***In vitro* methylation of *HpaII* sites in *Xenopus laevis* rDNA does not affect its transcription in oocytes**

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ABSTRACT

The effect of methylation of *X. laevis* rDNA on its transcription was tested by methylating all of the *HpaII* sites in a series of rDNA plasmids and injecting the methylated rDNA into *X. borealis* oocytes. Analysis of RNA transcribed from the injected plasmids showed that methylation of rDNA *in vitro* does not affect transcription of *X. laevis* ribosomal genes. This result suggests that despite the observed correlation between demethylation of rDNA and synthesis of ribosomal RNA in *X. laevis* embryos (1), methylation of rDNA does not regulate transcription of the gene.

INTRODUCTION

DNA containing the genes for the 18s, 5.8s, and 28s ribosomal RNAs of *Xenopus laevis* undergoes a specific pattern of demethylation during early embryonic development (1). In sperm the ribosomal DNA is almost completely methylated in both the spacer and gene regions (1). Near mid-blastula, shortly before ribosomal RNA synthesis can first be detected (2), specific *HpaII* and *AvaI* sites in the spacer become demethylated (1). Thus, there is a temporal correlation between demethylation of the rDNA spacer and the onset of transcription. This raises the possibility that demethylation of specific sites in the spacer may be required for ribosomal gene transcription to occur.

The region of the spacer which becomes demethylated is also of some interest for other reasons. The spacers of *X. laevis* ribosomal DNA are punctuated by two or more duplications of the gene promoter (the "Bam Islands" of Boseley, *et al.* (3)). Separating these duplicated promoters from each other and from the gene promoter are blocks of repetitive elements that are either 60 or 81 bp long (60/81 bp repeats). Figure 1 contains a diagram of

Figure 1 Organization of the non-transcribed spacer in the rDNA of *X. laevis*. The thick black bars represent transcribed regions. Within the spacer the gene promoter and the duplicated promoters are white bars. The small black bars denote the positions of the 60/81 bp repeats. *EcoRI* = ↓ ; *BamHI* = ↓ ; *HindIII* = ↑ .

X. laevis rDNA spacer organization. Recent work has shown that spacer sequences can strongly influence the activity of the gene promoter (4,5,6). Evidence has been presented that this spacer effect is due to the 60/81 bp repeats which may serve as binding sites for a factor that is essential for activating the gene promoter (6). Demethylation of the spacer occurs precisely within these 60/81 bp repeating sequences which further strengthens the correlation between demethylation and transcription.

If factor binding to the 60/81 bp repeats does activate the ribosomal gene promoter, then two alternate explanations of the correlation between demethylation of the 60/81 bp repeats and transcription of the gene are possible. 1) The 60/81 bp repeats must be demethylated so that factor can bind and activate the promoter. In this view methylation would act to regulate ribosomal gene transcription. 2) Binding of the factor to the 60/81 bp elements blocks methylation but transcription is insensitive to methylation. In this view it is transcription (i.e., factor binding) that regulates methylation. The experiments described in this paper show that the methylation state of rDNA does not affect its transcription which suggests that the latter alternative is the correct one.

MATERIALS AND METHODS

Structure of Injected Plasmids

The structures of pXlr315, 209, and Ψ 40 are diagrammed in Figure 3. The construction of pXlr315 is described by Sollner-Webb and McKnight (7). pXlr209 is described by Busby and Reeder (5), and the construction of Ψ 40 is described by Reeder

et al. (6).

Methylation of Plasmids

HpaII methylase was purchased from New England Biolabs. Ten micrograms of plasmid were incubated with 1 unit of HpaII methylase in a 100 microliter reaction in the buffers recommended by the supplier. After 24 hours at 37°C, 0.6 units of HpaII methylase were added, and the reaction was incubated 24 hours. The reaction was phenol extracted one time, and the DNA was ethanol precipitated and resuspended in TE buffer (10mM Tris HCl, pH 8; 1mM EDTA). Complete methylation was verified when HpaII did not cut the plasmid (as assayed by electrophoresis in a 1% agarose gel and staining with ethidium bromide).

Oocyte Injection

Xenopus borealis oocytes were isolated and injected according to the methods described by Reeder et al. (6). Injected oocytes were incubated overnight at 18-20°C. Healthy oocytes were pooled, and nucleic acids were extracted by the procedure of Busby and Reeder (5).

Transcription Assays

The primer extension assay was carried out as described by Reeder et al. (6).

RESULTS

The Effect of HpaII Site Methylation on Ribosomal Gene Transcription

One of the sites in the 60/81 bp repeating spacer elements that becomes demethylated during early cleavage is a recognition site for the methyl-sensitive restriction enzyme, HpaII (1,8,9,10). A ribosomal DNA repeat contains one HpaII site approximately every 50 bp, and each 60/81 bp repeat in the spacer has one HpaII site. We methylated all of the HpaII sites in two different ribosomal gene plasmids with HpaII methylase to test what effect methylation of these sites has on transcription of the adjacent ribosomal gene when the rDNA is injected into oocytes. Three different ribosomal gene plasmids were used for this test and their structures are shown in Figure 2. All three of these plasmids contain a miniature X. laevis ribosomal gene consisting of a promoter, a gene body that is 315 bp long, and a terminator. In pXlr209 this minigene is fused to a complete 3500 bp spacer

Figure 2 Structure of the rDNA plasmids injected into oocytes. The thick bars represent the 315 bp transcribed region of the minigene. The rest of the diagram designates non-transcribed spacer sequences and contains the elements described in Figure 1.

region that contains two duplicated promoters plus various other repetitive DNA elements and is typical of the average spacer region found in *X. laevis* ribosomal DNA. pXlr315 is identical to pXlr209 except that the left hand end of the spacer has been removed leaving only 1150 bp of spacer upstream of the initiation site. The reference plasmid, ψ40, is identical to pXlr315 except that 40 bp of linker DNA has been inserted into the gene at the +31 position. DNA inserted at this location has no effect on the gene promoter (6).

Transcription of these plasmids was assayed by injecting them into the nuclei of oocytes from a closely related species, *X. borealis*. After incubation overnight, RNA was extracted and hybridized with an end labeled 88 bp primer which hybridizes only to *laevis* transcripts and not to transcripts from *borealis*. Extension of this primer with reverse transcriptase when it is hybridized to a *laevis* transcript yields a 116 nucleotide product with transcripts from pXlr209 or pXlr315 and a 156 nucleotide product with transcripts from ψ40. Thus, ψ40 can be injected and assayed simultaneously with either of the other two plasmids (Figure 3, lanes 4 and 6).

A comparison of transcription from methylated and unmethylated plasmids reveals that methylation of *HpaII* sites has no detectable effect on transcription of these ribosomal gene plasmids. Figure 3 (lanes 3 and 7) shows that methylated pXlr315 and unmethylated

Figure 3 Transcription of methylated rDNA injected into oocytes. *X. borealis* oocytes were injected with 54×10^{-18} moles of one of the two methylated plasmids alone or with an equimolar amount of a methylated plasmid and the unmethylated reference plasmid, Ψ 40. Transcription was assayed by primer extension using total RNA isolated from 5-6 oocytes. The band at 156 bp is from Ψ 40. The band at 116 bp is from transcripts from the other plasmids, and the band at 88 bp is from unextended primer. Minor bands at other positions are due to premature termination by reverse transcriptase and do not affect the results. Lane 1, precursor rRNA from 0.1 *X. laevis* oocytes; lane 2, precursor rRNA from 0.5 *X. laevis* oocytes; lane 3, unmethylated Ψ 40 injected alone; lane 4, methylated pXlr209 plus unmethylated Ψ 40; lane 5, methylated pXlr209 alone; lane 6, methylated pXlr315 plus unmethylated Ψ 40; lane 7, methylated pXlr315 alone.

Ψ 40 give essentially the same signal when injected singly into oocyte nuclei.

A more sensitive test of transcriptional ability is to inject methylated pXlr209 or pXlr315 in a 1:1 molar mixture with unmethylated Ψ 40. Reeder *et al.* (6) have used this same set of plasmids to show that a promoter attached to a long spacer is transcriptionally dominant over a promoter attached to a short spacer when they are coinjected into oocytes. This dominance is apparently due to the fact that the longer spacer has more of the 60/81 bp elements and is a larger target for binding of a factor essential to activate the promoter (6). Figure 3 shows that the ability of spacer sequences to compete with each other is not affected by methylation. Methylated pXlr209, with seventeen 60/81

bp repeats, is dominant over unmethylated Ψ 40 with 10 such repeats (see lanes 3,4, and 5 in Figure 3). Methylated pXlr315 and unmethylated Ψ 40, each with 10 of the 60/81 bp repeats, give approximately equal signals (lanes 3,6 and 7). These results suggest that methylation affects neither the promoter nor binding of factor to the 60/81 spacer elements. Digestion of methylated rDNA with HpaII before its injection into oocytes does not affect transcription of the gene (data not shown). We also find, in agreement with the results reported by others (11), that the methylation state of the plasmid did not change while the plasmid was in the oocyte (data not shown). Thus, the RNA that is synthesized in oocytes injected with methylated rDNA is transcribed from methylated plasmids.

DISCUSSION

Despite the correlation between synthesis of ribosomal RNA and demethylation of the rDNA spacer that is observed in X. laevis embryos (1), we cannot detect any deleterious effect of methylation of all the HpaII sites in both the gene and the spacer on transcription of X. laevis ribosomal DNA injected into oocytes. To interpret this result it is important to know what rDNA sequences the oocyte requires for accurate transcription. Recent work has demonstrated that the gene promoter for X. laevis ribosomal DNA extends approximately from nucleotide -142 to +6 relative to the site of transcription initiation (12). Within this gene promoter there are at least two functional domains and in some oocyte injections only the domain near the initiation site was sufficient for accurate initiation. Additional experiments (Reeder and Roan, unpublished) have shown that the size of promoter utilized can vary, however, possibly depending on the physiology of the frogs. Whatever the reason for the variability, we have carefully and repeatedly determined that, under the precise injection conditions used in this study, oocytes require the full gene promoter for transcription initiation. As mentioned earlier in this paper, activity of the 60/81 bp spacer elements can also be readily assayed in oocytes. Thus all of the rDNA transcription control elements which we know how to assay, either in oocytes or in embryos, can be detected in oocyte injection

experiments and HpaII site methylation has no deleterious effect on any of them.

An average length spacer such as the one on pXlr209 (Figure 2) has about 280 CpG dinucleotides, 20 of which are in the gene promoter. Of these potential methylation sites, 98 are recognized by the HpaII methylase (7 in the gene promoter). Thus, we are sampling a large fraction of the total methylation sites, and the sites we sample are among those that are known to specifically demethylate during early cleavage. It is likely therefore that we are sampling sites that are biologically relevant. These results support the idea that, for RNA polymerase I, methylation of the DNA has no role in regulating polymerase activity. The results of Macleod and Bird (13) using sperm rDNA from X. laevis also support these conclusions.

It has previously been proposed that demethylation of the 60/81 bp repeats occurs because some protein binds specifically to those repeats during early cleavage and prevents access of the methylating enzyme after replication (1,10). Our results favor this interpretation. We would further suggest that the protein which prevents methylation is probably the transcription factor which we have elsewhere proposed binds to the spacer and is responsible for the fact that promoters attached to long spacers are dominant over promoters attached to short spacers (6).

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