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In vitro transcription of a cloned mouse ribosomal RNA gene

Yukio Mishima, Osami Yamamoto, Ryo Kominami and Masami Muramatsu

Department of Biochemistry, Cancer Institute, Japanese Foundation for Cancer Research, Kami-Ikebukuro, Toshima-ku, Tokyo 170, Japan

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ABSTRACT

An *in vitro* transcription system which utilizes cloned mouse ribosomal RNA gene (rDNA) fragments and a mouse cell extract has been developed. RNA polymerase I is apparently responsible for this transcription as evidenced by the complete resistance to a high concentration (200 μg/ml) of α-amanitin. Run-off products obtained with three different truncated rDNA fragments indicated that RNA was transcribed from a unique site of rDNA. The S1 nuclease protection mapping of the *in vitro* product and of *in vivo* 45S RNA confirmed this site, indicating that, in this *in vitro* system, transcription of rDNA started from the same site as *in vivo*. This site is located at several hundred nucleotides upstream from the putative initiation site reported by us (1) and by others (2). Some sequence homology surrounding this region was noted among mouse, *Xenopus laevis* and *Drosophila melanogaster*. The data also suggest that some processing of the primary transcript occurs in this *in vitro* system.

INTRODUCTION

The development of an *in vitro* transcription system is a prerequisite for the understanding of molecular mechanisms of gene regulation. Recent progress in this field has established cell-free systems for *in vitro* transcription with RNA polymerase III (3,4) and II (5,6). With these systems, identification of the sequences required for correct initiation etc. are coming out rapidly (7-10). At the same time, cofactors involved in transcription regulation are also being identified with these systems (11,12).

Attempts to develop an *in vitro* system for transcription of rRNA genes by RNA polymerase I have not been successful until the recently reported work of Grummt who described a mouse cell extract which could initiate transcription on a cloned mouse rDNA (13).

We have previously cloned a mouse rDNA fragment (14,15) and sequenced the region surrounding the transcription initiation site (1). We also determined a putative transcription initiation site by the method of S1 nuclease protection mapping (1). The *in vitro* initiation site reported by Grummt (13), which agreed with the 5'-end of *in vivo* 45S RNA determined by Bach *et al.* (2), was about 200 nucleotides upstream from our site.

We have now established an *in vitro* transcription system using mouse cell extract and the cloned rDNA, and determined the initiation site by the size measurement of the transcripts on different truncated templates and by the S1 nuclease protection mapping of those transcripts and of *in vivo* 45S RNA. The results indicate that RNA polymerase I initiates transcription *in vitro* at a site further upstream from the sites postulated by us and by others. We have recently obtained information that Miller and Sollner-Webb (16) and Onishi and Reeder (personal communication) came to a conclusion similar to us, independently.

MATERIALS AND METHODS

Preparation of cloned DNA template. The recombinant plasmid designated pMrSL-II, which was constructed by ligation of the SalI 3.2 kb fragment within the EcoRI 14.9 kb fragment (pMrEL-I) to the SalI site of pBR322 (14,15), was propagated in *E.coli*. HB101. The plasmid DNA was purified (17) and digested with XhoI and SalI. The SalI/XhoI 1.4 kb fragment was separated by agarose gel electrophoresis and recovered by the method of Tabak & Flavell (18). Recovered SalI/XhoI 1.4 kb fragment was redigested by PvuII or SmaI. Two fragments generated by digestion with SmaI were separated on an agarose gel and recovered as described above. About 1 pmol of these fragments prepared as above was used. For the transcription of the adenovirus type 2 (Ad2) major late SmaI-F fragment (pSmaF), SmaI-cleaved pSmaF DNA was used as a template. pSmaF recombinant was obtained from Dr. S. Hirose at the National Institute for Basic Biology.

Cell culture and preparation of cell extract. Mouse mammary tumor cell line FM3A cells were grown in suspension culture at 5 x 10^5 cells/ml and HeLa cells were cultured in monolayer conditions up to 80% confluence. Cells were harvested and

washed 3 times with phosphate buffered saline. S100 (100,000x g supernatant) from FM3A cells was prepared as described by Weil *et al.* (5) and that from HeLa cells prepared as by Talkington *et al.* (19). They were dialyzed against a buffer containing 20 mM Hepes (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, and 0.5 mM dithiothreitol for 5 hrs at 4°C. The protein concentration in these extracts was 20-25 mg/ml.

In vitro RNA synthesis and purification of RNA. The standard reaction mixture contained in 50 µl, 0.026 mM of α-^{32}P-CTP or α-^{32}P-UTP (15 Ci/mmol), 0.66 mM each of the three unlabeled ribonucleoside triphosphate, 10 mM HEPES (pH 7.9), 0.33 mM dithiothreitol, 80 mM KCl, and 1 pmol cloned DNA fragment. For the Ad2 SmaI-F transcription, reaction condition was adjusted as described by Weil *et al.*(5). The reaction was carried out at 30°C for 50 min after the addition of 15 µl of S100, and then terminated by the addition of 50 mM sodium acetate (pH 5.1), 0.3% sodium dodecyl sarcosinate, 0.15 M NaCl and 10 µg yeast RNA. The mixture was extracted with phenol mixture (phenol:methacresol:H_2O:8-hydroxyquinoline = 70:10:20:0.1) and precipitated with ethanol.

Glyoxalation and electrophoretic analysis of RNA. ^{32}P-RNA prepared as above was denatured in a solution containing 1 M glyoxal, 50% dimethylsulfoxide, 0.1 M HEPES (pH 7.9) at 37°C for 2 hrs as described by Weil *et al.* (5). The samples were collected by ethanol precipitation and subjected to electrophoresis on 4% polyacrylamide gel in 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA (pH 7.4). Size markers used were SmaI-cleaved pSmaF run-off 560 nucleotide product and end-to-end products generated from SmaI or PvuII cleaved SalI/XhoI 1.4 kb (316 and 454 nucleotides, respectively) and HinfI cleaved pBR322 DNA. In our gel electrophoresis conditions, glyoxalated RNA migrated by 30% more slowly than non-glyoxalated DNA fragments.

DNA Sequencing. DNA was sequenced by the method of Maxam and Gilbert (20).

45S RNA preparation. 45S RNA was prepared from the nucleoli of Ehrlich ascites tumor cells as described previously (21-23).

S1 nuclease mapping. *In vitro* synthesized RNAs or *in vivo* 45S RNA were hybridized with indicated DNA fragments shown in Figure

legends in 70% formamide, 0.5 M NaCl, 1 mM EDTA, 0.04 M Pipes-NaOH (pH 6.8) at 56°C for 2 hrs after heat treatment at 90°C for 3 min. The solution containing the hybrid was diluted 10 folds with S1 buffer (30 mM sodium acetate (pH 4.5), 0.22 mM $ZnCl_2$, 0.2 M NaCl) and treated with 50 units of S1 nuclease at 37°C for 1 h followed by ethanol precipitation (24). The samples were run on a neutral 4 or 5% polyacrylamide gel along with the HinfI cleaved pBR322 DNA or on the 8% sequencing polyacrylamide gel along with chemically cleaved coding strand DNA.

RESULTS

Analysis of in vitro rDNA transcripts with different truncated DNAs.

The genes coding for mouse ribosomal RNA are tandemly repeated as shown in Figure 1-a. We have cloned an EcoRI 14.9 kb fragment which carries the initiation site for the preribosomal RNA (14,15) as shown in Figure 1-b. From the data of S1 nuclease protection mapping with hybrids between restriction fragments and 45S RNA, we suggested that the transcription starts from about 2.4-2.5 kb upstream from the 3'-end of SalI 3.2 kb fragment (15). Further, we have sequenced this region and deter-

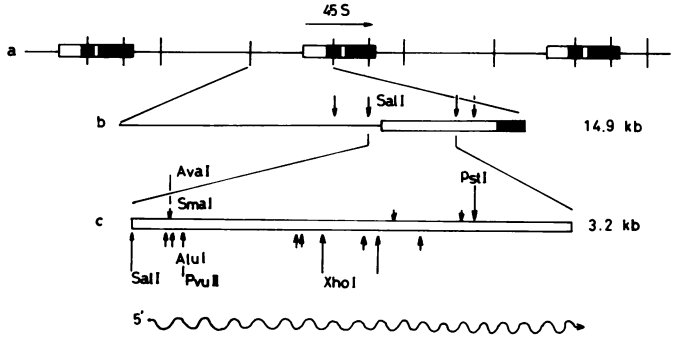

Figure 1. Maps of mouse ribosomal DNA surrounding initiation region. a. shows the EcoRI cleavage sites and ribosomal RNA genes. b. and c. show the SalI cleavage sites within EcoRI 14.9 kb fragment and various restriction endonuclease cleavage sites within SalI 3.2 kb fragment, respectively. Open and closed boxes in a. show the transcribed spacer, and 18S and 28S coding regions, respectively.

mined the putative initiation site (1).

We first investigated the transcripts generated when the SalI/XhoI 1.4 kb fragment (see Figure 1-c) was incubated with the S100 from the cultured mouse cells. Several descrete bands of 1300, 840, 640, 510, 420 and 200 nucleotides were generated in the absence and the presence of α-amanitin (Figure 2A lane 2-4), although the last two bands are too faint to be seen in this Figure. These bands were completely inhibited by actinomycin D (lane 5) and no bands were visible when DNA template was omitted (lane 1). These results indicate that the products were transcribed by RNA polymerase I on added DNA template. The major RNA band of 1300 nucleotides seemed to be the primary transcript and other minor bands possible processed products in this system from the data shown later. As a comparison we tested the transcription of the SmaI-cleaved pSmaF DNA by S100 prepared from HeLa cells. The 560 nucleotide "run-off" product was generated as shown in Figure 2B lane 1 but this product was completely eliminated by a low (1 μg/ml) concentration of α-amanitin (lane 2), demonstrating that the product was transcribed by RNA polymerase II. S100 prepared from the FM3A cells also could transcribe the SmaI-cleaved pSmaF DNA when RNA polymerase II was supplemented (Data not shown).

Next, we used the PvuII cleaved SalI/XhoI 1.4 kb DNA fragment. As shown in Figure 2C, only one band of about 300 nucleotide RNA was generated under appropriate DNA concentrations (lane 2 and lane 3), although end-to-end transcription products with the lengths of 454 and about 1100 nucleotides were also generated as the amount of DNA increased (lane 4 and lane 5). We again examined the effect of α-amanitin and actinomycin D using 14.4 μg/ml of PvuII cleaved SalI/XhoI 1.4 kb fragment, which was slightly less in molar concentration than that used in Figure 2C lane 3. As shown in Figure 2D lane 2-4, only 300 nucleotide RNA was generated in the absence (lane 2) and the presence of 1 μg/ml (lane 3) and 200 μg/ml (lane 4) of α-amanitin, respectively. This RNA synthesis was completely inhibited by actinomycin D (lane 5) and no bands were detected when DNA template was omitted (lane 1), although about 120 nucleotide product, which was DNA-independent, α-amanitin-insensitive and

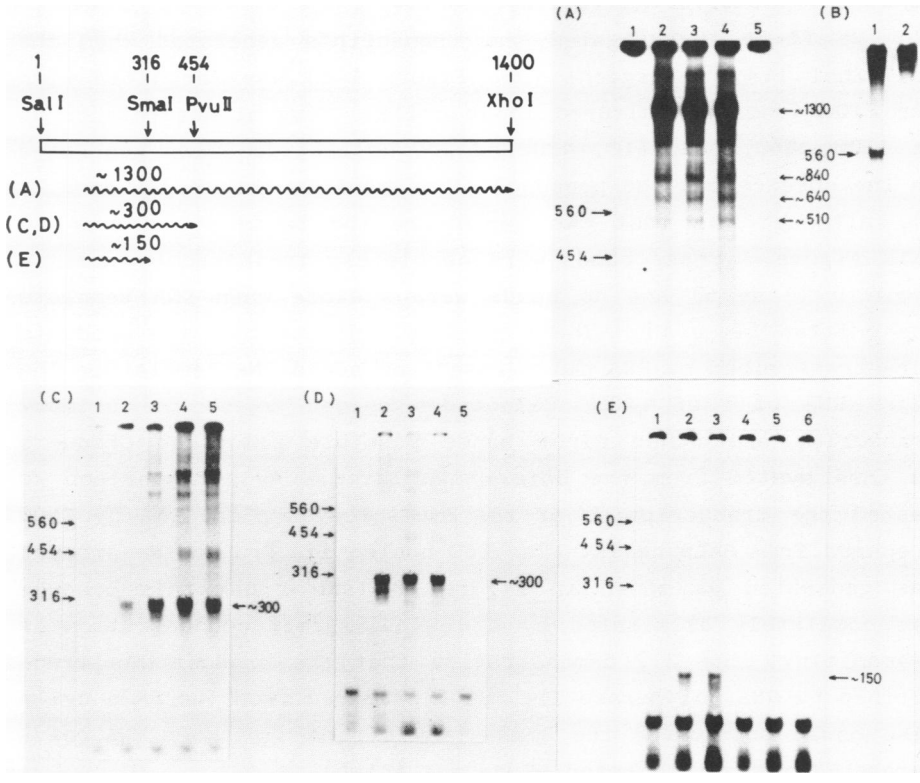

Figure 2. Electrophoretic analysis of RNAs transcribed *in vitro* from various truncated DNA templates.
(A) The SalI/XhoI 1.4 kb DNA template. lane 1: no DNA, lane 2: complete system without α-amanitin, lanes 3 and 4: plus 1 μg/ml and 200 μg/ml α-amanitin, respectively, lane 5: plus 20 μg/ml actinomycin D. 16 μg/ml DNA template and α-^{32}P-CTP were used.
(B) The pSmaF DNA template. lane 1: complete system for RNA polymerase II (5) with S100 fraction from HeLa cells, lane 2: plus 1 μg/ml α-amanitin.
(C) and (D) The PvuII cleaved SalI/XhoI 1.4 kb DNA template.
(C) Effect of DNA concentraion. DNA concentrations were 4,8,16, 24 and 32 μg/ml for lanes 1-5, respectively, plus 200 μg/ml α-amanitin. (D) The same conditions as in (A) for lanes 1-5, respectively, except for 14.4 μg/ml of DNA and α-^{32}P-UTP were used.
(E) The SmaI cleaved SalI/XhoI 1.4 kb DNA template. lanes 1-3: 2,4 and 6 μg/ml of SalI/SmaI 0.316 kb DNA fragment, respectively, lanes 4-6: 7.5, 15 and 22.5 μg/ml of SmaI/XhoI 1.1 kb DNA fragment, respectively, plus 200 μg/ml α-amanitin.

actinomycin D-insensitive, was observed when α-^{32}P-UTP was used as a radioactive precursor (lane 1-5). Similar spots had been noted by others and tentatively identified as 5S RNA to which labeled UMP was added at the 3'-end (25,26).

The above experiments strongly suggested that transcription was carrried out by RNA polymerase I and started at about 300 bp upstream the PvuII site of this truncated template. In order to confirm this, we next used another truncated template of this region. Here, we cleaved the SalI/XhoI 1.4 kb fragment by SmaI, whose site was located 138 bp upstream from the PvuII site (Figure 1-c), and the two fragments were separated by agarose gel to be used for DNA template. As shown in Figure 2E lane 1-3, about 150 nucleotide RNA was generated when SalI/SmaI 0.316 kb fragment was incubated with S100. The size of this product is exactly what is expected when the transcription started from the same site as determined by the previous templates. No bands were observed from SmaI/XhoI 1.1 kb fragment at three different concentrations of DNA (lane 4-6) suggesting that transcription did not start within this fragment in this *in vitro* system. Here also 120 nucleotide product was seen in every lane, showing their DNA-independent, α-amanitin-insensitive and actinomycin D-insensitive nature. Judging from the relative intensity of 150 nucleotide band to the 120 nucleotide product and the film exposure time (Figure 2D was 2 days and Figure 2E was 7 days), transcription efficiency was apparently lower when the SalI/SmaI 0.316 kb fragment was used than when PvuII cleaved SalI/XhoI 1.4 kb DNA was used as DNA template.

"Run-off" RNA products obtained with three different truncated DNAs thus indicate that rRNA transcription starts from about 170 bp downstream from the left-hand SalI site of the SalI 3.2 kb fragment (see Figure 1-c). This initiation site is about 650 bp upstream from the putative initiation site previously reported by us (1), and about 450 bp upstream reported by Grummt (13) and Bach *et al.*(2).

S1 nuclease protection mapping of the rDNA transcripts.

To verify the initiation point and orientation of the *in vitro* transcripts, we examined the ^{32}P-RNAs which had been transcribed from SalI/XhoI 1.4 kb DNA and from PvuII cleaved SalI/

XhoI 1.4 kb fragment by the technique of S1 protection mapping.

As shown in Figure 3A lane 2, several DNA-RNA hybrids of 1300, 840, 640, 510, 420, and 320 bp were protected from S1 nuclease digestion, when ^{32}P-RNA, which was synthesized on SalI/XhoI 1.4 kb DNA, was hybridized with the same fragment used for the template. ^{32}P-RNA alone did not produce any protected bands (lane 1) (See Discussion for detailed explanation of the bands).

On the contrary, when the product transcribed on PvuII

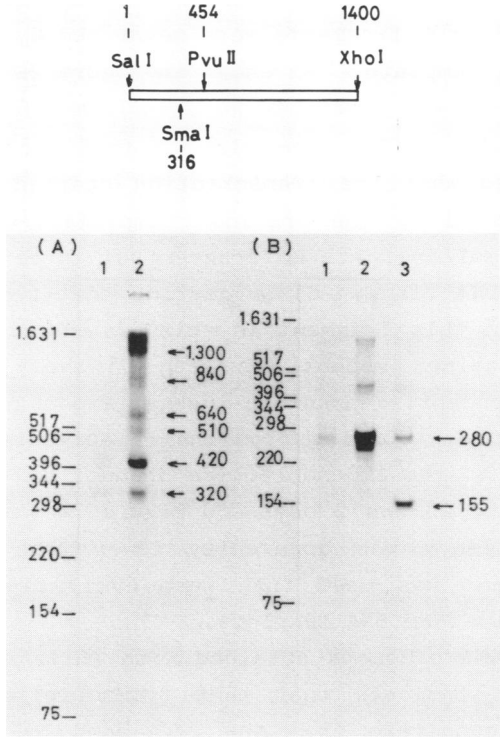

Figure 3. S1 nuclease protection mapping of *in vitro* transcripts. ^{32}P-RNA was synthesized in the standard reaction mixture containing 200 µg/ml α-amanitin and 100 µCi α-^{32}P-UTP. Templates used were SalI/XhoI 1.4 kb DNA (A) and PvuII cleaved SalI/XhoI 1.4 kb DNA (B). After treatment with iodoacetate-treated DNase I (27), ^{32}P-RNA was hybridized with the following DNAs; (A) lane 1, no DNA. lane 2, 0.5 µg of SalI/XhoI 1.4 kb DNA. (B) lane 1, no DNA. lane 2, 0.5 µg of PvuII cleaved SalI/XhoI 1.4 kb DNA. lane 3, 0.1 µg of SalI/SmaI 0.316 kb DNA. The hybrids were treated with 50 units of S1 nuclease, electrophoresed on 4% (A) and 5% (B) of polyacrylamide gel and autoradiographed. Size markers are HinfI cleaved pBR322 DNA.

cleaved SalI/XhoI 1.4 kb template was hybridized with the same DNA used for the template, only one band of 280 bp was detected as shown in Figure 3B lane 2. When ^{32}P-RNA was hybridized with SalI/SmaI 0.316 kb fragment, the size of the protected band decreased to 155 bp (lane 3). The weaker band of 280 bp probably represents the hybrid produced during transcription. ^{32}P-RNA alone did not give rise to any protected band when treated with S1 nuclease (lane 1) except for the weak 280 bp band as seen in lane 3. These results confirm that the transcription of rRNA starts from a unique site, which is indicated by the sizes of RNAs transcribed with different truncated DNA templates (Figure 2).

To locate the initiation sites of *in vitro* transcript and of *in vivo* 45S RNA more precisely, S1 nuclease protection mapping was carried out together with Maxam-Gilbert sequence ladder (Figure 4). A 316 bp SalI/AvaI fragment (AvaI recognition site is the same as SmaI) labeled at 5'-end of coding strand was recovered from 4% polyacrylamide gel and hybridized with either 45S RNA (lane 1) or *in vitro* transcripts which were generated in the presence (lane 2) or absence (lane 3) of PvuII cleaved SalI/XhoI 1.4 kb fragment. The S1 nuclease protected DNA fragments were electrophoresed in a sequence gel in parallel with the same 316 bp SalI/AvaI fragment cleaved by Maxam-Gilbert method. Protected bands appeared when either *in vivo* 45S RNA or *in vitro* synthesized RNA with the DNA template was used for protection. *In vitro* product in the absence of template did not give rise to any protected bands. The sizes of DNA protected by *in vivo* 45S RNA and by *in vitro* transcript were the same, indicating that *in vivo* and *in vitro* transcribed rRNA started from the same site. The 5'-end of these molecules comes to a T residue corresponding to -650 nucleotide reported previously (1), when corrections of $1\frac{1}{2}$ nucleotides were made according to Sollner-Webb and Reeder (28). Althought the first nucleotide of transcription initiation cannot be pinpointed by this experiment alone due to the inherent limitations of this technique, we conclude that the transcription of rRNA gene starts within a few nucleotides around this T residue (see Discussion).

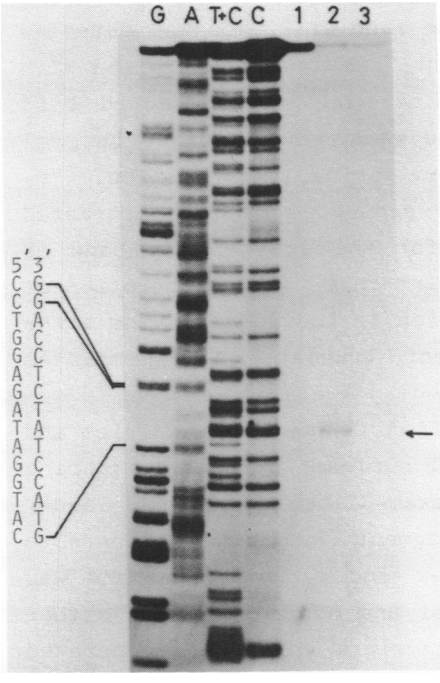

Figure 4. Localization of the transcription initiation site of *in vivo* and *in vitro* rRNAs by S1 nuclease protection mapping with sequencing ladders. 5'-end labeled coding strand of SalI/AvaI 0.316 kb fragment was hybridized with *in vivo* 45S RNA (lane 1) and *in vitro* transcription product which was generated in the presence (lane 2) or absence (lane 3) of PvuII cleaved SalI/XhoI 1.4 kb fragment. After digestion with 50 units of S1 nuclease, the protected fragments were denatured and electrophoresed on a 8% polyacrylamide/7M urea sequnding gel along with the same labeled fragments cleaved by base specific chemical modifications. Nucleotide sequences of the coding and non-coding strands are shown to the left of the gel. The sequence gel shown is not good in the important region - the bands in the A track are difficult to ascertain and the bands in the pyrimidine tracks are not all well separated. However there is no doubt from comparison with many other gels that this sequence is as designated. The sequence shown corresponds to -662 to -648 reported by Urano *et al.* (1). Arrow indicates the protected fragments.

DISCUSSION

In this paper, we described an *in vitro* system in which a cloned ribosomal RNA gene of the mouse was transcribed apparently faithfully with a mouse cell extract. Judging from the resistance to a high concentration of α-amanitin, the transcription must be mediated by RNA polymerase I. The system required an optimal concentration of template DNA (approx. 1 pmole/ 50 µl under these conditions) in order to suppress the *noise* which was caused mainly by the end-to-end transcription of the template.

Using this system, we estimated the initiation site of *in*

vitro rRNA transcription and compared it with that of *in vivo* 45S RNA. The largest *in vitro* transcript and the 45S RNA were found to start from the same site, suggesting strongly that this *in vitro* transcription system mimicked *in vivo* situation rather faithfully. The initiation site determined by the present technique was about 650 and 450 nucleotides upstream, respectively, from those reported by us (1) and Bach *et al.* (2). These sites in fact appear to be early processing sites, since the bands corresponding in size to transcripts starting from these sites could be detected in Figures 2(A) and 3(A). The dense band of 420 nucleotides in Figure 3(A) might be a processing product cleaved from the 1.3 kb primary transcript to produce 840 nucleotide intermediate. The abnormal intensity of this band may be explained by the high U content of this fragment due to the presence of U clusters. They do not seem to be extra initiation sites because these transcripts are not produced when SmaI/XhoI 1.1 kb fragment is used as template, albeit it has these sites (Figure 2E lane 4-6). In addition, preliminary experiments showed that the radioactivity in the largest band was chased out into bands of 840 and 640 nucleotides (data not shown). Whether or not other smaller bands are also natural processing products remains to be determined.

In our previous study (1), the major population of 45S RNA that was used for S1 nuclease mapping must have already been processed, having the 5'-terminus some 650 nucleotides shorter than the primary transcript. In the same vein, the initiation site reported by Grummt (13) and Bach *et al.*(2) also seems to be one processing site, although the whole processing scheme must be re-examined in more detail to clarify all these previous results.

In any event, both the faithful transcription initiation by RNA polymerase I and some initial stage of processing of the transcript seem to be taking place in this system, making it promising for further analysis mentioned in INTRODUCTION. We did not conclude the precise nucleotide from which the transcription started because, first there is some ambiguity in the determination by S1 nuclease protection mapping, second, starting from a pyrimidine (U) is rather unusual according to the

present knowledge of other transcription initiation. Structural analysis of the primary transcript as well as *in vivo* 45S RNA with respect to the 5'-ends are required to determine this point and are now under way.

When one compares the nucleotide sequence of the initiation region of the mouse with those of *Xenopus laevis* and *Drosophila melanogaster*, whose initiation sites are already identified by structural analysis of the transcripts (28,29), purine-TAGG near the initiation site and the CTTT at around -30 appear to be common to all these three genes. In fact, this sequence (CTTT) was also found in human as well as rat rDNA at a similar position (Financsek *et al.*, in preparation). Whether or not these sequences are the signals for transcription initiation has to be determined by using deletion mutants of these regions. Such studies are now in progress.

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