Nucleic Acids Research, 1998, Vol. 26, No. 24 **5573–5580**

Analysis of putative RNase sensitivity and protease insensitivity of demethylation activity in extracts from rat myoblasts

Jennifer F. A. Swisher, Eyal Rand[1], Howard Cedar[1] and Anna Marie Pyle*

Department of Biochemistry and Molecular Biophysics, Columbia University and Howard Hughes Medical Institute, New York, NY 10032, USA and [1]Department of Cellular Biochemistry, Hebrew University Medical School, Jerusalem 91120, Israel

Received September 23, 1998; Revised and Accepted October 14, 1998

ABSTRACT

The mechanism for demethylation of DNA in rat myoblasts has recently been studied using a new *in vitro* system that monitors demethylation in whole cell extracts. Previous investigations using this system had indicated that demethylation is resistant to conditions that are normally assumed to denature or digest proteins. Remarkably, it was reported that the activity appeared to be sensitive to the action of ribonuclease, suggesting a role for RNA in the demethylation of DNA. This manuscript reports that, upon further purification of the extract, demethylation activity has properties that are different. When subjected to more rigorous procedures for digestion of proteins, the demethylase activity disappears. Furthermore, RNase sensitivity of the extract disappears when a quantity of unmethylated competitor DNA is added to the reaction mix or when extracts treated with RNase are subsequently treated with protease. Although a role for RNA cannot be completely discounted, it is unlikely that this demethylation reaction involves RNA cofactors or ribozyme components. These results have important implications for the mechanism of DNA demethylation and they exemplify the potential pitfalls of experiments in which new biological roles for RNA are evaluated using RNase sensitivity experiments.

INTRODUCTION

The search for new RNA enzymes

In the early 1980s, the first examples of RNA molecules with catalytic activity were found in a self-splicing intron from *Tetrahymena thermophila* and in a tRNA processing enzyme called RNase P (1,2). Since then, numerous other catalytic RNAs (or ribozymes) have been discovered, and even more were created by *in vitro* selection techniques (3). Although natural catalytic RNAs range in size from a few to a few thousand nucleotides and can be divided into distinct structural and functional classes, most have been shown to play a similar role: cleavage, and often ligation, of RNA phosphodiester linkages. One explanation for this preponderance of RNA-cleaving ribozymes is that phosphodiester cleavage/ligation is the only role left by evolution for RNA enzymes, and that other reactions are performed more efficiently by protein enzymes. However, an alternate possibility is that catalytic RNAs possess a broader scope of enzymatic capabilities, but that inadequate search methods have limited efforts to identify them. Although directed molecular evolution and *in vitro* selection techniques have indicated that many catalytic roles are possible for RNA, none of these has been observed in nature until very recently. For example, directed molecular evolution was used to generate a ribozyme that readily cleaves DNA (4). Subsequently, it was found that a natural ribozyme derived from group II intron ai5γ could cleave DNA with high efficiency, and that the rates for DNA and RNA cleavage were remarkably similar. (5). This was consistent with the finding that DNA has actually been found to be a natural substrate for group II introns, which behave as mobile genetic elements by reverse splicing into duplex DNA (6). Furthermore, there is increasing evidence that peptidyl transfer activity in the ribosome may be an RNA-catalyzed process (7,8).

RNA implicated in DNA demethylation

In another area of nucleic acids research, there has been a long search for the *cis*- and *trans*-acting factors responsible for the demethylation of genes, a process which appears to be concomitant with transcriptional activation during development. Since the demonstration that methylation of a promoter region is sufficient to repress transcription of a downstream gene, and that genomic methylation patterns are clonally inherited, great effort has been directed toward uncovering the mechanism of regulated demethylation and *de novo* methylation during development (9,10). Until very recently, the factors important for demethylation activity were assumed to be proteins. However, studies on a demethylating activity from extracts of rat myoblast cells were found to be consistent with the possibility that the reaction is catalyzed by a ribozyme or some form of enzymatically active RNA–protein complex (11). If this hypothesis were correct, it would not only represent the first example of a catalytic RNA in the nucleus of a higher eukaryote and the second natural ribozyme to cleave a substrate other than RNA, but it might represent an entirely new type of ribozyme chemistry.

*To whom correspondence should be addressed at: Department of Biochemistry and Molecular Biophysics, Box 36, Columbia University, 630 W. 168th Street, New York, NY 10032, USA. Tel: +1 212 305 5430; Fax: +1 212 305 1257; Email: amp11@columbia.edu

Although a demethylase gene has never been cloned or isolated, demethylating activity has been detected in a variety of cell lines and in chick embryos (12–14). In some of these cases, a methylated, non-replicating plasmid carrying a specific sequence was demethylated upon introduction into cells (13). In addition, extracts from whole cells or embryos were found to demethylate plasmids, which enabled the development of an *in vitro* system for the analysis of demethylation activities. One such extract-based system was set up using whole cell extracts from L8 rat myoblasts, which are able to selectively demethylate a construct carrying the upstream control region of the rat α-actin gene (11). Upon applying various treatments to the active extracts, it was found that the demethylase activity was not only resistant to proteinase K treatment, but was augmented by it. Remarkably, treatment of active extracts with RNase prior to the *in vitro* demethylation reaction seemed to abolish demethylation activity entirely. This combination of results suggested that an RNA might be involved as an enzymatic component or cofactor of the demethylase activity.

Since this initial report, further experiments were performed with the goal of isolating an RNA component of the demethylase activity. However, consistent problems with the assay itself necessitated extensive modifications to the procedures used for analysis of demethylase activity. Unexpectedly, as reported in the following paper, these improvements caused the L8 extracts to behave differently than previously reported: it was found that, when carried out under more rigorous conditions, the demethylating activity can be abolished by protease treatment. Furthermore, it was found that the apparent inhibitory effects of RNase treatment may have been due to an artifactual effect of ribonuclease rather than enzymatic cleavage of putative RNA components of the demethylase enzyme itself. The findings reported here are not only important for understanding the mechanism of demethylation, but they also provide important guidelines for interpreting any experiment in which an activity is reported to be sensitive to ribonuclease.

MATERIALS AND METHODS

Preparation of whole cell extracts

Culture of mononucleated L8 rat myoblast cells was carried out as previously described (11). To prepare cells for extract, cells were grown to 70–80% confluence, trypsinized, and washed three times using Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline. Cells were then resuspended at ~10^6 cells (one 10 cm^2 tissue culture plate, 70–80% confluent) per 100 µl CE buffer [20 mM Tris–HCl, pH 7.5, 0.4 M KCl, 20% (v/v) glycerol, 2 mM DTT]. Cells were then stored in liquid nitrogen until needed (unless otherwise indicated), then thawed by centrifugation at 10 000 *g* for 15 min. The supernatant from this step was spun at 200 000 *g* for 3 h at 4°C. This supernatant was diluted with addition of 3 vol of distilled H_2O, and then treated with 200 µg/ml proteinase K (Sigma) at 56°C for 1 h before use (unless otherwise indicated). RNase treatments of extracts were carried out with 100 µg/ml RNase A (Sigma) or 1000 U/ml RNase T1 (Boehringer-Mannheim) or U2 (BRL) at 37°C for 1 h prior to the proteinase K digestion.

It should be noted that in the previous report (11), the protocol as stated in the Experimental Procedures gave the mistaken impression that RNase treatment of crude extracts was always followed by further incubation with proteinase K. In fact, the RNase A sensitivity experiment shown in figure 1B of Weiss *et al.* (11) was carried out without the addition of protease. For RNase T1 (figure 1D of Weiss *et al.;* 11), proteinase K was used, but further studies showed that the inhibition of demethylase activity in that case may have been due to the high salt concentration of the enzyme preparation. Thus, in the initial paper, RNase sensitivity was actually only tested in crude, non-protease-treated extracts.

DNA substrate preparation

An 810 bp fragment containing the rat α-actin sequence was removed from the pBS-α-actin plasmid (15) by digestion with restriction enzymes *Bam*HI and *Hin*dIII. The fragment was isolated on a 1% agarose gel, excised, and purified using the Qiaex DNA isolation system (Qiagen.) The 5′ phosphates were removed from the fragment by treatment with 4 U of calf intestinal phosphatase (CIP; 1000 U/ml; Boehringer-Mannheim) for 30 min at 37°C, followed by phenol–chloroform extraction and ethanol precipitation. An aliquot of 50 µg of this DNA fragment was then methylated *in vitro* by overnight incubation with *Hpa*II methylase (5 U/mg DNA; Fermentas) at 37°C. The provided buffer was supplemented with 160 µM S-adenosyl methionine, 0.1 mg/ml BSA and 10 mM EDTA. After overnight incubation, this reaction mixture was treated with 200 µg/ml proteinase K for 1 h at 56°C, then extracted with equal volumes of phenol and chloroform, and ethanol precipitated in the presence of 250 mM NaCl. The DNA fragment (1.6 µg) was 5′ end-labeled in a 100 µl reaction using 20 U T4 polynucleotide kinase (1000 U/ml; Boehringer-Mannheim) and 60 µCi [γ-^{32}P]ATP (150 mCi/ml; Dupont-NEN). Labeled DNA was either purified by electrophoresis on a 5% non-denaturing polyacrylamide gel or by gel filtration using a 10 ml Sephadex G100 column, equilibrated with 10 mM Tris–HCl, pH 7.5 containing 1 mM EDTA. Labeled, unmethylated DNA substrate (used in Fig. 3) was prepared in the same manner, except the *in vitro Hpa*II methylation reaction was omitted.

The demethylation assay

Approximately 20 ng ^{32}P-labeled DNA substrate was added to each milliliter of prepared cell extract, along with 60 µg unlabeled, unmethylated carrier DNA, unless otherwise indicated. Carrier DNA is comprised of unlabeled pBS-α-actin plasmid digested with *Bam*HI and *Hin*dIII. At various times throughout the reaction, aliquots were removed and the reaction terminated by mixture with an equal volume of phenol. Each reaction aliquot (usually 250 µl) was then phenol–chloroform extracted and ethanol precipitated. For control experiments in which carrier DNA was not used during the reaction, 15 µg of carrier DNA was added immediately prior to phenol extraction. Aliquots from each time point were then denatured for 2 min at 95°C in the presence of 1 mM EDTA, and then allowed to reanneal at 60°C for 90 min. To increase the sensitivity of this assay, substrate DNA in each of these samples was denatured and then reannealed in the presence of excess unmethylated fragment (carrier DNA) prior to the restriction enzyme digest; this step makes it possible to detect the removal of even a single methyl group from the double-stranded DNA substrate (11). Because *Hpa*II will only cleave unmethylated DNA, hemimethylated duplexes resulting from demethylation on only one strand would not be detectable with *Hpa*II treatment without this step. Finally, time points were each digested with *Hpa*II (20 U for 2 h at 37°C) and analyzed by electrophoresis on 1% agarose gels in the presence of ethidium

bromide. Before each gel was dried, cleavage of excess carrier DNA was assessed by UV visualization to ensure that the *Hpa*II digest of unmethylated DNA was complete. Gels were then dried and autoradiographed to quantitate the extent of demethylation.

RESULTS

Previous work established that extracts from L8 rat myoblasts selectively demethylate a DNA sequence from the control region of the rat α-actin gene, as indicated by increased sensitivity of the initially methylated DNA substrate to cleavage with *Hpa*II restriction enzyme (11). Based on this observation, an *in vitro* assay was developed to study demethylation (Fig. 1). The substrate for this assay is an 810 bp methylated fragment of the rat actin sequence, 5′ end-labeled with ^{32}P. This labeled fragment is placed in whole cell extracts from L8 rat myoblast cells and aliquots are taken at designated time intervals. Demethylation activity is measured with a restriction enzyme assay that is sensitive to removal of even a single methyl group from methylated or hemimethylated DNA. Finally, reaction products are subjected to electrophoresis on an agarose gel and activity is measured by the evolution of *Hpa*II cleavage products from the initially *Hpa*II-resistant substrate DNA (Fig. 1).

Improvements in cell extract preparation and assay conditions

The cell extract preparation used by Weiss *et al*. was performed by suspending L8 cells in CE buffer, freezing them for 60 min at –70 °C, then collecting the supernatant after a low speed (10 000 *g*) centrifugation step. This extract was then diluted and assayed for demethylase activity (11). However, when this procedure is followed, results can be difficult to assess due to a DNA degradation activity that is frequently associated with extracts prepared in this manner: upon exposure to extract, evolution of *Hpa*II-cleavable substrate is accompanied by the appearance of degradation products that co-migrate with DNA markers between 6 and 10 bp in size (Fig. 2). Evidence of this problem can be seen in figure 1B of Weiss *et al*. (11) as an apparent decrease in amount of substrate over time, and it is mentioned in the Materials and Methods section of the same paper. Since degradation of the methylated DNA substrate was often so extensive that it prevented detection of changes in *Hpa*II sensitivity (Fig. 2A, left), it was necessary to improve conditions so that product evolution could be detected and monitored.

The method that proved most successful was adding an excess (≥10 000-fold) of unlabeled, unmethylated DNA to the extract during the reaction (Fig. 2A, right). This treatment was expected to reduce degradation of the methylated substrate by providing a competitor for the nucleases, but not for the demethylase. The addition of carrier DNA to the extracts prior to reaction markedly increased the ability to detect demethylation while concomitantly reducing the effects of endonuclease activity on the assay (Fig. 2A, right). Carrier DNA may improve product detection by competing for the binding of non-specific DNA endonucleases, without competing for binding sites of the demethylase. The competitive effect was observed with several types of DNA, including single- and double-stranded DNA, oligos as short as 20 nt and plasmids several kb in size (data not shown). The results were interesting for several reasons. First, the fact that almost any carrier DNA can be used implies that there is no sequence specificity for the residual endonuclease activity. Second, the fact that carrier

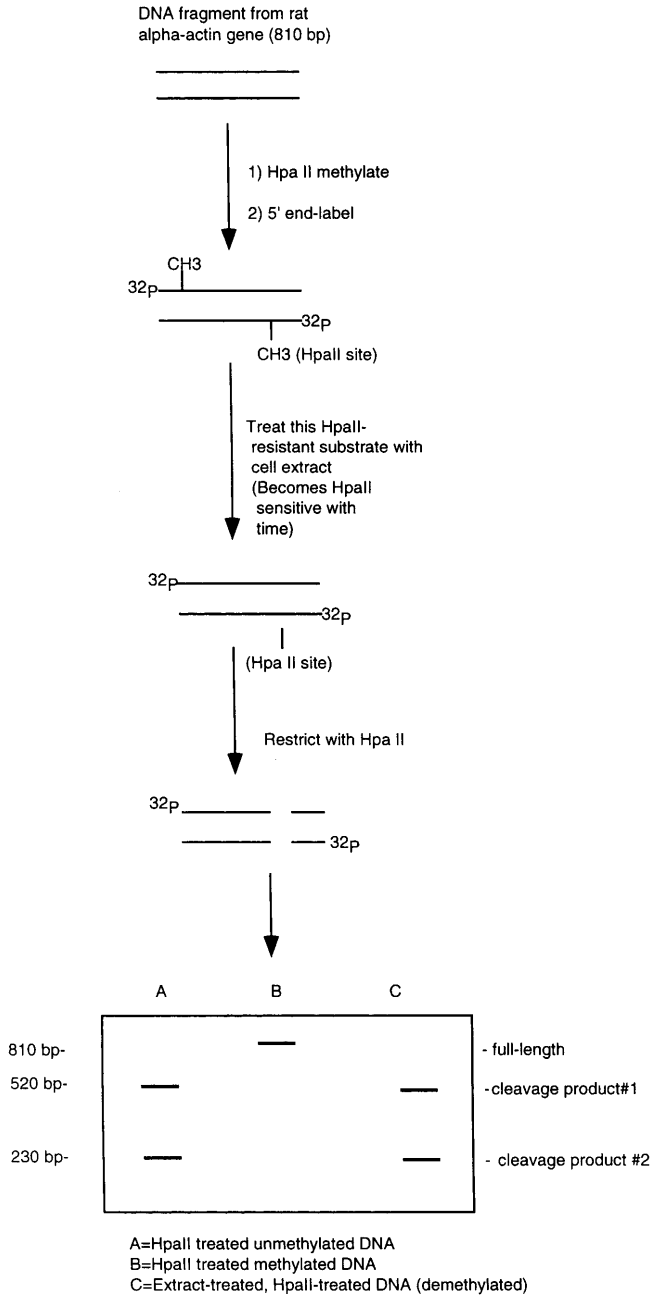

Figure 1. A scheme for monitoring demethylase activity in rat myoblast extracts.

DNA reduces degradation without reducing demethylation suggests that the two processes (degradation and demethylation) are completely uncoupled. This fact implies that the proteinase K treatments reported previously were insufficient for destroying activity of some DNA endonucleases in the cell. Finally, if DNA endonucleases were not completely destroyed by the proteinase K treatment, then it is possible that protein components of a demethylase enzyme also remained intact.

Another method that was found to reduce DNA degradation was exposure of the cell extracts to low temperature for extended periods of time. This improvement was initially discovered

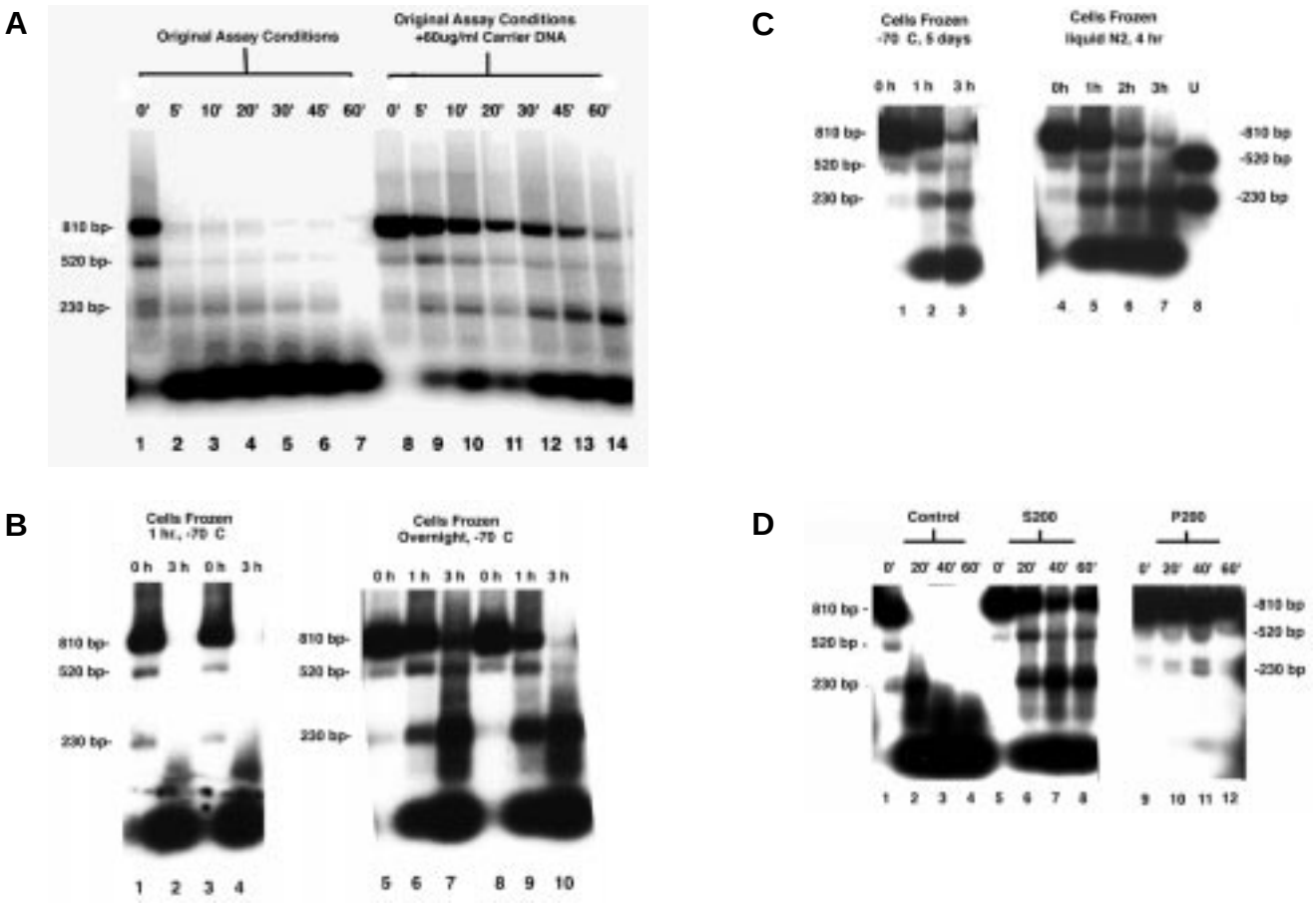

Figure 2. Altered conditions for extract preparation and demethylase reaction: new sensitivities to proteinase K and ribonuclease. Prepared extracts were assayed for demethylase activity after electrophoresis on a 1% agarose gel. The effect of each treatment on endogenous endonuclease activity was measured by taking the ratio of counts found in degradation products versus substrate bands. (**A**) Effects of added carrier DNA: lanes 1–7, labeled, methylated substrate was incubated with proteinase K-treated cell extracts for times indicated; lanes 8–14, identical reaction was supplemented with 60 µg/ml unlabeled, unmethylated DNA to titrate nuclease activity. (**B**) The effect of extended storage at –70°C; lanes 1–4, substrate was treated with extract prepared from frozen cells for 1 h at –70°C for times indicated; lanes 5–10, substrate exposed to extract made from cells left overnight at –70°C. Lanes 1 and 2 were treated with extract digested with 400 µg/ml proteinase K, whereas the extract used in lanes 3 and 4 was subjected to the standard 200 µg/ml proteinase K treatment. Lanes 5–7 and 8–10 represent two separate batches of extract, used in parallel reactions. (**C**) Effects of liquid nitrogen storage on extract activity: lanes 1–3, substrate was treated with extract prepared from cells stored for 5 days at –70°C; lanes 4–7, extract used was prepared from cells frozen for 4 h in liquid nitrogen. Lane U indicates labeled, unmethylated DNA marker which was digested with *Hpa*II. **(D)** Effects of ultracentrifugation on the extract activity: lanes 1–4, substrate exposed to extract prepared from cells stored in liquid nitrogen; lanes 5–8, substrate treated with supernatant of the same extract, subjected to centrifugation at 200 000 *g*; lanes 9–12, substrate exposed to resuspended pellet of 200 000 *g* (3 h) centrifugation of extract used in lanes 1–4. In these last four lanes, a doublet can be seen (230 and 290 bp) which represents demethylation of one, but not both, *Hpa*II sites on the substrate. (Two *Hpa*II sites on substrate are 60 bp apart; this doublet is not normally seen, implying that this activity normally fully demethylates a strand if it is demethylated at all.)

during attempts to store the active extract by freezing it under a variety of different conditions. Previous studies had required that a fresh demethylase extract be made for each experiment and the lack of effective storage conditions led to problems with reproducibility (11). To address this problem, an aliquot of trypsinized and washed L8 cells was left overnight at –70°C, rather than the 1 h incubation previously specified. Extract obtained from these cells was compared with extract from the same cells, frozen for 1 h (Fig. 2B). The data revealed that prolonged storage at –70°C was successful not only in preserving the demethylating activity, but in decreasing nuclease activity as well: after 3 h of incubation with extract that had not been subjected to prolonged freezing, substrate was completely degraded (Fig. 2B, left), whereas extract from cells frozen at –70°C overnight left a substantial amount of substrate intact, most of which was demethylated by activity in the extract (Fig. 2B, right). Because the –70°C freezing method was so successful, it was of interest to determine if the rapid freezing time and lower temperature afforded by liquid nitrogen might further improve storage efficiency and reduce nuclease activity. Extract made from cells kept at –70°C for 5 days has activity that is comparable to that of extracts frozen for 4 h in liquid nitrogen (Fig. 2C). While liquid nitrogen treatment was equally successful in preserving demethylase activity, the reduction in DNA degradation was slightly improved. Therefore, liquid nitrogen was added to the protocol as the preferred treatment and storage method.

The final method that was found to increase extract activity was ultracentrifugation. Initially, ultracentrifugation was employed as a crude separation method for attempting to remove DNA nuclease activity from extracts active in DNA demethylation. Unfortunately, the nuclease activity remained in the supernatant with the demethylase activity after a 3 h spin at 200 000 *g* (Fig. 2D,

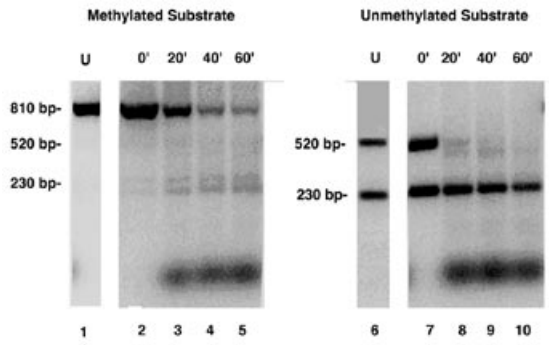

Figure 3. An extract-associated DNA degradation activity: labeled methylated and unmethylated DNA substrates were exposed to extract. Reaction products and extent of DNA degradation were analyzed by electrophoresis in a 1% agarose gel. Lane 1, labeled, methylated DNA substrate which has not been exposed to cell extract was treated with *Hpa*II endonuclease; lanes 2–5, methylated substrate was exposed to extract and aliquots taken at times indicated; lane 6, unmethylated substrate which has not been exposed to extract was treated with *Hpa*II endonuclease; lanes 7–10, unmethylated substrate, treated with extract for times indicated.

lanes 5–8) and was absent from the pellet (lanes 9–12), indicating that these two activities were not well separated. However, demethylase activity of the extracts was markedly improved in the supernatant (Fig. 2D, lanes 5–7). Furthermore, during ultracentrifugation a substantial amount of material was pelleted and removed from the extract, thereby providing a small degree of purification. It is well documented that membranes, organelles and microsomal components are pelleted at 100 000 *g*, as well as some large protein complexes (16). Consistent with this, there was a several-fold decrease in the A_{260} and A_{280} of the active extract after ultracentrifugation, while demethylase activity actually increased.

Based on the three improvements described above, a new protocol for extract preparation was developed containing the following features: (i) during the demethylation reaction, an excess of unmethylated carrier DNA is added to titrate nucleases and other DNA-binding proteins away from the substrate; (ii) extracts are made from large batches of prepared cells that have been treated and stored in liquid nitrogen; (iii) large organelles, membranes and protein aggregates are removed from the extract using ultracentrifugation.

It is important to note that, even with these improvements in extract preparation, the interfering nuclease activity was merely decreased but never completely eliminated in extracts active for DNA demethylation. If the degradation problem is due to the activity of DNA nucleases in the extract, then it can be assumed that the proteinase K digestion used to prepare this extract is incomplete, since the abundant DNA nucleases in most cells are proteins. Alternatively, it is possible that the degradation activity could be an intrinsic feature of the *in vitro* demethylation reaction. If the degradation was a byproduct of the demethylation reaction, it would be expected to act exclusively on methylated DNA. To explore this possibility, unmethylated substrate DNA was labeled and exposed to cell extract. A parallel reaction with labeled methylated substrate was performed under identical conditions (Fig. 3, lanes 7–10 and lanes 2–5, respectively). Both DNAs were found to be degraded with the same kinetics, suggesting that the degradation is not intrinsic to the DNA demethylation reaction. Although attempts to completely rid extracts of the DNase activity proved unsuccessful, the presence or absence of this activity proved to be instructive: experiments to confirm protease and RNase sensitivity of this active extract (described below) can be evaluated using the degradation activity as a marker for protein enzyme function. If a treatment extinguishes the nuclease activity, then it can be assumed that some (if not all) protein enzymes are inhibited, denatured, or digested in that extract.

Protease sensitivity of the demethylase activity

Previous reports suggested that the apparent demethylase activity was relatively protease resistant and RNase sensitive (11). Given the new optimized reaction conditions designed for assaying demethylation activity, it was of interest to repeat experiments on extract sensitivity to nucleases and proteases. Several additional conditions were used to test the effects of protein inactivation on extracts prepared by the new method. First, the extract was treated with proteinase K in the presence of 0.1% SDS (Fig. 4, lanes 6–8), which is the preferred method for complete digestion of proteins using proteinase K (17). Next, in place of proteinase K, active extract was heated at 60°C for 20 min (Fig. 4, lanes 9–11) or extracted with phenol and chloroform (Fig. 4, lanes 12–14). In all three cases, demethylation activity was extinguished (Fig. 4, compare lanes 3–5 with 6–14). The absence of nuclease activity after these treatments, when compared with the presence of that activity in figure 1 of Weiss *et al*., suggests a more thorough destruction of protein enzyme activity than was conferred by the same treatments in the previous report (11). Because all three modifications added to the protocol serve to remove organelles and proteins from the whole CE, the probable explanation for the difference in results is that in previous experiments, cellular materials buffered or physically protected the demethylase activity against the denaturing effects of heat treatment or digestion by protease in the presence of SDS. Therefore, these data suggest that rigorous protein denaturation or digestion removes all demethylase activity from these extracts.

Ribonuclease resistance of the demethylase activity

An apparent sensitivity of the demethylase activity to RNase was a pivotal point in the case that RNA is involved in demethylation (11). Because of the profound importance of this finding, RNase sensitivity experiments were repeated several times under a number of different reaction conditions (Fig. 5). During assays of RNase sensitivity described in previous work and in the experiments presented here, extracts are treated with 100 µg/ml RNase A. The potential problem with placing a trace quantity of ^{32}P-labeled DNA substrate into such an extract is that the DNA may become physically coated with RNase (18), perhaps rendering it inaccessible to the activity of interest (in this case, the demethylase activity).

Initial evidence for a possible DNA coating effect was apparent upon inspection of DNA degradation fragments obtained from reactions in which the extract had (Fig. 5, lanes 23–26), or had not (Fig. 5, lanes 15–18), been treated with RNase. When the extract has been treated with RNase, not only does the demethylase activity disappear, the DNA endonuclease activity also disappears. Just like a demethylase, a DNA endonuclease (which is likely to be a protein) must also gain access to DNA, and this may be blocked when the DNA is coated with RNase. This effect was apparent even in data from the previous study (figure 1b of Weiss *et al.*; 11), where protection of the substrate against ‘decrease in counts over

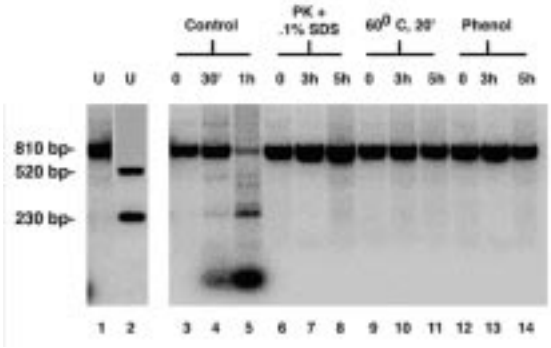

Figure 4. Effects of protein denaturation on demethylation activity: extracts were subjected to various treatments used to digest or denature proteins. Substrate was then added to each extract to measure demethylation activity. Reaction products were analyzed on a 1% agarose gel. Lane 1, labeled, methylated substrate was treated with *Hpa*II without exposure to cell extract; lane 2, labeled, unmethylated substrate fragment was *Hpa*II-treated without exposure to extract; lanes 3–6, extract was digested with 200 µg/ml proteinase K. Aliquots of 60 µg/ml carrier DNA and labeled, methylated substrate were added to cell extract and aliquots were removed at times indicated. Lanes 6–8, 0.1% SDS added to the proteinase K digestion step; lanes 9–11, extract treated at 60°C for 20 min following the proteinase K digestion; lanes 12–14, extract treated with phenol and chloroform following proteinase K digestion.

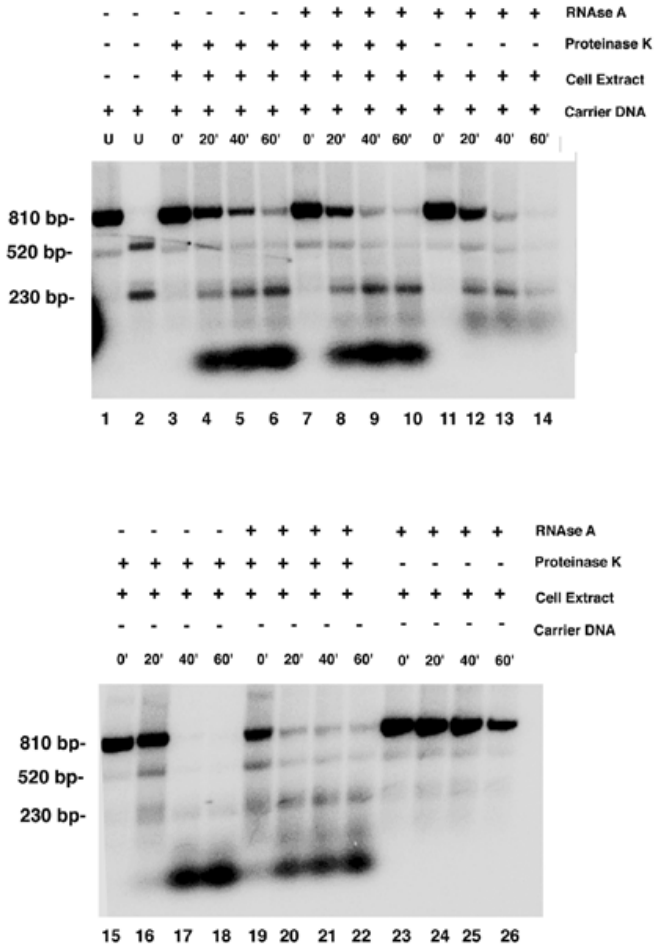

Figure 5. Effects of RNase treatment on demethylation activity: extracts were subjected to various treatments, as indicated, and incubated with labeled, methylated substrate for times shown. Lane 1 contains methylated substrate, *Hpa*II-treated without exposure to cell extract; lane 2 contains the unmethylated substrate. *Hpa*II-treated without exposure to cell extract. It should be noted that the disappearance of total counts seen in lanes 11–14 is seen reproducibly upon treatment of extracts with RNase which is allowed to remain in the extract. The counts were found to be in the phenol after the extraction step.

time' is an RNase-dependent phenomenon, just like the inhibition of demethylase activity.

Consistent with the notion that ribonuclease inhibition may be the result of a substrate DNA coating effect, it was observed that demethylase activity can be partially restored by the addition of 60 µg/ml carrier DNA together with the labeled substrate in order to titrate away RNase (Fig. 5, lanes 11–14) or by proteinase K treatment to digest the RNase after it is used to treat the extract (Fig. 5, lanes 19–22). As expected, demethylase activity is highest when both approaches were applied (Fig. 5, compare lanes 7–10 with 3–6), and when conditions used in the previous report are duplicated, activity disappears (Fig. 5, lanes 23–26; see Materials and Methods for comments on previous report). This effect of ribonuclease is observed using a broad spectrum of different enzymes, as the same patterns of reversible inhibition were observed when extracts were treated with RNase T1 or U2, which cleave at GpN and ApN, respectively, as opposed to the CpN and UpN linkages cleaved by RNase A. Taken together, these results suggest that the reported inhibition of demethylating activity by RNase seen in crude extracts is removable and may, in fact, represent an experimental artifact. This lack of true RNase sensitivity indicates that the putative demethylase is not a ribozyme and is unlikely to contain an RNA cofactor.

DISCUSSION

The discovery and analysis of apparent DNA demethylation activity

The regulation of tissue- and stage-specific gene expression via DNA methylation clearly requires changes in the genomic methylation pattern of cells throughout development, and this is brought about by programmed demethylation and *de novo* methylation events (19,20). Demethylation of specific DNA sequences has been recorded in several cell types (12–14). It has been observed that a methylated plasmid carrying the upstream control region of the rat α-actin gene is actively demethylated upon transient transfection into L8 rat myoblast cells (13). This demethylation is indicated by increased sensitivity of the substrate to *Hpa*II restriction enzyme cleavage, and the decrease in methylation state cannot be due to inhibition of the maintenance methylase because this plasmid does not undergo replication. Based on this observation, an *in vitro* assay was created using whole cell extracts to facilitate further study of this phenomenon (11). In this system, the 810 bp methylated DNA fragment from the rat α-actin gene control region is treated with extracts from L8 rat myoblast cells, and *Hpa*II sensitivity is used to measure active demethylation of the substrate DNA (Fig. 1).

One of the most remarkable results from the *in vitro* system was that the demethylase activity appeared to be resistant to protease and sensitive to ribonuclease, suggesting that an RNA was involved in demethylation. It was therefore of great interest to isolate the active RNA component from the extract and to characterize its behavior. However, it is necessary to have a robust

assay for monitoring activity before attempting to purify an enzyme, since dilute fractions from chromatographic separations will need to be analyzed. In addition, a strong, reproducible activity is important because different treatments of the extract (altering salt, pH, etc. for purification on different types of columns) may weaken activity further. To optimize activity and increase reproducibility of analysis, a series of improvements were made in the extract preparation and storage protocol. These improvements remove cellular debris which may complicate analysis, they reduce the presence of a contaminating DNA nuclease activity, and they allow for prolonged storage of active material.

Improved methodologies for monitoring demethylation

One of the most important improvements in the analysis of demethylation activity was the development of a low temperature extract treatment. This enables long-term storage of active extract and has the unexpected benefit of reducing adventitious DNA endonuclease activity from the L8 myoblast extracts. Just as previous reports showed that protease treatment improved the demethylation reaction, presumably by reducing the density of cellular protein components, deep freezing causes extensive fracturing of membranes and organelles, as well as the disruption of many proteins through misfolding, aggregation and precipitation (16). It is likely that the lengthened freezing time used herein completed the disruption and denaturation of cellular components which was only partial during the 1 h freezing time used previously. When followed by centrifugation, this step helps clarify the extract, reduce nuclease activity and remove many extract components without harming or removing the DNA demethylating activity.

In removing the cellular debris, ultracentrifugation eliminated substances that chemically or physically buffered the demethylation activity from the action of proteases. The third alteration to the reaction protocol involved the addition of large amounts of unlabeled, unmethylated DNA to the extract. Cell extracts are replete with proteins that, like DNA endonucleases, will bind DNA non-specifically through electrostatic interactions. When there is very little DNA to bind (as in this case), the labeled DNA substrate might be expected to be totally encased in protein from crude cell extract. Under this condition, added carrier DNA might behave as a molecular decoy, binding nucleases and other proteins that would otherwise associate with the trace amounts of methylated substrate. In the presence of excess carrier DNA, the methylated substrate DNA may now be more accessible to the activity of putative demethylase enzymes and, therefore, no longer rapidly degraded by nucleases in the extract. Despite these improvements to the assay, it is important to keep in mind that the whole cell extract still contains thousands of different components that can exert effects on the substrate or enzymatic activity, causing results to be misleading. As long as the activity is studied in a crude extract rather than a purified form, countless misinterpretations are possible which may be difficult to anticipate. Hopefully, the improved conditions described here will enable investigators to move forward in isolating and characterizing the specific demethylation activity.

Results are inconsistent with involvement of an RNA enzyme in demethylation

The most surprising result from the improved assay conditions was that the partially purified demethylase activity was found to be resistant, rather than sensitive, to ribonucleases (Fig. 5). Taken together with a variety of other results from this study we conclude that the behavior of the demethylase activity is inconsistent with the activity of a ribozyme and, unless it is completely encased in protein, may be inconsistent with a role for any RNA molecule.

Experiments with unmethylated DNA and with carrier DNA establish that extract which is active for demethylation also contains DNA endonucleases (Fig. 3). Furthermore, it was established that the small fragments of DNA that are always seen together with demethylation activity are not byproducts of the demethylation reaction. The fact that DNA endonucleases, which are most certainly proteins, are active under conditions of partial proteolysis (200 μg/ml proteinase K) suggests that a demethylase enzyme would not necessarily need to be composed solely of RNA in order to survive the proteolysis treatment. Ironically, similar conditions for proteinase K digestion have been employed in the purification of many proteins, including prions (21), which are proteinaceous disease-causing agents in which many individuals have sought (unsuccessfully) to identify a nucleic acid component. Thus, the lack of protease sensitivity observed under previously published reaction conditions is not necessarily indicative of an enzyme with an RNA component.

Finally, a number of other fundamental observations using the partially purified extracts are also inconsistent with the role of a ribozyme in the demethylation reaction. First of all, RNA enzymes are stable to heat (provided they are allowed to renature). In fact, most ribozymes are routinely heated to temperatures as high as 95°C so that they can slowly fold into the proper conformation prior to reaction. Although the demethylase activity as measured in crude extracts was resistant to heating at 70°C (11) it could be inactivated even at 60°C following treatment with proteinase K (Fig. 4, lanes 9–11). Ribozymes and other folded RNA molecules are not sensitive to SDS or phenol, whereas the demethylase activity is sensitive to both of these reagents (Fig. 4, lanes 6–8 and 12–14). Importantly, sensitivity to any of these three treatments (heat, SDS or phenol), is indicative of an activity composed of protein molecules.

Possible explanation for RNase sensitivity in crude extracts

One of the first observations that challenged the involvement of RNA was that under the original assay conditions the ribonuclease inhibited both the demethylase activity and the action of DNA endonucleases that are still in the extract [Fig. 5, lanes 23–26 herein; and figure 1b of Weiss *et al* (11)]. This strongly suggests that the previously reported inhibitory effects of ribonuclease were due to secondary effects. When the conditions used for ribonuclease treatment (100 μg/ml) in this assay were compared with conditions used for applying ribonuclease as a coating for DNA in electron microscopy, they were found to be the same (18). At the concentrations used in these assays, ribonuclease is therefore likely to coat the DNA substrate, perhaps making it unable to react with a demethylase or a DNA endonuclease. Thus, ribonuclease

may be having an indirect physical effect on the substrate, rather than a direct enzymatic effect on the demethylase enzyme.

Consistent with this theory, when initial ribonuclease treatment is followed by proteinase K digestion, the extract is reactive for demethylation (Fig. 5, lanes 19–22). This would not be possible if there was an enzyme in the extract that is inherently sensitive to ribonuclease. Similarly, when competitor DNA is added, RNase in the extract is sufficiently titrated away from the labeled, methylated substrate to reverse the RNase sensitivity of the demethylase activity (Fig. 5, lanes 11–14). When the above approaches are used in combination, demethylation activity is fully restored (and it is often improved) after treatment with ribonuclease (Fig. 5, compare lanes 7–10 with 3–6). This is reasonable given that cellular RNA molecules may electrostatically associate with the putative demethylase enzyme, thereby reducing its activity.

The fact that RNase treatment can indirectly inhibit the function of protein enzymes highlights the need for extreme caution when attempting to characterize any new enzymatic activity. In addition to cleaving RNA, a ribonuclease binds tightly to the surface of many different polyanions in an extract. The meshlike covering that it creates has been very useful during electron microscopy studies of DNA, which becomes substantially thicker and easier to visualize when treated with a coating of ribonuclease (18). Therefore, ribonucleases must be removed from the extract, or at least from the surface of the DNA substrate, before reactions of interest are performed. At a time when reports of putative ribonucleoprotein enzymes are growing more frequent, it is important that the misleading effects of RNase treatment be considered and that necessary precautions are taken so that RNase sensitivity experiments can be properly interpreted.

The results reported herein are most consistent with demethylation by a simple protein enzyme (or set of enzymes). Yet it is important to note that these results do not rule out the possibility that an RNA is involved in this reaction. Although agarose gels indicated that ribonuclease used in these experiments is fully active and degrades all the cellular RNA to small fragments (data not shown), it is possible that some undigested RNA remains below the detection limit of ethidium staining. Because enzymes are often amazingly efficient, measurable activity can be obtained from minuscule amounts. For example, the results of this study establish that digestion of the myoblast extracts with 200 µg/ml proteinase K does not remove all protein function from the extract. At the very least, the DNA nucleases are still present in sufficient amounts to digest the substrate. Despite this, SDS–PAGE gels indicated a total absence of protein in proteinase K-treated extracts, except for a single band corresponding to the size of proteinase K itself (data not shown). Although the removal of proteins by proteinase K is striking when visualized in this manner, calculations reveal that a protein could be present in the extract at a concentration of 0.05 µg/ml, at which many enzymes would be perfectly active, and still exist below the 0.3–1 µg/band limit of detection with Coomassie staining (22). Likewise, a gel validating activity of RNase added to this system does not necessarily rule out the involvement of an RNA in this reaction. It should be noted, in this regard, that a DNA demethylating activity found in chick embryos which is based on a glycosylase type reaction, indeed requires an RNA component (23) and it has recently been shown that this RNA is involved in recognizing the methylated DNA substrate (24).

ACKNOWLEDGEMENTS

We would like to thank Tim Bestor, Max Gottesman and Steve Goff for helpful discussions about this work, and Alona Weiss for technical assistance. This work was funded by NIH grants RO150313 to A.M.P. and 20483 to H.C. J.S. is supported by NIH grant T32AMO7328.

REFERENCES

1 Kruger,K., Grabowski,P.J., Zaug,A.J., Sands,J., Gottschling,D.E. and Cech,T.R. (1982) *Cell*, **31**, 147–157.
2 Guerrier-Takada,C., Gardiner,K., Marsh,T., Pace,N. and Altman,S. (1983) *Cell*, **35**, 849–857.
3 Eckstein,F. and Lilley,D.M.J. (1996) *Nucleic Acids and Molecular Biology, Volume 10: Catalytic RNA*. Springer-Verlag, Berlin.
4 Robertson,D.L. and Joyce,G.F. (1990) *Nature*, **344**, 467–468.
5 Griffin,E.A., Qin,Z.-F., Michels,W.A. and Pyle,A.M. (1995) *Chem. Biol.*, **2**, 761–770.
6 Zimmerly,S., Guo,H., Perlman,P.S. and Lambowitz,A.M. (1995) *Cell*, **82**, 545–554.
7 Noller,H.F., Hoffarth,V. and Zimniak,L. (1992) *Science*, **256**, 1416–1419.
8 Nitta,I., Kamada,Y., Noda,H., Ueda,T. and Wattanabe,K. (1998) *Science*, **281**, 666–670.
9 Busslinger,M., Hurst,J. and Flavell,R.A. (1983) *Cell*, **34**, 197–206.
10 Stein,R.Y., Gruenbaum,Y., Pollack,Y., Razin,A. and Cedar,H. (1983) *Proc. Natl Acad. Sci. USA*, **79**, 61–65.
11 Weiss,A., Keshet,I., Razin,A. and Cedar,H. (1996) *Cell*, **86**, 709–718.
12 Lichtenstein,M., Keini,G., Cedar,H. and Bergman,Y. (1994) *Cell*, **76**, 913–923.
13 Paroush,Z., Keshet,I., Yisraeli,J. and Cedar,H. (1990) *Cell*, **63**, 1229–1237.
14 Jost,J.P. and Jost,Y.C. (1994) *J. Biol. Chem.*, **269**, 10040–10043.
15 Melloul,D., Aloni,B., Calvo,J., Yaffe,D. and Nudel,U. (1984) *EMBO J.*, **3**, 983–990.
16 Scopes,R.K. (1994) *Protein Purification: Principles and Practice*. Springer-Verlag, New York.
17 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
18 Kleinschmidt,A.K. (1970) *Methods Enzymol.*, Vol. XII, pt. B, pp. 364.
19 Razin,A. and Cedar,H. (1991) *Microbiol. Rev.*, **55**, 451–458.
20 Jost,J.P. and Jost,Y.C. (1995) *Gene*, **157**, 265–266.
21 McKinley,M.P., Meyer,R.K., Kenaga,L., Rahbar,F., Cotter,R., Serban,A. and Prusiner,S.B. (1991) *J. Virol.*, **65**, 1340–1351.
22 Scopes,R.K. and Smith,J.A. (1995) In Ausubel,F., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds), *Current Protocols in Molecular Biology*. John Wiley and Sons, Inc., NY.
23 Fremont,M., Siegmann,M., Gaulis,S., Matthies,R., Hess,D. and Jost,J.P. (1997) *Nucleic Acids Res.*, **25**, 2375–2380.
24 Jost,J.-P., Fremont,M., Siegmann,M. and Hofsteenge,J. (1997) *Nucleic Acids Res.*, **25**, 4545–4550.