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The transcription termination site of the ribosomal RNA operon in yeast

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ABSTRACT

The site at which transcription of the ribosomal RNA operon in yeast is terminated was precisely localized. First, the exact position of the 3' end of the 26S rRNA gene was mapped on the rDNA on the basis of RNA- and DNA sequence data. Next, the 3' terminus of the primary transcript, 37S precursor rRNA, was established by hybridization experiments and sequence analysis. 37S pre-rRNA appears to be just 7 nucleotides longer at its 3' end than 26S rRNA. The non-coding strand around the termination site is extremely T-rich: 15 out of 18 nucleotides are T-residues. An extensive dyad symmetry is present in the sequence downstream from the termination site; a possible role of this structure in the regulation of transcription termination is discussed.

The 3'-terminal 110 nucleotides of yeast 26S rRNA have approx. 50% and 60% homology with the corresponding regions of *E. coli* 23S rRNA and *Xenopus laevis* 28S rRNA, respectively.

INTRODUCTION

In yeast the 17S, 5.8S and 26S rRNA molecules are transcribed as parts of a common precursor molecule, 37S pre-rRNA, which is processed in a number of steps to yield the mature RNA species (1). Recently, we have demonstrated that the 37S pre-rRNA of *S. carlsbergensis* contains a triphosphate group at its 5' terminus and a uridine-rich sequence at its 3' terminus (2). It thus can be considered as the primary transcription product.

To investigate the structure of the site at which transcription of the 37S pre-rRNA is terminated we have mapped the 3' terminus of this precursor RNA on the rDNA by hybridization experiments and DNA sequence analysis. We also have analyzed and mapped the 3'-terminal 25 nucleotides of 26S rRNA.

Our data indicate that the 37S pre-rRNA is just 7 nucleotides longer at its 3' end than 26S rRNA. Near the transcription termination site the DNA sequence exhibits an extensive dyad symmetry which may play a role in the regulation of transcription termination. Our data also show that the sequence

TTTCTTTTTTTTTTTTT
AAAGAAAAAAAAAAAAA'

which was found to be present in the rDNA of *Saccharomyces cerevisiae* about

220 base pairs downstream from the 3' end of the 26S rRNA cistron, and which was supposed to represent the terminator region by Valenzuela et al. (3) is partially deleted in the rDNA of S. carlsbergensis.

MATERIALS AND METHODS

Isolation of labelled RNAs

^{32}P-labelled 26S plus 5.8S rRNA was prepared from S. carlsbergensis ribosomes as described previously (4). Dissociation of the 26S - 5.8S rRNA complex was performed by heating at 53°C for 5 min in 0.1 M NaCl, 0.01 M EDTA followed by rapid chilling to 0°C. The 26S rRNA was then separated from the 5.8S rRNA by sucrose gradient centrifugation and recovered from the gradient by ethanol precipitation.

^{32}P-labelled 37S pre-rRNA was isolated in combination with 29S pre-rRNA as described by Klootwijk et al. (2).

Isolation of the "α-sarcin fragment" of 26S rRNA

^{32}P-labelled ribosomes, isolated from yeast cells (4) were suspended for 1 hr at 4°C in TMK buffer (0.01 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, 0.5 M KCl, 0.2% (w/v) Brij-58, 10 mM dithiotreitol, to which per litre were added 5 mg of puromycin (Sigma) and 3 ml of a macaloid suspension). The ribosome suspension was then subjected to linear sucrose gradient centrifugation (15-30% w/v sucrose in TMK buffer) at 20,000 rpm in a Spinco SW-27 rotor for 16 hr. The fractions containing 60S ribosomal subunits were pooled and centrifuged in a Spinco SW41 rotor for 20 hr at 40,000 rpm. The pellet was suspended in 3 ml TMK buffer at a concentration of about 0.65 mg/ml and incubated for 45 min at 37°C with 20 µg of the drug α-sarcin (generously provided by Dr. J.E. Davies, Madison, U.S.A.). Subsequently, the RNA was extracted with phenol plus 0.5% sodium dodecylsulphate and precipitated with ethanol. The RNA was dissolved in a 0.04 M Tris-acetic acid buffer (pH 8.0) and fractionated on a 8% polyacrylamide slab gel overlayered with 2 cm of 3% polyacrylamide spacer gel. After visualization by autoradiography the band containing the "α-sarcin fragment" was cut out from the gel, put into a 2 ml syringe and eluted with 2.5 ml of 1 M NaCl by recycling of the eluate for 16 hr at 4°C. After addition of unlabelled 26S yeast rRNA the eluate was dialyzed against distilled water and lyophilized.

RNA sequence analysis

To isolate 3'-terminal RNA fragments derived from 26S rRNA, about 1 mg of ^{32}P-labelled 26S rRNA was partially digested with RNAase T_1 (Calbiochem A.G. Lucerne, Switzerland) at an enzyme to RNA ratio of 1:500 (w/w) for 20 min at

0°C. The digestion products were fractionated by DBAE cellulose column chromatography (5). The polynucleotide material containing the 3'-terminal fragments was then fractionated two-dimensionally by electrophoresis on cellulose acetate strips at pH 3.5 followed by homochromatography on a thin-layer plate of DEAE-cellulose using a "homomix C" hydrolyzed for 9 min (5). Oligonucleotides recovered from the thin-layer plates were digested with 70 units of RNAase T_1 or 6 µg pancreatic RNAase (Sigma Chemical Co, St. Louis, Mo) per 200 µg of RNA for 7 hr at 37°C in 10 µl of 0.01 M Tris-HCl (pH 7.5) containing 1 mM EDTA (6). Sequence analysis of the oligonucleotides obtained after electrophoresis on DEAE-cellulose paper in 5% acetic acid was performed by standard techniques as described by Brownlee (6), using tertiary digestion with RNAase T_1, pancreatic RNAase, RNAase U_2 (Calbiochem) or 0.5 M KOH.

Isolation of rDNA fragments

We used the hybrid plasmids pMY1 and pMY60, constructed by Dr. J.H. Meyerink (7), which contain the Hind III fragment A and the complete ribosomal repeat of S. carlsbergensis, respectively, cloned in the vector pBR322. Plasmid DNA was isolated from E. coli K12 cells by the clear lysate method as described by Goebel and Bonewald (8). The procedure for the isolation of restriction fragments was described previously (9).

DNA sequence analysis

Sequence analysis was performed according to the method of Maxam and Gilbert (10) on 12% or 20% polyacrylamide - 7 M urea - citrate gels. DNA fragments were labelled at their 5' ends as described previously (9) except that the reaction mixture was incubated for 30 min at 37°C and fractionated on the Sephadex G50 column without prior heating.

Southern hybridization

2.5 µg of pMY60 DNA was digested with Hinf I, or Hae III plus Hinf I or EcoRI plus Hinf I. The digests were fractionated on a 3% agarose gel and transferred to a cellulose nitrate filter as described by Southern (11). The filters were hybridized in a tube with ^{32}P-labelled 37S pre-rRNA ($3x10^6$ cpm) in 1.5 ml of an aqueous solution containing 6x SSC, 0.3% ficoll, 0.3% polyvinylpyrrolidon, 50% formamide and 100 µg Bacillus RNA for 70 hr at 56°C. In some cases 200 µg of unlabelled 26S rRNA was added as competitor RNA. The filters were washed 3 times with 2x SSC, once with 2x SSC containing per ml 10 µg of pancreatic RNAase and 5 units of RNAase T_1 and finally with 2x SSC at 46°C for 1 hr. The filters were dried and exposed to X-Omat XRP 1 film (Kodak) using tungstate intensifying screens.

RESULTS

A. The 3' terminus of the 26S rRNA gene

In order to define the region on the ribosomal DNA which may contain the site of transcription termination we first mapped the 3' end of the 26S rRNA gene. Philippsen et al. (12) have previously shown that the 3' terminus of the 26S rRNA gene of S. cerevisiae is present on EcoRI fragment E by electrophoretic analysis of S_1 nuclease-treated rRNA/rDNA hybrids in alkaline agarose gels. To determine the exact position of the 3' end, it is necessary to compare rDNA and 26S rRNA sequences. We have previously established that 26S rRNA of S. carlsbergensis terminates in (A)UUUGU$_{OH}$ (2). As this sequence is present more than once on EcoRI fragment E (see below), we have to obtain more extended sequence information about the 3'-terminal part of 26S rRNA. We digested ^{32}P-labelled 26S rRNA partially with RNAase T_1 and fractionated the products by DBAE-cellulose column chromatography (5,13) to obtain a set of successively longer polynucleotide fragments derived from the 3' terminus. The fraction containing polynucleotides with a free 3'-OH end was then subjected to high-voltage electrophoresis and "homochromatography" as shown in Fig. 1. The autoradiograph displays a vast array of clear and weak spots. The polynucleotide material from the clear spots was divided in two portions and analyzed for the presence of both GU$_{OH}$ (by digestion with pancreatic RNAase)

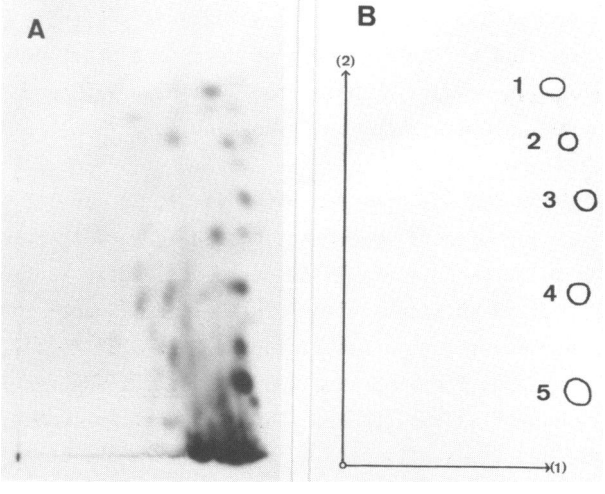

Fig. 1. Two-dimensional fractionation of the polynucleotide material containing 3'-terminal fragments of yeast 26S rRNA. (1) Electrophoresis on cellulose acetate at pH 3.5 and (2) "homochromatography". Products 1 to 5 were positively identified to be derived from the 3' end of 26S rRNA (cf. Table I).

and an oligonucleotide ending in ..(A)UUUGp (after digestion with RNAase T_1). The spots 1-5 (Fig. 1B) all fulfilled these conditions and therefore must represent oligonucleotides containing the 3' end of 26S rRNA. Products 1-5 were subjected to further sequence analysis (see Table I). These products can be arranged as a series of succesively longer polynucleotides; by going from fragment 1 to fragment 5 each nucleotide sequence contains one additional cleavage site for RNAase T_1 5'-distal to the previous one. This analysis leads to the sequence AUUAAGC(C,U,U,U)GUUGUCUGAUUUGU$_{OH}$ for the longest 3'-terminal fragment of 26S rRNA.

The DNA sequence of 416 base pairs upstream from the Hind III site in EcoRI fragment B was determined by the method of chemical modification (10); this sequence accounts for nearly half of EcoRI fragment E. Fig. 2 shows the rDNA map on which the sequenced region is indicated. The deduced sequence is shown in Fig. 3. The sequence from position -25 to position -1 unambiguously encodes the 3'-terminal fragment of 26S rRNA.

An independent approach to characterize the sequence at the 3' end of 26S rRNA was based upon the action of the drug α-sarcin. This drug effectively inhibits eukaryotic protein synthesis by causing a specific cleavage in the 26S rRNA near the 3' terminus (14). We have incubated yeast 60S ribosomal subunits with α-sarcin and fractionated the extracted RNA by polyacrylamide slab gel electrophoresis (Fig. 4). The autoradiograph reveals a band ('α.s') of roughly 300 nucleotides, which is absent in the control. To prove that this so-called α-sarcin fragment is derived from the 3' terminus of 26S rRNA, we checked for the presence of UUUGU$_{OH}$ by the procedure of kethoxal modification of guanosines and subsequent RNAase U_2 digestion in the same way as described previously for 26S rRNA (2). The proper sequence was found to be the only 3' end (data not shown). In tracking down nucleotide sequences characteristic for the 3'-terminal part of 26S rRNA the α-sarcin fragment was digested to

TABLE I. Sequences of 3'-terminal oligonucleotides of yeast 26S rRNA*

Fragment (cf. Fig. 1)	Sequence
1	AUUUG–U$_{OH}$
2	UCUG–AUUUG–U$_{OH}$
3	UUG–UCUG–AUUUG–U$_{OH}$
4	(C,C,U,U,U)G–UUG–UCUG–AUUUG–U$_{OH}$
5	AUUAAG–C(C,U,U,U)G–UUG–UCUG–AUUUG–U$_{OH}$
	25 20 15 10 5 1

* The complete analysis of these sequences is available upon request.

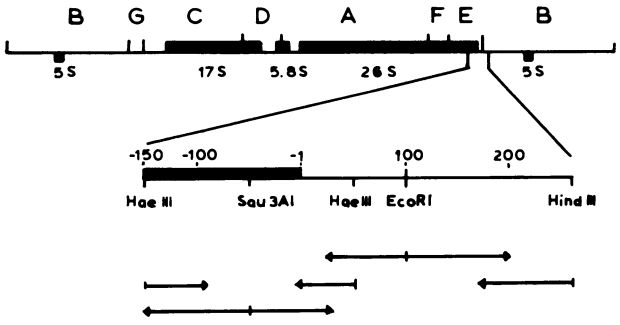

Fig. 2. Restriction enzyme sites in the region joining EcoRI fragments E and B of yeast rDNA. Sequence analysis was performed in the direction of the arrows.

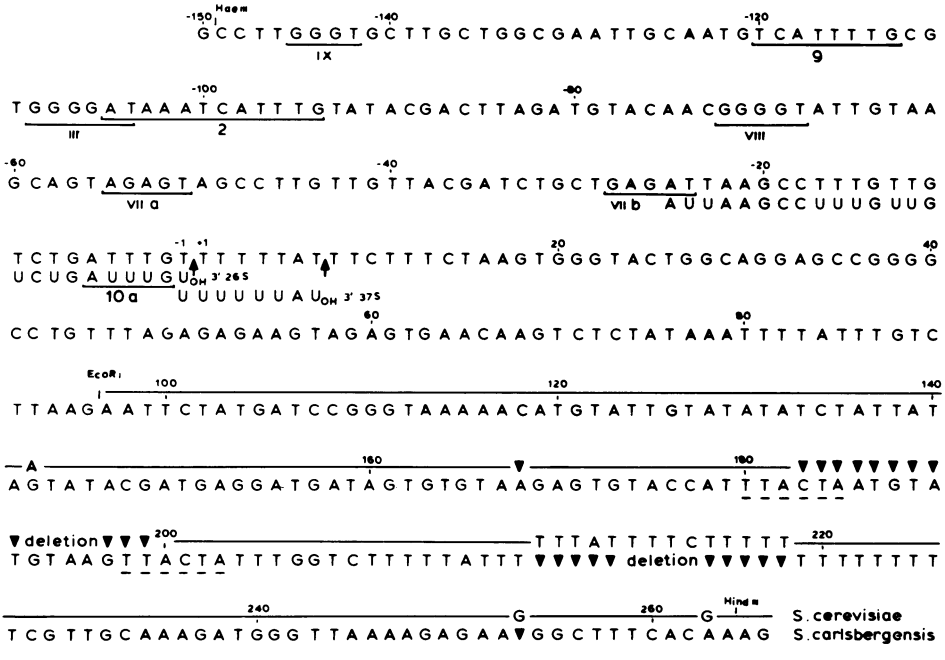

Fig. 3. Sequence of the non-coding strand around the transcription termination site of the yeast rDNA operon. Underlined sequences correspond to T_1- or pancreatic RNAase digestion products identified near the 3' end of 26S rRNA. The overlined sequence has been shown to be identical for a cloned ribosomal gene of S. cerevisiae as determined by Valenzuela et al. (4).

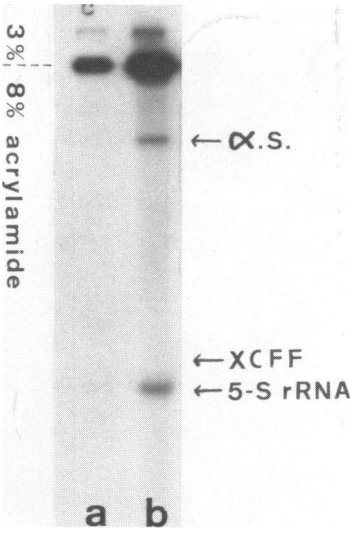

Fig. 4. Separation of a 3'-terminal fragment ('α.s') derived from 26S rRNA by the action of α-sarcin on 60S ribosomal subunits (lane b). Lane a shows a control of the RNA from a eight-fold lower amount of untreated ribosomes.

completion with either RNAase T_1 or pancreatic RNAase, followed by standard separation procedures as shown in Fig. 5A and C. Well-resolved oligonucleotides with a length of at least four nucleotides were subjected to further analysis. In principle, RNAase T_1 generated oligonucleotides were digested with RNAase U_2 and pancreatic RNAase, respectively, while the pancreatic RNAase generated oligonucleotides were treated with RNAase T_1. The results are given in Table II. The oligonucleotides 2, 8, 10a, III, VII a+b, VIII and IX are apparently encoded by the DNA sequence from position -150 to -1, as can be inferred from the underlined sequences in Fig. 3. The oligonucleotides 1, 3, 4, 6, 7, 12, 15, IV, V and VI are encoded further upstream into the 26S rRNA gene between position -350 to -150 (sequence not shown). These markers confirm unequivocally the deduced position of the 3' end of the 26S rRNA gene on EcoRI fragment E as shown in Fig. 3.

B. The site of transcription termination

The 3' terminus of the primary transcript, 37S pre-rRNA was previously determined as $U_{6-8}AN_{OH}$ (2). This terminus differs from the 3' terminus of mature 26S rRNA, which means that the transcription does not stop at the end of the 26S rRNA gene. To define the downstream limit for the site of transcription termination, we performed Southern hybridization of appropriate DNA fragments with ^{32}P-labelled 37S pre-rRNA. Hybrid plasmid pMY60 DNA was digested with either Hinf I, Hae III plus Hinf I or EcoRI plus Hinf I. The digestion products were fractionated on a 3% agarose gel as shown in Fig. 6A. Fig. 6C

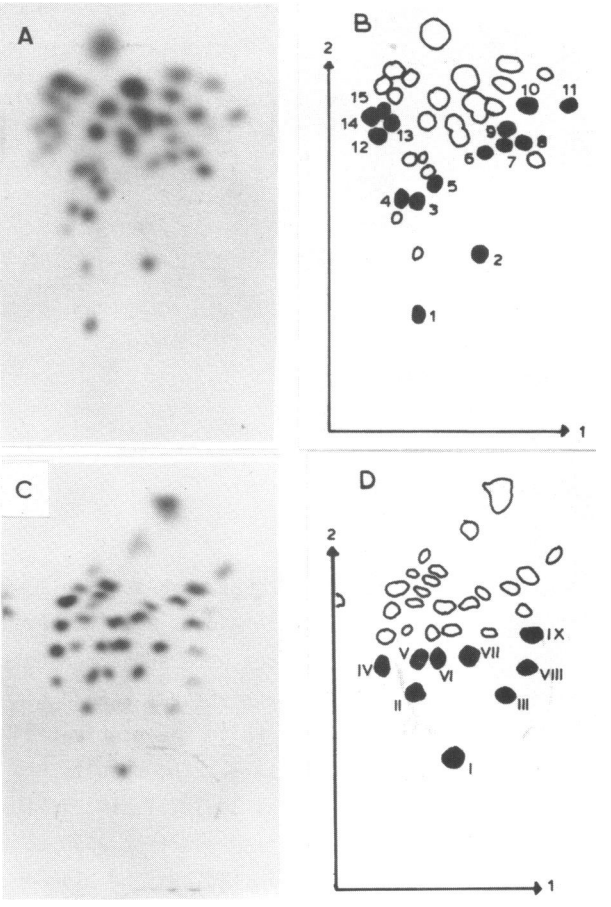

Fig. 5. 'Fingerprints' of the α-sarcin fragment. (A) Fractionation of the RNAase T_1 digestion products according to (6); (B) key to A; (C) fractionation of the pancreatic RNAase digestion products; (D) key to C.

shows the position of three relevant fragments on the ribosomal repeating unit. The DNA fragments were transferred to nitrocellulose filters and hybridized with ^{32}P-labelled 37S pre-rRNA in the presence of an excess of unlabelled 26S rRNA. The fragments 1 and 2, which do not code for 26S rRNA, also fail to hybridize with 37S pre-rRNA. Positive hybridization with 37S pre-rRNA, however, was observed with fragment 3 even in the presence of unlabelled 26S RNA. Hinf I fragment 4 (cf. Fig. 6A) serves as an internal control for the sensitivity of this experiment; it consists of a stretch of pBR322 DNA plus 150 base pairs of the "leader" sequence of the rRNA operon and

Table II. Catalogue of a number of oligonucleotide sequences present in the α-sarcin fragment (cf. Fig. 5).

Spot no.	Sequence
1	[C(C,C,U)A,UA,AUA,CA,CA]Gp
2	A(UCA,AA,UA)UUUGp
3	[CCA,(C,U)A](U,C,C)Gp
4	(AACC,AU)AGp
5	A(A,UCC)AUGp
6	A(A,U)AUUGp
7	(U,U,C)AU(U,C)Gp
8	UCAUUUUGp
9	AUUAUGp
10 a+b	AUUUGp + UAUUGp
11	UUUUUGp
12	AACAGp
13	CAAAGp
14	(AAC,C)Gp
15	AACGp
I	(AAAG,G,G,G)Cp
II	(AG,G)AACp
III	GGGGAUp
IV	AGAACp
V	(AAG,G)Cp
VI	AGAAUp
VII a+b	AGAGUp + (AG,G)AUp
VIII	GGGGUp
IX	GGGUp

it clearly hybridizes with 37S pre-rRNA (cf. Fig. 6B). From this experiment we may conclude that the site of termination will be present in the region ranging from the 3' end of 26S rRNA until some 50 base pairs beyond the Hae III recognition site at position +40 (cf. Fig. 3). The nucleotide sequence of this region reveals just one site which can code for the 3' terminus of 37S pre-rRNA, $(G)U_{6-8}AN_{OH}$, namely GTTTTTTAT from position -2 to +7. This putative termination site is only 7 nucleotides away from the 3' terminus of the 26S

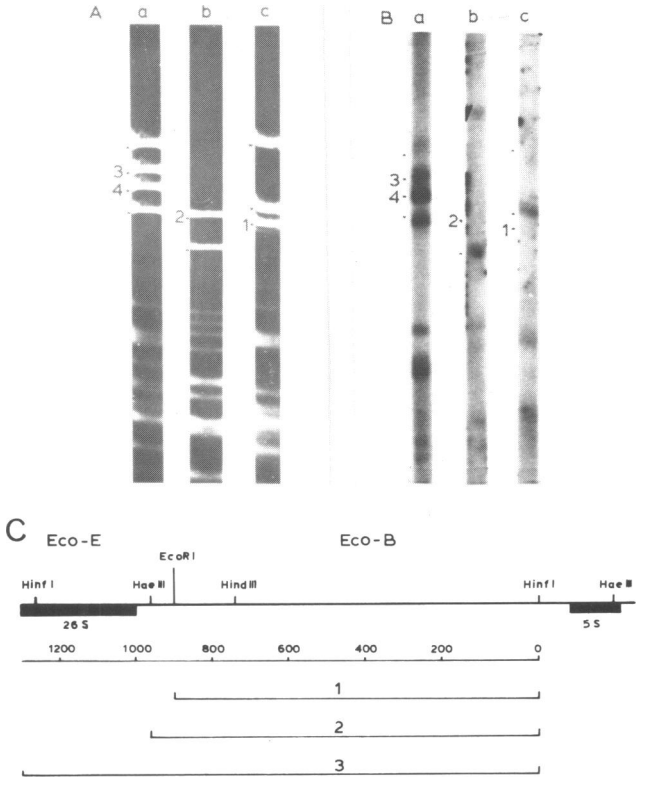

Fig. 6. Southern hybridization of DNA fragments with ^{32}P-labelled 37S pre-rRNA. A. pMY60 DNA was digested with various restriction enzymes and the fragments were fractionated on a 3% agarose gel. (a) Hinf I, (b) Hinf I plus Hae III, (c) Hinf I plus EcoRI. B. Autoradiograph after hybridization with 37S pre-rRNA. C. Map with the positions of fragments 1, 2 and 3. Fragment 4 is a stretch of pBR322 linked to 150 base pairs of the "leader" sequence of the rRNA operon.

rRNA gene. With such a short extra sequence at the 3' end of 37S pre-rRNA no hybridization of Hinf I fragment 3 with 37S pre-rRNA is expected in the presence of unlabelled 26S rRNA. Its positive hybridization therefore can be caused by a incomplete competition with unlabelled 26S rRNA and/or b the occurrence of a fraction of 37S pre-rRNA molecules with a somewhat longer 3'-terminal transcribed spacer sequence. If the latter possibility is true, this can only be a minor fraction since transcription beyond the putative terminator at position +7 will give rise to a characteristic RNAase T_1

generated oligonucleotide ($U_{13}A_3C_2G$, encoded from position -1 to +18), which we cannot detect among the major products in 'fingerprints' of 37S pre-rRNA (25).

DISCUSSION

We have mapped the 3' terminus of 26S rRNA on the ribosomal repeat of S. carlsbergensis. Sequence analysis of the 25 terminal nucleotides of 26S rRNA and a few oligonucleotide markers present in a 3'-terminal fragment generated by α-sarcin, supplied solid evidence to permit unequivocal mapping of the gene terminus on EcoRI fragment E, 97 bp upstream from the junction between EcoRI fragments E and B. Comparison of the 3'-terminal 100 nucleotides of the large subunit rRNA's of E. coli (15), yeast and Xenopus (16) reveals that E. coli 23S rRNA has a homology of about 50% with the corresponding region in both yeast and Xenopus 26/28S rRNA. The two eukaryotic sequences are more than 60% homologous (cf. Fig. 7). Although in general no significant sequence similarity was observed among the 3'-terminal oligonucleotides of 23/28S rRNAs from pro- and eukaryotes (17), the extended region of homology observed here indicates that the 3'-terminal region of the large ribosomal subunit rRNA has been fairly well conserved and therefore may play a vital role in the ribosome. In addition, this homology indicates that the increase in length of 28S rRNA of higher eukaryotes is a result of insertions at internal positions of the rRNA operon rather than at its 3' end.

To determine the transcription termination site the 3'-terminal sequence

```
          -140        -130        -120        -110        -100
            *           *           *           *           *
              UGAGUUCUCCUGACUCCUUGAGAGUCCUGAAGG--AA-C   E.coli
CCUUGGGUGCUUGCUGGCGAAUUGCAAUGUCAUUUUGCGUGGGGAUAAAUC   Yeast
GCCUCUCUCCGGAGCGCACCGCACGUUCGUGGGGAACCUGGUGCUAAAUC   X.laevis
          -90         -80         -70         -60         -50
            *           *           *           *           *
G-UUGAAGACGACGACGUUGAUAGGCCGGGUGU-GUAAGC-GCAG-GCA--   E.coli
AUUUGUAUACGACUUAGAUGUACAACGGGGUAUUGUAAGCAGUAGAGUAGC   Yeast
AUCGUAGACGACCUGAUUCUGGGUCAGGGUUUCGUGCGUAGCAGAGCAGC   X.laevis
          -40         -30         -20         -10         -1
            *           *           *           *           *
UGCGUUG-AGCUAACCGGUACUAAUGAA-CC-GUGAGGCUUAACCUU      E.coli
CUUGUUGUUACGAUCUG-CUGAGAUUAAGCCUUUGUUGUCUGAUUU-GU    Yeast
UACCUCGCUGCGAUCUA-UUGAAAGUCAUCCCUUGAGCCAAGCUUUUGU    X.laevis
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Fig. 7. Sequence comparison of the 3'-terminal regions of yeast 26S rRNA, E. coli 23S rRNA (15) and Xenopus laevis 28S rRNA (16). Homologous nucleotides are underlined. The numbering is according to Fig. 3.

of the primary transcript, (G)$U_{6-8}AN_{OH}$, was mapped on the rDNA by DNA sequence analysis and Southern hybridization. Transcription terminates just 7 nucleotides beyond the 26S rRNA gene with the sequence (G)$UUUUUUAU_{OH}$. Since the molar yield of this 3' end in 37S pre-rRNA is unknown, it cannot yet be concluded whether all RNA polymerases terminate at this site or a major fraction of them.

Inspection of this termination site for possibly relevant features for the termination of transcription by RNA polymerase A reveals that termination occurs in an (A+T)-rich region containing a long (15 out of 18 nucleotides at positions -5 to +13) stretch of T-residues in the non-coding strand. This cluster of T-residues has been observed at most other prokaryotic terminators and terminators of RNA polymerase C (18,19) and is thought to facilitate the release of the transcript (20). However, the 37S RNA termination site differs from most other termination sites by the lack of both a (G+C)-rich region preceding the T-cluster and a dyad symmetry in this region. On the other hand, the termination region of the yeast rRNA operon contains an extended dyad symmetry downstream from the termination site as illustrated in the form of a hairpin loop in Fig. 8A. Fig. 8B shows that a somewhat less extended dyad symmetry but at the same position towards the gene can be observed near the rRNA termination site of X. laevis (16). This structural similarity at two terminators of RNA polymerase A suggests that sequences beyond the site of termination are involved in the termination process. One can envisage that this palindromic region forms a specific tertiary structure, e.g. a four-stranded helix (21), which may be recognized by a termination factor. Leer et al. have isolated a protein factor which is involved in termination of rRNA transcription in nucleoli of Tetrahymena (22). In addition, it is interesting to note that distal sequences at the tryptophan operon of E. coli play a role in termination of transcription (23) and that dyad symmetries are also found downstream from some histon genes in sea urchin (24).

We have compared part of the DNA sequence of the non-transcribed spacers (residues +96 to +265) of S. carlsbergensis and S. cerevisiae (3). There are some remarkable differences in this region of the ribosomal repeat between the two closely related yeast species (Fig. 3). Single base changes or deletions occur at position +142, 168, 254 and 263. Moreover a stretch of 17 base pairs (from position 183 to 200) of S. carlsbergensis DNA is absent in S. cerevisiae DNA. This extra sequence may reflect either an insertion event or a deletion event. The extra sequence is flanked at both sides by the sequence TTACTA, which is present only once in the corresponding region of the S. cerevisiae

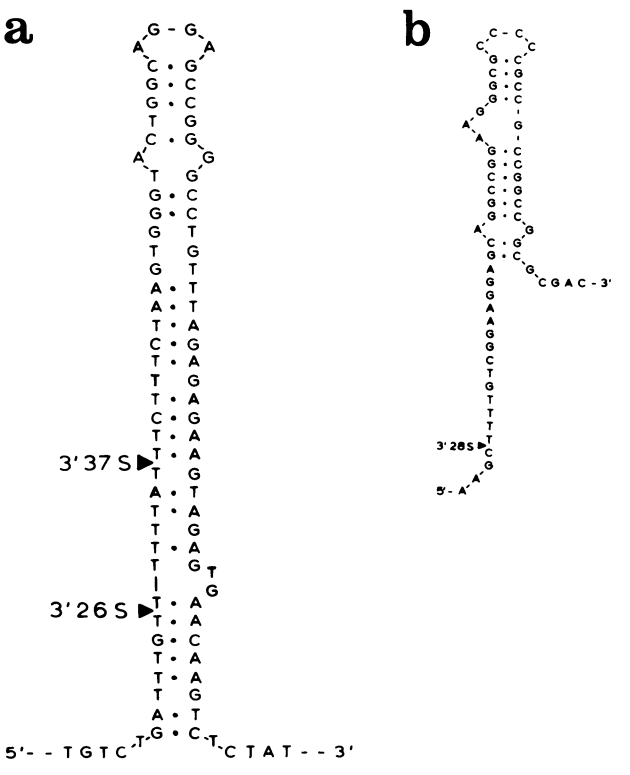

Fig. 8. Dyad symmetries present beyond the termination sites of the yeast and Xenopus laevis (16) rRNA operons. The non-coding strand is shown in the form of a hairpin.

DNA, suggesting that transposition is involved in this sequence difference between the two strains. Vice versa, near position 220 an extra stretch of 14 base pairs is present in the S. cerevisiae gene which is absent in S. carlsbergensis. This means that a part of the sequence

TTTCTTTTTTTTTTTTT
AAAGAAAAAAAAAAAAA

in S. cerevisiae DNA which was supposed to be part of the termination site by Valenzuela et al. (3) on the basis of homology with the termination region of the 5S rRNA gene is deleted in S. carlsbergensis. This fact together with the other data presented in this paper clearly refute the hypothesis of Valenzuela et al.

Our data indicated that very little processing is required at the 3' end of the primary transcript. Similar observations have been made for the pre-rRNAs of Xenopus laevis (16) and Tetrahymena thermophila (26).

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