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# A conserved sequence element is present around the transcription initiation site for RNA polymerase A in *Saccharomycetoideae*

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ABSTRACT

To identify DNA elements involved in the initiation of rRNA transcription in yeast we located the start site of the rRNA operon of Kluyveromyces lactis and Hansenula wingei, both members of the Saccharomycetoideae, by S1 nuclease analysis and determined the surrounding nucleotide sequences.

Comparison of these sequences with those of Saccharomyces carlsbergensis, S. cerevisiae and S. rosei (all belonging to the same yeast subfamily) reveals an identical sequence at the site of transcription initiation from position +1 to +7 which is part of a larger conserved region extending from position -9 to +23; the conserved heptanucleotide sequence is supposed to constitute an important part of the promoter for yeast RNA polymerase A. The non-transcribed spacers (NTS) upstream of position -9 have diverged strongly with the exception of two short elements around positions -75 and -135. The external transcribed spacer (ETS) downstream of position +23 is largely conserved between K. lactis, S. rosei and S. carlsbergensis except for a divergent region around position +75. On the other hand, the ETS of H. wingei has diverged significantly.

## INTRODUCTION

Initiation of transcription is an important level at which gene expression is controlled. Signals regulating the initiation of the mRNA synthesis by RNA polymerase B, and the 5S rRNA and tRNA synthesis by RNA polymerase C are now identified as short, conserved sequence elements (1-5). At present the knowledge about sequences important for controlling the initiation of transcription by RNA polymerase A, which synthesizes rRNA in the nucleolus, is much more limited. The transcription initiation sites of the rRNA operons have been determined for several species. Comparison of the nucleotide sequences of these initiating regions from yeast (6-8), mouse (9-11), Xenopus laevis (12-13) and Drosophila melanogaster (14) revealed little or no homology. This lack of homology suggests that an RNA polymerase A promoter evolves more rapidly than promoters for RNA polymerases B and C. The reason for this might be found in the fact that RNA polymerase A interacts with only one type of transcription unit whereas the B and C enzymes transcribe numerous different

genes. This should put a much higher evolutionary constraint upon the promoters for the latter two enzymes as compared to the promoter for RNA polymerase A. The recently observed species specificity of *in vitro* transcription of man, mouse and protozoan rDNA (15,16) suggests that the RNA polymerase A promoter co-evolves with one or more genes coding for (a) subunit(s) of the polymerase or (a) transcription factor(s). It has been proposed that the rate of this co-evolution is relatively high as a result of the homogenization process acting upon the array of rDNA units and hence upon the RNA polymerase A promoter (17). Identification of RNA polymerase A promoter elements by sequence comparison might therefore succeed only when the species analysed are sufficiently closely related. Indeed comparison of the appropriate sequences of three *Xenopus* species (18) revealed an identical sequence from position -9 to +4 in addition to homology further upstream. Also three mammals (mouse, rat and human) contain a highly conserved sequence extending from position -9 to +18 (19,20). Deletion analysis of the various rDNA templates has proven the functional significance of these conserved elements, which were found to be absolutely required for correct initiation (21-25). All these data suggest that, at least in vertebrates, the sequences around the initiation sites for RNA polymerase A play an important role in the promotion of transcription. Although the rDNA units of man and mouse have the same conserved sequence around the initiation sites of their rRNA operons the *in vitro* transcription of these units requires the complete homologous system (15,16). This suggests that the conserved sequence in the initiating region may not be the only promoter element. One or more additional elements may be present which have diverged too far to be revealed by sequence comparison. Such elements, whose existence indeed has been documented by deletion analysis (21-25), might be detected by analysing sequence conservation among a series of species whose evolutionary distance gradually increases. We have tried to apply this strategy in determining the structural elements involved in transcription initiation by RNA polymerase A in yeast. We initially compared the transcription starts of the rRNA operons of *S. rosei* and *S. carlsbergensis* (26). This comparison revealed some homologouw sequence elements upstream from the start site as well as an almost perfectly conserved sequence extending from position -9 to at least 120 bp into the external transcribed spacer (ETS). In order to reveal the parts of this extended homologous region with the highest evolutionary constraint we decided to include the more distantly related members *Kluyveromyces lactis* and *Hansenula wingei* of the subfamily of *Saccharomycetoideae* in our comparison (27). Restriction mapping and hetero-

duplex analysis of the cloned rDNA units of K. lactis and H. wingei had already indicated that these two yeast strains are indeed more divergent from S. carlsbergensis than S. rosei (M.Ph. Verbeet et al., submitted for publication).

This paper describes the identification of the transcription initiation site of the K. lactis and H. wingei rRNA operons and the surrounding nucleotide sequences. Comparison of these sequences for all four yeast species reveals a number of distinct conserved sequence elements.

## MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from Boehringer (Mannheim) and New England Biolabs. They were used under the conditions recommended by the suppliers. Calf intestine alkaline phosphatase, polynucleotide kinase, S1 nuclease and DNA polymerase were obtained from Boehringer. DNAase EP was obtained from Sigma. Radiochemicals were purchased from The Radiochemical Centre, Amersham.

DNA preparation. Plasmid DNA for sequencing and S1 nuclease mapping were isolated from Escherichia coli HB101 by the "cleared lysate" procedure (28) followed by caesium chloride-ethidium bromide centrifugation. The construction of the recombinants pKlR2, containing the K. lactis rDNA unit, and pHwR1, containing the H. wingei rDNA unit, will be described elsewhere (M.Ph. Verbeet et al., submitted for publication). DNA fragments used for nick-translation or 5'-end labelling were purified by polyacrylamide gel electrophoresis (8).

Isolation of RNA. Total RNA was isolated from K. lactis (NRRL, Y1140, Ill., USA) or H. wingei (NRRL 2340, strain 21, Ill., USA) by pouring a spheroplast suspension into liquid nitrogen and thawing in the presence of an equal volume of phenol containing 1% (w/v) SDS and 50 µM aurin tricarboxylic acid (an RNAase inhibitor). After phenol extraction and ethanol precipitation, high-molecular-weight RNA was obtained by fractionation on a linear 15-30% (w/v) sucrose gradient (29).

S1 nuclease mapping. 5'-end-labelled DNA fragments were run on 5% (w/v) polyacrylamide gels, containing 0.08% (w/v) N,N$^1$-methylene bisacrylamide in 50 mM Tris-boric acid (pH 8.3) and 1 mM EDTA, to separate the two strands. 50-200 ng single-stranded DNA was hybridized with different amounts of RNA in 80% (v/v) formamide, containing 0.4 M NaCl, 1 mM EDTA and 40 mM Pipes (pH 6.4), in the presence of 2 mg bacterial RNA per ml for 16 h at 30°C; prior to hybridization the mixtures were denatured for 15 min at 85°C. After the hybridization the samples were diluted 30-fold with an ice-cold solution containing 0.28 mM NaCl,

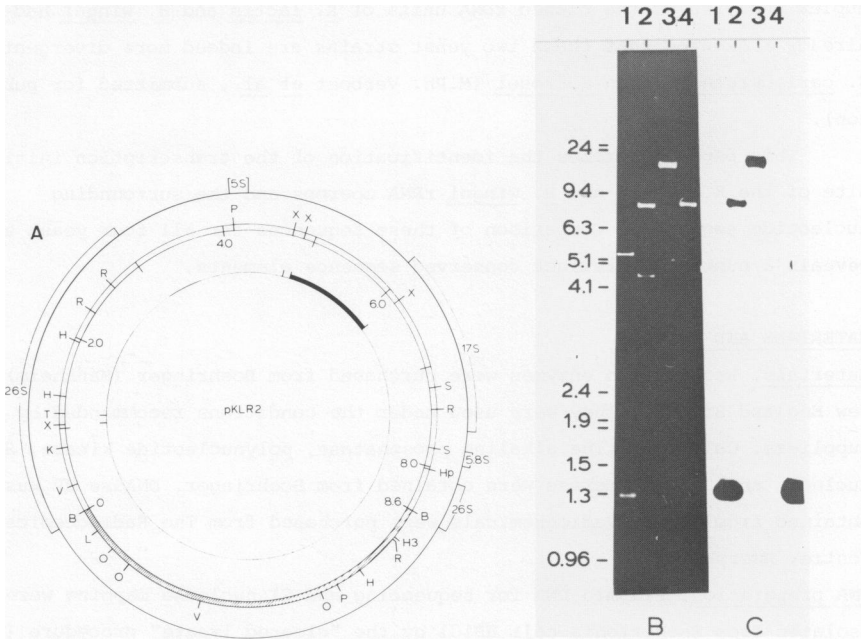
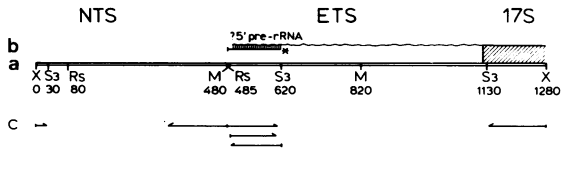



Fig. 1. Identification of rDNA fragments of K. lactis homologous to the initiation region of S. carlsbergensis rDNA.
(A) Physical and genetic map of pKlR2, containing the rDNA unit (8.6 kb) of K. lactis inserted in the BamHI site of pBR322. Restriction enzyme sites for BamHI (B), HindIII (H3), HindII (H), HpaI (Hp), SalI (L), SacI (S), EcoRI (R), XbaI (X), PstI (P), BglI (O), BglII (T), KpnI (K) and PvuII (V) are indicated. SmaI does not cut the rDNA of K. lactis. The pBR322 part of the plasmid is hatched. The genetic map (outer circle) shows the rRNA genes. The mapping by Southern blot hybridization of the fragments in the BglII plus XbaI digest of pKlR2 homologous to the initiation region of S. carlsbergensis rDNA is depicted in the inner circle by a black bar.
(B) The ethidium bromide-stained gel pattern of pKlR2 DNA digests fractionated on a 1% agarose gel. Lane 1: XbaI plus EcoRI; lane 2: BglII plus BamHI; lane 3: BglII; lane 4: BglII plus XbaI. The lengths of the marker fragments - λ DNA digested with HindIII and HindIII plus EcoRI - are indicated.
(C) Autoradiogram of the Southern filter blot (31) of the gel in panel (B) hybridized to a nick-translated (32) 730 bp long SmaI-HindIII fragment containing the initiation region of S. carlsbergensis rDNA. Hybridization was performed as described previously (26).

30 mM Na-acetate (pH 4.4), 4.5 mM Zn-acetate, denatured calf thymus DNA (20 µg/ml) and an amount of S1 nuclease (as specified in the legends). The mixtures were incubated for 2 h at 15°C. After precipitation of the DNA with 2-propanol, the protected DNA was analysed by electrophoresis on a 6% or 8% (w/v) polyacrylamide gel in 7 M urea (30).

DNA sequencing. DNA sequencing was performed by the chemical modification



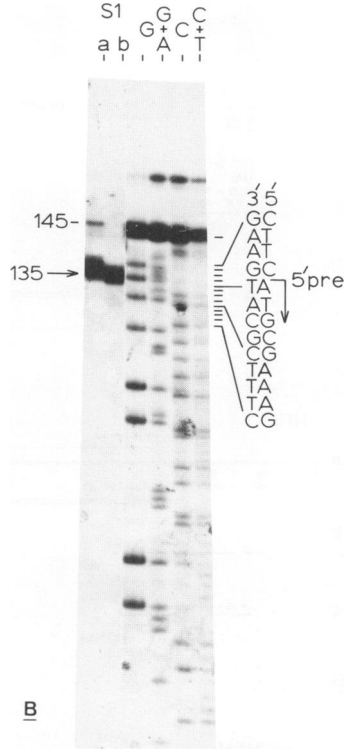




Fig. 2. Mapping of the transcription initiation site of the rRNA operon of K. lactis.
(A) This panel shows the fine map (a) of the 1280 bp long XbaI-generated fragment, indicating the location of the 17S rRNA gene, the external transcribed spacer (ETS) and the non-transcribed spacer (NTS), as well as the restriction enzyme sites for Sau3AI(S3), MspI (M) and RsaI (Rs). In addition, the pertinent DNA-fragment on which the 5'-end of 37S pre-rRNA was found (b) and the regions which have been sequenced (c) are indicated.
(B) Autoradiogram showing the results of the S1 nuclease mapping experiment. After hybridization of the strand-separated, $^{32}$P-end-labelled 145 bp long DNA-fragment (Sau3AxMspI) and high-molecular-weight RNA of K. lactis, the hybrids were digested with 150 U/ml (lane a) or 500 U/ml (lane b) S1 nuclease. The S1 nuclease-resistant DNA fragments were fractionated on an 8% (w/v) polyacrylamide gel containing 8 M urea (30). The sequence ladders prepared according to Maxam and Gilbert (30) from the same fragment are shown in the other lanes. The nucleotide sequence around the start site of pre-rRNA is given and the start site is indicated by 5'-pre.

method of Maxam and Gilbert (30). Sequencing products were analysed on 8% and 12% (w/v) polyacrylamide gels containing 8 M urea.

## RESULTS

### The transcription initiation region of Kluyveromyces lactis rDNA

To identify the region containing the transcription initiation site on K. lactis rDNA we performed Southern blot hybridization of digests of pKlR2 (see Fig. 1A), using as a probe the 730 bp long (SmaI-HindIII) fragment from the S. carlsbergensis rDNA unit, which spans the transcription start site from about -210 to +520. An analysis of heteroduplexed rDNA molecules of K. lactis and S. carlsbergensis had shown that a large part of the probe is homologous with sequences in the rDNA of K. lactis (results not shown). Fig. 1B and C show that in the XbaI plus EcoRI (lane 1) and in the XbaI plus BglII (lane 4)

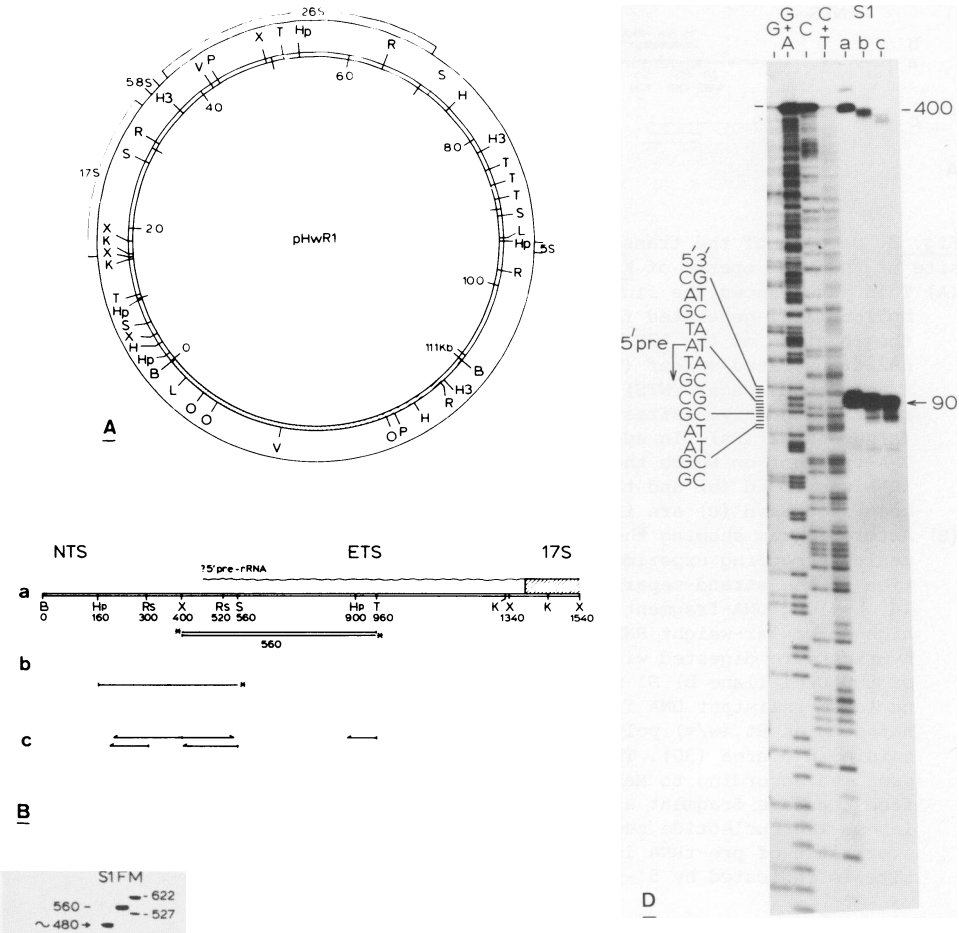



Fig. 3. Mapping of the transcription initiation site of the rRNA operon of H. wingei.

(A) Physical and genetic map of plasmid pHwR1, consisting of the rDNA unit of H. wingei (11.1 kb) inserted in the BamHI site of pBR322. The restriction enzyme sites are indicated as in Fig. 1A.

(B) The detailed map of the initiating region of H. wingei rDNA, including also some RsaI (Rs) sites, and showing the 5'-terminal part of the 17S rRNA gene, the external transcribed spacer (ETS) and the non-transcribed spacer (NTS). In addition, the DNA-fragments used for mapping of the 5'-end of 37S precursor rRNA, and the regions sequenced are indicated ((b) and (c), respectively).

(C) Autoradiogram showing the result of the mapping of the 5'-end of pre-rRNA by S1 nuclease analysis. Hybrids, obtained by hybridization of the strand-separated, $^{32}$P-end-labelled 560 bp long DNA fragment (XbaIxBglII) and high-molecular-weight RNA of H. wingei, were digested with S1 nuclease (1000 U/ml) in the presence of 10 µg

Bacillus RNA. The nuclease-resistant DNA fragments were fractionated (lane S1) on a 6% (w/v) polyacrylamide gel containing 8 M urea (30). The intact input DNA fragment (XbaIxBglII) was run in lane F. The products obtained by digestion of pBR322 with MspI were used as length markers in lane M.
(D) Autoradiogram showing the result of an S1 nuclease analysis using the strand-separated, $^{32}$P-end-labelled 400 bp long DNA-fragment (HpaIxSacI) as a probe. The amount of S1 nuclease used was 200, 700 and 2000 U/ml in lane a, b and c, respectively. The sequence ladders prepared according to Maxam and Gilbert (30) from the same fragment are shown in parallel. The sequence around the transcription start site is given and the start site is indicated by 5'-pre.

digests the sequences homologous with the probe are contained within a 1280 bp long XbaI-generated fragment, the map position of which is illustrated in Fig. 1A.

To establish the orientation of this XbaI-generated fragment with respect to the rRNA gene we constructed a detailed physical map (Fig. 2A) and subsequently sequenced both ends of the fragment. The nucleotide sequence (not shown) of the 150 bp long (Sau3A-XbaI) fragment (see Fig. 2A) was found to be almost identical with that at the 5'-end of the S. cerevisiae 17S rRNA gene (33). Therefore Fig. 2A shows the correct orientation of the 1280 bp long XbaI-generated fragment.

In order to locate the transcription start for the 37S pre-rRNA of K. lactis we performed an S1 nuclease experiment with a number of fragments of this XbaI-generated fragment hybridized to high-molecular-weight RNA of K. lactis. The protected hybrids were fractionated by gel electrophoresis and autoradiographed. Fig. 2B shows the result obtained with the 145 bp long Sau3A-MspI fragment (see Fig. 2A), $^{32}$P-labelled at its Sau3A 5'-end. Only one dominant protected fragment of about 135 bp is observed. To locate the 5'-end of the 37S pre-rRNA exactly we have to correct for the fact that the protected fragment migrates somewhat slower than the corresponding fragment in the sequence ladders obtained by the Maxam and Gilbert technique (see Ref. 13). Therefore, we place the 5'-end of the 37S pre-rRNA at the first A in the sequence 5'-CTTCATGCGAA-3'. We could not detect other protected products even when DNA fragments containing sequences upstream the assigned start position were used as a probe. The sequence around the presumed initiation site is identical with that around the single transcription start of the rRNA operon of S. carlsbergensis (8). From this analogy we conclude that the site mapped by S1 nuclease analysis is the true transcription initiation site of the K. lactis rRNA operon rather than a processing site.

We have determined the sequence from about 160 bp upstream to about 135 bp downstream of the transcription initiation site on K. lactis rDNA, as

shown in Fig. 4. This sequence has a striking homology with corresponding regions in S. carlsbergensis and S. rosei from position -11 to at least 130 bp downstream of the start site. On the other hand the NTS sequence of K. lactis, S. carlsbergensis and S. rosei have strongly diverged. Only a few short homologous stretches can be detected.

The transcription initiation region of Hansenula wingei rDNA

Preliminary analysis of heteroduplexed rDNA molecules of H. wingei and S. carlsbergensis suggested that the sequence conservation in the initiation region of the rRNA operon between these two species is confined to a short region of about 70 bp located about 300 bp upstream from the 5'-end of the 17S rRNA gene (results not shown). Since the ETS of the S. carlsbergensis rRNA is already 700 bp long we concluded that this conserved region is located within the ETS of the S. carlsbergensis rDNA and thus cannot be identical to the initiation region.

Southern blot hybridizations of digests of pHwR1, containing the complete rDNA unit (11.1 kb) of H. wingei (see Fig. 3A), using as a probe an S. carlsbergensis rDNA fragment reaching from 210 bp upstream to 520 bp downstream of the transcription start site, showed that a region of homology is present between the BglII site at position 960 and the XbaI site at position 1340, which is close to the 5'-end of the 17S rRNA gene. Since we could detect no difference in length between the 37S pre-rRNA of S. carlsbergensis and the presumed pre-rRNA of H. wingei (unpublished results) we expected the transcription initiation site of H. wingei rRNA operon to be located upstream from the BglII site at position 960 (see Fig. 3B) within a DNA region not hybridizing with an S. carlsbergensis rDNA probe.

We performed S1 nuclease mapping using the 560 bp long (BglII-XbaI) fragment (see Fig. 3B), $^{32}$P-labelled at its 5' ends, and high-molecular-weight RNA of H. wingei. Fig. 3C illustrates that there is only one dominant protected fragment of about 480 nucleotides. This result suggests that the transcription initiation site of the rRNA operon of H. wingei is located about 900 bp upstream from the 5'-end of the 17S rRNA gene (see Fig. 3B). For a more precise location of the initiation site(s) we performed an S1 nuclease experiment using the 400 bp long (HpaI-SacI) fragment (see Fig. 3B, line (b)), 5'-end-labelled at the SacI site. Only one dominant S1 nuclease-resistant fragment is visible in Fig. 3D, the 5'-end of which is located 90 bp from the SacI site. After correction (1.5 bp) for the difference in migration between the S1 nuclease-generated fragment and the corresponding fragment in the Maxam and Gilbert sequence analysis (13), the transcription initiation site of the

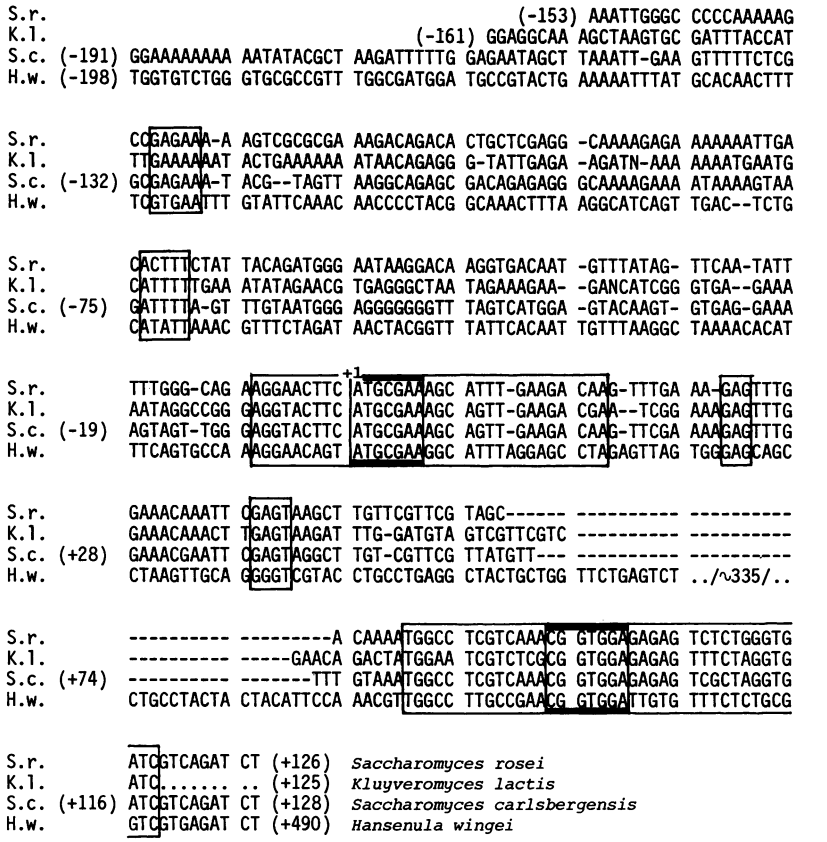



Fig. 4. Nucleotide sequence of the region surrounding the transcription initiation sites for pre-rRNA in S. carlsbergensis [(8); identical with S. cerevisiae (7)], S. rosei (26), K. lactis and H. wingei (this paper). Some small shifts (-) were introduced to increase homology at the aligned positions.
Sequences that are identical for at least 6 consecutive bp are qualified as 'highly conserved' and indicated by a box with thick, horizontal lines; sequences containing 3 or more bp identical out of 6 consecutive bp are described as 'moderately conserved' and indicated by fully thin-lined boxes.

rRNA operon in H. wingei can be mapped at the A, indicated by 5'-pre, in the sequence presented in Fig. 3D.

Fig. 4 shows the sequence of a 350 bp long fragment of H. wingei rDNA around the transcription start site (sequence strategy is indicated in Fig. 3B, line (c)), aligned with the corresponding sequences of three related yeast species. Fig. 4 shows that the sequences from position -155 to +70 contain one highly conserved region in addition to several short tracts of

moderate conservation. The highly conserved region surrounds the transcription initiation site from position -9 to +23 and comprises the perfectly conserved heptanucleotide ATGCGAA located at position +1 to +7. A similarly highly conserved region appears to be present downstream of position +460 of the H. wingei rDNA and downstream of position +80 of the other three yeast species.

## DISCUSSION

We wanted to identify promoter elements for yeast RNA polymerase A by comparing the appropriate sequences from a set of yeast species with increasing evolutionary distance. To this end we first determined the initiation site for transcription of the rRNA operon of K. lactis and H. wingei. The 5'-ends of pre-rRNA of these two species were mapped by S1 nuclease analysis at sequences identical to those identified as the 5'-terminal sequence of 37S pre-rRNA of S. carlsbergensis (8) and S. cerevisiae (7). Since it has been well-documented that the 37S pre-rRNA of S. carlsbergensis is a primary transcript of the rRNA operon (8,34) we feel confident that the sites identified in K. lactis and H. wingei represent sites of transcription initiation rather than of processing.

Sequence comparison of the initiating regions of the rRNA operons of S. rosei and S. carlsbergensis had previously shown an extensive homology downstream of position -9 into the ETS with a divergent region around position +75 whereas the homology in the NTS region is limited to a small number of short tracts (26). Comparison with the corresponding sequence of K. lactis, which is more distantly related to S. carlsbergensis than S. rosei (M.Ph. Verbeet et al., submitted for publication), also reveals a largely conserved region downstream of position -9 till far into the ETS. Therefore this region is clearly under evolutionary constraint. Likely candidates for components interacting with these sequences are RNA polymerase A, transcription factors and protein(s) assembling with the nascent pre-rRNA chain. Further comparison of this conserved region with the corresponding sequence of a still more distant species might pinpoint sequence elements that are more strongly conserved than other parts. Comparison with H. wingei rDNA indeed shows one region of striking sequence conservation between position -9 and +23, comprising an identical heptanucleotide sequence from position +1 to +7. We propose that this element is an important part of the promoter for yeast RNA polymerase A. An analogous sequence conservation around the transcription start site of the rRNA operon has been observed for two different groups of vertebrates (18,20) and by deletion analyses proven to be absolutely required

for correct initiation (21-25). These deletion analyses also revealed the presence of another promoter element(s) upstream of the conserved sequences. This latter element was not recognized as a conserved sequence, possibly because the species are either too closely related (the set of Xenopus species (18) and rat vs mouse (20)) or too different (human vs mouse and rat (15,16)). So far we have no data on the evolutionary distance of the four yeasts used in this comparison and on the species specificity of their RNA polymerase A promoters. If they are all species specific (part of) the relevant sequence constraint may be undetectable; if they are not it may be rewarding to screen the sequences for additional conserved features. Fig. 4 reveals two moderately conserved blocks at position -135 and -75 as possible candidates for an additional promoter element. Whether they contribute to the promoter function has to be tested by mutation analysis in an appropriate transcription assay system. Further comparison with more distant species like the slime mold Dictyostelium discoideum (35) and the protozoan Tetrahymena pyriformis (36,37) will not be useful. Recently Hoshikawa et al. (35) proposed a common, 9 bp long, element between these two species and S. cerevisiae but the position of this tract is very variable (positions +11, +20 and +40 respectively) and only 5 bases are conserved in the other yeast species studied in this paper.

The sequence of the ETS in H. wingei downstream from the conserved sequence around the transcription start is rather divergent. However, in the ETS of the H. wingei rRNA operon downstream from position +440 a sequence can be found which is strikingly homologous with a sequence downstream from position +80 in the ETS of the rRNA operon of the three other yeast strains. This implies that the H. wingei rDNA contains an "insertion" within the ETS of about 360 bp near position +75. It is remarkable that the sequences around this same position in the other three yeast species are also very divergent. In view of the large distance between the conserved sequence in the ETS and the start site of the H. wingei rRNA operon, it is very unlikely that this highly conserved ETS region has a function in transcription initiation. Rather we surmise that is plays some other role in ribosome biosynthesis.

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