A re-investigation of the ribonuclease sensitivity of a DNA demethylation reaction in chicken embryo and G8 mouse myoblasts

Jean-Pierre Jost*, Michel Siegmann, Stéphane Thiry, Yan-Chim Jost, Don Benjamin, Steffen Schwarz

Friedrich-Miescher-Institut, P.O. Box 2543, CH-4002 Basel, Switzerland

Received 9 March 1999

Abstract Recently published results (Nucleic Acids Res. 26, 5573–5580, 1998) suggest that the ribonuclease sensitivity of the DNA demethylation reaction may be an experimental artifact due to the possible tight binding of the nucleases to the methylated DNA substrate. Using an improved protocol we show for two different systems that demethylation of hemimethylated DNA is indeed sensitive to micrococcal nuclease, requires RNA and is not an experimental artifact. The purified 5-MeC-DNA glycosylase from chicken embryos and G8 mouse myoblasts was first incubated for 5 min at 37°C with micrococcal nuclease in the presence of Ca^{2+} in the absence of the DNA substrate. Upon blocking the nuclease activity by the addition of 25 mM EGTA, the DNA demethylation reaction was initiated by adding the labeled hemimethylated DNA substrate to the reaction mixture. Under these conditions the DNA demethylation reaction was abolished. In parallel controls, where the purified 5-MeC-DNA glycosylase was pre-incubated at 37°C with the nuclease, Ca^{2+} and EGTA or with the nuclease and EGTA, RNA was not degraded and no inhibition of the demethylation reaction was obtained. As has already been shown for chicken embryos, the loss of 5-MeC-DNA glycosylase activity from G8 myoblasts following nuclease treatment can also be restored by the addition of synthetic RNA complementary to the methylated strand of the substrate DNA. No reactivation of 5-MeC-DNA glycosylase is obtained by complementation with a random RNA sequence, the RNA sequence complementary to the non-methylated strand or DNA, thus ruling out a non-specific competition of the RNA for the binding of the nuclease to the labeled DNA substrate.

Key words: DNA demethylation; Ribonuclease sensitivity; Micrococcal nuclease

1. Introduction

In a series of studies on DNA demethylation carried out with L8 myoblasts and F9 teratocarcinoma cell lysate, Weiss et al. [1] showed that the reaction was sensitive to ribonucleases and insensitive to proteinase K. They concluded that DNA demethylation occurs through nucleotide excision catalyzed by a ribozyme. Later Swisher et al. [2] reported that the demethylation of DNA in L8 myoblasts was sensitive to proteinase K and that the ribonuclease sensitivity observed by Weiss et al. was an experimental artifact. In this case a very tight binding of the ribonuclease to the DNA substrate could possibly inhibit the demethylation reaction.

In chicken embryos we have previously shown [3] that the demethylation of a hemimethylated oligonucleotide requires both protein and RNA. RNA was isolated from SDS-PAGE purified enzyme and cloned [4]. We have also shown that the RNA tightly associated with 5-MeC-DNA glycosylase is very rich in CpGs and when added to the ribonuclease inactivated enzyme it could restore the full glycosylase activity. Further studies carried out with in situ hybridization with one representative RNA showed that it was only expressed in dividing and differentiating cells [5]. Colocalization of this RNA with DNA methyltransferase was also observed [5]. As we have already shown for chicken embryos, in growing mouse G8 myoblasts, demethylation of hemimethylated DNA is also ribonuclease sensitive and requires RNA. Additional controls rule out a non-specific effect of the nuclease on the DNA substrate.

2. Materials and methods

2.1. Purification of chicken embryo 5-MeC-DNA glycosylase

Purification of crude nuclear extracts from 12 day old chicken embryos and the various chromatographic steps were carried out as previously described [6].

2.2. Purification of mouse G8 myoblast 5-MeC-DNA glycosylase

Packed G8 myoblasts (4 g) were resuspended in 12 ml of 10 mM Tris pH 7.5, 1 mM EDTA, 5 mM DTT, 1 mM spermine, 0.25 mM spermidine and 2 mM benzamidine. The cell suspension was left on ice for 30 min and homogenized with 20 strokes in a Dounce glass homogenizer. Nuclei were lysed by adding slowly a 1/10 volume of 4 M $(NH_4)_2SO_4$ buffered with Tris to pH 8.0. The lysate was stirred on ice for 30 min and then centrifuged for 3.5 h at 50 000 rpm at 2°C in a SW 56 Beckman rotor. The supernatant was precipitated with solid ammonium sulfate (0.4 g/ml solution). After 1 h on ice the precipitate was sedimented by centrifugation at 30 000 rpm for 30 min in a SW 40 Beckman rotor. The sediment was dissolved in 3 ml of 20 mM HEPES pH 7.5, 10 mM EDTA, 100 mM KCl, 2 mM DTT, 2 mM benzamidine, 10% glycerol and dialyzed for 3-4 h at 4°C against two changes of 1 liter of the same buffer. Chromatography on heparin-Sepharose was carried out as previously described [6].

2.3. 5-MeC-DNA glycosylase assay

The standard assay of 5-MeC-DNA glycosylase was carried out as previously described [6]. The end-labeled DNA substrate was double stranded hemimethylated oligonucleotide with a methylated CpG on the lower strand 5′-TCACGGGATCAATGTGTTCTTTCAGCTCm-CGGTCACGCTGACCAGGAATACC-3′. The reaction product was analyzed on 20% polyacrylamide-urea sequencing gels. Gels were exposed for 30–60 min to X-ray films at −80°C. Various combinations of controls and micrococcal nuclease treatments are outlined in Table 1.

2.4. Complementation test of ribonuclease inactivated 5-MeC-DNA glycosylase with RNA

Table 1 shows the sequence of steps for the assembly of the pre-incubation mixture with active and inactivated micrococcal nuclease.

*Corresponding author. Fax: (41) (61) 721-4091.
E-mail: jost@fmi.ch

PII: S0014-5793(99)00454-8

Table 1
Assembly of the reaction mixtures 1–6 shown in Fig. 2

Incubation Mixture	10 min at 0°C Reaction mixture plus	Addition of enzyme E		Stop nuclease reaction all tubes on ice for 15 min		
	a	b	c	d	e	f
1	nuclease, EGTA, EDTA	E	Incubation 5 min at 37°C, then back at 0°C	---	Addition of labeled DNA substrate to all tubes	Incubate all tubes 1 hr at 37°C, then stop reaction
2	nuclease, Ca^{++} EGTA, EDTA	E		---		
3	EGTA, EDTA	E		---		
4	no addition	E		EGTA, EDTA		
5	nuclease, Ca^{++}	E		EGTA, EDTA		
6	no addition	---		---		

Pre-incubation before adding the labeled DNA substrate

Pre-incubation before adding the labeled DNA substrate consisted of a and b, where E is the purified 5-MeC-DNA glycosylase; c: the reaction mixtures were incubated for 5 min at 37°C. In d, EDTA and EGTA were added where indicated. In e, the reaction was initiated by the addition of the labeled hemimethylated DNA and all assay mixtures were incubated for 1 h at 37°C.

Micrococcal nuclease was preferred to ribonuclease A because its activity can be better controlled by Ca^{2+} and EGTA. Micrococcal nuclease was activated with 4 mM $CaCl_2$ and it was inactivated with 25 mM EGTA. As a further precaution, EDTA was added to a final concentration of 12.5 mM. For each reaction with 2 μg of purified 5-MeC-DNA glycosylase (fraction post carboxymethyl (CM)-Sepharose), 60 units of micrococcal nuclease were incubated for 5 min at 37°C or alternatively 30 μg of post heparin-Sepharose fraction was incubated with 10 units of micrococcal nuclease. The optimal concentration of micrococcal nuclease was determined experimentally. For example, Fig. 1 shows that a 5 min incubation at 37°C of 50 ng labeled RNA with 60 units of micrococcal nuclease is sufficient to completely digest the RNA (lane 2). When the same amount of RNA is incubated for 30 min at 37°C in the presence of 4 mM $CaCl_2$, 60 units of micrococcal nuclease, inactivated by the addition of 25 mM EGTA and 12.5 mM EDTA, very little degradation of the RNA was observed (lane 3). In addition, each assay received 100 units of recombinant porcine ribonuclease inhibitor which has been shown to reduce the background of ribonuclease activity in the 5-MeC-DNA glycosylase preparation. The basic reaction mixture consisted of 20 mM HEPES, pH 7.5, 50 mM NaCl, 4 mM Pefabloc, 50 μg enzyme grade BSA, 5 mM DTT, 100 units of recombinant porcine ribonuclease inhibitor in a total volume of 80 μl. Where indicated in Table 1 (a and b), 10–60 units of micrococcal nuclease, 4 mM $CaCl_2$ and/or 25 mM EGTA, 12.5 mM EDTA and 2 μg of purified chicken embryo 5-MeC-DNA glycosylase (fraction post CM-Sepharose) or 30 μg of purified G8 myoblast 5-MeC-DNA glycosylase (fraction post heparin-Sepharose) were added to the reaction mixture. The demethylation reaction was initiated by the addition of the labeled hemimethylated DNA substrate. Complementation assay of the nuclease treated 5-MeC-DNA glycosylase was carried out by adding appropriate RNA (5–10 μg) or DNA together with the labeled DNA substrate (10–50 ng substrate). The RNA sequence complementary to the methylated strand was 5′-GUGACCGGAGC-3′ whereas the sequence of the opposite non-methylated strand was 5′-GCUCCGGUCAC-3′. The random non-complementary sequence was 5′-CUCUCUCUCUU-3′. Upon incubation for 1 h at 37°C the reaction mixture was diluted to 150 μl with H_2O, phenol and chloroform extracted and precipitated with ethanol. Upon centrifugation the sediment was dissolved in 95% formamide dye, denatured for 5 min at 95°C and separated on a 20% polyacrylamide urea sequencing gel.

2.5. Chemicals and enzymes

Benzamidine was purchased from Fluka AG (Buchs, Switzerland). Pefabloc was obtained from Boehringer Mannheim; micrococcal nuclease (*Staphylococcus aureus*) was from Promega. Polynucleotide kinase and restriction enzymes were purchased from Biofinex (Praroman, Switzerland). [γ-^{32}P]ATP triethylammonium (3000 Ci/mmol) was purchased from Amersham, oligonucleotide RNAs were synthesized by Microsynth (Balgach, Switzerland) and all other oligonucleotides (methylated or otherwise) were synthesized in house by Mr. Peter Müller.

3. Results and discussion

3.1. Pre-incubation of purified 5-MeC-DNA glycosylase with micrococcal nuclease in the absence of DNA substrate abolishes subsequent demethylation reaction

In the upper panel of Fig. 2, reactions were carried out with purified 5-MeC-DNA glycosylase from chicken embryos whereas in the lower panel all reactions were done with a post heparin-Sepharose fraction prepared from mouse G8 myoblast cellular extracts. The first four lanes of Fig. 2 (upper and lower panels) are the controls as outlined in Table 1. Micrococcal nuclease is only active in the presence of Ca^{2+} (4 mM) and is inactivated by EGTA (25 mM). In lanes 1 and 2 prior to the addition of the purified 5-MeC-DNA glycosylase (enzyme E), micrococcal nuclease was present in its inactivated form. No nuclease but EGTA and EDTA were added to the reaction mixture of lane 3 whereas lane 4 had no further addition. In lane 5, micrococcal nuclease was activated by Ca^{2+} and lane 6 is a blank. After incubation for 10 min on ice, purified 5-MeC-DNA glycosylase was added to the assay mixtures 1–5 and samples were further incubated for exactly 5 min at 37°C. These conditions were sufficient to inactivate 5-MeC-DNA glycosylase (lane 5). All samples were then returned to ice for 15 min and where indicated in Table 1, EGTA and EDTA were added to the reaction mixture. Only at this point the end-labeled hemimethylated substrate was added to all samples. The reaction mixtures were then incubated for 1 h at 37°C. The results in Fig. 2, lanes 1 and 2 (upper and lower panels) clearly show that in the presence of micrococcal nuclease in its inactive form, no inhibition of DNA demethylation is observed. However, treatment for 5 min at 37°C with the active nuclease abolishes 5-MeC-

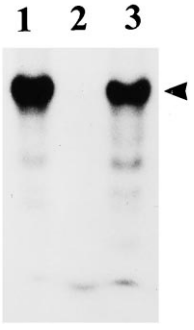

Fig. 1. Sensitivity of 50 ng end-labeled RNA: GUGACCGGAGC to 60 units of micrococcal nuclease. Lane 1 is the labeled RNA alone. In lane 2, the same RNA was treated for 5 min at 37°C with 60 units of micrococcal nuclease and in lane 3 the micrococcal nuclease was inactivated with 25 mM EGTA, 12.5 mM EDTA. Upon addition of the labeled RNA, incubation was continued for 30 min at 37°C. The reaction product was separated on a 20% polyacrylamide-urea gel. The arrowhead shows the intact labeled RNA.

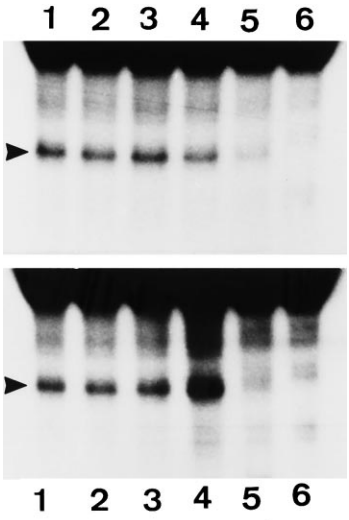

Fig. 2. Sensitivity of DNA demethylation reaction to micrococcal nuclease. In the upper panel reactions were carried out with 2 μg 5-MeC-DNA glycosylase (post carboxymethyl-Sepharose fraction) from chicken embryos whereas in the lower panel the reaction was performed with 30 μg 5-MeC-DNA glycosylase (post heparin-Sepharose fraction) from mouse G8 myoblasts. The conditions of reactions 1–6 are as outlined in Table 1 and in Section 2. The reaction product was separated on 20% polyacrylamide-urea gel. The arrowhead shows the correct position of the cleavage product.

DNA glycosylase activity (lane 5). In an additional control where 5-MeC-DNA glycosylase was pre-incubated at 37°C in the presence of only Ca^{2+} (prior to the addition of EGTA) or when the sequential addition of all components of the pre-incubation mixture was permutated, no inhibition of DNA demethylation was observed (results not shown). Since in all tubes the labeled DNA substrate was added only upon the inactivation of micrococcal nuclease, it cannot be argued that the difference obtained in lanes 1 and 2 and the micrococcal nuclease test in lane 5 is due to a coating of the DNA substrate with the nuclease. Similar observations were made in experiments where ribonuclease A activity was controlled by the porcine ribonuclease inhibitor. However, in these experiments very large quantities of the inhibitor were required for the complete inhibition of the nuclease [4].

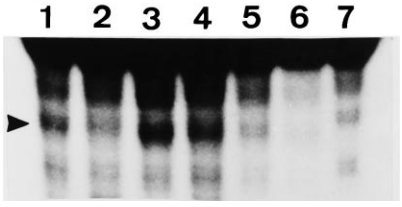

Fig. 3. Complementation test of nuclease inactivated DNA demethylation reaction with synthetic RNA. The reaction product was separated on a 20% polyacrylamide-urea gel. The arrowhead shows the correct position of the cleavage product. The experiment was carried out with 15 μg of post Heparin-Sepharose fraction from mouse G8 myoblast cellular extracts. Lane 1 is the positive control pre-incubated as in 1 of Table 1, in the absence of nuclease. Lane 2 is the enzyme treated with 10 units of micrococcal nuclease (details see Table 1, line 5). Lane 3 is as lane 2 except that following the pre-incubation with micrococcal nuclease, EGTA, EDTA and 10 μg of GUGACCGGAGC (2-*O*-methyl-ribose modified) were consecutively added and incubated for 1 h at 37°C in the presence of labeled substrate DNA. Lane 4 is as lane 3 with the same RNA sequence but unmodified. Lanes 5 and 6 are as lane 3 except that oligos GCUCCGGUCAC and CUCUCUCUCUU which are not complementary to the methylated strand of the hemimethylated substrate were added to the incubation mixture respectively. Lane 7 is a blank incubated with no further additions (see Table 1, line 6).

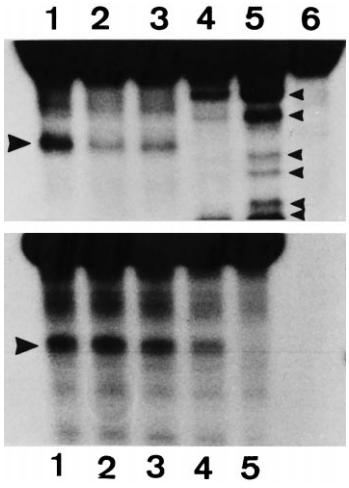

Fig. 4. The upper panel is a complementation test with short oligodeoxyribonucleotides (11 bases) with micrococcal nuclease inactivated 5-MeC-DNA glycosylase purified from mouse G8 myoblasts. Lane 1 is the positive control incubated with the intact enzyme. Lane 2 is the enzyme treated with micrococcal nuclease. Lanes 3–5 are as lane 2 except that EGTA, EDTA, the oligonucleotides and the labeled DNA substrate were added consecutively following the pre-incubation with micrococcal nuclease. The incubation conditions were basically the same as for Fig. 3. Lane 3 received 10 μg of GTGACCGGAGC (complementary to the methylated strand). Lane 4 received 10 μg of GCTCCGGTCAC (complementary to the non-methylated strand) and lane 5 received 10 μg of double stranded oligonucleotide (duplex of oligos of lanes 3 and 4). Lane 6 is a blank. The reaction products were separated on a 20% polyacrylamide-urea gel. The larger arrowhead indicates the correct position of the cleavage product and the small arrowheads show the non-specific cleavage products. The lower panel is an assay of 5-MeC-DNA glycosylase as in lane 1 of the upper panel except that oligonucleotides were added to the intact enzyme. Lanes 2–4 received respectively 10 μg of GTGACCGGAGC, GCTCCGGTCAC and a duplex of the two previous oligos. Lane 5 is a blank. Reaction products were analyzed as in the upper panel.

3.2. The nuclease inactivated 5-MeC-DNA glycosylase from G8 myoblasts inactivated by micrococcal nuclease can be selectively reactivated with a specific RNA sequence

We have previously shown that the purified 5-MeC-DNA glycosylase from chicken embryo, which had been inactivated by ribonuclease A, or micrococcal nuclease was reactivated by the addition of specific RNA [4]. Similarly the results shown in Fig. 3 strongly suggest that in mouse G8 myoblasts, the nuclease inactivated 5-MeC-DNA glycosylase is also capable of reactivation by the addition of synthetic RNA complementary to the methylated strand (lanes 3 and 4). In lane 3, 10 μg of 5′-GUGACCGGAGC-3′ (2-*O*-ribose methylated) and in lane 4, the same unmodified RNA were added together with the labeled DNA probe to the reaction mixture. In contrast, oligo RNA complementary to the opposite non-methylated strand showed no effect (lane 5). The same concentration of an oligo RNA with no complementarity to either strand was also ineffective (lane 6). Therefore, these results rule out a non-specific competition of the RNA for the binding of the nuclease to the labeled DNA substrate. In addition, the above results suggest that the demethylation of DNA observed in

mouse G8 myoblasts [7] may be very similar to that described for the developing chicken embryos. As a further test for a possible masking of the labeled DNA by micrococcal nuclease, a complementation test with synthetic oligodeoxynucleotides was carried out. The 5-MeC-DNA glycosylase inactivated by micrococcal nuclease was incubated with a 1000-fold weight excess of single stranded oligonucleotides complementary to the upper and lower strand of the DNA probe respectively. The results shown in Fig. 4 (upper panel), lanes 3–5, show that no complementation was achieved with either single or double stranded DNA. However, it is interesting to note that in lane 5, in the presence of a 1000-fold weight excess of non-labeled double stranded DNA, there is a dramatic increase in random nicks (small arrowheads) of the DNA probe. We have so far no explanation for this non-specific DNA degradation. In the present case it could still be argued that the non-labeled oligonucleotide could possibly compete with hemimethylated DNA substrate for the binding of 5-MeC-DNA glycosylase. The lower panel of Fig. 4 shows that the addition of a 1000-fold weight excess of either single or double stranded oligonucleotides (lanes 2–4) did not significantly compete for the activity of 5-MeC-DNA glycosylase. Therefore, it is logical to conclude that in the present reaction there is most probably no masking of the labeled DNA probe by the micrococcal nuclease. A similar conclusion was drawn from the results presented in the complementation assay of Fig. 3. In all assays presented here, the main difference between the test of Weiss et al. [1], Swisher et al. [2] and ours is the use of hemimethylated DNA as a substrate in the presence of EDTA, EGTA and the purified enzyme. In the presence of symmetrically methylated DNA substrate (methylated on both strands), the same protein fractions purified from G8 myoblasts and chicken embryos showed no significant activity. Therefore, the effect of the nuclease on the purified 5-MeC-DNA glycosylase was only tested on hemimethylated DNA substrate. We believe that the difference between our results and those of Swisher et al. [2] is probably due to a different type of mechanism of demethylation. A comparison between the two mechanisms will only be possible once the enzymes involved are purified, sequenced and cloned.

References

[1] Weiss, A., Keshet, I., Razin, A. and Cedar, H. (1996) Cell 86, 709–718.
[2] Swisher, J.F.A., Rand, E., Cedar, H. and Pyle, A.M. (1998) Nucleic Acids Res. 26, 5573–5580.
[3] Frémont, M., Siegmann, M., Gaulis, S., Matthies, R., Hess, D. and Jost, J.P. (1997) Nucleic Acids Res. 25, 2375–2380.
[4] Jost, J.P., Frémont, M., Siegmann, M. and Hofsteenge, J. (1997) Nucleic Acids Res. 25, 4545–4550.
[5] Schwarz, S., Frémont, M. and Jost, J.P., in preparation.
[6] Jost, J.P., Siegmann, M., Sun, L. and Leung, R. (1995) J. Biol. Chem. 270, 9734–9739.
[7] Jost, J.P. and Jost, Y.C. (1994) J. Biol. Chem. 269, 10040–10043.