Volume 14 Number 8 1986 Nucleic Acids Research

Processing in the external transcribed spacer of ribosomal RNA from *Physarum polycephalum*

Beat Blum, Gerard Pierron[1], Thomas Seebeck and Richard Braun

Institut für Allgemeine Mikrobiologie, University of Bern, Baltzerstrasse 4, 3012 Bern, Switzerland and [1]Institut de Recherches scientifiques sur le Cancer, BP no 8, 94802 Villejuif, Cedex, France

Received 19 March 1986; Accepted 2 April 1986

ABSTRACT

The rDNA of the myxomycete Physarum polycephalum is transcribed to give a 13.3 kb precursor of ribosomal RNA. At 1.7kb downstream of the primary initiation site there is a processing site or a second initiation site. This site was studied by S1-mapping, DNA sequencing and electron microscopy. None of these methods could conclusively distinguish between the two formal possibilities. However, capping experiments indicate that rapid processing is taking place at this site rather than reinitiation. In addition, primary transcripts and processed molecules were assayed throughout the synchronous mitotic cycle. During all interphase stages newly initiated transcripts of rDNA and products of the first processing step are present in similar amounts, indicating control of initiation and not of maturation as being the main regulatory step for the accumulation of mature rRNAs. During the brief period of mitosis the level of newly initiated rRNA precursors is lowered.

INTRODUCTION

In the slime mold Physarum polycephalum the ribosomal genes are arranged on extrachromosomal linear DNA molecules, of which about 200 copies are located in the nucleolus (1, 2). Each molecule is a large palindrome and carries two transcription units on either side of the axis of symmetry. As is the case for all eucaryotic organisms so far investigated, the ribosomal genes of Physarum are transcribed by RNA polymerase I, as shown by the α-amanitin sensitivity of the reaction (3). Each half palindrome is transcribed into a large precursor RNA, which is subsequently processed in a complex series of cleavage steps to give the mature 19S, 5.8S and 26S rRNAs (4). Early events in the maturation of the precursor-rRNA include splicing out introns from the 26S RNA coding region (5), which was also observed for other lower eucaryotes (6) and for organelles (7), and the release of an initial 1.7kb fragment from the external transcribed spacer (ETS) (4). The site underlying the latter process has been roughly mapped before and lies 1.7kb downstream of the primary initiation site (8). The putative processing site is the subject of the present paper. In vertebrates a site of rapid

processing has also been found in the external transcribed spacer, e.g. in the murine ribosomal RNA genes (9, 10, 11).

One of the attractive features of Physarum is the synchronous mitotic cycle studied extensively in macroscopic plasmodia (12). In each plasmodium, containing 10^8 or more nuclei, virtually all nuclei are at the same stage of their nuclear division cycle. In the present paper we further examined the question, if the early processing step at the 5'-moiety of the primary rRNA transcripts is regulated during the cell cycle.

MATERIAL AND METHODS

Growing of Physarum and isolation of RNA

Microplasmodia of Physarum strain M_3C VIII were grown in shake flasks at 25°C in N+C medium (13). From these cultures RNA was isolated during exponential growth. For experiments on the mitotic cycle a single synchronous surface plasmodium (14) was sectionally harvested for RNA preparation from different stages of the cell cycle (from mitosis II to mitosis III). Lysis of cells and purification of total RNA was done as described previously (4).

Restriction fragments and end-labeling

Construction of plasmids pPHR 11 and pPHR 103 has been described elsewhere (15, 8; see also figure 4A). Plasmid DNA was purified from cleared lysates by ethidium bromide-CsCl centrifugation and restriction fragments were isolated as indicated in (8) for large fragments or as in (16) for small fragments. DNA was end-labeled either at the 5'end using polynucleotide kinase and γ-^{32}P ATP or at the 3'end using terminal transferase and labeled cordycepin (α-^{32}P 3'-dATP) according to the manufacturer's instructions.

S1-mapping

S1-mapping experiments were performed as described previously (17). For a typical assay 10'000 cpm of labeled DNA (appr. 50 ng) was hybridized to 10µg of RNA. S1-resistant DNA was size-fractionated either on alkaline agarose gels or 8% sequencing gels depending on the size of the DNA. One S1 unit given in this text is defined as the amount of enzyme required to degrade 1 µg of denatured DNA in 30 min at 37°C.

Sequencing

Chemical cleavage was performed according to (16). M13 sequencing in either direction from the PstI-site (indicated in figure 2A) was achieved as described in (18).

Electron microspopy

Chromatin of plasmodia in the G2-phase was spread according to (19)

except that the 1% ethanolic phosphotungstic acid staining was omitted and the rotary shadowing was performed using platinum only.

Capping assay

Total plasmodial RNA from Physarum (4) was capped using Vaccina capping enzyme according to (20) with the exception that no urea and no carrier E. coli tRNA was added at the end of the reaction. Hybridization to plasmid-DNA and S1 digestion were performed as described above. For eachbatch 2500 units of S1 nuclease were used. Protected RNA was size-fractionated on 8% sequencing gels.

Sources of enzymes and isotopes

Restriction enzymes, calf alkaline phosphatase, polynucleotide kinase and S1 nuclease were obtained from Boehringer-Mannheim. Terminal transferase and Vaccina capping enzyme were from BRL. The radioactively labeled precursors γ-^{32}P ATP (3000 Ci/mmol), α-^{32}P 3'-dATP (5000 Ci/mmol) and α-^{32}P GTP (600Ci/mmol) were obtained from New England Nuclear.

RESULTS

Identification of the processing site in the ETS

In a previous paper we located the initiation site of the primary transcript by S1-mapping and determined the surrounding sequence (8). This initiation site is 4 kb upstream from the 18S RNA coding region. Performing further S1-mapping experiments we determined a second 5'end of RNA, laying 1.7 kb downstream of the primary initiation site of transcription. In order to characterize this further, a cloned 2.1 kb SalI-fragment from the ETS (see fig.1A) was labeled at the 5'ends and hybridized to total plasmodial RNA prior to digestion with the single strand specific nuclease S1 and electrophoresis. The resulting autoradiograph of the gel shows two protected fragments (fig. 1B, lane 1). One fragment consists of the full-length input DNA, which is protected by the full size primary transcript. The 0.7 kb fragment on the other hand corresponds to a second 5'end of the pre-rRNA, which could be generated by cleaving the 1.7kb 5'-moiety off the primary transcript. If such a processing of the RNA does actually take place at this site, it should also be possible to find the corresponding 3'end of RNA upstream from the processing site. In order to probe for the presence of such a 3'end, further S1-mapping experiments were done, using the same 2.1kb SalI-fragment as above, but with its 3'ends labeled. The resulting autoradiograph of the gel shows, beside the fully protected fragment, two smaller bands of 1.4 kb and 1.1 kb in length (fig. 1B, lane 5). The 1.4kb

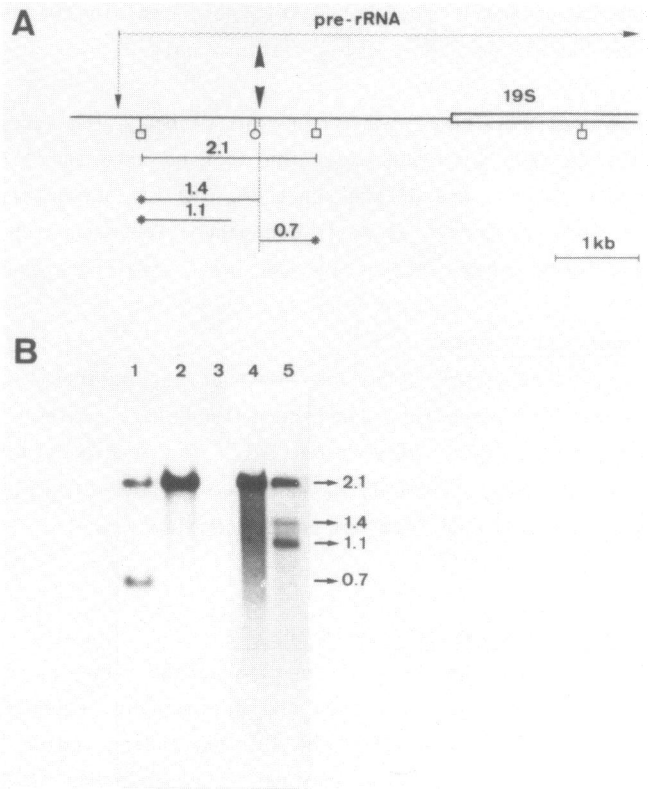

Figure 1
Location of the processing site
The 2.1kb SalI-fragment was labeled at the 5'- or at the 3'ends prior to hybridization to total RNA and digestion with nuclease S1. The products were sized on alkaline agarose gels. (A) Schematic outline of the experiment. The ETS and part of the 19S gene of the rDNA transcription unit are shown. The initiation site of transcription (small arrow) and the processing site in the ETS (large arrows) are indicated. The primary RNA transcript is shown by a wavy line. Input fragment (⊢——⊣) and S1-protected fragments with the respective endlabel (——*: 5'/*—— :3') are drawn beneath. The restriction sites for SalI (⏄) and PstI (⏃) are given. (B) Autoradiograph of sized fragments. Lanes 2 and 4, input DNA fragments labeled at the 5'ends (2) or 3'ends (4); lanes 1 and 5, protected fragments after hybridization and digestion with 10'000 units of S1 nuclease ((1) 5'end and (5) 3'end labeled fragments); lane 3, no RNA was added to the 5'end labeled DNA fragment prior to digestion with 10'000 units S1. Sizes are indicated in kilobases.

band indeed maps the 3'end of a RNA to about the same position where the processed 5'-end was found. The 1.1kb, which is much heavier, could correspond to a more stable processing product of the 1.7kb RNA fragment. Thus, we have roughly mapped a presumptive processing site.

Sequencing and exact position of the processing site

In order to map the identified processing site more precisely, the surrounding sequence was determined by two independent methods (fig. 2). S1-mapping experiments in parallel to chemical cleavage (16) of the same single stranded fragment were performed. The resulting autoradiographs allow one to directly map the RNA-ends at the sequence level. It is clear that an inaccuracy of 1-2 nucleotides is still possible, since the electrophoretic properties of chemical cleavage products and S1-protected fragments are somewhat different (21). The 5'end of the processed RNA was mapped by 5'end labeling a 222bp HpaII-fragment (see fig. 2A), of which the coding strand was either chemically cleaved or used for an S1-experiment. As figures 2B and 2C show, the processed RNA starts with the sequence 5'GGCCTTC..... The corresponding 3'end of the released 1.7kb RNA fragment was mapped by 3'end labeling a 560bp HaeII-fragment. This fragment was then asymmetrically cleaved with AluI. Chemical cleavage and the S1-experiment were performed with the isolated 390bp HaeII/AluI-fragment (see fig. 2A). The 3'end, which is slightly heterogeneous, maps just downstream of a small T-cluster in the non-coding strand (fig. 2B/C). Surprisingly there is a distance of about 27 nucleotides between the observed 3'- and the 5'ends. In this apparent spacer sequence, one can recognize a small inverted repeat just downstream from the 3'end.

Electron microscopic studies

Actively transcribing chromatin of Physarum was spread under conditions, where one can visualize nascent RNA chains attached to the DNA template. The rDNA was identified as a 60 kb linear molecule with two large symmetrical transcription units on either side of the centre of the palindrome. One such rDNA transcription unit is shown in figure 3. The total length of the transcribed area is in good agreement with the length of RNA measured by northern blotting (4). Enlargement of the 5'end of the transcription unit shows the structure at the inset of figure 3. The RNA chain begins to grow from the primary startpoint of transcription. At about 0.36µm (1.3 kb) downstream from this site, an abrupt transition point is apparent, after which no more growing chain can be observed. Further downstream, chain growth resumes and proceeds without interruption through the remainder of the transcribed region. These observations suggest that the cleavage of the primary transcript at the site identified above occurs very rapidly and while RNA chain elongation is still in progress.

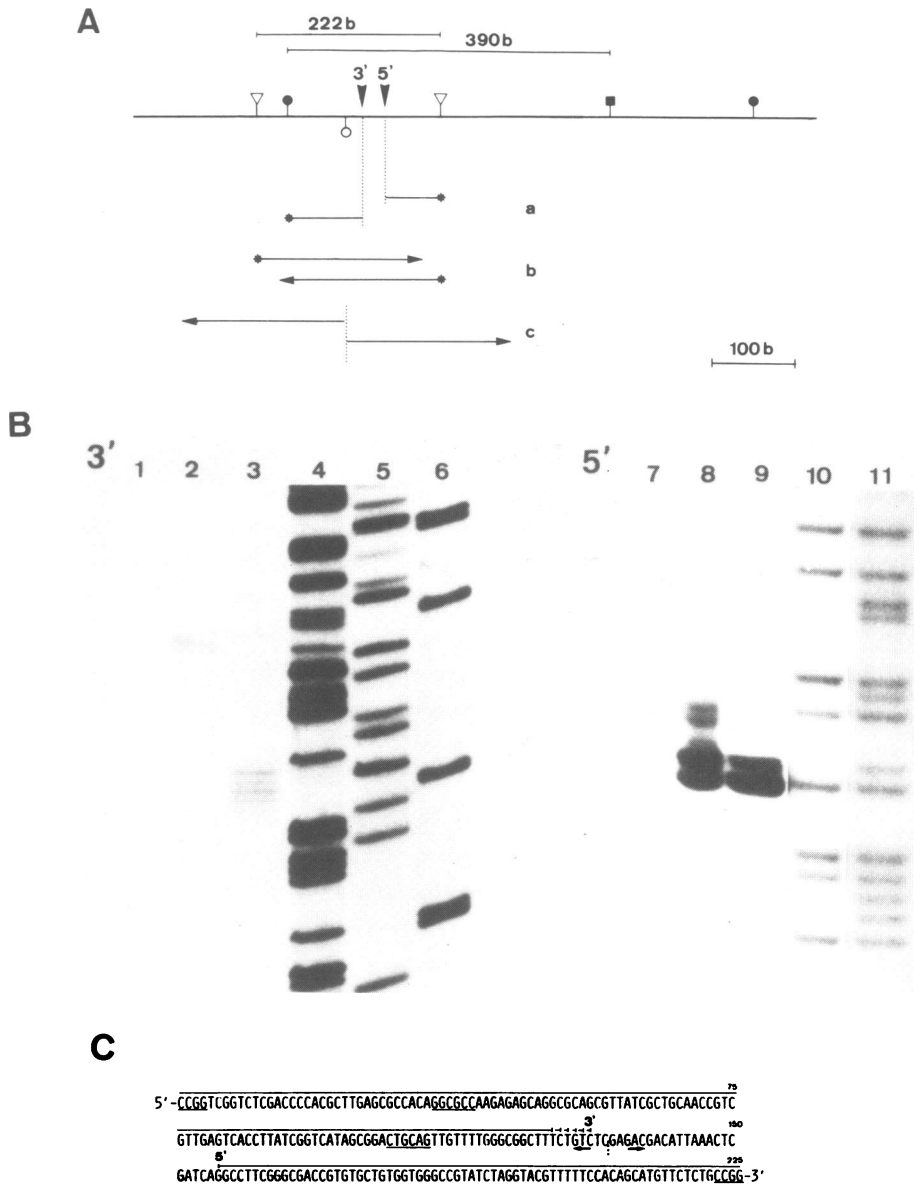

Figure 2
Mapping of the processing site and the surrounding sequence
The 390bp HaeII/AluI-fragment, 3'-labeled at the HaeII-site or the coding strand of the 222bp HpaII-fragment labeled at the 5'end, were hybridized to total RNA prior to digestion with nuclease S1. The products were run on 8% sequencing gels together with samples of the chemically cleaved input

fragments. The DNA sequence of the 222bp HpaII-fragment was determined by the chemical cleavage technique. This was confirmed by M13 sequencing from the PstI-site in either direction. (A) Scheme of the S1-mapping and sequencing. Only the DNA around the processing site is drawn. For this the scale from fig.1 was 10 times enlarged. Protected fragments (a) and the sequencing strategy by chemical cleavage (b) orvia M13 analysis (c) are outlined beneath. The 3'end and the 5'end of RNA are indicated by arrows. Relevant restriction sites: PstI (♁), HpaII (▽), HaeII (♀), AluI (▼). Labeled ends are given by an asterix. (B) Autoradiograph of 8% sequencing gels; (3') mapping of the RNA 3'end and (5') of the RNA 5'end, respectively. (1,7) No RNA was added prior to digestion with 10'000 units of nuclease S1; DNA/RNA hybrids were digested with 1000 units (2,8) and 10'000 units (3,9) of S1; products of sequencing reactions at T+C (4), G (5,10) and A+G (11); (6) PBR/HpaII size marker (from up to down: 122, 110, 90, 76 bases). (C) DNA sequence of the 222pb HpaII-fragment (non-coding strand). RNA is shown as a line above the sequence; heterogeneous 3'ends and the 5'end are indicated. Restriction sites used in this study are underlined. A small palindromic sequence is marked by inverted arrows and a dotted line shows its center.

Capping experiments

The observed 5'- and 3'ends of RNAs in the ETS could also, formally, be generated by another process, namely by premature termination followed by reinitiation of transcription. If indeed reinitiation takes place, it might be possible to label the second de novo 5'end by the Vaccina capping enzyme (21). In order to examine this question, total RNA was capped in presence of α-^{32}P GTP. The labeled RNA was then hybridized to specific plasmid-DNA either overlapping 500 bases from the primary initiation site (pPHR 11) or 800 bases from the presumptive second initiation site (pPHR 103)(for detailed information see references 8 and 15 and fig. 5A). The resulting hybrids were digested with nuclease S1 and protected RNA was size-fractionated on denaturing gels. On the autoradiograph a labeled RNA fragment of about the expected size (500b) can be seen for the primary initiation site (see fig. 4, lane 2). On the other hand no capped RNA of expected size (700b) could be detected from the second site (see fig. 4, lane 1). Thus, the site discussed in this paper most probably is a real processing rather than a reinitiation site. The difference in size between the expected (500b) and actually measured size (640b, see fig. 4, arrow) of RNA from the primary initiation site is most likely due to irregular migration properties of large RNA molecules in sequencing gels or to incomplete S1 digestion of the RNA because of internal secondary structure. Smaller RNA bands, which are seen equally in both experiments (fig. 4, lanes 1 and 2) can be explained by self-hybridisation events among capped RNA molecules leading to protection from S1 digestion. These bands are present in all experiments, irrespective of the presence or absence of DNA, and irrespective of the type of DNA used.

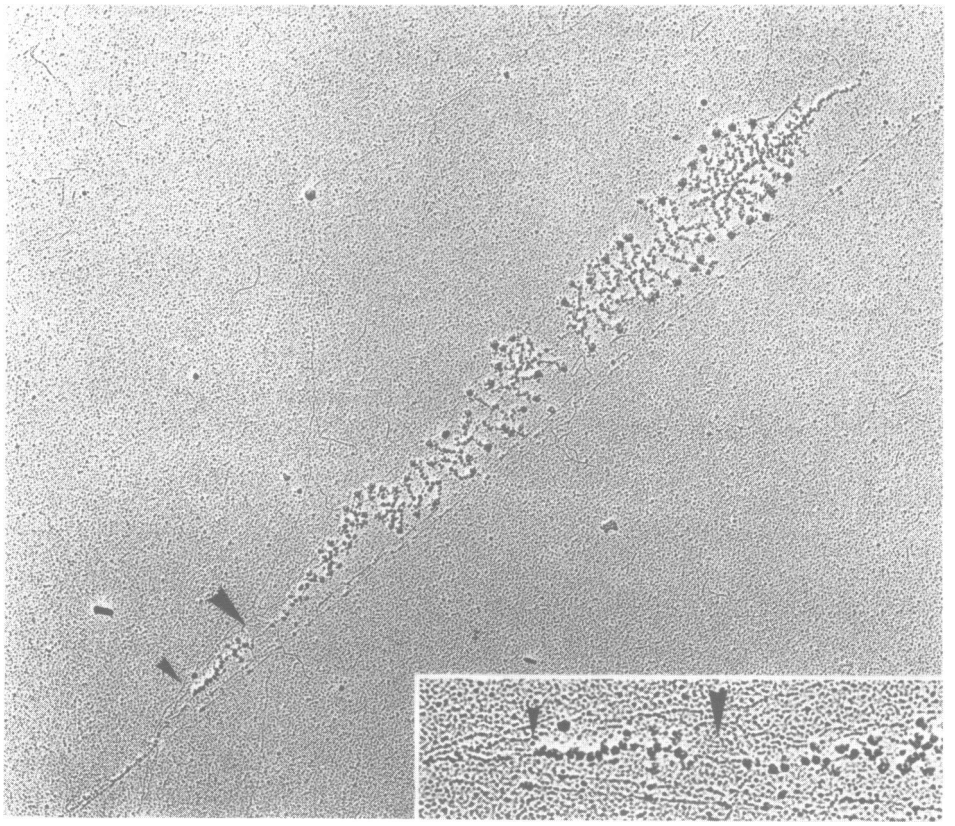

Figure 3
Electron micrograph of actively transcribing ribosomal chromatin
One rDNA transcription unit is shown. Approximate location of the initiation site of transcription (small arrow) and the processing site (large arrow) are indicated. The inset shows an enlarged section around the initiation and processing sites. Magnification: 39'000x; inset: 78'000x.

Processing during the cell cycle

The steady state amount of RNA downstream of the transcription initiation site was compared to the amount of RNA downstream of the processing site at different stages of the cell cycle. Total RNA from a synchronous plasmodium was isolated at twelve succesive stages from one mitosis to the next. The RNA of each stage was then analysed by S1-mapping, using an excess of two different DNA fragments, which were equally labeled at their 5'ends. A 3.1kb MboI-fragment overlaps the initiation site of transcription, a 2.1kb SalI-fragment spans the processing site (fig. 5A).

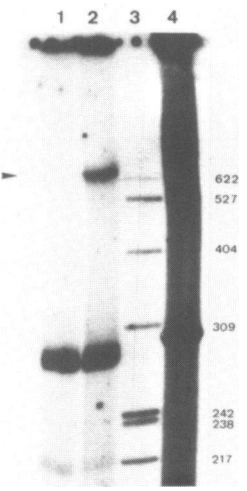

Figure 4
Identification of in vivo initiation sites using Vaccina capping enzyme
Total RNA was capped in the presence of α-^{32}P GTP and hybridized to cloned DNA overlapping the pre-rRNA 500 bases downstream from the primary initiation site (pPHR 11) or 700 bases downstream from the putativesecond initiation site (pPHR 103). Hybrids were digested with 2500 units of S1 nuclease and protected RNA was size-fractionated in 8% sequencing gels. The autoradiograph shows capped RNA at the primary initiation site (lane 2, arrow) but none at the putative second initiation site (lane 1). 5'-labeled Hpa II-fragments of pBR 322 were added as size markers (lane 3). Lane 4 shows total capped RNA. Sizes are indicated in nucleotides.

Thus, full size S1-protected fragments of 2.1kb correspond to newly initiated read-through transcripts, which are not yet processed. The S1-protected fragments of 0.5kb and 0.7kb correspond to 5'RNA-ends from the initiation site and the processing site, respectively. The strong band at the bottom of the autoradiograph (0.2kb) comes from a DNA/DNA overlap between the two labeled fragments and serves as an internal standard for the quality of the S1-experiment.

The steady state level of both initiated and processed RNA 5'ends is quite constant over most of the cell cycle (compare S1-protected fragments of 2.1kb and 0.7kb, shown in fig. 5B, lanes 2 to 11). Only around mitosis the amount of newly initiated RNA is very small. This is not surprising, since one knows that transcription virtually stops during mitosis (3). At this stage, the remaining pool of primary transcripts is rapidly processed into the intermediates, of which the molecules downstream of the processing site are more stable than the 1.7kb cleaved-off RNA (compare 0.7kb to 0.5kb band

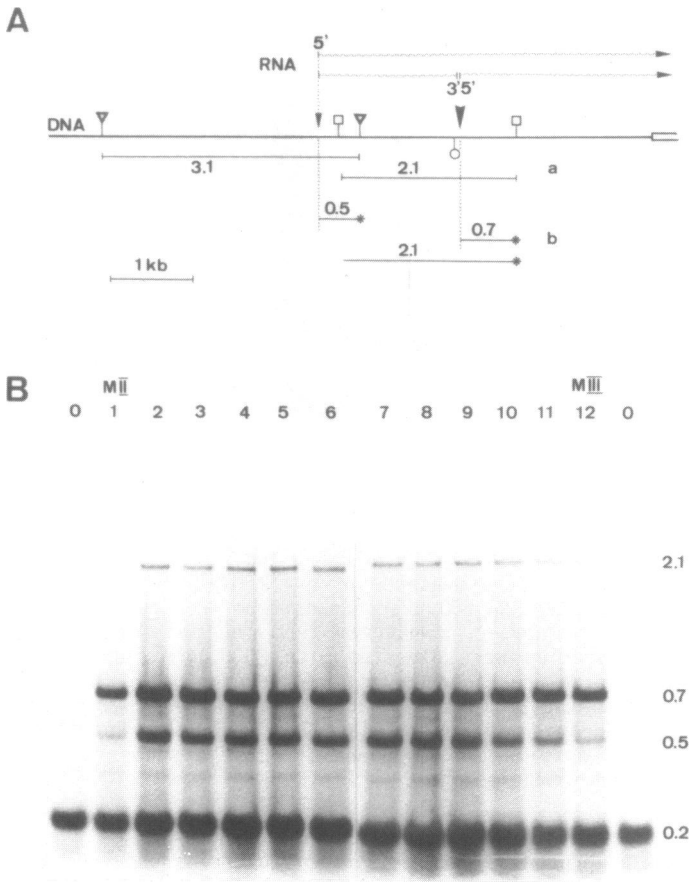

Figure 5
Steady state transcripts during the cell cycle
Two different DNA fragments (a 3.1kb MboI- and a 2.1kb SalI-fragment) were separately and equally labeled at the 5'end and mixed prior to hybridization to total RNA from different stages of the cell cycle. DNA/RNA hybrids were digested with nuclease S1 and protected fragments were sized in alkaline agarose gels. (A) schematical outline of the experiment. The ETS of the rDNA transcription unit is shown. Presumptive RNA species are lined up at the top (wavy line); input DNA fragments (a) and protected fragments (b) are indicated. A small and a large arrow mark the initiation and the processing sites. Relevant restriction sites: PstI ( ), MboI ( ), SalI ( ). Labeled ends are given by an asterix. (B) autoradiograph of sized fragments. RNA of mitosis II (lane 1) and from subsequent stages: 20 minutes (2), 40 min. (3) 100 min. (4), 160 min. (5), 220 min. (6) 280 min. (7), 340 min. (8), 400 min. (9), 460 min. (10), 520 min. (11) and 555 min.= mitosis III (12) were hybridized to the combined DNA input fragments. Hybrids were digested with 10'000 units of S1 nuclease. Lanes 0, no RNA was added prior to digestion with S1. Sizes on the gel are indicated in kilobases.

in fig. 5B, lanes 1 and 12). It is also noteworthy that throughout interphase the total amount of RNA from downstream of the processing site is larger than that from downstream of the initiation site. In particular, read-through molecules are very scarce (2.1kb band). This is a further indication for a rapid processing step and/or for rapid degradation of the first RNA segment from the ETS.

DISCUSSION

In a previous paper concerning the ribosomal genes of Physarum polycephalum we have mapped the primary initiation site of transcription (8). We have now identified and precisely mapped a probable processing site lying in the ETS of the rDNA and located 1.7 kb downstream from the primary initiation site. A short stretch of surrounding sequence was determined and found to agree well with recently published data from another laboratory (22). The putative processing site was studied both by electron microscopy of actively transcribing chromatin and also by detailed localization of various steady state RNA molecules on the DNA map by S1-mapping.

For electron microsocopy whole chromatin was spread and the actively transcribing ribosomal chromatin could easily be identified by the length of its DNA and by its transcriptional arrangement (fig. 3). About 1.5kb downstream of the primary visible initiation site the nascent RNA seems to fall off the DNA and a new initiation of transcription appears. These observed structures can be interpreted in two alternative ways. There is either a processing site, where the primary transcript is cleaved immediately after it has been synthesized, or alternatively a termination event occurs, which is followed immediately or after a short interval by a second initiation event (e.g. as in 23). The biochemical data confirm the ultrastructural observations and in addition favor the first of these two interpretations.

If processing does occur at the site under debate, there has to be a RNA fragment with the 5'end downstream of the processing site and a fragment with the 3'end upstream of this site. Such molecules have in fact been found by S1 mapping experiments. What is puzzling, though, is that the 3'end and the 5'end do not on the DNA map line up to immediately adjacent nucleotide pairs, but rather that a gap of 27 nucleotides is seen. This unexpected gap between the two processing intermediates requires several comments. The simplest explanation for the observed gap, an inaccuracy of the S1-mapping, can be

ruled out, since the experimental results were fully reproducible and since high-resolution sequencing gels were used to characterize the DNA fragments obtained after S1 cleavage. Next, the gap could easily be accounted for if the putative processing site was in fact no processing site, but if the DNA contained a termination site followed about 27 nucleotide pairs downstream by a reinitiation site. There are several arguments in favor of this possibility. Abortive transcription followed by reinitiation has been proposed for other rRNA transcription systems (24, 25, 26). Also, we find a T-cluster just upstream of the putative termination site and a small inverted repeat just downstream of it, a sequence arrangement thought to have termination function in polymerase I transcription (27, 28). In addition, some sequence homologies are present at the primary initiation site (8) and putative reinitiation site discussed here.

Despite these arguments we favor the hypothesis that the putative processing site is actually a real processing site. This conclusion is supported by the following observations. Vaccina capping enzyme recognizes and modifies the 5'end of the RNA from the primary transcription site, but not the 5'end of the RNA from the putative reinitiation site (see fig. 4). Thus, no reinitiation process is indicated by this kind of assay. In addition, the S1-experiments clearly show the presence of at least some read-through RNA molecules, in which the DNA at the processing site has been transcribed into a contiguous RNA molecule spanning this site. This is most simply be accounted for as a single primary transcript, which subsequently is processed. Alternatively, a certain proportion of transcription events could give rise to read-through transcripts, while on other rDNA molecules termination and reinitiation could occur. This model, which implicates two alternative pathways, is difficult to eliminate completely, but again the capping data do not favor it to any considerable degree.

If indeed the secondary 5'end is due to processing, the curious gap of about 27 nucleotides in the RNA still requires some rationalization. Three possibilities come to mind. Firstly, the primary transcript could be cut by a specific RNase at the processing site and subsequently about 27 nucleotides would rapidly be cut off from the 3'end by another activity. Secondly, the primary transcript could be cut twice at the same time with the two cuts lying about 27 nucleotides apart. An oligonucleotide of this length would escape detection in the hybridization assays. Thirdly, the apparent gap in the RNA could in fact not be a gap at all, but an unusual RNA structure leading, upon hybridization with DNA, to S1 nuclease sensitivity. At the

present time it is not possible to distinguish between these formal possibilities.

A semi-quantitative analysis of the amount of primary transcripts and initial processing intermediates throughout the cell cycle shows that the relative concentrations are maintained during most of the cycle. From this result and the known quantitative changes of rRNA synthesis during the mitotic cycle, we conclude that processing at the site described in this study is not a rate limiting step used to regulate the accumulation of mature rRNA. Rather, the frequency of initiation at the primary start site of transcription is likely to be the main regulatory step determining how much rRNA is synthesized at different times of the cell cycle and under different environmental conditions. In agreement with earlier reports,which have noted the absence of RNA synthesis during mitosis (3), we found only few newly initiated transcripts during mitosis. In contrast, the level of the initial processing intermediates remains unaltered throughout all stages of the cell cycle.

ACKNOWLEDGMENTS

We would like to thank Valentin Küng for the gift of isolated RNA from different stages of the cell cycle of Physarum. Our thanks go also to Kathryn Behrens for help in cultivating Physarum and to Hans-Peter Amstutz for his patient introduction to the art of M13 sequencing. This research was supported by the Swiss National Science Foundation grant 3.650-0.84.

REFERENCES

1. Vogt, V.M. and Braun, R. (1976) J. Mol. Biol. 106, 567-587
2. Molgaard, H.V., Matthews, H.R. and Bradbury, E.M. (1976) Eur. J. Biochem. 68, 541-549
3. Seebeck, T. and Braun, R. (1980) Advances in Microb. Physiol. 21, 1-46
4. Gubler, U., Wyler, T., Seebeck, T. and Braun, R. (1980) Nucl. Acids Res. 8, 2647-2664
5. Gubler, U., Wyler, T. and Braun, R. (1979) FEBS-Letters 100, 347-350
6. Wild, M.A. and Gall, J.G. (1979) Cell 16, 565-573
7. Tabak, H.F., Van der Horst, G., Osinga, K.A. and Arnberg, A.C. (1984) Cell 39, 623-629
8. Blum, B., Seebeck, T., Braun, R., Ferris, P. and Vogt, V.M. (1983) Nucl. Acids Res. 11, 8519-8533
9. Miller, K.G. and Sollner-Webb, B. (1981) Cell 27, 165-174
10. Grummt, I. (1981) Nucl. Acids Res. 9, 6093-6102
11. Fetherston, J., Werner, E. and Patterson, R. (1984) Nucl. Acids Res. 12, 7187-7198
12. Aldrich, H.C. and Daniel, J.W., eds. (1982) Cell Biology of Physarum and Didymium, Vol.I. Published by Academic Press, New York
13. David, J.W. and Balwin, H.H. (1964) Methods Cell Physiol. 7, 9-41

14. Guttes, E. and Guttes, S. (1964) Methods Cell Physiol. 1, 4-8
15. Ferris, P.J. and Vogt, V.M. (1982) J. Mol. Biol. 195, 359-381
16. Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, 499-560
17. Din, N., Engberg, J., dKaffenberger, W. and Eckert, W.A. (1979) Cell 18, 525-535
18. Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467
19. Scheer, U., Zentgraf, H. and Sauer, H.W. (1981) Chromosoma 84, 279-290
20. Saiga, H., Mizumoto, K., Matsui, T. and Higashinakagawa, T. (1982) Nucl. Acids Res. 10, 4223-4236
21. Sollner-Webb, B. and Reeder, R.H. (1979) Cell 18, 485-499
22. Hattori, M., Ljljana, A. and Sakaki, Y. (1984) Nucl. Acids Res. 12, 2047-2053
23. Trendelenburg, M.F. (1982) in Embryonic Development, Part A, 199-210. Published by Alan R. Liss Inc., New York
24. Kister, K.-P., Müller, B. and Eckert, A. (1983) Nucl. Acids Res. 11, 3487-3502
25. Moss, T. (1983) Nature 302, 223-228
26. Miller, J.R., Hayward, D.C. and Glover D.M. (1983) Nucl. Acids Res. 11, 11-19
27. Kukita, T., Sakaki, Y., Nomiyama, H., Otsuka, T., Kuhara, S. and Takagi, Y. (1981) Gene 16, 309-315
28. Trendelenburg, M.F. (1982) Chromosoma 86, 703-715