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Inhibition of promoter activity by methylation: Possible involvement of protein mediators

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ABSTRACT To study the relationship between DNA methylation and promoter activity we have methylated *in vitro* the promoters of the mouse metallothionein I gene and the herpes simplex virus thymidine kinase gene. We have transiently transfected these promoters fused to the human growth hormone in their methylated or unmethylated state into mouse L or F9 cells. Promoters methylated by methylase (M.) *Hpa* II and M.*Hha* I caused inhibition of reporter gene expression in L cells but not in F9 cells, while methylation of all CpGs by *M.Sss* I caused inhibition in both cell lines. Repression of promoter activity by M.*Hpa* II and M.*Hha* I methylation, but not by M.*Sss* I methylation, could be alleviated by cotransfection with an excess of untranscribable DNA methylated with M.*Sss* I. The methylated sites in nuclei isolated from the transfected L cells, but not F9 cells, were found to be protected from *Msp* I digestion. Taken together these results suggest that a factor present in L cells and missing in F9 cells mediates the methylation-directed inhibition of promoter activity. The ability of methylated DNA to overcome the inhibition seems to reflect competition for the mediator factor. Interestingly, treatment with Zn^{2+} ions brought about activation of the methylated promoter of the metallothionein gene. Similarly, butyrate could override the repression of the thymidine kinase methylated promoter. These activations were not accompanied by demethylation of the promoter or displacement of the mediator factor.

It is now well established that in mammals and other vertebrates DNA methylation plays a role in gene expression. Several observations indicating that methylation at the promoter region is generally associated with transcriptional inactivity (1) led to a series of studies on the effect of promoter methylation on the binding of transcription factors. *In vitro* optimal expression of genes encoding the adenovirus late major protein and of other cellular genes (2) requires binding of the HeLa cell major late transcription factor (MLTF), and it has been shown that methylation of a single CpG centrally located in the recognition sequence for MLTF (3) will hinder MLTF binding. Similarly, methylation of the sequence TGACGTCA present in the cAMP responsive element of certain promoters abolished both the binding of transcription factors and transcription (4). The effects of the methylation of the chloramphenicol acetyltransferase transcription factor (CTF) and Spl binding sites of the herpes simplex virus thymidine kinase promoter depend on the experimental situation: *in vivo* expression of the gene is repressed 50-fold but *in vitro* methylation had no effect on the binding of CTF to the CTF recognition region (5). Finally, Spl transcription factor binds and activates transcription even when the binding site is methylated (6). It has been suggested that the lack of effect of methylation on the binding of Spl transcription factor may reflect the fact that the Spl factor appears to be involved in the expression of housekeeping genes that are not methylated at their 5′ end. On the other hand, cAMP responsive element is typically associated with tissue-specific genes that are always methylated at their 5′ end except in those cell types that express the gene (1, 7). Clearly, as these studies indicate, the relationship between promoter methylation and transcription factor binding is quite complex.

Two alternative models have been proposed to explain the mechanism whereby DNA methylation might repress gene transcription (8). Promoter methylation might hinder the binding of transcription factors thereby directly inhibiting gene expression, as has been shown in the case of MLTF and cAMP responsive element (3, 4), or it might act indirectly through a mediator capable of binding to methylated sites in the promoter thus preventing the formation of the transcription complex.

The recent identification of proteins capable of binding specifically to methylated DNA (9, 10) favors the hypothesis that the methylation-directed inhibition of transcription may be mediated by a repressor factor. The data presented here add further support to this possibility.

METHODS AND MATERIALS

Cell Constructs and Cell Treatments. In our experiments, we used the mouse Ltk^- line and the murine F9 teratocarcinoma cells. Cells were grown in 5% CO_2/95% humidified air at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Biological Industries, Beit-Haemek, Israel). For the F9 cell culture, the serum was inactivated at 55°C for 33 min. $ZnSO_4$ (Sigma) was added to a final concentration of 100 μM and sodium butyrate (Fluka) at 10 mM was added to the respective culture medium for 14 h, 2 h after transfection. All enzymes were from New England Biolabs, except *Msp* I, which was purchased from Boehringer Mannheim. The constructs used here were from Nichols Diagnostics (San Juan Capistrano, CA) and have been described by Selden *et al.* (11).

Transfections and Growth Hormone Assays. Logarithmic-phase cells were split and plated on 60-mm tissue culture dishes at 8×10^5 cells per plate. After 16 h of incubation at 37°C, cells were transfected with the appropriate amount of DNA (0.7 μg for Ltk^- cells and 2 μg for F9 cells) with the transfection reagent *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) from Boehringer Mannheim according to the instructions of the manufacturer. Transfection mixture was removed after 8 h and fresh medium was added and changed every 24 h. In experiments that took longer than 72 h, cells were split after 48 h and plated onto 100-mm tissue culture dishes.

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Abbreviations: MLTF, major late transcription factor; CTF, chloramphenicol acetyltransferase transcription factor; DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride; M., methylase.

Growth hormone assays were carried out with the Nichols Diagnostics kit. Briefly, 100-μl aliquots were withdrawn from the tissue culture medium, and 100 μl of ^{125}I-labeled antibody solution was added and mixed. Avidin-coated beads were added, and the mixture was incubated at room temperature for 4 h on a horizontal shaker at 180 rpm. The beads were washed twice and counted in a γ-counter. The combination of the DOTMA transfection procedure and the growth hormone assay yielded highly sensitive and reproducible results.

The level of secreted growth hormone was previously shown to be directly proportional to the transcriptional activity (11).

Nuclei Isolation and *Msp* I Digestions. Cells were washed twice with phosphate-buffered saline. To lyse the cells, 0.75 ml of reticulocyte standard buffer (RSB) (10 mM Tris·HCl, pH 7.5/50 mM NaCl/3 mM $MgCl_2$) plus 0.5% Nonidet P-40 was added and the cells were scraped off, resuspended, and left for 7 min on ice. Nuclei were precipitated by centrifugation at 750 × *g* for 10 min and washed once with RSB. The washed nuclei were resuspended in *Msp* I buffer and digested with *Msp* I as indicated in the legend to Fig. 3. The reactions were stopped with an equal volume of 2 times stop buffer (50 mM Tris·HCl, pH 7.5/0.3 M NaCl/50 mM EDTA/1% SDS), and proteinase K was added to a final concentration of 300 μg/ml and incubated at 55°C for 1 h.

RESULTS

Inhibition of Promoter Activity by Methylation Is Blocked by Competition with Excess Methylated DNA. As a first step in our effort to elucidate the mechanism whereby DNA methylation inhibits gene expression, we adopted the following strategy: constructs of the human growth hormone gene fused to methylated or unmethylated promoters such as the herpes simplex virus thymidine kinase promoter or the mouse metallothionein I promoter were transfected into Ltk⁻ or F9 cells. In this system, the human growth hormone gene served as a reporter and the level of the growth hormone secreted in the medium is an excellent representation of the promoter activity. The assay of human growth hormone was performed on aliquots taken from the culture medium, and, therefore, DNA methylation and gene expression could be assayed on the same cell culture plates.

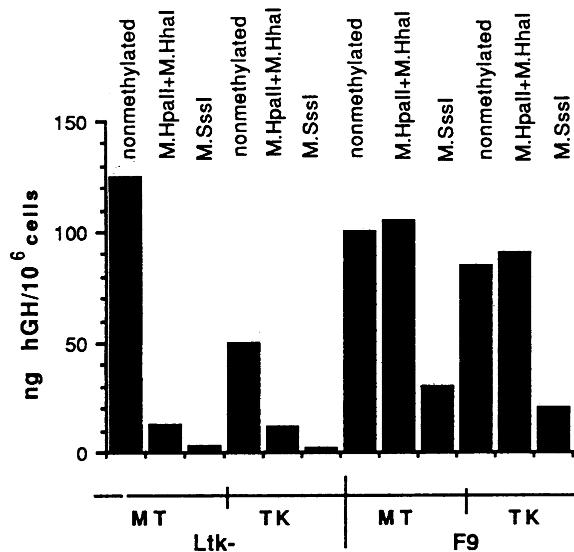

FIG. 1. Methylation-imposed inhibition of transcription. Nonmethylated and M.*Hpa* II plus M.*Hha* I or M.*Sss* I methylated constructs were transfected into Ltk⁻ and F9 cells. The constructs used were structural sequences of human growth hormone gene (hGH) fused to a 1.8-kilobase *Eco*RI/*Bgl* II fragment containing mouse metallothionein I promoter and upstream region (MT) or a 0.2-kilobase *Pvu* II/*Bgl* II fragment of the herpes simplex virus thymidine kinase promoter region (TK) (11). hGH was assayed in the culture medium 48 h posttransfection.

As shown in Fig. 1, the expression of the reporter gene is significantly affected in both Ltk⁻ and F9 cells transfected with constructs containing promoters in which all CpGs have been methylated by methylase (M.) *Sss* I (12). In contrast, however, when ≈10% of the CpG residues in the promoters are methylated by M.*Hpa* II and M.*Hha* I there is no effect on reporter gene expression in F9 cells, but in Ltk⁻ cells reporter gene expression is significantly affected. These results suggest that in Ltk⁻ cells the repression of gene expression is quantitatively related to the extent of promoter methylation. To rule out the possibility that the lack of repression in F9 cells results from demethylation (13), we analyzed the methylation status of the promoters at the end of the experiment and found no demethylation (data not shown). This observation together with the report that F9 cells are practically devoid of MeCP.1 (9), a protein capable of binding to methylated DNA, suggested that the inhibition of gene expression by promoter methylation might be due to a factor capable of binding specifically to methylated DNA. To explore this possibility we cotransfected Ltk⁻ cells with the methylated constructs and with various amounts of methylated untranscribable (pBR322 or ϕX174) DNA. The competition effect shown in Fig. 2 was linear with respect to the amount of added methylated untranscribable DNA. The possibility that specific sequences in the competitor DNA may be responsible for the effects shown in Fig. 2 is very unlikely since practically the same results were obtained when pBR322 or ϕX174 replicative form was used as competitor DNA, and we conclude that the effect implies the involvement of a general kind of repression.

Is a Mediator Factor Bound to Methylated DNA in Ltk⁻ Cells but Not in F9 Cells? To probe for the presence of a factor(s) capable of binding to methylated DNA, we used the *Msp* I limited digestion of nuclei as described (14). In the presence of *S*-adenosylmethionine M.*Hpa* II methylase will convert CCGG to C^{m}CGG and the restriction enzyme *Msp* I will cleave the CpG bond or the ^{m}CpG bond equally well. The results shown in Fig. 3 indicate that the amount of enzyme needed to cleave methylated templates transfected into Ltk⁻ cells is >10 times greater than the amount needed to cleave

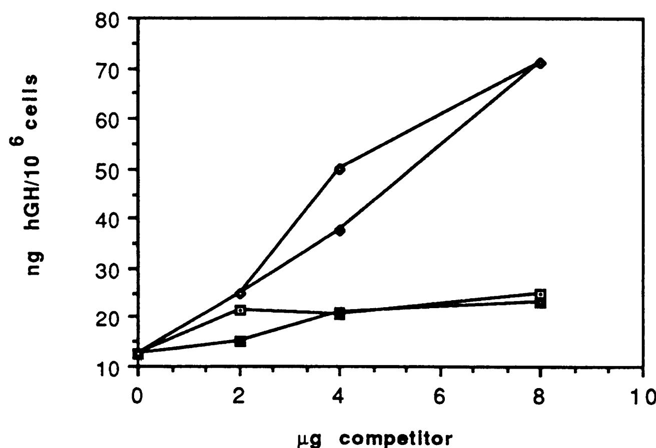

FIG. 2. Reactivation of a methylated gene by an excess of untranscribable DNA. M.*Hpa* II plus M.*Hha* I methylated metallothionein construct (0.7 μg) was cotransfected with the amounts indicated of nonmethylated pBR322 (■) or ϕX174 (□), or with M.*Sss* I methylated pBR322 (◆) or ϕX174 (◇). The human growth hormone gene (hGH) was assayed 72 h posttransfection.

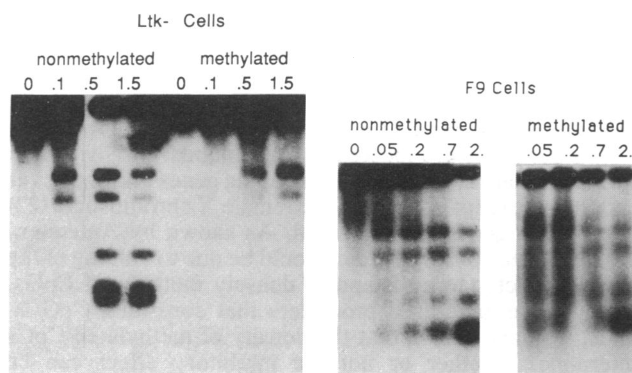

FIG. 3. *Msp* I digestion of transfected Ltk$^-$ and F9 nuclei. Ltk$^-$ and F9 cells were transfected with 0.7 μg of methylated or nonmethylated metallothionein construct. Nuclei were isolated 48 h posttransfection and incubated with the indicated number of units of *Msp* I for 45 min. Total DNA was purified and digested with *Kpn* I/*Eco*NI, electrophoresed on 1% agarose gels, alkali blotted to Zeta-Probe nylon membranes (Bio-Rad), and hybridized to a radioactive 750-base-pair *Kpn* I/*Dra* III fragment from the metallothionein promoter region.

unmethylated templates when tested in intact nuclei. This effect is obtained as early as 9 h posttransfection (data not shown). In contrast, we found that in F9 cells the *Msp* I-catalyzed cleavage of the methylated or unmethylated templates requires the same enzyme concentration. These results suggest that in Ltk$^-$ cells but not in F9 cells the resistance of the methylated constructs to *Msp* I digestion may be due to their protection by a "factor" that binds to methylated DNA. In light of the fact that in embryonic cells, like F9, methylated and unmethylated templates were equally sensitive to *Msp* I digestion, together with the fact that embryonic cells have very low levels of extractable MeCP.1 (9), it is reasonable to propose that a protein analogous to MeCP.1 might have played the role of mediator in the methylation-directed inhibition of promoter activity discussed above.

Enhancing Promoter Activity Overrides the Methylation-Directed Inhibition. Is it possible to override the inhibitory effect of promoter methylation by means other than competition by untranscribable methylated DNA? To answer that question, we used Zn^{2+} ions, which are known to enhance metallothionein gene activity (15), or butyrate, which is believed to cause activation of genes by affecting the assembly of chromatin structure in the promoter region (16). Addition of Zn^{2+} ions to Ltk$^-$ cells transfected with constructs containing the M.*Hpa* II plus M.*Hha* I-methylated metallothionein promoter resulted in activation of the promoter (Fig. 4). The same results were obtained by brief treatment of Ltk$^-$ cells with butyrate after transfection with thymidine kinase promoter methylated by M.*Hpa* II plus M.*Hha* I. No activation was observed when the promoters had been methylated with M.*Sss* I methylase. Southern blot analysis revealed that the activation by Zn^{2+} ions and butyrate are not associated with demethylation of the promoter region (Fig. 4): transcription *per se* is therefore not sufficient to cause demethylation. This result confirms and extends earlier observations showing that reactivation of methylated inactive adenovirus type 2 promoter by E1A functions does not involve promoter demethylation (17).

In addition, the *Msp* I protection assay performed after Zn^{2+} ion induction or butyrate activation (data not shown) reveals that the protective effect against digestion of the methylated metallothionein promoter in transfected L cells remains unchanged (Fig. 5), indicating that butyrate and zinc can override the inhibitory effect of methylation without displacing the DNA binding protein. This is formally analogous to the passing of the transcription apparatus through histones without histone displacement (18).

DISCUSSION

While the effect of methylation on promoter activity is well established, the mechanism that underlies this phenomenon is still obscure and several hypotheses have been advanced to explain it. The experiments described above show that the inhibitory effect of promoter methylation on the activity of transfected metallothionein and thymidine kinase promoters is neutralized by cotransfection with an excess of methylated untranscribable DNA. It was also shown that the effects of promoter methylation on gene expression are cell type de-

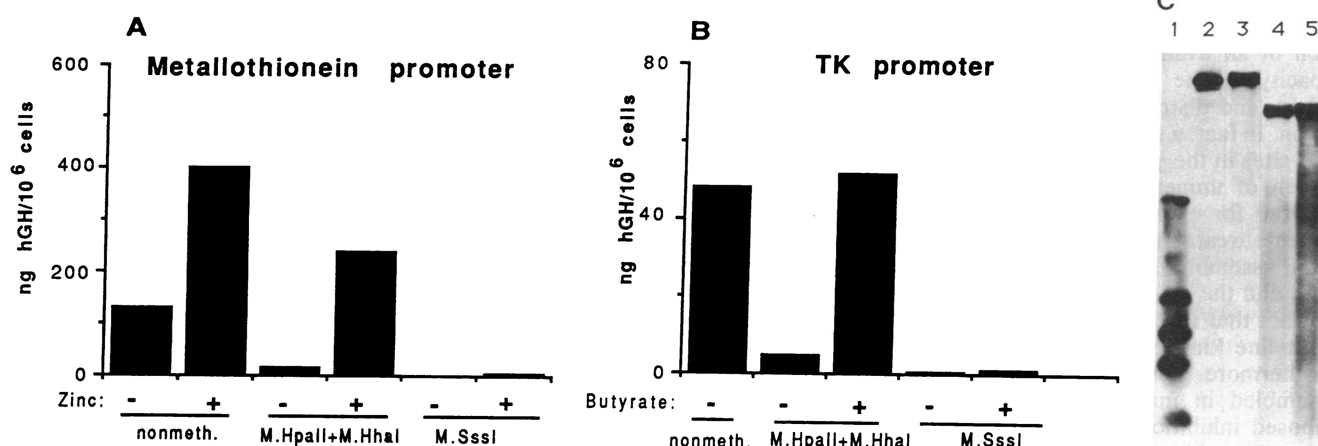

FIG. 4. Activation of methylated genes by butyrate or zinc. (*A*) Ltk$^-$ cells were transfected with 0.7 μg of methylated or nonmethylated metallothionein construct. At 12 h posttransfection $ZnSO_4$ was added to a final concentration of 100 μM (where indicated) and 36 h later cells were split and replated. The human growth hormone gene (hGH) was assayed 96 h posttransfection. (*B*) For butyrate treatment, 0.6 μg of methylated or nonmethylated thymidine kinase (TK) construct was transfected into Ltk$^-$ cells. Sodium butyrate was added, and assays of hGH were performed 72 h posttransfection. (*C*) To test the status of methylation after activation, DNA was prepared from the corresponding cells at the time of hGH assay, digested with M.*Hpa* II plus *Eco*RI (metallothionein; lanes 1–3) or M.*Hpa* II plus *Hin*dIII plus *Nco* I (TK; lanes 4 and 5), Southern blotted, and hybridized with a radioactive probe spanning the hGH gene. These digestions excise the hGH gene and upstream sequences from the vector. Lanes: 1, metallothionein, not methylated; 2, metallothionein, methylated; 3, metallothionein, methylated, plus Zn^{2+}; 4, TK, methylated; 5, TK, methylated, plus butyrate.

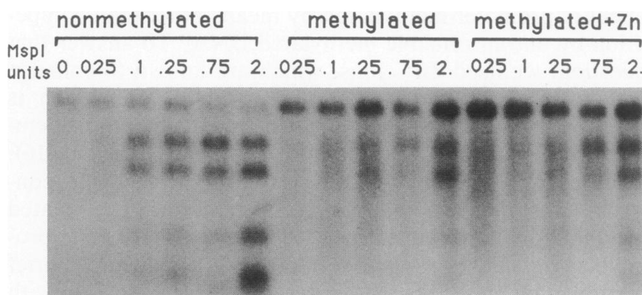

FIG. 5. *Msp* I digestion assay after Zn^{2+} activation of the metallothionein promoter. Ltk^- cells were transfected with M.*Hpa* II plus M.*Hha* I, methylated and nonmethylated metallothionein construct as described in Fig. 3, and $ZnSO_4$ was added where indicated. At 100 h after transfection, nuclei were isolated and digested with *Msp* I as described in Fig. 3. Total DNA was extracted, digested, electrophoresed, blotted, and hybridized as described in the legend to Fig. 3.

pendent; limited promoter methylation inhibits the expression of transfected genes in L cells but is without effect in F9 cells. This observation taken together with the fact that a protein (MeCP.1) capable of binding to methylated DNA has been identified in the nuclei of somatic cells of the mouse and other mammals but shown to be almost absent in embryonic cells (9) supports the idea that MeCP.1 or a similar factor may participate as mediator in the inhibitory effects of promoter methylation (14). The experiment showing that methylated promoters are 10 times more resistant to *Msp* I digestion in L cells than in F9 cells adds further weight to the idea that a mediator factor is involved in the inhibition process. This conclusion is based on the fact that cleavage of naked DNA by *Msp* I is not affected by the presence of ^{m}C within the CCGG sequence. The resistance of methylated promoters in intact nuclei can, therefore, be safely interpreted to mean that, when methylated, this particular sequence is protected in the nuclei of L cells, presumably by a bound protein.

The experiments showing that Zn^{2+} ions or butyrate can overcome the repression of promoter activity caused by M.*Hpa* II plus M.*Hha* I methylation are of great interest because they indicate that reactivation does not involve demethylation of the promoter or displacement of the bound mediator. The fact that Zn^{2+} ions and butyrate were unable to overcome the inhibition of repression caused by methylation of all available CpG sites suggests that the binding capacity of the repressor protein is correlated with the density and distribution of methyl groups in the promoter region. In fact, a study of the effect of methylation of specific CpG sites in the γ-globin promoter revealed that a minimum stretch of unmethylated DNA in the promoter region is required for expression (19). It should also be noted that butyrate treatment was performed during the time of chromatin assembly (16). A relationship between DNA methylation and the assembly of active chromatin is suggested by the fact that the methylation-dependent inactivation of the thymidine kinase gene requires chromatin formation (20). Furthermore, methylated DNA is known to be preferentially assembled in inactive chromatin (21). The methylation-imposed inhibition of promoter activity may therefore involve a combination of three mechanisms: (*i*) inhibition of binding of transcription factors to their corresponding binding sites, (*ii*) interference in transcription initiation by methylated DNA binding proteins, and (*iii*) methylation-directed formation of inactive chromatin structure at the promoter region. While the first mechanism would act on specific promoters, the other two can serve as general repression mechanisms.

To evaluate the significance of our observations to the *in vivo* situation, it should be remembered that the promoters used in our study are located in CpG islands. *In vivo*, these CpG islands are typical of housekeeping genes and are never methylated except in the inactive female X chromosome (22) where these genes are repressed. As shown by Antequera (14), resistance to *Msp* I attack could be due to binding of the repressor factor to an island of densely methylated CpGs. Although we have used promoters that constitute CpG islands, we have shown that the density of methylated CpGs determines whether or not the inhibitory effect can be overcome: with promoters methylated with M.*Hpa* II and M.*Hha* I, for instance, the inhibitory effect could be alleviated, whereas promoters methylated with M. *Sss* I cannot be reactivated. These observations might explain why in tissue-specific genes, where the number of CpGs is relatively small, the inhibitory effect of methylation can be overcome by various activators, such as the estrogens in the case of the chicken vitellogenin gene (23).

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1. Cedar, H. & Razin, A. (1990) *Biochim. Biophys. Acta* **1049**, 1–8.
2. Carthew, R. W., Chodesh, L. A. & Sharp, P. A. (1988) *Genes Dev.* **1**, 973–980.
3. Watt, F. & Molloy, P. L. (1988) *Genes Dev.* **2**, 1136–1143.
4. Iguchi-Ariga, S. & Schaffner, W. (1989) *Genes Dev.* **3**, 612–619.
5. Ben-Hattar, J., Beard, P. & Jiricny, J. (1989) *Nucleic Acids Res.* **17**, 10179–10190.
6. Hoeller, M., Westin, G., Jiricny, J. & Schaffner, W. (1988) *Genes Dev.* **2**, 1127–1135.
7. Becker, P. B., Ruppert, S. & Schutz, G. (1987) *Cell* **51**, 435–443.
8. Boyes, J. & Bird, A. (1991) *Cell* **64**, 1123–1134.
9. Meehan, R. R., Lewis, J. D., McKay, S., Kleiner, E. L. & Bird, A. P. (1989) *Cell* **58**, 499–507.
10. Huang, L. H., Wang, R., Gama-Sosa, M. A., Sheney, S. & Ehrlich, M. (1984) *Nature (London)* **308**, 293–295.
11. Selden, R. F., Burke Howie, K., Rowe, M. E., Goodman, H. M. & Moore, D. D. (1986) *Mol. Cell. Biol.* **6**, 3173–3179.
12. Renbaum, P., Abrahamove, D., Fainsod, A., Wilson, G., Rottem, S. & Razin, A. (1990) *Nucleic Acids Res.* **18**, 1145–1152.
13. Frank, D., Keshet, I., Levine, A., Shani, M., Razin, A. & Cedar, H. (1991) *Nature (London)* **351**, 239–241.
14. Antequera, F., Macleod, D. & Bird, A. P. (1989) *Cell* **58**, 509–517.
15. Hamer, D. H. (1986) *Annu. Rev. Biochem.* **55**, 913–952.
16. Reeves, R., Gorman, C. M. & Howard, B. (1985) *Nucleic Acids Res.* **13**, 3599–3615.
17. Weisshar, B., Langer, K.-D., Juettermann, R., Mueller, V., Zock, C., Klimkait, T. & Doerfler, W. (1988) *J. Mol. Biol.* **202**, 255–270.
18. Nacheva, G. A., Guschin, D. Y., Preobrazhenskaya, O. V., Karpov, V. L., Ebralidse, K. K. & Mirzabekov, A. D. (1989) *Cell* **58**, 27–36.
19. Murray, E. J. & Grosveld, F. (1987) *EMBO J.* **6**, 2329–2335.
20. Buschhausen, E., Wittig, B., Graessmann, M. & Graessmann, A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 1177–1181.
21. Keshet, I., Lieman-Hurwitz, J. & Cedar, H. (1986) *Cell* **44**, 535–543.
22. Bird, A. P. (1986) *Nature (London)* **321**, 209–213.
23. Wilks, A., Seldram, M. & Jost, J. P. (1984) *Nucleic Acids Res.* **12**, 1163–1177.