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Spacer sequences downstream of the 28S RNA coding region are part of the mouse rDNA transcription unit

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ABSTRACT

Evidence is presented that more than 300 bp of spacer sequences downstream of the 28S RNA coding sequence are part of the mouse rDNA transcription unit. Studies in two cell-free transcription systems as well as analysis of cellular RNA indicate that RNA polymerase I does not terminate within the 334 bp 3' terminal spacer sequences contained in the rDNA clone used. Quantitative hybridization data, S1 mapping experiments and Northern analysis of nuclear RNA showed that the 14 kb pre-rRNA molecules hybridize with the same efficiency to both the 28S and the 3' NTS specific DNA probe. This indicates that the rRNA precursor contains both at the 5' and 3' end several hundreds bases of external transcribed spacer sequences which are eliminated in subsequent processing reactions.

INTRODUCTION

The 3' end of the ribosomal transcription unit has been reported to be in the immediate vicinity of the 28S RNA terminus in several eukaryotes. In Xenopus laevis (1) and Drosophila melanogaster (2) the 3' end of pre-rRNA coincides with the 3' end of mature 28S RNA. In other species, including yeast (3), Tetrahymena (4) and mouse (5) the precursor RNA has been shown to be only a few nucleotides longer at its 3' end than the mature large rRNA. These data were interpreted to mean that RNA polymerase I terminates transcription directly or shortly behind the rRNA coding sequences and that only a limited trimming is involved in the generation of mature 3' ends.

However, the 3' end of the pre-rRNA may not be necessarily coincident with the termination site of transcription. Processing of the nascent RNA chains may occur before the RNA polymerase has reached the end of the transcription unit or very rapid processing at the 3' end may take place. In this case

3' mapping experiments will only identify the first semistable processing intermediates and not the primary terminated transcript.

In this paper we report the sequence of 339 nucleotides of the 3' "non-transcribed spacer" adjacent to the 28S RNA coding sequence and show that these sequences are contained within the primary transcripts of mouse rDNA. This implies that in this system termination of transcription by RNA polymerase I occurs more than 300 bp further downstream than reported before(5). This finding together with the heterogeneity of 45S pre-rRNA identified previously (6, 7) suggests that processing and/or trimming of both the 5' end 3' end of the primary transcript precedes the cleavage of "45 S pre-rRNA" to mature rRNA.

MATERIALS AND METHODS

Plasmid Constructions

The recombinant plasmid pMrSP has been described before (8). It contains a 461 bp SalI-PvuII fragment from the 5' region of mouse rDNA extending from position -169 to +292 relative to the transcription initiation site. The clone pMrT contains a 918 bp EcoRI-BamHI fragment derived from the 3' end of the ribosomal transcription unit (see Fig. 1). The EcoRI- BamHI fragment from pMrT was cleaved with BstNI and the resulting 445 bp BstNI-BamHI fragment was inserted into the PvuII site of pMrSP yielding the "minigene" pMrPT. This minigene contains 169 bp of the non-tran-scribed spacer (NTS) 5' of the initiation site, 292 bp of the external transcribed spacer (ETS), 112 bp of the 28S RNA and 334 bp spacer sequences beyond the 3' end of 28S RNA. From pMrPT two subclones were generated. pUCMrSS contains the 306 bp SmaI-SmaI fragment in the vector pUC9, pUCMrSB was obtained by inserting the 277 bp SmaI-BamHI fragment into pUC9.

In vitro Transcription Systems

Both types of cell-free transcription systems used in this study have been described before (9-11). In the nucleolar transcription system nucleoli isolated from Ehrlich ascites cells were used. They were incubated in a final volume of 250 µl containing 0.1 M Tris-HCl (pH 8.5), 5 mM $MgCl_2$, 6 mM NaF, 50 mM $(NH_4)_2 SO_4$, 5 mM dithiothreitol, 50 units RNasin,

0.3 mM ATP, CTP, UTP, 0.012 mM GTP and 10 /uCi ^{32}P-GTP. After incubation for 20 min at 37° C, the labelled RNA was extracted by phenol and chloroform treatment. Sedimentation analysis was carried out by centrifugation in a SW41 rotor for 15 h at 25 000 rpm at 5°C on 15-30% linear sucrose gradients containing 10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 10 mM EDTA, 0.2% SDS and 0.1% sodium deoxycholate. The fractions containing the 45S RNA were pooled, ethanol precipitated and used for hybridization.
In the soluble cell-free transcription system the plasmid pMrPT was used as template. It was truncated with BamHI and 0.15 - 0.2 /ug were incubated in a 50 /ul assay in the presence of 30 /ul of either cytoplasmic (S-100), nuclear or nucleolar extracts. The concentration of components in the assay were: 12 mM Hepes (pH 7.9), 85 mM KCl, 0.12 mM EDTA, 0.5 mM DTT, 5 mM $MgCl_2$, 10 mM creatine phosphate, 0.6 mM each of ATP, CTP and UTP, 12.5 /uM GTP and 2 /uCi α-(^{32}P) GTP (spec. act. 400 Ci/mmole). The mixture was incubated for 60 min at 30°C and processed for gel analysis as described before (9, 10). The nuclear or nucleolar extracts were prepared according to Dignam et al. (12).

Hybridization analysis

The hybridization probes used in this study were subclones derived from pMrPT. pUCMrSS contains a 306 bp SmaI fragment consisting of 137 bp of the 5' ETS, 112 bp of 28S RNA and 57 bp of 3' flanking sequences (see Fig. 1). pUCMrSB contains a 279 bp SmaI-BamHI fragment from the 3' spacer region. DNA or RNA immobilized on nitrocellulose filters was prehybridized for 2 hours and hybridized for 16 hours in a buffer containing 50% formamide, 5x SSC, 5x Denhard's reagent, 20 mM sodium phosphate buffer (pH 6.8) and 100 /ug/ml denatured E. coli DNA. After hybridization the filters were washed under stringent conditions (0.2x SSC, 0.1% SDS,30 min at 60° C). If 45S RNA synthesized in vitro was used as hybridization probe, the filters were treated with RNase before autoradiography.
For S1 mapping 20 /ug of nuclear RNA were hybridized in 25 /ul of 80 % formamide, 0.4 M NaCl, 40 mM PIPES, pH 6.4, 1 mM EDTA to either the 445 bp BstNI-BamHI fragment from pMrT or to the SmaI-BamHI 3' spacer fragment which had been 3' labelled at the

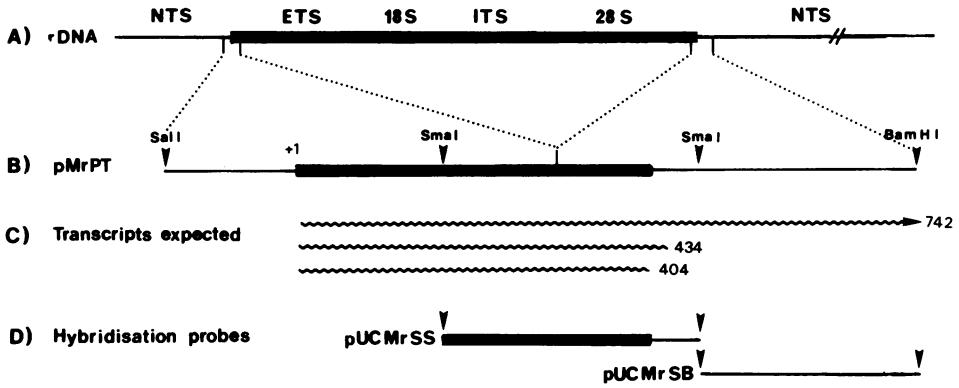

Fig. 1 Structure of the ribosomal transcription unit (A) and the ribosomal "minigene" (B). The transcripts expected to be synthesized in the cell-free system are shown in C: run-off transcripts should be 742 nucleotides long, transcripts terminated at the 3' end of the 45S precursor according to Kominami et al. (5) should be 434 nucleotides long while the generation of the 28S RNA 3' terminus would correspond to a 404 nucleotides long RNA species.
D) Subclones of pMrPT used as hybridization probes. pUCMrSS is a 306 bp SmaI fragment cloned into pUC9, which hybridizes to ETS and 28S RNA sequences. pUCMrSB contains a 279 bp SmaI-BamHI fragment derived from spacer sequences downstream of the 28S coding region.

SmaI site by T4 DNA polymerase. After hybridization for 3 h at 64°C, the hybrids were diluted with 250 µl S1 buffer, treated for 30 min at 37°C with 60 units S1 nuclease and subjected to electrophoresis on 6 % denaturing polyacrylamide gels.

RESULTS

Transcription of the Ribosomal "Minigene" in vitro

Previous studies in cell-free transcription systems have shown that cytoplasmic or nuclear extracts derived from exponentially growing Ehrlich ascites cells contain both RNA polymerase I and the transcription factors required for the faithful initiation of pre-rRNA synthesis (9, 10). To study the transcription termination process *in vitro* we have constructed the artificial ribosomal "minigene" pMrPT, which contains 169 bp of 5' flanking sequences, 403 bp between the initiation site and the 3' end of 28 S RNA as well as further 334 bp of 3' terminal spacer sequences. pMrPT was truncated with BamHI and used as

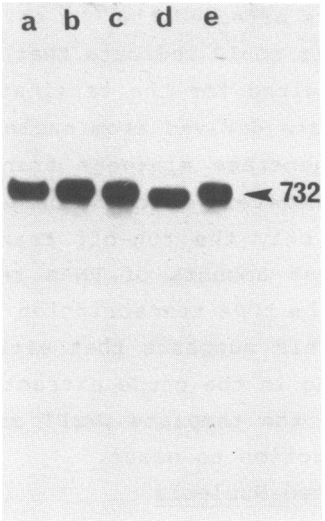

Fig. 2 Transcription of pMrPT *in vitro*
pMrPT was cleaved with Bam HI and used as template in the cell-free transcription system. Each 50 µl assay contained 0.15 µg of template DNA and 30 µl of extract.
a) 30 µl S-100 extract, b) 30 µl nuclear extract, c) 15 µl each of S-100 and nuclear extract, d) 15 µl each of S-100 and nucleolar extract, e) 15 µl each of nuclear and nucleolar extract.

template in the soluble cell-free transcription system containing S-100 extracts. If RNA polymerase I stops at the presumptive termination site 30 nucleotides behind the 3' end of 28 S RNA (5), a 434 nucleotide RNA should be synthesized. If trimming of the 3' end takes place and the genuine end of 28S RNA is generated *in vitro* a 404 base transcript should be observed. However, no RNA molecules of the predicted sizes could be detected in the S-100 system. The vast majority of transcripts were approximately 740 bases long, a size which closely corresponds to the distance from the initiation point to the artificial termination site created by cleaving the template with BamHI (Fig. 2 lane a). This failure to produce RNA molecules with the expected 3' termini was observed under a variety of experimental conditions. We have tested the effects of varying the incubation time, temperature, KCl and $MgCl_2$ concentrations, or using different amounts of template DNA.

None of these parameters affected the RNA pattern qualitatively (not shown). This result could indicate that the S-100 extracts lack the factor(s) required for the termination reaction. We therefore tested extracts derived from nuclei or nucleoli (12) for their ability to generate minigene transcripts with the expected 3' termini. However, also in the presence of nuclear or nucleolar extracts only the run-off transcripts were synthesized. No significant amounts of RNAs terminating at the presumptive 3' end of the rDNA transcription unit were observed (Fig. 2, lanes b-e). This suggests that either some essential component(s) are missing in the crude extracts, or that the DNA sequences contained in the template pMrPT are not sufficient for the termination reaction to occur.

Transcription in Isolated Nucleoli

In order to overcome the inability of the soluble transcription system to generate the expected 3' termini of rRNA we used an alternate transcription system which could have retained the factor(s) potentially involved in transcription termination. Isolated nucleoli have been shown to synthesize distinct classes of high molecular weight RNA the sedimentation coefficient of which correspond to 45S RNA and defined processing intermediates (14). This RNA synthesis is mainly due to the elongation of growing RNA chains, since no significant initiation takes place in this system (13). In order to ascertain how far the 45S RNA synthesized in isolated nucleoli extends beyond the 3' end of 28S RNA, nucleoli were incubated in the presence of α-(^{32}P)-GTP and RNase inhibitor under standard transcription conditions (Fig.3). The labelled RNA from the 45S region was pooled and hybridized to either the probe from the coding region (pUCMrSS) or a downstream "NTS" probe (pUCMrSB). As shown in the inset of Fig.3 hybridization signals obtained with each probe were roughly identical indicating that termination of 45S RNA in isolated nucleoli must have taken place further downstream than inferred from a previous *in vivo* study (5).

Transcription into the 3' NTS also Occurs in vivo

To find out whether the longer transcripts are also synthesized *in vivo*, two different hybridization methods were applied. Fig. 4 shows the result of a quantitative dot-hybridization.

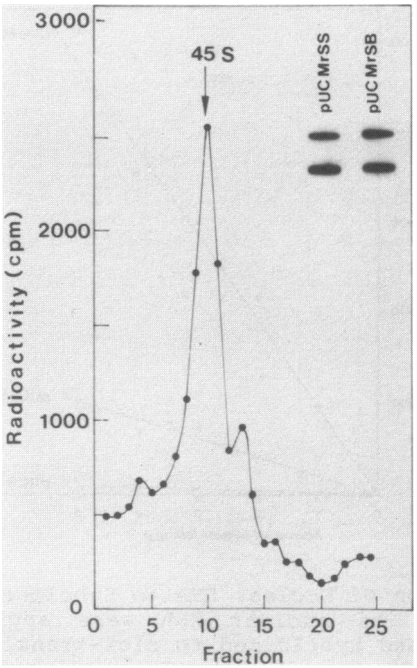

Fig. 3 Hybridization of in vitro Synthesized Nucleolar 45S RNA to Subclones of pMrPT
Nucleoli isolated from Ehrlich Ascites cells were incubated as described in Material and Methods. The labelled RNA was extracted, sedimented in a sucrose gradient, the 45S RNA (fractions 9-11) was pooled and hybridized to 1 ug pUCMrSS or pUCMrSB DNA immobilized on a nitrocellulose filter (duplicate slots were tested for each DNA sample, as shown in the inset).

Increasing amounts of total nuclear RNA were applied onto nitrocellulose filters and hybridized against nick-translated probes pUCMrSS and pUCMrSB, respectively (see Fig.1). Quantitation of the hybrids by counting the radioactivity of the filters revealed significant hybridization of nuclear RNA to pUCMrSB. As much as one fifth RNA hybridized to the "NTS" probe pUCMrSB as compared to pUCMrSS. This indicates that a substantial steady-state level of 3' spacer transcripts is present in cellular RNA. To make sure that the RNA molecules detected with the 3' "NTS"probe pUCMrSB were in fact pre-rRNA molecules and had not been initiated within the 3' spacer, a Northern analysis was carried out. Nuclear RNA was separated on a 0.7%

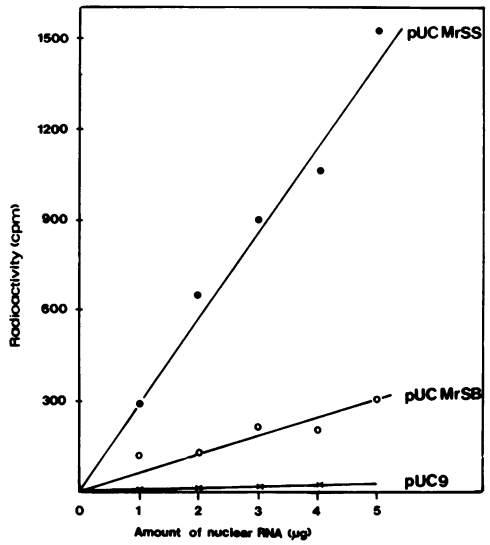

Fig. 4 Hybridization of Nuclear RNA to Subclones of pMrPT. Increasing amounts of nuclear RNA were applied to nitrocellulose filters and hybridized to nick-translated rDNA fragments cloned in pUC9 (see Fig.1). The radioactive dots were punched out and quantitated by scintillation counting.

agarose-formaldehyde gel, transferred to nitro cellulose filters and hybridized to the two labelled DNA probes, pUCMrSS and pUCMrSB, respectively (Fig. 5). As expected, the pUCMrSS probe hybridized to 45S RNA, nuclear 28S RNA and its direct precursor 32S rRNA. The "downstream" probe pUCMrSB hybridized predominantly to the 45S species. It is noteworthy that the intensities of the signals obtained for 45S RNA are roughly similar with both probes.This result indicates that part of the so-called 3' "non-transcribed" spacer does belong to the rDNA transcription unit. It implicates that RNA polymerase I transcribes *in vivo* beyond the SmaI site at position +37 relative to the 3' end of 28S RNA.

S1 Mapping Experiments using the 3' Spacer Fragment

In a previous study Kominami et al. (5) have demonstrated that the 3' terminus of 45S RNA maps 30 bp downstream of the 3' end of the 28S RNA gene. This result was obtained by S1 mapping experiments using 28S and 45S RNA labelled with ^{32}P-pCp at the

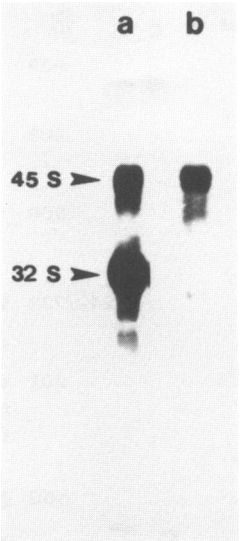

Fig. 5 Hybridization of Nuclear RNA to pUCMrSS and pUCMrSB
10 µg of nuclear RNA from 3T6 cells were denatured and run on a 0.7% agarose-formaldehyde gel for 300 volt-hours. The RNA was transferred to a nitrocellulose filter and hybridized to nick-translated pUCMrSS (a) or pUCMrSB (b) DNA(spec.act. 2×10^7 cpm/µg).

3' termini. In an analogous experiment we have used a 445 bp BstNI-BamHI fragment derived from pMrT which extends from position -111 to +334 relative to the end of 28S RNA. After hybridization of 45S nucleolar RNA to this 3' labelled probe a 140 bp S1 nuclease resistant fragment as well as a large amount of full-length fragment was observed (Fig. 6A). This finding confirms the previous reports on the existence of pre-rRNA molecules 30 bases longer than 28S RNA and suggests that there is a large proportion of molecules which extend further downstream. If the SmaI -BamHI fragment of the spacer probe pUCMrSB was 3' end-labelled at the SmaI site and hybridized to nuclear RNA the full length fragment was protected from S1 digestion. This indicates that the primary mouse rDNA transcript contains several hundred nucleotides of 3' terminal spacer sequences which seem to be eliminated in a subsequent processing reaction. Obviously the product of such an early processing reaction is a 45S RNA species, the 3' terminus of which extends 30 nucleotides beyond the 3' end of 28S RNA.

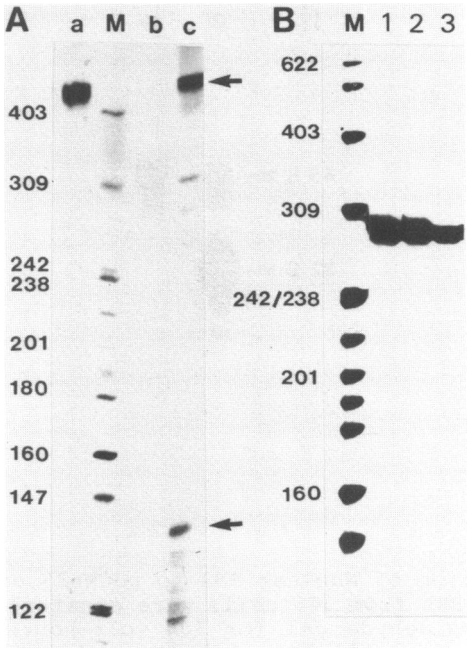

Fig. 6 S1 Mapping of pre-rRNA
Nucleolar (A) or nuclear RNA (B) from Ehrlich ascites cells was hybridized to 3' labelled fragments derived from the 3' end of the ribosomal transcription unit, treated with S1 nuclease and analyzed on a 6% polyacrylamide sequencing gel along with a pBR322/HpaII size marker (M).
A) Hybridization to the 445 bp BstNI-BamHI fragment. Lane a) untreated probe, lane b) hybridization to 18S rRNA, lane c) hybridization to 45S RNA pooled from sucrose gradients.
B) Hybridization of 20 µg nuclear RNA to the 306 bp SmaI-BamHI spacer fragment 1) no S1 nuclease, 2) 60 units S1 nuclease, 3) 180 units S1 nuclease.

DISCUSSION

In this study we have reinvestigated the question whether the 3' end of mouse rRNA is generated by a genuine transcription termination process or by a processing reaction. In several species the end of the rDNA transcription unit has been demonstrated to coincide with the 3' end of the large rRNA (1, 2). In mouse the 3' end of 45S pre-rRNA has been reported to be 30 nucleotides longer than 28S RNA (5). We intended to study the termination reaction in a crude cell-free system which has been shown to be capable of accurate initiation on the rDNA

start site (10). A ribo somal "minigene" was constructed and used as a template in the cell-free system. We have neither observed any termination of transcription nor processing of read-through transcripts into RNA molecules with correct 3' termini, irrespective which kind of extract or experimental conditions were used. Even if we investi gated processing in an uncoupled system, i.e. after isolation of the 742 bases run-off transcript and incubation under different conditions (varying the salt concentrations, temperature, incubation time, type of extracts) no processing of the transcripts into the 434 or 404 nucleotides RNA species was observed (not shown).

This finding suggested that (I) either the cell extracts lack the proteins required for transcription termination and/or processing or(II) that the DNA template used in these studies did not contain the sequences required for termination. The second possibility is much more likely since we have shown that in isolated nucleoli and also in vivo RNA polymerase I transcribes beyond the BamHI site located at position +334 relative to the 3' end of 28S RNA. Thus the primary transcript of mouse rDNA contains 3' spacer sequences which have been believed not to be transcribed. We therefore conclude that in mouse the 45S RNA species, the 3' end of which was mapped 30 nucleotides downstream of the 3' end of 28S rRNA must represent a processing intermediate rather than a primary transcript. This situation is very reminiscent of the processing events taking place at the very 5' end of pre-rRNA. A 5' terminal processing site in the ETS 650 bp downstream of the initiation site has been identified by SI mapping data of mouse rRNA transcripts synthesized either in vivo or in vitro (10, 14, 15). The 5' terminal processed 45S RNA has been shown to be in tenfold excess over the unprocessed one (15).

Certainly the finding that in mouse the rDNA transcription unit extends several hundred nucleotides into the 3' spacer cannot be generalized. Mapping experiments in Xenopus (1), Tetrahymena (4) and yeast (3) suggest that the ribosomal transcription unit does not extend much further downstream. Particularly, it has been shown recently that a yeast rDNA fragment extending from position -36 to +101 relative to the 3' end of the 26S rRNA gene

contains the sequence information to direct transcripts with correct 3' ends after introduction of an artificial rDNA minigene into yeast (16).

The present results do not permit any valid conclusions regarding the DNA sequences involved in the termination reaction of RNA polymerase I. We are now using further downstream regions to identify the terminus of the primary rDNA transcript at the nucleotide level in order to investigate the molecular mechanisms involved in the generation of correct 3' ends of rRNA.

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