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Role of the 5.8S rRNA in ribosome translocation

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ABSTRACT

Studies on the inhibition of protein synthesis by specific anti 5.8S rRNA oligonucleotides have suggested that this RNA plays an important role in eukaryotic ribosome function. Mutations in the 5.8S rRNA can inhibit cell growth and compromise protein synthesis *in vitro*. Polyribosomes from cells expressing these mutant 5.8S rRNAs are elevated in size and ribosome-associated tRNA. Cell free extracts from these cells also are more sensitive to antibiotics which act on the 60S ribosomal subunit by inhibiting elongation. The extracts are especially sensitive to cycloheximide and diphtheria toxin which act specifically to inhibit translocation. Studies of ribosomal proteins show no reproducible changes in the core proteins, but reveal reduced levels of elongation factors 1 and 2 only in ribosomes which contain large amounts of mutant 5.8S rRNA. Polyribosomes from cells which are severely inhibited, but contain little mutant 5.8S rRNA, do not show the same reductions in the elongation factors, an observation which underlines the specific nature of the change. Taken together the results demonstrate a defined and critical function for the 5.8S rRNA, suggesting that this RNA plays a role in ribosome translocation.

INTRODUCTION

Although initially thought of as simply structural components of the ribosome, many now view the ribosomal RNAs as major functional constituents. Indeed recent studies by Noller and co-workers (1) have shown that the 23S rRNA participates in the peptidyl transferase function and aminoacyl esterase activity in the *Tetrahymena* ribozyme (2) suggests that the first aminoacyl tRNA synthetase could have been an RNA molecule.

The cytoplasmic ribosomes of eukaryotes contain one additional integral RNA component, the 5.8S rRNA, which forms an RNA–RNA complex with the 25–28S rRNA of the large ribosomal subunit (3–5). Although it is clear that in other ribosomes this sequence is present as part of the large subunit rRNA (6,7), the reason for this evolutionary change is unknown. Speculation regarding the function of this sequence has focused on a role in tRNA binding (8). Indeed, accessibility studies indicate that the molecule may be localized in the ribosomal interface (9,10) and other studies utilizing temperature denaturation (11) or affinity chromatography have revealed ternary (12) or quaternary (13) complexes of the 5S and 5.8S rRNAs as well as ribosomal proteins or tRNA. Furthermore, analyses based on chemical cross-linking (14) show that some 5.8S rRNA binding proteins are proximal to the A-site.

Direct evidence for a functional role was first provided by a study utilizing antisense oligonucleotides which were complementary to specific exposed regions in the ribosome-associated 5.8S rRNA (15). These antisense probes demonstrated a significant and specific inhibition of protein synthesis *in vitro*, consistent with an important role in ribosome function. More recent analyses utilizing efficiently expressed mutant 5.8S rRNAs (16) have confirmed a functional role, demonstrating little or no effect on initiation, but providing *in vivo* evidence for a role in protein elongation or termination.. These mutants were characterized by a consistent increase in the size of the polyribosomes, as well as elevated levels of ribosome associated tRNA.

In the present study this defect was examined further when mutant ribosomes were tested with respect to their sensitivity to specific inhibitors of protein synthesis. The results confirm a defect in the elongation cycle and suggest that the 5.8S RNA plays a direct role in ribosome translocation.

MATERIALS AND METHODS

Strains, plasmids and expression of mutant rRNA genes

All studies utilized transformants of *Schizosaccharomyces pombe*, strain h$^-$ leu-32 ura4-D18 (17), prepared by using recombinants of pFL20, a high copy yeast shuttle vector (18) containing mutant rDNA transcriptional units (Table 1). Cells were grown with aeration under selective conditions in minimal medium (0.67% nitrogen base containing 2% dextrose, 80 µg/ml leucine and 200 µg/ml asparagine); growth was determined using the absorbancy of cultures at 550 nm. Specific mutations were introduced into the 5.8S rRNA sequence of the *S.pombe* rDNA transcriptional unit using a two-step PCR-based strategy (19). The changes were subcloned into the pFL20 shuttle vector for *S.pombe* transformation and expression as previously described (16). The *S.pombe* cells were transformed by electroporation as described by Prentice (20). For electroporation the cells were grown in YED medium (3% dextrose, 0.5% yeast extract and 0.5% KH_2PO_4), collected by centrifugation, washed three times with cold 1.2 M sorbitol and suspended in sorbitol solution with plasmid DNA (10 µg/200 µl). The cells were pulsed with 1800 V using an Invitrogen Electroporator (Invitrogen Corp., San Diego, CA), diluted further with 0.5 ml of cold 1.2 M sorbitol, placed on ice for 5 min and finally plated on minimal medium plates for incubation at 30°C (4–6 days).

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Table 1. Effect of 5.8S rRNA mutations on the growth rate of transformed *S.pombe*

Transforming plasmid[a]	Plasmid copy no.[b]	Doubling time (h)[c]	Percent mutant 5.8S rRNA[d]
pFL20/SprDNA[e]	84	4.3	0.0
pFL20/Sp5.8A13Δ4	60	4.5	4.3
pFL20/Sp5.8A18Δ3A44i5	80	4.7	nd
pFL20/Sp5.8C32i4	86	4.7	5.6
pFL20/Sp5.8T40G41T42C43T44	87	5.6	nd
pFL20/Sp5.8A44i5	78	6.2	43.5
pFL20/Sp5.8A44i5G104C105	78	9.2	5.1
pFL20/Sp5.8A44i5A146A147C148C149C150	82	5.8	0.7
pFL20/Sp5.8A44i5A153G154T155A156A157	90	6.2	40.7
pFL20/Sp5.8A105i4	55	6.3	4.4
pFL20/Sp5.8A146A147C148C149C150	83	5.1	4.5
pFL20/Sp5.8G123i2A131i2	74	4.2	55.4

[a]The mutation is identified beginning with the residue number where the changes begin; insertion is indicated by i and deletion is indicated by Δ.
[b]Relative copy number as determined in Materials and Methods.
[c]Hr/doubling for logarithmically growing cells in selective medium as determined from the absorbancy at 550 nm.
[d]Percent mutant 5.8S rRNA taken from density measurements of total cellular RNA that was fractionated and stained with methylene blue.
[e]Normal 5.8S rRNA sequence.
nd, none detected.

The expression of mutant RNA usually was confirmed and quantified using polyacrylamide gel electrophoresis to separate the endogenous and mutant 5.8S rRNA (16); after staining with methylene blue, the image was captured using a Gel Doc 1000 system (Bio-Rad Laboratories, Richmond, CA) and analyzed with Molecular Analyst/PC software. For transformants with little or no detectable mutant 5.8S rRNA, the expression of the mutant rDNA transcriptional unit was confirmed by northern gel hybridization analysis. Total RNA was extracted from transformed cells and fractionated by electrophoresis on 1.4% agarose gels containing 0.8% formaldehyde (21). The RNA was transferred to Zetabind nylon membrane (CUNO Inc., Meriden, CT) and incubated for 18 h at 45°C in hybridization buffer (6× SSPE, 1% SDS, 2× Denhardt's solution, 0.3 μg/ml denatured salmon sperm DNA) containing 5′-labelled oligonucleotide probe as previously described (22). The filters were washed three times in 6× SSPE at 55°C. The oligonucleotide used to make the mutant 5.8S rRNA was used as a specific probe in each instance.

Assay for *in vitro* protein synthesis

Cultures of normal or transformed cells were grown to an absorbancy of 0.6 at 550 nm, harvested by centrifugation (3000 *g*) for 5 min at 4°C and washed with water followed with extraction buffer (0.1 M NH_4Cl, 2 mM Mg acetate, 2 mM dithiothreitol, 20% glycerol in 20 mM HEPES/KOH, pH 7.4). The pellet was suspended in cold extraction buffer (1 ml/g wet weight) and the cells were broken by vortexing with an equal volume of glass beads (0.5 mm) using five 30 s pulses at maximum speed (16). Cell debris was removed by centrifugation at 15 000 *g* for 10 min (4°C); the supernatant was removed avoiding the top lipid layer and further cleared by centrifugation at 15 000 *g* for 15 min. The resulting extract was then diluted with homogenization buffer to 50 A_{260} units/ml prior to assay.

The assay protocol was essentially that of Picard and Wegnez (23). Cell-free extract (40 μl) was added to 60 μl of reaction mix (125 mM NH_4Cl, 3 mM Mg acetate, 0.5 mM ATP, 0.1 mM GTP, 25 mM creatine phosphate, 20 μl/ml creatine phosphokinase, 10% glycerol, 20 M HEPES/KOH, pH 7.4) containing 40 μM of each amino acid except leucine and 2 Ci [^{3}H]leucine (1.46 μM). After incubation at room temperature, aliquots (20 μl) were removed at appropriate times, mixed with 1.5 ml of cold 10% TCA and heated for 3 min at 90°C before being cooled on ice and diluted further with 1.5 ml of 10% TCA. 0.5 ml of a 3% solution of Casamino acids was added to each sample; the mixture was precipitated on ice for 10 min and applied to Whatman GF/C glass filters (Maidstone, UK). The acid-insoluble precipitate was washed twice with 10% TCA and once with ethanol before the membranes were dried at 80°C and the radioactivity was determined by liquid scintillation counting. To normalize the incorporation profiles for differences in ribosome concentration, RNA was extracted from aliquots of each extract with SDS/phenol and fractionated on 8% polyacrylamide gels to determine the relative ribosome concentration, assuming one molecule of 5 and 5.8S rRNA per ribosome (16).

Preparation and analysis of ribosomal protein

Protein was extracted from purified polyribosomes or washed ribosomal subunits based on procedures described by Torano and co-workers (24) and Van Ryk and Nazar (25). Normal or transformed yeast cells were grown with constant shaking at 30°C in minimal medium broth to an absorbancy of 0.4–0.6 at 550 nm. The pellet was resuspended in 1 ml/g wet weight of cold

(4°C) lysis buffer (0.1 M NaCl, 30 mM $MgCl_2$, 10 mM Tris–HCl pH 7.4), mixed with an equal volume of glass beads and the cells were broken by vortex with eight 20 s pulses separated by 20 s pauses on ice. Triton X-100 was added to a final concentration of 1% and the lysate was cleared of cellular debris by centrifugation at 15 000 *g* for 15 min in a Beckman (Palo Alto, CA) JA-21 rotor (4°C). For polyribosomes, the supernatant was cleared a second time and layered on a 30% sucrose cushion or 5–40% sucrose gradient. When layered on 30% sucrose, the polyribosomes were collected by centrifugation for 2 h at 50 000 r.p.m. in a Beckman 70.1 Ti rotor; when fractionated on a sucrose gradient, centrifugation was for 2 h at 27 000 r.p.m. in a Beckman SW41 Ti rotor. For washed ribosomes, the cell pellet was initially resuspended in a high salt buffer (0.8 M KCl, 20 mM dithiothreitol, 12 mM Mg acetate, 50 mM Tris–HCl pH 7.4) and after the cells were broken by vortex, the debris again was cleared twice by centrifugation at 15 000 *g*. This supernatant was layered on a 10% sucrose cushion and the ribosomes were collected by centrifugation for 2 h at 50 000 r.p.m. in a Beckman 70.1 Ti rotor. Pellets were resuspended in high salt buffer and incubated on ice for 10 min. The suspension was cleared again by centrifugation at 15 000 *g*, layered on a second 10% sucrose cushion and collected by centrifugation as described above.

The resultant ribosome or polyribosome pellets were suspended in 0.5 ml of water and the protein was extracted by mixing on ice with 0.4 vol 1 M $MgCl_2$ and 2 vol glacial acetic acid (26). After 30 min, the protein extract was cleared by centrifugation for 15 min at 15 000 *g* and the supernatant was dialyzed against 10 mM Tris–HCl pH 7.4 at 4°C for 24 h with constant stirring before being freeze dried.

For electrophoretic analyses, the protein was dissolved in loading buffer and aliquots were fractionated in one-dimension on 10% SDS/polyacrylamide gels as described by Laemmli *et al.* (27) or by two-dimensional gel-electrophoresis as described by Geyl *et al.* (28) and Kaltschmidt and Wittmann (29). Electrophoresis was performed using a mini 2-D gel electrophoresis system (Bio-Rad laboratories, Hercules, CA); all gels were stained with Coomassie Brilliant Blue R. For quantitative studies, images were captured using a Gel Doc 1000 system (Bio-Rad Laboratories, Hercules, CA) and analyzed using Molecular Analyst/PC software.

Labelling of EF2 by ADP-ribosylation

Elongation factor 2 was quantified by using ADP-ribosylation to specifically label the protein (30). Ribosome protein was prepared from polyribosomes as described above and 35 µg aliquots were incubated for 5 min at 0°C in 50 µl 50 mM Tris–HCl pH 7.4, containing 50 mM dithiotheritol, 25 µg of bovine serum albumin, 2–4 µmol [adenine-2, 8-^{3}H]NAD (ICN Pharmaceuticals, Costa Mesa, CA) and 15 LF units (31) of diphtheria toxin (Connaught Laboratories, Toronto, Canada). The reaction mixture was fractionated on 8% SDS/polyacrylamide gels and the labelled bands were visualized by autoradiography.

RESULTS

As a basis for functional and structural analyses, in this and previous studies, mutations were introduced into the 5.8S rRNA sequence at sites which have been highly conserved in the course of evolution or have exhibited unusual reactivity to chemical probes (e.g., ref. 9). Subsequent analyses on the effect of mutant

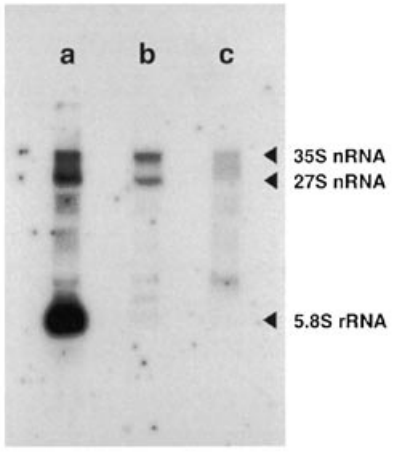

Figure 1. Hybridization analysis of mutant rRNA transcripts in *S.pombe*. Total cellular RNA was prepared from *S.pombe* cells transformed with pFL20/Sp5.8A44i5 (lane a), pFL20/Sp5.8A44i5G104C105 (lane b) or pFL20/SprDNA (lane c), fractionated on a 1.4% agarose gel containing 0.8% formaldehyde and transfered onto a nylon membrane. The membrane was hybridized with 5′ end-labeled oligonucleotide probe (5′-TTTCGCTGCGGA-TCCTTCTTCATC-3′) specific for the A44i5 sequence mutation (22). The positions of the mature 5.8S rRNA and nucleolar precursor RNAs (nRNA) are indicated on the right.

5.8S rRNA expression indicate a wide range of influence on the growth rate (Table 1). Since the mutant rDNAs have been shown to be efficiently expressed (16,32), the effects do not correlate with the plasmid copy number which, as anticipated, is relatively constant. Instead the effects must result from changes in ribosome structure or function. Indeed a detailed analysis (16) of one of these mutants (Sp5.8A44i5) has documented an inhibition of protein synthesis, both *in vivo* and *in vitro*; with 40–50% mutant RNA, the cellular growth and protein synthesis rates also were reduced by ~40%.

As further shown in Table 1, analyses of additional mutants in the present study indicate at least four categories of mutation. One of the mutants, Sp5.8G123i2A131i2, was very efficiently expressed and had no effect on the growth rate, characteristics which are ideal for a structural marker in studies of rDNA expression (32). On the other hand, mutants such as Sp5.8A44i5 (GGAUC insert after A44) and Sp5.8A44i5A153 G154T155A156A157 resulted in substantial amounts of ribosomal mutant RNA, with intermediate effects on the growth rate; mutants such as Sp5.8A44i5G104C105 resulted in small amounts of ribosomal mutant RNA, but severe effects on the growth rate; and, mutants such as Sp5.8C32i4 or Sp5.8A44i5A146A147C148C149C150 which resulted in traces or no detectable mutant 5.8S rRNA and little or no effect on the growth rate. As noted initially, since all the basic rDNA constructs remain constant, all RNAs should be expressed with equal efficiency, the reduced amounts of mutant RNA reflecting RNA instability and rapid degradation. To confirm this, mutations with severely reduced amounts of RNA were further examined by northern gel hybridization analysis. As illustrated by the example shown in Figure 1, these analyses confirm an efficient transcription of the mutant rDNA with a highly unstable product which is largely or completely degraded during RNA processing and ribosome assembly. In this example, the initial 35S nRNA transcript is essentially equally intense with both mutants (lanes a and b), but the 5.8S rRNA is clearly absent

in the unstable mutant (lane b). In some cases this severely reduced level of mutant ribosome actually proves to be advantageous to the cell. Changes such as the multiple mutations in Sp5.8A44i5G104C105 clearly would be essentially lethal, but the instability of the assembled ribosomal subunit results in a sufficiently low mutant population, (5–10%) to permit some cell growth (one doubling per 9.2 h) and survival.

Although the results shown in Table 1 and the previously detailed characterizations of Sp5.8A44i5 suggested the 5.8S rRNA plays an important role in protein elongation (16), the evidence is largely circumstantial and a specific step was not identified. To provide more direct evidence, in this study inhibitors of protein synthesis were incubated with mutant ribosomes to search for a step which was unusually sensitive to a specific type of inhibitor. Since protein synthesis is fundamentally a series of enzyme catalyzed reactions, a step made defective by mutation would become rate limiting and, therefore, is likely to become more sensitive to smaller amounts of inhibitor than in normal ribosomes. In the present studies, sufficient inhibitor was used to inhibit cell free protein synthesis with normal ribosomes by ~50%. It was anticipated that this level of inhibition would clearly compromise ribosomal function, but permit sufficient protein synthesis to allow the detection of any significant effect by the mutant RNA. In this survey, seven different antibiotics were examined, representing a broad range of specificity. As shown by the example synthetic profiles in Figure 2 (left), and the quantitative results summarized in Table 2, all the results were consistent with a defect in the elongation cycle. The mutant ribosomes were not unusually sensitive to antibiotics such as aurintricarboxylic acid or emitine which inhibit initiation (33) or act on the 40S ribosomal subunit (see ref. 34), but clearly were more sensitive to the other antibiotics which are known to inhibit elongation and act on the 60S ribosomal subunit. Of these, the mutant cell extracts were most inhibited by the two antibiotics (cycloheximide and diphtheria toxin) which are known to act primarily or entirely on translocation (see ref. 34). This was emphasized further with still lower concentrations of antibiotic. As shown in Figure 2 (right) and quantified in Table 3, when the concentration of anisomycin, a specific inhibitor of the transpeptidation reaction (see ref. 34), and diphtheria toxin, a specific

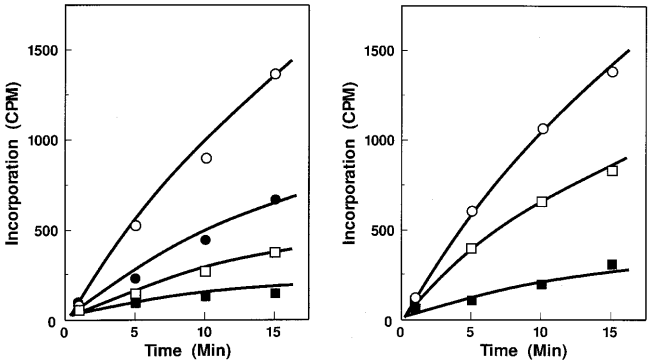

Figure 2. Effect of antibiotics on ribosomes containing mutant 5.8S rRNA. (**Left**) A cell free extract was prepared from yeast transformed with pFL20/Sp5.8A44i5 and assayed for *in vitro* protein synthetic activity in the absence (open circles) and presence of aurintricarboxylic acid (50µg/ml; closed circles), anisomycin (50 µg/ml; open squares) or diphtheria toxin (20 LF/ml; closed squares). The incorporation of [^{3}H]leucine into acid-precipitable counts was determined by scintillation counting. (**Right**) Extracts were assayed in the absence (open circles) and presence of anisomycin (0.1 µg/ml; open squares) or diphtheria toxin (0.04 LF/ml; closed squares).

inhibitor of ribosome translocation (see ref. 34) was substantially reduced, the differences became even more striking. Extracts from normal cells were essentially uninhibited, the inhibition with mutant extract and anisomycin was <50%, but the mutant extract remained very sensitive to diphtheria toxin with only a slight increase in activity to ~15% of the normal level.

The unusual sensitivity to diphtheria toxin was further underlined when the same antibiotics were used with extracts containing mutant 5S rRNA. Previous studies on this RNA have identified mutations which also adversely affect protein synthesis, *in vitro* (25). As illustrated in Figure 3, when extracts containing such a mutant 5S rRNA (Y5U90i5) were treated with anisomycin or diphtheria toxin, the elevated sensitivity to diphtheria toxin was obviously absent; with both antibiotics the synthetic activity was reduced by ~50%. The differential effect with mutant 5.8S rRNA clearly was specific to this RNA molecule.

Table 2. Effect of 5.8S rRNA mutation on inhibitor sensitivity

Inhibitor	Target[a]		Concentration	Relative rate of protein synthesis[b]	
	Subunit	Step		pFL20/SprDNA	pFL20/Sp5.8A44i5[c]
None	–	–	–	100%	100%
Anisomycin	60S	transpeptidation	50 µg/ml	48.5 ± 3.2	20.2 ± 4.1
Aurintricarboxylic acid	–	initiation	10 µg/ml	49.3 ± 4.2	54.1 ± 7.8
Cycloheximide	60S	translocation	10 µg/ml	42.7 ± 6.3	13.5 ± 2.5
Diphtheria toxin	60S	translocation	20 LF/ml	56.8 ± 6.8	10.5 ± 4.2
Emitine	40S	translocation	50 µg/ml	37.2 ± 9.6	46.1 ± 8.2
Fusidic acid	60S	tRNA binding	5 µg/ml	48.0 ± 5.6	25.2 ± 3.6
Puromycin	60S	transpeptidation	50 µg/ml	45.2 ± 5.3	23.6 ± 3.5

[a]Primary or only site of inhibition.
[b]Incorporation rate relative to extracts without inhibitor; values are averages of three to four determinations.
[c]The activity of this extract relative to an extract of pFL20/SprDNA transformed cells was ~48% (16).

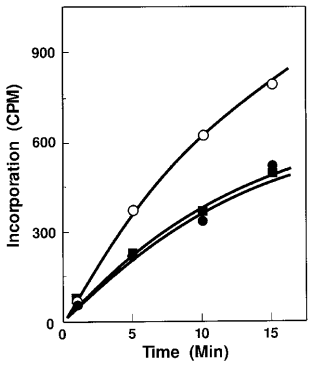

Figure 3. Effect of antibiotics on ribosomes containing mutant 5S rRNA. Cell free extracts were prepared from yeast transformed with pYFU90i5 (25) and assayed for *in vitro* protein synthetic activity in the absence (open circles) and presence of anisomycin (50 µg/ml; closed squares) or diphtheria toxin (20 LF/ml; closed circles). The incorporation of [^{3}H]leucine into acid-precipitable counts was determined by scintillation counting.

Table 3. Effect of 5.8S rRNA mutations at low inhibitor concentrations

Inhibitor	Concentration	Relative rate of protein synthesis[a]	
		pFL20/SprDNA	pFL20/Sp5.8A44i5
None	–	100%	100%
Anisomycin	0.1 µg/ml	98	56.2 ± 5.2
Diphtheria toxin	0.4 LF/ml	93	14.2 ± 6.3

[a]Incorporation rate relative to extracts without inhibitor.

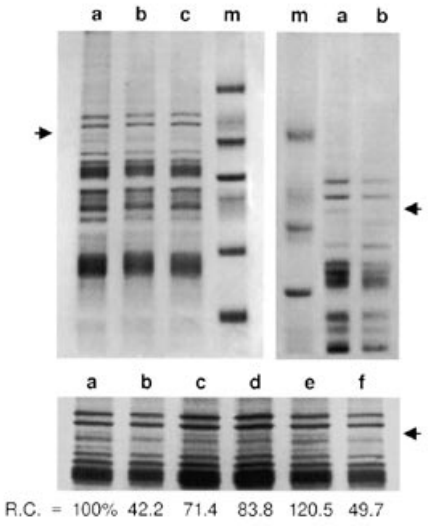

Figure 4. Elongation factor 1 polypeptides in polyribosomes from *S.pombe* cells expressing mutant 5.8 rRNAs. (**Top left**) Polyribosomes were prepared from cells transformed with pFL20/SprDNA (a) pFL20/Sp5.8A44i5 (b) or pFL20/Sp5.8A44i5G104C105 (c) as previously described (16), the ribosomal proteins were extracted (26) and fractionated together with a molecular weight standard (m) on 10% SDS/polyacrylamide gels as described by Laemmli *et al.* (28) and stained with commasie blue. (**Top right**) Polyribosomes were prepared from cells transformed with pFL20/SprDNA (a), washed with high salt buffer (b), and the ribosomal proteins at each step were extracted and fractionated together with a molecular weight standard (m) by SDS/polyacrylamide gel electrophoresis. (**Bottom**) Cell free extracts were prepared from cells transformed with pFL20/Sp5.8A44i5 and incubated for 5 min with antibiotics as described in Figure 2. Ribosomal proteins were extracted and fractionated by SDS/polyacrylamide gel electrophoresis. The image was captured using a Gel Doc 1000 (Bio-Rad Laboratories, Richmond, CA) and the EF1 polypeptides were quantified using Molecular Analyst/PC software. Lanes a–f contain ribosomal proteins from normal ribosomes, mutant ribosomes and ribosomes treated with anisomycin, cycloheximide, puromycin and diphtheria toxin, respectively. The relative EF1 concentrations (R.C.) representing averages for two determinations are presented below.

A number of antibiotics such as diphtheria toxin act indirectly to interfere with the binding of translation factors or other components of the synthetic reaction. To identify effects of this nature and perhaps even to localize the specific mechanistic failure, analyses of the constituents of active ribosomes were undertaken. Previous studies (16) had already shown normal levels of rRNA and elevated levels of ribosome-associated tRNA, observations which eliminated the possibility of RNA loss or a defect in the initial binding of tRNA to ribosomes. In this study the protein constituents were examined including the elongation factors, EF1 and EF2. Attention was focused on two specific mutations, Sp5.8A44i5, the mutant which was used for the antibiotic analyses and is present in 40–50% of the ribosome population as well as Sp5.8A44i5G104C105, a mutant which more severely inhibits protein synthesis, but which is present in only 5–10% of the ribosomes. Together, these mutants also permitted differentiation between direct effects which would only be observed in 40–50% of the population with Sp5.8A44i5 and indirect effects of inhibited protein synthesis in general, which would be observed in the entire population, even with Sp5.8A44i5G104C105 which constitutes <10% of the ribosome population. In the second instance the effect would also be proportional to the inhibition in cell growth and protein synthesis. Two-dimensional gels, which, generally, were somewhat subject to some technical variation, revealed no reproducible changes in the ribosomal proteins (results not shown). In contrast, as shown in Figure 4, one-dimensional analyses did reveal reproducible differences in at least one minor band which was consistent with elongation factor 1 (EF1). As shown in the upper panels, a band of 38 000 molecular weight was substantially and reproducibly reduced in the Sp5.8A44i5 mutant, but only slightly lower with Sp5.8A44i5G104C105 (left panel). Several other variations in band intensity are present but none were found to be reproducible in replicate experiments. The size of the EF1 polypeptide was consistent with the previously described subunit of the yeast EF1 (35). Furthermore, when purified ribosomes are washed with high salt buffer, a treatment which removes translation factors (24), the same band was essentially absent (right panel), confirming its identity as an EF1 polypeptide. The presence of substantial amounts of this EF1 polypeptide in the slower growing mutant, however, strongly suggests that EF1 is largely or entirely absent only in ribosomes containing mutant RNA rather than all the ribosomes. When protein synthesis is even more severely compromised by a small number of defective ribosomes, this large drop in EF1 polypeptide is not observed.

To further evaluate the significance of a reduction in EF1, active ribosomal preparations also were examined after treatment with antibiotics. As shown in Figure 4 (lower panel), only cycloheximide and diphtheria toxin resulted in reductions to the EF1 bands. As indicated below the gel insert, the changes in EF1, which are observed with diphtheria toxin when protein synthesis

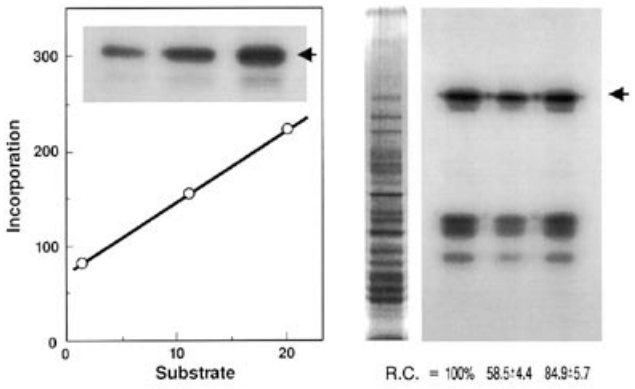

Figure 5. Elongation factor 2 polypeptides in polyribosomes from *S.pombe* cells expressing mutant 5.8S rRNAs. (**Left**) Polyribosomes were prepared from cells transformed with pFL20/SprDNA and the EF2 factor in varying aliquots of protein was labelled with [adenine-2, 8-^{3}H]NAD as described in Materials and Methods. The labelled mixture was fractionated by SDS–polyacrylamide gel electrophoresis before autoradiography (see insert) and the determination of radioactivity in the labelled bands. (**Center**) Ribosomal protein was extracted from polyribosomes which were prepared from cells transformed with mutant rDNA and labelled with NAD^{+} as described above, fractionated on 10% polyacrylamide gels and stained with coomassie blue. (**Right**) The stained gel fractionations for protein extracts of cells transformed with pFL20/SprDNA, pFL20/Sp5.8A44i5 or pFL20/Sp5.8A44i5G104C105 (shown left to right) were dried and exposed to film. The radioactivity was determined by scintillation counting or image analysis and the relative EF2 concentrations representing averages of three determinations are presented below. In all panels the intact EF2 factor is indicated by the arrow.

is inhibited to approximately the same degree as with mutant RNA, are also quantitatively similar, again raising a special relationship between the effects of 5.8S rRNA mutation and the action of diphtheria toxin.

Diphtheria toxin is known to specifically inhibit translocation by modifying elongation factor 2 (EF2), a form which no longer binds to ribosomes. To search for changes in EF2 in the mutant ribosomes, EF2 was specifically labelled and quantified by ADP-ribosylation (30). As shown in Figure 5 (left panel), when increasing amounts of extracted polyribosomal protein were added to the reaction mixture, linear incorporation was observed over a wide concentration range. When this assay was further applied to extracts of cells expressing mutant 5.8S rRNA significant differences were again observed. As also indicated in Figure 5 (right panel), the level of EF2 was substantially reduced in cells containing the Sp5.8A44i5 mutant RNA, but again was only slightly lower with Sp5.8A44i5G104C105. It is equally important to note that both changes again approximate the amount of mutant RNA which is present in the ribosome (Table 1), rather than the degree to which protein synthesis is inhibited. This indicates that the reduced level of EF2 is only found in the defective ribosomes.

DISCUSSION

The present study strongly supports previous suggestions that the 5.8S rRNA plays a critical role in protein elongation. Mutations such as Sp5.8A44i5G104C105, which result in severe reductions in protein synthesis even though <10% of the RNA is mutant, illustrate the critical nature of this RNA, while the substantial differences in antibiotic sensitivity illustrate the specific nature of the defect. While the action of an antibiotic is sometimes not direct or specific, the use of several antibiotics at each step of protein synthesis (Table 2) has provided a greater degree of certainty. Taken together, therefore, the antibiotic studies confirm a role in protein elongation and suggest that the ribosomes actually are defective in translocation. This is entirely consistent with previous analyses which demonstrated elevated polyribosome profiles in cells expressing the same mutant RNAs (16). As summarized in Tables 2 and 3, the mutant ribosomes were generally more sensitive to antibiotics which act on the ribosomal 60S subunit during protein elongation, but were most sensitive to antibiotics which were partially or entirely specific to translocation. This was especially true of diphtheria toxin, an antibiotic which is highly specific for translocation.

Previous studies on cells expressing Sp5.8A44i5 also indicated elevated levels of polyribosome-associated tRNA (16), an observation which suggested that the synthetic cycle was compromised after the incoming charged tRNA is bound, but before the uncharged ribosome-associated molecule is released. Protein analyses in this study are again consistent with this suggestion. As shown in Figure 5, the mutant ribosomes contained reduced levels of EF1, consistent with a release of factor after the binding of charged tRNA (36). Similarly, the reduced levels of EF2, illustrated in Figure 5, are consistent with a defect prior to or during EF2 binding but before the release of uncharged tRNA (37,38). The fact that the levels of factor correlate with the amount of mutant RNA and not the degree of inhibition makes it very likely that the changes reflect only the mutant ribosomes and not just general changes in protein synthesis. All of the results are consistent with a defect in EF2 binding, although other possibilities remain.

Ribosome translocation has been extensively studied in bacteria (39) and, more recently, the equivalent steps have been examined in considerable detail in eukaryotes, particularly in mammalian cells (40). These include the binding of charged tRNA in the presence of EF1, the formation of a peptide bond, the release of uncharged tRNA and the induction of ribosome translocation after the binding of EF2. Such assays will have to be adapted for use with *S.pombe* in order to verify the specific step which is compromised with defective 5.8S rRNA. In the interim, the present results continue to implicate the 5.8S rRNA in protein elongation and serve to focus defining assays as they can be applied to studies in *S.pombe*.

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