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Specific cleavage of ribosomal RNA caused by alpha sarcin

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ABSTRACT

Alpha sarcin causes the specific cleavage of RNA from 80S ribosomes and 60S subunits of yeast, but not from the 40S subunits to produce a small RNA fragment. The fragment was also produced on treatment of the 60S subunits of wheat germ ribosomes. The fragment has a molecular weight of 100,000 and is a cleavage product of the large RNA species in the 60S subunits. The fragment is not derived from the 5'end of the yeast 25S RNA nor does it bind to 5.8S RNA and we propose that the fragment represents the 3' terminal 320 nucleotides of 25S rRNA. The ability to produce fragment could not be separated from the ability of alpha sarcin to inhibit protein synthesis. Alpha sarcin also causes the specific cleavage of the 23S RNA of the E. coli subunit to produce a smaller fragment of RNA than that produced from eukaryote ribosomes.

INTRODUCTION

Alpha sarcin is a potent inhibitor of eukaryotic protein synthesis which apparently blocks the ribosome-dependent elongation factor 1-GTPase reaction.[1] It is a basic protein with a molecular weight of about 16,000 which was isolated from a mold, Asperigillus giganteus[2]. Because it inhibits protein synthesis at very low concentrations, it is thought to act enzymatically, perhaps to inactivate a component involved in translation. There are several other eukaryotic protein synthesis inhibitors which are known to act catalytically. They are ricin, abrin[3], pokeweed antiviral protein (PAP)[4], diptheria toxin[5], and Pseudomonas aeruginosa toxin[6]. Only in the case of the latter two inhibitors is the molecular basis of inactivation known. They inactivate elongation factor II by ADP-ribosylation. We have investigated how alpha sarcin might inhibit protein synthesis, but were unable to inactivate any specific cellular fraction or ribosome subunit, probably because of difficulty in removing alpha sarcin from the treated fractions; preincubation did not increase the inhibition. However, when analyzing the ribosomal RNA from ribosomes which had been treated with alpha sarcin, we observed the appearance of a cleavage product of ribosomal RNA. We show here that alpha sarcin induces a specific

cleavage in the largest RNA species of the 60S subunit of the ribosomes of yeast and wheat germ. This cleavage produces a fragment about 300 nucleotides long, probably at the 3' end. The fragment does not bind to 5.8S RNA. Alpha sarcin also induces a specific cleavage in the largest RNA species of E. coli 50S subunits to produce a fragment which is smaller than that from eukaryotes.

MATERIALS AND METHODS

RNA Gel Electrophoresis. For routine analysis of RNAs larger than 6S (e.g. the fragment), 20 µl of ribosomes or subunits at a concentration of 50-150 OD_{260} were treated as described in the legends or in the text and the reactions were terminated by adding 20 µl of 10 M urea and 5 µl of 10% sodium dodecyl sulfate (SDS). The mixture was allowed to stand at room temperature for 15 min with occasional shaking. Bromphenol blue in 60% glycerol (5 µl) was added and the sample layered on a 5 mm diameter cylindrical polyacrylamide gel. Acrylamide gels were prepared as described by Peacock and Dingman[7] except the gels contained 0.1% SDS. The acrylamide concentration was usually 3.5%. Electrophoresis was carried out at 120 v at room temperature until the bromphenol blue ran off the end of the gel; the gels were stained with methylene blue. On occasion, gels containing less than 3.5% acrylamide were used and then 0.5% agarose was added. For the analysis of low molecular weight RNA, the RNA was extracted and run on gels as described by Rubin[9] and stained with Stains-all[10].

Preparation of Radioactive RNA. The yeast cells were grown in low phosphate medium[9] to a density of about 1.5×10^7 cells/ml. They were then incubated with 10 mC/250 ml of carrier free $H_3{}^{32}PO_4$ for 3-4 hr. Subunits were prepared as described previously[11]. The alpha sarcin induced RNA fragment was obtained by incubating 20 µl of labelled 60S subunits for 10 min at 30C with alpha sarcin at a concentration of 2-4 µg/ml. Gels (3 mm diameter) were run as described above using 2.5% acrylamide - 0.5% agarose. This gel was embedded in the top of a 7% acrylamide gel slab and run as above until 30 min after the tracking dye ran off the bottom of the gel. The slab was autoradiographed and the extra spot on the alpha sarcin treated RNA containing gel was cut out. The gel was ground with a glass rod and eluted for at least 5 hr at room temperature with 3 ml of 0.3 M NaCl. For preparation of the 25S RNA, the origin was cut out, frozen, wrapped in siliconized plastic, and smashed with a hammer. It was then eluted overnight as described above. The eluate was filtered through a glass fiber filter and a nitrocellulose membrane (0.45 µ), carrier yeast RNA (20 µg per fingerprint) was added, and the RNA was precipitated with 2 volumes of ethanol. The RNA precipitate was washed with cold ethanol and stored frozen in distilled water.

OTHER METHODS AND MATERIALS

Fingerprinting with T_1RNAse and pancreatic RNase was according to the method of Sanger[12] or by homochromatography[14]. Homomixture b was used.

Digestion with T_2 nuclease and separation of the digestion products were carried out as described by Hashimoto and Muramatsu[15].

Yeast ribosomes and subunits were prepared as described previously. Wheat germ subunits were the gift of Li-Ming Chang Chien and E. coli subunits were the gift of Heinz Kurt Hochkeppel and Gary Craven. E. coli ribosomes were the gift of Stanislaw Perzynski.

Alpha sarcin was obtained from B. H. Olson as a white, lyophilized powder. It was very soluble in water. It was found to be indefinitely stable in water solution at a concentration of 10 mg/ml and stored at -20C, even without rapid freezing. The solid was stored at 4C. Restrictocin and mitogillin were also obtained from B. H. Olson.

RESULTS

Incubation of yeast 80S ribosomes with alpha sarcin produces an extra RNA fragment when the RNA species are separated by gel electrophoresis. This is shown in Figure 1. The molecular weight of the fragment based on its mobility in relation to yeast 18S, 5.8S, and 5S RNA is 100,000 (about 320 nucleotides). When the low molecular weight RNAs were analyzed, no additional fragments were seen in RNA extracted from ribosomes treated with alpha sarcin (data not shown). Incubation of separated 40S and 60S subunits from yeast with alpha sarcin showed that the fragment produced was from the 60S subunit, while the 40S subunit was unaffected (Figure 1). Similar results were obtained with wheat germ subunits. For an example, see Figure 5. Treatment of free ribosomal RNA from wheat germ and yeast with alpha sarcin did not produce any fragmentation. We assume that the fragment comes from one end of the ribosomal RNA; the other cleavage product is about 1.1×10^6 daltons and is not separated from the uncleaved ribosomal RNA. We were unable to separate this larger fragment from the uncleaved RNA using a variety of electrophoresis conditions, even when urea was included in the gel.

Alpha sarcin causes the production of a fragment of RNA from the 50S subunit of E. coli, but not from the 30S subunit. The fragment is smaller than the corresponding fragment from wheat germ or yeast ribosomes (Figure 1). In contrast, we have never observed a fragment of RNA produced when rat liver polysomes were used as the substrate.

The RNA of yeast was radioactively labelled with ^{32}P and the alpha sarcin

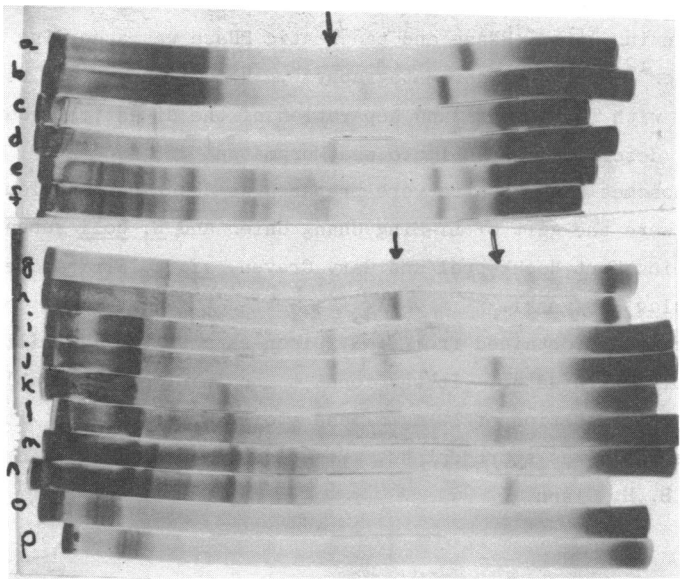

Figure 1. RNA of ribosomes after treatment with alpha sarcin; 2-3 OD_{260} units of ribosomes or subunits in TKM were incubated at 30C for 10 min (a-f) or 60 min (g-0) with or without alpha sarcin and the RNA species were separated as described in Materials and Methods. The E. coli subunits were in 10 mM Tris-HCl - 20 mM $MgCl_2$.

a) 40S yeast subunits
b) 40S yeat subunits + alpha sarcin (2 µg/ml)
c) 60S yeast subunits
d) 60S yeast subunits + alpha sarcin (2 µg/ml)
e) 80S yeat ribosomes
f) 80S yeast ribosomes + alpha sarcin (2 µg/ml)
g) 60S yeast subunits
h) 60S yeast subunits + alpha sarcin (2 µg/ml)
i) 70S E. coli ribosomes
j) 70S E. coli ribosomes + alpha sarcin (20 µg/ml)
k) 30S E. coli subunits
l) 30S E. coli subunits + alpha sarcin (20 µg/ml)
m) 50S E. coli subunits
n) 50S E. coli subunits + alpha sarcin (20 µg/ml)
o) rat liver polysomes
p) rat liver polysomes + alpha sarcin (20 µg/ml)
The arrows points to the position of the RNA fragments produced by alpha sarcin treatment.

fragment was prepared and purified by gel electrophoresis. The fragment and 25S RNA were treated with T_1 ribonuclease and the digestion products were separated by electrophoresis in the first dimension and homochromatography in the second dimension. The fingerprint pattern of the T_1 digestion products of the 25S RNA was complex but the large, presumably unique, spots were clearly

resolved, as can be seen in Figures 2a and b. The fragment contains only a subset of those spots found in the total 25S digest. Fingerprints of 25S RNA from 60S ribosomes subunits untreated and treated with alpha sarcin were identical, suggesting that the cleavage is inefficient; nucleotide spot intensities in the 25S RNA from ribosomes treated with alpha sarcin were not significantly fainter than the untreated. Since we were not able to separate the large alpha sarcin cleavage product from complete 25S RNA, the fingerprint of the treated 25S RNA was taken from RNA that did not migrate into the gel and contained the uncleaved 25S RNA also.

A T_1 ribonuclease digest of the alpha sarcin induced fragment was separated by electrophoresis in two dimensions according to the method of Sanger and found to have a marked reduction in complexity compared to the 25S pattern. The complexity of the patterns were consistent with a size of 100,000 daltons. These are shown in Figures 2c and d. The spot corresponding to the 5' end, pUpUpGp[16], is clearly missing from the alpha sarcin fragment. We were not able to locate a nucleotide spot corresponding to the GpU in the pancreatic ribonuclease digest, even in the 25S RNA. This dinucleotide is the 3' end of the 25S RNA[17]. We have no explanation for this failure.

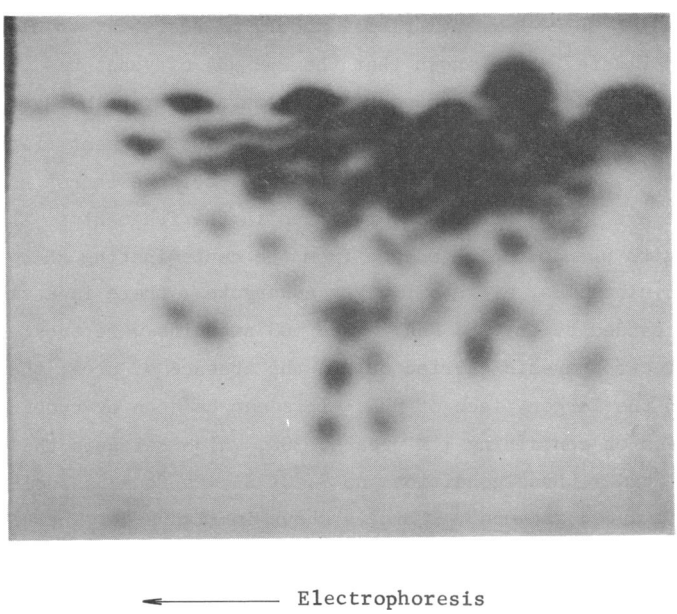

Figure 2a. Separation of the T_1 ribonuclease products: An autoradiogram of the fingerprint of the T_1 ribonuclease digest of the 25S RNA separated by electrophoresis in the first dimension and homochromatography in the second dimension.

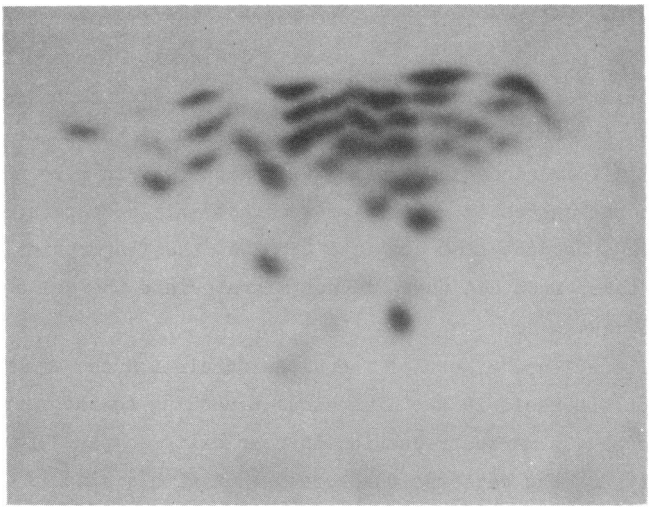

Figure 2b: An autoradiogram of the fingerprint prepared, as above of the fragment produced by alpha sarcin treatment.

When ribosomal RNA was digested with T_2 ribonuclease, only the phosphate linkages adjacent to 2'-O-methyl ribose should be resistant to cleavage. Klootwijk and Planta[18] have shown that the 25S RNA of yeast has 37 methylated riboses. When the digestion products were separated by thin layer chromatography, no di- or tri-nucleotides were evident in the fragment (Figure 3a), whereas in the 25S RNA many are present (Figure 3b). We cannot say definitely whether there are any pseudouridine residues in the fragment as the spot corresponding to pseudouridine may be from the contaminating RNA.

In yeast ribosomes, as in all other eukaryotes, there is a small (5.8S) RNA hydrogen bonded to the 25S RNA. When 60S subunits were treated with alpha sarcin and electrophoresis carried out in the absence of urea, the 5.8S RNA migrates with the larger piece of RNA. This can be seen by running the gel in a second dimension containing 7 M urea at 30C, which releases the 5.8S RNA from the 25S RNA. Under these conditions no 5.8S RNA was found associated with the alpha sarcin induced fragment. This is shown in Figure 4. Therefore, the 5.8S RNA is not bound to the 25S RNA sequence containing the alpha sarcin induced fragment.

A crucial question concerns the purity of the alpha sarcin preparation. Can the cleavage be attributed to a contaminating nuclease? Firstly, our preparation of alpha sarcin showed only two proteins when electrophoresed on

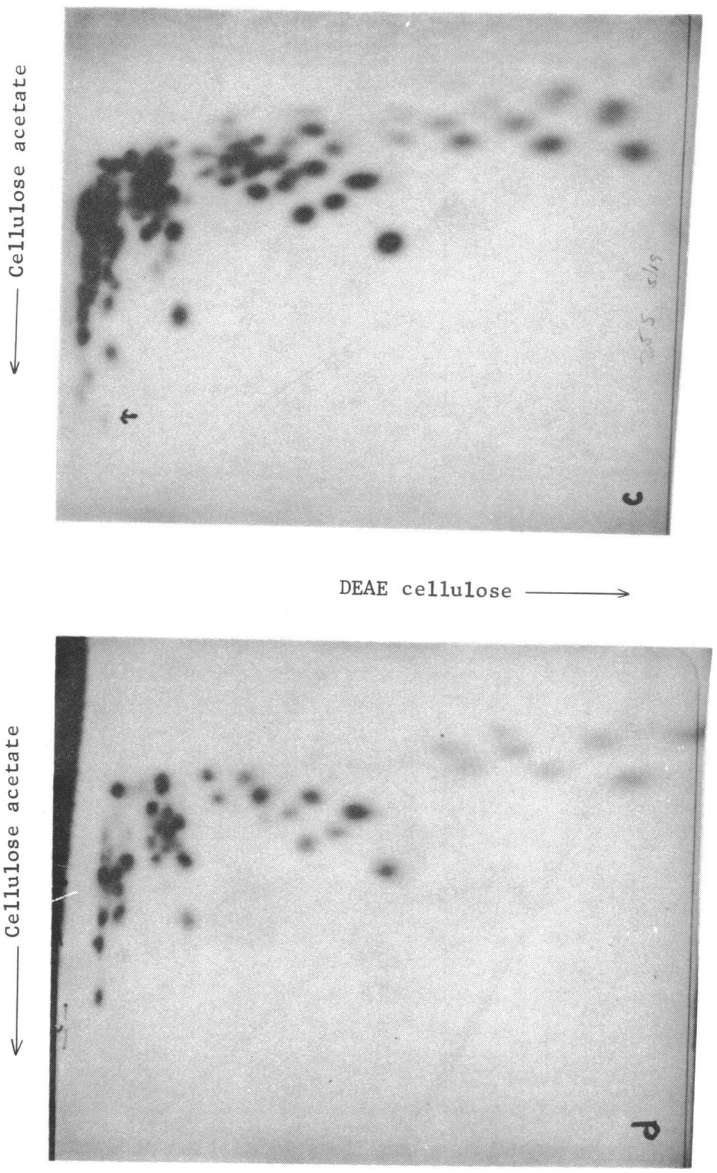

Figure 2c and d: Autoradiograms of fingerprints of T_1 ribonuclease digest as above but separated by electrophoresis in both dimensions. The arrow in 2c indicates pUpUpGp, the 5' end of the 25S RNA.

polyacrylamide gels in the presence of sodium dodecyl sulfate, with about 90-95% of these proteins in a single band. As can be seen in Figure 5, when alpha sarcin was repurified using a Sephadex C-25 column a fraction containing

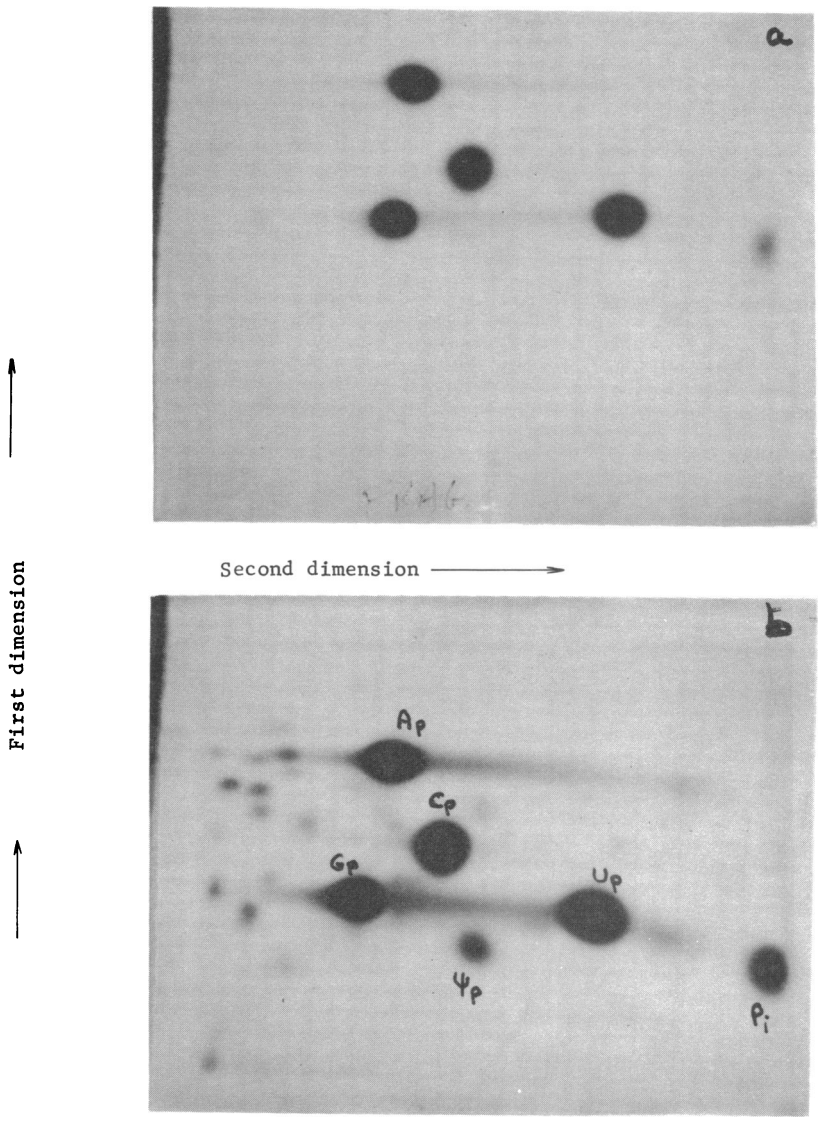

Figure 3a, 3b: Separation of T_2 ribonuclease digestion products.

a single band on SDS gels both inhibited protein synthesis and caused the production of the fragment. Secondly, treatment of yeast ribosomes with different concentrations of pancreatic or T_1 RNase did not produce a product that corresponded to the alpha sarcin induced fragment although they produced a variety of cleavage products. This indicates that the fragment is not simply the result of cleavage at a nuclease sensitive site. When the ability of

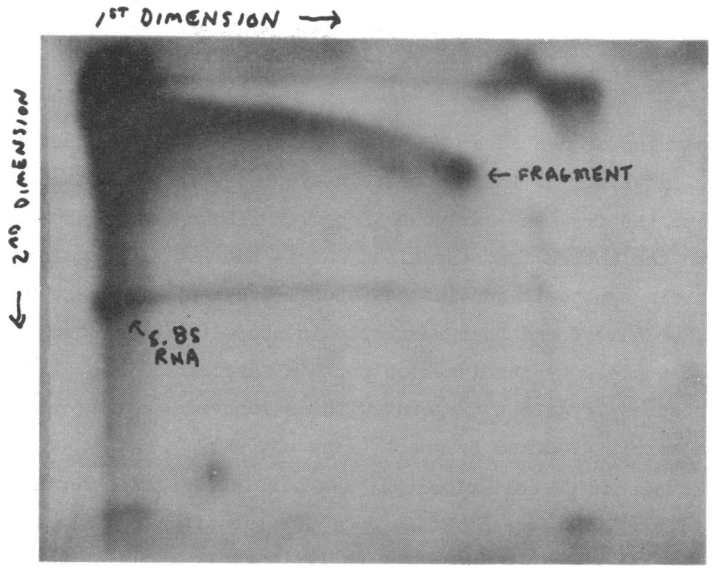

Figure 4. Two dimensional gel demonstration that 5.8S RNA is not bound to the fragment produced by alpha sarcin. The first dimensional gel was 2.5% acrylamide - 0.5% agarose. After electrophoresis it was soaked in electrode buffer containing 7 M urea for 30 min at room temperature. Then it was embedded in a slab gel containing 7% acrylamide and 7 M urea and electrophoresed in the same buffer as the first dimension.

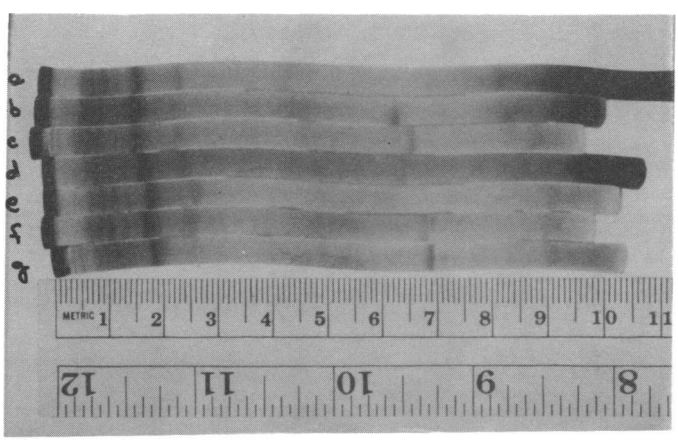

Figure 5. Assay of heat treated alpha sarcin for fragment producing ability. (Reading from top to bottom the gels are 60S wheat germ subunits treated for 10' at 30C with a) no α-sarcin, b) 10 µg/ml untreated alpha sarcin, c) µg/ml alpha sarcin heated to 80C for 10 min in TKM buffer, d) 10 µg/ml of alpha sarcin heated at 80C for 10 min in 3 mM KOH, e) 10 µg/ml of alpha sarcin heated at 80C for 10 min in water, g) 10 µg/ml of untreated alpha sarcin.

alpha sarcin to inhibit protein synthesis was destroyed by heating at 90C for 10 min in 3 mM KOH, the alpha sarcin induced fragment-producing activity was destroyed also.

When alpha sarcin was heated at 90C for 10 min in 3 mM HCl, conditions under which most of activity to inhibit protein synthesis remains, the alpha sarcin still produces the fragment on incubation with ribosomes (Figure 6, Table 1). This supports the notion that the alpha sarcin and not a contaminant both inhibits protein synthesis and induces the cleavage of the large ribosomal RNA. In addition, we have found that restrictocin and mitogillin, two other potent inhibitors of protein synthesis which contain sarcinine, also cleave the RNA in yeast ribosomes to give a fragment with an identical electrophoretic mobility as the alpha sarcin produced fragment (data not shown).

The cleavage of E. coli ribosomal RNA was investigated further. As can be seen in Figure 7, treatment of E. coli 50S subunits with alpha sarcin gives rise to a fragment which is about 2.8 kb (kilobases) assuming the 23S RNA of E. coli is 3.0 kb. The smaller fragment of about 0.2 kb can be seen in Fig. 1. It has been shown by Allet and Spahr[22] that pancreatic ribonuclease produces a break in the 23S RNA of the 50S subunit to produce two fragments, one of which is 1.9 kb in length (18S) and the other 1.1 kb (13S). They showed that the 18S fragment contains the 3' end of the 23S RNA. Figure 7

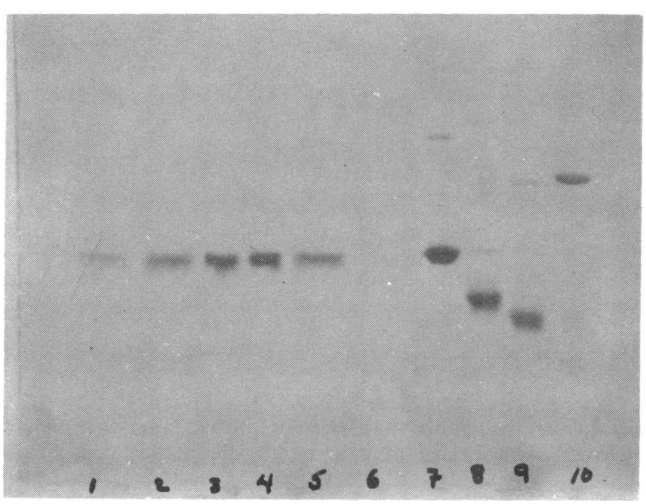

Figure 6. SDS gels of repurified alpha sarcin. Lanes 1-6 are fractions from a C-25 column, lane 7 is the crude alpha sarcin and lanes 8-10 are lysozyme, bovine hemoglobin, and chymotrypsinogen, respectively. The contaminant in the crude alpha sarcin is in lanes 5 and 6.

TABLE 1: Inactivation of alpha sarcin's ability to inhibit protein synthesis.

	Experiment 1		Experiment 2	
Concentration of alpha sarcin	4 ng/ml	1 ng/ml	4 ng/ml	1 ng/ml
Treatment of alpha sarcin				
None	87%	59%	59%	29%
Heat in TKM	76%	31%	80%	47%
Heat in 3 mM HCl	74%	25%	72%	41%
Heat in 3 mM KOH	5%	4%	6%	4%
Heat in H_2O	58%	20%	56%	25%

Effect of various treatments on the ability of alpha sarcin to inhibit protein synthesis. Samples of an 0.1 mg/ml solution of alpha sarcin were treated as described in Figure 5 and then tested for inhibitory activity on cell-free protein synthesis in wheat germ extracts. Results are expressed as percentage inhibition of ^{14}C-leucine incorporation.

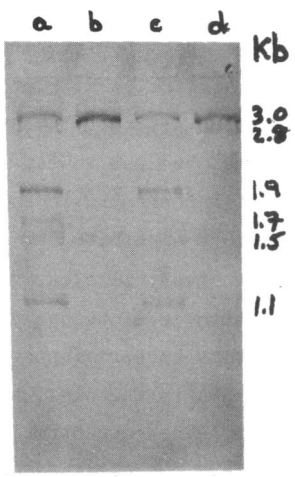

Figure 7. Cleavage of E coli 50S subunits; The RNA species were separated as described in Materials and Methods except the gel was 3.0% acrylamide-0.5% agarose, was electrophoresed for 4 hours at 4C, and stained with Stains-all. The equivalent of 0.1 OD_{260} of 50S was run in each lane. a) 50S subunits + alpha sarcin (100 ug/ml, 60 minutes)+ pancreatic RNAse (1 ng/ml, 30 minutes) b)50S subunits c)50S subunits+ pancreatic RNAse d) 50S subunits + alpha sarcin. Incubations were at 37C.

shows that when 50S are treated with pancreatic ribonuclease and alpha sarcin, a 1.7 kb fragment is produced which is presumably the 18S fragment which has had its 3' end cleaved off by alpha sarcin. The band just below the 1.7 kb fragment is contaminating 16S RNA (1.5 kb). It is clear that the cleavage is not quantitative and we have not found conditions under which it is quantitative.

DISCUSSION

We have shown that alpha sarcin induces a specific cleavage in the largest RNA species of the 60S subunits of the ribosomes of yeast and wheat germ. This cleavage produces a fragment about 300 nucleotides long, probably at the 3'end. The fragment does not bind to 5.8S RNA. The major obstacles in showing that this cleavage is responsible for protein synthesis inhibition are that we have been unable to inactivate ribosomes in vitro and that the concentration of alpha sarcin needed to produce the ribosomal RNA fragment is 100-500 fold higher than that needed to inhibit protein synthesis. We do not obtain quantitative cleavage even when the reaction is carried out in the presence of all components necessary for protein synthesis. However, it is possible that a direct relationship between the two events could exist if only a small fraction of ribosomes are active in protein synthesis in our cell-free systems and these are inactivated.

The cleavage may have nothing to do with protein synthesis inhibition as cleavage and inhibition can be separated. The RNAs in E. coli, wheat germ, and yeast ribosomes are cleaved, but not in rat liver. E. coli protein synthesis is inhibited only at high concentrations of alpha sarcin and wheat germ, and rat liver protein synthesis are inhibited at low concentrations of alpha sarcin.

Although a specific endonucleolytic cleavage has not been shown to be the mode of action of any eukaryotic protein synthesis inhibitor, it is in this way that colicin E3 inhibits bacterial protein synthesis, causing the cleavage of the 16S ribosomal RNA in the small subunit at a position about 50 nucleotides from the 3' end. The concentrations needed to obtain cleavage are comparable to those necessary for cleavage of eukaryotic 25S RNA with alpha sarcin although with colicin E3 cleavage is quantitative[19]. Alpha sarcin is 500 times more active than colicin E3 as an inhibitor of protein synthesis. It may be worth mentioning that colicin E3 has been reported to inactivate mouse ascites ribosomes for the EF-1 dependent binding of aminoacyl-tRNA, the same step inhibited by alpha sarcin, although other steps in eukaryotic protein synthesis were not tested for their sensitivity to colicin E3; the 60S subunit was more sensitive than the 40S subunit to colicin E3 inactivation[21].

However, it has not been demonstrated if colicin E3 immunity protein blocks the action of colicin E3. Colicin E3 and alpha sarcin may act in the same manner on eukaryotic ribosomes. In our hands, purified colicin E3 did not inhibit the wheat germ protein synthesizing system. This suggests that a nuclease in the crude colicin E3 preparation may be responsible for the observed inhibition and emphasizes the importance of demonstrating the purity of alpha sarcin. Nucleases have been found in crude colicin E3 preparations (A. E. Dahlberg, personal communication).

We cannot state with certainty that alpha sarcin is a nuclease, or even that the cleavage is catalytic. The amount of 25S RNA fragment produced has not been shown to be greater than the amount of alpha sarcin present; it is possible that alpha sarcin binds to the ribosome and activates an endogenous nuclease, although incubation of 60S subunits without alpha sarcin for up to 1 hour did not produce the fragment. Even preparations of ribosomes containing extensively degraded RNA did not have an RNA fragment with the same electrophoretic mobility as the alpha sarcin fragment; these preparations of ribosomes can be induced to produce the fragment only when treated with alpha sarcin. This, and the fact that the fragment can be produced from salt washed yeast subunits and with wheat germ subunits argues against the cleavage being the product of an endogenous nuclease.

In spite of the reservations noted above, with respect to the relationship between cleavage and inhibition, alpha sarcin may prove useful in preparing a specific fragment of the large ribosomal RNA for sequencing, hybridization and reconstitution studies of eukaryotic ribosomal RNA.

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