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The 5' terminus of the precursor ribosomal RNA of Saccharomyces cerevisiae

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ABSTRACT

The 5' terminus of Saccharomyces cerevisiae 35S pre rRNA was mapped on the rDNA using two methods: 1) Suitable restriction endonuclease fragments were hybridized to total high molecular weight RNA and extended with reverse transcriptase to the 5' end of the RNA template. 2) Other restriction fragments spanning the 5' terminus of 35S pre rRNA and radioactively labeled at their ends were hybridized to high molecular weight RNA and the non hybridized nucleic acids were digested with S1 nuclease. On the basis of these experiments, the 5' terminus of 35S pre rRNA was placed approximately 670 nucleotides upstream from the 17S rRNA coding region. The exact position was determined by reverse transcription as above, but in the presence of dideoxyribonucleoside triphosphates, which served as a way of sequencing the 5' terminal region. 35S pre rRNA synthesis is initiated at a site in EcoRI restriction fragment B which is 48 nucleotides upstream from the EcoRI cleavage site in the coding strand.

INTRODUCTION

Approximately 140 tandemly repeated genes code for ribosomal RNA in the yeast Saccharomyces cerevisiae. Each repeat contains one unit for the synthesis of 5S RNA and one for the synthesis of large precursor RNA which is processed to yield 25S, 17S and 5.8S rRNA. The longest precursor rRNA that could be detected has a sedimentation constant of 35S (7, 26) and it contains a triphosphate group at its 5' terminus (15). It thus most likely represents the primary transcription product.

The ribosomal genes have been cloned (14, 19, 20) and extensively analysed. Transcription of a single 35S pre rRNA molecule proceeds in the sequence 5'-17S-5.8S-25S-3' (8, 14, 20) (see Fig. 1). 5S and large ribosomal genes are interspersed and are transcribed in opposite directions.

We were interested in precisely localizing the site at which transcription of the 35S pre rRNA is initiated. A long stretch of DNA upstream from the 17S rRNA coding region, which is expected to contain the site at which initiation of transcription occurs, has been sequenced (27). No striking sequence homologies could be found, however, between this sequence and the

analogous region in Xenopus laevis DNA, the only genome for which the site of initiation of pre rRNA synthesis is known (24). The DNA sequence thus does not, by itself, tell us where the initiation site is located. In this report we locate the probable transcription initiation site by mapping the 5' end of S. cerevisiae 35S pre rRNA on the rDNA.

MATERIALS AND METHODS

Growth of cells and RNA purification. The diploid S. cerevisiae strain M25 (29) was grown at 30°C in MV-A minimal medium (11) containing 2% glucose to a density of 10^7 cells/ml. Labelling was done at that density for 5 min with 50 μCi/ml [^{3}H] uracil (23 Ci/mmol; New England Nuclear, Boston, MA). Cells were concentrated by centrifugation, washed and resuspended in a small volume of extraction buffer (10 mM Na acetate pH 5.2, 10 mM EDTA, 100 mM NaCl, 1 mM aurin tricarboxylic acid). Glass beads (0.45 mm) were added to the suspension and the cells were broken by agitating on a vortex mixer for 1.5 min. The RNA was extracted from the cleared supernatant with phenol-chloroform (12) and precipitated with ethanol. Two mg of RNA were loaded onto a 38 ml linear 10-30% sucrose gradient in 100 mM Tris acetate, pH 7.4, 10 mM EDTA, 0.1% sodium dodecylsulfate and centrifuged in a Beckman SW 27 rotor for 19 hr at 26,000 rpm at 4°C. RNA bigger than 25S was pooled, precipitated with, and stored in, ethanol. This RNA, which is about 20-fold enriched in 35S pre rRNA, is referred to as high molecular weight RNA. For analytical purposes RNA was glyoxylated and separated on 1.2% agarose gels according to the procedure described by McMaster and Carmichael (16). The gel was soaked in acetone for one hour with one change of this solvent and subsequently in a solution of 5% PPO in acetone for 2 h. The PPO in the gel was precipitated with water (3). The gel was dried and exposed to X-Omat XR5 film (Kodak).

Isolation of restriction fragments. The plasmid G12 containing part of the ribosomal repeat cloned in pMB9 (18) (see Fig. 1) was generously provided by T. Petes. It was purified by preparing a cleared supernatant as described by Clewell and Helinski (5) followed by two rounds of banding in CsCl-ethidium bromide gradients. Restriction endonucleases were obtained from New England Biolabs, Inc. (Beverley, MA) and BRL, Inc. (Rockville, MD) and restrictions were performed according to the recommendations of the manufacturers. Restriction fragments were electrophoretically separated on agarose gels run in E buffer (40 mM Tris acetate, pH 7.9, 20 mM Na acetate, 1 mM EDTA). The DNA fragments were visualized by staining with ethidium bromide and cut out of the gel. Gel pieces were fragmented and embedded with agarose in a tube and

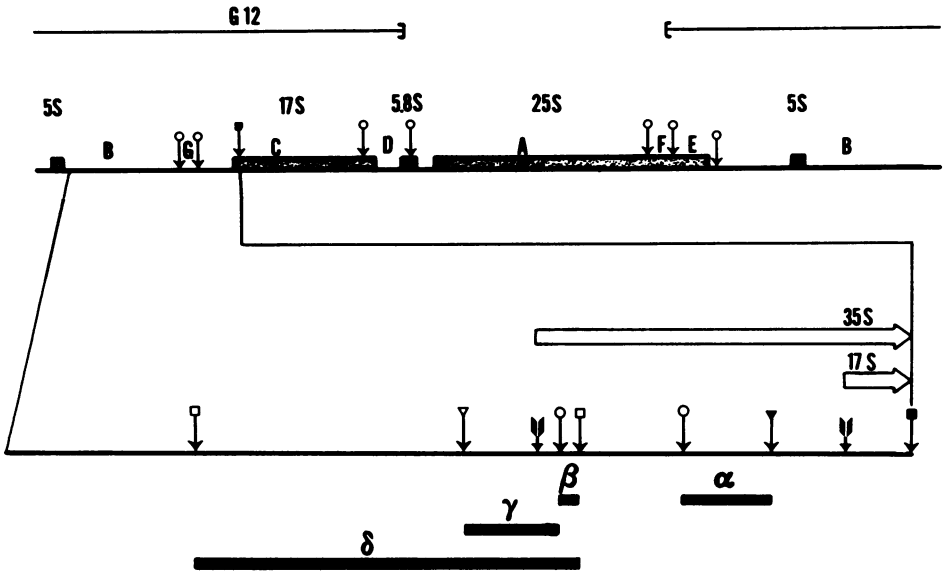

Fig. 1. The yeast ribosomal repeat. The fragments created by EcoRI cleavage (O) are named A through G following Philippsen et al. (20). The top line indicates the regions of the ribosomal repeat contained in the plasmid G12 (18, and S. Fuhrman, personal communication). The bottom line represents the DNA region around the transcription initiation site. Restriction endonuclease fragments α, β, and γ were used as primers to be extended by reverse transcriptase using 35S pre rRNA as template. Fragment δ was used in the S1 protection experiment. Restriction endonuclease cleavage sites: Alu I (▽), Hae III (□), Hind III (▼), Eco RI (○), Xba I (■). ↓ indicates the 5' ends of 35S pre rRNA and 17S rRNA.

overlaid with a small quantity of DE 52 cellulose (Whatman). The tube was placed in a tube gel apparatus and the restriction fragments were electrophoresed into the DE 52 cellulose. Subsequently the latter was extensively washed with E buffer and the DNA was eluted with 1 M LiCl, precipitated with ethanol and redissolved in the appropriate buffer for subsequent manipulations.

Primer extension and sequencing. Three pmoles of restriction fragments were hybridized to high molecular weight RNA extracted from 1.5 x 10^8 cells in 30 μl 70% deionized formamide, containing 400 mM NaCl and 10 mM Pipes, pH 6.4 at 52°C for 16 hr (4). The hybrid was diluted ten times in 10 mM Tris HCl, pH 7.5 and precipitated with isopropanol (Isopropanol precipitation was chosen to avoid precipitating EDTA along with the nucleic acids). Reverse transcription

was done in 10 µl of 50 mM Tris HCl, pH 8.3, 60 mM NaCl, 6 mM Mg acetate, 20 mM dithiothreitol, 1 mM each dATP, dGTP, dTTP, 100 µg/ml actinomycin D, containing 1 µl AMV reverse transcriptase (the generous gift of I. Verma), 5 µCi [α-^{32}P]-dCTP (400 Ci/mmol, NEN) and 10% of the recovered hybridization mixture. After incubation at 42°C for 30 min. 1 µl of cold 6 mM dCTP was added and the reaction was continued for 20 min. The RNA was digested by adding NaOH to a concentration of 100 mM and boiling for 3 min. After neutralization with HCl, tRNA was added as carrier and the nucleic acids were ethanol precipitated. For the terminator sequencing procedure (23), the conditions used were as for the above described primer extension reaction with the following changes: the concentration of each of the two driver deoxynucleoside triphosphate (neither labeled nor in competition with the dideoxynucleoside triphosphate) was 10 µM, the concentration of the deoxynucleoside triphosphate which was competed for by the corresponding dideoxynucleoside triphosphate was 5 µM, ddATP and ddGTP were 20 µM, ddCTP and ddTTP, 15 µM, respectively. In order to determine the penultimate nucleotide, the ddATP concentration was increased to 50 µM. The label was [α-^{32}P]-dCTP except in the reaction with ddCTP, where it was [α-^{32}P]-dGPT.

S1 protection experiments. Restriction fragments were dephosphorylated in 20 µl of a buffer containing 10 mM Tris HCl, pH 9.5, 1 mM Spermidine, 0.1 mM EDTA, using 0.3 units of calf intestinal alkaline phosphatase (Boehringer) per pmol of 5' ends. The enzyme was inactivated by boiling for 3 min. The kinase reaction was done in 40 µl of a buffer containing 50 mM Tris HCl, pH 9.5, 10 mM $MgCl_2$, 5 mM dithiothreitol with a 3 fold excess of [γ-^{32}P]-ATP (7000 Ci/mmol; ICN, Irvine, CA) over 5' ends and 30 units of T4 polynucleotide kinase (prepared by S. Brennan) per pmol of 5' ends. Hybridization of kinase-labeled DNA to high molecular weight RNA and S1 digestion was done as described by Berk and Sharp (1). Analysis of all DNA was done on 0.3 mm thick polyacrylamide gels as described by Sanger and Coulson (22).

RESULTS

1) The size of 35S pre rRNA. The aggregate length of 25S, 17S and 5.8S RNA together with their intervening transcribed spacer regions is 5850 nucleotides (20). We determined the size of the 35S pre rRNA in a first experiment in order to obtain an idea of how long the external transcribed spacer is. Most of the external transcribed spacer is expected to be located upstream from the 17S rRNA coding region since it has been found that the 25S rRNA coding region is closely followed by a very AT rich sequence most likely

representing the terminator of transcription (27). The size of 35S pre rRNA would thus give an indication of where its 5' terminus is located. Unfortunately, published size estimates of 35S pre rRNA vary between 2.8 x 10^6 (7) and 2.5 x 10^6 (26), and none of these size estimates is based on analysis of RNA under fully denaturing conditions. If 2.8 x 10^6 were the correct molecular weight one would have to assume that the promoter for the large ribosomal unit is located upstream from the 5S gene which then would be transcribed in both orientations.

Total yeast RNA, pulse labeled during 5 min with tritiated uracil was fractionated on a linear 10-30% sucrose gradient, portions of different regions of the gradient were denatured with glyoxal and run on an agarose gel. The fluorogram, shown in Fig. 2, reveals that: 1) there exist two high molecular weight RNAs which are rapidly labeled (Fig. 2B, lane a) and which probably are both precursor rRNAs as results of kinetic experiments suggest (data not shown); 2) the molecular weights of the two high molecular weight precursors are approximately 2 x 10^6 and 1.8 x 10^6 daltons, taking the yeast 25S and 17S rRNA as molecular weight standards and 3) there are two immediate precursors to 25S rRNA (Fig. 2B, lane b) and one to 17S rRNA (Fig. 2B, lane c).

The longer one of the two high molecular weight precursors contains approximately 700-800 nucleotides of external transcribed spacer. If all of this extra sequence is located upstream from 17S RNA, as we assume, the site of transcriptional initiation is expected to be located near the Eco RI site that separates fragment B from fragment G (see Fig. 1).

The question of whether the smaller one of the 2 high molecular weight precursors represents a processing intermediate or a primary transcript initiated at a different promoter is being investigated.

2) Mapping of the 5' end of the largest precursor rRNA. The following strategy was chosen for the mapping of the 5' end of 35S pre rRNA. A 200 nucleotide long EcoRI-HindIII restriction fragment (fragment α, Fig. 1) which is expected to map downstream from the 5' end of 35S pre rRNA was hybridized to high molecular weight yeast RNA (see Materials and Methods). The restriction fragment served as the primer for extension by reverse transcriptase, hopefully to the 5' end of the template 35S pre rRNA. As it turned out, many different extension products were produced (Fig. 3, lane b) probably due to retardation and release of the polymerase at secondary structures of the template RNA. The longest extension product was approximately 510 nucleotides long and its 3' end maps in EcoRI fragment B, not far away from fragment G. The 40 nucleotide long EcoRI-HaeIII restriction fragment (fragment β, Fig. 1)

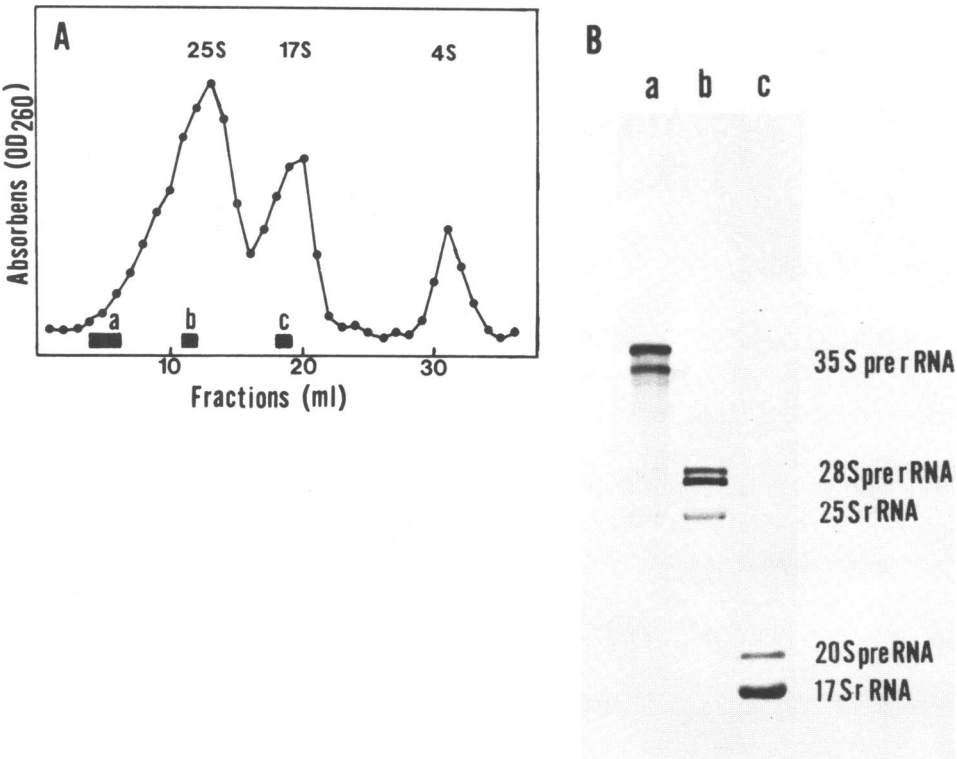

Fig. 2. Determination of the size of 35S pre rRNA. Total yeast RNA, pulse labeled for 5 min with [^{3}H]-uracil was fractionated on a 10-30% sucrose gradient (panel A). Portions of the regions indicated by bars were glyoxylated and electrophoresed on a 1.2% agarose gel. The fluorogram of the gel is shown in panel B. The molecular weights of 17S and 25S rRNA are 0.53 and 1.03 x 10^6 according to Philippsen et al. (20).

which maps downstream from that site was hybridized to high molecular weight RNA and extended by reverse transcriptase. The extension product was approximately 50 nucleotides longer than the primer (Fig. 3, lane e). The AluI-EcoRI restriction fragment (fragment γ, Fig. 1) could not be extended (result not shown). The result of this experiment suggests that the 5' terminus of the 35S pre rRNA maps approximately 50 nucleotides upstream from the EcoRI fragment G. But it is also possible that the longest extension product in that experiment is due to a very extensive secondary structure of the template RNA which results in premature termination of transcription.

We chose an S1 protection experiment as a second way to look for the 5'

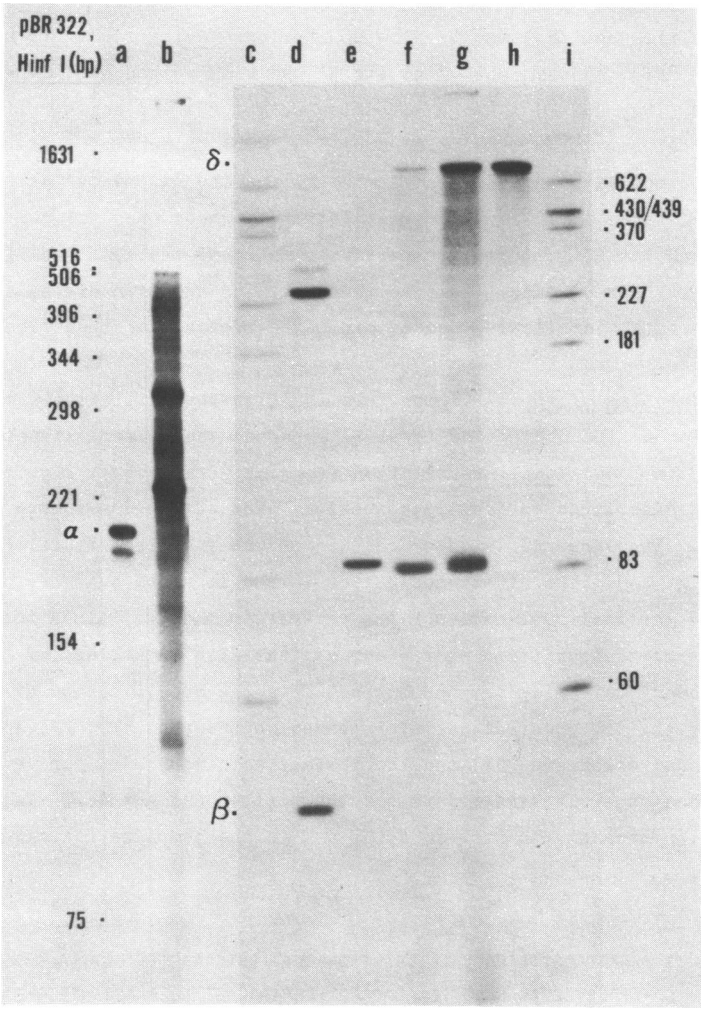

Fig. 3. Mapping of the 5' end of 35S pre rRNA. Lane a: kinase labeled restriction fragment α; lane b: fragment α hybridized to 35S pre rRNA and extended by reverse transcriptase; lanes c and i: molecular weight markers (pBR322, Hae II digested); lane d: kinase labeled EcoRI restriction fragment G, HaeIII digested (lower band = β); lane e: fragment β hybridized to 35S pre rRNA and extended by reverse transcriptase; lane f: kinase labeled restriction fragment δ, hybridized to 35S pre rRNA followed by digestion with 50 units S1 nuclease; lane g: as lane f but with 20 units S1 nuclease; lane h: kinase labeled fragment δ.

terminus of 35S pre rRNA because, with this method, RNA secondary structure should not influence the result. The 800 bp long HaeIII restriction fragment (fragment δ, Fig. 1) was labeled at its 5' end with polynucleotide kinase and [γ-^{32}P]-ATP, hybridized to high molecular weight RNA and non-hybridized nucleic acids were digested with S1 nuclease. The product of this reaction has the same mobility on a polyacrylamide gel as the extended fragment (compare Fig. 3, lanes e and f). The restriction fragment used for the primer extension and the S1 protection experiments both had the same HaeIII restriction site at their 5' ends. Reverse transcription and S1 nuclease digestion must have created identical 3' ends, since the products of these reactions are equal in size.

3) Sequencing of the 5' region. To localize the initiation site for transcription at the nucleotide level we repeated the primer extension reaction using the EcoRI/HaeIII restriction fragment (fragment β, Fig. 1) in the presence of dideoxynucleoside triphosphates. The obtained sequence is shown in Fig. 4B. The sequence is identical to the one published by Valenzuela et al. (27, Fig. 4A). The dideoxynucleotide method locates, but does not identify the 3' terminal nucleotide of the reverse transcript, since termination of the reverse transcription must occur at that site regardless of the presence of dideoxynucleoside triphosphates. Thus the specification of the 5' terminus of the 35S pre rRNA, as A, is based on the published DNA sequence of Valenzuela and coworkers (27) and the perfect coincidence of our reverse transcription sequence with their data for the next 43 nucleotides. Initiation of transcription thus starts with an A (at a T in the template DNA sequence)

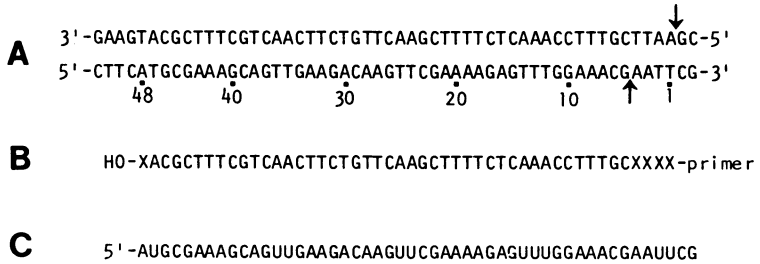

Fig. 4. A: Sequence of the DNA upstream from the EcoRI site which separates fragments B and G, determined by Valenzuela et al. (27). The EcoRI site is indicated by arrows. B: Sequence of the DNA complementary to the 5' terminal region of 35S pre rRNA. X indicates nucleotides which were not determined. C: The deduced 35S pre rRNA sequence.

which is 48 nucleotides upstream from the EcoRI site in the coding strand.

DISCUSSION

This report describes the mapping of the 5' terminus of the longer 35S precursor rRNA onto rDNA. Veldman and coworkers have also investigated this question (28). Two independent methods were used in our work: reverse transcription from the 3' hydroxyl end of a DNA primer to the 5' end of the 35S pre rRNA template; protection from digestion with S1 nuclease by hybridization with a DNA fragment spanning the 35S pre rRNA 5' terminus. With both methods the conclusion was reached that the 5' end of 35S pre rRNA maps in EcoRI fragment B approximately 50 nucleotides away from the EcoRI restriction fragment G. Sequencing of the extension product maps the initiating nucleotide to position 48 from the EcoRI B/G site. 35S pre rRNA of S. cerevisiae was shown to start with an A or G (17). The precursor RNA used in these studies was isolated on sucrose gradients and no separation of the two high molecular weight precursors was obtained. It is conceivable that both the longer and the smaller high molecular weight precursors are primary transcripts and that they start with an A and a G respectively. We are currently analysing this problem. The methods used in the present study only identify the farthest upstream end of a transcript. They do not distinguish downstream promoters from sites of RNA processing. It is, however, clear from the S1 protection experiments described here and another one using EcoRI fragment G (data not shown) that the initiation site we have mapped is not closely followed by another one as has been described for the ribosomal genes in E. coli (6, 9, 10, 30).

The 5' sequence of 35S pre rRNA isolated from S. carlsbergiensis has been shown to start with an A followed by a U (15). The same 5' dinucleotide was found in our study.

35S pre rRNA was shown to contain a polyphosphate terminus and we therefore assume that this RNA represents a primary transcript. The possibility cannot, however, be excluded that an enzyme activity, which phosphorylates 5' monophosphates as described for vaccinia virus (25) also exists in yeast. Experiments are being done to investigate that point.

The site at which RNA polymerase I of Xenopus laevis initiates transcription of large ribosomal RNA has been described recently (24). We cannot detect any resemblance between that sequence and the one described in this report. Perhaps common features among different RNA polymerase I initiation sites will be detected when more extensive comparisons, including more closely

related organisms, become available. It is conceivable that the sequences recognized by the proteins involved in initiation of ribosomal transcription have greatly diverged in very different organisms. Alternatively, the control of ribosomal transcription could be asserted at sites that are located considerably up or downstream from the initiating nucleotide (2, 21).

Our assumption that most of the external transcribed spacer is derived upstream from the 17S rRNA coding region appears to be correct. There is an extra sequence of approximately 670 nucleotides at the 5' end of 35S pre rRNA which corresponds well to the difference in size between 35S pre rRNA on the one hand and the sum of the mature ribosomal RNA species together with their internal transcribed spacer regions.

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