The primary transcript of the ribosomal repeating unit in yeast

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ABSTRACT

      Endgroup analysis of 37S ribosomal precursor RNA from Saccharomyces
carlsbergensis has revealed that the major 5' endgroup is pppA-Up, with a
molar yield of 0.8. This shows that most, if not all, 37S RNA molecules
have preserved a transcriptional initiation sequence. Analysis of the
3' terminus of 37S RNA has shown the presence of a uridine rich oligo-
nucleotide, tentatively identified as U_{6-8}-A-N_{OH}. This long stretch of
uridines at the 3' end of 37S RNA may represent a transcriptional
termination site. The two sets of data on the terminal sequences suggest
that 37S ribosomal precursor RNA, if not already spliced, is a primary
transcription product.
      Since the 3' terminus of 26S rRNA, U-U-U-G-U_{OH}, appears to be clearly
different from the 3' end of 37S RNA, we conclude that 37S ribosomal
precursor RNA contains additional nucleotides 3'-distal to the 26S rRNA
sequence.

INTRODUCTION

      Both in bacteria and eukaryotic cells ribosomal RNA (rRNA) components
are synthesized as part of a common transcript, containing also non-
ribosomal sequences. This socalled ribosomal precursor RNA (pre-rRNA) is
processed in a number of steps into the mature rRNAs. Since processing
takes place immediately after or even during transcription it is often
difficult to say whether the largest pre-rRNA, which can be detected in
pulse labelling experiments, is indeed the primary transcript of the
ribosomal repeating unit.

      Recently Batts-Young and Lodish (1) detected pppAp at the 5' end of
the immediate precursor to 17S rRNA from Dictyostelium and this observation
indicates that 37S pre-rRNA, from which the precursor to 17S rRNA
originates, has preserved an initiation sequence at its 5' end. In
addition, Reeder et al. (2) recently described an indirect method to
detect 5' termini bearing a di- or triphosphate, using vaccinia capping
enzymes. This assay clearly showed that such polyphosphate-containing ends

are present in 40S pre-rRNA of Xenopus laevis. These recent data suggest that in these two cases the largest and first-labelled pre-rRNA is likely to be a primary transcript.

For yeast, Retèl and Planta (3) have described a 3.1×10^6 dalton molecule, the socalled 42S RNA, as the initial ribosomal RNA precursor; this RNA species was observed only after methionine deprivation of a methionine-requiring mutant of S. carlsbergensis. In wild type cells of S. carlsbergensis, however, the same group found a 37S RNA with a molecular weight of about 2.8×10^6 dalton as the first detectable pre-rRNA (4,5), and an initial pre-rRNA of similar size was reported for S. cerevisiae (6). These data led Planta et al. (7) to the assumption that the 42S RNA is the primary transcript of the ribosomal repeating unit in yeast, which accumulates after a period of methionine starvation but is converted very rapidly into 37S pre-rRNA under normal conditions.

This hypothesis can be verified in principle by endgroup analysis to establish whether transcriptional initiation and termination sequences are present or not at the termini of both RNAs. As 37S RNA, in contrast with 42S RNA, can be labelled sufficiently high now, we decided to perform this type of analysis for 37S pre-rRNA.

In enzymatic digests of 37S RNA we detected pppA-Up as the major 5' endgroup with a molar yield of 0.8. Qualitative analysis of the 3' end revealed a uridine rich nucleotide sequence, which may represent a transcriptional termination site. We therefore conclude that 37S RNA is most likely the primary transcript of the ribosomal repeating unit in yeast under normal conditions.

Whether 42S RNA represents either an undermethylated conformomer of 37S pre-rRNA or an abarrant ribosomal transcription product remains to be established.

MATERIALS AND METHODS

Isolation of labelled RNAs

Cells of Saccharomyces carlsbergensis, NCYC S74, were grown and converted into protoplasts as described by Retèl and Planta (8). Protoplasts were suspended in a phosphate-free medium (8) containing 120 g/l of mannitol and incubated for 30 min at 29°C to permit recovery from the hemicellulase treatment. 10 min before label was added a shift-up situation was created (9) and pulse labelling for 15-25 min was then performed after cooling the suspension to 15°C (10) and adding (^{32}P)ortho-phosphate (The Radiochemical Centre, Amersham, UK) to a final concentration

of 0.25 mCi/ml. The protoplast suspension was then poured into a two-fold
volume of ice-cold citrate buffer (10 mM sodium citrate pH 7.5 containing
5 mM $MgCl_2$ and 120 g/l of mannitol), followed by centrifugation during
5 min at 4,000 x g at 2^OC. A nuclear fraction was prepared (10) and RNA
was extracted from the nuclei with phenol-sodium dodecyl sulphate at 35^OC
(8). 37S RNA was purified by two cycles of sucrose gradient centrifugation
(11) and its purity was checked by electrophoresis on 2.6% polyacrylamide
gels (10).

^{32}P-labelled 26S plus 5.8S rRNA was prepared from yeast ribosomes as
described earlier (11).

Digestion of the RNA preparations and fractionation of the digests

For the analysis of 5'-terminal structures RNA was digested with T_1
plus pancreas RNase at an enzyme to substrate ratio of 1:10 (w/w) each, in
0.01 M Tris-HCl, pH 7.5, containing 1 mM EDTA, for 45 min at 37^OC. The
digest was then fractionated by two-dimensional high-voltage electro-
phoresis (12). The molar yield of spot X (cf. Fig. 1) was calculated
after determination of the radioactivity per phosphate group averaged
over a number of oligonucleotides of known sequence and stoechiometry.
The yields of those oligonucleotides present in both 37S RNA and 29S RNA
were corrected for the contamination with 29S RNA (up to 20%).

Analysis of 3' termini was performed by column chromatography of
enzymatic digests on DBAE-cellulose as described (13,14). To this end the
RNA's were digested with T_1 RNase at an enzyme to substrate ratio of 1:20
(w/w) during 30 min at 37^OC. Modification by kethoxal and subsequent
digestion with U_2 RNase was performed as follows: RNA was dissolved
(100 µg/240 µl) in 0.42 M kethoxal (2-keto-3-ethoxy-n-butyraldehyde,
Nutritional Biochem. Corp.), 0.1 M sodium acetate, 0.1 M EDTA (pH 5.0) and
heated in a sealed tube for 5 min at 75^OC, followed by an incubation for
1 h at 37^OC. These conditions allow modification of at least 80% of all
guanines. After two cycles of ethanol precipitation RNA was digested with
0.5 unit of U_2 RNase (Calbiochem) in 40 µl of 0.1 M sodium acetate,
1.5 mM EDTA (pH 4.5) for 2.5 h at 37^OC. Removal of the substituted
kethoxal groups was performed by adding 150 µl of a solution containing
0.125 M $MgCl_2$, 1.25 M NaCl and 10 ml morpholine per liter and incubating
the mixture for 45 min at 37^OC. After addition of 50 µl dimethylsulfoxide
and 300 µl of solution A (13) the sample was applied to the column. The
material of the socalled 'peak C' in the eluate (14) was pooled, dialyzed
against distilled water, lyophilized and fractionated according to
Brownlee and Sanger (15).

Nucleotide sequence analysis of oligonucleotides

        Oligonucleotides obtained after electrophoresis on DEAE-cellulose
paper were digested using standard procedures with T_2 RNase (25 mU in
10 μl of 0.1 M ammonium acetate, pH 4.5, during 2 h at $37^{\circ}C$), endonuclease
P_1 (0.1 μg in 10 μl of 0.05 M ammonium acetate, pH 5.3, containing 0.1 mM
$ZnSO_4$ for 2 h at $37^{\circ}C$) or 0.5 M KOH (16 h, $37^{\circ}C$). The products were
identified after paper electrophoresis in 5% (v/v) acetic acid, pH 3.5,
either on DEAE-cellulose or on Whatman 3 MM. Oligonucleotides recovered
from DEAE-cellulose thin-layer plates were digested either with 70 units
of T_1 RNase, 6 μg of pancreas RNase or with 0.025 units of U_2 RNase per
200 μg of RNA for 7 h at $37^{\circ}C$ in the appropriate buffers (16).

RESULTS
Analysis of the 5' terminus of 37S RNA
        We applied the pancreatic plus T_1 RNase 'fingerprint' procedure
which easily separates most oligonucleotides with 5'-(poly)phosphate ends
from oligonucleotides derived from internal positions. Fig. 1 shows such
a 'fingerprint' of yeast 37S pre-rRNA. The pattern comprises three series
of standard products, $(Ap)_nUp$, $(Ap)_nGp$ and $(Ap)_nCp$ (n being 0,1,2.....),
in addition to an array of non-standard products. Most of these non-
standard products comprise 2'-O methylated oligonucleotides, already
present in 37S RNA within the sequences of mature rRNA (17,18).
        Inspection of Fig. 1 reveals one significant non-standard product,
spot X, which is definitely lacking in the corresponding 'fingerprints' of
mature 26S and 17S rRNA (17), and therefore, most likely, belongs to the
non-conserved sequences in 37S pre-rRNA. Its position in the 'fingerprint'
indicates a highly negative charge, suggesting a high phosphate content.
Digestion of the oligonucleotide material of spot X by T_2 RNase and
subsequent electrophoresis on Whatman 3MM at pH 3.5 reveals 2 products,
X_1 and X_2 (cf. Fig. 2A). X_1 coelectrophoreses with uridine-2',3'-mono-
phosphate, whereas X_2 migrates rather fast and streaky. This latter
behaviour is supposed to be characteristic for a polyphosphorylated
nucleotide (16). All ^{32}P-label present within X_1 and X_2 can be converted
into $^{32}P_i$ by treatment with alkaline phosphatase (cf. Fig. 2B). This
shows that no internucleotide phosphate is present either in X_2 or in X_1
and therefore both X_1 and X_2 are single nucleotides. X_2 is most likely an
adenosine derivative since T_2 RNase apparently has cleaved a phosphodiester
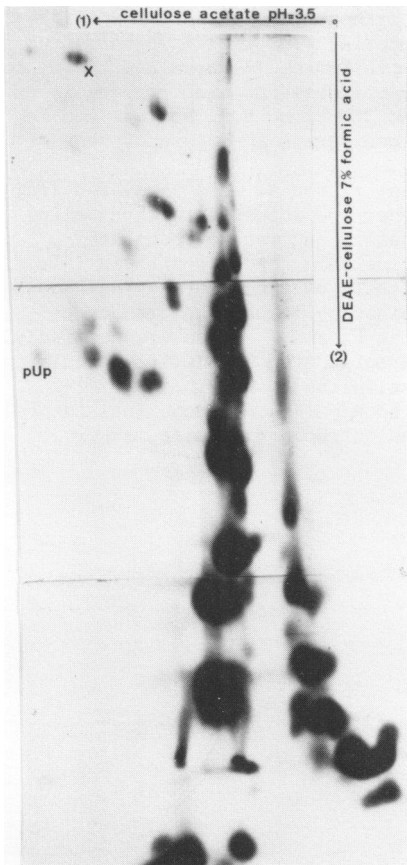bond which was not cleaved by T_1 plus pancreatic RNase. Its electrophoretic

Fig. 1. Fractionation of a pancreatic plus T_1 RNase digest of yeast 37S pre-rRNA. 123 μg RNA containing 12.6×10^6 cpm ^{32}P 37S RNA was digested to completion with pancreatic plus T_1 RNase and subjected to two-dimensional electrophoresis (a) on Cellogel (Chemotron, Italy) from right to left and (b) on DEAE-cellulose paper (downwards).

mobility (see Fig. 2A) as well as its relative amount (in terms of radio-activity in comparison with X_1, viz. 3.5:1) suggest that product X_2 is identical with (p)ppAp (16), and therefore the sequence (p)ppA-Up can be proposed for product X. From the data of Jordan et al. (19) it can be calculated that ppA-Up and pppA-Up have an electrophoretic mobility on DEAE-paper in 7% formic acid of 0.23 and 0.10, respectively (relative to the blue marker dye). As product X appears to have an electrophoretic mobility of 0.10 in this system (cf. the second dimension in Fig. 1) its sequence is most likely pppA-Up.

To confirm the assignment of pppA-Up to product X we applied digestion with endonuclease P_1. We expect as digestion products pppA, pU and P_i and indeed all three products are found after electrophoresis on DEAE-paper at pH 3.5 (cf. Fig. 2C), on which (γ-^{32}P)labelled pppA was
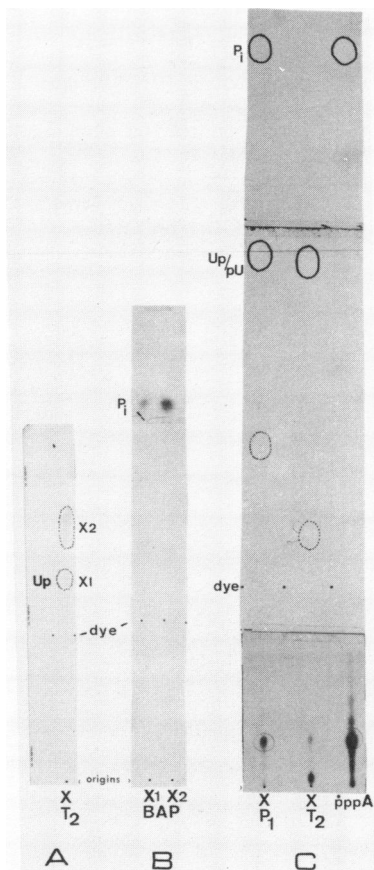
Fig. 2.   Analysis of the oligonucleo-
tide corresponding to spot X in Fig. 1.
(A) Material from spot X was digested
to completion by T_2 RNase and the digest
was fractionated by electrophoresis on
Whatman 3MM at pH 3.5. Spot X_1 and X_2
contained 70 cpm and 248 cpm, respec-
tively.
(B) Spot X_1 and X_2 were recovered from
the paper and incubated with alkaline
phosphatase and reelectrophoresed
under the same conditions as in (A).
(C) Digests of the material from spot
X obtained by incubation with endo-
nuclease P_1 and T_2 RNase, respectively,
were fractionated by electrophoresis on
DEAE-cellulose at pH 3.5; (γ-^{32}P)-
labelled ATP (The Radiochemical Centre)
was coelectrophoresed as a marker.

coelectrophoresed as a marker. A T_2 RNase digest of product X was
coelectrophoresed as well and the spot close to the origin has an
electrophoretic mobility, which may be expected for pppAp (1). This set
of data shows that product X has the sequence pppA-Up.

Next we calculated the molar yield of spot X relative to a set of
oligonucleotides of known sequence and molar yield (17). We obtained an
apparent molar yield of 0.8 in two independent 'fingerprints' of 37S RNA
preparations, which had been pulse-labelled for 25 min. This value of 0.8
may be considered as the true molar yield of product X since the specific
activities of the phosphates in the respective positions are close to
equilibration after this period of labelling; this latter conclusion has
been drawn from nucleotide sequence analysis of oligonucleotides in
'fingerprints' of these 37S RNA preparations (18,20) and from the ratio of

radioactivity between Up and pppAp.

The data presented so far show that pppA-Up is the major 5' terminus of 37S pre-rRNA. A few minor spots have been observed close to spot X which could be minor 5' termini but their low yield have precluded further analysis so far. In addition some pUp is always present in the T_1 plus pancreatic RNase 'fingerprints' of 37S RNA. This is predominantly caused by contamination with 29S pre-rRNA which has pUp as the major 5' endgroup (20).

Analysis of the 3' end of 37S RNA

A successful identification of a 3'-terminal oligonucleotide from a ^{32}P-labelled RNA digest depends both on the choice of the correct ribonuclease and on the method for its selective isolation. Only after digestion of 37S pre-rRNA with T_1 RNase and subsequent chromatography of the digest over DBAE-cellulose columns we were able to locate and identify a 3'-terminal fragment. Fig. 3 shows a 'homochromatogram' of the oligonucleotide material in the socalled 'peak C' of the eluate, which is supposed to contain the 3'-terminal oligonucleotides (cf. Fig. 1 of ref. 14 for a typical elutionprofile of a DBAE-column). The autoradiogram reveals one intense spot, indicated by the arrow, superimposed on a background of spots. This background can also be observed in the pattern obtained for an unfractionated T_1 RNase digest of 37S RNA (20), and, therefore, is most probably caused by contamination of the sample with material from the preceding peak 'B' in the eluate. Nevertheless, all predominant spots of the background were analysed and all appeared to
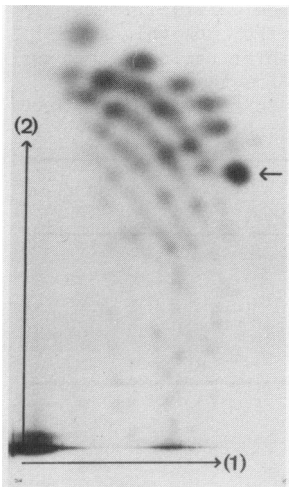

Fig. 3. Two-dimensional fractionation of material possibly containing the 3'-terminal oligonucleotide of 37S pre-rRNA. 37S RNA (27x10^{6}cpm ^{32}P) was digested to completion with T_1 RNase and fractionated on a DBAE-cellulose column (13,14). Material of the socalled 'peak C' (see text) was subjected to high-voltage electrophoresis on cellulose acetate at pH 3.5 in 7 M urea (1) and then to homochromatography (2) using a homomix C hydrolyzed for 15 min (15). The dark spot marked by the arrow has been further analyzed as described in Table 1.

contain a 3'-terminal Gp and are, therefore, no 3' termini.

The results of the sequence analysis of the indicated intense spot of Fig. 3 are summarized in Table 1. Pancreatic RNase produced mainly uridine residues whereas after U_2 RNase digestion only one oligonucleotide was observed terminating in an adenosine residue. This adenosine residue must therefore be present in the other pancreatic RNase digestion product, tentatively identified as A-N$_{OH}$ (cf. Table 1). After re-electrophoresis of this latter product on Whatman 3 MM paper at pH 3.5 it was found between the positions of Ap and Gp. At this stage of the analysis the radioactivity in this product was too low to perform further identification. The data in Table 1 support the deduction of the sequence U_{6-8}-A-N$_{OH}$. Although the identity of N$_{OH}$ is unclear as yet, we conclude that this T_1 RNase generated oligonucleotide must have been derived from the 3' end of 37S RNA. This conclusion is based upon the pertinent absence of a guanosine-3'-phosphate in this product and further supported by its relative blackening in Fig. 3. Although we do not know its molar yield, we think that U_{6-8}-A-N$_{OH}$ represents the predominant 3' end of 37S RNA as no other 3'-terminal product could be found neither in Fig. 3 nor after other digestion procedures, i.e. with pancreatic RNase or U_2 RNase after modification of the RNA by kethoxal (21).

Analysis of the 3' end of 26S rRNA

To establish the presence of additional nucleotides in 37S pre-rRNA 3'-distal to the 26S rRNA sequence we also analyzed the 3' end of mature

TABLE 1. SEQUENCE ANALYSIS OF THE OLIGONUCLEOTIDE POSSIBLY CONTAINING THE 3' END OF 37S PRE-rRNA FROM YEAST. The oligonucleotide material from the spot indicated by the arrow in Fig. 3 was recovered from the thin layer plate and treated with pancreatic or U_2 RNase. The molar ratio of the two pancreatic RNase digestion products was determined from their ratio of radioactivity, taking the underlined product as a reference (2 determinations).

Enzyme used for digestion	Products obtained	Molar ratio	Sequence deduced
pancreatic RNase	A-N$_{OH}$[*]	1.0	
	Up+U>p	6.1-7.7	
U_2 RNase	Y-U$_{5-7}$-Ap[**]		U_{6-8}-A-N$_{OH}$

[*] see text for identification.
[**] identified after digestion with endonuclease P_1; the number of uridine residues was based upon 2 determinations of the radioactivity ratio between pA plus pAp and pU.

26S rRNA.Shine et al. (22) have reported (Y)-G-U_{OH} as the 3' end of 26S
rRNA of S. cerevisiae using in vitro labelling with (^{3}H) isoniazid. We
have confirmed this sequence for 26S rRNA of S. carlsbergensis using the
'diagonal procedure' of Dahlberg (23) applied to a pancreatic RNase digest
of 26S rRNA (unpublished results). To obtain a longer 3'-terminal nucleo-
tide sequence we performed specific cleavage behind adenosine residues
after modification of guanosines by kethoxal and subsequent digestion with
U_2 RNase (21). Since the 'diagonal procedure' failed to reveal a 3'-
terminal product we applied DBAE-column chromatography for the enrichment
of 3'-OH containing oligonucleotides. To circumvent partly the disadvantage
of the column procedure that no molar yield of the 3' terminal fragments
can be calculated we used the homogeneous 3' end of 5.8S rRNA as an
internal reference. The material in the socalled peak 'C' (14) was
fractionated as described above for the T_1 RNase digest of 37S RNA by
electrophoresis and 'homochromatography' (cf. Fig. 4). The pattern
reveals two clear spots (1 and 2); the two spots were subjected to
further analysis as summarized in Table 2. These results allow us to
deduce the sequences U-U-U-G-U_{OH} and U-U-U_{OH} for spot 1 and 2, respectively.
As 5.8S rRNA is expected to have the 3'-terminal sequence U-U-U_{OH} (24),
the product U-U-U-G-U_{OH} must represent the 3' end of 26S rRNA. The relative
intensities of spot 1 and 2 strongly suggest that U-U-U-G-U_{OH} is the
predominant 3' endgroup of 26S rRNA. This result clearly shows that the
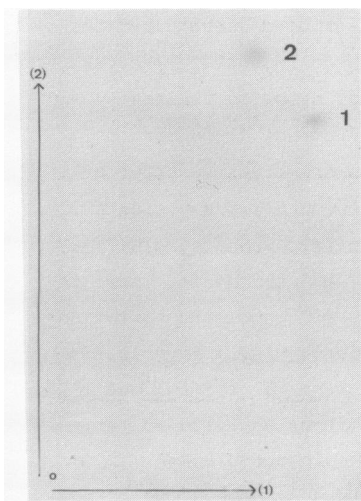3' termini of 37S pre-rRNA and 26S rRNA are different and therefore the

Fig. 4. Two-dimensional fractionation
of the 3'-terminal oligonucleotides
from 26S plus 5.8S rRNA.
26S plus 5.8S rRNA was treated with
kethoxal and subsequent digested with U_2
RNase. After removal of the substituted
kethoxal groups the digest was
fractionated over a DBAE-cellulose
column (13,14). The material of
'peak C' was then subjected to (1)
electrophoresis on cellulose acetate
at pH 3.5 and (2) homochromatography
on a thin layer plate of DEAE-cellulose
using a homomix C hydrolyzed for 30 min.
(15).

TABLE 2. SEQUENCE ANALYSIS OF THE 3'-TERMINAL OLIGONUCLEOTIDES PRESENT IN YEAST 26S PLUS 5.8S rRNA. The material from spots 1 and 2 (Fig. 4) was analyzed by digestion with T_1 RNase or pancreatic RNase. The molar ratios were determined using the underlined product as a reference.

Spot	Enzyme used for digestion	Products obtained	Molar ratio	Sequence deduced
1	pancreatic RNase	$G\text{-}U_{OH}^a$	1.0	
		Up	3.0	
	T_1 RNase	U-U-U-Gp	–	$U\text{-}U\text{-}U\text{-}G\text{-}U_{OH}$
2	pancreatic RNase	Up	–	
	T_1 RNase	$U\text{-}U\text{-}U_{OH}^{a,b}$	–	$U\text{-}U\text{-}U_{OH}$

a identified by digestion with T_2 RNase and snake venom phosphodiesterase.
b identified by its electrophoretic mobility on DEAE-cellulose paper at pH 3.5 relative to Gp.

maturation of 37S RNA into 26S rRNA must involve the removal of sequences present at the 3' terminus of 37S pre-rRNA.

DISCUSSION

The results described in this paper provide strong evidence that yeast 37S pre-rRNA is a primary transcript. The finding of pppA-Up in a close-to-one molar yield shows the preservation of the transcriptional initiation sequence in this RNA species. So far 5'-polyphosphate termini of eukaryotic ribosomal precursor RNA have only been reported by Reeder et al. (2) for the 40S pre-rRNA of Xenopus laevis, using an indirect method, and by Batts-Young and Lodish (1) for the direct precursor to 17S rRNA of Dictyostelium. In all three cases the primary transcript of the ribosomal repeating unit appears to start with pppA-, suggesting that the eukaryotic RNA polymerase A uses an adenosine triphosphate rather than guanosine triphosphate for the initiation of RNA synthesis. There is some evidence that the 5' terminus of the primary ribosomal transcription product is heterogeneous (see Ref. 2 and this paper). Fig. 1 reveals a few weak spots close to product X which could be minor 5' termini but their low yield of radioactivity has precluded further analysis so far. Only pUp can be found as an additional 5' endgroup in our 37S RNA preparations (cf. Fig. 1) with a molar yield up to 0.2. However we cannot

distinguish as yet whether pUp represents a genuine minor 5' endgroup of 37S pre-rRNA or an artefact caused by hidden breaks in the RNA chain.

The finding of U_{6-8}-A-N_{OH} in 37S RNA suggests the preservation of a transcriptional termination sequence. This suggestion is based upon the detection of stretches of 5 to 8 uridines at the 3' termini of a number of bacterial primary transcripts (25). In addition, DNA sequencing data of the genes coding for yeast 5S rRNA (26,27), yeast tRNA[phe] (28) and yeast tRNA[tryp] (29) have revealed significant stretches of deoxyadenosine residues downstream the coding sequences and these stretches have been designated as putative transcription termination sites.

The conclusion that yeast 37S RNA represents a primary transcript presupposes that this RNA is not a "splicing" product of an initial transcript. So far we cannot exclude completely that "splicing" of the primary transcript of the ribosomal repeating unit in yeast is part of the rRNA maturation process. However, the absence of intervening sequences in at least the rRNA regions of the yeast ribosomal repeat seems to be well established (30,33). Furthermore, our observations rather suggest that the first nucleolytic event in the maturation of yeast rRNA is the conversion of 37S pre-rRNA into 29S and 18S pre-rRNA, a step which occurs relatively late after transcription and which is preceded by the bulk of the 2'-O-methylations (18), pseudouridylations (20) and the assembly with a large number of proteins into a 90S preribosomal particle (10).

The nature of the previously found, rapidly labelled 42S RNA (3) is uncertain. This RNA has been observed only by pulse-labelling after methionine starvation of a methionine-requiring mutant (3). In addition in vitro transcription of total yeast nuclear DNA with purified yeast RNA polymerase A produces ribosomal transcript with molecular weights up to about 3×10^6 dalton (i.e. equal to the size of a 42S RNA). Furthermore, it was found that RNA polymerase A, before reading into the 17S rRNA sequences, transcribes a stretch of DNA corresponding with a RNA leader sequence of about 1×10^6 dalton (31,32). Therefore, it remains possible that 42S RNA is an aberrant ribosomal transcription product which arises by initiation of transcription at a position upstream of the initiation site for 37S pre-rRNA. Another possibility is that the 42S RNA is an undermethylated conformomer of 37S pre-rRNA.

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