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3′-End formation of transcripts from the yeast rRNA operon

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Deletion analysis of artificial rRNA minigenes transformed into *Saccharomyces cerevisiae* revealed that a 110 bp long fragment corresponding to positions −36 to +74 relative to the 3′-end of the 26S rRNA gene, is both necessary and sufficient for obtaining transcripts whose 3′-termini are identical to those of 26S and 37S (pre-)rRNA. These termini are produced via processing of longer transcripts because in an *rna 82.1* mutant the majority of the minigene transcripts extend further downstream. Since the *rna 82.1* mutation inactivates an endonuclease involved in the 3′-processing of 5S pre-rRNA it is concluded that the maturation of 37S- and that of 5S pre-rRNA requires a common factor. Comparison of the spacer sequences between *Saccharomyces carlsbergensis*, *Saccharomyces rosei* and *Hansenula wingei* revealed several conserved sequence blocks within the region between +10 and +55. These conserved sequence tracts, which are part of a longer region showing dyad symmetry, are supposed to be involved in the interaction with the processing component(s). Deletion of the sequences required for the formation of the 3′-ends of 26S rRNA and 37S pre-rRNA revealed a putative terminator for transcription by RNA polymerase I situated at position +210. This site maps within a DNA fragment that also contains the enhancing element for rDNA transcription by RNA polymerase I.

Key words: processing/ribosomal DNA/RNA polymerase I/termination/yeast

Introduction

Regulation of rRNA synthesis has been the subject of extensive studies in a number of organisms (reviewed by Mandal, 1984; Moss *et al.*, 1985). The majority of these studies have been focused on initiation of transcription by RNA polymerase I (Pol I) and have revealed a wealth of details about this process and its regulation.

In contrast, our knowledge of the processes involved in the generation of the 3′-ends of Pol I transcripts, which may also play a regulatory role in rRNA production, is still very limited. Until recently, it was generally assumed that the 3′-ends of the largest pre-rRNA molecules identified reflected the site of transcription termination. These 3′-ends either coincide with the 3′-terminus of 26S rRNA in *Xenopus* and *Drosophila* (Sollner-Webb and Reeder, 1979; Mandal and Dawid, 1981) or extend a few nucleotides beyond it in yeast, mouse and *Tetrahymena* (Veldman *et al.*, 1980; Kominami *et al.*, 1982; Din *et al.*, 1982).

Our primary aim in this study was the identification of the sequence element(s) involved in the formation of the 3′-termini of 37S pre-rRNA, the longest Pol I transcript detectable in yeast (Veldman *et al.*, 1980). Furthermore, we wanted to distinguish between processing and termination as the mechanism responsible for the generation of these termini.

To answer the first question two different approaches were used that previously have been successfully employed in the analysis of the signals involved in initiation of rRNA transcription. The first consisted of deletion analysis of rRNA minigenes that, after transformation into yeast cells, give rise to transcripts having discrete 3′-ends (Kempers-Veenstra *et al.*, 1984, 1985). In order to substantiate and extend the results of this analysis we searched for evolutionary conserved sequences in the relevant regions of the rDNA units of several Saccharomycetoideae (Verbeet *et al.*, 1984a).

Introduction of the minigenes into an *rna 82.1* mutant (Piper *et al.*, 1983), carrying a mutation that inactivates an endonuclease involved in maturation of 5S rRNA, provided evidence that the 3′-end of 37S pre-rRNA is formed by processing rather than by termination. Furthermore, the mutants in which this processing is abolished by removal of the required signals revealed a putative terminator at +210. Interestingly, this site maps within a DNA fragment that also contains the enhancing element for initiation of transcription by Pol I (Elion and Warner, 1984). The observation that Pol I terminates at a significant distance downstream of the 26/28S rRNA cistron has recently also been made in studies on transcription of mouse rDNA (Grummt *et al.*, 1985a,b). Similar results have been obtained in *Xenopus laevis* (Labhart and Reeder, 1985, 1986; Moss *et al.*, 1985). Therefore, as in yeast, the previously reported 3′-termini of the respective pre-rRNAs must be the result of rapid processing of a longer transcript.

Results

3′-Ends of minigene transcripts

Figure 1 depicts the minigene constructs used in this study. Briefly, these minigenes consist of two yeast rDNA fragments, one containing the Pol I promoter, the other comprising the signals required for formation of transcripts having discrete 3′-termini. To allow detection of the transcripts the major part of the minigene is made up of a fragment derived from the DNA of *Spirodela oligorhiza* chloroplasts (Kempers-Veenstra *et al.*, 1984).

Using pARES9 we first determined the position of the 3′-ends of the minigene transcripts by S1 nuclease mapping. When RNA isolated from cells transformed with pARES9 was probed with the *Xho*I plus *Hin*dIII fragment (Figure 1) two closely spaced signals were observed (Figure 2, lane 2). The major signal corresponds closely to the distance between the labelled *Xho*I site and the 3′-end of the 26S rRNA sequence (designated position −1), while the other signal represents a transcript that is ~10 nucleotides (nt) longer. These results are in good agreement with earlier conclusions that an unknown proportion of the 37S pre-rRNA molecules in yeast has a 3′-terminal extension of 7 nt relative

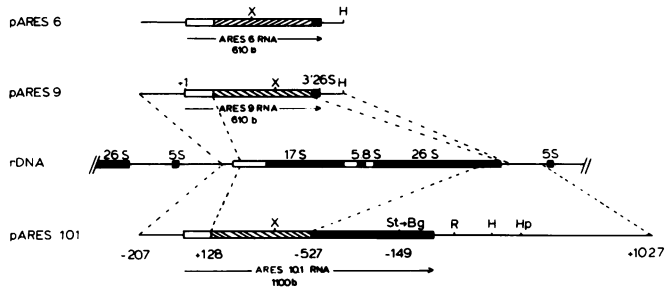

Fig. 1. Constructs used in this study. ARES minigenes are cloned into pJDB207 (Beggs, 1981). The promoter fragment in all three ARES genes is identical, ranging from −207 to +128 relative to the transcription start. pARES6 and 9 contain a 140 bp 3′-end-generating fragment from −36 to +101 relative to the 3′-end of 26S rRNA; the 1550 bp 3′-end-generating fragment in pARES10.1 contains sequences between −527 and +1027. The only difference between pARES6 and 9 is in the orientation of the marker DNA fragment. Open bars (□) refer to transcribed spacer sequences, closed bars (■) represent the rDNA coding regions and shaded bars (▨) indicate the *Spirodela oligorhiza* marker DNA. X = *Xho*I, H = *Hin*dIII, Hp = *Hpa*I, R = *Eco*RI, St = *Stu*I, Bg = *Bgl*II.

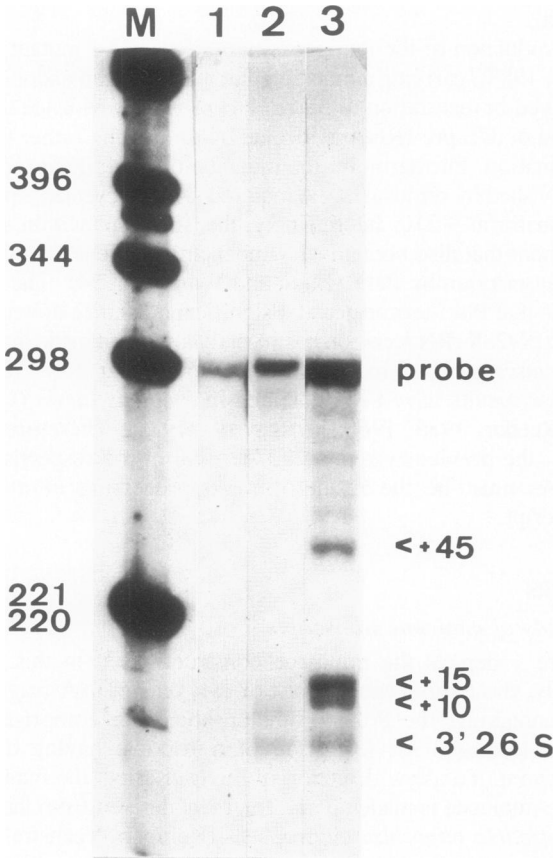

Fig. 2. 3′-End analysis of pARES9 transcripts by S1 nuclease mapping. The probe used is the *Xho*I plus *Hin*dIII fragment (Figure 1) labelled at the *Xho*I site. 25 μg of RNA isolated from *S. cerevisiae*. YT6-2-1L **(lane 2)** or L16 **(lane 3)** transformed with pARES9, or from untransformed YT6-2-1L **(lane 1)** were hybridized to the probe. Samples were subsquently digested with 75 U of S1 nuclease. **Lanes 'G'** and **'G+A'** represent chemical modifications of the probe according to Maxam and Gilbert (1977).

to the 26S rRNA sequence (Veldman *et al.*, 1980). Clearly the sequence between positions −36 and +101 with respect to the 3′-end of 26S rRNA present in pARES9 is not only sufficient for generating discrete, but also the correct 3′-ends.

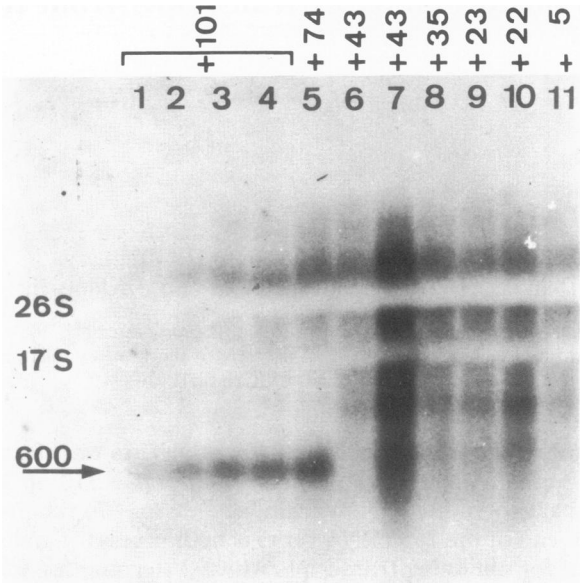

Fig. 3. Northern blot hybridization of RNA isolated from yeast cells transformed with 3′-deletion mutants of pARES6. 20 μg of RNA was fractionated on a 1.6% agarose gel and blotted onto DBM paper. The probe used was the *Spirodela* marker DNA cloned into M13mp9 (M13SoA), labelled with Klenow DNA polymerase. **Lanes 1–4** contain a calibration series of pARES6 RNA (5, 10, 15 and 20 μg respectively). In **lane 7** double the amount of RNA was applied compared with **lane 6**.

In order to further delineate the elements involved in formation of the 3′-end of 26S rRNA, we constructed a series of 3′-deletion mutants starting from the unique *Hin*dIII site in pARES6 (Figure 1). The numbering of these mutants refers to the last nucleotide of yeast rDNA still present next to the re-inserted *Hin*dIII linker. Figure 3 shows that deletion mutant +74 still gives rise to transcripts with discrete 3′-ends. Deletion to +43, however, virtually abolishes the ability of the minigene to direct formation of such discrete transcripts. In the mutants with a deficient 3′-end-generating fragment minigene transcripts of heterogeneous lengths are observed. A small proportion accumulates in a band of ~1000 nt corresponding to a 3′-end mapping within the vector. From these data we conclude that the 3′-boundary of sequences involved in formation of the 3′-ends at positions −1 and +10 is situated between +43 and +74.

3′-Ends of −1 and +10 are formed by processing

The results presented in Figures 2 and 3 do not tell us whether the 3′-termini at positions −1 and +10 are due to termination or are the result of processing of a longer transcript. In order to distinguish between these two possibilities we mapped the 3′-ends of pARES9 transcripts produced in the yeast *rna 82.1* mutant which is deficient in 3′-processing of 5S rRNA (Piper *et al.*, 1983). Figure 2 (lane 3) shows that although S1 signals corresponding to 3′-termini at −1 and +10 can still be detected in this host, the major signal now is produced by a transcript extending 15 nt beyond the 26S rRNA sequence. Thus, yeast Pol I does not terminate at position −1 and +10 but transcribes into the spacer sequences. Transcription proceeds to at least a point 45 nt downstream of the 26S sequence as witnessed by the prominent S1 signal corresponding to this position (Figure 2, lane 3). Apart from showing that the 3′-termini at −1 and +10 are formed by processing these results also indicate that the 3′-processing reactions of 5S rRNA and the Pol I transcript share

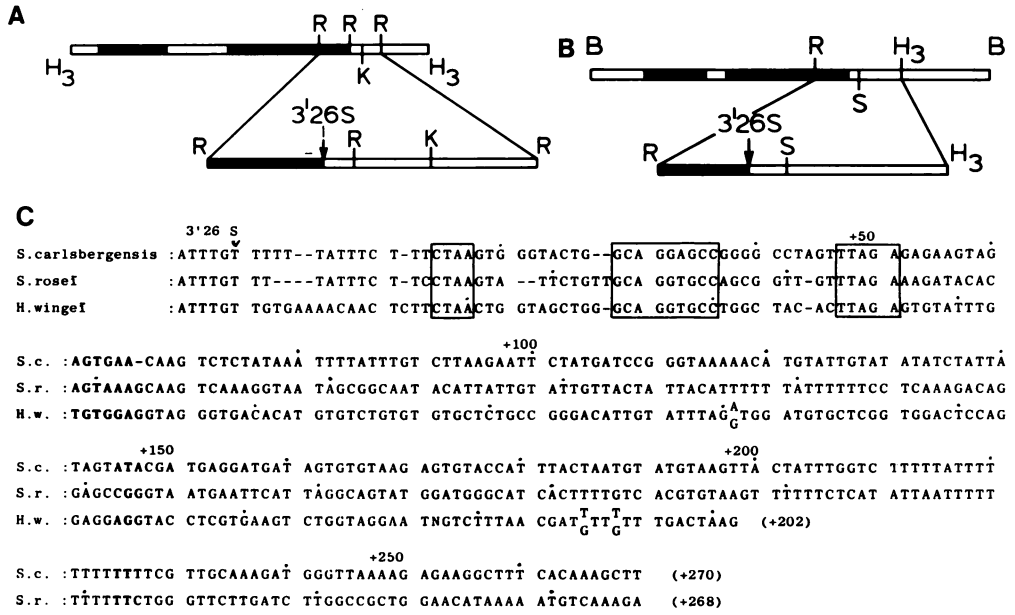

Fig. 4. Sequence comparison around the 3'-end of 26S rRNA in three Saccharomycetoideae. **(A)** Map of the rDNA repeat of *S. rosei* (Verbeet *et al.*, 1983). The black boxes represent the mature 17S and 26S rRNA genes. The *Eco*RI fragment containing the 3'-end of 26S rRNA was sequenced. The symbols are explained in Figure 1. **(B)** Map of the rDNA repeat of *H. wingei* (Verbeet *et al.*, 1984b). The *Eco*RI plus *Sac*I (S) fragment was sequenced. **(C)** Nucleotide sequence of the region surrounding the 3'-end of 26S rRNA in three Saccharomycetoideae; the non-coding strand is shown. Conserved elements are boxed. Shifts (−) were allowed to maximize homology. Nucleotides of *S. carlsbergensis* are numbered relative to the 3'-end of 26S rRNA at −1. Dots indicate multiples of 20 nt beyond the 3'-end of 26S rRNA for all three sequences. Note the addition of an A at position +44 in the *S. carlsbergensis* sequence as compared with data from Veldman *et al.* (1980).

at least one component that is affected, directly or indirectly, by the *rna 82.1* mutation.

Sequence comparison of the 3'-end generating region of three Saccharomycetoideae

In order to ascertain whether the ability of the region between −36 and +74 to direct formation of discrete 3'-ends could be traced to specific signals in the nucleotide sequence, we determined the primary structure of the corresponding positions of the rDNA from *Saccharomyces rosei* and *Hansenula wingei*. The pertinent restriction fragments were isolated using the restriction maps of the respective rDNAs determined by Verbeet *et al.* (1983, 1984b). In both cases the position of the 3'-end of the 26S rRNA gene was located on the basis of the nearly perfect sequence homology between the mature rRNA sequences of *S. rosei*, *H. wingei* and *S. carlsbergensis* (J.Oliemans, unpublished observations).

Comparison of the two newly determined sequences with that of *S. carlsbergensis* (cf. Figure 4) revealed that significant sequence homology is confined to three short tracts located between positions +10 and +55. Deletion of sequences downstream of position +43, which abolishes the formation of transcripts ending at −1 and +10 (Figure 3) removes the 3'-distal member of this set of conserved sequence tracts which strongly suggests its involvement in the processing reaction.

5'-Deletion analysis of the 3'-end-generating region

The 5'-boundary of the *cis*-acting element(s) involved in the processing reaction(s) was determined by introducing deletions extending downstream from position −149 of the 26S rRNA sequence. The starting point for the construction of the 5'-deletion mutants was pARES10.1 (Figure 1). This plasmid was derived from pARES9 by replacing the 137 bp 3'-end-generating fragment with a 1554 bp fragment extending from 527 bp upstream of the 3'-end of 26S rRNA to the 5S gene (see Materials and methods). 500 bp of mature 26S sequences were included to confer more stability to transcripts upon deletion of the 3'-part of 26S rRNA, while 1000 nt of spacer sequences were present to allow detection of the putative termination site. The *Stu*I site in the 26S sequence (position −149) was changed into a unique *Bgl*II site by linker insertion. This site was then used for generating 5'-deletions by *Bal*31 digestion. In addition to locating the 5'-boundary of the processing signals we hoped that the deletions inactivating these signals might unmask the true transcription termination site. Figure 5A summarizes the mutants obtained, which are designated by the coordinates of the sequence having been deleted.

Northern blot analysis of the transcripts produced by the mutants carrying deletions −149/−100 and −149/−71 (Figure 5B) demonstrates that these deletions do not interfere with normal 3'-end generation. In each case the size of the transcript is slightly below that observed for the pARES10.1 transcript in accordance with the length of the sequence deleted. Longer deletions, however, e.g. those extending to +18 and +30, cause the appearance of two transcripts ending roughly at positions +250 and +50 downstream of the 26S rRNA sequence, respectively. The ratio between the two varied to a certain extent with the individual RNA preparation even from cells transformed with the same minigene. Although this variability points towards endonucleolytic cleavage of the '+250 transcript' at a specific site during the isolation procedure, it cannot be excluded that some portion of the observed '+50 transcripts' is already produced by inefficient *in vivo* processing of the longer molecules.

The cleavage site at +50 is expected to be eliminated in mutant −149/+111 which indeed produces only the '+250 transcript'. This transcript, due to the large deletion, moves at about the same position as that derived from the undeleted minigene (Figure 5B).

Finally, mutant −149/+282 in which the site at +250 is elim-

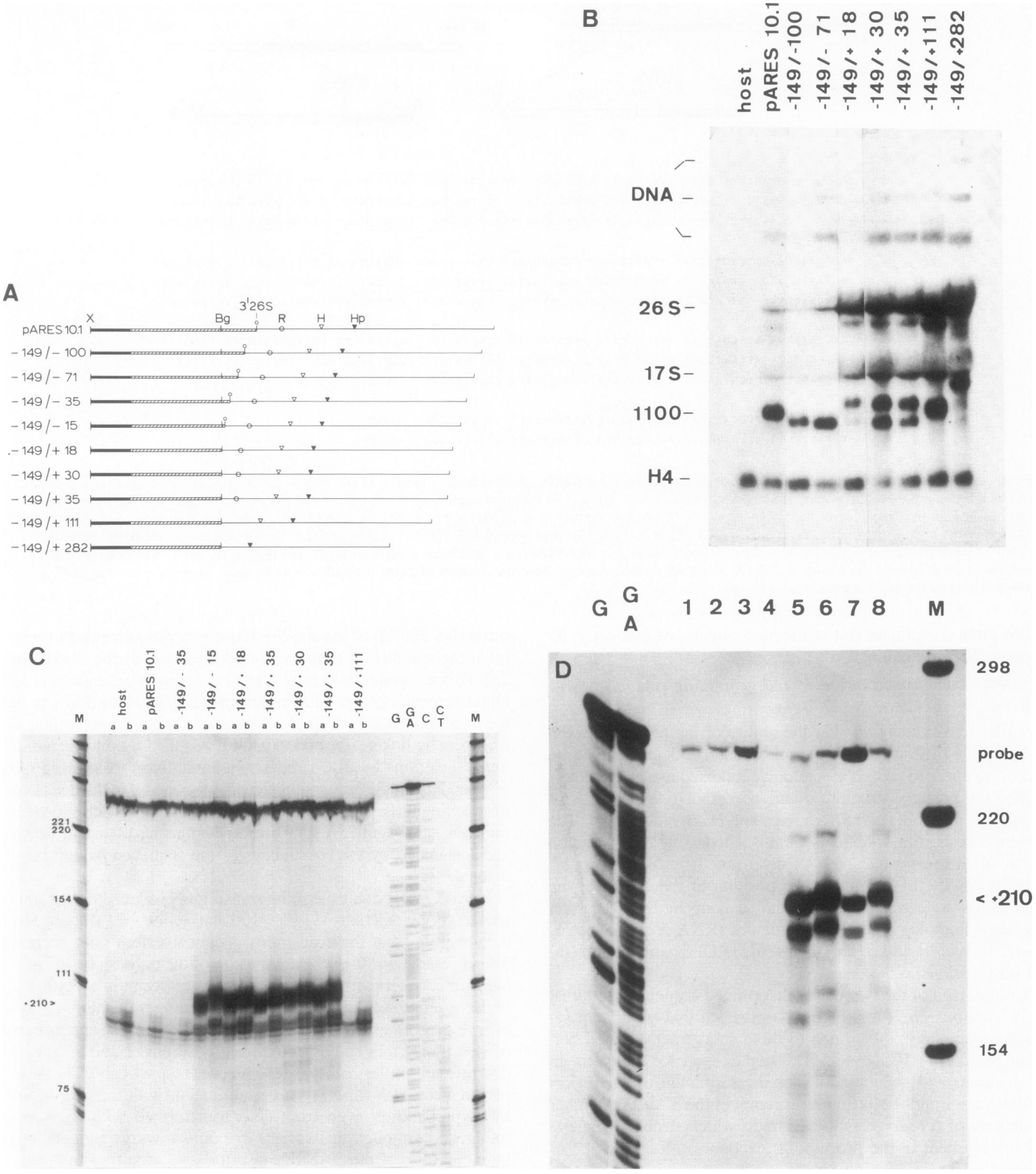

Fig. 5. Analysis of 5′-deletion mutants. **(A)** Map of the 3′-end-generating region of pARES10.1 and the deletion mutants. The *Bgl*II site was used for *Bal*31 digestion, followed by re-insertion of a *Bgl*II linker. Numbering indicates the borders of the deleted sequence. Symbols are described in Figure 1. **(B)** Northern blot hybridization of RNA isolated from yeast cells transformed with several 5′-deletion mutants. 20 μg of RNA was fractionated on a 1.2% agarose gel and blotted onto Hybond-N. Probes used were M13SoB (Figure 3) and M13mp8 containing part of the yeast histone H4 gene (Woudt *et al.*, 1983). **(C)** S1 nuclease mapping of the 3′-ends of transcripts from several 5′-deletion mutants using a general rDNA probe. 25 μg of RNA was hybridized to the *Eco*RI plus *Hpa*I fragment (A) and subsequently digested with 60 U **(lanes a)** or 20 U **(lanes b)** of S1 nuclease at 16°C. **Lanes 'G', 'G+A', 'C'** and **'C+T'** are described in Figure 2. **(D)** S1 nuclease mapping of the 3′-ends of transcripts from mutant −149/+18 using a plasmid-specific probe. 25 μg of *Bacillus licheniformis* RNA **(lanes 1** and **2)**, host RNA **(lanes 3** and **4)**, −149/+18 RNA **(lanes 5** and **6)** and −149/+18 RNA, treated with DNase **(lanes 7** and **8)** were hybridized to the corresponding *Bgl*II plus *Hin*dIII fragment, labelled at *Bgl*II **(A)**. Subsequently, samples were digested with 225 U **(lanes 1, 3, 5, 7)** or 75 U **(lanes 2, 4, 6, 8)** of S1 nuclease. **Lanes 'G'** and **'G+A'** are described in Figure 2.

inated produces a discrete transcript of ~1400 nt moving slightly ahead of the (aspecifically labelled) 17S rRNA (Figure 5B) thus revealing a further 3'-end-generation site located approximately at position +750.

It should be noted that the yield of the minigene transcripts in this experiment cannot be inferred directly from the intensity of the signals in Figure 5B, since they have to be corrected for recovery (cf. the signal for histone H4 mRNA), copy number of the plasmid and differences in turn-over rate.

A more detailed analysis of the 5'-boundary of the processing signals as well as the position of the 3'-end of the '+250 transcript' was carried out by S1 nuclease mapping using the *Eco*RI plus *Hpa*I fragment as a probe under conditions of RNA excess. This probe is expected to detect host rDNA (spacer) as well as minigene transcripts. As can be seen in Figure 5C total host RNA gives rise to a signal at +200. However, this signal disappears upon DNase treatment of the RNA preparation (data not shown). Therefore it must be due to the extensive sequence mismatch between *S. cerevisiae* host rDNA units and the *S. carlsbergensis* probe starting at position +200 (Skryabin *et al.*, 1984). A similar picture emerged for *S. cerevisiae* transformed with pARES10.1 or mutants carrying deletions extending down to −35. Longer deletions caused the appearance of a strong signal at +210 which could not be eliminated by DNase treatment of the RNA sample, whereas now the +200 signal becomes partially resistant to DNase (data not shown; cf. Figure 5D). From these results we conclude that the 5'-boundary of the element(s) involved in generating the 3'-end of 26S rRNA and the '+10 transcript' is situated between −35 and −15. The inactivation of this element(s) allows the formation of longer transcripts, the majority of which have their 3'-end at position +210 while a small proportion end at position +200. These 3'-termini remain unchanged upon transformation of the deletion mutants into an *rna 82.1* strain (data not shown). We therefore consider the site at +210 to be a putative termination site.

Our conclusions were confirmed with mutant-specific probes. Figure 5D shows one example in which transcripts produced by the deletion mutant −149/+18 were probed with the *Bgl*II plus *Hin*dIII fragment from this mutant (Figure 5A). The probe was labelled at its 3'-end on position +18. In this case, as expected, the host RNA (lanes 3 and 4) does not give rise to any signal whereas RNA from the transformant −149/+18 specifies two signals at +210 and +200 respectively (lanes 5 and 6). Both signals are still present after extensive treatment of the RNA with DNase (lanes 7 and 8).

Discussion

Here we show that ARES minigenes, used previously to study initiation of transcription of the large rRNA precursor by yeast Pol I (Kempers-Veenstra *et al.*, 1984, 1985; Elion and Warner, 1984) also are suitable for studying 3'-end generation. The starting point for these studies was the observation that transcripts derived from the ARES9 minigene (Figure 1) have 3'-termini corresponding to those observed previously (Veldman *et al.*, 1980) for 37S pre-rRNA and 26S rRNA (Figure 2). Therefore, all information required to form these termini must be contained within the rDNA fragment extending from 36 bp upstream to 101 bp downstream of the 3'-end of the 26S sequence.

Evidence that the 3'-termini of the ARES9 transcripts, and hence those of *in vivo* 26S rRNA and 37s pre-rRNA, arise by processing is provided by the analysis of the products of ARES9 transcription in the yeast *rna 82.1* mutant (Piper *et al.*, 1983). As shown in lane 3 of Figure 2 the majority of the transcripts produced in this mutant extend to position +15, slightly farther downstream than the longest transcript detected in the wild-type host. Moreover, at least a portion of the Pol I molecules appears to transcribe even farther downstream as concluded from the presence of an additional S1 signal mapping at position +45 (Figure 2). Since the *rna 82.1* mutant carries a single nuclear lesion that inactivates an endonuclease involved in 3'-processing of 5S pre-rRNA, we conclude that the 'wild-type' 3'-termini of the ARES9 transcripts are produced by processing.

Deletions extending from position −149 downstream past position −35, but leaving all of the spacer sequence intact, abolish the formation of 3'-termini at −1 and +10. Instead transcripts extending to positions +210 and, to a lesser extent, +200 accumulate (Figure 5). From this result we conclude that the 3'-ends at +15 and +45 observed in the *rna 82.1* strain transformed with pARES9 (Figure 2) are not due to termination at this position. Instead they must also be formed by processing of a longer transcript which is dependent upon elements whose 5'-boundary is located between positions −35 and −15. The observation that information required for processing is present downstream of +43 (Figure 3) strongly suggests that on the undeleted minigene transcription also proceeds beyond this position.

Taken together, the data indicate that the mechanism for formation of the 3'-end of 26S rRNA consists of at least two steps. Transcription to a point beyond position +43 appears to provide a recognition signal for a processing enzyme involved in the first nucleolytic attack on the 3'-terminal sequence of the primary rRNA transcript at position +45 and/or +15. After this obligatory primary cut there is at least one step involving the *rna 82.1* endonuclease, viz. the conversion of the +45 and/or +15 transcript into the +10 transcript and/or 26S rRNA. As is evident from the deletion mapping experiments shown in Figures 3 and 5 the sequence elements involved in the processing are encoded within a 110 bp long region extending from −35 to +74 and thus comprising both 26S rRNA and spacer sequences. Recently Labhart and Reeder (1986) showed that in *Xenopus* the element(s) required for the corresponding processing reaction is contained within a much shorter region extending from position −2 to position +17. Therefore, the requirements for processing in yeast appear to be more complex than those in *Xenopus*.

The yeast spacer sequences required for processing encompass several short tracts, located between +10 and +55, that constitute the only significant sequence conservation within the 200 bp region adjacent to the 26S gene in several members of the Saccharomycetoideae. Presumably, therefore, these conserved sequences are important recognition elements for the components of the processing machinery although rigorous proof for this notion has still to be provided.

Although the sequence conservation points towards a requirement for specific primary structures, secondary structure may also be involved. The conserved sequences in all three yeast strains studied are part of an (imperfect) inverted repeat (Figure 6) previously described for *S. carlsbergensis* (Veldman *et al.*, 1980). Since, as discussed above, transcription is likely to proceed beyond +43, formation of this stem−loop structure in the transcript should indeed be possible. Such a structure might then act as (part of) the signal recognized by the processing machinery involved in the primary cut. In fact, 3'-deletion to position +43, by which processing is abolished, removes one of the conserved tracts and will partially disrupt the stem−loop structure (Figure 6) thus supporting its involvement in the processing reaction. On the other hand, comparison of the three structures shown in Figure 6 reveals several non-compensatory base changes in the

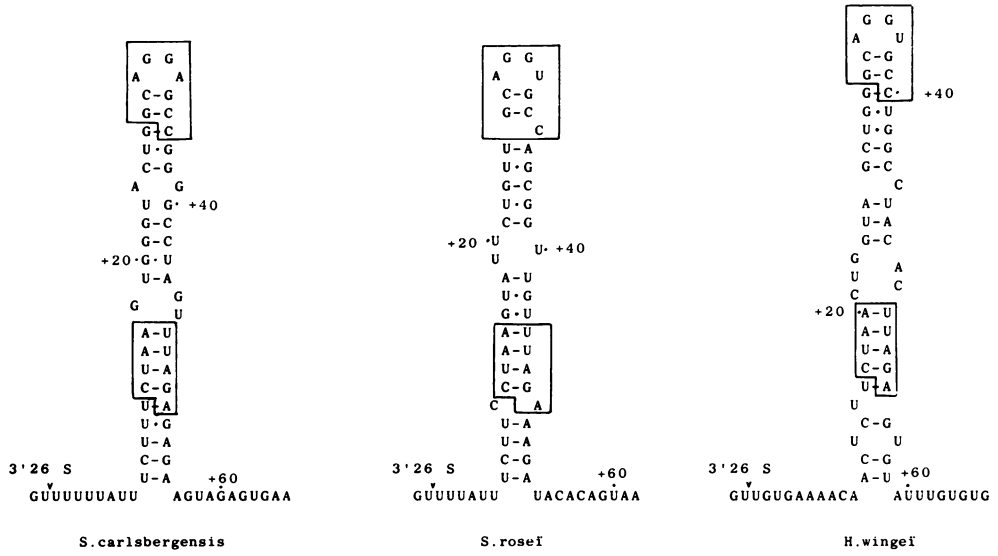

Fig. 6. Dyad symmetry beyond the 3'-end of 26S rRNA in three Saccharomycetoideae. The RNA is shown in the form of a hairpin. Conserved sequences are boxed.

stems. Also the thermodynamic stability of the base pairing (Tinoco *et al.*, 1973) varies widely. More detailed studies, therefore, are required to establish whether the stem−loop structure indeed plays a role in the formation of the 3'-end of 26S rRNA.

The recognition elements for the RNA 82.1 endonuclease-dependent processing at −1 and +10 remain obscure. We notice a very limited sequence homology around the cleavage sites in pre-5S rRNA (UCU↓UU) and the Pol I transcript (UGU↓UU and UCU↓UU). Also, the 3'-terminal extensions of both pre-5S and 37S pre-rRNA are very U rich. There is no clear secondary structure homology in or around the regions containing the cleavage sites.

With respect to the exact site of transcription termination by yeast Pol I our results leave three options. (i) The +45 site (T1) cannot altogether be ruled out as a site of termination rather than processing. In view of the results presented in Figure 5, however, the former could only be explained by assuming that processing at −1 and +10 is strictly dependent on termination at +45 and that deletion of sequences located within the 26S rRNA gene, between −35 and −15, inactivates a signal required for this termination. We deem the possibility that transcription terminates at +45 unlikely, the more so because in *Xenopus* processing at −1 clearly does not depend on termination (Labhart and Reeder, 1986). (ii) The results in Figure 5 show that removal of elements located between −35 and +74 abolishes processing and causes the accumulation of transcripts ending at +210 (T2). As expected, the generation of this 3'-end does not depend on the presence of an intact RNA 82.1 gene product (data not shown). We consider T2 to be the most likely candidate for the main site of termination by yeast Pol I. (iii) If, however, T2 is still another processing site, the site at +750 (T3), revealed by deletion mutant −149/+282 (Figure 5B), might act as the genuine terminator. On the other hand T3 may be a fail-safe terminator preventing a small number of Pol I molecules that have failed to terminate at T2 from transcribing through the 5S rRNA gene. Preliminary studies in permeabilized yeast cells indicate a strong attenuation of transcription between ~+100 and +300 and reveal no evidence for transcription by Pol I through the 5S gene. Therefore, we deem the presence of a terminator downstream of T3 to be highly unlikely. So far we have no direct proof that T2

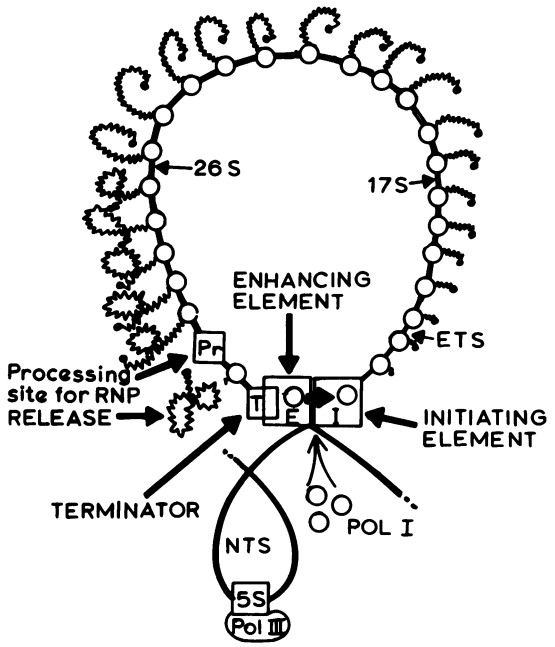

Fig. 7. Model of an actively transcribing rDNA unit (the 'ribomotor').

acts as the main terminator in chromosomal rDNA *in vivo*. Therefore, we are now studying the effects of inserting DNA fragments containing T1, T2 and T3 respectively into an appropriate rDNA transcription unit. In addition, 'run-on' experiments using short spacer probes are in progress to determine the degree of transcription attentuation at T1, T2 and T3 more precisely.

In our conception of yeast T2 as a genuine, though maybe slightly leaky, terminator it is not equivalent to T2 in *Xenopus* rDNA at +235 (Labhart and Reeder, 1986) which forms the boundary between a relatively stable and a highly unstable portion of the Pol I transcript but does not cause release of Pol I molecules. The yeast T2 could, however, be functionally homologous to position +565 in mouse rDNA which has been proposed as a termination site both *in vivo* and *in vitro* (Grummt *et al.*, 1985b). In mouse this site is followed at a slight distance by a T-rich

sequence (Grummt *et al.*, 1985b). In yeast T2 is located immediately upstream of a stretch of six T residues in the non-coding strand. Recent data point towards a role for such T-rich stretches in termination by all three types of eukaryotic RNA polymerase (Platt, 1986).

It is striking that T2 maps within a DNA element (coordinates +100 to +270) that enhances transcription initiation by yeast Pol I (Elion and Warner, 1984). This observation leads us to propose that a functional linkage exists between termination at +210 and transcription initiation. In our model, as visualized in Figure 7, each rDNA transcription unit forms a loop which juxtaposes the promoter and the terminator/enhancer element. RNA Pol I molecules having terminated at T2 are then transferred directly to the promoter by the action of the enhancing element. This mechanism maintains a high loading density of the transcription unit by ensuring efficient recycling of the RNA Pol I molecules without these molecules entering the free pool. We would, therefore, propose the name 'ribomotor' for the complex of the rDNA promoter, enhancer and terminator. An alternative, though in our view less attractive, possibility is that the promoter in this ribomotor complex is the one belonging to the next downstream transcription unit. This could be accomplished by looping out the spacer sequences, including the 5S rRNA gene, instead of the transcription unit.

In the model presented in Figure 7, the +45 site is considered to be required for the release of the (partially) assembled preribosomal particle.

Support for our model may be found in the observation that active rDNA units in both *Bombyx mori* and *Drosophila melanogaster* have been visualized as loops separated by intergenic spacers (Hamkalo, 1985). *Xenopus*, on the other hand, may have evolved a different way of ensuring efficient transcription of the rRNA genes (Labhart and Reeder, 1986). In this organism the terminator for Pol I appears to be located shortly upstream of the promoter of the next rDNA unit. Although this arrangement clearly is different from that in yeast, it may also channel polymerases directly to the promoter after termination, bypassing the free pool of Pol I molecules. It seems, therefore, that different eukaryotes have evolved alternative strategies for ensuring efficient transcription of their rRNA genes.

Materials and methods

Enzymes and strains

Restriction enzymes were purchased from Boehringer (Mannheim) or New England Biolabs. Polynucleotide kinase, DNA polymerase I (Klenow enzyme) and T4 DNA ligase were obtained from Boehringer, S1 nuclease from Sigma, *Bal*31 from Bethesda Research Laboratories, Zymolyase-100 T from Seikagaku Kogyo Co. (Tokyo) and helicase from Industrie Biologique Francaise (Clichy). *Escherichia coli* HB101, DH1 and JM101 were used for transformation and propagation of plasmid and M13 phage DNAs. *S. cerevisiae* YT6-2-1L (*cir°, a, leu2-3,112; his4-519, can1*) and L16 (*rna 82.1, leu2-3, 112; his3-Δ1, trp1-289*) were used for expression of ARES genes. *S. rosei* (CBS817, Delft, The Netherlands) and *H. wingei* (NRRL2340, strain 21, IL, USA) were used to isolate full length rDNA clones (Verbeet *et al.*, 1983, 1984b).

Construction of artificial minigenes

The construction of the pARES6 plasmid, which contains an rRNA minigene consisting of 445 bp of *S. oligorhiza* chloroplast DNA flanked by two *S. carlsbergensis* rDNA fragments responsible for correct initiation of Pol I and for generation of transcripts having discrete 3'-termini respectively (cf. Figure 1) has been described previously (Kempers-Veenstra *et al.*, 1984). pARES9 is identical to pARES6 except for the reversed orientation of the *Spirodela* marker DNA fragment which creates a unique *Bgl*II site immediately 3' to this fragment. pARES10.1 was derived from pARES9 by replacing the *Bgl*II plus *Hin*dIII fragment extending from positions −36 to +101 relative to the 3'-terminus of the 26S rRNA sequence with a *Bam*HI plus *Hin*dIII fragment from M13mp10, in which was cloned a *Taq*I fragment (coordinates −527 to +1027) isolated from plasmid pMY60 (Meyerink, 1979). In addition a synthetic *Bgl*II linker was inserted into the *Stu*I site at position −149.

Construction of the deletion mutants

3'-Deletion mutants were constructed by cutting pARES6 at the unique *Hin*dIII site downstream of the 26S rRNA sequence and digestion of the plasmid DNA with *Bal*31 as described previously (Kempers-Veenstra *et al.*, 1985). The digested plasmid was re-circularized after repairing the ends with Klenow polymerase and inserting a synthetic *Hin*dIII linker (Bethesda Research Labs). The deleted minigenes were recovered as *Bam*HI plus *Hin*dIII fragments which were inserted into pJDB207 (Beggs, 1981) digested with *Bam*HI plus *Hin*dIII.

5'-Deletion mutants were obtained by *Bal*31 digestion starting at the unique *Bgl*II site of pARES10.1. A synthetic *Bgl*II linker was inserted after digestion and the large *Bgl*II plus *Xho*I fragment, extending downstream of the 26S rRNA sequence up to the marker fragment, was recovered. In order to ensure that the sequences adjoining the deletion were identical in all mutants this *Bgl*II plus *Xho*I fragment was recombined with the small *Xho*I plus *Bgl*II fragment isolated from pARES10.1 (cf. Figure 1). The recombinant molecules now contain only deletions from the *Bgl*II site towards the 3'-end of 26S rRNA. The size of both the 3'- and 5'-deletions was estimated from analysis of the plasmid DNA on 2% agarose gels. Deletions of selected mutants were mapped accurately by sequence analysis according to Maxam and Gilbert (1977).

RNA isolations

RNA from 3'-deletion mutants was isolated as described previously (Kempers-Veenstra *et al.*, 1984). RNA from 5'-deletion mutants was isolated according to Kief and Warner (1981) with some minor modifications.

Northern blotting

RNA from 3'-deletion mutants was denatured by gentle boiling for 2 min, then chilled on ice, fractionated on a 1.6% agarose gel and blotted onto DBM paper (Alwine *et al.*, 1977). RNA of 5'-deletion mutants was analysed according to Thomas (1980). In this case Hybond N (The Radiochemical Centre, Amersham, UK) was used.

S1 nuclease mapping

Analysis of 3'-ends of pARES9 RNA in different hosts (Figure 2) was performed as described previously (Kempers-Veenstra *et al.*, 1984). For mapping of the transcripts of 5'-deletion mutants (Figure 5C) 25 μg of RNA was hybridized to the probe at a temperature decreasing linearly from 43 to 25°C over a period of 16 h. S1 nuclease treatment was at 16°C for 2 h, using 60 U ('high') or 20 U ('low') of S1 nuclease. In some cases RNA preparations for S1 nuclease mapping were treated with 40 U/ml of DNase I (RQ1, Promega) for 15 min at 37°C.

Miscellaneous

Spirodela marker DNA probes were obtained by cloning the *Spirodela* chloroplast DNA fragment in both orientations in M13mp9 (M13SoA, M13SoB). Methods for probe labelling and strand separation were described previously (Kempers-Veenstra *et al.*, 1984). Sequencing of 3'-end-generating regions of *S. rosei* and *H. wingei* was performed according to Sanger (1977).

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