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A novel promoter in the mouse rDNA spacer is active *in vivo* and *in vitro*

Anne Kuhn and Ingrid Grummt

Institut für Biochemie der Universität Würzburg, Röntgenring 11, 8700 Würzburg, FRG

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We have identified a novel RNA polymerase I (pol I) transcription initiation site within the 'non-transcribed' spacer of mouse rDNA. This spacer promoter is located about 2 kb upstream of the 45S pre-rRNA promoter and directs specific transcription initiations both in a cell-free system using truncated templates and *in vivo* after transfection into mouse cells. The spacer promoter contains an 11 out of 16 bases match to the core element of the major ribosomal gene promoter and is oriented in the same direction. It exerts a significantly lower transcriptional activity as compared to the 45S pre-rRNA promoter. The elongation of transcripts initiated at the spacer promoter is stopped at a termination signal located 170 bp upstream of the pre-rRNA start site. Since it has been previously shown that, in addition to its terminator function, the same sequence motif acts as an upstream element of the adjacent gene promoter, the function of the spacer promoter may be to capture free pol I molecules and drive them to the gene promoter in order to achieve the high level of transcription characteristic of eukaryotic rRNA genes.

Keys words: ribosomal genes/promoter/spacer/cell-free transcription/upstream terminator

Introduction

The spacer regions separating the tandemly repeated ribosomal RNA genes differ profoundly, both in length and sequence, even in closely related species (Brown *et al.*, 1972; Schibler *et al.*, 1975). Increasing experimental evidence has demonstrated that the spacers serve important biological functions. They contain essential signal sequences required for transcription initiation (Grummt, 1981; Sollner-Webb *et al.*, 1983; Skinner *et al.*, 1984; Kohorn and Rae, 1982; Yamamoto *et al.*, 1984; Learned *et al.*, 1983), transcription termination signals (Grummt *et al.*, 1985, 1986a and b; Labhard and Reeder, 1986), enhancer-like sequences (Moss, 1983; Labhard and Reeder, 1984; Elion and Warner, 1984, 1986; De Winter and Moss, 1986a; Cassidy *et al.*, 1986), and hotspots for recombination (Voelkel-Meiman *et al.*, 1987). Furthermore, the rDNA spacer of both *Xenopus laevis* and *Drosophila melanogaster* has been shown to contain duplicated RNA polymerase I 'spacer promoters' whose biological function is still unknown (Kohorn and Rae, 1982; Moss, 1983; Morgan *et al.*, 1983; Miller *et al.*, 1983; Murtif and Rae, 1985). It has been suggested that the spacer promoters and the repetitive 60/81 bp elements present in the rDNA spacer of *X. laevis* deliver RNA polymerase I (pol I) or transcription factors to the major promoter thus facilitating the high loading density of pol I at the ribosomal transcription unit (Moss, 1983). On the other hand, both in frog and Drosophila the entire ribosomal gene repeating unit appears to be transcribed as part of the primary transcript except for about 200 bp upstream of the transcription start site (Labhard and Reeder, 1986; De Winter and Moss, 1986b; Tautz and Dover, 1986). It is therefore difficult to imagine exactly how extra spacer transcription may contribute to the efficiency of pre-rRNA transcription initiation.

Recently we have shown that in the mouse rDNA transcription unit 18 bp repeated elements (termed '*Sal*I box') in the 3′ terminal spacer starting about 585 bp downstream of the 28S RNA terminus constitute a termination signal for pol I (Grummt *et al.*, 1985, 1986a). An apparently structurally and functionally analogous '*Sal*I box' is also located nearly 30 000 bp further downstream at position −171, just upstream of the gene promoter (Grummt *et al.*, 1986b). The presence of this upstream terminator (T_0) whose sequence and location relative to the transcription initiation site is highly conserved in several mammals has been shown to be required for efficient initiation at the adjacent gene promoter (Grummt *et al.*, 1986b; Henderson and Sollner-Webb, 1986; McStay and Reeder, 1986) and thus appears to constitute an essential upstream promoter element.

In addition to its properties as promoter element the upstream terminator also serves a function in terminating spacer transcripts. In this study we show that these spacer transcripts have been started from a promoter located about 2 kb upstream of the pre-rRNA initiation site. We report on the structural and functional characterization of this novel mammalian spacer promoter and compare its properties with the major ribosomal gene promoter of mouse.

Results

A 5′ terminal spacer fragment directs pol I-transcription initiation in vitro

In previous studies we have analyzed the distribution of elongating pol I molecules along different regions of the ribosomal transcription unit by nucleolar run-on experiments. We have shown that there is little if any transcription in the 3′ spacer extending approximately 3700 bp downstream of the termination site, but significant levels of RNA hybridizing to 5′ terminal spacer fragments which encompass a region of about 5.6 kb preceding the pre-rRNA initiation site. This discontinuity in the distribution of elongating pol I molecules in different parts of the spacer suggests that transcription initiation must occur within the 5′ spacer. Since sequences contained in pMrD, a plasmid covering the spacer region from −170 to −1894, are not transcribed under conditions optimal for utilization of the authentic rRNA promoter (Wood *et al.*, 1984; Grummt *et al.*, 1986b) we have investigated the ability of the adjacent spacer region to promote transcription initiation in the cell-free system. The plasmid pMrA contains a 3.8 kb rDNA insert extending from an *Eco*RI site ~5700 bp upstream of the initiation site to a *Sal*I site at position −1895 (Figure 1A). In the cell-free transcription system truncated transcripts with apparent mol. wts of ~80 and 260 nucleotides are synthesized from pMrA after cleavage with *Hin*dIII or *Pvu*II, respectively (Figure 1B, lanes 1 and 3). The lengths of these run-off transcripts place the initiation site of spacer transcripts about

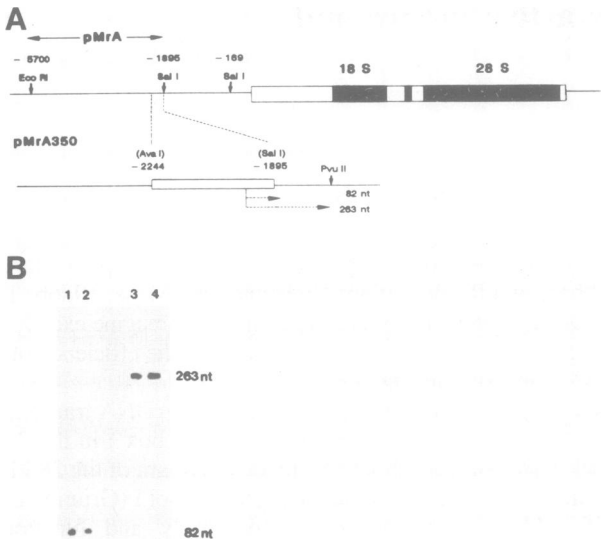

Fig. 1. (**A**) Schematic representation of the mouse rDNA gene unit and the spacer regions contained in the plasmids pMrA and pMrA350.
(**B**) Transcripts synthesized in the cell-free system in the presence of 300 ng pMrA (**lanes 1** and **3**) or pMrA350 (**lanes 2** and **4**) that have been truncated with *Hin*dIII (**lanes 1** and **2**) or *Pvu*II (**lanes 3** and **4**).

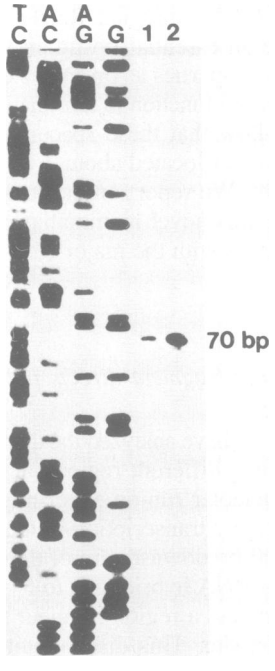

Fig. 2. S1 nuclease mapping of spacer transcripts. Transcripts were synthesized in a 125 μl assay containing 1 μg of template DNA pMrA350 truncated with *Hin*dIII (**lane 1**) or *Pvu*II (**lane 2**). The RNA was hybridized at 51°C to a 164 bp *Bst*NI−*Sal*I fragment (−2059 to −1895) derived from pMrA that had been 5′ labelled at the *Sal*I site. After S1 nuclease treatment the protected fragment was analyzed on 6% sequencing gels. For precise sizing of the protected fragment, probe DNA was subjected to chemical sequence analysis (Maxam and Gilbert, 1980) and run in parallel to the S1 treated fragments. The G, G+A, C+A and T+C refer to the base specificities of the cleavage reactions.

60−70 bp upstream of the *Sal*I site of the 3.8 kb spacer fragment. We then constructed a subclone which contains a 349 bp *Ava*I−*Sal*I fragment (from −2244 to −1895) (Figure 1A). This subclone pMrA350 yields identical run-off transcripts as pMrA (Figure 1B, lanes 2 and 4) indicating that the sequences responsible for the synthesis of spacer transcripts are contained within this 350 bp region.

5′ end mapping of spacer transcripts synthesized in vitro

To map the start site of spacer transcripts at the nucleotide level S1 nuclease protection experiments were performed. RNA synthesized *in vitro* from pMrA350 were hybridized to a 5′ labelled 164 bp *Bst*NI−*Sal*I (−2059 to −1895) fragment derived from pMrA. The mobility of S1 nuclease-resistant fragments was compared with a Maxam−Gilbert sequencing ladder of the same DNA on high resolution gels (Figure 2). The position of the protected band indicates that spacer transcripts have been started with an adenine at position −1960.

The spacer promoter shares sequence homology to the pre-rRNA promoter

In order to identify the nucleotide sequences which are required for initiation of spacer transcripts and to find out whether the spacer promoter shares structural homologies to the major promoter, the nucleotide sequence of the subclone pMrA350 was determined. The sequence was compared with the corresponding region of the rat rDNA spacer (Financsek *et al.*, 1986) and with the mouse core promoter — a region of about 50 bp adjacent to and including the major transcription initiation site (Figure 3A and B). There is a striking homology of about 75% between the mouse and the rat sequence, indicating a strong selective pressure to conserve these sequences in both species. An alignment of sequences preceding the initiation site of pre-rRNA and spacer transcripts, respectively, reveals an 11 out of 16 bases match from positions −3 to −18 with respect to the transcription start site. This region immediately upstream of the initiation site has previously been shown to be essential for the assembly of functional transcription complexes (Clos *et al.*, 1986a). A detailed mutational analysis of the core promoter has revealed two functionally distinct domains, a distal sequence motif from −22 to −16 which is recognized by the species-specific initiation factor TIF-IB, and a proximal motif from −15 to −1 which appears to play a role in the assembly of functional initiation complexes (Clos *et al.*, 1986b). Whereas most point mutations within the core promoter have little or no effect on template activity, substitutions of the guanines at −7 and −16 severely impair transcription. The proximal part of the core promoter (including the essential G at −7) is well conserved in the spacer promoter; the distal part, however, shows less sequence conservation except the functionally important bases at −16 and −18. These differences in the nucleotide sequence may be responsible for the low transcriptional activity of the spacer promoter. It is also worth noting that the sequence GGAGATAG of the proximal part of the mouse rDNA core promoter (from −10 to −3) is not only conserved at a similar position in front of the spacer transcription initiation site but is also found further upstream at positions −2088 and −2019 relative to the major start site. The functional relevance of this sequence conservation is not yet clear.

Transcriptional activity of the spacer promoter in vivo and in vitro

The limited sequence homology of the spacer promoter to the pre-rRNA promoter implies a different ability to direct transcription initiation. To quantitatively compare the strength of both promoters, their transcriptional activities were determined *in vitro* and *in vivo*. Increasing concentrations of template DNA containing either the authentic pre-rRNA promoter (pMr600/*Eco*RI) or the spacer promoter (pMrA350/*Pvu*II) were transcribed in the extract system and the amount of run-off transcripts was determined by densitometry. As shown in Figure 4A, the two con-

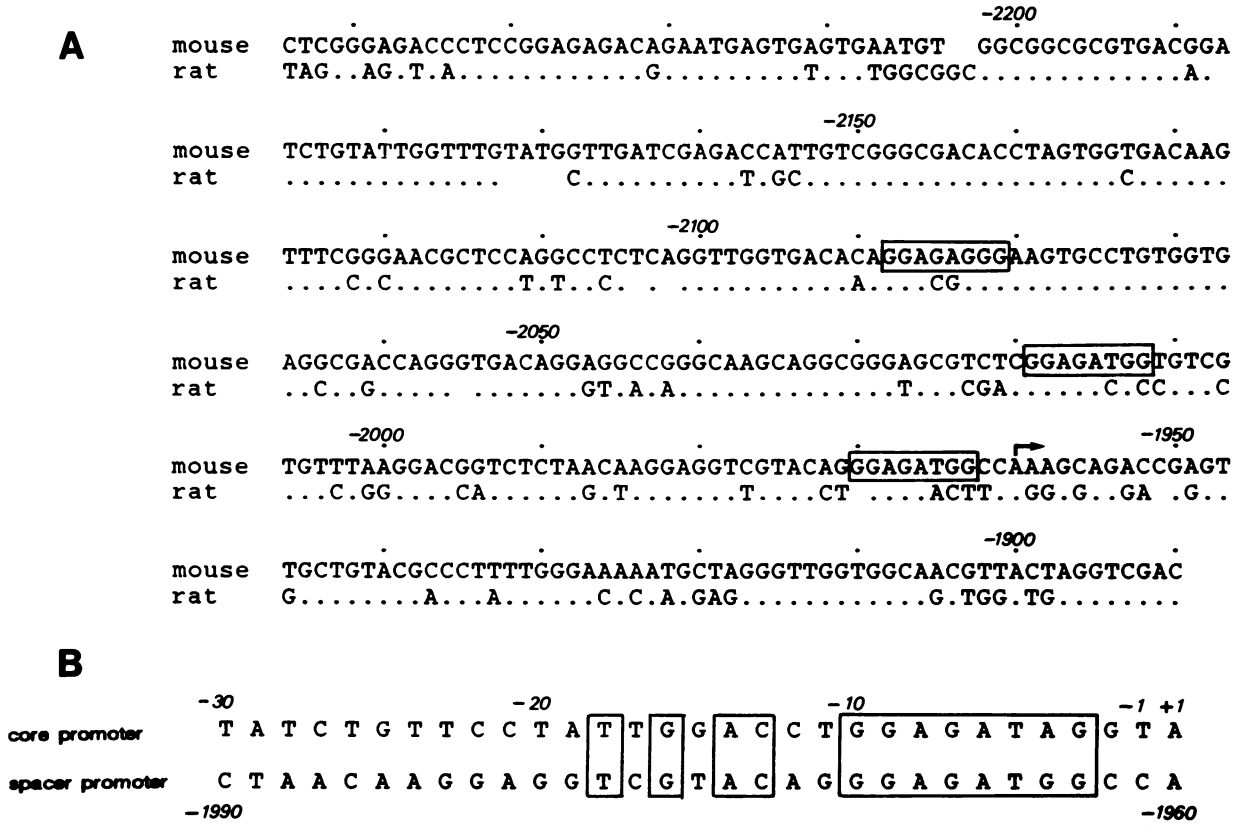

Fig. 3. Nucleotide sequence of the spacer promoter. (**A**) The DNA sequence of the 349 bp *AvaI*−*SalI* fragment from the mouse rDNA spacer is shown and compared with the corresponding sequence from rat (Financsek *et al.*, 1986). Numbers refer to the position of nucleotides with respect to the 45S pre-rRNA initiation site. Nucleotides that are identical in the mouse and rat sequence are indicated with dots, the GGAGAT$^{A}_{G}$G motif which is homologous to nucleotides −10 to −3 of the mouse rDNA promoter is boxed. The arrow marks the 5′ end of spacer transcripts. (**B**) Comparison of the core sequence of the 45S pre-rRNA promoter and the spacer promoter. Homologous nucleotides are boxed.

structs require different DNA concentrations to reach maximal transcriptional activity. The amount of RNA synthesized at optimal DNA concentrations is ~10-fold higher from pMr600 as compared to pMrA350. A less pronounced difference in promoter strength was observed *in vivo*. A fragment from the bacterial chloramphenicol acetyltransferase (CAT) gene was fused to the two promoters yielding the recombinant plasmids pMrCAT-2 and pMrA350-CAT, respectively. These constructs were transfected into 3T6 cells together with pSV2-CAT DNA as internal marker. The transcripts were mapped by primer extension of a synthetic oligonucleotide from the CAT-coding region (Figure 4B). RNA that has been initiated at the pre-rRNA start site yields a 222 nucleotide cDNA product, RNA molecules derived from the spacer promoter generate 146 nucleotide reverse transcripts. Quantitation of the rDNA specific cDNAs reveals a 2- to 3-fold stronger signal in RNA from cells transfected with pMrCAT-2 than cells transfected with pMrA350-CAT.

For a more precise evaluation of the amounts of transcripts synthesized *in vivo* from the spacer as compared to the gene promoter, quantitative hybridization experiments were performed. Two concentrations of RNA derived from cells transfected with pMrCAT-2 and pMrA350-CAT, respectively, were immobilized onto nitrocelllose filters and hybridized to a labelled CAT-probe. As shown in Figure 4C there are clear differences in the hybridization signals from both RNAs demonstrating a 60% reduced transcriptional activity of the spacer promoter as compared to the gene promoter. Interestingly, this difference in the strength of both promoters is much less pronounced *in vivo* than *in vitro*. This may be due to the fact that the assay conditions of the *in vitro* system which have been optimized for maximal transcription from the gene promoter may not be optimal for the spacer promoter.

Transcripts initiated at the spacer promoter terminate at the upstream termination site T_o

Previously we have shown that a transcriptional terminator is located in close proximity of the ribosomal gene promoter and that this upstream terminator is required for efficient pre-rRNA synthesis (Grummt *et al.*, 1986b). The presence of this terminator in front of the transcription start site suggests that in addition to its effect on the promoter activity it may play a role in terminating spacer transcripts. To demonstrate that in fact pol I molecules that have started at the spacer promoter are stopped at the upstream site T_o, the spacer sequences contained in pMrA350 were fused to a rDNA fragment encompassing sequences from −328 to +292 with respect to the pre-rRNA initiation site including the T_o-box extending from −171 to −154. *In vitro* this truncated template (pA350P600/*SmaI*) directs the synthesis of three distinct transcripts 155 nucleotides, ~220 nucleotides and 562 nucleotides long (Figure 5B). The 155 nucleotide RNA represents run-off transcripts initiated at the pre-rRNA start site, the 220 nucleotide molecules are transcripts initiated at the spacer promoter and terminated at the upstream site T_o, and the 562 nucleotide bands are transcripts that did not terminate at T_o and therefore run off the template at the *SmaI* site (Figure 5A). The relative intensities of the bands representing terminated and readthrough spacer transcripts varied between different experiments depending on the extract and the template con-

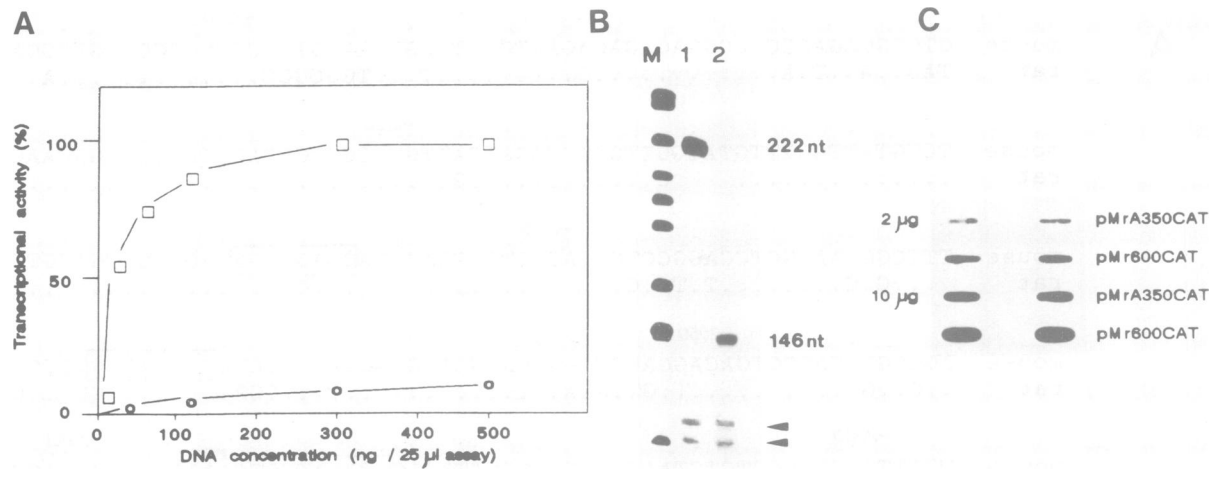

Fig. 4. Comparison of the strength of the spacer and the 45S rRNA promoter. (**A**) Different amounts of template DNA pMr600/*Eco*RI (□----□) and pMrA350/*Pvu*II (○----○) were transcribed in the *in vitro* system and the amount of the 297 nucleotide and 263 nucleotide run-off RNAs were determined by densitometry. The relative transcriptional activities (in percent) were plotted against the DNA concentration in the assays. (**B**) Primer extension analysis of RNA synthesized *in vivo* from rDNA-CAT fusion genes. 20 µg of pMrCAT-2 (**lane 1**) or pMrA350-CAT (**lane 2**) were co-transfected with 2.5 µg of pSV2-CAT into 3T6 cells and the transient expression of CAT-specific RNA was measured by primer extension. 50 µg of cellular RNA were hybridized to a 20 nucleotide 5′ labelled primer which is complementary to nucleotides 4936 to 4955 from pSV2-CAT (Gorman, 1985). Hybridization was performed for 3 h at 37°C and primer extension was carried out as described before (Grummt and Skinner, 1985). The arrows indicate the positions of the reverse transcripts derived from the SV40 early cap sites. Pol I-specific transcripts initiated at the gene promoter are 222 nucleotides long, transcripts initiated at the spacer promoter are 146 nucleotides long. (**C**) Quantitation of transcripts expressed in cells transfected with pMrA350-CAT and pMrCAT-2. 3T6 cells were transfected with 20 µg of pMrA350-CAT and pMrCAT-2, respectively. 2 µg or 10 µg of cellular RNA were applied in duplicates onto nitrocellulose filters using the Schleicher and Schuell Minifold II apparatus. Hybridization was for 20 h at 42°C against the nick-translated 1632 bp *Hin*dIII−*Bam*HI fragment from pSV2-CAT.

centration used. This is due to the fact that efficient termination requires low amounts of template DNA and high levels of termination factors (Grummt *et al.*, 1985, 1986a).

Next we studied whether the termination of spacer transcripts at site T_0 can also be demonstrated *in vivo*. The construct pA350P600 was transfected into 3T6 cells and its transient expression was analyzed by primer extension and S1 protection assays. To map the 5′ end of spacer transcript a 72 bp *Dde*I−*Sau*3A fragment (from −247 to −318) was hybridized to RNA extracted from transfected cells and used as primer for a reverse transcriptase reaction. As shown in Figure 5C, both RNA synthesized *in vivo* or *in vitro* yielded an 165 bp cDNA indicating that transcripts with identical 5′ ends are synthesized *in vivo* and *in vitro*.

The 3′ end of spacer transcripts was mapped by hybridization of RNA to a 3′ labelled *Bam*HI−*Hin*dIII fragment and analysis of the S1 nuclease-resistant hybrids on a sequencing gel (Figure 5D). Transcripts synthesized both *in vitro* or in transfected cells yielded identical hybridization signals which map the 3′ end of spacer RNA to position −185. The 3′ ends of RNA synthesized both *in vivo* and *in vitro* are very heterogeneous. This suggests that either termination at T_0 yields RNA with heterogeneous 3′ ends or spacer transcripts are preferentially degraded.

Discussion

We have identified a novel promoter in the spacer region of mouse rDNA which directs pol I-specific transcription initiation 1960 bp upstream of the 45S pre-rRNA initiation site. This spacer promoter is functionally active both in a crude or partially purified (not shown) cell-free transcription system and after transfection into 3T6 cells. In both assay systems the spacer promoter exerts a lower strength as compared to the authentic gene promoter. A comparison of the sequences preceding the two initiation sites reveals an 11 out of 16 bases match from positions −3 to −18 relative to the transcription start site. This region includes two evolutionarily conserved guanines at positions −7 and −16 which have been shown to serve an important function in the assembly of pre-initiation complexes (Clos *et al.*, 1986b). Unexpectedly, the distal region of the core promoter (from −16 to −22), which is recognized by the mouse rDNA-specific transcription initiation factor TIF-IB, shows more sequence variation than the proximal part (from −3 to −10). Probably this sequence difference accounts for the weak transcriptional activity of the spacer promoter. When we compared the sequence of the spacer fragment with the analogous region from the rat rDNA spacer (Financsek *et al.*, 1986) we found a remarkable sequence conservation between both species. In contrast, there is no significant homology in the spacer region of both species beyond the 3′ end of 28S RNA and the site of transcription termination 565 bp further downstream (Grummt *et al.*, 1985; Yavachev *et al.*, 1986) which indicates a different selective pressure to maintain different spacer sequences between related species. We conclude that this striking sequence conservation about 2 kb upstream of the gene promoter reflects an important biological function of this gene region and predict that the analogous region from the rat gene may also contain a functional promoter.

So far, spacer promoters have been identified in yeast (Swanson *et al.*, 1985), Drosophila (Kohorn and Rae, 1982; Miller *et al.*, 1983) and all three Xenopus species (Morgan *et al.*, 1983; Bach *et al.*, 1981), but not in mammals. The yeast spacer promoter maps to a position 2.2 kb upstream from sequences which encode the 5′ terminus of the major 35S rRNA precursor. A 190 bp region of yeast spacer rDNA which contains this promoter sequence has been shown to 10- to 30-fold enhance the synthesis of 35S pre-rRNA (Elion and Warner, 1984 and 1986). It is not yet clear whether the spacer promoter plays a role in this transcriptional enhancement, or whether different sequences independently direct the synthesis of spacer transcripts and the enhancement of pre-rRNA synthesis.

More detailed studies to establish the precise relationship bet-

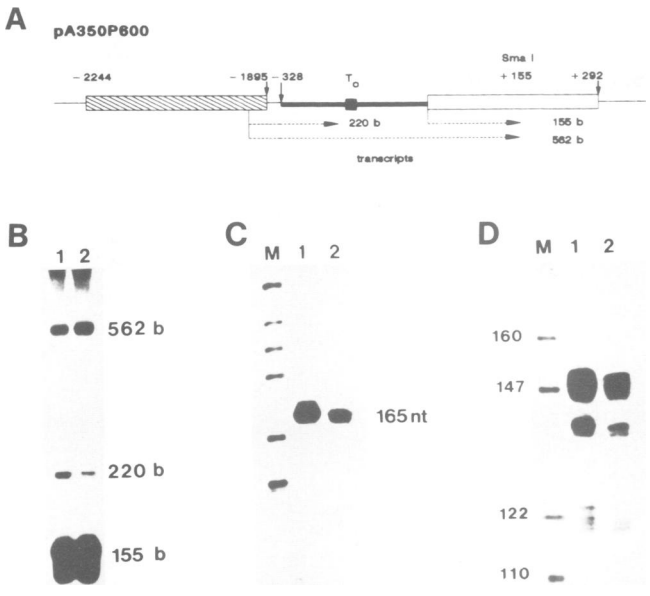

Fig. 5. Termination of spacer transcripts. (**A**) Diagram of the plasmid pA350P600. The region encompassing the 349 bp *AvaI*−*SalI* spacer fragment is represented by the hatched bar, the 292 bp of the coding region by the open bar. The thicker line marks 328 bp of spacer sequences which flank the pre-rRNA initiation site and contain the upstream termination site T_0. The transcripts generated from this DNA are indicated by dotted lines. The numbers mark the length of transcripts synthesized *in vitro*. (**B**) Autoradiogram of *in vitro* transcripts derived from pA350P600. pA350P600 DNA was linearized with *SmaI* and 30 μg (**lane 1**) and 60 μg (**lane 2**) of template DNA were incubated in the standard transcription assay containing a mixture of S-100 and nuclear extracts. The numbers mark the lengths of the transcripts. (**C**) 5′ end mapping of spacer transcripts synthesized *in vitro* and *in vivo*. Transcripts synthesized in a 100 μl transcription assay in the presence of 120 ng of pA350P600 (**lane 1**), or 50 μg RNA isolated from cells transfected with the same plasmid (**lane 2**), were hybridized to a 72 bp 3′ labelled *DdeI*−*Sau*3A fragment (from −247 to −318) derived from pMr600. After denaturation at 75°C for 5 min hybrids were formed by incubation for 3 h at 43°C. The hybrids were transcribed by reverse transcriptase and the cDNAs were analyzed on a 6% polyacrylamide/urea gel. (**D**) 3′ end mapping of spacer transcripts synthesized *in vitro* and *in vivo*. The same transcripts used for 5′ mapping were hybridized to a 642 bp *Bam*HI−*Hin*dIII fragment derived from pA350P600 which had been 3′ labelled at the *Bam*HI site. After hybridization for 3 h at 54°C the hybrids were treated with nuclease S1 and analyzed on a 6% sequencing gel. **Lane 1**: transcripts synthesized *in vitro* from pA350P600, **lane 2**: RNA from cells transfected with pA350P600.

ween the rDNA spacer sequences required for initiation and those required for enhancing pre-rRNA synthesis have been carried out with the rDNA spacer of *X. laevis*. The duplicated spacer promoters and an array of repeated 60/81 bp promoter-related sequences which enhance transcription from the 40S pre-rRNA promoter have been shown to act together. Whether or not enhancing activity can be uncoupled from spacer promoter activity is still a matter of dispute (Labhard and Reeder, 1984; De Winter and Moss, 1986a).

The biological function of the promoter in the mouse rDNA spacer is not yet known. It is conceivable that pol I molecules that have transcribed one rDNA unit can be captured by the spacer promoter(s) and passed to the next gene unit. This process of 'readthrough enhancement' (Moss, 1983) may be facilitated by the upstream terminator T_0 which appears to serve a dual function. It stops polymerases that have been initiated within the spacer region and, in addition to its terminator function, acts as an upstream element of the adjacent ribosomal gene promoter (Grummt *et al.*, 1986b; Henderson and Sollner-Webb, 1986). Both effects are mediated by a nuclear protein that binds to the termination signal sequence, the '*SalI* box'. Probably a mechanism exists for passing the polymerase from the upstream terminator to the promoter without releasing it from the DNA. However, the precise molecular mechanism by which both termination of spacer transcripts and the enhancing effect on the initiation reaction is brought about remains to be established.

Materials and methods

rDNA plasmids

The plasmid pMrA contains a ~3.8 kb *Eco*RI−*SalI* fragment from the mouse rDNA spacer covering sequences from −1895 to about −5700 relative to the transcription start site at +1. A subclone extending from the *AvaI* site at −2244 to the *SalI* site at −1895 was designated pMrA350 (Figure 1A). The 45S pre-rRNA promoter is contained in clone pMr600 extending from −328 to +292 with respect to the initiation site. Truncation with *SmaI* or *Eco*RI yields 155 nucleotide or 297 nucleotide run-off RNAs in the cell-free system. A fusion of the sequences contained in pMrA350 and pMr600 was obtained by insertion of the 620 bp *PvuII* fragment into the *HindII* site of pMrA350 resulting in the recombinant plasmid pA350P600 (Figure 5A). pMrCAT-2 has been described before (Grummt and Skinner, 1985). It contains rDNA sequences from −171 to +155 fused to the *Hin*dIII−*Bam*HI fragment from pSV2-CAT. The analogous construct with the spacer promoter is pMrA350-CAT. Here the CAT-coding region is inserted into the *Hin*dII site of pMrA350.

In vitro transcription assay

S-100 and nuclear extracts were prepared from cultured Ehrlich ascites according to Weil *et al.* (1979) and Dignam *et al.* (1983). For *in vitro* transcription, the amount of template DNA indicated in the figure legends was incubated in a 25 μl assay in the presence of a mixture of nuclear and S-100 extracts. The reaction mixture contained 12 mM Hepes (pH 7.9), 85 mM KCl, 0.12 mM EDTA, 5 mM $MgCl_2$, 10 mM creatine phosphate, 0.6 mM each of ATP, CTP, and UTP, 12.5 μM GTP, and 1 μCi of [α-^{32}P]GTP. The mixture was incubated for 60 min at 30°C and was processed for gel analysis as previously described (Grummt, 1981). To prepare unlabelled RNA the assay was scaled up to 125 μl and transcription was performed in the presence of 0.6 mM of each of the four ribonucleoside triphosphates. Samples to be analyzed by S1 mapping were treated with DNase before phenol extraction and ethanol precipitation.

Transfection of cells and preparation of cellular RNA

Subconfluent cultures of 3T6 cells were transfected with 20 μg of pMrCAT-2 or pMrA350-CAT DNA together with 2.5 μg of pSV2-CAT DNA, which served as an internal control for the quantitation of transcripts (Grummt and Skinner, 1985). The cells were harvested 44−48 h after transfection. RNA was isolated by the method of Chirgwin *et al.* (1979).

RNA analysis by S1 mapping and primer extension

RNA from a preparative (125 μl) *in vitro* transcription assay or 50 μg of cellular RNA extracted from transfected cells was mixed with the labelled hybridization probe, precipitated with ethanol and dissolved in 25 μl hybridization buffer [80% formamide, 0.4 m NaCl, 0.04 M Pipes (pH 6.4) and 1 mM EDTA]. After hybridization for 3 h at the appropriate temperature (see figure legends) the hybrids were treated with S1 nuclease (Berk and Sharp, 1977) or were reversely transcribed into cDNA (Grummt and Skinner, 1985). The hybrids were purified by extraction with phenol−chloroform and analyzed on 6% sequencing gels along with size markers (pBR322/*Hpa*II) or a Maxam−Gilbert sequencing ladder of the probe DNA.

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Note added in proof

After submission of this manuscript a paper has been published describing a similar promoter in the spacer of rat rDNA [Cassidy,B.G., Yang-Yen,H.-F. and Rothblum,L.I. (1987) *Mol. Cell. Biol.*, **7**, 2388–2396].