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Transcription of an artificial ribosomal RNA gene in yeast

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We constructed an artificial yeast rRNA gene and studied its transcription after introduction into a recipient yeast strain. The artificial gene comprised a fragment containing the sequence from position −207 to +128 relative to the site of initiation of *Saccharomyces carlsbergensis* 37S pre-rRNA, followed by a marker fragment from *Spirodela oligorhiza* chloroplast DNA and finally a fragment containing the sequence from position −36 to +101 relative to the 3′ end of the 26S rRNA gene. The resulting construct was cloned into the yeast-*Escherichia coli* shuttle vector pJDB207. Both Northern blot hybridization and R-loop analysis of RNA from transformed *Saccharomyces cerevisiae* cells revealed a discrete transcript of the expected length. S1 nuclease mapping as well as primer extension analysis showed that the major proportion of the transcripts was initiated at exactly the same site as 37S pre-rRNA. These results show that the respective rDNA fragments contain the information for correct initiation of transcription and formation of the 3′ end. A minor proportion of the transcripts was initiated at a number of sites between positions −1 and −100 upstream of the predominant start. The proportion and the pattern of these upstream starts is affected by the vector context of the artificial rRNA gene.

Key words: ribosomal DNA/RNA polymerase A/transcription/yeast transformation

Introduction

The biosynthesis of ribosomes is a complicated process which requires the coordinate expression of the genes coding for the four rRNAs and ~80 ribosomal proteins. Regulation of the overall process is most likely exerted at the level of initiation of transcription of the large rRNA operon by RNA polymerase A, as may be inferred from the response of yeast to nutritional shifts (Planta *et al.*, 1980; Warner, 1982). Therefore we want to identify the DNA elements and transcription factors involved in the regulation and initiation of transcription of the large rRNA operon. In yeast this operon is contained, together with the 5S rRNA gene, within a 9.1-kb rDNA unit which is tandemly repeated on chromosome XII 100 – 140 times (Retèl and Planta, 1968; Petes, 1979; Kaback *et al.*, 1980). Transcription initiates at a site ~700 bp upstream of the 17S rRNA gene (Klemenz and Geiduschek, 1980; Bayev *et al.*, 1980; Klootwijk *et al.*, 1984). Comparison of the initiating regions of a number of *Saccharomycetoideae* revealed significant sequence conservation around the transcription initiation site between positions −9 and +23. In this region the heptanucleotide from positions +1 to +7 is perfectly conserved (Verbeet *et al.*, 1984). This heptanucleotide sequence is supposed to constitute an important part of the promoter for yeast RNA polymerase A. Unfortunately, as yet, no faithful *in vitro* transcription system for yeast RNA polymerase A has been developed which could be used to verify the supposed role of this sequence and to identify other possible promoter elements. The yeast transformation technique, however, in principle offers the opportunity for studying initiation of transcription from an individual promoter *in vivo*. By employing *in vitro* mutagenization the DNA elements of interest can then be identified. Here we describe the construction and transcriptional analysis of an Artificial Ribosomal Extrachromosomal gene of *Saccharomyces carlsbergensis* (ARES). We demonstrate that this gene is correctly transcribed in transformed yeast cells. This means that the information required for faithful transcription initiation is contained within the 335 bp long 5′ portion of the ARES gene which corresponds to positions −207 to +128 of the original rRNA operon. Moreover the information contained within a 137-bp fragment at the 3′ end of the ARES gene, corresponding to the region around the 3′ terminus of the original 26S rRNA gene, clearly suffices for generation of the 3′ end of the ARES transcript. This system therefore allows the identification of DNA elements involved *in vivo* in the regulation of initiation and termination of transcription by yeast RNA polymerase A.

Results

Construction of an artificial yeast rRNA gene (ARES6)

For the study of the transcription of an individual rRNA gene we prepared a construct consisting of the presumed initiating and 3′ end generating DNA regions of a cloned rDNA unit of *S. carlsbergensis* separated by a marker DNA fragment (Figure 1A). Since there is ample evidence placing the transcription initiation site of the large rRNA operon in *S. carlsbergensis* at a position ~700 bp upstream of the 17S rRNA gene (Klootwijk *et al.*, 1984) we selected a (*Sma*I + *Bgl*II) fragment extending from position −207 to +128 relative to the start site as the initiating fragment. As a presumed 3′ end generating fragment we took an (*Sau*3AI + *Eco*RI) fragment extending from position −36 to +101 relative to the 3′ end of the 26S rRNA gene. This fragment comprises the 3′ end of 37S pre-rRNA mapped by Veldman *et al.* (1980) at position +7, and contains an extended dyad symmetry around position +31 which was suggested to be involved in the generation of the 3′ end of 37S pre-rRNA (Veldman *et al.*, 1980). Both fragments were kept relatively short to suppress homologous recombination events with the repeated chromosomal rDNA units (Szostak and Wu, 1979).

To be able to analyse the transcription signals present on the rDNA fragments against the background of rRNA transcription from chromosomal genes, the two fragments were separated by a piece of marker DNA. We chose a 450 bp long

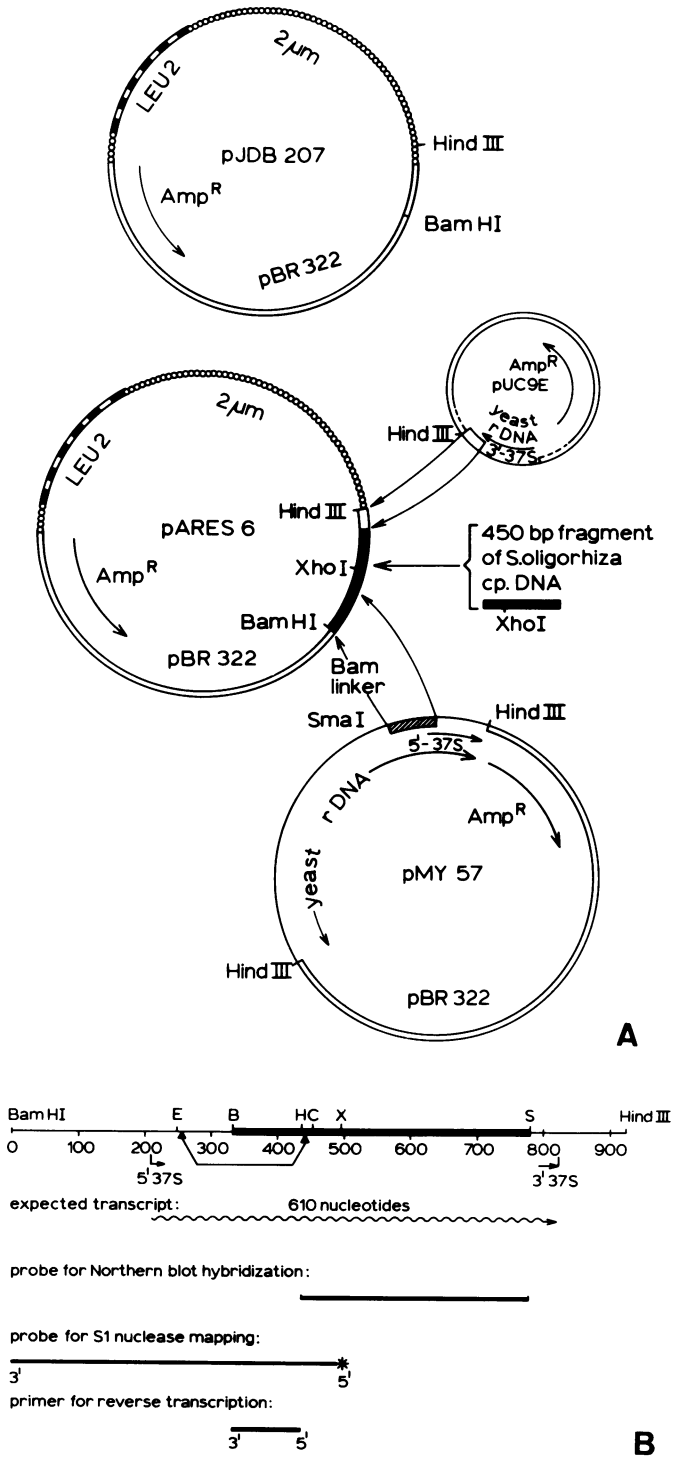

Fig. 1. Construction of pARES6. **(A)** The presumed initiating and 3′ end generating fragments of the yeast rRNA operon were isolated from pMY57 and pUC9E, respectively, as described in Materials and methods. Both fragments were cloned into pJDB207 separated by a 450-bp fragment from *S. oligorhiza* chloroplast DNA. The resulting plasmid (pARES6, 7670 bp) retains the amp^R and LEU^2 selection markers. **(B)** Schematic representation of ARES6 and 7. The artificial rDNA gene (ARES6) is predicted to yield a transcript of 610 nucleotides. The sequence between the arrows at positions 260 and 430 has been deleted in pARES7A (and 8A and B). The probes for the Northern blot hybridization and the S1 nuclease mapping as well as the primer for the reverse transcription extension are indicated.
E = *Eco*RI, B = *Bgl*II, H = *Hha*I, C = *Cla*I, X = *Xho*I and S = *Sau*3AI.

*Sau*3AI-generated fragment from *Spirodela oligorhiza* chloroplast DNA, which contains a number of useful restriction sites for probing and does not contain transcription termination or initiation signals (H.Lustig, personal communication). Northern blot hybridization (see Figure 3, lane A) revealed that in *Saccharomyces cerevisiae* no transcripts are detectable using this marker sequence as a probe.

The construct, ARES6, is shown in detail in Figure 1B; it was cloned in the tet^R gene of the yeast *Escherichia coli* shuttle vector pJDB207 (Beggs, 1981), consisting of the bacterial vector pAT153 (Twigg and Sherrat, 1980) plus the small *Eco*RI fragment of form B of 2 μm DNA, in which the LEU2 gene has been inserted. pJDB207 is present in ~15 copies per cell in *cir*^{o} strains (Broach, 1983).

Analysis of the transcription of ARES6 in vivo

The *S. carlsbergensis* rDNA fragments present in the ARES minigenes are >98% homologous with their counterparts in a cloned rDNA unit from *S. cerevisiae* (compare Bayev *et al.*, 1980, 1981). Moreover, recent revisions in yeast taxonomy have classified *S. carlsbergensis* as a synonym of *S. cerevisiae* (Barnett *et al.*, 1983). Therefore analysis of the transcription of the ARES genes in a suitable transformed *S. cerevisiae* strain is pertinent to the identification of transcriptional signals affecting RNA polymerase A. A 5–15S RNA fraction was isolated from *S. cerevisiae* cells transformed with pARES6. After subjecting this RNA to R-loop analysis with pARES6 DNA we frequently observed R-loops that correspond to the expected ARES6 transcript in both size and location (Figure 2A). Figure 2B schematically shows the position of the R-loops on 39 randomly chosen molecules. These data are compiled in Figure 2C which allows us to position both end points of the presumed ARES6 transcript on the ARES6 DNA. The length of the transcript appears to be 596 (±48) nucleotides which is very close to the expected size of ~600 nucleotides (see Figure 1B). In addition, to the distance of 195 (±33) bp separating the R-loop from the *Bam*HI site is very close to the number of base pairs (214) between the *Bam*HI site and the start site of 37S pre-rRNA. These data suggest that ARES6 is correctly and relatively frequently transcribed in the yeast cell.

To ascertain whether the correct strand of ARES6 was transcribed, we performed Northern blot hybridization using the coding strand of the marker DNA as a probe. Lane B of Figure 3 reveals that total RNA from pARES6-transformed cells contains an RNA species of ~600 nucleotides to which the marker DNA hybridizes. This transcript is not present in cells transformed with the vector pJDB207 (lane A) and it is strongly enriched in a 5–15S RNA fraction from pARES6 transformed cells (lane C). The strong hybridization signals visible in the upper part of the gel are due to plasmid DNA present in the RNA preparation since they disappear upon DNase treatment (lane D). This Northern blotting experiment confirms the conclusion from the R-loop study that a fairly discrete RNA of ~600 nucleotides is transcribed from ARES6 and that it is derived from the correct strand.

The initiation site of the ARES6 transcript was located more precisely by means of S1 nuclease mapping using a (*Bam*HI + *Xho*I) fragment (see Figure 1B) annealed to the 5–15S RNA fraction. Correct initiation will result in a protected DNA fragment of ~290 bases after S1 nuclease treatment. Figure 4A indeed shows such a fragment as the major product. In addition a number of minor products are present,

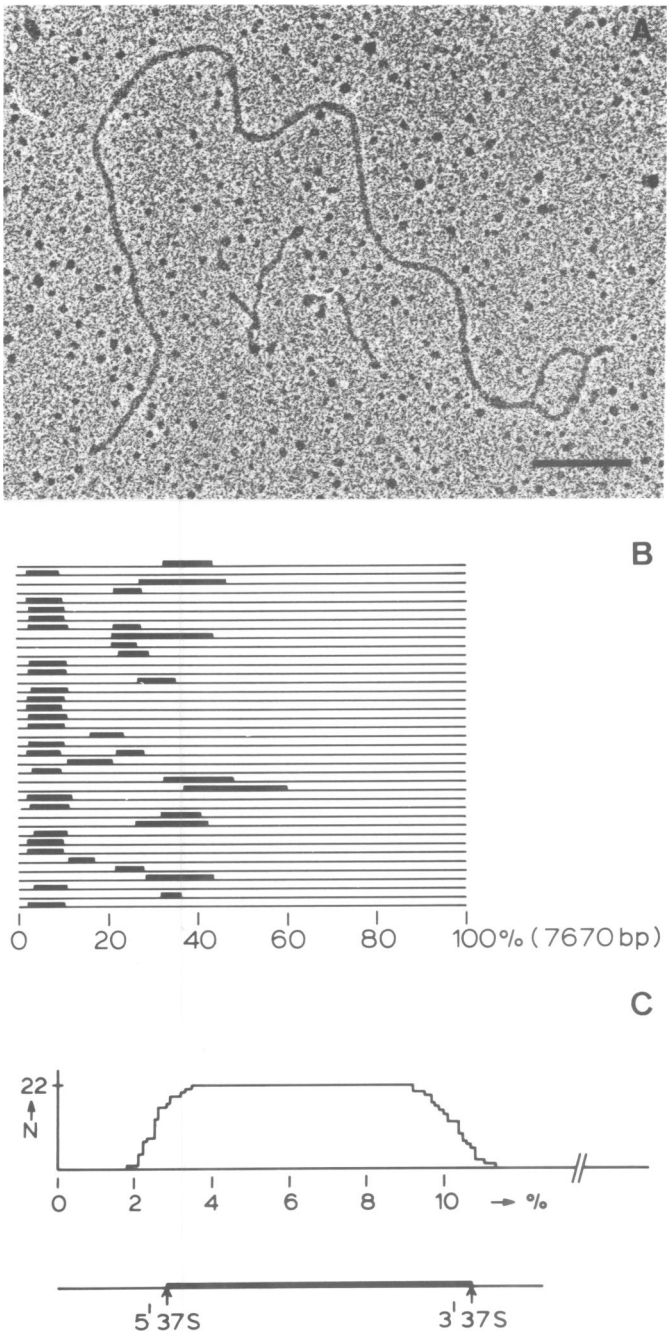

Fig. 2. Electron microscopic analysis of ARES6 transcripts. **(A)** Electron micrograph of an R-loop formed between the 5–15S RNA fraction from pARES6-transformed yeast cells and *Bam*HI-linearized pARES6-DNA. The bar represents 0.2 μm. **(B)** A compilation of R-loops observed on 39 randomly chosen *Bam*HI-linearized pARES6 molecules. Only one orientation is given. The loops between 2 and 10% **(C)** correspond in length and position with transcripts from the ARES6 region.

suggesting the presence of transcripts starting upstream from position +1 up to position −100.

To enable us to locate the site(s) of initiation more precisely primer extension in the presence and absence of ddNTPs was carried out. The primer used was an 83 nucleotides long single-stranded (*Hha*I + *Sau*3AI) fragment from the marker DNA (see Figure 1B). When this primer was annealed to the 5–15S RNA fraction of pARES6-transformed cells and then extended, a major product of 210 bases was observed (Figure 4B, lane 6). This corresponds to the predicted length of the

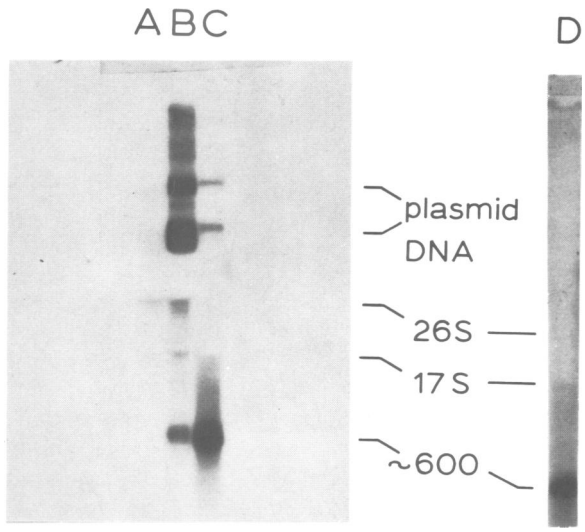

Fig. 3. Northern blot analysis of RNA from pARES6-transformed yeast cells. RNA was fractionated on a 1.6% agarose gel, transferred to nitrocellulose and hybridized with the probe depicted in Figure 1B. **Lane A**: RNA from yeast cells transformed with pJDB207. **Lane B**: total RNA from pARES6-transformed cells. **Lane C**: 5–15S RNA fraction from pARES6-transformed cells. **Lane D**: as **C** but after DNAase treatment. DNA fragments were used as mol. wt. 'markers'.

product formed on a correctly initiated transcript. In fact the results of the extension experiments in the presence of ddNTPs (Figure 4B, lanes 2–5) position the 5′ end of the corresponding template RNA exactly at nucleotide +1, the start of the 37S pre-rRNA. Many shorter extension products are also present in lane 6. Since no corresponding signals can be seen in the S1 nuclease mapping experiments, these products do not represent genuine 5′ ends of template RNAs. Rather they must be caused by stopping or pausing sites for reverse transcriptase and by extension of degraded primer molecules. The presence of some length heterogeneity in the (internally labelled) primer used is evident from the smear below the position of the primer in Figure 4B. Fragments longer than the main product are also present, indicating starts mainly from the region between positions −5 and −45. Even larger transcripts initiated in the vector sequence (the non-coding strand of the tet^R gene) can be seen in the upper part of Figure 4B.

To establish whether the vector context influences the pattern of initiation on the yeast rDNA promoter fragment, we changed both the vector and the orientation of ARES in the vector. To simplify the analyses we first moved the *Xho*I site, used in preparing the probe, closer to position +1. This was achieved by cutting at the *Bgl*II site (see Figure 1B) and removing 76 bp of the yeast external transcribed spacer (ETS) as well as 88 bp of the adjoining marker DNA by treatment with *Bal*31 exonuclease. The transcription of the resulting plasmid, pARES7A, showed a pattern of upstream starts similar to that of pARES6 upon both S1 nuclease mapping and primer extension (Figure 5B, lanes 2, 4 and 5). The number of extension products shorter than the main product is much lower than observed with pARES6, due to the deletion of a large number of stopping or pausing sites (Figure 5B, lane 2).

To study the effect of the vector context on transcripton of the ARES gene, the *Hind*III site in pARES7A was converted into a *Bam*HI site. The ARES insert was then excised with

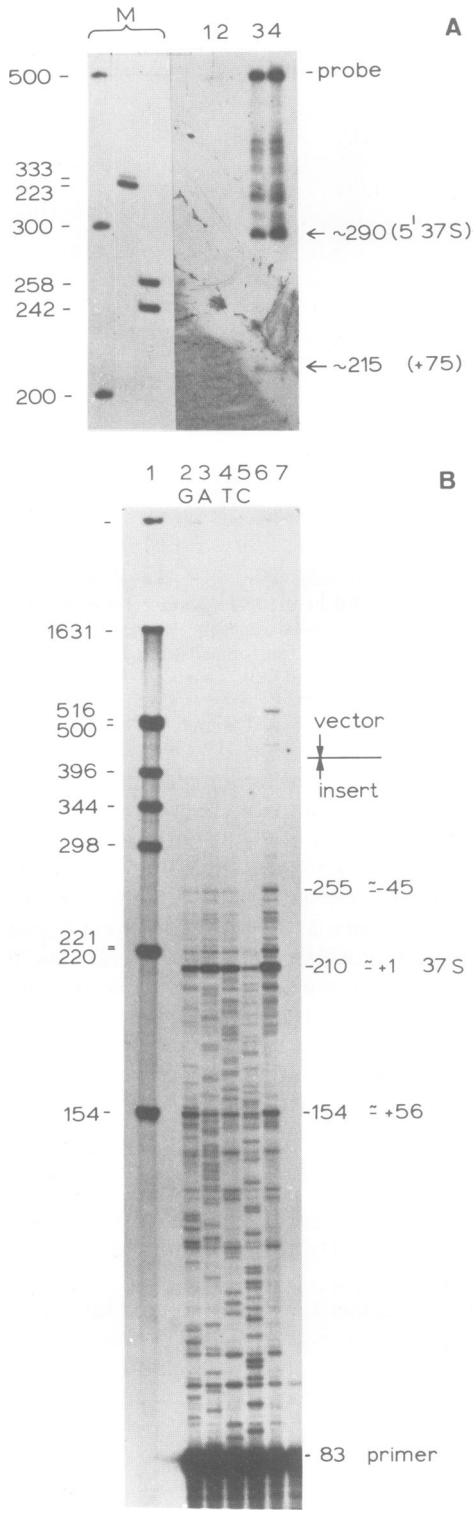

Fig. 4. 5′ End analysis of ARES6-directed transcripts. **(A)** S1 nuclease mapping. The probe (see Figure 1B) was hybridized with 25 μg of a 5 – 15S RNA fraction from pJDB207 transformed cells **(lanes 1** and **2)** or from pARES6 transformed cells **(lanes 3** and **4)**. The samples were subsequently treated with 75 U **(lane 1** and **3)** or with 25 U **(lane 2** and **4)** S1 nuclease. Markers were obtained from the double-stranded probe by cutting with various restriction enzymes. **(B)** Primer extension analysis. The *Hha*I-*Sau*3AI fragment (see Figure 1B) was annealed to a 5 – 15S RNA fraction from pARES-transformed cells and subjected to primer extension in the presence of ddGTP **(lane 2)**, ddATP **(lane 3)**, ddTTP **(lane 4)**, ddCTP **(lane 5)** or without ddNTPs **(lane 6)**. **Lane 7** shows the control reaction without prior annealing of the primer to the RNA; **lane 1:** pBR322 digested with *Hinf*I.

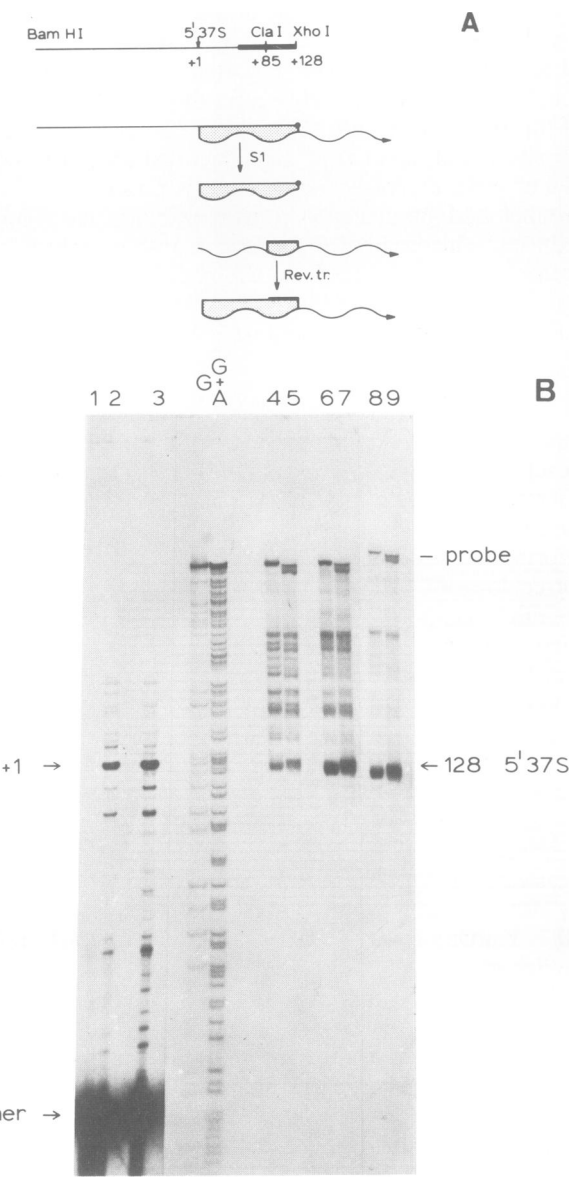

Fig. 5. 5′ End analysis of pARES7A-, 8A- and 8B-directed transcripts. **(A)** Experimental outline showing the structure of the initiating region plus a part of the adjoining marker DNA (thick bar) together with the 5′-labelled (——●) (*Bam*HI plus *Xho*I) probe for S1 nuclease mapping and the internally labelled (——) primer for the extension reaction. **(B)** The (*Xho*I + *Cla*I) fragment was subjected to primer extension without annealing to RNA **(lane 1)** or after annealing to 5 – 15S RNA fractions from pARES7A **(lane 2)** or pARES8A **(lane 3)** transformed cells. S1 nuclease mapping was performed with 25 μg of RNA from pARES7A **(lanes 4** and **5)**, pARES8A **(lanes 6** and **7)** and pARES8B **(lanes 8** and **9)** transformed cells. Hybrids were digested with 75 U **(lanes 4, 6** and **8)** or 25 U **(Lanes 5, 7** and **9)** S1 nuclease. **Lanes 'G'** and **'G + A'** represent chemical modifications of the same DNA fragment according to Maxam and Gilbert (1977). **Lanes 8** and **9** are taken from a 'twin' gel and have been exposed for the same time.

*Bam*HI and cloned in both orientations in the *Bam*HI site of the related (high-copy number) vector pL620; this vector is very similar to pJDB219 (Beggs, 1978) and contains pBR325 in the *Eco*RI A site of 2 μm DNA (form B). pARES8A has the same orientation as pARES6 and 7A with the same tet^R sequences linked to the yeast rDNA initiating fragment. pARES 8B has the opposite orientation. Both S1 nuclease mapping and primer extension of pARES8A indicate a higher yield of transcripts starting at position +1 (Figure 5B, lane 2

versus 3, 4 and 5 *versus* 6 and 7) as compared with pARES7A; this effect is caused by the higher copy number of pL620 as compared with pJDB207 (C.P.Hollenberg, personal communication). However, the pattern and frequency of 'upstream starts' are very similar. Lanes 8 and 9 of Figure 5B show that upon reversing the orientation of ARES the higher yield of correct transcripts is preserved whereas the number and frequency of upstream starts are significantly reduced. This comparison shows a major influence of the vector context of ARES on the phenomenon of 'upstream starts'. Initiation at position +1, however, appears not to be influenced by the vector context.

Discussion

This paper shows that an artificial, extrachromosomal rRNA gene from *S. carlsbergensis* when introduced into the very closely related *S. cerevisiae* is able to direct the product of a discrete transcript initiated at the correct position. Therefore, the selected, relatively small, initiating fragment contains all information necessary to guide the RNA polymerase to the correct location. The RNA polymerase involved is most likely the nucleolar polymerase A. This notion is supported by *in vitro* transcription experiments in lysates of pARES-transformed yeast cells (W.Musters, unpublished observations). Apparently the RNA polymerase A promoter is located between positions −207 and +52 relative to the start of the 37S pre-rRNA. This fragment contains the promoter element near position +1 proposed earlier on the basis of its conservation in several members of the *Saccharomycetoideae* (Verbeet *et al.*, 1984). The boundaries of the yeast promoter are consistent with those, inferred for the RNA polymerase A promoter in human (Learned *et al.*, 1983), mouse (Grummt, 1982; Yamamoto *et al.*, 1984), *Xenopus laevis* (Moss, 1982; Reeder *et al.*, 1982) and *Drosophila melanogaster* (Kohorn and Rae, 1983), which in all cases are located between positions −150 and +40.

The data with respect to the 3′ end of the ARES transcript so far are less detailed. Clearly the rDNA fragment extending from positions −36 to +101 relative to the 3′ end of the 26S rRNA gene contains sufficient information to specify a 3′ end for the ARES transcript. Whether the 3′ end is generated by termination or by processing cannot be deduced from our analysis. The R-loop experiment shown in Figure 2, as well as preliminary S1 nuclease mapping experiments (data not shown), indicate that the 3′ termini of the ARES transcript map near the position of the ends of 26S rRNA and 37S pre-rRNA. Whether they coincide with one of these ends, however, has not yet been established. No estimate can be given as yet of the relative efficiency of the extrachromosomal rDNA promoter of ARES nor can the stability of its transcript be assessed. The interpretation of a kinetic study will be hampered in any case by our inability to determine the proportion of plasmid molecules used as a template for RNA polymerase A in the cell.

Although the majority of the ARES transcripts is initiated at the correct position, additional signals were observed in the two 5′ end mapping assays (Figures 4 and 5). The signals of the short products visible after primer extension can be ignored as far as initiation of transcription is concerned since they are not confirmed in the S1 nuclease mapping experiments. Rather than pointing to initiation downstream of position +1 they must be caused by premature termination of the reverse transcriptase. This phenomenon, occurring mostly at positions requiring the incorporation of dTTP, has also been observed by others (e.g., Boss *et al.*, 1981). The S1 nuclease signal at position +75 (Figure 4A) also is not confirmed by the second 5′ end mapping assay. This signal is likely to be due to an S1 nuclease sensitive site at the T5 tract in the ETS around this position.

A number of additional signals upstream of position +1 are relevant since they occur in both independent assays and thus indicate genuine 5′ ends of ARES transcripts. The proportion of these longer transcripts relative to the total number of ARES transcripts seems to be much higher in the S1 nuclease analysis than in the primer extension experiments (Figures 4 and 5). Since S1 nuclease mapping is known (Miller and Sollner-Webb, 1981) to over-estimate the relative amount of longer transcripts in a mixed population, the results of the extension analysis are probably closest to the actual distribution. This conclusion is consistent with the R-loop data which do not reveal a major class of transcripts with a 5′ end upstream from position +1. It is remarkable that the multiple minor 5′ ends originate from a distinct region of the initiating yeast rDNA fragment. Furthermore it is striking that both the pattern and the relative proportion of these 'upstream 5′ ends' is strongly affected by the vector sequences adjoining the initiating region. Since sequences outside ARES rather than the rDNA sequence itself affect the genesis of these minor 5′ ends, it is unlikely that they have arisen by processing of longer transcripts. Possibly heterogeneity is present in the assembly of pARES with histones or transcription factors depending on the nuclear localization of the plasmid and/or the expression of other genes on the plasmid. Further variation may shed light on the factors that contribute to these 'upstream starts'. Since the major fraction of products is synthesized from the correct start site, the pARES plasmids will allow in principle the elucidation of DNA elements that are involved *in vivo* in the regulation, initiation and termination of transcription by yeast RNA polymerase A.

Materials and methods

Enzymes and other chemicals

Restriction enzymes were purchased from Boehringer (Mannheim) or New England Biolabs and used as recommended by the manufacturer. Polynucleotide kinase, DNA polymerase I (Klenow enzyme) and T4 DNA ligase were obtained from Boehringer, S1 nuclease and DNAase, EP, from Sigma, reverse transcriptase from Dr. J.W.Beard (Life Science Inc.), *Bal*31 exonuclease from Bethesda Research Lab., zymolyase 60.000 from Kirin Brewery (Japan) and helicase from Industrie Biologique Française (Clichy). Radiochemicals were purchased from The Radiochemical Centre (Amersham).

Strains and plasmids

E. coli C600, HB101 and JM101 were used for transformation and propagation of plasmid and M13 phage DNAs. *S. cerevisiae* YT 6-2-1L *(cir°, a, leu 2-3, 112, his 4-519, can 1)* (Erhart and Hollenberg, 1981) obtained from Dr. C.P.Hollenberg (Düsseldorf), was used for transformation.

Fragments were isolated from pUC9E, containing the *Eco*RI-E fragment of the *S. carlsbergensis* (NCYC74) rDNA unit (Veldman *et al.*, 1980) in pUC9 (Messing, 1981), from pMY57 (Meyerink, 1979) containing the *Hind*III-B fragment of the same rDNA unit in pBR322 and from pHH14, containing the *Pst*I-G fragment of *S. oligorhiza* chloroplast DNA (De Heij *et al.*, 1983, obtained from H.Lustig). Fragments were cloned in the yeast-*E. coli* shuttle vectors pJDB207 (Beggs, 1981) and pL620 (kindly provided by Drs. E.Erhart and C.P.Hollenberg).

Yeast transformation

Spheroplasts of *S. cerevisiae* were transformed using the method of Beggs (1978) with some minor modifications. The presence of plasmid DNA in transformed cells was checked by Southern blot hybridization and a β-lactamase assay (Chevallier and Aigle, 1979).

Isolation of RNA

Transformed yeast cells were grown in selective medium. At a cell density of 2−3 x 10^7 cells/ml, the culture was given a nutritional shift-up by adding 0.25

volume of 5-times concentrated rich medium (containing per liter 11 g glucose, 3 g yeast extract, 3 g malt extract and 5 g neutralized bactopeptone). The culture was grown for two generations in this medium before the cells were harvested. RNA was isolated as described earlier (Verbeet *et al.*, 1983). A 5–15S RnA fraction was obtained by sucrose gradient centrifugation (Brand *et al.*, 1977).

Preparation of single-stranded probes

Probes for S1 nuclease mapping were labelled at the 5′ end. Strands were separated as described previously (Verbeet *et al.*, 1983). Probes for filter hybridization and primer extension were prepared according to Leer *et al.* (this laboratory, in preparation) using single-stranded M13 mp9 containing the 450-bp marker DNA from *S. oligorhiza* as a template and a pentadecamer (synthesized by Dr. J.H.van Boom, Leiden) as a primer for Klenow DNA polymerase. The reaction was performed for 15 min at 25°C in the presence of [α-^{32}P]dATP followed by a 'chase' of 15 min. The probe was isolated by phenol extraction, digestion with restriction enzymes and strand separation (Verbeet *et al.*, 1983).

Northern blotting

Fractionation and blotting of RNA to nitrocellulose and hybridization was performed essentially as described by Thomas (1980).

S1 nuclease mapping

S1 nuclease mapping was carried out according to Verbeet *et al.* (1983) except that the total amount of nucleic acid present in each sample during hybridization and S1 nuclease digestion was 25 μg. S1 nuclease digestion was performed using 25 U ('low') or 75 U ('high') for 2 h at 27°C.

Primer extension

About 1 ng of primer was incubated with 85 μg of RNA in 50 mM Tris-HCl, pH 8.0, containing 6 mM $MgCl_2$ and 50 mM KCl, for 30 min at 65°C in a glass capillary. After rapid cooling to 0°C, the mixture was divided into five aliquots. Four 'sequence reactions' were performed in the same buffer plus 100 μM of each dNTP, 4 mM dithiothreitol, 0.06 mg/ml of actinomycin D, 6 U reverse transcriptase and 100 μM of one of the four ddNTPs, respectively. To the fifth aliquot no ddNTP was added while a separate control reaction was performed in which the probe was incubated without prior annealing to RNA. Incubation was carried out for 30 min at 42°C. After hydrolysis of the RNA the cDNA was precipitated and analyzed on a 6% (w/v) polyacrylamide-7 M urea gel (Maxam and Gilbert, 1977).

Construction of an artificial rRNA minigene (ARES).

The construction is illustrated in Figure 1A. The 3′ part of the minigene was a 140 bp long *Sau*3AI-generated fragment from pUC9 E, obtained by electroelution from a 5% (w/v) polyacrylamide gel. It contains the 3′ ends of the 26S rRNA and 37S pre-rRNA genes and has an *Sma*I site between the *Eco*RI and *Bam*HI (*Sau*3AI) cloning sites from pUC9 (Messing, 1981). This fragment was inserted into the unique *Bgl*II site of a pMY57 derivative in which the *Sma*I site at position −207 relative to the transcription start of 37S pre-rRNA was changed into a *Bam*HI site by inserting a *Bam*HI linker (kindly provided by Dr. J.H.van Boom, Leiden). The introduction of the 3′ end generating fragment in the correct orientation restores the *Bgl*II site at position +128 relative to the initiation site. Next a *Hind*III linker (BRL) was inserted in the *Sma*I site within the 3′ end generating sequence and the resulting rDNA minigene (ARES5) was transferred as a (*Bam*HI plus *Hind*III)-generated fragment to pJDB207 digested with *Hind*III plus *Bam*HI (pARES5). A 450-bp *Sau*3AI-generated fragment from the cloned *Pst*I-G fragment of *S. oligorhiza* chloroplast DNA (de Heij *et al.*, 1983) was subsequently inserted into the *Bgl*II site of pARES5, separating the initiating and 3′ end generating regions of the gene. The resulting plasmid, pARES6, retains a *Bgl*II site at position +128 of the initiating sequence. pARES7A was obtained by digesting pARES6 with *Bgl*II and removing 76 bp of the ETS and 88 bp of the adjoining marker sequence by subsequent incubation with *Bal*31, Klenow DNA polymerase and DNA ligase. The *Hind*III site downstream of the rDNA minigene in pARES7 was replaced by a *Bam*HI site, using synthetic *Bam*HI linkers, to allow excision of ARES7 by *Bam*HI. The *Bam*HI-generated fragment was cloned into the *Bam*HI site of the high-copy-number vector pL620 in both possible orientations (pARES8A and 8B).

Electron microscopic R-loop analysis

pARES6 DNA (0.25 μg) was digested with *Bam*HI and hybridized with 3.5 μg of 5–15S pARES6 RNA in 0.1 M Pipes buffer (pH 7.8) containing 10 mM EDTA, 0.4 M NaCl and 70% (v/v) formamide at a temperature decreasing linearly from 54°C to 48°C over a period of 2 h. After quenching in ice, glyoxal was added to a final concentration of 1 M and the mixture was incubated for 2 h at 12°C to stabilize the R-loops (Kaback *et al.*, 1979). The sample was then passed through a Sephacryl S1000 column (50 x 2.5 mm) equilibrated with 0.05 M Tris-HCl (pH 8), 1 mM EDTA. Electron microscopy was performed as described earlier (Veldman *et al.*, 1981).

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