
The *in vivo* and *in vitro* initiation site for transcription of the rRNA operon of *Saccharomyces carlsbergensis*

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ABSTRACT

We have performed a detailed analysis of the transcription initiation of the rRNA operon in the yeast *Saccharomyces carlsbergensis*. Electron microscopic analysis of R-looped pre-rRNA molecules together with a very sensitive S1-nuclease mapping showed the use of only a single transcription start at about 700 bp upstream of the 17S rRNA gene and not of the minor start sites proposed for the very closely related species *S. cerevisiae* by others [Bayev *et al.* (5), Swanson and Holland (6)]. The sequence of 730 bp of the initiating region is presented. *In vitro* transcription in concentrated lysates of yeast spheroplasts in the presence of (γ -SH)ATP or (γ -SH)GTP, followed by purification of the *in vitro* initiated RNA via Hg-agarose, revealed that on the endogenous template exactly the same site is used for transcription initiation as *in vivo*.

INTRODUCTION

In eukaryotic cells the ribosomal operons are transcribed by a separate RNA polymerase A or I (1). This transcription is efficiently regulated as may be inferred for instance from the response of yeast to external stimuli like nutritional shift-up and shift-down (2). The predominant control is most likely exerted at the transcription initiation level. Therefore we want to elucidate the structural elements within the ribosomal DNA which are involved in the regulation and promotion of ribosomal RNA transcription in yeast. As a first step we need an unambiguous identification of the transcription start site(s) of the yeast rRNA operon. We have previously shown (3) that pppA-Up is the predominant 5'-terminal sequence of 37S pre-rRNA of *Saccharomyces carlsbergensis*. Therefore, mapping of the 5'-end of 37S pre-rRNA pinpoints the transcription start point(s). Both Klemenz and Geiduschek (4) and Bayev *et al.* (5) have mapped the 5'-end of 37S pre-rRNA in the very closely related yeast species *Saccharomyces cerevisiae* at about 700 bp upstream of the 17S rRNA gene. However, Bayev *et al.* (5) suggested the occurrence of an additional, minor pppG-start slightly (28 bp) upstream of the major one. Recently, still another minor start site was proposed to

be located between the genes for 26S and 5S rRNA at about 2900 bp upstream of the 17S rRNA gene (6). We have carried out a detailed analysis of the initiation site(s) for RNA polymerase A in S. carlsbergensis using R-loop analysis and S1-nuclease mapping and could detect only a single site for transcription initiation corresponding to the major one in S. cerevisiae. We have extended this analysis by developing an in vitro transcription system based on a lysate of yeast spheroplasts. Analysis of the start sites used by RNA polymerase A on the endogenous template in this in vitro system demonstrated that initiation takes place at the same site as in vivo. This lysate might be very useful in developing a specific, template-dependent in vitro transcription system for yeast RNA polymerase A.

MATERIALS AND METHODS

Isolation of DNA fragments

We used the recombinant plasmids pMY57 and pMY60, which contain the 2700 bp HindIII-generated fragment B and the entire rDNA unit of Saccharomyces carlsbergensis (NCYC, S74) rDNA respectively (see Fig. 1) in pBR322 (7). Plasmid DNA was isolated from E. coli K12 cells by the clear lysate method (8), followed by CsCl gradient purification. DNA fragments were isolated from 3-5% (w/v) polyacrylamide gels by electroelution. Strand separation of (5'-³²P)-labeled fragments was achieved on 5% polyacrylamide gels containing 0.08% N,N'-methylene bisacrylamide in 50 mM Tris-boric acid (pH 8.3), 1 mM EDTA.

Isolation of RNA

Total RNA was isolated as described previously (9). An RNA fraction enriched in 37S pre-rRNA was obtained from a crude nuclear pellet (3,10).

R-looping and electron microscopy

R-looping, spreading and preparation for electron microscopy was performed as described previously (9).

S1-nuclease mapping and DNA sequencing

5'-Ends of DNA fragments were labeled with (γ -³²P)ATP and polynucleotide kinase (Boehringer, Mannheim) DNA sequence analysis was performed according to Maxam and Gilbert (11). Excess 5'-labeled strand-separated DNA was hybridized with various amounts of RNA in 80% formamide, containing 0.4 M NaCl, 1 mM EDTA and 40 mM Pipes (pH 6.4) for 16 h. S1-nuclease digestion was performed at 15°C with 400 units in the presence of 20 μ g/ μ l calf thymus DNA as described previously (9).

Preparation of lysates of yeast

Exponentially growing cells of S. carlsbergensis were converted to

spheroplasts with zymolyase (Kirin Brewery, Japan) in a solution containing 13% (w/v) mannitol, 1 mM $MgCl_2$ and 10 mM Na-citrate at 25°C. The incubation was stopped with an excess of the same solution, containing 1 mM PMSF at 0°C. Spheroplasts were spun down in a Labofuge (Heraeus) at 2000 rpm for 5 min. 1 gram (wet weight) of spheroplasts was suspended in 2 ml of a solution containing 30% (w/v) Ficoll 400, 20% (v/v) glycerol, 20 mM Hepes, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 10 mM DTT and 1 mM PMSF (pH 7.9). Cells were lysed at 0°C by two strokes in a potter with a close-fitting pestle. The extent of lysis was checked microscopically and at completion one tenth volume of a solution containing 200 mM Hepes, 45 mM $MgCl_2$ and 500 mM KCl (pH 7.9) was added. The lysate could be stored at -70°C, in which case it was frozen quickly in liquid nitrogen.

Transcription and Hg-agarose chromatography

Transcription was performed for 10 min at 25°C after a two-fold dilution of the lysate into an appropriate solution. The final concentrations of the components during transcription were 30 mM Hepes, 5 mM $MgCl_2$, 40 mM KCl, 0.1 mM EDTA, 0.5 mM PMSF, 6 mM DTT, 10% glycerol, 15% Ficoll, 1 mM ATP or (γ -SH)ATP, 1 mM GTP or (γ -SH)GTP, 0.01-0.1 mM CTP, 0.5 mM UTP, 0.1 mM vanadyl ribonucleoside complex (Bethesda Res. Lab.) and 1 U/ μ l RNasin (Biotec) (pH 7.9). (α -³²P)CTP (The Radiochemical Centre, Amersham) was either added at 1 μ Ci/ μ l or in trace amounts; incidentally (α -³²P)UTP was used in which case the concentrations of UTP and CTP were reversed. Transcription was stopped by adding aurin tricarboxylic acid and SDS to final concentrations of 0.4 mM and 0.25% (w/v), respectively. Then proteinase K (Boehringer) was added at 0.1 mg/ml and incubated for 5 min at 37°C. Nucleic acids were extracted by phenol/chloroform/isoamylalcohol (50:50:1, v/v/v) in the presence of 0.2 M Na-acetate and 1% (w/v) SDS. Hg-agarose was prepared according to Reeve *et al.* (12) starting from CNBr-activated Sepharose-4B (Pharmacia). The binding of ethylenediamine was measured using the method of Inman and Dintzis (13) and the binding of p-chloromercuribenzoate was estimated by the method of Sluyterman and Wijdenes (14). RNA was applied to a column (1 ml, ϕ = 0.5 cm) of Hg-agarose after dissolving the ethanol-precipitated RNA in TNES (10 mM Tris-HCl, 0.1 M NaCl, 10 mM EDTA and 0.5% (w/v) SDS, pH 7.9). The column was eluted with TNES until no more RNA was washed off. RNA bound to the column was eluted with TNES plus 10 mM β -mercaptoethanol (15) and subsequently analysed by hybridization with filter-bound *S. carlsbergensis* rDNA fragments according to Southern (16) or by S1-nuclease mapping.

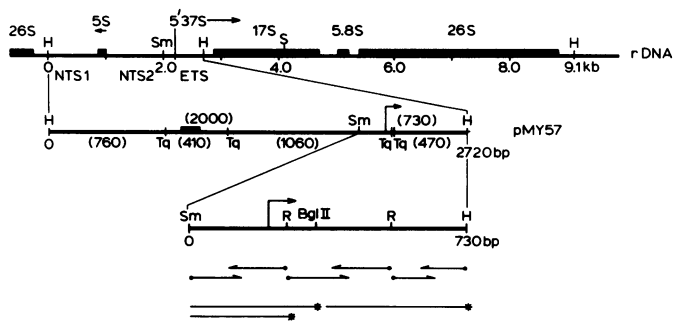


Fig. 1. Map of the rDNA unit of *S. carlsbergensis*. The upper line shows one complete rDNA unit with the restriction enzyme sites for HindIII (H), SmaI (Sm) and SacI (S). The HindIII-generated fragment B cloned in pBR322, is enlarged and the TaqI (Tq) sites in this fragment are indicated. The (SmaI + HindIII)-fragment of 730 bp was sequenced; the strategy is given below. Single-stranded probes for S1-nuclease mapping are indicated. The 5'-label (asterisk) is present at the EcoRI (R), BglIII or HindIII site.

RESULTS

Mapping of the in vivo initiation site(s) of the rRNA operon

Figure 1 shows a map of the rDNA unit of *S. carlsbergensis*. The small HindIII-generated fragment (2.7 kb) has been cloned in pBR322 and the resulting plasmid, pMY57 (7), has been used throughout this study. This cloned fragment contains most of the nontranscribed spacer (NTS) sequences of the rDNA unit together with the 5S rRNA gene and the major part of the external transcribed spacer (ETS) (4,17). We used pMY57 for an R-loop analysis with high-molecular-weight RNA enriched in nuclear RNA. Since the plasmid does not contain any mature 17S and 26S rRNA sequences nor the internal transcribed spacer sequences, we will only detect pre-rRNA molecules that have a sufficiently long ETS sequence.

Figure 2A shows a typical example of such an R-loop as visualized by electron microscopy. The site at which the long (single-stranded) RNA-chain is protruding from the R-loop marks, in principle, the HindIII site at the junction of the yeast DNA and pBR322. To provide a second point of reference R-looping was performed with a plasmid linearized by cutting with SmaI within the yeast DNA (see Fig. 1). The result of such an experiment is shown in Fig. 2B. By measuring the lengths of the R-loops and their distance towards the SmaI-generated end the 5'-end of the RNA can be mapped rather precisely.

Figure 3 shows the histogram of the location of 56 R-loops. There is apparently one major if not unique initiating region at about 220 bp downstream of the SmaI site and at about 700 bp upstream of the 17S rRNA gene. We

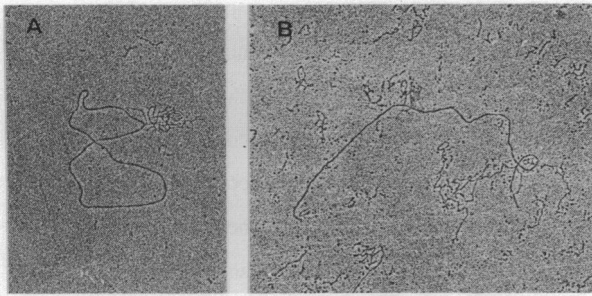


Fig. 2. Electron micrographs of an R-loop formed between 37S pre-rRNA and pMY57 DNA (a), or pMY57 DNA linearized with SmaI (b).

screened a large number (N = 335) of SmaI-linearized, R-looped molecules for the presence of a fork at the end of the molecule instead of a loop; such a fork will result from a pre-rRNA that has been transcribed from a start upstream of the SmaI-site as suggested by Swanson and Holland (6). No such molecule could be found, neither in RNA preparations enriched in 37S pre-rRNA nor in total unfractionated RNA.

Figure 4 shows the sequence of the 730 bp long fragment, generated by digestion with SmaI plus HindIII (see Fig. 1). Apart from a few single base differences, the sequence is identical to the corresponding DNA segment of *S. cerevisiae* as published by Bayev *et al.* (5,18). To pinpoint the 5'-end of 37S pre-rRNA exactly, we performed S1-nuclease mapping, using the (SmaI + BglII)- and the (BglII + HindIII)-fragments as probes (see Fig. 1). We hybridized an excess of these single-stranded, (5'-³²P)labeled probes with various amounts of RNA.

Figure 5 (lane 2-4) shows one protected band of an estimated length of 128 bases for the (SmaI + BglII)-probe whereas the (BglII + HindIII)-probe (lane 5-7) is entirely protected; the intensity of the protected fragment is proportional to the amount of input RNA. At 128 bp upstream of the BglII site there is an A-T-G sequence (see Fig. 4), which corresponds to the pppA-U

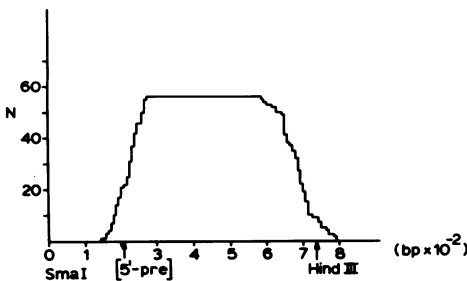


Fig. 3. Histogram of the length and the position relative to the SmaI site of 56 R-loops formed between pre-rRNA molecules and pMY57 DNA. The HindIII site does not coincide exactly with the average position of right-hand end of the R-loops, possibly due to some branch migration of the protruding RNA chain.

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GGGCACCGT CACTTGGAA AAAAAAATA TACGCTAAGA TTTTGGAGA ATAGCTTAA (-148)
TTGAAGTTT TCTCGGCAG AAATACGTAG TTAAGGCAGA GCGACAGAGA GGGCAAAGA (- 88)
AAATAAAGT AAGATTTAG TTTGTAATGG GAGGGGGGT TTAGTCATGG AGTACAAGTG (- 28)
TGAGAAAAG TAGTTGGGAG GACTTCATG+1 CGAAAGCAGT TGAAGACAAG TTCGAAAAGA (+ 33)
GTTTGGAAAC GAATTCGAGT AGGCTTGTCTG TFCGTTATGT TTTGTAAAT GGCCTCGTCA (+ 93)
AACGGTGGAG AGAGTCGCTA GGTGATCGTC AGATCTGCT AGTCTCTATA CAGCGTGTTC (+153)
AATTGACATG GGTGATGCG TATTGGGAGA TACAATTTGG GAAGAAATTC TCAGAGTGTG (+213)
TTTCTTTTGC GTTAACTTG AACAGTCTCA TCGTGGGCAT CTTGCGATTC CATTGGTGAG (+273)
CAGCGAAGGA TTTGGTGGAT TACTAGCTAA TAGCAATCTA TTTCAAAGAA TTCAAACCTG (+333)
GGGGAATGCC TTGTGAATA GCCGTCGCA AGACGTGATT CTTCAAGTGT AACCTCTCT (+393)
CAAATCAGCG ATATCAAACG TACCATTCCG TGAACACCG GGTATCTGT TTGGTGAAC (+453)
CTGATTAGAG GAAACTCAA GAGTGTATG GTATGGTGAC GGAGTGTGCT GGTCAAAGAGT (+513)
GTAAAAGCTT

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Fig. 4. Sequence of the (SmaI + HindIII)-fragment of 730 bp containing the initiating region of the rRNA operon of *S. carlsbergensis*. The sequence of the non-coding strand is shown; the transcription start is numbered +1.

sequence found at the 5'-end of 37S pre-rRNA after digestion with T_1 plus pancreatic RNAase (3). Preliminary experiments in which 37S pre-rRNA was digested by T_1 RNAase only and poly-phosphate containing oligonucleotides were purified on hydroxyapatite, showed the presence of an oligonucleotide with the sequence pppA-U-G. These data unambiguously map the transcription start at position +1 in Fig. 4.

Figure 5 (lane 2) shows a few very weak bands which are even more faint than the main signal in lane 4 where a 100-fold lower amount of input RNA was used. Therefore the yield of these fragments is less than 1% compared to the yield of the dominant protected fragment of 128 bases in lane 2. This is too low to consider them to be indicative for the presence of pre-rRNAs with distinct 5'ends. None of these fragments corresponds to the minor protected fragment observed by Bayev *et al.* (5). Furthermore, some protection of the entire (SmaI + BglII)-probe is observed. This cannot be due to pre-rRNA molecules longer than the 37S pre-rRNA (see Fig.3) but is most likely caused by the presence of nuclear DNA in our RNA preparation. In lane 5 (Fig.5) the (BglII + HindIII)-probe gives rise to a minor signal of about 300 bases in addition to the fully protected probe. The most probable explanation is the occurrence of some S1-nuclease digestion of the hybrid at the (A+T)-rich tract shortly down-

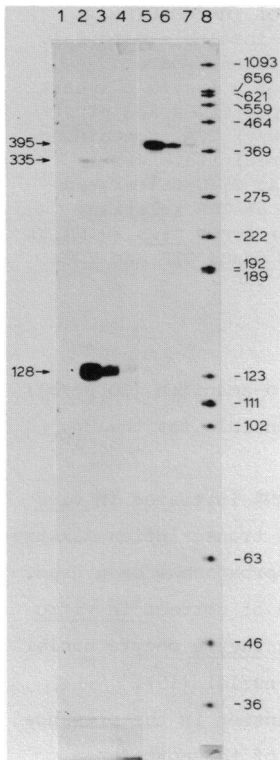


Fig. 5. S1-nuclease mapping of the 5'-end of 37S pre-rRNA. (5'- 32 P)labeled single-stranded (SmaI + BglII)-fragment (335 bases, lane 1-4) and (BglIII + HindIII)-fragment (395 bases, lane 5-7) were hybridized with 1 μ g *Bacillus* RNA (lane 1), or with 1 μ g (lane 2 and 5), 0.1 μ g (lane 3 and 6) or 0.01 μ g (lane 4 and 7) high-molecular weight *S. carlsbergensis* RNA; see Fig. 1 for the location of the fragments used as probe. S1-nuclease protected fragments were analysed on a 6% (w/v) polyacrylamide gel containing 6 M urea, using polyoma DNA digested with DdeI as a marker (lane 8).

stream of the BglII site. We therefore conclude that only a single transcription start for RNA polymerase A is present on *S. carlsbergensis* rDNA under our growth and labeling conditions.

In vitro transcription in lysates of yeast spheroplasts

Purification of the yeast RNA polymerase A transcription complex leads to a loss of specificity: neither purified RNA polymerase A nor the so-called S100 fraction is able to initiate transcription on added yeast rDNA templates at the 5'-end of 37S pre-rRNA (6,19). A study of the in vitro transcription on the endogenous template in an unfractionated system might provide insight into the parameters which are relevant for the preservation of faithful transcription. Highly concentrated lysates are able to incorporate labeled UTP into TCA-insoluble material (see Fig. 6). This incorporation is template-dependent as inferred from its sensitivity to actinomycin D. The relatively high incorporation in the first 10 min is thought to reflect mainly elongation of pre-existing transcription complexes. After 10 min incorporation continues at a lower rate, likely due to transcription that initiated in vitro. Since

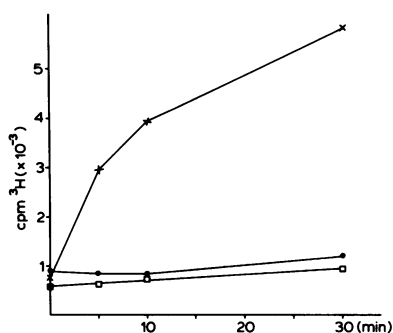


Fig. 6. Time course of (α - 32 P)UTP incorporation in TCA-insoluble material. 10 μ l aliquots were taken from a transcription assay of a lysate of yeast spheroplasts at various times and pipetted into 200 μ l 5% (w/v) TCA, containing 10 mM $\text{Na}_4\text{P}_2\text{O}_7$. The precipitate was collected on a Whatman GF/C filter and washed with the same 5% TCA solution. x—x standard assay; ●—● plus 20 μ g/ml actinomycin D; □—□ minus CTP and GTP.

the incorporation of UTP was assayed in the presence of α -amanitin (20 μ g/ml) either RNA polymerase A and/or C are thought to be responsible for the observed activity.

To separate RNA that was initiated in vitro from RNA initiated in vivo we included the (γ -SH) analogue of ATP and/or GTP in the transcription mixture and fractionated the RNA on a Hg-agarose column. This approach has been used successfully in several cases, e.g. in the demonstration of correct in vitro initiation by RNA polymerase A in homogenates of *Xenopus laevis* oocyte nuclei (20) and for RNA polymerase C studies in isolated yeast nuclei (15).

In vitro initiated RNA was prepared after transcription in the presence of (α - 32 P)CTP and either (γ -SH)ATP or (γ -SH)GTP instead of the normal triphosphates. The RNA was hybridized to filter-bound fragments of yeast rDNA (see Fig. 7). In Fig. 7A hybridization with rDNA fragments obtained by digestion of pMY60, a plasmid consisting of one complete rDNA unit of *S. carlsbergensis* in vector pBR322 (7) with HindIII plus SmaI plus SacI is shown. Using RNA started in vitro with (γ -SH)ATP a very strong hybridization is obtained with the 730 bp long fragment, which contains the in vivo initiating region plus most of the ETS (cf. also Fig. 1). This fragment gives only a very weak signal when RNA starting with (γ -SH)GTP is used. This suggests that transcription initiation of the rRNA operon occurs in the correct region using predominantly ATP. The hybridization to the adjacent 1490 bp (HindIII + SacI)-fragment is rather weak. This indicates that the in vitro initiated RNA molecules, selected by the column, are relatively short, probably as a result from RNAase activity in the lysate. The weak hybridization to the 4880 bp (SacI + HindIII)-fragment is likely to be due to some aspecific binding by the Hg-agarose column of RNA fragments without a (γ -SH) group. These labeled sequences are overrepresented, because of elongation of RNA chains initiated in vivo. The 2000 bp (HindIII + SmaI)-

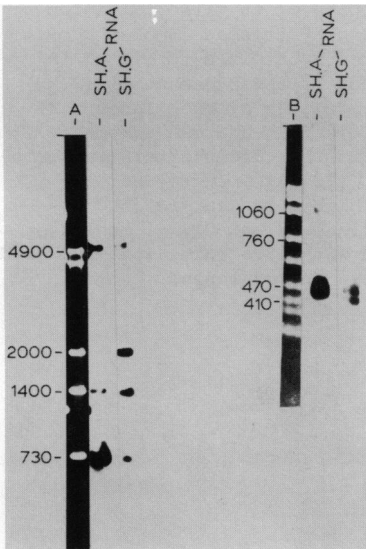


Fig. 7. Southern hybridization of *in vitro* initiated RNA. *In vitro* initiated RNA was isolated from transcription mixtures containing (α - 32 P)CTP and either (γ -SH)ATP ("SH-A RNA") or (γ -SH)GTP ("SH-G RNA"), purified by Hg-agarose affinity chromatography and hybridized to filter-bound digests of plasmids containing yeast rDNA.

(A) pMY60, containing one complete rDNA unit of *S. carlsbergensis* (see Fig. 1), digested with HindIII plus SacI plus SmaI.

(B) pMY57, containing the small HindIII-generated fragment of the rDNA unit, digested with TaqI plus HindIII (see Fig. 1).

The size of the yeast rDNA fragments is indicated; the remaining fragments are derived from the vector pBR322.

fragment hybridizes only with RNA initiated with (γ -SH)GTP. Since this fragment contains the 5S rRNA gene (*cf.* Fig. 1), transcription of which starts with pppG *in vivo* (21), the hybridization signal most probably reflects transcription of this 5S rRNA gene. In Fig. 7B shorter fragments of the NTS and ETS of the rDNA are hybridized with the same Hg-agarose selected RNAs as in Fig. 7A. We observe that RNA initiated with (γ -SH)ATP hybridizes strongly with an ETS-fragment of 470 bp. Hybridization with a 1060 bp fragment, which contains only 24 bp of the ETS and most of the NTS2 (see Fig. 1) is weak. RNA initiated with (γ -SH)GTP hybridizes (relatively weakly) with the 470 bp fragment but also with the 410 bp fragment which carries the 5S rRNA gene. These results confirm the suggestion that RNA polymerase A initiates in the correct region using ATP and that transcription of the 5S rRNA gene by RNA polymerase C in the lysate takes place. It is unlikely that RNA polymerase C is also responsible for the transcription of the ETS sequences in the 730 bp (Fig. 7A) or 470 bp fragment (Fig. 7B). An elevated salt concentration during transcription, which inhibits RNA polymerase A but not C (22), strongly reduces the hybridization of RNA initiated with both (γ -SH)ATP and (γ -SH)GTP to the 730 bp (SmaI + HindIII)-fragment, but not that of RNA initiated with (γ -SH)GTP to the 2000 bp (HindIII + SmaI)-fragment (data not shown). It should be noted that the 760 fragment in Fig. 7B does not hybridize at all. Since the transcription start found by Swanson and Holland (6) in S100 extracts on exogenous cloned rDNA is immediately upstream of the sequences in this fragment,

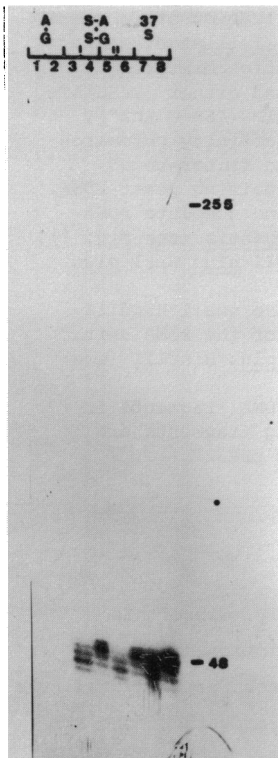


Fig. 8. S1-nuclease mapping of in vitro initiated RNA. (5'-³²P)labeled, single-stranded (SmaI + EcoRI)-fragment (see Fig. 1) was hybridized to either in vitro initiated RNA, made in the presence of (γ-SH)ATP plus (γ-SH)GTP and isolated by one (lanes 3,4) or two cycles (lanes 5,6) of Hg-agarose chromatography or to RNA bound to the column after transcription in the absence of (γ-SH)ATP plus (γ-SH)GTP (lanes 1 and 2), or to high-molecular-weight S. carlsbergensis RNA (lanes 7 and 8). S1 nuclease digestion was performed with 18.5 units (odd-numbered lanes) or 6 units (even-numbered lanes).

we have to conclude that this start is hardly used, if at all, on the endogenous template.

Finally, we mapped the 5'-end of the in vitro initiated RNA using a 255 bp (SmaI + EcoRI)-fragment as a probe (cf. Fig. 1). S1-nuclease digestion of hybrids formed between this fragment and 37S pre-rRNA gives rise to a protected DNA fragment of 48 bases as shown in lanes 7 and 8 of Fig. 8. Exactly the same fragment is obtained with RNA initiated in vitro with (γ-SH)ATP plus (γ-SH)GTP purified by either one (lanes 3 and 4) or two (lanes 5 and 6) cycles of Hg-agarose column chromatography. This signal is not caused by aspecific binding of 37S pre-rRNA to the column. RNA bound to the column after transcription in the absence of (γ-SH)ATP and (γ-SH)GTP does not give rise to a protected DNA fragment (lanes 1 and 2). Therefore, we conclude that RNA polymerase A starts in vitro at the same position as in vivo.

DISCUSSION

We have unambiguously mapped the transcription initiation site of the rRNA operon of S. carlsbergensis. Only one start appears to be used by RNA

polymerase A under our growth and labeling conditions. The R-loop analysis (Figs. 2 and 3) shows only one region for transcription initiation just downstream of the SmaI site. Within this region only one start site could be mapped by S1-nuclease analysis (Fig. 5). This start site is identical to the one reported by Klemenz and Geiduschek (4) and Bayev et al. (5) for the very closely related species S. cerevisiae except that Bayev et al. detected a second protected fragment 28 bases longer than the main product. A similar signal is definitely absent in S. carlsbergensis (see Fig. 5). The difference may be explained either by the different growth conditions used, or by the different isolation procedure of the RNA, or by an artefact of the S1-nuclease analysis performed by Bayev et al. (5). We favour the third explanation, since they do not find this putative start site in their reverse transcription experiments. The extra start site proposed by Swanson and Holland (6) at 2900 bp upstream of the 17S rRNA gene has also not been observed in S. carlsbergensis. Not a single R-loop corresponding to such a pre-rRNA was found in the large number (335) of R-loop complexes analysed. Neither could any transcription initiation in vitro be observed at that site (see Fig. 7). Swanson and Holland (6) based their proposal for an alternative start site mainly upon in vitro transcription of exogenous templates in a crude yeast extract (an "S100" fraction). If this site would appear to be a genuine start site, it is apparently fully repressed under the conditions we use for culturing S. carlsbergensis as well as for in vitro transcription of the endogenous template in the lysate.

In vitro transcription in lysates of yeast spheroplasts appears to mimic in vivo transcription faithfully according to the criteria tested so far. All three RNA polymerase activities (A, B and C) can be demonstrated in the lysate. Addition of α -amanitin (20 μ g/ml) reduces the incorporation of labeled UTP by 30% (data not shown), apparently by eliminating RNA polymerase B activity. Southern hybridization of RNA initiated in vitro in the presence of α -amanitin shows that transcription of 5S RNA starts with GTP and that of 37S pre-rRNA with ATP, as in vivo. Transcription initiation of the 5S rRNA gene by yeast RNA polymerase C on both endogenous and exogenous templates has been demonstrated by others as well (15,24,25,26). Our S1-nuclease mapping analyses also indicate that yeast RNA polymerase A starts correctly in vitro on the endogenous template (see Fig. 8). The weak hybridization of RNA initiated with (γ -SH)GTP with the ETS-containing fragments in Fig. 7 is not due to an alternative pppG-start of 37S pre-rRNA but to some transfer of the (γ -SH) group to ADP in the lysate since S1-nuclease mapping of the RNA initiated with (γ -SH)GTP produces a protected fragment of identical length as

observed for RNA initiated with (γ -SH)ATP plus (γ -SH)GTP or (γ -SH)ATP only (result not shown). The results shown in Fig. 8 cannot be explained by a transfer of the (γ -SH) group to pre-existing 37S pre-rRNA, since it has been shown already (15) that in isolated nuclei the (γ -SH) group is not transferred to elongated transcripts. Moreover, addition of actinomycin D to the transcription mixture containing (γ -SH)ATP plus (γ -SH)GTP suppresses the S1-nuclease signal (data not shown). The mapping of the in vitro start site is fully consistent with our in vivo data. Therefore, the lysate system provides an essential starting point for the study of parameters involved in the transcription initiation of the yeast rRNA operon.

The sequence of the S. carlsbergensis promoter region in Fig. 4 has been compared with that of two S. cerevisiae rDNA clones (5,22). It appears to be virtually identical with the sequence reported by Bayev et al. (5) whereas it deviates to some extent from the one determined by Valenzuela et al. (22). Most of these latter differences are located between position -47 and -110 of the sequence in Fig. 4; altogether 25 basepairs of the S. carlsbergensis sequence within this region are lacking from the S. cerevisiae sequence. This entails, that the exact spacing of promoter sequence elements upstream of position -47, if present, with respect to the transcription start site is not critical. Sequence comparison with putative RNA polymerase A promoters of other species is only useful with species, that are not too distant (9). We found a conservation of about 30 bp around the start site (position -9 to +23) of 37S pre-rRNA in four different yeast species, all belonging to the subfamily of the Saccharomycetoideae (Verbeet et al., submitted for publication). This conserved initiating sequence is supposed to constitute an important part of the promoter for yeast RNA polymerase A.

Further inspection of the sequence of the initiating region reveals the presence of four directly adjacent stretches of about 25 bp which are 50-65% mutually homologous (Fig. 9). They can be considered as 'old' duplications within the transcription start region. The transcription initiation is contained within the second stretch; the first one, directly upstream, was

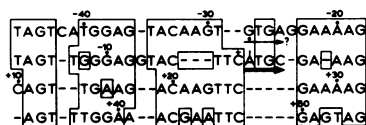


Fig. 9. 'Old' duplications within the initiating sequence of the S. carlsbergensis rRNA operon. The sequence from position -46 to +55 (see Fig. 4) is presented as four imperfect direct repeats. Shifts (--) are introduced to maximize homology.

already noticed by Bayev *et al.* (5) and contains the putative, minor pppG-start in *S. cerevisiae* (but not in *S. carlsbergensis*) from a homologous position. The third and fourth stretch, downstream of the start, are inactive in both yeasts; they both lack the A-T-G-C sequence used for the start of transcription in the second stretch. It is striking that the length of the repeat (see Fig. 9) matches rather well the length of the afore mentioned, conserved sequence at the start site of four *Saccharomycetoideae*. This parallel suggests that this putative element has been subjected to duplication and supports a previous suggestion of Moss *et al.* (23) that the external transcribed spacer of the rRNA operon might partly arise from a balance between duplications of the functional promoter and inactivation by subsequent mutations of one of the two repeats.

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