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Some characteristics of processing sites in ribosomal precursor RNA of yeast

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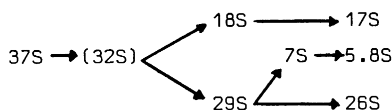
ABSTRACT

The DNA sequence of the intergenic region between the 17S and 5.8S rRNA genes of the ribosomal RNA operon in yeast has been determined. In this region the 37S ribosomal precursor RNA is specifically cleaved at a number of sites in the course of the maturation process. The exact position of these processing sites has been established by sequence analysis of the terminal fragments of the respective RNA species.

There appears to be no significant complementarity between the sequences surrounding the two termini of the 18S secondary precursor RNA nor between those surrounding the two termini of 17S mature rRNA. This finding implies that the processing of yeast 37S ribosomal precursor RNA is not directed by a double-strand specific ribonuclease previously shown to be involved in the processing of *E. coli* ribosomal precursor RNA (see Refs 1,2). The processing sites of yeast ribosomal precursor RNA described in the present paper are all flanked at one side by a very (A+T)-rich sequence. In addition, sequence repeats are found around the processing sites in this precursor RNA. Finally, sequence homologies are present at the 3'-termini (6 nucleotides) and the 5'-termini (13 nucleotides) of a number of mature rRNA products and intermediate ribosomal RNA precursors. These structural features are discussed in terms of possible recognition sites for the processing enzymes.

INTRODUCTION

In yeast the four rRNA species, *viz.* 26S, 5.8S, 17S and 5S rRNA, are all encoded by a genetic unit which is repeated more than hundredfold on the genome (3). From this repeated unit 5S rRNA is transcribed as well as a 37S ribosomal precursor RNA, which contains single copies of each of the 17S, 5.8S and 26S rRNA sequences. This precursor RNA molecule is subsequently modified and cleaved to yield the three mature rRNA species, as follows (4):



The various steps in this processing pathway apparently involve highly specific recognition and cleavage of the ribosomal precursor RNA by endo-

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nucleases (processing enzymes). In an attempt to understand the structural basis of these specific protein-nucleic acid interactions we have sequenced the transcribed spacer region between the 17S rRNA and 5.8S rRNA gene. In this region at least 3 processing sites are present, leading to the formation of 18S and 29S precursor rRNA, and subsequently to the formation of 17S rRNA and 7S precursor rRNA. The exact position of these processing sites has been established by sequence analysis of the terminal fragment of the RNAs concerned.

The sequences surrounding the cleavage sites in the yeast ribosomal precursor RNA have a number of conspicuous features, which may serve as possible recognition sites for the processing enzymes involved. At any rate the sequence data shows that the mechanism of primary processing of yeast ribosomal precursor RNA differs fundamentally from the way in which ribosomal precursor RNA of Escherichia coli is processed. In this prokaryotic organism the sequences flanking both the 16S rRNA gene and the 23S rRNA gene are complementary, and therefore may base pair in the 30S precursor rRNA to form duplex structures, which are cleaved by the double-strand specific RNAse III (1,2). Such a sequence complementarity cannot be found in the regions flanking the 17S rRNA gene or the 18S precursor rRNA sequence in yeast.

A preliminary account of the work described in this paper has been presented elsewhere (5).

MATERIALS AND METHODS

Isolation of the rDNA fragment

For this study we used the hybrid plasmid pMY1, constructed by Dr. J.H. Meyerink (6) in our laboratory, being a recombinant of the plasmid pBR322 and a large fragment of the ribosomal repeating unit of Saccharomyces carlsbergensis, generated by the endonuclease Hind III. This yeast DNA fragment contains the 17S, 5.8S and 26S rRNA genes and the intergenic region (6).

Plasmid DNA was isolated from E. coli K12 cells by the clear lysate method as described by Goebel et al. (7). For the isolation of the intergenic region 50 µg of plasmid DNA was digested with 50 units of the endonuclease EcoRI (Boehringer, Mannheim) in 50 µl of 6 mM Tris-HCl (pH 7.5) containing 50 mM NaCl, 6 mM $MgCl_2$ and 6 mM 2-mercaptoethanol for 3 hr at 37^oC. The digest was subjected to electrophoresis on a 3% polyacrylamide gel according to Yang et al. (8) and the fragments were visualized with the aid of

ethidiumbromide. The fragment containing the 17S-5.8S rRNA intergenic region (i.e. fragment D, see Ref. 9) was recovered from the excised gel segment by electrophoresis. The fragment was further purified by filtration over a GF/C filter, followed by phenol extraction and ethanol precipitation.

Isolation of labelled RNAs

^{32}P-labelled 17S and 26S plus 5.8S rRNA were prepared from S. carlsbergensis ribosomes as described previously (10). Isolation of ^{32}P-labelled 18S RNA was performed as described by de Jonge et al. (11). ^{32}P-labelled 29S RNA was isolated in combination with 37S RNA as described by Klootwijk et al. (12).

DNA sequence analysis

The chemical modification method of Maxam and Gilbert (13) was used. Aliquots of the reaction samples were loaded on a 12% or 20% polyacrylamide-7 M urea-citrate gel.

DNA fragments were labelled with (γ-^{32}P)ATP (The Radiochemical Centre, Amersham) and polynucleotide kinase (Boehringer, Mannheim) essentially as described by Maxam and Gilbert (13). The reaction mixture (25-50 µl) was incubated for 30 min at 0°C, then for 45 min at 37°C and finally for 2 min at 100°C. The mixture was fractionated on a Sephadex G50 column (1x10 cm) by elution with 5 mM Tris-HCl (pH 8.0) containing 0.5 mM EDTA. Fractions containing labelled DNA were pooled, lyophilized, extracted with phenol and digested with a second restriction endonuclease.

Digestions with restriction endonucleases were generally performed in a final volume of 20 µl for 3 hr at 37°C, except for the digestion with Taq I which was performed at 60°C. Incubations with Hinf I (isolated by Mr. R.J. Leer of our laboratory) were in 20 mM Tris-HCl (pH 7.5) containing 20 mM $MgCl_2$ and 50 mM NaCl; with Sau 3A (Bethesda Research Laboratories) in 6 mM Tris-HCl (pH 7.6) containing 15 mM $MgCl_2$, 60 mM NaCl and 6 mM 2-mercaptoethanol; with Hae III and Taq I (New England Biolabs) in 6 mM Tris-HCl (pH 7.4) containing 6 mM $MgCl_2$, 6 mM NaCl, 6 mM 2-mercaptoethanol and 100 µg/ml gelatine.

RNA sequence analysis

40-80 µg of rRNA were digested with RNAse T_1 (Calbiochem A.G.) at an enzyme to substrate ratio of 1:20 (w/w) in 0.01 M Tris-HCl (pH 7.5) containing 1 mM EDTA for 30 min at 37°C. The digest was fractionated by electrophoresis on a cellulose acetate strip at pH 3.5 followed by homochromatography on a thin-layer plate of DEAE-cellulose (Polygram Cel 300 DEAE/HR-2/15) using a "homomix C" hydrolyzed for 12-15 min (14). Separate oligonucleotides were eluted with 30% triethylaminebicarbonate (pH 8.3) as described by Brownlee

(15), and the material of a single spot (about 200 μg) was digested either with 6 μg of pancreatic RNAse (Worthington) in 10 μl of 0.01 M Tris-HCl (pH 7.5) - 1 mM EDTA, or with 0.025 units of RNAse U_2 (Calbiochem) in 10 μl of 0.1 M Na-acetate (pH 5.0) - 1.5 mM EDTA for 7 hr at 37°C. The digests were fractionated by electrophoresis on DEAE-cellulose paper in 5% acetic acid (pH 3.5). The base composition of the various oligonucleotide fragments was determined after complete digestion either with 0.5 M KOH (16 hr at 37°C) or with RNAse T_2 (in 0.1 M ammonium acetate, pH 4.5, at 37°C) and subsequent separation of the nucleotides by electrophoresis on Whatman 3 MM paper in 5% acetic acid.

RESULTS

The intergenic region between the 17S rRNA gene and the 5.8S rRNA gene of yeast is present on a fragment of about 660 bp (6,16) which is produced by digestion of yeast ribosomal DNA with EcoRI. This fragment (D) was isolated from a hybrid plasmid pMY1, which consists of plasmid pBR322 and the complete ribosomal RNA operon of yeast (6). A restriction map of the intergenic fragment D for the restriction enzymes Hinf I, Taq I, Sau 3A and Hae III is shown in Fig. 1. Using appropriate subfragments the sequence of the intergenic region was determined by the method of Maxam and Gilbert (13). This sequence (from residue 201 to residue 623) is presented in Fig. 2.

The 3'-end of 17S rRNA can be unambiguously mapped at position 224, since the previously in this laboratory determined 3'-terminal sequence: -$Gm^{6}_{2}Am^{6}_{2}$ACUCGCGGAAGGAUCAUUA$_{OH}$ (Ref. 17) is fully present at position 204 to

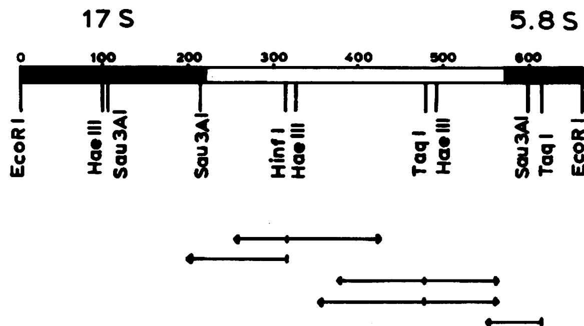

Fig. 1. Restriction map of the EcoRI fragment D of the ribosomal repeating unit from yeast. Sequences read from the gels are indicated by arrows.

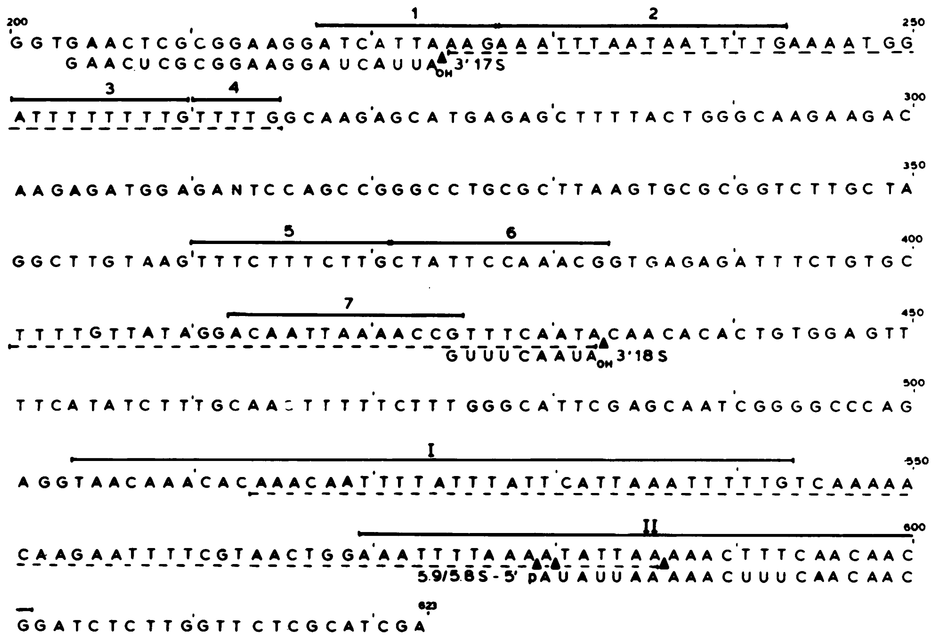

Fig. 2. Sequence of the 17S-5.8S rRNA intergenic region (only the noncoding strand is shown). (A+T)-rich sequences adjacent to the cleavage sites are underlined: the stretch of 40 nucleotides downstream the 3'-end of the 17S rRNA gene contains 88% (A+T), the 33 3'-terminal nucleotides of the 18S RNA region are for 76% (A+T) and the 73 nucleotides upstream the 5'-end of the 5.8S rRNA gene are for 84% (A+T).
Nucleotide sequences which give rise to characteristic oligonucleotides after digestion of the corresponding RNAs with ribonuclease T_1 are overlined and numbered in accordance with the numbering in Figs 4 and 5.

224. Also the 3'-end of the 18S precursor rRNA can be precisely located at position 433 of the sequence in Fig. 2, as the 3'-terminal sequence of 18S RNA: $-GUUUCAAUA_{OH}$ (Ref. 11) can be found only at position 425 to 433. The presence of this nucleotide sequence (in the coding strand) is documented in Fig. 3B which shows the gel pattern reading from position 453 to 350. The DNA sequence between position 224 (the 3'-end of 17S rRNA) and position 433 (the 3'-end of 18S RNA) was confirmed by a comparative sequence analysis of 17S rRNA and 18S RNA. "Fingerprints" of the two ^{32}P-labelled RNAs are shown in Fig. 4. In the digest of 18S RNA a number of products can be observed which are absent in that of 17S rRNA. These products were subjected to (partial) sequence analysis as summarized in Table I. The oligonucleotide no. 1

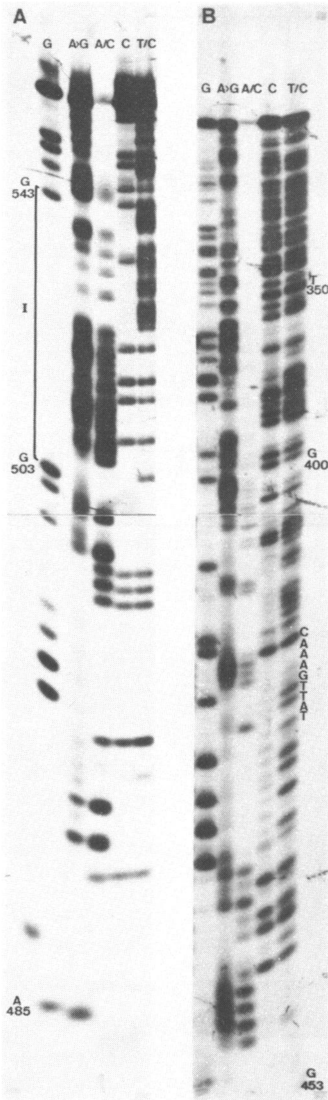

Fig. 3. Autoradiograms of DNA sequencing gels for two adjacent fragments of the intergenic region, both labelled at the Taq I site at position 482/483.
Gel A is a 20% polyacrylamide gel, which permits reading of the sequence from position 485 to 543. This sequence results on the RNA level in a very long ribonuclease T_1-generated oligonucleotide which was found to be present in a digest of 29S precursor rRNA (Spot I in Fig. 5).
Gel B is a 12% polyacrylamide gel, which permits sequence reading from position 453 to 350 and which shows the position of the 3'-end of 18S RNA.

apparently bridges the 3'-end of 17S rRNA and the adjacent stretch of non-conserved RNA; its sequence corresponds exactly with the DNA sequence from position 218 to 227. The other precursor-specific oligonucleotides, 2-7, also fit in with the DNA sequence presented in Fig. 2. All these products, except no. 3, are present in the digest of 18S RNA in a nearly unimolar amount indicating that this transcribed spacer region is essentially homogeneous.

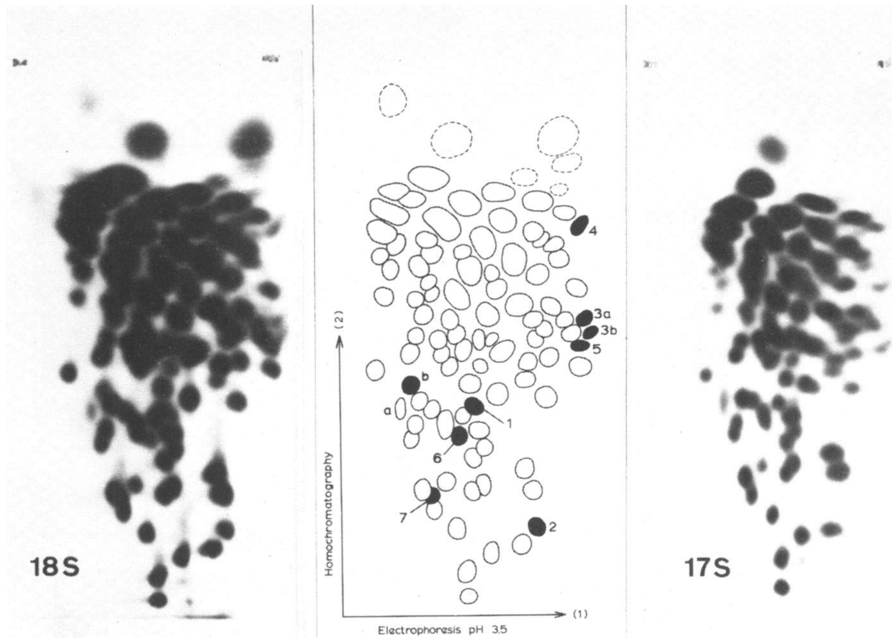

Fig. 4. Two-dimensional separation of the ribonuclease T_1 digestion products of 18S precursor RNA and 17S rRNA by high-voltage electrophoresis on cellulose acetate at pH 3.5 and homochromatography. Products present in the digest of 18S RNA but lacking in that of 17S rRNA are indicated by filled spots in the key to the figure.

The products 3a and 3b were recovered in submolar yields; exact values could not be determined due to contamination by neighbouring spots. The two products apparently differ by only one uridine residue and may be both derived from position 251 to 260 if a minor heterogeneity is assumed to be present among the ribosomal repeats. Product c, i.e. AAAAUGp, which has been observed in another type of "fingerprint" of 18S RNA (see Plate I in Ref. 18) is also predicted by the DNA sequence. All other precursor-specific products expected on basis of the DNA sequence in 18S RNA most probably comigrate with products derived from the 17S rRNA sequence. Therefore, one may conclude that the sequence from position 225 to 433 corresponds completely to the non-conserved part of 18S RNA. Since all 18S RNA-specific products can be accommodated by this sequence, the combined sequence data support the conclusion previously drawn (11) that all extra nucleotides in 18S precursor RNA are present at the 3'-end of the molecule. This means that the non-

TABLE I. SEQUENCES OF OLIGONUCLEOTIDES DERIVED FROM THE 3'-NON-CONSERVED PART OF 18S RNA

Spot number	Ribonucleases used	Nucleotide sequence	Molar yield**
1	panc(±CMCT)*, U_2	AUCAUUAAAGp	0.9
2	panc, U_2, T_2	$[A_5,U_4]$AAUUUUGp	0.9
3a	panc, U_2, T_2	A-$[U_{6-8}]$Gp	not.det.
3b	panc, U_2, T_2	A-$[U_{7-9}]$Gp	not.det.
4	T_2	UUUUGp	1.0
5	panc, T_2	$[C_2,U_{7-8}]$Gp	0.8
6	panc	$[AAACp,AUp,C_{2-3},U_{2-3}]$Gp	0.8
7	panc, U_2	[AAAACp,AAUp,ACp,U,C]Gp	1.0
b	panc, U_2	[AACp,ACp,ACp,$m^1\Psi$-Cp]Gp	0.7
c***	panc, T_2	AAAAUGp	not.det.

* panc stands for pancreatic RNAse, and CMCT indicates N-cyclohexyl-N^1-(2-(4-β-morpholinyl)-ethyl)carbodiimide methyl-p-toluene sulphonate, which was used according to Brownlee (15).

** The molar yield was calculated from the relative amount of label in the product compared with that of some unimolar products derived from 17S rRNA.

*** This product was detected in another type of "fingerprint", applying electrophoresis on DEAE-paper instead of homochromatography (cf. Plate I in Ref. 18).

conserved stretch in 18S RNA comprises 209 nucleotides, and that is less than previously calculated from the estimated molecular weights of 18S and 17S RNA (19). Product "b" in the "fingerprint" of 18S RNA is derived from the sequence of 17S rRNA and is identical to product "a" of 17S rRNA, except for a difference in modification ($m^1\Psi$ instead of amΨ; see Ref. 18).

As 29S precursor rRNA is generated simultaneously with 18S RNA from their common precursor, the possibility exists that the 5'-end of 29S RNA is joined directly to the 3'-end of 18S RNA in the 37S precursor RNA. However, the 5'-terminal part of 29S RNA has not yet been elucidated, and therefore the exact position of the 5'-end of 29S RNA on the map in Fig. 2 is still unknown. Kinetic experiments (20) have indicated that 7S RNA, the immediate precursor of 5.8S rRNA, arises from 29S RNA. This finding suggests that the 5'-end of 29S RNA is located between position 433 (the 3'-end of 18S RNA) and position 587 (the 5'-end of 5.8S rRNA (see Ref. 21) and also the 5'-end of 7S RNA (see Ref. 22)). To provide structural proof for the presence of the

7S RNA sequence in 29S RNA, "fingerprints" of 29S RNA were examined on the presence of a long marker sequence of 7S RNA, viz. the ribonuclease T_1-generated oligonucleotide UCAUUUCCUUCUCAAACAUUCUGp which overlaps the 3'-end of the mature 5.8S rRNA (22). In Fig. 5 the "fingerprints" of 29S RNA and of 26S rRNA plus 5.8S rRNA are shown. Comparison with the "fingerprint" of 7S RNA (not shown) indicated that spot III is the most likely candidate for the selected marker sequence of 7S RNA. This assumption was shown to be correct by (partial) sequence analysis of the relevant extra spots in the 29S RNA "fingerprint" (Table II).

We then investigated the possibility that the 5'-end of 29S RNA is identical with that of 7S RNA by screening "fingerprints" of 29S RNA on the presence of a spot corresponding to the oligonucleotide: pAAACUUUCAACAACGp, that is the 5'-end of both 7S RNA and 5.8S rRNA. As can be seen in Fig. 5 this oligonucleotide, which is represented by spot IV in the "fingerprint" of 26S plus 5.8S rRNA, is absent in the digest of 29S RNA. Instead the "fingerprint" of 29S RNA contains a number of additional spots which are lacking in the pattern representative for 26S plus 5.8S rRNA. Two of these spots (spots I and II in Fig. 5) must correspond to rather long oligonucleotides. Partial sequence analysis (cf. Table II) of the oligonucleotide

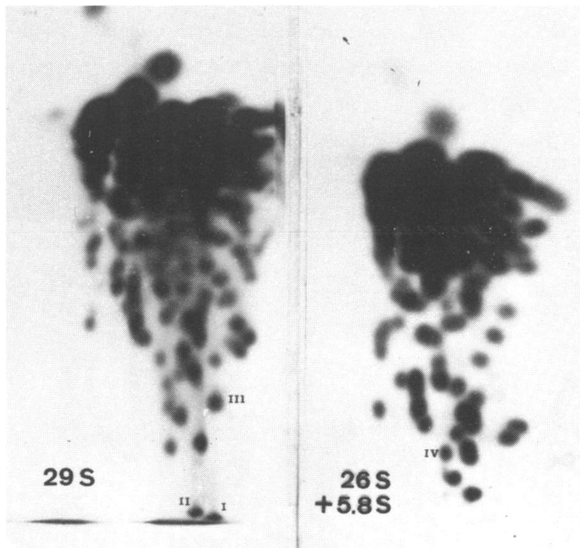

Fig. 5. Two-dimensional separation of the ribonuclease T_1 digestion products of 29S RNA and of 26S plus 5.8S rRNA.

TABLE II. SEQUENCE DATA OF THREE LONG OLIGONUCLEOTIDES DERIVED FROM THE NON-CONSERVED PART OF 29S RNA (*cf.* FIG. 5)

Spot number	ribonucleases used	products (molar yield)
I*	panc	AAAUp,AAACp,AAUp,AACp,AUp,ACp, Gp,Cp,Up
	U_2	two long unidentified products, UUUAp,(U_2,C)Ap,UAp,CAp,Ap
II*	panc	AAAAACp,AAAAUp,AAAUp,AAUp,AACp, AUp,ACp,Gp,Cp,Up
	U_2	(C_2,U_3)Ap,UUUUAp,UUAp,UAp,CGp, CAp,Ap
III	panc	AAACp(1),AUp(2),Gp(1),Cp(5-6), Up(6-8)
	U_2	(U_{4-6},C_{3-4})Ap(1),(C,U_3)Gp(1), (U,C)Ap(1),CAp(1),Ap(2)

* Due to a too low amount of radioactivity at this stage of the analysis the molar yields of the various digestion products could not be reliably established.

material in these spots revealed that spot II corresponds to a long oligonucleotide which overlaps the 5'-end of 7S (and 5.8S) RNA as predicted by the DNA sequence shown in Fig. 2, whereas spot I corresponds to an oligonucleotide of 40 nucleotides which is encoded by the DNA sequence from position 504 to 543 (see Fig. 2). Fig. 3A illustrates the large gap in the "G-lane" corresponding to this long oligonucleotide. The presence of this oligonucleotide in a digest of 29S RNA implies that the 5'-end of the 29S RNA region must be located between position 434 and 503 of the DNA sequence in Fig. 2. The possibility that the 5'-end of 29S RNA joins directly the 3'-end of 18S RNA in the 37S precursor RNA still exists.

DISCUSSION

We have determined the DNA sequence of the spacer region between the 17S and 5.8S rRNA genes in the ribosomal operon of the yeast *Saccharomyces carlsbergensis*. This spacer is 362 bp long and, - as judged from the precursor rRNA sequence data presented in this paper - is virtually homogeneous throughout all ribosomal repeats in this organism. Comparison of this spacer sequence with the one recently published by Skryabin *et al.* (23) for *S. cerevisiae* reveals sequence differences at 8 different sites. These differences could

reflect either differences between the two yeast strains, or gene heterogeneity or experimental errors. At least one microheterogeneity appears to be present around position 255 (see Fig. 2) where a stretch of 7 thymidine residues is found in S. cerevisiae as against 8 thymidine residues in S. carlsbergensis. This assumption is strongly supported by the finding that a T_1 RNAse digest of 18S precursor RNA contains two products in submolar amounts with the sequence AU_7G and AU_8G, respectively (spot 3a and 3b in Fig. 4).

Comparison of the DNA sequence in Fig. 2 with that of the corresponding region in the ribosomal RNA operon in E. coli (1) reveals that, apart from the extensive homology between the 3'-terminal regions of yeast 17S rRNA and E. coli 16S rRNA, the two intergenic regions have a completely different sequence. Likewise, if we compare the sequence around the 5'-end of the 5.8S rRNA gene with that of the corresponding region in Neurospora crassa (24) a strong conservation of the mature 5.8S rRNA sequence beside large sequence differences in the transcribed spacer region can be noticed.

Searching for structural characteristics which may act as recognition sites for the processing enzymes we compared the nucleotide sequences surrounding a number of processing sites in 37S precursor rRNA. In the rRNA operons of E. coli complementary sequences flank both the 16S and 23S rRNA genes (1,2). These complementary sequences may base pair to form a giant stem and loop structure in the 30S precursor rRNA; the so formed double-stranded regions include the sites of cleavage by the double-strand specific RNAse III. Therefore, it was particularly interesting to see whether a similar sequence complementarity is present in the regions flanking the mature rRNAs in the yeast 37S precursor rRNA. However, we could not detect any significant base complementarity between the sequence surrounding the 3'-end of 18S RNA (cf. Fig. 2) and the sequence surrounding the 5'-end of 18S RNA (or 17S rRNA) as published for S. cerevisiae (25). There is also no complementarity between the sequence which contains the 5'-end of 29S RNA (and the 3'-end of 18S RNA; Fig. 2) and the sequence flanking the 3'-end of 37S or 29S precursor RNA (26). Finally, the intergenic region as presented in Fig. 2 does not contain complementary sequences of sufficient length to form a secondary RNA structure within this region itself. Therefore, it may be concluded that the processing of ribosomal precursor RNA in yeast (and presumably in eukaryotes in general) proceeds in a way which differs fundamentally from the mechanism of processing of E. coli ribosomal precursor RNA.

A further examination of the sequences surrounding the cleavage sites

in 37S precursor rRNA reveals a number of interesting features. As indicated in Fig. 2 the three processing sites in the intergenic region are all flanked at one side by a very (A+T)-rich region. A similar (A+T)-rich sequence is found at the 3'-end of the 26S rRNA gene (Ref. 26, and G.M. Veldman, unpublished results) and at the 5'-end of the 17S rRNA gene (25), but not at the 3'-end of the 5.8S rRNA gene (22). Whether these (A+T)-rich sequences are functionally involved in the specific interaction between the precursor rRNA substrate and the processing enzymes cannot be concluded yet. In addition, frequently sequence repeats of 3 to 6 nucleotides are observed around the bond to be cleaved, as indicated (by dashed lines) in Fig. 6. In all cases the cleavage site is present in a sequence which is repeated at a short distance. Again, the function of these sequence repeats in the processing is not yet clear.

Comparison of the 3'-terminal sequences of the three mature rRNAs and 18S RNA reveals a fairly homologous sequence of nucleotides, viz. GNNUCAUU$^{A}_{U}$, near or at the 3'-end (cf. Fig. 6). This homology strongly suggests that this sequence might serve as a recognition site for the processing enzyme(s), and in addition that a common or strongly related processing enzyme is involved in the generation of the various 3'-termini. An even more

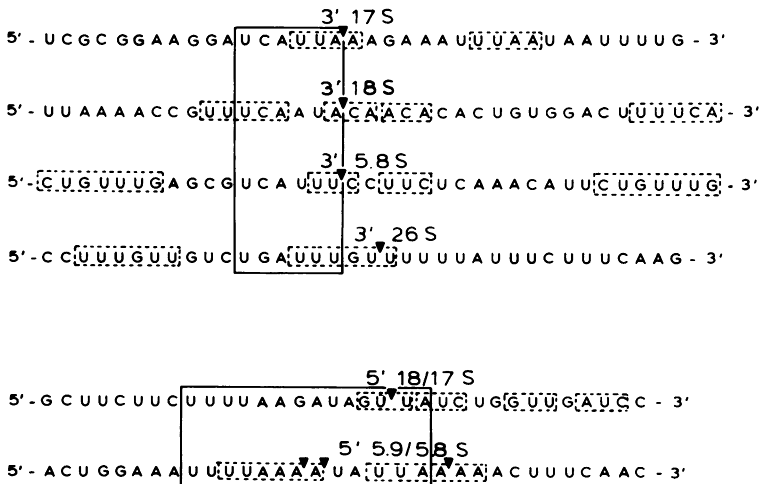

Fig. 6. Nucleotide sequences around various cleavage sites in yeast precursor rRNA. Sequence data at the 3'-end of 5.8S rRNA are taken from refs 21 and 22, at the 5'-end of 18S and 17S RNA from ref. 25 and at the 3'-end of 26S rRNA from ref. 26.

striking homology is observed at the 5'-ends of 18S (and 17S) RNA and 7S (and 5.8S) RNA comprising a nearly identical sequence of 13 or 14 nucleotides (cf. Fig. 6). This long common sequence strongly suggests that it forms part of a recognition site for a processing enzyme which generates both termini. Both 17S rRNA and 5.8S rRNA are formed via precursor RNAs (viz. 18S and 17S RNA, respectively) which do possess already a mature 5'-end. This fact is consistent with the suggestion that both 5'-termini are generated by the same processing enzyme by recognition of (part of) the common nucleotide sequence.

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