Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 4684–4688, May 1993
Biochemistry

Nuclear extracts of chicken embryos promote an active demethylation of DNA by excision repair of 5-methyldeoxycytidine

(hemimethylated DNA/mCpG endonuclease/DNA polymerase β)

JEAN-PIERRE JOST

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

Communicated by Diter von Wettstein, February 9, 1993

ABSTRACT **Here I show that nuclear extracts of chicken embryos can promote the active demethylation of DNA. The evidence shows that in hemimethylated DNA (i.e., methylated on one strand only) demethylation of 5mCpG occurs through nucleotide excision repair. The first step of demethylation is the formation of specific nicks 5′ from 5-methyldeoxycytidine. Nicks are also observed *in vitro* on symmetrically methylated CpGs (i.e., methylated on both strands) but they result in breakage of the oligonucleotide with no repair. No specific nicks are observed on the nonmethylated CpG. Nicks are strictly 5mCpG specific and do not occur on 5mCpC, 5mCpT, 5mCpA, or 6mApT. The effect of nonspecific nuclease(s) has been ruled out. The nicking of mCpG takes place in the presence of 20 mM EDTA irrespective of the nature of the sequence surrounding the 5mCpG. No methylcytosine glycosylase activity could be detected. The repair is aphidicolin and *N*-ethylmaleimide resistant, suggesting a repair action by DNA polymerase β. In extracts of chicken embryos, the excision repair of mCpG is highest between the 6th and the 12th day of development, whereas it is barely detectable in nuclear extracts from different organs of adults. The possible implications of 5mCpG endonuclease activity in active demethylation of DNA during differentiation is discussed.**

DNA methylation provides a basis for the heritable epigenetic system (1–3). Through DNA methylation it is possible to change the information content of DNA and affect differentiation and development (1–3). The dynamic changes of DNA methylation are dictated in part by the combination of maintenance and/or *de novo* methylation and by the demodification of the mCpGs (4–6). The pivotal role of DNA methyltransferase in embryonic development has recently been demonstrated by targeted disruption of the DNA methyltransferase gene in mice, where the loss of function causes lethality of homozygous embryos at early stages of development (7). Changes in the level of DNA methyltransferase activity, however, may not be sufficient to achieve a temporal modification of the epigenetic blueprint of DNA methylation. Other reactions such as passive and/or active demethylation of mCpGs may also be operational. In addition to the classical model of passive demethylation (4–6), there is cumulative evidence suggesting the existence of an active demodification system of mCpGs (4–6, 8–14). For example, in mouse embryos the *ApoAI* gene appears to be fully methylated at the 8-cell stage but is completely unmethylated one division cycle later at the 16-cell stage (14). Hypomethylation of the promoter region in the avian vitellogenin II gene occurs under the influence of estradiol in the presence of inhibitors of DNA synthesis (15). In other systems, such as in teratocarcinoma cells and Friend erythroleukemia cells (11–13), there is upon initiation of differentiation a rapid and transient genome-wide demethylation of DNA occurring in the absence of apparent DNA replication, suggesting an active process of demethylation. I report here evidence in differentiating chicken embryos for the existence of an enzymatic system capable of demethylation of mCpG by nucleotide excision repair.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Preparation of Nuclear Extracts. Chicken embryos (3–20 days old) were minced with a Waring Blender and homogenized (four to six strokes) with a glass/Teflon homogenizer in 4 vol of buffer A containing 10 mM Hepes (pH 8), 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% (vol/vol) glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.15 mM spermine, and 0.5 mM spermidine. The homogenate was centrifuged at 1000 × *g* for 10 min, and the nuclei sediment was gently resuspended in the same volume of buffer A and centrifuged as described above. The crude nuclei pellet was resuspended in a minimal volume of buffer A and 4 M ammonium sulfate was slowly added to a final concentration of 0.4 M. After centrifugation for 1 hr at 300,000 × *g* at 0°C to remove chromatin, the supernatant fraction was stored in small aliquots at −80°C. Protein concentration was 7–20 μg/μl. Nuclei from different organs from adult roosters were prepared as described by Sierra (16). Nuclei from chicken erythrocytes were prepared according to McGhee *et al.* (17).

Demethylation Assay. Demethylation activity was measured with a 100-μl reaction mixture containing 3–5 ng of end-labeled DNA (10^8 cpm/μg) and 18–36 μg of protein in 25 mM Hepes, pH 7.5/0.5 mM EDTA/0.01 mM $ZnCl_2$/0.5 mM 1,4-dithio-DL-threitol/40 mM KCl/1 mM $MgCl_2$/20 mM creatine phosphate/0.5 mM ATP/1 mM each deoxyribonucleoside triphosphate. After incubation at 37°C for 3–9 hr, the reaction mixture was mixed with 150 μl of 20 mM EDTA and extracted three times with 150 μl of phenol saturated with 1 M Tris·HCl (pH 8). After two additional extractions with chloroform the supernatant fraction was adjusted to 0.3 M sodium acetate and DNA was precipitated with 3 vol of ethanol in the presence of 30 μg of Dextran T40 as carrier. Samples were kept 5 min at −80°C and centrifuged 10 min at 30,000 × *g*. Sediments were dissolved in 200 μl of 0.3 M sodium acetate (pH 5) and reprecipitated with 600 μl of ethanol as described above. The sediments were dissolved in 80 μl of *Hpa* II digestion buffer as recommended by the supplier (Boehringer Mannheim), 40 μl was incubated without any enzymes and 40 μl was incubated with 5 units of *Hpa* II. Upon 1 hr of incubation at 37°C, samples were directly ethanol precipitated as indicated above and the sediment was dissolved in 30 μl of 95% formamide containing 5 mM EDTA and 0.05% each bromphenol blue and xylene cyanol. Aliquots of each sample containing 50,000 cpm were heated at 90°C for 1 min and separated on a 10% or 20% polyacrylamide/urea sequencing gel (18). Gels were either dried or directly exposed for autoradiography.

DNA Nicking Assay. The reaction mixture with a vol of 100 μl contained 3–5 ng of end-labeled DNA (10^8 cpm/μg), 18–36 μg of protein in 25 mM Hepes, pH 7.5/20 mM EDTA. Samples were incubated and processed as indicated for the demethylation assay (except they were not digested with *Hpa* II).

DNA Substrates. The substrates used in assays were end-labeled 50-bp oligodeoxynucleotides containing a single *Hpa* II site, which was either unmethylated, hemimethylated, or fully methylated. Substrate A has the sequence (only the upper strand is shown) 5′-GGTATTCCTGGTCAGCGT-GACCGGAGCTGAAAGAACACATTGATCCCGTG-3′; substrate B has the unrelated sequence 5′-GAGAGC-CCTATTCACCTTGCGCTATGAGGGGGATCATACTG-GCATTATGGT-3′ containing an *Hha* I site methylated symmetrically with *Hha* I methylase. All nonmethylated and methylated oligonucleotides were synthesized on an Applied Biosystems 300 synthesizer. The purified oligonucleotides were end-labeled either with [γ-^{32}P]ATP by means of the kinase reaction or with [α-^{32}P]dATP by means of Sequenase version 2 (19). All DNA sequencing reactions were carried out as described by Maxam and Gilbert (18).

Chemicals and Enzymes. [α-^{32}P]dATP, [γ-^{32}P]ATP (triethylammonium salt) (3000 Ci/mmol; 1 Ci = 37 GBq), and *S*-adenosyl[^{3}H]methionine (60 Ci/mmol) were purchased from Amersham. *Hpa* II, *Hpa* II methylase, *Msp* I, and *S*-adenosylmethionine were from Boehringer Mannheim, whereas all other restriction enzymes and polynucleotide kinase were obtained from Biofinex Praroman (CH 1724, Switzerland). Aphidicolin and *N*-ethylmaleimide were obtained from Sigma.

RESULTS

Presence of High Levels of DNA Demethylating Activity in Chicken Embryonic Extracts. During early and late embryonic development, genes are selectively methylated or demethylated according to a specific program of development (1–6). For this reason, it was of interest to test developing chicken embryos for the presence of DNA demethylating activity. As a simple and reliable assay, a 50-nucleotide synthetic end-labeled oligonucleotide hemimethylated on either the upper or the lower strand at the *Hpa* II site was used as a substrate. Upon replacement of 5-methyldeoxycytidine by deoxycytidine, the oligodeoxynucleotide becomes sensitive to digestion with *Hpa* II, yielding a specific band (30 bp) on a sequencing gel, whereas the parallel controls, if repaired, should not in the absence of *Hpa* II digestion show any nick at the CpG. Fig. 1 shows evidence that in the 4-day embryos there are already traces of demethylation activity. The highest level of demethylation is observed between the 6th and 12th days of development, after which it decreases. In the controls incubated with the nuclear extracts but not digested with *Hpa* II, no specific band could be observed (Fig. 1*B*). This means that the specific bands observed in Fig. 1*A* were not due to unrepaired nicks. Under the same assay conditions, only traces of demethylation activity could be detected in nuclear extracts prepared from organs (testis, liver, kidneys, and erythrocytes) of 4-month-old roosters (Fig. 2). No activity was detected in nuclear extracts of cultured HeLa cells (results not shown). Fig. 3 shows that the repairing activity of mCpG depends on protein concentration; controls incubated with the extracts but not digested with *Hpa* II show, as for Fig. 1*B*, that the specific nicks have been repaired. The time course of the reaction is shown in Fig. 4. The repair has reached a maximum by 3 hr of incubation at 37°C and did not increase further after 9 hr of incubation. The controls not digested with *Hpa* II (lanes 7–10) show that with longer incubation times, there is a

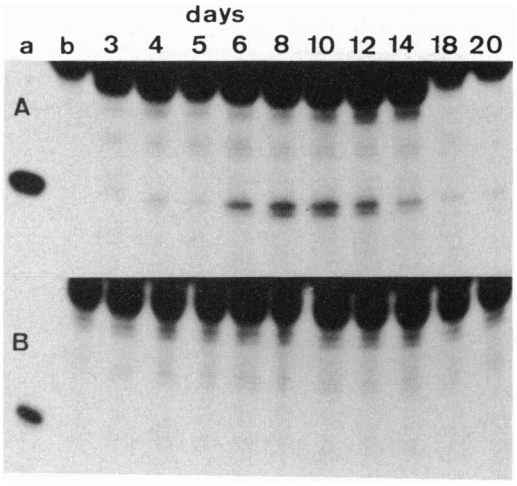

FIG. 1. Occurrence of mCpG excision repair activity in chicken embryonic nuclear extracts. (*A*) Presence of excision repair activity in embryos of different ages. (*B*) Controls incubated with nuclear extracts but not digested with *Hpa* II. DNA template is a 50-bp end-labeled oligonucleotide A hemimethylated at the *Hpa* II site. The substrate is resistant to *Hpa* II digestion if hemimethylated and becomes sensitive to that enzyme upon replacement of 5-methylcytosine by cytosine. For each time point, 20–40 embryos were used. Each incubation mixture had 36 μg of nuclear extract per 100 μl and the incubation was for 3 hr at 37°C. Lanes a and b, hemimethylated template digested with *Msp* I and *Hpa* II, respectively.

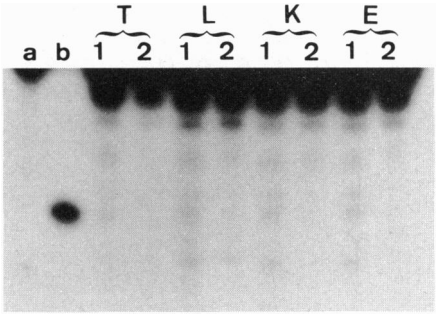

FIG. 2. Occurrence of mCpG excision repair activity in organs of 4-month-old roosters. Lanes a and b, hemimethylated end-labeled DNA template A (as for Fig. 1) digested with *Hpa* II and *Msp* I, respectively. Lanes 1, samples were incubated with 36 μg of nuclear extract per 100 μl for 6 hr at 37°C and the reaction product was subjected to *Hpa* II digestion; lanes 2, controls incubated with extracts but not subjected to *Hpa* II digestion. T, testis; L, liver; K, kidney; E, erythrocytes.

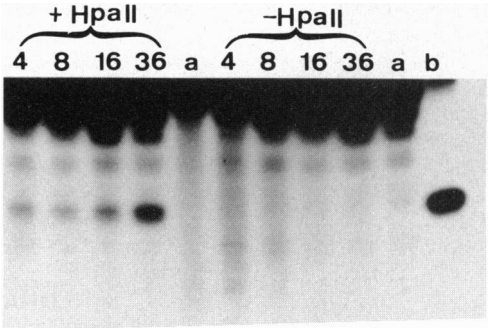

FIG. 3. Protein concentration dependence (μg per 100 μl) of mCpG excision repair from 6-day embryos. Hemimethylated end-labeled substrate A (as for Fig. 1) was incubated for 5 hr at 37°C and the reaction product was purified and tested with *Hpa* II (+Hpa II). Controls were also incubated with nuclear extracts but not subjected to *Hpa* II digestion (−Hpa II).

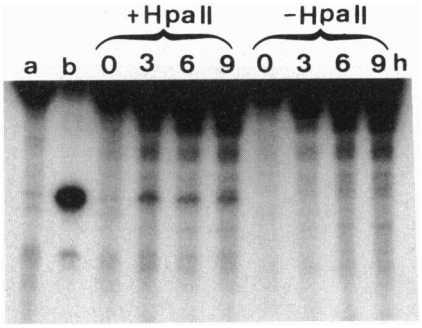

FIG. 4. Time dependence of mCpG excision repair of hemimethylated end-labeled DNA substrate A incubated with nuclear extract of 4-day embryos (36 μg per 100 μl). Reaction product was purified and tested with *Hpa* II endonuclease (+Hpa II), whereas incubated controls were not subjected to *Hpa* II digestion (−Hpa II). Lanes a and b, hemimethylated DNA template digested with *Hpa* II and *Msp* I, respectively.

drastic increase in nonspecific degradation of the labeled oligonucleotide template. Identical results were obtained with oligonucleotides hemimethylated on the upper or lower strand. However, no specific demethylation could be observed on the bifilarly methylated DNA, where symmetrical double-stranded cuts resulted in breakage of the oligonucleotide (data not shown).

Nicking at 5-Methyldeoxycytidine Is Specific for mCpG. The first step in replacing 5-methyldeoxycytidine by deoxycytidine is either the removal of the methylated base with subsequent cleavage of the apyrimidinic sugar or the direct nicking at the 5-methyldeoxycytidine with its subsequent replacement by deoxycytidine. The presence and specificity of the nicking activity at mCpG was studied on a hemimethylated substrate and nonmethylated DNA substrate labeled at either the upper or the lower DNA strand. Fig. 5 shows that in the presence of 20 mM EDTA specific nicking is only observed for the methylated CpG and not for the nonmethylated CpGs. Since it was shown by Fox (20) that mCpG increased the susceptibility of the DNA to DNase I (0.01 unit/ml) it was important to rule out any nonspecific nuclease activity. Using the same assay conditions but in the presence of 60 units of DNase I per ml no trace of nicking of the labeled methylated DNA substrate was observed even after 6 hr of incubation at 37°C (Fig. 5). Furthermore, nicking

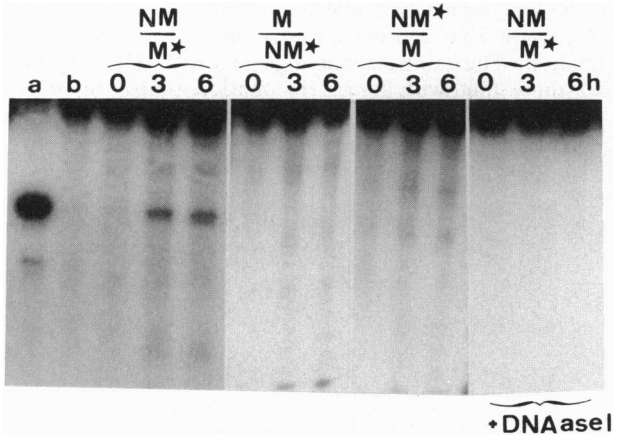

FIG. 5. Nicking at CpG occurs only if CpG is methylated. NM, nonmethylated DNA strand (oligonucleotide A); M, methylated strand. Star indicates which strand has been end-labeled. Upon purification of the reaction product, DNA was denatured and separated on a 10% acrylamide sequencing gel. Lanes a and b, hemimethylated end-labeled substrate digested with *Msp* I and *Hpa* II, respectively. The two rightmost lanes were incubated with DNase I.

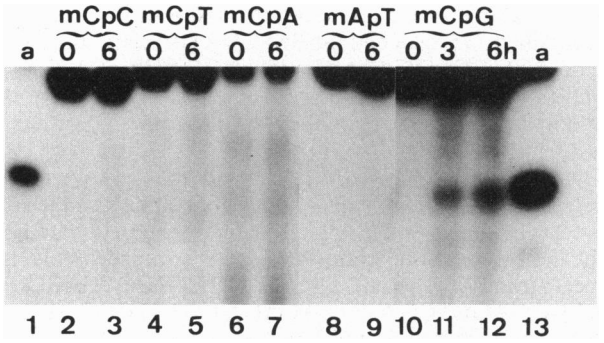

FIG. 6. Nicking of hemimethylated DNA is specific for mCpG. Experimental conditions were the same as for Fig. 5 except that mCpC, mCpT, mCpA, and mApT replaced mCpG at the *Hpa* II site of the same oligonucleotide A. Lanes 10–12, oligonucleotide B with a methylated *Hha* I site was used. After 6 hr of incubation at 37°C with 36 μg of 8-day embryonic extracts, the reaction product was purified, heat denatured, and separated on a 10% acrylamide sequencing gel. Lane a, hemimethylated end-labeled DNA template digested with *Msp* I (30-bp marker).

(Fig. 6) at the 5-methyldeoxycytidine is CpG specific and is not observed for mCpC, mCpT, mCpA, and mApT, suggesting that the cleavage is strictly mCpG specific. Moreover, specific nicking of mCpG is also observed in the presence of the methylated oligonucleotide B (see lanes 10–12 of Fig. 6, where the sequence surrounding the mCpG is different from the one of Fig. 5). Taken together these results indicate that the formation of the nicks is not due to nonspecific endonucleolytic processing of the DNA containing a helical distortion but is rather a specific incision targeted to the mCpG.

The precise position of the nicks was investigated by separating the 3′ and 5′ end-labeled nicked oligonucleotide A on a 20% sequencing gel. As shown in Fig. 7 for both the 3′ and 5′ end-labeled oligonucleotides, the nicks are located 5′ of the mCpG. Treatment of the nicked mCpG end-labeled oligonucleotides with sodium hydroxide or piperidine gave no faster moving band (Fig. 7, compare lanes 5 and 6 with lanes 11 and 12), thus giving indirect evidence that there is no

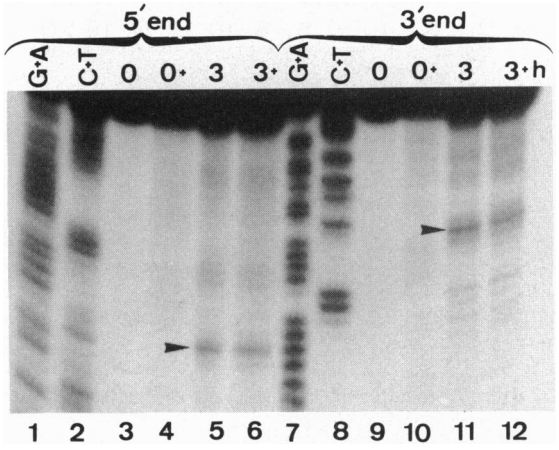

FIG. 7. Position of nicks on the hemimethylated oligonucleotide A. Hemimethylated oligonucleotide duplex was labeled at either the 3′ or the 5′ end. Upon incubation of the DNA substrate with 6-day embryonic nuclear extracts for 0 or 3 hr, respectively, DNA was isolated and half of it was treated with 1 M piperidine at 95°C for 30 min (lanes 4, 6, 10, and 12). Upon repeated lyophilization, samples were dissolved in 95% formamide dye and separated on a 20% acrylamide sequencing gel. The rest of the DNA was not treated with alkali and was separated on the same gel (lanes 3, 5, 9, and 11). The 5′ and 3′ end-labeled oligonucleotides sequenced according to Maxam and Gilbert (18) were used to locate precisely the positions of the nicks.

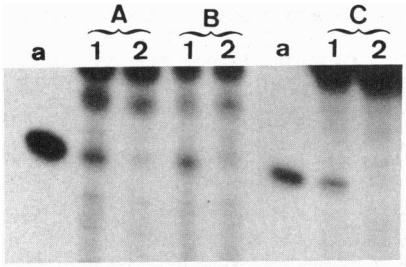

FIG. 8. Repairing activity by DNA polymerase is insensitive to aphidicolin (lanes B) and *N*-ethylmaleimide (lanes C). Lanes A, control incubated with protein but with no inhibitor. Lanes a, hemimethylated DNA substrate digested with *Msp* I. Lanes 1, tests incubated for 3 hr with the nuclear extracts (4-day embryos) and digested with *Hpa* II; lanes 2, parallel controls not digested with *Hpa* II. Experiments were carried out as described. Aphidicolin and *N*-ethylmaleimide were used at final concentrations of 0.3 and 5 mM, respectively.

alkali-sensitive apyrimidinic site at the position of the cleavage. A direct test using a [^{3}H]methyl group on the mCpG showed no traces of release of 5-methylcytosine (data not shown). Therefore, most probably the demethylation occurs not through a methylcytosine glycosylase but rather through excision and replacement of the nucleotide.

Identity of the DNA Polymerase Repairing Activity. Short patch DNA repair is known to be catalyzed by DNA polymerase β (21, 22). DNA polymerase β can be distinguished from the other three polymerases by its lack of sensitivity to both aphidicolin and *N*-ethylmaleimide (21). Since *N*-ethylmaleimide strongly inhibits the nicking reaction, the experiment was carried out in two steps. First the nicking of the labeled methylated oligonucleotide was achieved by incubating for 3 hr with embryonic extract in the presence of 20 mM EDTA. DNA was then phenol extracted, precipitated, and incubated in the complete system with the nuclear extracts plus aphidicolin or *N*-ethylmaleimide. The results of Fig. 8 show clearly that neither aphidicolin (lanes B) nor *N*-ethylmaleimide (lanes C) inhibited the repair reaction, suggesting that DNA polymerase β is the repairing enzyme.

DISCUSSION

Changes in the methylation pattern of a given DNA sequence can occur by two different pathways: the selective site-specific inhibition of the maintenance methylase following several cycles of replication (passive demethylation) (4–6) and the active demethylation of mCpGs by an enzymatic reaction (4–6). In the first case, the selective inhibition of the maintenance methylase could take place by the binding of a hypothetical determination protein to DNA. In the second case, the demethylation could occur in the absence of DNA replication (8–14) or after a single cycle of replication (14). So far the only detailed study of active demethylation has been done with differentiating Friend erythroleukemia cells (13, 23). By using the technique of double labeling of DNA with a density label and a radioactive deoxycytidine, it was concluded that demethylation of DNA during differentiation is achieved by an enzymatic replacement of 5-methylcytosine by cytosine (13). However, the nature of the enzymatic reaction leading to demethylation of mCpG has so far remained obscure. The results presented here show evidence for an enzymatic system replacing 5-methyldeoxycytidine by deoxycytidine. Its selectivity for mCpG and not for the other mCpN suggests an important role of this enzyme in the active demodification of DNA. Since the enzyme demethylates only hemimethylated DNA, this could explain why in specific cases it suffices to have only one cycle of replication to achieve a complete demethylation of mCpGs (14). In this case, the newly replicated DNA in the hemimethylated form provides an excellent substrate for the enzyme. The simultaneous *in vitro* demethylation of both mCpGs in the bifilarly methylated DNA is probably impossible, since the double-strand nicks break the DNA into two pieces unless there is an *in vivo* mechanism for holding the two fragments of DNA together. In fact, under my *in vitro* experimental conditions no demethylation of such a substrate has been observed (data not shown). However, it is also conceivable that only one strand at a time gets demethylated, yielding a hemimethylated DNA intermediate (24, 25). The hemimethylated DNA obtained postreplicatively should, however, provide the best substrate for both the demethylation and the maintenance methylase. However, the essential factor(s) determining which of the two reactions will predominate is not known. From the kinetics of demethylation of the avian vitellogenin gene in response to estradiol, it is clear that demethylation of specific mCpGs in the promoter region occurs very early but proceeds rather slowly (26). This suggests that demethylation of DNA is a consequence of a structural change in the chromatin brought about by the steroid hormone. Demethylation of the gene decreases the affinity of DNA for histone H1 (27) and helps to maintain the chromatin in the open form that can be modulated by a whole series of transcription factors. According to Razin *et al.* (28), the demethylating activity is already in the cells before differentiation, since inhibitors of protein synthesis did not prevent the genome-wide demethylation of differentiating erythroleukemia cells. In this context, it would be quite interesting to measure in such differentiating systems the change in the level of demethylating activity. During the differentiation of chicken myoblasts to muscle fibers, there are transient single-strand DNA breaks that need ADP ribosyltransferase (ADPRT) for their repair (29). The causality between these single-strand DNA breaks–repairs and differentiation was established by using specific inhibitors of ADPRT (29). Similar observations were made with differentiating lymphocytes (30). In the first case, the possibility of the single-strand DNA breaks being the result of a defective DNA repair system has been ruled out. Therefore, it is tempting to speculate that the transient single-strand breaks represent the mCpG sites undergoing active demethylation. This hypothesis is consistent with results showing that differentiating cells have a transient genome-wide demethylation (11–13). Whether the enzymatic activity described in this paper has any relevance to this phenomenon or whether it is related to the protein that induces cell differentiation while causing nicks in double-stranded DNA (31) remains to be shown. It is also interesting to note that in the mycoplasma *Acholeplasma laidlawii* JA1 (32) there is a restriction endonuclease capable of cleaving 5-methylcytosine irrespective of the DNA sequence. Whether there is any evolutionary relationship between this enzyme and the chicken embryo mCpG endonuclease remains to be shown. The specificity of these enzymes would have a variety of experimental uses, particularly in studies on the role of methylation of cytosine in gene regulation processes.

I thank Drs. F. Meins, J. M. Zingg, and T. Raizis for critically reading the manuscript and Y. C. Jost for typing it.

1. Holliday, R. (1989) *Sci. Am.* **260**, 40–48.
2. Holliday, R. (1990) *Biol. Rev.* **65**, 431–471.
3. Holliday, R. (1987) *Science* **238**, 163–170.
4. Razin, A., Cedar, H. & Riggs, A., eds. (1984) *DNA Methylation: Biochemistry and Biological Significance* (Springer, New York).
5. Adams, R. L. P. & Burdon, R. H. (1985) *Molecular Biology of DNA Methylation* (Springer, New York).
6. Jost, J.-P. & Saluz, H. P. (1993) *DNA Methylation: Molecular Biology and Biological Significance* (Birkhaeuser, Basel).
7. Li, E., Bestor, T. H. & Jaenisch, R. (1992) *Cell* **69**, 915–926.

8. Paroush, Z., Keshet, I., Yisraeli, J. & Cedar, H. (1990) *Cell* **63,** 1229–1237.
9. Trasler, J. M., Hake, L. E., Johnson, P. A., Alcivar, A. A., Millette, C. F. & Hecht, N. B. (1990) *Mol. Cell. Biol.* **10,** 1828–1834.
10. Razin, A., Webb, C., Szyf, M., Yisraeli, J., Rosenthal, A., Naveh-Many, T., Sciaky-Gallili, N. & Cedar, H. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 2275–2279.
11. Young, P. R. & Tilghman, S. M. (1984) *Mol. Cell. Biol.* **4,** 898–907.
12. Bestor, T. H., Hellewell, S. B. & Ingram, V. M. (1984) *Mol. Cell. Biol.* **4,** 1800–1806.
13. Razin, A., Szyf, M., Kafri, T., Rool, M., Giloh, H., Scarpa, S., Carotti, D. & Cantoni, G. L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2827–2831.
14. Shemer, R., Kafri, T., O'Connell, A., Eisenberg, S., Breslow, J. L. & Razin, A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 11300–11304.
15. Wilks, A., Seldran, M. & Jost, J.-P. (1984) *Nucleic Acids Res.* **12,** 1163–1177.
16. Sierra, F. (1990) in *A Laboratory Guide to in vitro Transcription*, eds. Azzi, A., Polak, J. M. & Saluz, H. P. (Birkhaeuser, Basel).
17. McGhee, J. D., Wood, W. I., Dolan, M., Engel, J. D. & Felsenfeld, G. (1981) *Cell* **27,** 45–55.
18. Maxam, A. M. & Gilbert, W. (1980) *Methods Enzymol.* **65,** 499–560.
19. Jost, J.-P. & Saluz, H. P., eds. (1991) in *A Laboratory Guide to in Vitro Studies of Protein-DNA Interactions* (Birkhaeuser, Basel).
20. Fox, K. R. (1986) *Biochem. J.* **234,** 213–216.
21. Wang, T. S. F. (1991) *Annu. Rev. Biochem.* **60,** 513–552.
22. Wiebauer, K. & Jiricny, J. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 5842–5845.
23. Razin, A., Levine, A., Kafri, T., Agostini, S. & Cantoni, G. L. (1988) *Gene* **74,** 139–142.
24. Saluz, H. P., Jiricny, J. & Jost, J.-P. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 7167–7171.
25. Paroush, Z., Keshet, I., Yisraeli, J. & Cedar, H. (1990) *Cell* **63,** 1229–1237.
26. Jost, J.-P., Saluz, H. P. & Pawlak, A. (1991) *Nucleic Acids Res.* **19,** 5771–5775.
27. Jost, J.-P. & Hofsteenge, J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9499–9503.
28. Razin, A., Levine, A., Kafri, T., Agostini, S., Gomi, T. & Cantoni, G. L. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 9003–9006.
29. Farzaneh, F., Zalin, R., Brill, D. & Shall, S. (1982) *Nature (London)* **300,** 362–366.
30. Johnstone, A. P. & Williams, G. T. (1982) *Nature (London)* **300,** 368–370.
31. Weisinger, G., Korn, A. P. & Sachs, L. (1986) *FEBS Lett.* **200,** 107–110.
32. Sladek, T. L., Nowak, J. A. & Maniloff, J. (1986) *J. Bacteriol.* **165,** 219–225.