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# Negative and positive effects of CpG-methylation on *Xenopus* ribosomal gene transcription in vitro

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**Abstract** Methylation of cytosine-residues in the sequence CpG affects the expression of many genes and generally correlates with reduced transcription. The ribosomal genes of *Xenopus laevis* were among the first genes to be studied with respect to their DNA methylation, and a loss of methylation during embryonic development correlated with the onset of transcription. Nevertheless, highly methylated ribosomal genes were transcribed at normal levels when injected into oocyte nuclei, and thus transcription of these genes was generally assumed to be insensitive to CpG-methylation. Here I show that *Xenopus* ribosomal gene transcription can be repressed by cellular factors binding to $^{me}$CpG, similarly as it has been described for transcription by RNA polymerase II. In the absence of these repressors, however, CpG-methylation has a direct positive effect on RNA polymerase I-promoter activity.

*Key words:* RNA polymerase I; *Xenopus laevis*; DNA methylation; Ribosomal; Transcription

## 1. Introduction

Effects of DNA methylation on transcription have been reported in numerous publications over the last 10–15 years. While in some cases no effect was detected, methylation of DNA on cytosine in the sequence CpG was generally found to be associated with repression of transcription. As discussed in several recent reviews, the available experimental data indicate that $^{me}$CpG can inhibit transcription by both direct and indirect mechanisms [1–3]. In the direct mechanism the methylation prevents the binding of a transcription factor; in the indirect mechanism transcription is shut off because of the binding of repressor proteins which specifically recognize $^{me}$CpG.

The ribosomal genes of *Xenopus laevis* are very rich in CpG and have long been known to occur both in methylated and unmethylated forms [4]. They were also among the first genes on which the methylation sites have been mapped [5]. Work by A. Bird and colleagues showed [6] that most of the CpG-sites are methylated but that there is an undermethylated region in the rDNA spacer. This region corresponds to the enhancer for ribosomal gene transcription [7]. Despite a striking correlation between the appearance of this undermethylated region during embryonic development of *X. laevis* and the onset of transcription [6], the involvement of CpG-methylation in the regulation of the ribosomal genes remained unclear. The discovery that in *X. borealis* a corresponding region in the rDNA spacer was undermethylated in sperm indicated that lack of methylation is not sufficient to turn on transcription [8]. On the other hand, fully methylated *X. laevis* sperm rDNA or cloned ribosomal genes methylated with *Hpa*II-methylase were transcriptionally competent when injected into oocyte nuclei [9,10], showing that $^{me}$CpG per se does not interfere with ribosomal gene transcription. Since those early studies, the focus has shifted towards the study of genes transcribed by RNA polymerase II (polII), and due to lack of further work on polI the 'conventional wisdom' has been accepted that polI-transcription is not affected by DNA methylation (see e.g. [11]). Nevertheless, it remained possible that CpG-methylation had an effect on ribosomal gene transcription given the right conditions and in the presence of the right regulatory factors.

Here I present evidence that – as described for polII-genes – transcription by polI can be fully blocked by $^{me}$CpG-binding repressor proteins. I also show, however, that when these putative repressors are sequestered away from the template, $^{me}$CpG in the promoter actually stimulates ribosomal gene transcription.

## 2. Materials and methods

Plasmids MH-T3wt and P-T3wt were described previously [12]. *Sss*I methylase (New England BioLabs) was used to methylate CpG-sites on the template DNA according to the manufacturer's protocol. For the construction of the chimeras, both the unmethylated and methylated *Hin*dIII–*Bam*HI fragment (317 base pairs [bp]; 43 CpG-sites) and *Bam*HI-*Eco*RI fragment (429 bp; 38 CpG-sites) were isolated from an 1.4% agarose gel by electroelution onto DE81-paper (Whatman). The isolated fragments were mixed in the four relevant combinations, and after ligation with T4 DNA ligase the DNA was digested with *Hin*dIII and *Eco*RI to release the 746 bp fragment containing the reassembled chimeric and control minigenes. The 746 bp fragment was separated from the other ligation- and restriction-products on 1.4% agarose and again electroeluted onto DE81-paper. The DNA concentration of the final preparation was estimated by running a fraction on a gel along with DNA-standards of known concentration followed by ethidium bromide staining.

The S-100 extract was prepared as described [12,13]. For in vitro transcription, 10 μl of S-100 extract was preincubated with 200 ng of unmethylated or methylated pGem4 (Promega) for 10 min on ice. 10 ng of template DNA was then added and after an additional 10 min on ice transcription was started as described [12]. Transcription reactions were for 2 h. All reactions contained 2 mM 6-dimethylaminopurine to inhibit a transcription-repressing kinase [12] and 100 μg/ml α-amanitin to inhibit polII and III. Transcription was resistant to up to 500 μg/ml α-amanitin (data not shown). Transcripts were either labeled during transcription with [α-$^{32}$P]CTP or analyzed with an S1-protection assay [14].

For electrophoretic mobility shift assays, a 63 bp *Pst*I/*Bgl*II fragment of MH-T3wt carrying a *Bgl*II-linker scanner mutation in the upstream T3 [15] was used (see Fig. 1). The fragment, methylated and unmethylated, was end-labeled with T4 polynucleotide kinase and [γ-$^{32}$P]ATP. 1 μl of S-100 extract (15 mg/ml) was preincubated in a 20 μl reaction with 500 ng of *Hpa*II-digested Bluescript-plasmid (Stratagene) under

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the same buffer conditions as used for transcription. After addition of the labeled fragment and incubation at room temperature for 15 min, the sample was directly loaded onto a pre-electrophoresed 5% polyacrylamide gel in 50 mM Tris-borate-EDTA-buffer at 4°C. The same result was obtained when poly[d(IC)] was used as a non-specific competitor.

## 3. Results

To investigate the effect of CpG-methylation on polI-transcription in vitro, plasmid DNA containing a ribosomal minigene was methylated with *Sss*I methylase. Two functionally equivalent minigenes were used. MH-T3, shown in Fig. 1, had 81 CpG-sites in the minigene-insert, i.e. from the *Hin*dIII-site 270 bp upstream from the initiation site to the *Eco*RI-site ca. 50 bp downstream from the T3 terminator, where RNA 3′ ends of the 425 nucleotide mini-rRNA were formed (Fig. 1). The second minigene, P-T3wt (not shown; [12]), had 97 CpG-sites in the minigene-insert and produced a T3-terminated transcript of 410 nt. Treatment of both plasmids with *Sss*I methylase rendered the DNA completely resistant to digestion with *Hpa*II, indicating that CpG-methylation was complete. Control DNA, treated identically except that methylase was omitted, could be fully digested with *Hpa*II (data not shown).

The two minigenes were linearized with *Ssp*I and co-transcribed in an S-100 extract from *Xenopus* tissue culture cells [13]. RNA was labeled during transcription with [α-$^{32}$P]CTP. Preliminary experiments showed that the effect of methylation on ribosomal gene transcription depended strongly on both template concentration and the presence and type of non-specific competitor DNA. I therefore preincubated the extract either with the control plasmid vector pGem or with methylated pGem ($^{me}$pGem) prior to addition of either control or methylated template DNA. Transcription of the two unmethylated minigenes with unmethylated pGem as non-specific competitor yielded similar amounts of the two expected transcripts of 410 and 425 nt (lane 1). Because the sequence where the RNA 3′ ends are formed (ca. 15 bp upstream from the terminator element [16]) is different in the two constructs, the RNA 3′ ends were more heterogeneous with P-T3wt (lower band) than with MH-T3 (upper band). Due to efficient termination at T3 in this system, the run-off transcripts to the *Ssp*I-site were barely detectable. If one of the two plasmids was methylated, transcription of the methylated plasmid was severely repressed, while transcription of the unmethylated plasmid remained unchanged (lanes 2 and 3). If both plasmids were methylated, transcription from both plasmids was repressed (lane 4).

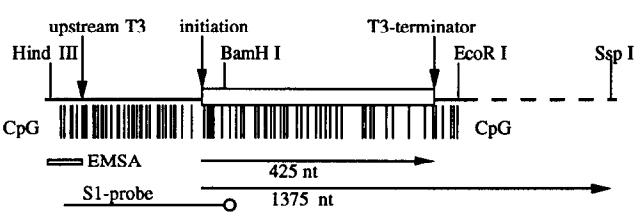


Fig. 1. Map of the ribosomal minigene MH-T3wt. The *Hin*dIII to *Eco*RI insert (drawn to scale) contains a ribosomal gene promoter including the upstream T3-element [13], a minigene of 425 bp (open box), and the T3-terminator and was cloned in the plasmid vector pGem4. The vertical lines below the map indicate the positions of CpG-sites in the sequence from the *Hin*dIII- to the *Eco*RI-site. The location and size of the T3-terminated transcript and of the run-off transcript are indicated below the map (arrows). Also shown is the S1-probe with the $^{32}$P-label (circle) at the *Bam*HI-site and the probe used for electrophoretic mobility shift assays (EMSA).

A very different result was obtained upon preincubation with $^{me}$pGem (lanes 5–8). While transcription from the control templates was essentially the same as in the presence of the control pGem (lane 5, compare to lane 1), CpG-methylation now stimulated transcription of the methylated minigene (lane 8). Co-transcription of methylated and unmethylated DNA showed that the stimulation was strictly a *cis*-effect (lanes 6, 7). Densitometric analysis showed that the $^{me}$CpG-induced stimulation in this experiment was 2-fold for MH-T3wt (upper band) and 3.2-fold for P-T3wt (lower band).

The results described so far suggest that in the extract there is a $^{me}$CpG-binding protein that blocks transcription by binding to the methylated template, but that can be removed by excess methylated competitor DNA. The presence of such a factor in the S-100 extract is demonstrated in Fig. 2B. A 63 bp fragment from a region upstream of the T3-promoter element having 9 CpG-sites (see Fig. 1) was end-labeled and used in an electrophoretic mobility shift assay. In the present S-100 extract, this region does not reveal any regulatory function for polI-transcription, but has been shown to bind transcription factor xUBF [17]. If used in its unmethylated form, this DNA fragment formed several complexes of different mobilities with proteins in the S-100 extract (lane 1). If the CpG-sites were methylated, the same complexes were still seen, but one additional very strong complex was formed (lane 2). Since the only difference between the two DNA-probes was the absence or presence of $^{me}$CpG, this result shows that there is a cellular factor in the S-100 extract that specifically binds to $^{me}$CpG.

To find out whether methylation in the promoter and in the vicinity of the initiation site would be sufficient to see the negative and positive effects of methylation on polI-transcription, chimeras were constructed. The unmethylated or methylated *Hin*dIII–*Bam*HI fragment containing the promoter plus the first 50 bp of the transcribed sequence was ligated in all four possible combinations to the methylated or unmethylated *Bam*HI–*Eco*RI fragment containing the minigene body and the T3-terminator (see Fig. 1). The chimeras were transcribed with control pGem or with $^{me}$pGem as non-specific competitors. In this experiment, the transcripts were analyzed with an S1-protection assay. The results showed (Fig. 3A) that the effects on transcription were predominantly determined by the methylation status of the promoter fragment. Thus with control pGem as non-specific competitor (lanes 1–4), transcription was inhibited in the construct having only the promoter fragment methylated (lane 2) as well as in the fully methylated construct (lane 4). The chimera which was methylated in the gene only (lane 3) was transcribed only slightly less than the fully unmethylated construct (lane 1). After preincubation with $^{me}$pGem, even transcription from the control template was stimulated in this experiment (lane 5, compare to lane 1), but both the promoter-methylated chimera as well as the fully methylated construct showed an additional modest increase in transcription (lanes 6 and 8). Methylation in the gene-region only, on the other hand, gave the same result as no methylation at all (lanes 5 and 7).

Fig. 3B shows the densitometric analysis of two additional independent assays of the chimeras. The data confirm that methylation in the promoter plus immediately downstream from the initiation site is sufficient for both the repression (bars

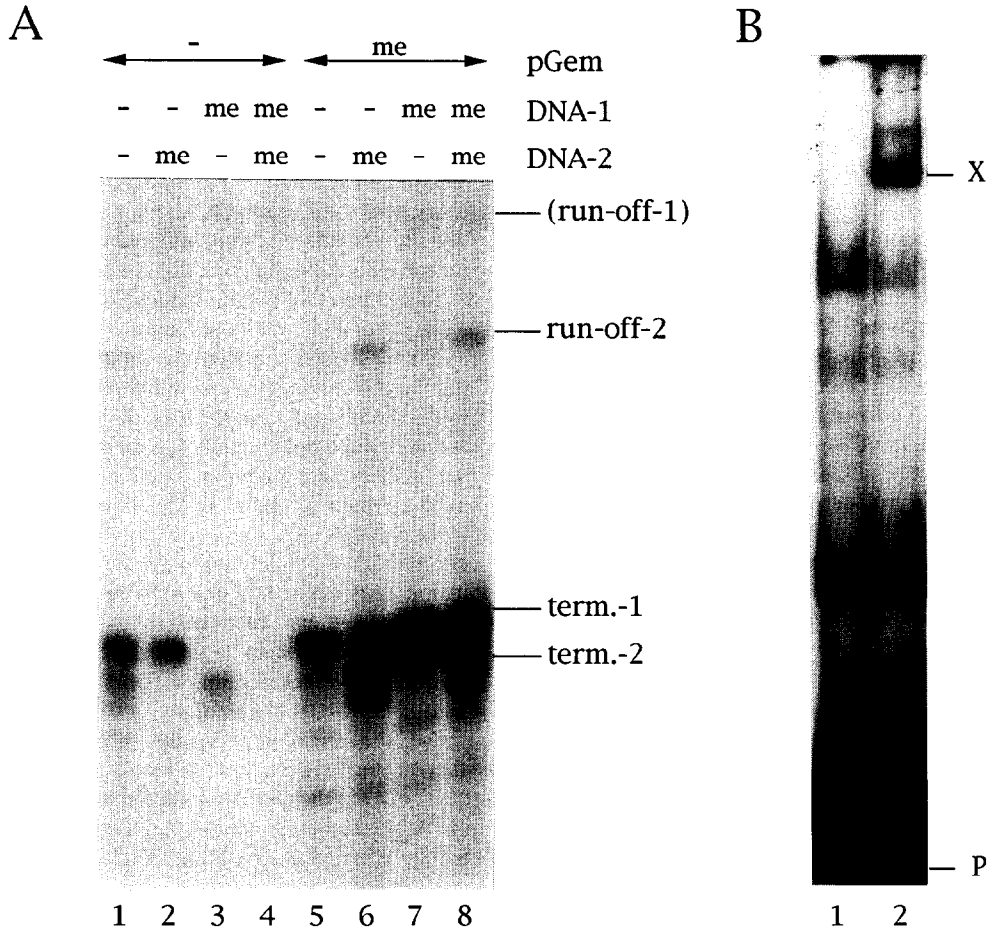


Fig. 2. Effect of CpG-methylation on in vitro transcription by polI and evidence for a $^{me}$CpG-binding protein in the transcription extract. (A) Equimolar amounts of linear minigenes MH-T3wt (DNA-1) and P-T3wt (DNA-2) were transcribed after preincubation of the S-100 extract with unmethylated pGem (lanes 1–4) or $^{me}$pGem (lanes 5–8). The minigenes were unmethylated (−) or methylated (me) as indicated above the lanes. term.-1, T3-terminated transcript from MH-T3wt; term.-2, T3-terminated transcript from P-T3wt; run-off-2, run-off transcript from P-T3wt; (run-off-1), approximate location of the run-off transcript from MH-T3wt (not visible). (B) Evidence for a $^{me}$CpG-binding protein in the S-100 transcription extract. P, end-labeled DNA probe, unmethylated (lane 1) or methylated (lane 2). X, specific complex between a cellular factor and methylated DNA.

2) and the stimulation (bars 6). The data also suggest that the $^{me}$CpG in the gene region might have an additional repressive effect (compare bars 1 and 2 to 3 and 4) or might neutralize some of the stimulation by $^{me}$CpG in the promoter (bars 8). However, I cannot rule out that minor changes in the signals are due to small differences in the template concentration, which for the gel-purified chimeras could not be determined as precisely as for the plasmids.

## 4. Discussion

The present study closes a gap in our knowledge about the effect of CpG-methylation on transcription of eukaryotic genes. While a large number of polII-genes have been analyzed in detail and generally found to be repressed by CpG-methylation, the ribosomal genes transcribed by polI have not been studied in this respect since the papers by Macleod and Bird [9], who reported that fully methylated rDNA was normally transcribed when injected into *Xenopus* oocytes, and by Pennock and Reeder [10], who obtained the same result with *Hpa*II-methylated cloned ribosomal genes. Thus, the polI-promoter was generally accepted to be insensitive to CpG-methylation, which was somewhat surprising considering the fact that there are 18 CpG-sites in the 140 bp promoter of *Xenopus*, which should be good binding sites for $^{me}$CpG-binding proteins [18,19]. Here I show that the polI-promoter is no exception in that it is strongly inhibited by CpG-methylation. The finding that the repression is not seen in the presence of methylated non-specific competitor DNA suggests an indirect mechanism involving a $^{me}$CpG-binding repressor. The inability to see an effect of methylation on ribosomal gene transcription in oocytes most likely means that there was not enough $^{me}$CpG-binding protein in the oocyte nucleus to quantitatively cover the injected DNA. Indeed, if the present transcription reactions in the S-100 extract were carried out with a high template concentration (10–20 ng/μl) and no non-specific competitor DNA, the methylated template was not inhibited, indicating that the ratio of $^{me}$CpG-binding protein to template DNA is critical (data not shown). Template concentration and methylation-status of non-specific competitor DNA were also found to affect the $^{me}$CpG-induced inhibition of various polII-genes [20], suggesting that a similar mechanism of repression is at work. Further-

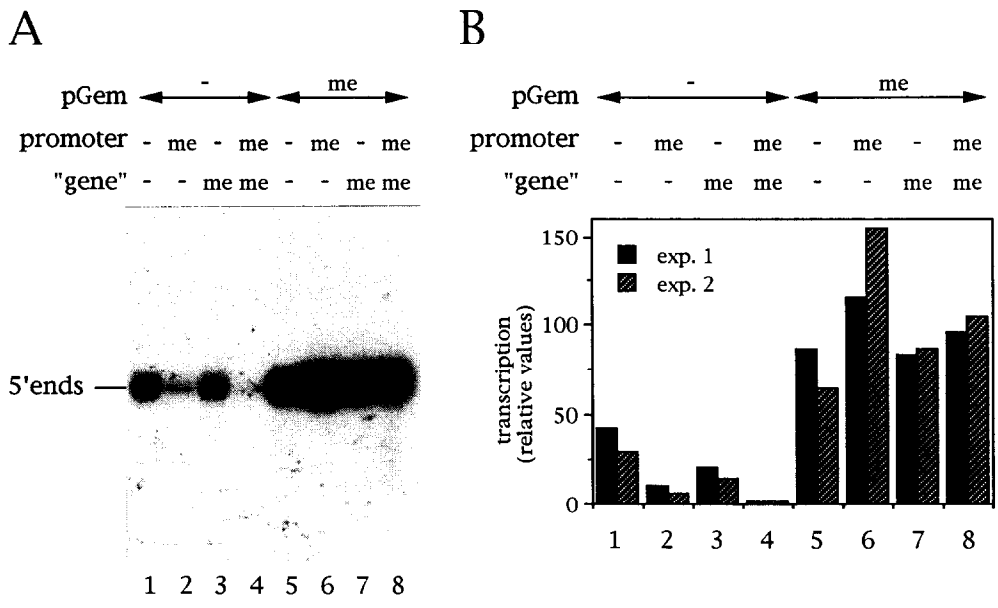


Fig. 3. Transcription of chimeras. (A) S1-assays of in vitro transcription reactions after preincubation of the S-100 extract with unmethylated pGem (lanes 1–4) or $^{me}$pGem (lanes 5–8). The chimeras were unmethylated or methylated in the promoter (*Hin*dIII to *Bam*HI, see Fig. 1) or in the 'gene' (*Bam*HI to *Eco*RI), as indicated above the lanes. 5′ ends, 51 nucleotide protection of the probe from S1-digestion indicative of transcripts starting at the promoter. (B) Densitometric analysis of the S1-signals from two additional experiments (exp. 1 and exp. 2) as in A.

more, the results with the chimeras indicate that $^{me}$CpG-sites in the proximity of the promoter and the initiation site are most effective in repressing transcription. Again, similar results have been obtained with polII-transcribed genes [21,22].

A novel observation is the finding that CpG-methylation can have a direct positive effect on transcription. To my knowledge a similar observation has not been made with any of the analyzed polII-genes. This increased transcription of a methylated promoter could indicate that 5-methylcytosine induces a conformational change in the promoter DNA and that such a conformation is bound more avidly by ribosomal transcription factors or allows better promoter clearance by polI. It is well known that $^{me}$CpG can have major effects on DNA conformation. It can induce the transition from the B- to the Z-form [23] or can increase or decrease DNA-bending in synthetic oligonucleotides [24] or in polII- and polIII-promoter DNA [25]. Note that all the present experiments were performed with linear template DNA, thus a change in torsional stress cannot be responsible for the increased promoter activity. Alternatively, the presence of $^{me}$CpG in promoter DNA could directly increase the binding of one of the ribosomal transcription factors. Copenhaver et al. [26] have investigated the effect of CpG-methylation on xUBF binding and could not detect any change in the binding efficiency.

The present results suggest that CpG-methylation of ribosomal genes, especially in the promoter, can contribute to their inactivation in vivo by attracting $^{me}$CpG-binding repressor proteins. Several studies have reported that repression of ribosomal gene transcription correlated with their methylation and that demethylation can lead to reactivation of ribosomal genes (e.g. [6,27,28]). Whether such a repression occurs would depend on the abundance and availability of $^{me}$CpG-binding proteins in the cell. On the other hand, the in vivo significance of the $^{me}$CpG-induced promoter stimulation is less certain. The promoter is outside the undermethylated region identified by Bird et al. [6], and thus at least some of the rRNA synthesis in the cell appears to be driven by methylated promoters. However, the increased strength of a methylated promoter might be neutralized by $^{me}$CpG-binding proteins. Furthermore, it is worth noting that the amplified ribosomal genes in *Xenopus* oocytes, which are maximally active, have no detectable 5-methylcytosine [4].

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