# Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription

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CpG methylation in vertebrates correlates with alterations in chromatin structure and gene silencing[1–4]. Differences in DNA-methylation status are associated with imprinting phenomena and carcinogenesis[5–10]. In *Xenopus laevis* oocytes, DNA methylation dominantly silences transcription through the assembly of a repressive nucleosomal array[11]. Methylated DNA assembled into chromatin binds the transcriptional repressor MeCP2 which cofractionates with Sin3 and histone deacetylase. Silencing conferred by MeCP2 and methylated DNA can be relieved by inhibition of histone deacetylase, facilitating the remodelling of chromatin and transcriptional activation. These results establish a direct causal relationship between DNA methylation-dependent transcriptional silencing and the modification of chromatin.

Methylated DNA microinjected into mammalian cells or *Xenopus* oocytes requires nucleosome assembly for transcriptional silencing[11,12]. MeCP2 is a methylation-specific transcriptional repressor that is stably incorporated into chromatin and chromosomes[13,14]. MeCP2 is also essential for embryonic development in the mouse[15]. We investigated whether MeCP2 might contribute, together with nucleosome assembly, to the transcriptional silencing of methylated DNA in *Xenopus* oocytes. We cloned a *Xenopus Mecp2* cDNA by PCR amplification, expressed a fusion protein and raised polyclonal antibodies against *Xenopus* MeCP2 (A.P.W. *et al.*, manuscript submitted). The antibodies were used to monitor the biochemical fractionation of MeCP2 (Fig. 1a,b). Gel-filtration chromatography of *Xenopus* oocyte extracts revealed that MeCP2 and a *Xenopus* homologue of the corepressor Sin3 (ref. 15) copurify at a molecular weight of 700 kD, suggesting that MeCP2 and Sin3 associate *in vivo*. An alternative purification procedure utilizing ion-exchange
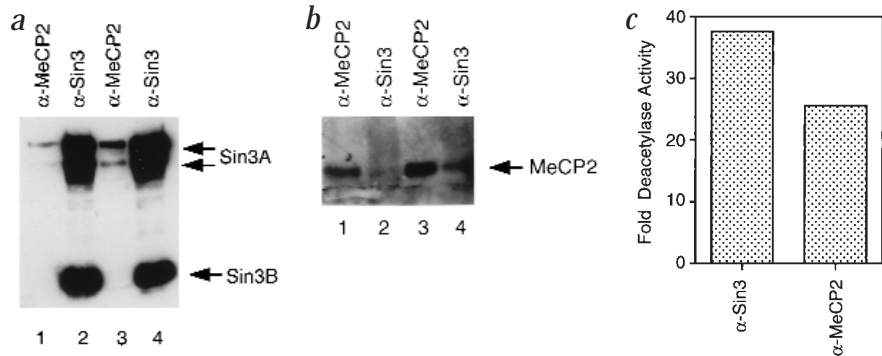
chromatography showed that MeCP2 cofractionates with Sin3 and eight additional polypeptides (Fig. 1b,c). Western-blot analysis of extracts from across each purification gradient indicated that more than 90% of *Xenopus* MeCP2 cofractionates with Sin3 in oocytes (data not shown). Co-elution, however, is not precise, and this might be explained by some dissociation of the proteins
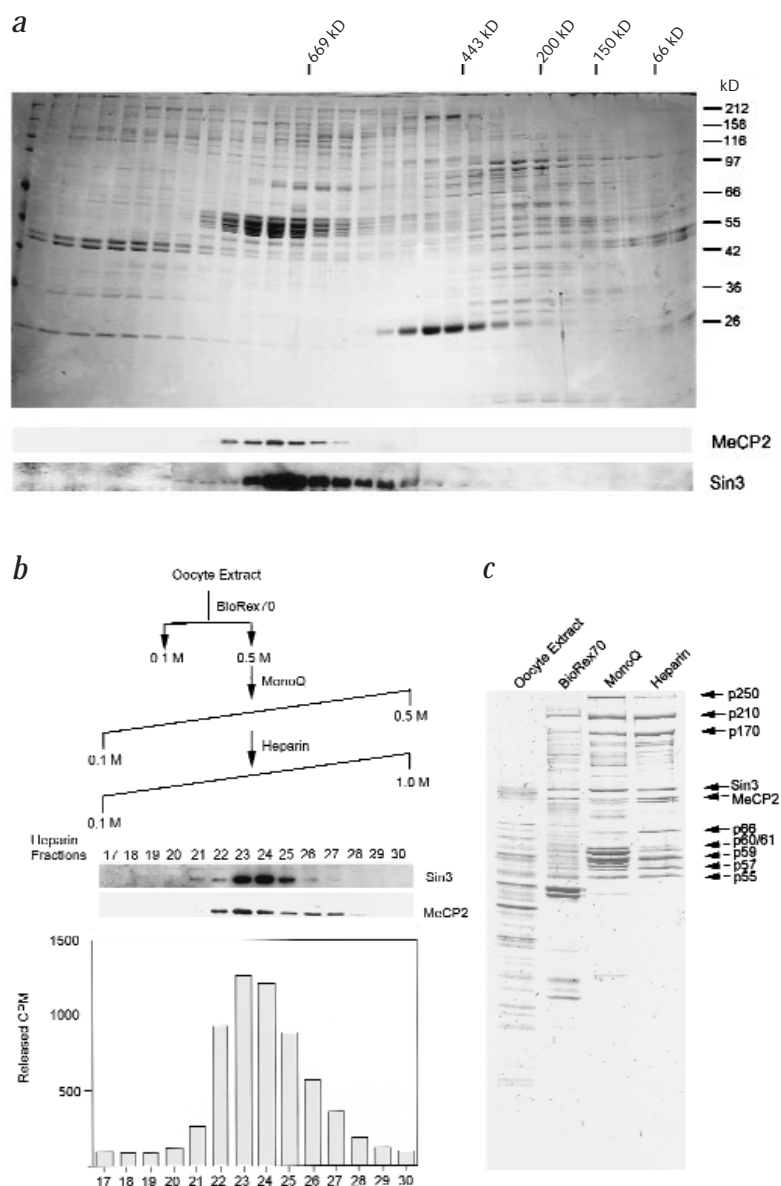
Fig. 1 Cofractionation of a histone deacetylase with MeCP2 and Sin3. *a*, Size fractionation of *Xenopus* oocyte extract stained with Coomassie Blue (upper panel) and assayed by western-blot analysis (lower panel) for *Xenopus* MeCP2 and Sin3. *b*, Flow chart showing the fractionation of MeCP2 and Sin3. MeCP2 and Sin3 protein were followed through each step of purification by western-blot analysis using antibodies against MeCP2 and Sin3 and assays of histone-deacetylase activity. A representative purification assayed through heparin is shown. *c*, Coomassie Blue-stained gel with each lane containing an equal amount of protein (7.0 µg) from each step of *Xenopus* extract purification. The apparent molecular weights for each of the polypeptides copurifying with MeCP2 and Sin3 are listed on the right.

Fig. 2 MeCP2, Sin3 and histone-deacetylase activity coimmunopricipitate. Immunoprecipitations of oocyte extract using antibodies against either MeCP2 (α-MeCP2; *a* and *b*, lanes 1 and 3) or Sin3 (α-Sin3; *a* and *b*, lanes 2 and 4) were analysed by western-blotting and probed for *a*, Sin3; or *b*, MeCP2. Immunopreciptiations were carried out in either 5 mM KCl (lanes 1 and 2) or 50 mM NaCl (lanes 3 and 4). Sin3A and SinB and MeCP2 are indicated. Pre-immune sera did not immunoprecipitate any detectable MeCP2 or Sin3 (data not shown) *c*, Immunoprecipitations were carried out from the BioRex 0.5 M elution using either Sin3 or MeCP2 antibodies and assayed for histone-deacetylase activity (shown as fold activity over background).

during chromatography. The salt elution profile from a heparin column shows that, in addition to Sin3, *Xenopus* MeCP2 also cofractionates with histone-deacetylase activity (Fig. 1*b*). Sin3 and histone deacetylase are components of multiple corepressor complexes[16–19]. Thus, the potential association of MeCP2, Sin3 and histone deacetylase might explain the requirement for chromatin assembly to direct transcriptional silencing on methylated DNA (refs 11,12), as histone deacetylase requires a nucleosomal infrastructure to silence transcription[20].

To further examine whether MeCP2 might associate with Sin3, we performed co-immunoprecipitation experiments. Dependent on the ionic conditions, polyclonal antibodies against MeCP2 immunoprecipitate the *Xenopus* Sin3A variant but not the Sin3B variant from oocyte extracts (Fig. 2*a*). The characterization of both *Xenopus* Sin3A and Sin3B variants[15] will be described elsewhere (P.L.J. & A.P.W., manuscript in preparation). Conversely, antibodies against Sin3 immunoprecipitate MeCP2 (Fig. 2*b*). Both MeCP2 and Sin3 polyclonal antibodies immunoprecipitate histone deacetylase activity (Fig. 2*c*). These results are consistent with the existence of a soluble complex containing MeCP2, Sin3 and histone deacetylase.

Xenopus MeCP2 is very similar to the mammalian MeCP2 proteins (Fig. 3*a*) in both the methyl-CpG binding domain[21] and the transcriptional-repression domain[13]. The *Xenopus* protein is present in oocytes and gastrula-stage embryos with the anticipated molecular weight of 81 kD (Fig. 3*b*). *Xenopus* MeCP2 purified from bacteria selectively binds to methylated DNA (Fig. 3*c*) and stably associates with methylated nucleo-
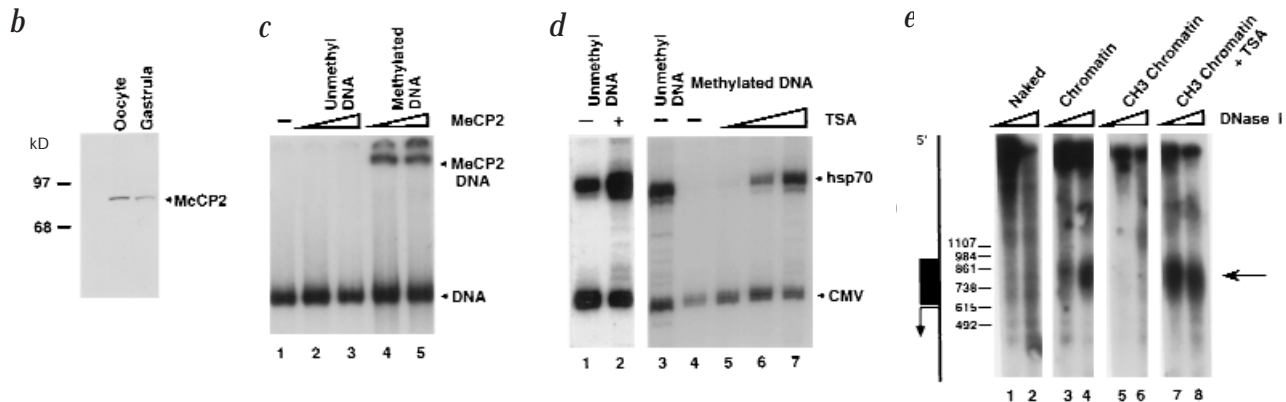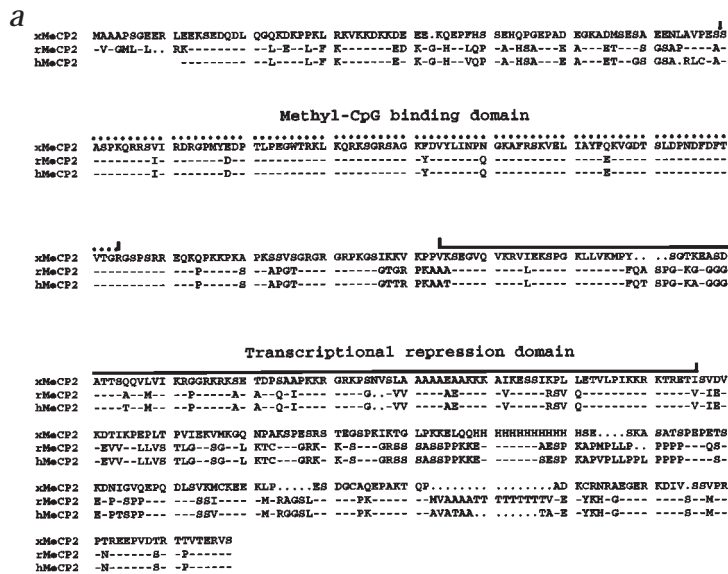
Fig. 3 Properties of *Xenopus* MeCP2. *a*, Sequence comparison of *Xenopus* MeCP2 (xMeCP2), rat MeCP2 (rMeCP2) and human MeCP2 (hMeCP2) proteins. Identical amino acids are indicated by dashes, and gaps in the sequence by dots. The extent of the methyl-CpG binding domain and the transcriptional repression domain are indicated[13,21]. *b*, Western blot of proteins isolated from a single oocyte or gastrula embryo from *Xenopus* probed with polyclonal antibodies against xMeCP2. The MeCP2 protein is indicated. *c*, Gel-shift experiments with MeCP2 and unmethylated or methylated 40-mer oligonucleotide probes Lane 1, no addition; lanes 2 and 4, 0.5 ng MeCP2; lanes 3 and 5, 2 ng MeCP2. *d*, Methylated or unmethylated pHSP-CAT plasmid was injected into oocyte nuclei and transcription from the *hsp70* promoter was assayed 10 h after injection. Injection of an unmethylated pCMVCAT (0.25 ng/oocyte), 3 h before oocyte isolation and RNA extraction, serves as an internal standard. In lanes 1, 3 and 4, no TSA was added; in lanes 2, 5, 6 and 7, TSA was added to 300, 3, 30 and 300 nM, respectively. The positions of transcripts derived from the *hsp70* and CMV promoters are indicated. *e*, Methylated CH3 (lanes 5–8) or mock-methylated (control) plasmid pHSP-CAT (lanes 3 and 4) was injected into oocyte nuclei and assayed for the presence of DNaseI-hypersensitive sites. Oocytes were incubated in the presence (lanes 7 and 8) or absence of 30 nM TSA (lanes 3–6). The plasmid was linearized with *Nco*I and the Southern blot was probed with a *Nco*I-*Eco*RI fragment (nucleotides +313 to +616 relative to the start site of transcription). The arrow indicates the major site of hypersensitivity.

Fig. 4 The MRD effectively represses transcription in frog oocytes in a TSA-sensitive manner. *a*, Gal4-MRD represses transcription from the HSV tk promoter. Oocytes were injected with 30, 100 and 300 pg of RNA encoding the Gal4 DNA binding domain (lanes 2-4), Gal4-MRD fusion protein (lanes 5–7) or no RNA (lane 1). After 4 h, pCMV-CAT and pG5HSVtk-CAT (the latter plasmid contains five Gal4 sites upstream of the promoter) was injected and RNA was isolated and analysed 18 h later. Endogenous histone-H4 RNA was used for normalized quantitation of RNA transcribed from the injected CMV and HSV tk promoters. *b*, TSA relieves transcriptional repression induced by Gal4-MRD. Oocytes were injected with 200 pg of Gal4-MRD RNA (lanes 5–8), or not injected (lanes 1–5), and subsequently incubated for 4 h, injected with promoter construct, and incubated for 18 h in the absence (lanes 1 and 5) or presence of 3 nM (lanes 2 and 6), 30

nM (lanes 3 and 7) or 300 nM (lanes 4 and 8) of TSA. *c*, Graphic representation of result shown in *b*. 30 nM (or more) of TSA partially relieves repression of transcription by Gal4-MRD. The level repression was calculated by comparing the normalized levels of RNA transcribed from the HSV tk promoter in lanes 1–4 and 5–8.
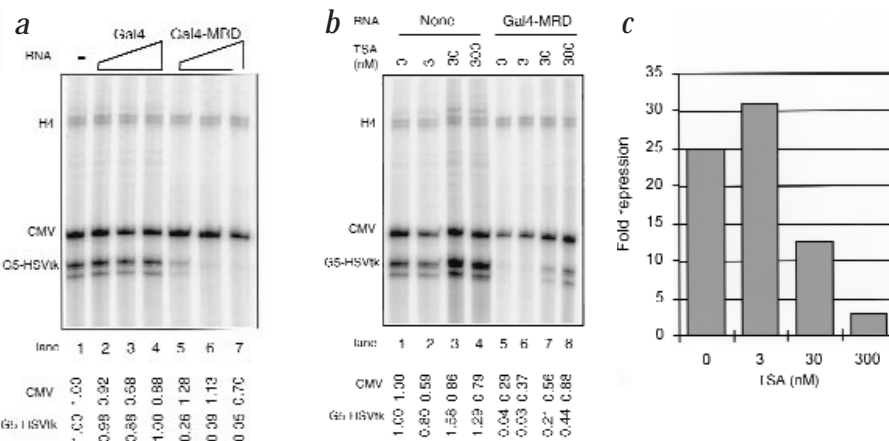
somal DNA (data not shown; refs 13,21). The role of histone deacetylase in transcriptional repression of methylated DNA following chromatin assembly was assayed using the inhibitor Trichostatin A (TSA; ref. 22). Concentrations of TSA between 30 and 300 nM inhibited histone deacetylase activity in the MeCP2 complex by 80 and 100%, respectively (data not shown). Microinjection of unmethylated *Xenopus hsp70* promoter-containing templates into *Xenopus* oocytes results in strong transcription after an overnight incubation period (10 h) during which chromatin is assembled (Fig. 3*d*; ref. 22). The addition of 300 nM TSA modestly enhances transcription by two- to three fold compared with a non-chromatinized, unmethylated human cytomegalovirus (CMV) promoter (Fig. 3*d*). Complete CpG methylation of the *hsp70* promoter using *Sss*I methyltransferase leads to transcriptional silencing of greater than 100-fold after an overnight incubation compared with that of a CMV promoter control (Fig. 3*d*). The addition of 3, 30 and 300 nM TSA progressively relieves transcriptional repression on the methylated template relative to the CMV control (Fig. 3*d*). Transcription from the methylated *hsp70* promoter was enhanced more than 100-fold, indicating that histone deacetylase has an active role in repressing transcription on methylated DNA. This effect of TSA is not a general response of weak promoters because the weak adenovirus E4 or herpes simplex virus thymidine kinase (HSV tk) promoters do not show major stimulatory effects on unmethylated templates transcribed in the presence of TSA (Fig. 4; and data not shown). The *Xenopus hsp70* promoter is assembled into a DNaseI-hypersensitive site within chromatin following microinjection into *Xenopus* oocyte nuclei (Fig. 3*e*; ref. 23). Methylation of the promoter leads to the loss of hypersensitivity, but the presence of 30 nM TSA is sufficient to restore hypersensitivity (Fig. 3*e*). Thus, inhibition of histone-deacetylase activity facilitates the remodelling of chromatin structure, concomitant with transcription competence on methylated DNA templates.

The capacity of MeCP2 to bind to methylated DNA in chromatin (data not shown) coupled with the release of transcriptional silencing and remodelling of chromatin on methylated DNA on inhibition of histone deacetylase (Fig. 3*d,e*) led us to test directly whether the MeCP2-repression domain was silencing transcription dependent on deacetylase activity. We fused the MeCP2 repression domain to the DNA-binding domain of Gal4 and microinjected mRNA encoding either the Gal4 DNA-binding domain or a Gal4-MeCP2 repression domain (Gal4-MRD) fusion into *Xenopus* oocyte cytoplasm. After an

incubation period (4 h) sufficient to allow synthesis of these DNA-binding proteins, we injected the template DNA containing the HSV tk promoter containing five upstream Gal4 DNA-binding sites, together with an internal CMV control. Western-blot analysis using anti-Gal4 antibodies showed that the Gal4 and Gal4-MRD proteins were approximately equally abundant in the oocyte following injection of the mRNA (data not shown). For quantification purposes, we assayed both the activity of the CMV promoter and the abundance of endogenous histone H4 RNA as internal controls. The Gal4 DNA-binding domain has no effect on transcription in the oocyte (Fig. 4*a*). In contrast, the Gal4-MRD protein is a potent silencer of transcription in the oocyte. Transcription from the HSV tk promoter is repressed more than 20-fold following injection of as little as 300 pg of RNA encoding Gal4-MRD (Fig. 4*a*). We next injected 200 pg of Gal4-MRD mRNA into oocytes, and examined the effects of TSA on transcription in these oocytes compared with transcription in oocytes not containing the Gal4-MRD protein. TSA reduces the ability of the Gal4-MRD to confer transcriptional repression by more than tenfold (Fig. 4*b,c*). The remaining repressive influence might be explained by the existence of parallel repression mechanisms conferred by other repressive components associated with MeCP2.

Our results suggest that the methylation-specific repressor MeCP2 will recruit the Sin3-histone deacetylase complex to promoters by binding methylated DNA through its methyl-CpG binding domain. As MeCP2 recognizes methylated DNA in a nucleosome and not all methyl CpGs will be exposed in chromatin, the incorporation of MeCP2 into chromatin potentially accounts for the strong threshold dependence of transcriptional silencing on methyl-CpG density *in vivo*[12,24]. The recruitment of Sin3 and histone deacetylase also explains the dependence of transcriptional silencing on chromatin assembly[11,12] and the capacity of methylated DNA segments to confer silencing at a distance in *cis*[12,25]. These experiments further emphasize the potential causal action of DNA methylation in directing transcriptional silencing within chromatin. Maintenance of DNA methylation occurs at the replication complex before chromatin assembly is complete[26,27]. Thus, subsequent sequestration of MeCP2 into nascent chromatin might help to establish a stable state of histone deacetylation which would further repress gene activity[28]. Thus, DNA methylation could make effective use of chromatin modification to maintain a stable state of gene expression. The additional proteins that cofractionate with MeCP2 and Sin3 may also contribute

to this regulatory process. DNA methylation might directly influence other aspects of chromatin function such as nucleosome positioning and mobility[29,30]. The potential causal action of DNA methylation on transcription through modification of histone-acetylation status provides an additional link between carcinogenesis, chromatin and DNA-methylation status[6,9,28].

Methods

Protein purification. Oocyte extracts were prepared from six mature female *Xenopus* per purification. The oocytes were collected and collagenase-treated as previously described[22,23], washed twice in extraction buffer (20 mM HEPES pH 7.5, 10 mM β-glycerophosphate, 5 mM KCl, 1.5 mM MgCl$_2$, 1 mM EGTA, 1 mM PMSF, 0.5 mM DTT, 10% glycerol), centrifuged at 38,000 rpm for 1 h at 4 °C (Beckman SW41-Ti), and the soluble fraction collected. The extract was loaded onto a BioRex70 (Na+) (BioRad) column equilibrated with Buffer A (100 mM NaCl, 20 mM Hepes pH 7.5, 10 mM β-glycerophosphate, 1.5 mM MgCl$_2$, 1 mM EGTA, 1 mM PMSF, 0.5 mM DTT, 10% glycerol) at 10 mg protein per ml packed column volume (cv), washed with 3 cv Buffer A (0.1 M), and step eluted with Buffer A (0.5 M). For gel filtration analysis, the 0.5 M eluate was centrifuged at 12,000 g for 20 min, loaded onto a Superose6 HR10/30 gel filtration column (Pharmacia Biotech) at 2 mg protein in 500 μl Buffer A and fractionated by fast protein liquid chromatography (FPLC) at 0.1 ml/min with 250 μl fractions. For ion-exchange chromotography, the 0.5 M eluate was dialysed against Buffer A (50 mM) for 90 min and centrifuged at 12,000 g for 20 min. The supernatant was fractionated by FPLC over MonoQ HR10/10 (Pharmacia Biotech) in a 20 cv linear gradient from Buffer A (0.1 M) to Buffer A (0.5 M) and collected in 4 ml fractions. All fractions were analysed by western blotting and histone-deacetylase activity as described below. The fraction containing the peak of MeCP2 and Sin3 was dialysed and centrifuged as before, fractionated by FPLC over a 1 ml Heparin HiTrap (Pharmacia Biotech) column in a 10 cv linear gradient from Buffer A (0.1 M) to Buffer A (1 M) and collected in 0.4 ml fractions. All fractions were analysed by western blotting and assayed for histone deacetylase activity.

Antibodies, western blotting and immunoprecipitations. Protein samples were separated by SDS-PAGE and transferred to ECL nylon membrane (Amersham Life Science) and western blotting was performed as described[13]. The Sin3 antibody was a rabbit polyclonal derived from a bacterially expressed GST fusion with *Xenopus Sin3* cDNA (P.J. & A.P.W. unpublished data). The MeCP2 antibody was a rabbit polyclonal derived from bacterially expressed full-length *Xenopus* cDNA encoding MeCP2. Sin3, MeCP2 or pre-immune sera were coupled to CNBr-activated sepharose (Pharmacia Biotech) as per the manufacturers' instructions. Bound antibodies were incubated with 100 μg of oocyte extract in 5 mM KCl or 50 mM NaCl, or 100 μg of the BioRex 0.5 M fraction in 100 mM NaCl, prepared as above, for 2 h at 4 °C with rotation. The immunoprecipitations were washed three times with Buffer A (0.1 M) and assayed by western-blot analysis or histone deacetylase assays. NP-40 to 0.01% was added at all steps.

Histone-deacetylase assays. Chicken histone octamers (20.25 nmoles) were acetylated using recombinant yeast HAT1p (kind gift of M. Parthun) and ^{3}H-acetyl CoA (5.3 nm) followed by a cold acetyl CoA (100 nm) chase for complete acetylation. Deacetylation of the sample (5 μl) was carried out in a reaction (200 μl) containing 25 mM Tris (pH 8.0), 50 mM NaCl, 1 mM EDTA, 10% glycerol, and 1 μg ^{3}H-histone octamer substrate. Reactions were incubated at 30 °C for 60 min, stopped by adding 50 μl stop solution (0.1 M HCl and 0.16 M HAc), and extracted with 600 μl ethyl acetate with 450 μl of the aqueous layer being measured. TSA was used at 30 nM and added at the time of sample.

Electrophoretic mobility shift experiments using naked DNA and nucleosomes. MeCP2 was purified from *E. coli* as a GST-fusion protein, binding to naked DNA used the GAM12 probe and protocols previously described[13,21]. Radiolabelled 40-bp oligonucleotides (0.1 ng) plus unlabelled poly dI/dC (10 ng) were incubated with 0, 0.5 and 2 ng of purified MeCP2 protein. Briefly, DNA-protein binding reactions were carried out in 20 mM HEPES (pH 7.9), 1 mM EDTA, 3 mM MgCl$_2$, 10 mM 2-mercaptoethanol, 10% glycerol and 0.1% Triton X-100. Labelled DNA was incubated with different amounts of protein on ice for 30 min and then electrophoresed on a 5% polyacrylamide gel in 6.7 mM Tris-HCl (pH 7.9), 3.3 mM sodium acetate and 1 mM EDTA for 4 h at 100 V at 4 °C. Cytosines in the CpG sequences were methylated with the CpG methylase *Sss*I (New England Biolabs). Incubation was as recommended by the manufacturer except that the enzyme concentration was increased five-fold. The extent of methylation was monitored by resistance to cleavage with *Hpa*II (New England Biolabs) and by Maxam and Gilbert cleavage chemistry. Methylation reactions were carried out until complete resistance to cleavage was achieved, then phenol-chloroform-extracted and ethanol-precipitated. Mock methylation reactions were carried out in the absence of *Sss*I.

Transcription and DNaseI-hypersensitivity analysis. The *Xenopus hsp70* promoter and human CMV and G5-HSVtk promoters have been previously described[12,22,23]. Plasmids were methylated *in vitro* using *Sss*I under conditions recommended by the manufacturer (New England Biolabs). DNA solution (9.2 μl containing 0.5 ng per oocyte) was injected into *Xenopus* oocyte nuclei as described[22]. Oocytes (30–40) were collected and homogenized in 20 mM Tris-HCl (pH 8.0), using 10 μl per oocyte. This homogenate was used for both RNA and DNA analysis. RNA was isolated using RNAzol (Cinna Scientific). A 30-mer oligonucleotide (3′–TAC-CTCTTTTTTTAGTGACCTATATGGTGG–5′) complementary to *CAT* mRNA was used for primer extension. Extension products were separated on polyacrylamide 6% sequencing gels and visualized by autoradiography. DNaseI hypersensitivity was analysed 10 h after injection by homogenization of injected oocytes (35 per sample) in buffer E (70 mM KCl, 20 mM HEPES pH 7.5, 1 mM DTT and 5% sucrose), using 20 μl per oocyte; MgCl$_2$ was added to a final concentration of 5 mM and the homogenate divided into four aliquots. DNaseI (10, 15, 20 and 30 U) was added and the samples were incubated for 5 min at RT. The reaction was stopped by addition of an equal volume of buffer containing SDS, EDTA and proteinase K, to final concentrations of 0.5%, 15 mM and 500 μg/ml, respectively. Purification and concentration of DNA samples was carried out as before. The DNA was then linearized with *Nco*I and separated on a 1.6% agarose gel in TAE buffer. Southern-blot analysis was performed using an *Nco*I–*Eco*RI fragment from the plasmid, adjacent to the promoter sequence. The pGAL4-MRD encoding the yeast Gal4 DNA-binding domain (amino acids 1–147) fused to the MeCP2 transcription repression domain (amino acids 204 KSEGVQ...RKTRET 310) was obtained by PCR amplification of the MeCP2 Repression Domain (MRD) and cloning of the fragment into the *Xba*I and *Bam*HI sites of pMSIIGal4. The pGal4 control construct is identical to pGAL4-MRD except for a 16-bp insert between the Gal4 and MRD coding sequences, causing a stop codon to terminate translation directly 3′ of the Gal4 sequence.

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