo drug treatment was not due to premature release of peptidyl-tRNA from polysomes.

The translation of polysomes from mutants resistant to PM and HM showed substantial resistance to HM inhibition during chain elongation. The inhibition of these drug-resistant polysomes at concentrations of PM greater than 10 μ M was not significantly different from the inhibition of wild-type polysomes, further suggesting that any effects obtained with these relatively high concentrations of PM may not reflect the primary mechanism of action on protein synthesis in vivo. We recently reported that misreading during aminoacyl-tRNA selection in vitro is maximal at 10 μ M PM with *T. thermophila* ribosomes (6), demonstrating that a specific interaction of PM with ribosomes occurs at concentrations less than 10 μ M. This result, coupled with the clear shift of ribosomes from polysomes to monosomes and a relative lack of effect on in vitro chain elongation, strongly suggests that the target of PM (and G418) action is at a stage of translation

before initiation, the initiation stage, or a very early stage of elongation. Precedents for aminoglycoside inhibition of initiation have been reported in procaryotes (7, 10, 11). Using translation systems derived from *E. coli*, these investigators have found that SM inhibits the initiation of translation, among its other effects on translation. Similar activities by aminoglycoside antibiotics on the initiation of translation in a eucaryotic organism have not been reported. PM and G418 activity in *T. thermophila* represent the first examples of initiation inhibition by aminoglycoside antibiotics in a eucaryotic organism.

The results from *in vitro* translation of polysomes from wild-type cells and aminoglycoside-resistant mutants demonstrated that HM resistance was displayed at the level of chain elongation. With wild-type polysomes, HM concentrations in only a twofold excess over the ribosome concentration caused an 80% inhibition of polysome translation, suggesting a highly specific interaction of this drug on the ribosome. With the reasonable assumption that leucine comprises about 6% of the total amino acids polymerized by these polysomes, the number of amino acids incorporated per ribosome was between one and two, suggesting that about one round of aminoacyl-tRNA binding and incorporation occurred before HM inhibited elongation.

Analysis of the partial reactions of elongation revealed that peptide bond formation was not inhibited by concentrations of HM up to 10 μ M. Furthermore, translocation of peptidyl-tRNA on polysomes was only modestly inhibited by 10 μ M HM (37% inhibition). Our interpretation of these findings is that HM did not bind to polysomes that had peptidyl-tRNA bound to the A site. As a result, A site-bound peptidyl-tRNA translocated to the P site, HM then bound to the ribosome, a new aminoacyl-tRNA bound to the A site, and a peptide bond formed. The resultant (HM-ribosome-peptidyl-tRNA) complex could not be translocated to the P site again for a second round of elongation.

We noted that there was a correlation between the effects of aminoglycoside antibiotics on the misreading caused by these drugs on *T. thermophila* ribosomes (6) and apparent inhibition of initiation (or early elongation steps). PM and G418, which are disubstituted 2-deoxystreptamine-containing aminoglycoside antibiotics with 6' hydroxyl groups, are the most potent misreaders with *T. thermophila* ribosomes (6) and also gave inhibition of initiation. In contrast, HM, a monosubstituted 2-deoxystreptamine-containing aminoglycoside antibiotic, is a poor misreader (6) and preferentially inhibited chain elongation. These results are the inverse of the relationship between misreading and elongation inhibition found in *E. coli* (9, 19).

The existence of a class of mutants (exemplified by HYR3) which was selectively highly resistant to HM was consistent with a different mechanism of action for HM and PM (and G418). That another class of mutants (HYR16) could be obtained which was concomitantly resistant to low PM, G418, and HM does, however, suggest that the specific binding sites for these antibiotics either overlap each other or are proximal. Regardless of the potential proximity of the binding sites for these antibiotics, the mechanisms of inhibition of protein synthesis for these aminoglycosides differ significantly.

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LITERATURE CITED

1. Albright, E. B., T. S. Nowak, and H. N. Munro. 1978. Assessment of counting efficiencies for labeled protein and other macromolecules in filter disk assays. *Anal. Biochem.* **91**:258-263.
2. Barbacid, M., M. Fresno, and D. Vazquez. 1975. Inhibitors of polypeptide elongation on yeast polysomes. *J. Antibiot.* **28**:453-462.
3. Calzone, F. J., R. C. Angerer, and M. A. Gorovsky. 1982. Regulation of protein synthesis in *Tetrahymena*: isolation and characterization of polysomes by gel filtration and pH 5.3 precipitation. *Nucleic Acids Res.* **10**:2145-2161.
4. Contreras, A., and D. Vazquez. 1977. Cooperative and antagonistic interactions of peptidyl-tRNA and antibiotics with bacterial ribosomes. *Eur. J. Biochem.* **74**:539-547.
5. Eustice, D. C., F. J. Kull, and A. Shrift. 1981. Selenium toxicity: aminoacylation and peptide bond formation with selenomethionine. *Plant Physiol.* **67**:1054-1058.
6. Eustice, D. C., and J. M. Wilhelm. 1984. Fidelity of the eukaryotic codon-anticodon interaction: interference by aminoglycoside antibiotics. *Biochemistry* **23**:1462-1467.
7. Lando, D., M. A. Cousin, and M. P. de Garilhe. 1973. Misreading, a fundamental aspect of the mechanism of action of several aminoglycosides. *Biochemistry* **12**:4528-4533.
8. Lando, D., M. A. Cousin, T. Ojasoo, and J. P. Raynaud. 1976. Paromomycin and dihydrostreptomycin binding to *Escherichia coli* ribosomes. *Eur. J. Biochem.* **66**:597-606.
9. Laughrea, M. 1981. Speed-accuracy relationships during *in vitro* and *in vivo* protein biosynthesis. *Biochimie* **63**:145-168.
10. Lelong, J. C., M. A. Cousin, D. Gros, M. Grunberger-Mango, and F. Gros. 1971. Streptomycin induced release of fmet-tRNA from the ribosomal initiation complex. *Biochem. Biophys. Res. Commun.* **42**:530-537.
11. Modell, J., and B. D. Davis. 1970. Breakdown by streptomycin of initiation complexes formed on ribosomes of *E. coli*. *Proc. Natl. Acad. Sci. U.S.A.* **67**:1148-1155.
12. Orias, E., and P. J. Bruns. 1976. Induction and isolation of mutants in *Tetrahymena*. *Methods Cell Biol.* **13**:247-282.
13. Palmer, E., and J. M. Wilhelm. 1978. Mistranslation in a eukaryotic organism. *Cell* **13**:329-334.
14. Palmer, E., J. M. Wilhelm, and F. Sherman. 1979. Phenotypic suppression of nonsense mutants in yeast by aminoglycoside antibiotics. *Nature (London)* **277**:148-149.
15. Pestka, S. 1972. Studies on transfer ribonucleic acid-ribosome complexes. *J. Biol. Chem.* **247**:4669-4678.
16. Skogerson, L. 1979. Separation and characterization of yeast elongation factors. *Methods Enzymol.* **60**:676-685.
17. Sutton, C. A., M. Ares, and R. L. Hallberg. 1978. Cycloheximide resistance can be mediated through either ribosomal subunit. *Proc. Natl. Acad. Sci. U.S.A.* **75**:3158-3162.
18. Vazquez, D. 1978. Translation inhibitors. *Int. Rev. Biochem.* **18**:169-232.
19. Vazquez, D. 1979. Inhibitors of protein synthesis. *Mol. Biol. Biochem. Biophys.* **30**:1-306.
20. Vazquez, D., E. Zaera, H. Dolz, and A. Jimenez. 1982. Protein biosynthesis in eukaryotes, p. 311-337. In R. Perez-Bercoff (ed.), NATO Advanced Study Institute on Protein Biosynthesis in Eukaryotes. Plenum Publishing Corp., New York.
21. Wilhelm, J. M., J. J. Jessop, and S. E. Pettitt. 1978. Aminoglycoside antibiotics and eukaryotic protein synthesis: stimulation of errors in the translation of natural messengers in extracts of cultured human cells. *Biochemistry* **17**:1150-1153.
22. Wilhelm, J. M., S. E. Pettitt, and J. J. Jessop. 1978. Aminoglycoside antibiotics and eukaryotic protein synthesis: structure-function relationships in the stimulation of misreading with a wheat embryo system. *Biochemistry* **17**:1143-1149.
23. Wormbach, P., and K. H. Nierhaus. 1983. The inhibition pattern of antibiotics on the extent and accuracy of tRNA binding to the ribosome, and their effect on the subsequent steps in chain elongation. *Eur. J. Biochem.* **130**:9-12.