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Expression of an mRNA coding gene under the control of an RNA polymerase I promoter

Sabine Fleischer and Ingrid Grummt*

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

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We have placed a 225-bp fragment from the 5′ end of the mouse rDNA transcription unit (from −169 to +56) in front of the SV40 tumor antigen coding sequence. After microinjection of this chimeric plasmid into nuclei of mouse L-cells expression of SV40 large T antigen has been observed. The expression of T antigen was dependent on the correct orientation of the rDNA fragment relative to the T antigen-coding region and was seen only in mouse cells. This indicates that the 225-bp rDNA fragment contains the sequence information required for pre-rRNA transcription and demonstrates for the first time that a protein-coding gene can be transcribed and expressed under the control of an RNA polymerase I promoter.

Key words: fusion genes/microinjection/ribosomal promoter/SV40 T antigen

Introduction

The nucleotide sequence requirements for the specific initiation of transcription of genes transcribed by RNA polymerase II and III have been investigated in detail *in vitro* and *in vivo* (for review, see Shenk, 1981). These studies have identified an intragenic control region within the polymerase III structural genes which is essential for transcription initiation. Genes transcribed by RNA polymerase II have been characterized as having several control regions upstream of the site at which transcription is initiated (for review, see Breathnach and Chambon, 1981). Certain regions like the CAAT and TATA-box are conserved in sequence and location in diverse eukaryotes. Though the TATA-box seems to be sufficient for correct transcription initiation *in vitro*, DNA segments much further upstream have been shown to be important for gene expression in several *in vivo* studies (Benoist and Chambon, 1982; McKnight and Kingsbury, 1982; Dierks *et al.*, 1983).

The DNA sequences that constitute the RNA polymerase I promoter are not definitively known yet. It has been shown that DNA sequences indispensable for correct and quantitative transcription of rDNA *in vitro* lie upstream of the site of transcription initiation (Grummt, 1982; Kohorn and Rae, 1983; Learned *et al.*, 1983). A major component of the promoter of RNA polymerase I activity involves the region −39 to −13 on mouse rDNA (Grummt, 1982), and −43 to −27 on *Drosophila* rDNA (Kohorn and Rae, 1983). In addition to this essential region, sequences further upstream also affect the efficiency of initiation *in vitro* (Grummt, 1982). Furthermore, the region around the start site also seems to serve a function in promoting transcription of rRNA (Kohorn and Rae, 1983; Learned *et al.*, 1983).

Whether the same regions that are required for transcription *in vitro* are sufficient for transcriptional activity *in vivo* is unknown. Before a detailed mutational analysis of the rDNA promoter *in vivo* can be performed, a functional assay for detecting transcription from a cloned rDNA promoter fragment above the background of endogenous cellular RNA has to be developed. Here we describe an approach to identify transcripts of a fusion gene consisting of the putative mouse rDNA promoter fragment and the coding region of SV40 T antigen.

*To whom reprint requests should be sent.

Results

Expression of T antigen in pMrSV-injected LTK⁻ cells

In vitro studies have demonstrated that species-specific factors play an important role in the transcription initiation process (Grummt, 1981; Grummt *et al.*, 1982; Mishima *et al.*, 1982; Kohorn and Rae, 1982). Therefore it is necessary to investigate the promoter activity of cloned rDNA fragments *in vivo* in homologous cells. To identify the level of transcription from introduced rDNA fragments above the background of endogenous cellular pre-rRNA transcripts derived from the exogenous rDNA molecules, we have joined the putative rDNA promoter to the coding region of SV40 T antigen. Thus the expression of the chimeric gene can be easily monitored by staining the cells with immunofluorescent antibodies. Figure 1 shows the construction of the two hybrid genes we used in which the cap site and promoter sequences of the SV40 early region were replaced by two tandem copies of the mouse rDNA fragment inserted into the same (pMrSV) or opposite (pMr*SV) direction relative to the T antigen sequence.

To determine whether the hybrid gene pMrSV is expressed in mouse cells, we microinjected the DNA into the nuclei of LTK⁻ cells. Two days after injection the cells were fixed, incubated with an antibody against T antigen and stained with a fluorescein-conjugated second antibody. The plasmid pBSV3x (Schaffner, 1980) which contains three tandem copies of SV40 DNA integrated into the *Bam*HI site of pBR322 was used as a positive control for the microinjections. The vector pEMP (Benoist and Chambon, 1982), which has no promoter sequences in front of the SV40 early region, served as a negative control. After injection of pBSV3x DNA into the nuclei of mouse cells, 70–80% of the cells showed nuclear fluorescence characteristic of T antigen (Table I). No fluorescent cells were found after microinjection of the vector pEMP. When cells were injected with the fusion gene pMrSV, 25–50% of the nuclei were fluorescent, that is, about half the amount of the SV40 positive control. Although the absolute percentage of positive cells varied from one experiment to another, cells injected with pMr*SV were always clearly negative. This indicates that a significant portion of cells expressed T antigen if the rDNA promoter was fused to the SV40 sequences in the same orientation as the early transcription unit.

Hence, T antigen expression in the chimeric rDNA-SV40 plasmids absolutely depends on the orientation of the rDNA fragment relative to the T antigen-coding region. To show

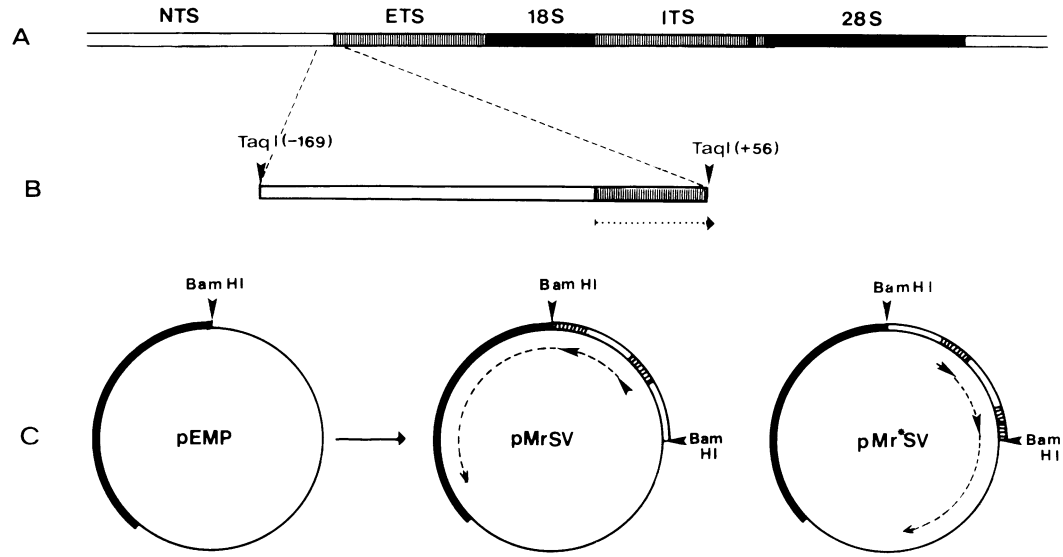

Fig. 1. Construction of the recombinant plasmids pMrSV and pMr*SV. A 225-bp *Taq*I fragment (**B**) from the 5′ end of the rDNA repeat (**A**) was isolated and inserted into the unique *Bam*HI site of pEMP by blunt-end ligation (see Materials and methods). The vector pEMP contains genomic sequences of SV40 (from position 2469 to 5163) which code for T antigen. The recombinant plasmid containing two tandem copies of the rDNA fragment in the same orientation as the SV40 early region was designated pMrSV, in the opposite orientation pMr*SV. The thin line of the recombinant plasmids represent pBR322 sequences, the thick solid line SV40 sequences.

Table I. SV40 T antigen expression in mouse LTK cells injected with different SV40 recombinant plasmids

Plasmid injected	Number of DNA molecules injected per nucleus	DNA concentration (mg/ml)	Percentage of fluorescent nuclei
pBSV3x	250	0.4	70–80 (8)
pEMP	700	0.5	0 (8)
pMrSV	650	0.5	25–50 (8)
pMr*SV	650	0.5	0 (8)
pBSV3x + pMR*SV	450	0.25 + 0.25	60–80 (2)

100 cells were injected with each plasmid DNA within a marked area on separate Petri dishes. Microinjections were carried out essentially as described (Graessmann and Graessmann, 1976). Concentrations of injected DNA varied between 0.2 and 0.5 mg/ml. 30 h after injection the cells were rinsed with PBS and fixed with methanol at −20°C. T antigen was detected by indirect staining of hamster serum against SV40 T antigen with fluorescein-labeled rabbit anti-hamster serum (Miles). The relative amount of T antigen expression was determined indirectly by counting the number of immunofluorescent cells. The degree of fluorescence was not taken into account. The numbers in brackets represent the number of experiments.

that the inability of pMr*SV to support T antigen expression is in fact due to the wrong orientation of the rDNA promoter and is not caused by the presence of toxic substances in the individual plasmid preparations, pBSV3x and pMr*SV were mixed in a molar ratio of 1:1 and injected into mouse cells. The ratio of T antigen-positive nuclei did not decrease compared with the ratio obtained with pBSV3x alone (Table I), indicating that the absence of T antigen expression in pMr*SV injected cells is indeed due to the wrong orientation of the rDNA promoter relative to the SV40 early region. This, therefore, supports the conclusion that transcription of T antigen sequences in pMrSV-injected cells started from the rDNA initiation site. Due to the limited number of injected cells it has not yet been possible to make transcriptional mapping studies, which would demonstrate without ambiguity whether transcription was started accurately from the rDNA initiation site. We therefore have to rely on indirect experimental evidence to show that T antigen mRNA has been transcribed from the rDNA promoter by RNA polymerase I.

Effect of α-amanitin

The three classes of cellular RNA polymerases can be distinguished *in vitro* by their differential sensitivities against the toxin α-amanitin. Polymerase III from animal cells is inhibited by high levels (10–20 μg/ml for 50% inhibition) of α-amanitin, distinguishing it from polymerase I which is insensitive and polymerase II which is sensitive to low levels (0.01–0.05 μg/ml for 50% inhibition) of the toxin (reviewed by Roeder, 1976). To prove directly that pMrSV DNA was transcribed from the rDNA promoter by RNA polymerase I, we co-injected α-amanitin (1 μg/ml final concentration) together with the chimeric plasmids into the cell nuclei. However, due to the harsh effect of α-amanitin on the physiological state of the cells, these experiments gave no clear, reproducible results. Injection of α-amanitin resulted in morphological changes and death of the majority of cells. Nevertheless, we could observe a differential effect of α-amanitin on T antigen expression directed by either pBSV3x or pMrSV. T antigen expression was almost completely inhibited (2–4% positive nuclei) in cells injected with pBSV3x, whereas there was still a significant percentage (12–15%) of fluorescent cells after microinjection of pMrSV in the presence of α-amanitin. This 5-fold difference in the number of fluorescent nuclei suggests that transcription of T antigen sequences in pMrSV-injected cells is brought about by RNA polymerase I.

Species specificity of pMrSV-directed T antigen synthesis

Because of the ambiguity of these results we used another approach to demonstrate transcription of pMrSV by RNA polymerase I. Previous experiments in cell-free transcription systems have suggested that species-specific factors are re-

Table II. Expression of T antigen after microinjection of pBSV3x and pMrSV into nuclei of different cell types

Cell type	Species	Percentage of fluorescent nuclei after injection of plasmid DNA	
		pBSV3x	pMrSV
LTK$^-$	Mouse	60	29
HeLa	Human	45	0
CVI	Monkey	53	0

Experiments were performed as described in Materials and methods. The number of plasmid molecules injected into each nucleus was ~240 for pBSV3x and 650 for pMrSV, respectively.

quired for accurate transcription initiation by RNA polymerase I. Such a species specificity has not been observed for transcription from RNA polymerase II or III promoters. Therefore, if SV40 mRNA has been transcribed from the mouse rDNA promoter by RNA polymerase I in pMrSV-injected cells one would expect pMrSV-directed T antigen expression to occur only in homologous mouse cells and not in cells from other species. As shown in Table II this prediction proves to be true. The control DNA pBSV3x or the fusion plasmid pMrSV were injected into mouse (LTK$^-$), human (HeLa) and monkey (CV1) cells. Injection of the control plasmid resulted in approximately the same percentage of fluorescent nuclei in all three cell types. In contrast, pMrSV-directed T antigen expression could only be detected in mouse cells. This finding is in accord with the suggested species specificity of the mouse RNA polymerase I transcription factor(s) and supports the conclusion that RNA polymerase I has initiated on the rDNA fragment and transcribed the SV40 early region. This indicates that the 225-bp *Taq*I fragment (-169 to $+56$) from the 5′ end of the rDNA gene contains the sequence information required for pre-rRNA transcription and demonstrates for the first time that a protein coding gene can be transcribed and expressed under the control of an RNA polymerase I promoter.

Discussion

Here we have shown that sequences from the 5′ end of mouse rDNA are capable of promoting transcription of mRNA-coding sequences. A ribosomal gene fragment encompassing the region from -169 to $+55$ of the rDNA repeating unit was fused upstream of the SV40 early gene region and the resulting chimeric plasmid was microinjected into nuclei of mouse LTK$^-$ cells. As shown by immunofluorescence, a significant percentage (25 – 50%) of cells produced T antigen after injection of the rDNA-SV40 fusion gene. However, the efficiency of this construct pMrSV was always a factor of two lower than that of T antigen expression from the control plasmid pBSV3x which contains the SV40 promoter including the 72-bp repeat enhancer sequence. Whether this lower amount of positive cells is due to a lower transcriptional efficiency of the ribosomal *versus* the SV40 promoter or due to different rates of post-transcriptional processes remains to be investigated.

The following observations support the conclusion that transcription of T antigen sequences in pMrSV-injected cells started at the rDNA initiation site by RNA polymerase I. Firstly, T antigen synthesis was only detectable if the rDNA promoter was in the same transcriptional orientation as the SV40 early region. If the rDNA frgment was linked to the T antigen sequences in the opposite orientation (pMr*SV) no T antigen was expressed. Secondly, after administration of α-amanitin, the percentage of fluorescent cells was 5-fold higher in pMrSV-injected cells as compared with cells which have been microinjected with pBSV3x. Although this result must be treated with caution due to the toxic effect of the drug on the cells it would seem to indicate that the T antigen mRNA has been transcribed by RNA polymerase I. Thirdly, the synthesis of T antigen was only seen when pMrSV DNA was microinjected into mouse cells and not into cells of other species. The SV40 control plasmid promoted T antigen synthesis in all three cell types tested. This suggests an involvement of different cellular transcription initiation factor(s) in rDNA promoter recognition *in vivo*, such as have been identified in cell-free systems.

Our data support the conclusion that mRNA-coding genes can be transcribed by a heterologous RNA polymerase. However, the rDNA-directed synthesis of T antigen was less efficient than that from the SV40 promoter. Whether this difference in the percentage of fluorescent cells is due to differences in transcriptional activity, processing or translational efficiency is unknown. Furthermore, it is not known whether the transcription of the fusion gene by RNA polymerase I in transfected cells occurs extrachromsomally or in close association with the nucleolar structure. Experiments from this laboratory indicate that the percentage of fluorescent nuclei was not increased when the cells were reversibly arrested within mitosis by treatment with colcemid (S. Fleischer, unpublished results). The rationale of this experiment was to test whether in mitotic cells, where the nucleolus has disintegrated, RNA polymerase I and transcription factors are more easily accessible for exogenous rDNA molecules than in interphase cells. This negative result indicates that the physiological state of the nucleolus obviously does not affect the rate of SV40 early gene expression in pMrSV-injected cells.

A number of questions arise from this study. Is the primary transcript of the fusion gene correctly initiated at the 45S pre-rRNA initiation site? Is this pre-mRNA that has been synthesized by RNA polymerase I capped and polyadenylated? What fraction of the primary transcripts is properly spliced? To answer these questions, detailed biochemical analyses of the RNA synthesized must be carried out. Preliminary experiments to map the 5′ end of the transcripts by the nuclease S1 technique have been unsuccessful, presumably because the amounts of SV40-specific RNA in the injected cells are too low. We suspect that analysis of SV40 RNA initiated from this RNA polymerase I promoter will require transfection of large numbers of cells. To this end, experiments in which the rDNA-SV40 fusion gene and other similar constructs are transfected into mouse L-cells via the calcium phosphate technique are in progress. Despite the lack of RNA analysis, the phenotypes of the injected cells show that *bona fide* SV40 T antigen is synthesized after microinjection of pMrSV, indicating that the RNA polymerase I promoter can give rise to some properly spliced and translatable mRNA. The expression of mRNA genes by a heterologous RNA polymerase will not only provide a useful system for a detailed analysis of the rDNA promoter but also represents a new and profitable approach to the study of mRNA processing and translation.

Materials and methods

Construction of the rDNA-SV40 fusion genes

pEMP and pBSV3x were generously provided by P. Chambon and W. Schaffner, respectively. The plasmids pMrSV and pMr*SV were constructed by standard cloning procedures. For pMrSV a 225-bp *Taq*I fragment from the 5′ end of the rDNA repeat (see Figure 1) was isolated and inserted into the unique *Bam*HI site of pEMP (Benoist and Chambon, 1982) by blunt-end ligation after filling-up the ends by the Klenow fragment of DNA polymerase in the presence of all four deoxynucleoside triphosphates. The vector pEMP contains genomic sequences of SV40 (from position 2469 to 5163) which code for T antigen. The recombinant plasmid containing two tandem copies of the rDNA fragment in the same orientation as the SV40 early region was designated pMrSV, in the opposite orientation, pMr*SV. Plasmid extractions and purifications were carried out by centrifugation of a detergent-cleared lysate in caesium chloride-ethidium bromide. All DNA preparations were dialysed extensively against 10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA before injection.

Microinjection into cell nuclei

LTK$^-$ cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum. For microinjection, the cells were seeded on Petri dishes at a density of 10^5 cells/ml. Microinjections were carried out essentially as described by Graessmann and Graessmann (1976). One hundred cells were injected with each plasmid DNA within a marked area on separate Petri dishes. Concentrations of injected DNA varied between 0.2 and 0.5 mg/ml. Thirty hours after injection the cells were rinsed with phosphate-buffered saline (PBS) and fixed with methanol at −20°C. T antigen was detected by indirect staining of hamster serum against SV40 T antigen with fluorescein-labeled rabbit anti-hamster serum (Miles).

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