Localization and DNA sequence around the initiation site of ribosomal RNA transcription in *Physarum polycephalum*

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ABSTRACT

We have used S1 nuclease to map the initiation site of ribosomal RNA transcription in the acellular slime mold *Physarum polycephalum*, and we have determined the sequence of 1011 nucleotides surrounding the start site. Consistent with others' observations, there is little homology with the comparable region of other species. As predicted by previous restriction mapping, direct repeats roughly 30 base pairs in length are present upstream of the initiation site and a 148 base pair duplication occurs in the external transcribed spacer. The results also suggest the presence of a processing site within the external transcribed spacer of the ribosomal transcription unit.

INTRODUCTION

Transcription of nuclear genes in eukaryotic cells is effected by three different RNA polymerases. The nucleolar RNA polymerase I transcribes the DNA (rDNA) coding for 18S, 28S and 5.8S ribosomal RNAs; RNA polymerase II transcribes the genes coding for proteins, and RNA polymerase III transcribes DNA coding for small RNAs. For all three polymerases, the control of initiation is probably an important element in the overall regulation of transcription. In the case of polymerase II, several conserved sets of nucleotide sequences have been implicated in the precise definition of the transcriptional start, and a region of up to several hundred base pairs upstream from the actual start of transcription appears to be functionally important (1,2). In contrast, signals governing the activity of RNA polymerase III reside within the genes (3,4,5).

Much less is known about the signals necessary for correct transcriptional initiation of the ribosomal RNA genes by RNA polymerase I. In vitro systems able to faithfully transcribe rRNA genes have been developed only recently and, in marked contrast to polymerase II in vitro systems, are capable of transcribing only homologous DNA, i.e. DNA from the same species as the RNA polymerase-containing extract (6,7,8). The DNA sequences around the

initiation site for rRNA transcription have been determined for a number of organisms (9-23), and recent studies have functionally defined a ribosomal RNA promoter region (24,25,26,26a). So far, no regulatory sequences have been exactly defined. In Drosophila melanogaster, some ribosomal RNA genes contain interrupted sequences. These interrupted genes are not transcribed, although when compared with the normally transcribed, uninterrupted genes, they have identical nucleotide sequences around their initiation sites (21). These findings suggest that segments of the gene other than those immediately surrounding the initiation site might also be important for the onset of transcription.

We are investigating the structure and function of the ribosomal RNA genes of the acellular slime mold Physarum polycephalum. This organism offers particular advantages in that its plasmodia, containing $10^8 - 10^9$ nuclei each, proceed through the cell cycle with a high degree of natural synchrony, which facilitates an investigation of the regulation of ribosomal RNA transcription throughout the cell cycle (27). Each diploid nucleus of Physarum contains about 200 copies of the ribosomal RNA genes. These are located on linear, extrachromosomal DNA molecules of 60 kilobases (kb) in length (28,29). They are transcribed into precursor molecules of 13.4 kb in length, which are subsequently processed via several intermediate steps into the mature rRNA species (30). The site of initiation of transcription has been tentatively located by several techniques (30,31,32) at a position just downstream from a conspicuous stretch of highly reiterated DNA (33). The DNA coding for 26S RNA contains two introns, which are transcribed into the precursor rRNA and later excised from it (30,34). Termination of transcription appears to be coincident with or close to the 3' end of the mature 26S rRNA (30,35). Large portions of Physarum rDNA have recently been sequenced (35-39).

In the present communication, we report the results of S1 nuclease protection experiments to localize the initiation site of transcription, and we present the DNA sequence surrounding it. The results of the S1 experiments also corroborate the presence of a processing site 1.6 kb downstream from the initiation site.

MATERIALS AND METHODS

Growth of cells and isolation of total or nuclear RNA

The Physarum strain M₃C VIII was grown as microplasmodia in shake flasks at 25°C in N+C medium (40). Only cultures in the early exponential phase (1 ml of packed cells per 100 ml culture) were taken for the isolation of RNA.

Total plasmodial RNA was isolated from whole plasmodia as described previously (30). For the preparation of nuclear RNA, nuclei were isolated from surface plasmodia by the Mohberg procedure (41) with the modification that 0.1% diethylpyrocarbonate was added to every solution.

Isolation of restriction fragments

Plasmid pPHR103 contains a SalI fragment from within the external transcribed spacer (ETS) of the rRNA transcription unit from Physarum polycephalum strain M₃C inserted into pBR322 (Amstutz, personal communication). The construction of plasmid pPHR11.3 (also from strain M₃C) has been described (33). Plasmid pPHR25 contains the BclI fragment spanning the transcription start site from strain ATCC 24466 inserted into the BamHI site of pBR322. The locations of all three plasmids are indicated in Figure 1.

Plasmid DNA was isolated from cleared lysates by ethidium bromide-CsCl centrifugation. Restriction fragments were electrophoretically separated on agarose gels. After visualization by ethidium bromide staining, the fragments were recovered by electrophoresis into DEAE cellulose. The cellulose slurry was subsequently transferred to a small column and was extensively washed with low salt buffer (0.15 M NaCl, 50 mM Tris HCl pH 8.0, 1 mM EDTA). Finally, DNA was eluted from the column with high salt buffer (1 M NaCl, 50 mM Tris HCl pH 8.0, 1 mM EDTA) and precipitated with ethanol.

S1-mapping

S1-mapping experiments were performed exactly as described (42). For a typical S1 assay, 5,000 cpm of labeled DNA (specific activity 10^5 cpm/ μ g, labeled at the 5' end with polynucleotide kinase) and 10 μ g of RNA were used. S1-resistant DNA fragments were size-fractionated on alkaline agarose gels or on polyacrylamide gels containing urea. The units for S1 are those given by the manufacturer; one unit degrades 1 μ g of denatured DNA in 30 min at 37°C.

Sequencing

DNA sequencing was performed using the chemical cleavage method (43 as modified by 44). DNA was labeled either at the 3' end by filling in a 5' protruding sticky end using reverse transcriptase and the appropriate α - 32 P-dNTP (44), or at the 5' end using polynucleotide kinase and γ - 32 P-ATP (according to the manufacturer's instructions) after removal of the 5' phosphate with calf alkaline phosphatase (prepared as in 45). The sequencing strategy is shown in Figure 1.

Sources of enzymes and isotopes

Restriction enzymes and polynucleotide kinase were obtained from New

Figure 1

Restriction map of rDNA and the sequencing strategy

The upper part of the figure shows half of the palindromic rDNA from *Physarum polycephalum* strain ATCC 24466. The primary transcript is drawn as a heavy line, with the final rRNA products shown as solid blocks. The open blocks indicate the positions of the two introns in the 26S gene. Transcription proceeds to the right. The arrow indicates the center of the rDNA, the open triangle marks the location of a processing site in the external transcribed spacer. The regions contained within the various plasmids are indicated, as are the positions of restriction sites for *Bcl*I (■) and *Xho*I (○). The lower part shows a detailed restriction map of the section of pPHR25 (24466) and pPHR11.3 (M₃C) that was sequenced (the adjoining pBR322 DNA is shown as a dotted line), and the strategy used to determine the sequence. The fragments used for sequencing extend from an asterisk (3' labeled) or open circle (5' labeled) to an arrowhead, with the section that gave useful data indicated as a solid black line. The three fragments labeled at the *Xho*I site are shown below the *Taq*I map (*Xho*I sites are marked X, the *Sal*I site is marked S). A large arrowhead marks the transcription start site.

England Biolabs, Boehringer Mannheim and BRL. Reverse transcriptase was a gift from J.W. Beard, Life Sciences, Inc. α -³²P-dNTP (3000 Ci/mmol) and γ -³²P-ATP (3000 Ci/mmol) were obtained from Amersham. Calf alkaline phosphatase (Grade I, suspension in ammonium sulfate) and S1 nuclease were from Boehringer Mannheim.

Figure 2

S1-mapping using pPHR103

The insert (2.1 kb) of pPHR103 was isolated and labeled at its 5' ends. It was then hybridized to total plasmodial RNA. After S1 digestion, DNA was sized on an alkaline agarose gel. The autoradiogram of the dried gel is shown.

HindIII-digested λ DNA was used as a marker.

(a) 1000 units of S1, (b) no S1, (c) as in (a), but no RNA present during hybridization.

RESULTS

Identifying an rRNA processing site

A tentative location of the initiation site of ribosomal transcription was obtained earlier by several techniques other than S1-mapping (30,31,32). The results from these experiments suggested that transcription might start within the rDNA fragment of plasmid pPHR103 (Fig. 1). Therefore, we decided to use this fragment for a first S1-mapping experiment. After being labeled at its 5' ends with polynucleotide kinase, the fragment was hybridized to total plasmodial RNA. The reaction mixture was then treated with various concentrations of the single strand-specific nuclease S1. Finally, the S1-resistant DNA that had been protected by RNA was size-fractionated on a denaturing agarose gel (Fig. 2).

Panel a of Figure 2 shows two discrete bands of radioactive DNA generated by the S1 digestion. The more slowly migrating band corresponds to the completely protected fragment (2.1 kb), showing that the start of transcription is beyond the boundary of this fragment. The second S1-resistant band, 0.7 kb in size, is absent without S1 treatment (panel 2b) or without RNA during hybridization (panel 2c). Thus, it appears to result from protection by a subpopulation of RNA molecules that are shorter than the primary transcript. We have previously described ribosomal RNA precursors of

Figure 3

S1-mapping using pPHR11.3

The insert (3.1 kb) of pPHR11.3 was isolated, labeled at its 5' end and hybridized to total plasmodial RNA.

After S1 digestion, DNA was sized on an alkaline agarose gel. The autoradiogram of the dried gel is shown.

HindIII-digested λ DNA and HpaII-digested pBR322 DNA were used as markers.

(a) No S1 added, (b) 1000 units of S1, (c) 5000 units of S1, (d) as in (c), but no RNA present during hybridization.

this size (30), which we interpret to arise from a processing step that cleaves a 5'-terminal fragment off the primary transcript. The location of this presumptive processing site corresponds to the position of the site detected by S1-mapping. However, neither the S1-mapping nor the direct length measurements of RNA molecules (30) distinguishes between a processing site and a secondary transcription initiation site. It is also possible that the 0.7 kb protected fragment is generated by S1 cleavage at a run of AT residues in the DNA, although this would not explain the previously observed molecules.

Identifying the transcription start site

The experiments described above suggest that the initiation of transcription takes place upstream from pPHR103. Hence, the S1-mapping was repeated with a DNA fragment that partly overlaps pPHR103, but extends a further 2.9 kb upstream (plasmid pPHR11.3; see Fig. 1). The patterns of S1-resistant DNA are displayed in Figure 3. Panel a, representing a control hybridization without S1 digestion, shows the position of the full length fragment (3.1 kb). Panels b and c display the band pattern obtained after digestion of the hybrids with different concentrations of S1 nuclease. A single DNA band 0.5 kb in length is clearly visible, while no bands of lower mobility are detectable, even when the gels are overexposed. This result

Figure 4

A) Localization of the initiation site of transcription

The 5'-labeled coding strand of the 120 bp AluI-TaqI fragment containing the initiation site was hybridized to nuclear RNA, digested with different concentrations of S1-nuclease and electrophoresed on a sequencing gel. (b) no S1 added, (c) 1000 units S1, (d) 10,000 units, (e) 40,000 units, (a) as in (e) but no RNA present during hybridization. The same single strand was exposed to different sequencing reactions and the resulting cleavage products were run in parallel on the same gel. (f) C reaction, (g) C+T reaction, (h) A+G reaction, (i) G reaction. Only the relevant part of the gel is shown.

B) Nucleotide sequence surrounding the initiation site of transcription.

suggests that the observed single band reflects the start of transcription on the pPHR11.3 fragment.

Recent S1 experiments with mouse rDNA have suggested that AU-rich DNA-RNA hybrids, even if they form perfectly double-stranded structures, can be attacked by S1 nuclease, when the digestion is performed under our standard conditions at 45°(6). Therefore, we have also performed S1 digestions at room temperature. The results obtained were identical to those presented in Figure 3 in all cases. When isolated nuclear RNA, instead of total cellular RNA, was

T
 AAAAAATTTTCGGCTCTCA..GTTAAACGGGGCG ⁻³⁵⁰ Map I
 **..AAAAATTTTCGGCTCTCA..GTTAAACGGGGCG ^{Map I}
 **..AAAAATTTTCGGCTCTCA..GTTAAACGGGGCG ⁻³⁰⁰ Map I
 AAAAAATTTT.CCGGCTCTCA..GTTAAACGGGGCG ^{Map I}
 AAAAAATTTT.CCGGCTCTCA..GTTAAACGGGGCG
 ..AAAAATTTTCGGCTCTCAAAACATACGGGA... ^{**}
 AATTCGATTTTCGGCTCTCA..ATTAAACGGGG... ^{****}
 ****..AAATTTTCGC.....CATAGATTTTTCGCAAAACCTAAGTCAAAAAATCTATCGGAAGCTTTTGGCCCGCTCTCAACATAACGGGAAAAATTTTTCGC ⁻¹⁵⁰
CCGCTCTCA..TTTAAACGGGGCG ^{Map I}
 AAAAAATATTTT.....TCAGCGCGCATAAATCCGGCGCACCGCTATGCTTTTAAAGAGAAACCCAGAGATACATATAGGGGGGGTATGAGG ⁻⁵⁰ Alu I
 TGTGGCAGCGCTCATACAGGTACGGGAAATCGCTCTTATCGCGGTTCTGACACGGTCAGATCGCGCTCGACACGGTAAACGCTGTTTTCACCTTTGCCACGGC ⁵⁰ Tsp I
GTATAGGGGAGCCCGCTTTTCAAAAATTTTCAAGCGCGCAACCGGATTACAGCTGTG ¹⁵⁰ Ava II
 ATACGCAACGCCCTGATAGGGCGCCCGCTTTTTCAAAAATTTTCGGCGGGTGACTTGAAGACGGGCGAACGCCATGGCGAACGCCGAAATCTTGGAGAGTTCGAGCTGGGGCGGAAACGGTAGACACACGCCCTGATGCTGCTCGCGGTGCGGAA ²⁰⁰ Tsp I
 ATAGCCAATGCCCTGTGTAGGGCGCCCGCTTTTTCAAAAATTTTCGGCGGGTGACTTGCAGACGGCGCGAACGCCATGGCGAACGCCGAAATCTTGGAGAGTTCGAGCTGGGGCGAAGACGGTAGACACACGCCGGAAGAGTGGTCCCGTGTGGGA ³⁰⁰ Tsp I Alu I
 TCGACCGGTATACACGGCGAGTCAACCAACGTCGTGGGGAAGCGCGCTCTCGCTGGAAAAAACCGGACCAACGCGTGGGGTG ³⁵⁰ Xho I
 BstE II Map I ⁴⁰⁰ Map I ⁴⁵⁰ Map I
 CGTAAACGGGCTTGGGCAACCCGGTTAGCTGGGCAATTATGGCGGGCAAAAGTCCGGCTGGGTGGGCGGCAACCGGCTTGATCA ⁵⁰⁰ Bcl I
 GCAACCGGCTTATACACGGCGAGTCAACCAACGTCGTGGGGAAGCGCGCTCTCGCTGGAAAAAACCGGACCAACGCGTGGGGTG ⁵⁵⁰

used for hybridization, again no difference was found in the pattern of the S1-resistant DNA (data not shown).

The results presented so far localize the initiation site of transcription on the pPHR11.3 fragment. A more precise mapping of this site was obtained by a further S1 experiment, using a DNA fragment extending from the TaqI site immediately downstream from the suspected site of initiation to 2.5 kb upstream from it. Sizing of the S1-resistant fragment localized the 5' end of the primary transcript to 90 ± 10 base pairs (bp) from the rightward TaqI site (results not shown).

Sequencing and exact position of the initiation site

In order to locate the precise start of transcription within the DNA sequence, the AluI/TaqI fragment (Fig. 1), which the above experiments showed to contain the initiation site, was sequenced (Fig. 4B), and S1-mapping was again performed. The coding strand of the 120 bp AluI/TaqI fragment, labeled at its 5' end, was hybridized to nuclear RNA and the hybrids were digested with different concentrations of S1 nuclease. The digests were electrophoresed on a sequencing gel in parallel with the chemical cleavage products obtained from the same single strand (Fig. 4A). At the highest concentration of S1 used, the protected DNA migrated predominantly as a triplet of bands. We interpret the 3' end of the smallest of these to correspond to the transcription start site. Since chemically generated fragments of DNA migrate $1\frac{1}{2}$ nucleotides faster than the corresponding S1 generated fragments (22), we infer from the DNA sequence ladder that the transcript begins with the sequence 5'-AGGGGGGUAAU... encoded by the 3'-TCCCCCCCATA-5' sequence of the coding DNA strand (Fig. 4B). Consistent with this conclusion is the observation that γ -[S]-ATP but not γ -[S]-GTP efficiently labels rRNA transcripts in isolated Physarum nuclei (31). An adenosine residue at the 5' terminus of rRNA primary transcripts has been

Figure 5

DNA sequence around the transcription start site

The DNA sequence of the non-coding strand is numbered relative to the transcription start site, indicated by the arrowhead, which is position 1. The 30 bp repeats are aligned one above the other to show homology. The first repeat is arbitrarily defined as the prototypic sequence, and variations from this sequence in the other repeats are marked by asterisks. The two 148 bp repeats in the ETS are aligned, as well as the partial repeat of 29 bp. The sequence from 183-330 is considered the prototypic sequence, and deviations from it are marked with asterisks. The sequence from 154-182 is underlined to indicate that it has no homology with the repeat. For the change of a G to a C at position 281, see the text. Three short repeats near the downstream end of the sequence are underlined with a solid line. Dots in the sequence indicate gaps introduced to allow alignment of the repeats.

found in all species examined, except rat and human.

We have previously used the Physarum strain ATCC 24466 for studies on the structure of the rDNA central spacer region (33) and on rDNA inheritance (46). To gain further information on rDNA structure, we sequenced the DNA around the start of transcription in this strain. The sequenced region is contained on plasmid pPHR25, and extends 1011 bp - from the BclI site at one end of the clone, which is within the ETS, to a point within the 30 bp direct repeats located upstream of the start site. The sequencing strategy is shown in Figure 1, and the sequence in Figure 5. The stretch of 117 nucleotides around the RNA start site that was sequenced in strain M₃C is identical in strain 24466.

DISCUSSION

We have examined the start point of rRNA transcription in Physarum polycephalum by S1-mapping and have sequenced 117 bp (from -32 to +85) in strain M₃C VIII. The results confirm and extend earlier observations from this and other laboratories and indicate that initiation occurs about 60 bp downstream from a region of highly repeated sequences on the rDNA. An array of repetitive sequences upstream from the start of ribosomal transcription has previously been found in Xenopus laevis (47). We have also determined 1011 bp of the sequence around the start site in another strain, ATCC 24466. Previously, sequences have been determined for the region around the site of termination of transcription (35), for the 26S gene, including the two introns (36,37,38) and for the 5.8S gene and flanking regions (36,39). Consequently, some 23% of the rDNA has been sequenced, or roughly 45% of the transcribed region. Of the transcribed region, some of the internal transcribed spacer, the 19S gene and about 3.8 kb of the external transcribed spacer remain to be sequenced.

S1-mapping experiments can lead to erroneous conclusions regarding transcription start sites. First, only the strong bands originating from relatively stable processing intermediates may be visible on autoradiograms that show the protected DNA fragments. We have tried to enhance any minor bands that could originate from unstable, larger processing intermediates by performing S1-hybridizations also with nuclear RNA, which is enriched for ribosomal precursor RNAs and should therefore allow the detection of rare precursor species. However, no additional bands could be detected, even on heavily overexposed autoradiograms. An additional difficulty arises due to the sensitivity of AU-rich RNA-DNA hybrids to S1 digestion (6), which can lead

to S1 cleavage within a perfect duplex and hence to an erroneous localization of the initiation of transcription. We have tried to minimize this problem by also carrying out S1 digestions at room temperature (instead of the usual 45°).

Since the same smallest S1-resistant fragment was obtained under all conditions, we interpret the end of this fragment to correspond to the start of transcription, opposite the T residue defined as +1 in Figure 4. This placement is consistent with the observation that rRNA synthesis starts with an A residue (31). However, since we cannot exclude nibbling of the S1 enzyme at the end of the RNA/DNA duplex, it is also possible that transcription is initiated several nucleotides upstream, opposite the T-residues at -2, -4, or -6.

In addition to locating the initiation sites, S1-mapping experiments also suggest the presence of a processing site within the external transcribed spacer, and allow its localization about 1.6 kb from the start site. Physarum represents an exception among the eukaryotes in this respect, since the generally accepted schemes of eukaryotic rRNA processing have assumed the primary transcripts to be converted into "41S" rRNA via a single processing step, which involves the loss of the entire transcribed spacer (48). Our results suggest that an additional processing step may first remove a portion of the external transcribed spacer. Mouse rDNA transcripts have been found to be processed at an analogous site (6).

The 1 kb DNA sequence around the initiation site from strain 24466 confirms several inferences made previously about this region of Physarum rDNA. The sequence is in complete agreement with the published restriction map (33). When the sequence is compared with the restriction map of rDNA from strain M₃C, two differences are apparent. First, around position 280 a TaqI and an AluI site occur in strain 24466, while in rDNA of strain M₃C a SalI site occurs instead. These differences can be explained by a change of the G residue at position 281 to a C in the rDNA of M₃C, as indicated in Figure 5. Second, the HpaI site at -256 present in 24466 is absent in M₃C.

Restriction mapping indicated that a 2 kb region composed of direct repeats of a unit roughly 30 bp long is present upstream of the transcription start site. As can be seen in Figure 5 where these repeated sequences are aligned one above the other, six of these repeats are present at the upstream end of the sequence, separated by 83 bp from a single full repeat. Short homologies to the repeat unit can be seen within the 83 bp as well. Within the direct repeat regions, we had observed that the MspI sites adjacent to

HaeIII sites were cleaved more slowly in partial digestion experiments (33). Consequently, we predicted that the sequence CCGGCC would be part of the repeat unit, and that prediction is borne out. According to Pauli *et al.* (49) the 30 bp repeat contains a site sensitive to cleavage by staphylococcal nuclease. Staphylococcal nuclease is reported (50,51) to cleave within AT-rich sequences flanked by GC-rich sequences, suggesting that the staphylococcal nuclease is recognizing either the GTTAAC or the GA₍₅₋₇₎^T₍₃₋₄₎^C sequence.

The presence of a duplication within the ETS predicted from the restriction map also is confirmed by the sequence. The duplication is 148 bp long, with 8 differences between the two repeats. In addition, 29 of the 148 bp are repeated a third time. These repeats are shown aligned one above another in Figure 5. At the downstream end of the sequence are a number of MspI sites, consistent with the observation that the 500 bp (800 bp in strain M₃C) of the ETS downstream of the BclI site are composed of repeated sequences containing MspI sites every 10-15 bp (unpublished observations). Three blocks of DNA with similar sequences (and each containing an MspI site) are underlined in Figure 5. These may represent the repeat unit that makes up the following 500 bp. The presence of repetitive sequences within the ETS has not been reported in other species, except for several repeats 20-30 bp long in mouse and rat (9,12).

The start site for rRNA transcription has been identified in 12 species: rat (9,10,10a), mouse (13), human (14,15), Dictyostelium discoideum (11), Saccharomyces cerevisiae (18,19,20), S. carlsbergensis (20a), S. rosei (20a), Drosophila melanogaster (21), Xenopus laevis (22), X. clivii (23) and Physarum polycephalum. The start site in Tetrahymena pyriformis has been localized to two separate positions (16,17), and a region of the Xenopus borealis rDNA believed to contain the start site has been sequenced (23). Comparison of these sequences reveals little homology among the different species, except those that are closely related (e.g. Xenopus spp.; rat, mouse and human).

Although inter-species sequence comparisons have not helped to elucidate the signals important in rRNA transcription initiation, results from *in vitro* transcription of truncated templates indicate that sequences near the start site are important for initiation. In mouse, sequences upstream of -39 are not essential for transcription (26) and in Xenopus essential sequences extend upstream of -35, but not necessarily much further, and certainly not beyond -145 (25). In Drosophila, sequences upstream of -27, but not beyond -43, are necessary for *in vitro* transcription (26a). Of course, these results don't

rule out the possibility of more distant regulatory elements influencing transcriptional efficiency. For example, mouse rDNA templates containing longer non-transcribed spacer (NTS) sequences compete preferentially with rDNA containing a shorter NTS sequence in an in vitro transcription system, even though either template alone is transcribed equally well (26). A similar result has been obtained in Xenopus laevis by microinjecting combinations of plasmids with varying lengths of NTS sequences into Xenopus oocytes (52). In Xenopus, it has been suggested that the direct repeat sequences in the spacer may be serving to bind RNA polymerase I molecules, increasing their concentration near the transcription initiation point. One could speculate that the repeats immediately upstream of the Physarum start site serve a similar function.

Financsek et al. (15) observed that the primary transcript just downstream of the start site in mouse, rat and human contains sequences that can be drawn in a stem and loop structure. Such structures can be drawn for Physarum (bases 8 to 15 and 25 to 32 form a perfectly paired, 8 bp stem), as well as for Xenopus laevis and X. clivii. A similar structure can be made for Drosophila, although in this case the stem begins at the start site rather than slightly downstream. No such structure can be drawn for Saccharomyces, Tetrahymena or Dictyostelium. Whether this structure is important for the initiation of transcription, for processing of the primary transcript or is just fortuitous is at present unknown.

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