Volume 269, number 2, 358-362 FEBS 08821 September 1990

DNA methylation inhibits transcription by RNA polymerase III of a tRNA gene, but not of a 5S rRNA gene

Daniel Besser, Frank Götz, Kai Schulze-Forster, Herbert Wagner, Hans Kröger and Dietrich Simon

Robert Koch-Institut, Abteilung Biochemie, Nordufer 20, D-1000 Berlin 65, Germany

Received 12 July 1990

Methylation of cytosine in the DNA inhibits the transcription by RNA polymerase II in higher eukaryotes, but has no influence on RNA polymerase I transcription. The effect on RNA polymerase III was unknown, so far. Two polymerase III genes: a type 1 5S rRNA gene and a type 2 tRNA gene were methylated in vitro with a purified eukaryotic DNA methyltransferase (EC2.1.1.37) and their transcription was analyzed in *Xenopus* oocytes. The 5S rRNA gene, an oocyte 5S rRNA gene from *X. laevis* which is subject to developmental inactivation, was not affected by methylation. Conversely, transcription of the tRNA gene was 80% inhibited by methylation with the eukaryotic methyltransferase. *Hha*I and *Hpa*II methylation left its transcription unaffected.

DNA methylation; RNA polymerase III; $tRNA^{lys}$ gene; 5S rRNA gene; Transcription; Oocyte

1. INTRODUCTION

It has been established over the last 10 years that methylation of polymerase II genes, preferably in the promotor region, leads to their shut off from transcription (for reviews see [1,2]). Conversely, polymerase I genes are not affected by methylation as has been shown by injection of methylated and unmethylated rRNA genes into *Xenopus* oocytes [3]. Whether polymerase III genes are regulated by methylation is not yet known.

Polymerase III transcribes two types of genes which differ in their requirement for transcription factors: type 1 comprises the 5S rRNA genes, type 2 the tRNA genes, the adenovirus VA-genes, the Epstein Barr EBER-genes, the human 7SL, 4.5S and Alu genes [4]. The type 1 5S genes in *X. laevis* which can be subdivided in oocyte and somatic 5S genes undergo an interesting developmental regulation [5]. Whereas both 5S genes are transcribed in oocytes, the oocyte 5S genes are shut off during embryogenesis and only the somatic 5S genes remain active in somatic tissues. A role for histone H1 [6] and for the distribution of the transcription factor TFIIIA [7] in the inactivation of the oocyte 5S genes is being discussed. Whether DNA methylation in analogy to the polymerase II genes, could be a factor in the final inactivation process has not yet been sufficiently explored. Miller et al. [8] from sequencing data reported a high degree of CpG methylation in oocyte 5S DNA isolated from frog erythrocytes and discussed an influence of methylation on transcription as being improbable. An analysis with the methylation sensitive restriction enzymes *Hpa*II and *Hha*I showed no difference in the methylation pattern between oocyte and somatic 5S genes in somatic tissues [9]. However, *Hpa*II and *Hha*I sites constitute only about 8% of the CpG methylation sites in eukaryotes.

Transcription regulation of type 2 tRNA genes has only been reported in a few cases [10-12] and received little attention. Inconclusive differences in the methylation pattern of tRNA genes between sperm and somatic tissues on the basis of *Hpa*II and *Hha*I cutting have been described [13]. In order to decide whether DNA methylation can regulate polymerase III transcription, we have analysed the transcription of an oocyte 5S gene and of a chicken $tRNA^{lys2}$ gene in oocytes after in vitro methylation with prokaryotic or eukaryotic methyltransferases.

Correspondence address: D. Simon, Robert Koch-Institut, AIV2, Nordufer 20, D-1000 Berlin 65, Germany

Abbreviations: TFIIIA/B/C, transcription factors A/B/C for polymerase III; SAM, S-adenosyl-L-methionin; NP, nucleotide position

2. MATERIALS AND METHODS

2.1. *Plasmids and enzymes*

The plasmid pXls11 containing a somatic 5S rRNA gene from *X. laevis* in the *Hin*dIII cloning site of M13mp9 [14] and pXlo31 containing an oocyte 5S rRNA gene in the *Hin*dIII cloning site of pBR322 [15] were donated by T.Pieler, Berlin. Ch A2 lys2 containing a $tRNA^{lys2}$ gene from chicken cloned into the *Bam*HI site of pSP64 [16] was a gift from B.Wittig, Berlin (Fig. 1). For injection into oocytes, the cloned inserts were purified from vector sequences by agarose gel electrophoresis and circularized by ligation using standard methods [17].

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00145793/90/$3.50

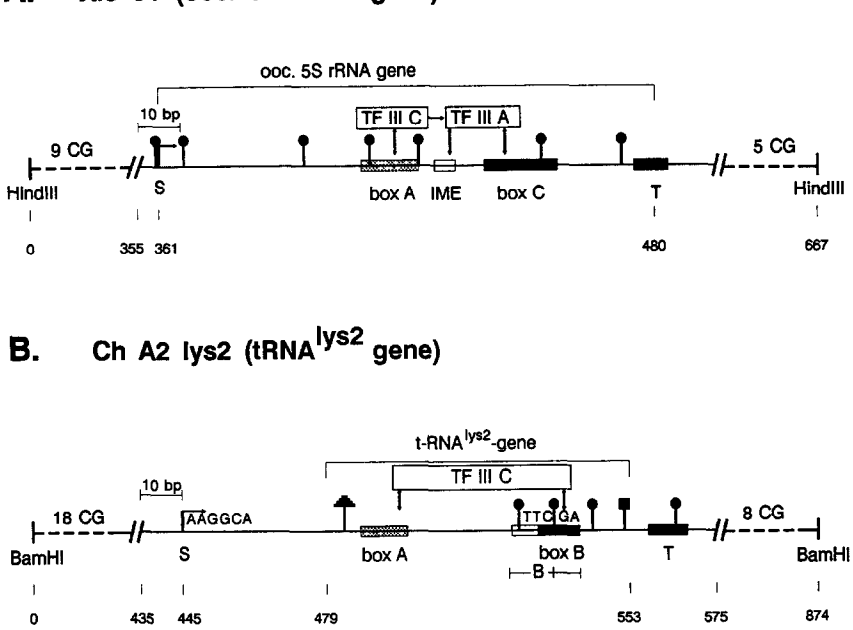

Fig. 1. Representation of the oocyte 5S rRNA gene and the $tRNA^{lys2}$ gene used. Transcription factors, TFIIIA and TFIIIC, and their target sequences, transcription start sites (horizontal arrows) and terminators (T) are shown. Methylation sites are represented by filled circles (CG), squares (CCGG) and triangles (GCGC) above the line.

A eukaryotic DNA methyltransferase was purified from regenerating rat liver [18,19]. The prokaryotic *Hpa*II and *Hha*I methyltransferases were obtained from New England Biolabs, other DNA modifying enzymes from Gibco-BRL.

2.2. *Methylation of DNA*

The DNAs were methylated as described [20] with minor modifications. Methylation with the rat liver methyltransferase was performed at a DNA concentration of 1–2 ng/μl with 1000 units of enzyme/μg DNA at 30°C for 40 h. The conditions were: 20 mM Tris-HCl, pH 7.8, 5 mM EDTA, 40 mM KCl, 0.5 mM dithiothreitol, 10% glycerol, 0.05% Triton-X100, 200 μg/ml bovine serum albumin, 70 μM SAM. The reaction mixture for *Hpa*II and *Hha*I methylation contained 10 μg/ml DNA, 0.5 units/μg DNA of M · *Hpa*II or 0.5 units/μg DNA of M · *Hha*I, 50 mM Tris-HCl, pH 7.8, 5 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 200 μg/ml bovine serum albumin, 30 μM SAM and was incubated for 16 h at 33°C. Mock-methylated DNA was obtained by incubation in the absence of SAM.

The DNAs were purified from the reaction mixtures by treatment with 1% lauroyl sarcosinate and 250 μg/ml proteinase K for 40 min at 37°C and two phenol extractions, followed by dialysis over two G-25 spin-columns. DNAs methylated with the bacterial methyltransferases, after purification, were incubated with the corresponding restriction enzyme at 10 units/μg DNA for 2 h at 37°C and repurified. The efficiency of methylation was controlled by the incorporation of radioactive methylgroups into the DNA in a 20 μl parallel methylation incubation with [^{3}H]SAM (15 Ci/mmol). An aliquot of the methylated, ^{3}H-labeled DNA was incubated with the methylation-sensitive restriction enzymes *Hpa*II or *Hha*I and the fragments were separated on an agarose gel. After equilibration with ^{3}H-Enhance (NEN) a fluorography of the dried gel was performed with Kodak X-Omat film.

2.3. *Injection into oocytes and RNA detection*

Groups of 25–30 stage VI *Xenopus* oocytes were injected [21] with 25 nl/oocyte containing: 120 nCi [α-^{32}P]GTP (NEN: 800 Ci/mmol) and 2 ng Ch A2 lys2/tRNA gene, 2 ng Xls11 gene or 5 ng Xlo31 gene. Four groups of oocytes were injected with each DNA in one experiment and 3 independent experiments were performed with each DNA. The results described below were reproducibly obtained in all the experiments.

After 18–24 h of incubation at 18°C the oocytes were homogenized by pipetting in 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 10 mM NaCl, 1% NaDoSO4 and 500 μg/ml proteinase K [22] and incubated for 2 h at 37°C. After the addition of 400 mM NaCl the RNA was purified by 3 phenol/chloroform extractions and 2 ethanol precipitations and analyzed on 8% acrylamide-urea gels. *Hha*I fragments of pBR322^{32}P-end-labeled with T4 polynucleotide kinase were used as markers. An autoradiography of the dried gel was performed with Kodak X-Omat film.

3. RESULTS

3.1. *Expression of methylated 5S genes*

A possible role of DNA methylation in the developmental inactivation of oocyte type 5S rRNA genes was evaluated by comparing the expression of methylated and unmethylated 5S genes after injection into oocytes. A cloned oocyte type 5S gene after excision from the vector, was extensively methylated with a DNA methyltransferase from rat liver. Under the conditions used, the enzyme methylates cytosine in every CpG and a small amount of the cytosine in CpA and CpT in addition. This has been demonstrated by Maxam-Gilbert sequencing of in vitro methylated SV40 DNA [20] and TK DNA (unpublished results), as well as by determination of the percentage of CpG methylation and analysis with methylation sensitive restriction enzymes of different in vitro methylated DNAs [19,23,24]. To check the efficiency of methylation a fraction of the incubation mixture was incubated with [^{3}H]SAM as a methyldonor. The oocyte 5S gene contains 3.2% CpGs. As we found an incorporation of

4.7% methylgroups and as the enzyme is known to have an at least 50 fold preference for CpG over CpA and CpT methylation [20], all CpGs should have been completely methylated.

After injection into oocytes (Fig. 2A) together with [α-^{32}P]GTP to label the RNA, no significant differences in expression were observed between methylated (lanes 3,4) and mock-methylated (lanes 1,2) oocyte 5S genes. As Fig. 2B shows, somatic 5S genes were also not inhibited by methylation (lane 1: mock-methylated; lane 2: methylated). The extra bands of lower MW could be caused by degradation during the RNA isolation or by premature termination of RNA synthesis. No labeled RNA was visible when [α-^{32}P]GTP was injected without DNA (Fig. 2B, lane 3).

3.2. *Expression of the methylated tRNA^{lys2} gene*

The tRNA^{lys2} gene (Ch A2 lys2) was purified from the vector and circularized by ligation. Subsequent methylation with the rat liver methyltransferase led to the incorporation of 5.2% methylgroups. With a CpG content of 3.6% this indicated methylation of all the CpGs and some CpA and CpT. As two of the CpGs form part of *Hpa*II sites (CCGG at NPs: 175, 482) and further two of *Hha*I sites (GCGC at NPs: 352, 552), they could also be tested for complete methylation with the corresponding methylation sensitive restriction enzymes. Neither enzyme cleaves a DNA, if its recognition sequence is methylated at the inner cytosine. For that purpose, tRNA^{lys2} DNA methylated with [^{3}H]SAM was mixed with an excess of unmethylated control DNA and incubated with *Hpa*II or *Hha*I, and additionally with *Bam*HI to linearize the DNA. Fig. 3A shows complete restriction of the unmethylated control DNA with *Hpa*II (lane 1) and *Hha*I (lane 2) in the ethidium-bromide stain, but no restriction of the methylated DNA (Fig. 3B) with *Hpa*II (lane 1) or *Hha*I (lane 2) in the fluorogram, indicating complete methylation of these restriction sites. Concerning the distribution of the supercoil and open ring form, a similar picture on agarose was obtained for mock-methylated (Fig. 3C, lane 1) and methylated DNA (lane 2) before injection.

Transcription of the tRNA^{lys2} gene, starting at AAG-GCA (Fig. 1), results in an 105 bp precursor tRNA which is rapidly processed to the final 74 bp product [16]. A time-course experiment in oocytes showed a steady state level of mature product with a small amount of precursor from 12–24 h after injection (data not shown). The tRNA^{lys2} gene was transcribed about 20 times more efficiently than the oocyte 5S gene in oocytes. Methylation with the eukaryotic enzyme inhibited its transcription by about 80% (Fig. 4A, lanes 3,4) in comparison with the mock-methylated control DNA (lanes 1,2). Similar results were obtained in three independent experiments with three different DNA preparations. In contrast to eukaryotic methylation, methylation with the *Hpa*II (Fig. 4B, lanes 3,4) or *Hha*I methyltransferase (Fig. 4C, lanes 3,4) or both together (not shown) dit not affect tRNA^{lys2} gene transcription (see Fig. 4B, lanes 1,2; 4C, lanes 1,2 for mock-

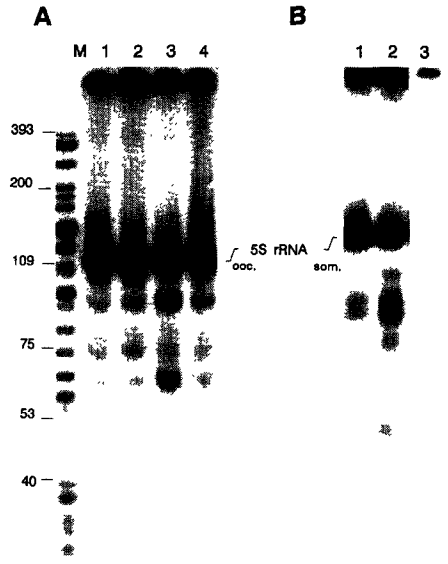

Fig. 2. Transcription in oocytes of 5S rRNA genes methylated with a eukaryotic DNA methyltransferase. 5 ng oocyte 5S gene or 2 ng somatic 5S gene were injected per oocyte together with [α-^{32}P]GTP. The RNA from 5 oocytes was separated on an 8% acrylamide-urea gel.(A) oocyte 5S gene mock-methylated (lanes 1,2) and methylated (lanes 3,4); M: pBR322/*Hha*I fragments, ^{32}P-end-labeled. (B) somatic 5S gene mock-methylated (lane 1) and methylated (lane 2). Injection of [α-^{32}P]GTP alone (lane 3).

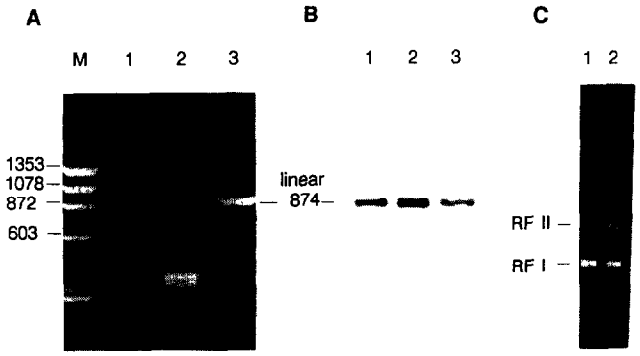

Fig. 3. In vitro methylation of a tRNA^{lys2} gene with a eukaryotic methyltransferase: test for methylation efficiency. 5 ng tRNA^{lys2} DNA methylated with the eukaryotic enzyme in the presence of [^{3}H]SAM together with 100 ng unlabeled DNA was incubated (2 h, 37°C) with *Bam*HI and the methylation sensitive enzymes *Hpa*II or *Hha*I (2U) and separated on 1.4% agarose. A: ethidiumbromide stain: *Bam*HI/*Hpa*II (lane 1); *Bam*HI/*Hha*I (lane 2); *Bam*HI (lane 3). B: ^{3}H-fluorogram of the same gel. C: agarose (1.4%) gelelectrophoresis of mock-methylated (lane 1) and methylated (lane 2) tRNA^{lys2} DNA (100 ng) without restriction enzyme treatment. M: ΦX174/*Hae*III fragments.

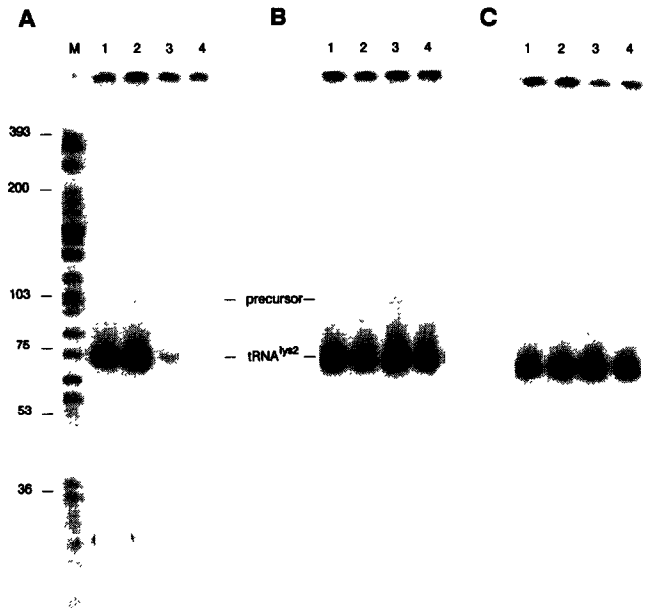

Fig. 4. Transcription in oocytes of a tRNA^{lys2} gene methylated with eukaryotic or prokaryotic DNA methyltransferase, 2 ng mock-methylated or methylated tRNA^{lys2} DNA were injected per oocyte together with [α-^{32}P]GTP. The RNA of 5 oocytes was separated on an 8% acrylamide-urea gel. A: DNA mock-methylated (lanes 1,2) or methylated (lanes 3,4) with a rat liver methyltransferase; B: DNA mock-methylated (lanes 1,2) or methylated (lanes 3,4) with M · *Hpa*II; C: DNA mock-methylated (lanes 1,2) or methylated (lanes 3,4) with M · *Hha*I; M: pBR322/*Hha*I fragments, ^{32}P-end-labeled.

methylated controls). Each enzyme has one methylation site in the tRNA gene (Fig. 1) and an additional one in the upstream region (see above).

4. DISCUSSION

In vitro methylation of somatic 5S DNA from *X. laevis* does not influence its transcription in *Xenopus* oocytes and we conclude that DNA methylation is not involved in the permanent shut off of these genes during *X. laevis* embryogenesis. Transcription of a tRNA^{lys2} gene, on the other hand, is inhibited by 80% under similar experimental conditions. These differences in the response to DNA methylation can probably be explained by different requirements for the formation of a transcription complex which precedes transcription initiation by RNA polymerase III.

In the case of RNA polymerase III type 1 genes, as 5S rRNA genes, binding of transcription factor TFIIIA to box C and IME and additionally to TFIIIC to box A (Fig. 1) results in an active transcription complex. With RNA polymerase III type 2 genes, to which tRNA genes, VA genes, EBER genes, human 7SL, 4.5S genes and the Alu gene family belong, factor TFIIIC has to bind to the B(B+) and to the A box to form stable complexes. For tRNA genes additional binding of TFIIIB is also necessary [4,25].

From RNA polymerase II genes it is known that methylation in the recognition sequence of a transcription factor can reduce its binding and inhibit transcription [26,27]. But this is not the only mechanism for transcription inhibition by DNA methylation. There is also evidence for a repressor protein which binds with enhanced affinity to methylated DNA [28]. A third possibility is the alteration of the binding or distribution of nucleosomes in the promotor region by methylation [29]. Generally speaking, methylation in different ways influences the equilibrium between activators and repressors of transcription in favour of the latter ones and thus inhibits transcription of RNA polymerase II genes.

Similar mechanisms could also apply to the inhibition of tRNA^{lys2} gene transcription by methylation. The tRNA^{lys2} gene itself including the main regulatory elements for transcription contains five CpGs (Fig. 1). Methylation of two of them forming part of a *Hpa*II and a *Hha*I site have no influence on transcription (Fig. 4). The remaining three CpGs, one of them in and two immediately adjacent to box B ICR, are therefore good candidates to be responsible for the inhibition of transcription by methylation. They could act by altering the binding equilibrium between TFIIIC and nucleosomes or other repressors. The central one of the CpGs in the sequence TTCGA is of special interest because this sequence is conserved in 65% of the eukaryotic tRNA genes. Another 9% of tRNA genes have the closely related sequence ATCGA at this position [30].

As, however, for some tRNA genes an additional influence of 5′ flanking sequences up to −70 on transcription has been demonstrated [4] a role of two CpGs in the upstream region of the tRNA^{lys} gene (NPs: −27, −36) in the inhibition effect cannot be excluded at present. Further experiments will have to show whether other tRNA genes and polymerase III type 2 genes can also be repressed by DNA methylation and whether this regulatory mechanism is exploited by the cell.

Acknowledgements: The authors wish to thank T. Pieler and B. Wittig for helpful discussion and for providing the plasmids. This research was supported by the Deutsche Forschungsgemeinschaft.

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