Suppressors *suaC109* and *suaA101* of *Aspergillus Nidulans* Alter the Ribosomal Phenotype *in vitro*

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A new homologous, cell-free system for protein synthesis has been devised for use with ribosomes and elongation factors from *Aspergillus nidulans.* Ribosome preparations from strains with either the *suaA101* or *suaC109* mutations have a higher misreading ratio (non-cognate:cognate amino acid incorporation) in the presence of hygromycin than controls. They can be classed as fidelity mutants. These results also prove that the mutations must be in genes coding for ribosomal proteins or enzymes which modify ribosomal proteins post-translationally. Alternatively, the genes could code for translation factors.

**INTRODUCTION**

Allele-specific suppressor mutations were isolated by Roberts *et al.* (1979). Some of these had properties corresponding to those of the ribosomal ambiguity mutants (*ram*) of *Escherichia coli* (Rosset and Gorini, 1969), i.e. recessive in diploids, wide spectrum of suppression and hypersensitivity to ribosomal antibiotics (Martinelli, 1984). Proof was sought that these suppressor mutations were located in genes coding for ribosomal components but neither mutation altered the two-dimensional gel patterns of ribosomal proteins from these strains (C. Harvey and S. D. Martinelli, unpublished results). So, we devised a cell-free system for protein synthesis using ribosomes and elongation factors extracted from *Aspergillus nidulans.* This enabled us to examine ribosomes from wild-type and mutant strains, *in vitro,* to determine whether they were hypersensitive to antibiotics and whether the intrinsic misreading capacity of the ribosomes had been changed.

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Misreading of the genetic code is a property of the ribosome and can be demonstrated \textit{in vitro} by substituting the non-cognate amino acid leucine for phenylalanine and measuring the amount of leucine incorporation using poly(U) as substrate (Davies \textit{et al.}, 1966). Hygromycin was added since the suppressor strains were known to be hypersensitive \textit{in vivo} (Martinelli, 1984) and since this type of misreading is known to be stimulated by a number of aminoglycoside antibiotics including hygromycin (Wilhelm \textit{et al.}, 1978). Also, this stimulation is often more pronounced in strains containing suppressor mutations, such as \textit{ramC} (Cabezon \textit{et al.}, 1976) in \textit{E. coli}.

In preliminary experiments, the method devised for \textit{Podospora anserina} was followed exactly (Coppin-Raynal, 1977; Crouzet \textit{et al.}, 1978). No incorporation of amino acids into protein was seen. Methods devised for a range of other organisms (Adrian Hobden, unpublished results) were also used, with similar lack of success. In order to obtain active elongation factors, it was absolutely necessary to precipitate the factors from the inactive S100 using ammonium sulphate. Other methods of purifying factors, such as desalting through a Sephadex G25 column, were unsuccessful.

This \textit{in vitro} system was reported to the Genetical Society of Great Britain (Zamir and Martinelli, 1986).

\textbf{METHODS}

\textbf{Strains}

Roberts \textit{et al.} (1979) selected the \textit{suaCl09} mutation by coreversion of \textit{alX4} and \textit{sB43}, and \textit{suaA101} by coreversion of these two alleles plus \textit{alcR125}. Gene designations are described in Roberts \textit{et al.} (1979) or Clutterbuck (1974). Glasgow strain 051 (\textit{biA1}) was used for the preparation of the S100 so that the same factor preparation could be used for all ribosome work regardless of the ribosomal mutations carried. Birkbeck 390 and 391 are the strains from which the suppressor strains were derived. Full genotypes follow:

\begin{verbatim}
390  alX4; sB43; pabaA1; fwA1.
391  alX4; sB43; pabaA1; fwA1; alcR125.
380  alX4; sB43; pabaA1; fwA1; suaC109.
370  alX4; sB43; pabaA1; fwA1; suaA101; alcR125.
\end{verbatim}

Strain 390 was used, \textit{in vitro}, as the control for both suppressor strains. The \textit{alcR125} mutation of 391 (not in 390) codes for a positive acting regulatory protein for expression of the alcohol dehydrogenase gene and should not alter the interpretation of results.

This is described in full by Martinelli (1976), except that 2 L flasks were used containing 200 ml of minimal medium. When cultures reached mid-log phase (about 15 h at 37°C for wild-types), sodium azide was added (to 1 mM) to inhibit protein synthesis and increase the yield of monosomes in the extract (Zeijst, 1972).
In vitro Protein Synthesis

The buffers and centrifuge regime of Coppin-Raynal (1977) and Crouzet et al. (1978) were followed. Brief details have been given here.

Washed mycelium was frozen in liquid nitrogen and stored at -80°C. The brittle mycelium was ground with alumina and the absolute minimum of extraction buffer (0.44 M sucrose; 0.05 M Tris-HCl, pH 7.8; 0.01 M magnesium acetate; 0.025 M potassium chloride; 5 mM β-mercaptoethanol) in order to keep the extract very concentrated. A second grinding of the same pellet was used to extract more ribosomes. Cell debris was removed at 10,000 g for 10 minutes, then 20,000 g for 20 minutes.

After the ribosomes had been removed by centrifugation at 100,000 g for 3 hours, the supernatant (S100) was drawn off minus the top lipid layer and the bottom layer of ribosomes and membranes. Only the supernatant from the first grinding was used as a source of elongation factors and synthetases. The factors were precipitated by saturated ammonium sulphate solution (40–70% wt/vol), followed by dialysis against extraction buffer for 24 h at 4°C. The factors must be dissolved in a minimal amount of extraction buffer after precipitation. Ribosomes were cleaned by centrifugation through a sucrose cushion (50% wt/vol).

For cell-free protein synthesis (after Coppin-Raynal, 1977), exogenous amino acids and tRNA were added and the system depended on the addition of exogenous message, in this case polyuracil. 12.5 μL of dialysed ammonium sulphate precipitated factors were added per 125 μL of assay. [14C]phenylalanine (specific activity 450 mCi/mmol) or [15C]leucine (specific activity 300 mCi/mmol) were purchased from Amersham International. Incorporation of radio-label into TCA-precipitated material was monitored in omniflor scintillant (NEN). Hygromycin B was a generous donation from Eli-Lilly of Indiana and paromomycin from Parke-Davis, Wales. Other chemicals were purchased from Sigma, including tRNA extracted from Baker’s yeast.

All assays were performed in duplicate. At least 6 different extracts were made from each strain for the tests. Overall trends were similar between experiments but numerical values and ratios varied with the S100 and ribosome preparations used. Results were closely comparable when high levels of incorporation were used and results from single experiments of this type are illustrated.

RESULTS

Before using the assay routinely for our mutant strains, the system was tested with varying concentrations of all the main ingredients to see if it was optimised for use with extracts of wild-type Aspergillus strains. The system performed at or near optimum for each additive so that no alteration was made to the assay designed for Podospora anserina.

Hygromycin depressed the incorporation of phenylalanine into protein for all strains and at all concentrations (Fig. 1), but particularly by ribosomes from the mutants (Fig. 2a and 2b). However, at very high concentrations (Fig. 1) there was a stimulation of polypeptide synthesis compared with that seen at low concentrations.
Leucine incorporation was very low in the absence of hygromycin, but markedly stimulated in its presence (Fig. 2c,d). Incorporation increased with the amount of hygromycin added up to 250 µg/ml. More hygromycin depressed incorporation (data not shown) so that very high concentrations were not routinely used. All strains reacted in a broadly similar manner but suaA101 caused more and suaC109 caused less incorporation than control.

The major difference between the strains lay in the ratio of leucine incorporation compared with phenylalanine incorporation. Both suppressor strains had a greater misincorporation (or misreading) ratio than the control strain (Fig. 2e,f), but in the case of suaC109 this could be attributed to the lower phenylalanine incorporation rather than higher leucine incorporation. In another experiment (data not shown), there was a further increase in misreading at 500 µg/ml hygromycin, with ribosomes from both strain 390 and strain 380. Concentrations of hygromycin as low as 10 µg/ml or even 1 µg/ml were sufficient to differentiate between 380 and control. 370 was not tested at these concentrations.

Similar results were obtained for strains 370 and 390 with paromomycin, another aminoglycoside antibiotic (Fig. 3a,b,c). There was no stimulation of phenylalanine incorporation at very high concentrations, in contrast to hygromycin.

**DISCUSSION**

Ribosomes of prokaryotes and eukaryotes have an intrinsic misreading rate which can be increased or decreased by mutation. These mutations can be detected in vivo by suppression of point mutations in other loci and in vitro by increased misincorporation of a non-cognate amino acid. Notable examples of decreased ribosomal fidelity are the ramC (Cabezon et al., 1976) and ramA (Rosset and Gorini, 1969) suppressor mutations in *E. coli*; the sul-60 and sul2-2 in *Podospora anserina* (Coppin-Raynal, 1982; Dequard-Chablat and Coppin-Raynal, 1984); and SUP46 in *Saccharomyces cerevisiae* (Masurekar et al., 1981). Examples of increased fidelity include the restrictive strA and neaA mutations in *E. coli* (Ozaki et al., 1969 and Bollen...
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et al., 1975, respectively), and antisuppressors *AS7-1* (Coppin-Raynal, 1982), *AS1-1* and *AS6-1* (Picard-Bennoun, 1981) of *Podospora anserina*. All these suppressors have been shown to incorporate non-cognate amino acid in a simple cell free system for protein synthesis based on poly-uracil as mRNA. Conversely, the restrictive mutations decrease misreading in vitro. In one unusual case, the *sul2* locus of *P. anserina* was shown to be able to mutate to both increased or decreased misreading (Dequard-Chablat, 1986).
Fig. 3. Effect of the suaA101 suppressor mutation on paromomycin-induced misreading of poly(U) by ribosomes in vitro. Incorporation of amino acid is expressed as a percentage of the drug-free control. Maximum incorporation in pmoles per A260 unit of ribosomes for strain 391 (sua+; ○ --- ○) was phe 18.08 and leu 3.54. For strain 370 (suaA101, ▲ --- ▲) incorporation was phe 13.73 and leu 4.18.

The suppressor mutations isolated in Aspergillus nidulans by Roberts et al. (1979) fell into two distinct classes—semi-dominant mutations which suppressed a narrow range of mutations and altered the overall phenotype very little (genes suaB and suaD) and those which were recessive, altered the growth rate on all media, the antibiotic sensitivity and the ability to grow at low temperatures (genes suaA and suaC). The latter were assumed to result from alterations in genes coding for ribosomal proteins and the former semi-dominant mutations to be in genes coding for tRNAs. In the cell-
free assay for misincorporation of amino acid into protein, both mutations (suaAl01 and suaC109) caused higher misreading of the genetic code than the control strain and this parallels their ability to suppress a wide variety of mutations in vivo. This is proof that suaA and suaC mutations are fidelity mutations and suggests that they alter ribosome structure and function, although the possibility that the code for translation factors cannot be excluded because unwashed ribosomes were used.

It remains to be seen whether suaA and suaC are the actual structural loci for ribosomal proteins. The possibility exists that suaA and suaC may code for enzymes which modify the ribosomal proteins during or after ribosome biogenesis, rather than directly for a ribosomal component. Electrophoresis of ribosomal protein preparations would normally distinguish between these two possibilities. However, Bratt (1986) has recently shown that other mutations in both the suaA and suaC genes can alter electrophoretograms of 40 S proteins to increase the amount of protein S5a relative to S5. The relationship between the genes and protein alterations is undergoing further investigation.

The response of all the ribosomes to hygromycin was complex. There was a decrease in phenylalanine incorporation in the presence of the drug, followed by an increase as the concentration was raised. This multiphasic response has been seen with other fungal ribosomes in vitro (Adoutte-Panvier and Davies, 1984), but the effect has not been satisfactorily explained. In bacterial systems, hygromycin inhibits protein synthesis in a monophasic manner. This is thought to indicate that there is only one site for inhibitory action by this antibiotic on the ribosome (Zierhut et al., 1979). Conversely, a multiphasic response may indicate multiple binding sites for the drug. The response to paromomycin was simpler but could be connected with the greater insensitivity of Aspergillus nidulans to paromomycin found in vivo (Martinelli, 1984).

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