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The effects of protein synthesis inhibition, and of mutations *rna1.1* and *rna82.1*, on the synthesis of small RNAs in yeast

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Strains of *Saccharomyces cerevisiae* have been constructed that possess temperature-sensitive defects in tRNA precursor (pre-tRNA) splicing and which also lack the processing endonuclease that acts at the 3′-terminus of 5 S rRNA and 35 S rRNA precursors (pre-rRNAs). The unspliced pre-tRNAs accumulated by such strains at the nonpermissive temperature are identical in structure to those accumulated by pre-tRNA splicing-defective strains with a functional pre-5 S RNA processing enzyme. The pre-RNA processing activity is therefore not obligatorily involved in maturation of several yeast tRNAs. However, gels of the pulse-labelled RNAs of RNA82$^+$ and *rna82.1* strains provide evidence that this enzyme acts upon a few small unstable transcripts that are not 5 S RNA forms. The most prominent of these transcripts on gels was, in wild-type strains, an RNA 145 ± 2 nucleotides in length.

RNA processing; Endonuclease defect; 5 S rRNA synthesis; tRNA synthesis; Cycloheximide; (*Saccharomyces cerevisiae*)

1. INTRODUCTION

In *Drosophila* and yeast primary transcripts of 5 S ribosomal RNA (5 S RNA) genes are a few nucleotides longer than mature 5 S RNA. Their maturation involves the removal of 7–15 3′-terminal nucleotides through a single and generally very rapid endonucleolytic cleavage [1–5]. In *Xenopus* species and mammalian cells 5 S RNA gene transcripts are only 0–3 nucleotides longer than mature 5 S RNA and do not require this endonucleolytic processing ([5] and references cited therein). The 5 S RNA processing endonuclease of *Drosophila* becomes inactivated when cells are subjected to heat shock [1,2]. The corresponding enzyme of yeast is not inactivated during heat shock and would appear to be nonessential for viability since we have isolated a *Saccharomyces cerevisiae* mutant that lacks this processing activity and yet displays unaltered growth properties [4]. No other mutation causing a complete, rather than a conditional, loss of an RNA-processing enzyme has yet been reported in yeast. This does not necessarily mean that this enzyme is unimportant, as when an RNA-processing activity becomes inactivated another processing enzyme frequently deputises in its role to suppress, to a variable extent, the phenotypic consequences of this inactivation [6]. Thus bacterial strains that completely lack the activity of an important RNA-processing enzyme can sometimes be viable. Processing of the normal substrates of this enzyme in such strains relies upon RNA cleavages that can be expected to differ from those in the wild-type parent, a reflection of the fact that they are being conducted by enzymes of different reaction specificity [6].

The 5 S rRNA-processing endonuclease of yeast has recently been implicated in maturation of the 3′-termini of 35 S pre-rRNA and mature 25 S rRNA [19]. We have investigated whether this enzyme plays any major role in maturation of the

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3′-terminal sequences upon yeast tRNAs. 5 S RNA and tRNA are the two major forms of RNA synthesised from transcripts made by RNA polymerase III in eukaryotic cells [7–9].

Under all growth conditions the *rna82.1* mutant accumulates forms of 5 S RNA which, compared to the 5 S RNA of wild-type yeast, have short 3′ sequence extensions. These extra nucleotides remain for a considerable period at the 3′-end of 5 S gene transcripts owing to the lack of the processing enzyme, being subjected in vivo to a very slow exonucleolytic removal. This removal generates at least one 3′-terminal sequence that acts as a substrate for a poly(A) polymerase [4]. Comparison of the pulse-labelled small RNAs of mutant and wild-type cells on polyacrylamide gels revealed that certain small RNAs of different lengths from 5 S RNA were a few nucleotides longer in *rna82.1* cells. These RNAs were synthesised in much smaller amounts than forms of 5 S RNA. Moreover, they were all comparatively unstable so that after pulse labelling and a chase period the only labelled small RNA of the mutant found to be larger than normal, albeit by only a few nucleotides, was 5 S RNA. Here, we describe experiments which, whilst not identifying the nature of these RNAs, render highly improbable the possibilities that they are unspliced precursors of tRNAs (pre-tRNAs) or are derived from rRNA gene transcripts.

2. EXPERIMENTAL

Yeast strains, culture, labelling and chase conditions, preparation of small RNAs, gel separations and the detection of tritium by fluorography were all as described previously [4].

3. RESULTS AND DISCUSSION

The small RNAs other than 5 S RNA which display small length increments in *rna82.1* as compared to RNA82$^+$ strains are between 90 and 150 nucleotides in length, and are labelled A–E in fig.1a of [4]. Not only are they made in much smaller amounts than 5 S RNA but they are also unstable. This was apparent when a chase with cold uridine followed a pulse labelling, whereupon the only labelled RNAs in the 90–150 nucleotide region of the gels were forms of 5 S RNA (fig.1).

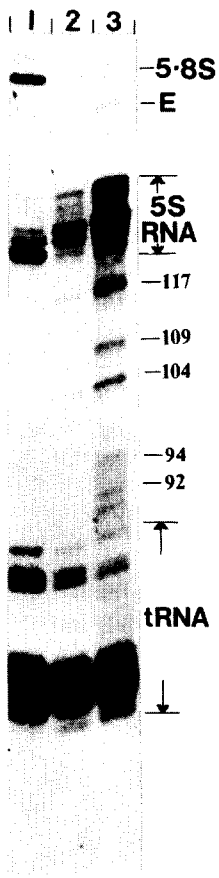

Fig.1. [^{3}H]RNAs labelled during a 10 min, 25°C, [^{3}H]uridine pulse labelling of cells of the *rna82.1* strain P46 [4] that lack the endonuclease normally involved in 5 S RNA maturation, separated on an 8% gel. Slots 1–3 reveal the effect of applying, immediately following this labelling period and also at 25°C, a chase with nonradioactive uridine for 120 min (slot 1), 20 min (slot 2) or 0 min (slot 3). The regions occupied by mature tRNAs (74–85 nucleotides) and 5 S RNAs (121–134 nucleotides) of this strain are indicated. The numbers 92, 94, 104, 109, and 117 denote the lengths in nucleotides of unstable RNAs that probably represent specific unspliced pre-tRNAs, namely pre-tRNA^{Tyr} and pre-tRNA^{Phe} (92 and 94 nucleotides, respectively [14]), pre-tRNA^{Ser} (104 nucleotides [20,21]), pre-tRNA^{Trp} (109 nucleotides [15]) and pre-tRNA$_3^{Leu}$ (117 nucleotides [16]). Pulse labelling and uridine chase were as in [4].

The other minor pulse-labelled RNAs extended in the *rna82.1* mutant have half-lives of no more than a few minutes and are, as a result, not readily amenable to analysis. It is improbable that they are

snRNA precursors as yeast snRNAs are longer than this, except for a 'spot 13' RNA [23], and much more stable. The possibilities that we considered were: (i) that they correspond either to degradation products of 5 S RNA or incorrectly initiated transcripts of 5 S RNA genes; (ii) that they correspond to pre-tRNAs; or (iii) that they are unrelated to either 5 S RNA or tRNAs. Of these minor RNAs extended in *rna82.1* cells a species which has been estimated to be 145 ± 2 nucleotides long in wild-type cells from its mobility on denaturing polyacrylamide gels (band E [4]) is the most clearly discernable on gels of small RNAs. Unlike other bands designated A–D in [4], this band E RNA is longer than 5 S RNA and cannot therefore be a degradation product of the latter.

S. cerevisiae shows a very tightly coordinated synthesis of the components required for ribosome assembly [22]. Probably as a result of a rapid depletion of pools of free ribosomal proteins, the protein synthesis inhibitor cycloheximide causes rRNA synthesis to decline rapidly by at least 80%, whilst having little immediate effect on tRNA synthesis [12]. To determine whether the unstable RNAs extended in the mutant were subject to these controls over rRNA we investigated the effects of cycloheximide on the synthesis of small RNAs. Fig.2 shows the pulse-labelled small RNAs of RNA82$^+$ and *rna82.1* cells synthesised before and after cycloheximide addition. The inhibitor rapidly blocked formation of mature 5.8 S rRNA. It also quickly reduced 5 S RNA synthesis to less than 10% of its former level. The relative proportion of the residual labelled 5 S RNA existing as forms identified [4] as pre-5 S RNAs apparently increased in *rna82.1* cells after cycloheximide was added (fig.2). This indicates that what little 5 S RNA was being made in the presence of the inhibitor was either being processed more slowly than usual or being degraded fairly rapidly. Possibly the synthesis of 5 S RNA in yeast is controlled by the concentration of free 5 S gene-specific transcription factor. In the absence of ribosomal protein synthesis and ribosome assembly 5 S RNA synthesis may be inhibited by the sequestration of this transcription factor [13] in the form of transcription factor-5 S RNA complexes. Such sequestration controls 5 S RNA synthesis in *Xenopus* [7,9].

Synthesis of tRNA declines much more slowly

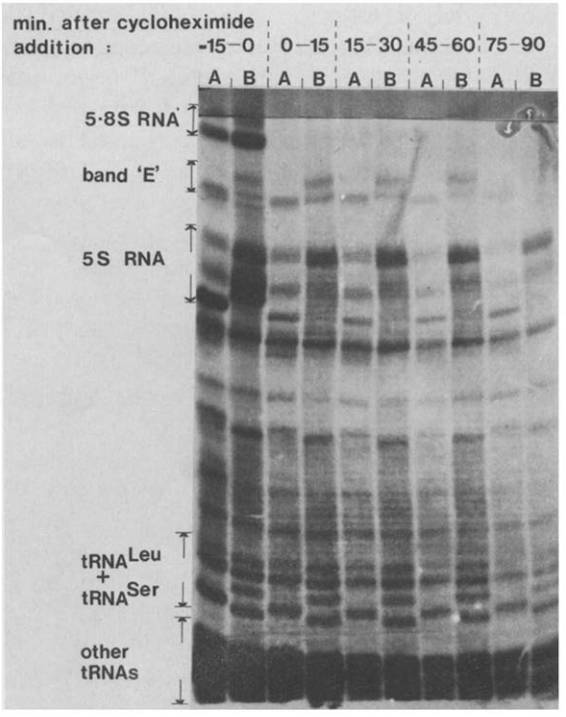

Fig.2. Effect of cycloheximide on the synthesis of small RNAs in yeast. Cells of the RNA82$^+$ strain M304E (A) and the *rna82.1* strain P46tsE (B) growing exponentially in minimal synthetic medium were pulse labelled with [^{3}H]uridine for 15 min at 25°C, both immediately before, and at intervals after the addition of cycloheximide (100 μg/ml). Their small RNAs were then extracted, fractionated on an 8% denaturing gel, and tritium detected as in [4]. Both strains have *rna1.1*, but the phenotype of this mutation is not expressed at 25°C.

than synthesis of rRNAs in response to cycloheximide addition. The gel in fig.2 also revealed that synthesis of the band E RNA is declining as slowly as tRNA synthesis in both strains, an indication that its synthesis is not subject to the rRNA synthesis controls. Also RNA fingerprints of band E RNA (not shown) indicate that it does not have 5 S RNA sequences.

The sizes of the unstable RNAs displaying small length increments *rna82.1* compared to RNA82$^+$ strains suggested that they might correspond to unspliced yeast tRNA precursors (pre-tRNAs). However, those bands on the gels of 74–85 nucleotides, the region occupied by mature tRNAs, were practically identical in both *rna82.1* and wild-type RNA samples irrespective of the

labelling period (see, e.g. fig.1a of [4]). Short pulse labellings sometimes revealed an altered gel pattern of tRNAs in the $tRNA^{Ser}$ and $tRNA^{Leu}$ region (see figs 2,3). This alteration did not correlate with the presence or absence of *rna82.1* in the strains examined. Also, fingerprint and minor base analysis of these tRNAs as in [18,20] revealed mature 3′-termini and virtually complete modification in both *rna82.1* and $RNA82^+$ strains (Piper, P.W., unpublished). The difference in the $tRNA^{Ser}$ and $tRNA^{Leu}$ region in figs 2 and 3 is not therefore a processing defect and might represent a difference in the rate of execution of a tRNA minor base modification in the strains concerned. Since RNA polymerase III generally terminates transcription upon yeast tRNA genes only a very small number of nucleotides downstream of the tRNA coding sequence [8], the absence of *rna82.1*-associated alterations in the tRNA region on gels suggested that maturation of the 3′-terminus of tRNAs may not be retarded in *rna82.1* cells.

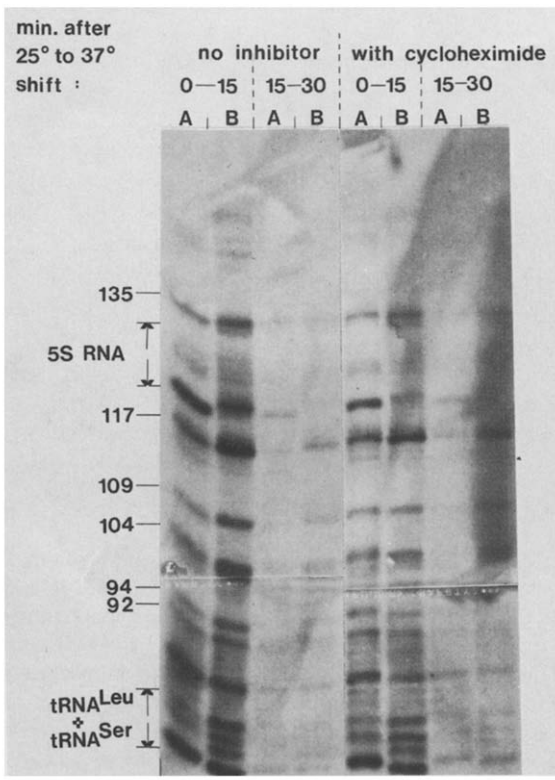

Fig.3. Cycloheximide and *rna82.1* have no appreciable effect on the accumulation of unspliced pre-tRNAs by *rna1.1* cells at the nonpermissive temperature. The four RNA samples shown fractionated on the left side of this 8% gel were from cells of the $RNA82^+$ strain M304E (A) and the *rna82.1* strain P46tsE (B) initially growing at 25°C and then pulse labelled with [^{3}H]uridine 0–15 min and 15–30 min after a 25 to 37°C temperature shift. RNA synthesis at 25°C prior to the temperature shift was as in the first two sample tracks of fig.2. The four RNA samples on the right-hand side of the gel were prepared identically except that cycloheximide (100 μg/ml) was added 10 min prior to temperature shift-up. The numbering of the bands denotes the lengths in nucleotides of unspliced pre-tRNAs (see fig.1 legend) that are accumulated as part of the phenotype of the *rna1.1* mutation at 37°C. The band marked 135 is a 135-nucleotide long pre-$tRNA^{Ile}$ [17] that is not visible in fig.1. Mature tRNAs other than those with a long extra arm ($tRNA^{Leu}$ and $tRNA^{Ser}$ forms) have been run off the bottom of the gel.

Some of the unstable RNAs 92–135 nucleotides long in yeast have been shown to correspond to the unspliced precursors to specific tRNA species. These are normally processed rapidly, yet accumulate at the nonpermissive temperature in cells bearing temperature-sensitive mutations that affect pre-tRNA splicing (*rna1.1* [10] and *los1.1* [11]). These precursors, as accumulated by these splicing-defective strains at high temperature, have mature 5′- and 3′-termini [14–17,20,21]. RNA bands of identical lengths are also clearly discernable upon gels of the small RNAs of wild-type cells labelled during very short pulses. This indicates that the removal of the intervening sequence is generally the slowest step of maturation of intron-containing pre-tRNAs [10,11].

To resolve this issue of whether *rna82.1* directly affected tRNA maturation we constructed two haploid strains, each of which have two mutations affecting RNA processing. These were P46tsE which possesses *rna82.1* and *rna1.1*, and P46tsLS which has *rna82.1* and *los1.1* [4]. In both of these strains 3′ sequence maturation upon 5 S RNA during a 1 h period after a shift from 25 to 37°C was similar to that observed at a temperature (25°C) at which the *rna1.1* and *los1.1* mutations are not expressed [4]. This experiment in fig.3 shows that at the higher temperature both M304E and P46tsE accumulate an identical series of unspliced pre-tRNAs as judged by one-dimensional gel analysis. Accumulation of these unspliced pre-tRNAs at 37°C occurs mainly during the first 15 min after temperature shift-up, and is largely unaffected by *rna82.1* or cycloheximide (fig.3).

We have investigated the structure of these unspliced pre-tRNAs of P46tsE in greater detail by isolating them, uniformly ^{32}P-labelled, from M304E cells that have *rna1.1* but are RNA82^{+}, and from P46tsE cells (*rna1.1* plus *rna82.1*). Prior to the temperature up-shift and labelling with [^{32}P]orthophosphate the cultures of both M304E and P46tsE were incubated for 30 min at 25°C in low-phosphate YEPD medium containing 100 μg/ml cycloheximide. The effect of such a preincubation with cycloheximide prior to increasing the temperature and pulse labelling was to render the pre-tRNA bands more prominent on a one-dimensional gel of labelled small RNAs especially in the 5 S RNA region of the gel by suppressing 5 S RNA formation (figs 2,4c).

The cycloheximide-treated cultures of M304E and P46tsE were labelled with [^{32}P]or-

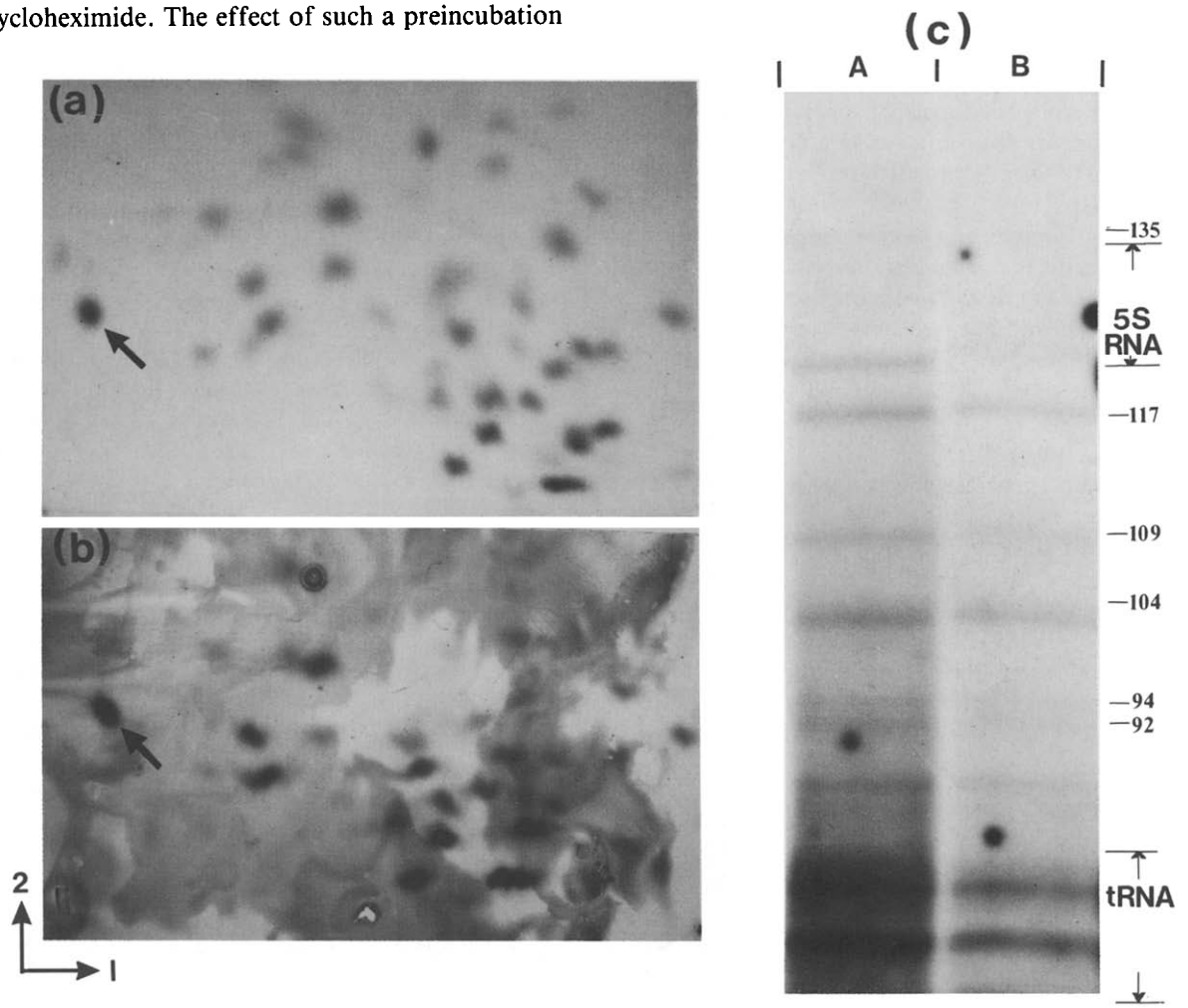

Fig.4. (a,b) Fingerprints of ribonuclease T_1 digests of uniformly ^{32}P-labelled pre-tRNA^{Leu} accumulated by cells of strains M304E (a) and P46tsE (b) at 37°C. Oligonucleotide separation in the first (1) and second (2) dimensions was as in [18]. The fragment arrowed is: CAACCACCA, the T_1 ribonuclease digestion product derived from the 3′-terminus of pre-tRNA^{Leu} [16]. This was identical in both the RNA82^{+} (a) and *rna82.1* (b) strain-derived samples. (c) Small RNAs labelled during a 5 min pulse labelling of M304E cells (A) and P46tsE cells with [^{32}P]orthophosphate. Labelling (0.1 mCi/ml) was in low-phosphate YEPD medium [4] between 15 and 20 min after expression of *rna1.1* was induced by a temperature shift from 25 to 37°C. The regions of the gel occupied by mature tRNAs and 5 S RNA forms are indicated, although those tRNAs of less than 80 nucleotides have been run off the bottom of the 12% denaturing gel. Individual pre-tRNAs are indicated as in figs 1 and 3.

thophosphate for 20 min immediately after increasing their temperature from 25 to 37°C. The pre-tRNA^{Tyr} [14] and pre-tRNA^{Phe} [14], pre-tRNA^{Trp} [15], pre-tRNA$_3^{Leu}$ [16] and pre-tRNA^{Ile} [17] of each culture were then isolated from a one-dimensional preparative 8% gel and fingerprinted as described by Squires et al. [18]. The pre-tRNA^{Ser} isolated this way was not pure enough for fingerprint analysis. These fingerprints revealed that each of these precursors was of identical structure and possessed the 3′ CCA terminus of mature tRNA in both M304E and P46tsE. Two representative fingerprints, those of pre-tRNA^{Leu} from both strains, are shown in fig.4a and b. There was also no difference between these two strains with regard to the minor base modifications of these precursors. Even during very short pulse labellings of these strains the unspliced pre-tRNAs have identical lengths. Fig.4c shows these precursors as synthesised during a 5 min labelling with [^{32}P]orthophosphate. This was the shortest pulse labelling for which we were still able to detect these RNAs on gels, although they were insufficiently labelled for fingerprint analysis.

The finding that unspliced pre-tRNAs labelled during such short pulses are of the same sizes irrespective of the presence or absence of *rna82.1*, and the structural analysis of these pulse-labelled RNAs together indicate that the processing endonuclease of 5 S RNA precursors in RNA82$^+$ cells is not essential for the maturation of the 3′-termini upon most tRNA precursors. The pulse-labelled small RNAs other than 5 S rRNA that are slightly lengthened in *rna82.1* compared to RNA82$^+$ cells are not intron-containing pre-tRNAs and, from the cycloheximide experiments, are unlikely (at least in the case of band E) to be the products of rRNA genes. Since they are synthesised in such small amounts compared to tRNA and 5 S RNA and are highly unstable they are not readily amenable to further analysis without clones of their genomic sequences.

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