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A preference of histone H1 for methylated DNA

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We have identified a clear preference of histone H1 for CpG-methylated DNA, irrespective of DNA sequence. The conditions under which this preference is observed allow cooperative binding of H1; the H1–DNA complexes formed were shown earlier to be 'tramlines' of two DNA duplexes bridged by an array of H1 molecules, and multiples of these. The preference for methylated DNA is clear in sedimentation assays, which also show that the preference is greater with increased methylation level, and in gel retardation assays with an oligonucleotide containing a single methyl-CpG pair; it is shared by the globular domain which also binds cooperatively to DNA. A small intrinsic preference of H1 for methylated DNA is also apparent in Southwestern assays where the immobilized H1 presumably cannot bind cooperatively. Methylated DNA in H1–DNA complexes was partially protected (relative to unmethylated DNA) against digestion by *Msp*I but not by enzymes whose cutting sites were not methylated, consistent with a direct interaction of H1 with methylated nucleotides; this was also true of GH1–DNA complexes. H1 variants (spH1 and H5) from transcriptionally repressed nuclei have a stronger preference than H1 for methylated DNA, suggesting that this may be relevant to the stabilization of chromatin higher order structure and transcriptional repression.

Keywords: cooperativity/DNA methylation/histone H1/ H1–DNA complexes/H1 variants

Introduction

Methylated DNA stably transfected into cultured cells becomes resistant to transcription and acquires the DNase I resistance characteristic of inactive chromatin, whereas unmethylated DNA is transcribed and shows general DNase I sensitivity. This suggests that the methylated DNA becomes packaged into a more stable chromatin structure, and that the repressive effect of methylation on transcription is a direct reflection of this (Keshet *et al.*, 1986; Buschhausen *et al.*, 1987; Cedar, 1988). A more stable chromatin structure could arise from tighter/altered binding of an intrinsic chromatin protein (e.g. histone H1, which has a role in chromatin folding) and/or from the binding of some protein with a general preference for methylated DNA. Previous studies have identified such proteins, for example MDBP (Supakar *et al.*, 1988), MeCP1 (Meehan *et al.*, 1989) and MeCP2 (Lewis *et al.*, 1992; Meehan *et al.* 1992). MeCP2 is relatively abundant; it can bind to DNA containing a single methyl-CpG pair in gel retardation assays (Meehan *et al.*, 1992) and it is associated with chromatin (Lewis *et al.*, 1992).

Since histone H1 has a role both in sealing the nucleosome and in the formation of higher order structure (Thoma *et al.*, 1979), it is of some interest to determine whether H1 binding to DNA is also affected by methylation and whether it therefore might be involved in the tighter packing of chromatin containing methylated DNA. The evidence on this point is conflicting. After micrococcal nuclease digestion, 5-methylcytosine is located preferentially in mononucleosomes that contain H1 (Ball *et al.*, 1983); more recently, on the basis of band shift assays, a chicken H1-like protein, MDBP-2, was reported to bind preferentially to methylated DNA (Jost and Hofsteenge, 1992), and in filter binding assays calf thymus H1 similarly showed a preference for methylated DNA (Levine *et al.*, 1993). The early observation that H1 binds more strongly to calf thymus DNA (heavily methylated) than to *Escherichia coli* DNA (no CpG methylation) may also reflect a preference for methylated DNA, rather than a preference for AT-rich versus GC-rich DNA as originally suggested (Renz, 1975). Moreover, DNA methylation promotes the inhibition by H1 of transcription from DNA *in vitro* (Levine *et al.*, 1993; Johnson *et al.*, 1995). However, no preference of mouse liver H1 for methylated DNA was detected in filter binding assays, although H1 did protect some methylated sites from digestion with *Msp*I (Higurashi and Cole, 1991), or of calf thymus H1 for the methylated 5S rRNA gene in a gel shift assay (Nightingale and Wolffe, 1995).

We have argued (Clark and Thomas, 1986, 1988) that H1 (or H5)–DNA complexes, which have been well characterized, are a good model system for the study of some aspects of the interaction of H1 within chromatin. The complexes [and the morphologically similar complexes containing only the isolated globular domains, GH1 and GH5 (Draves *et al.*, 1992; Thomas *et al.*, 1992)], consist of 'tramlines' of two DNA duplexes bridged by an array of cooperatively bound protein molecules and indicate the existence of two DNA binding sites on the globular domains, apparently mirroring the situation at the H1 binding site on the nucleosome. This is also suggested by the X-ray crystal structure of GH5 (Ramakrishnan *et al.*, 1993), and the similar NMR structure of GH1 (Cerf *et al.*, 1994). H1 cooperativity is salt-dependent and is greatest for H1 variants (such as H5) found in transcriptionally inert chromatin, suggesting that the assembly of the histone–DNA complexes does indeed reflect some aspects of H1(H5)–DNA interaction in chromatin.

We have examined the effect of DNA methylation on

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the formation of these complexes and asked whether there are differences in behaviour between different histone variants. We have also used a Southwestern assay to compare the intrinsic affinities of H1 for methylated and unmethylated DNA, and restriction endonuclease digestion to determine whether there is selective protection of methylated CpG dinucleotides by H1.

Results

Under conditions that permit cooperative binding, H1 discriminates in favour of methylated DNA as judged by sedimentation analysis

To test whether H1 can distinguish between methylated and unmethylated DNA, chicken erythrocyte H1 was added to an equimolar mixture of the two and the products were analysed by sedimentation in sucrose gradients. The DNA (~420 bp) was from a dinucleosome fraction from unmethylated sea urchin sperm chromatin (see Materials and methods). It was used for the sedimentation experiment as a 1:1 mixture of unmethylated DNA and DNA methylated at every CpG dinucleotide with *Sss*I methylase. We chose to use bulk DNA sequences in order to avoid sequence-specific effects, since we are asking about the general, genome-wide, recognition (or not) of methylated DNA by H1.

H1–DNA complexes were formed at an input ratio (H1:DNA) of 40% (w/w) (~5 mol H1/mol DNA) in buffer containing 40 mM NaCl. Sedimentation resulted in two peaks (Figure 1A), the faster sedimenting material (peak b) containing all the H1, as judged by SDS–PAGE, and the slower sedimenting material (peak a) being free DNA (Figure 1A), indicative of cooperative binding of H1 (Clark and Thomas, 1986, 1988). DNA extracted from these two peaks had the same size distribution (Figure 1B). Most of the free DNA peak was sensitive to digestion with *Hpa*II (Figure 1B, lane 4) showing that it was very largely non-methylated. In contrast, the DNA from the faster sedimenting fraction was resistant to digestion by *Hpa*II (lane 6), although completely digested with *Msp*I (lane 7), showing that the DNA complexed with H1 is methylated. Hence, under these conditions, where binding is cooperative, H1 shows a clear preference for methylated DNA.

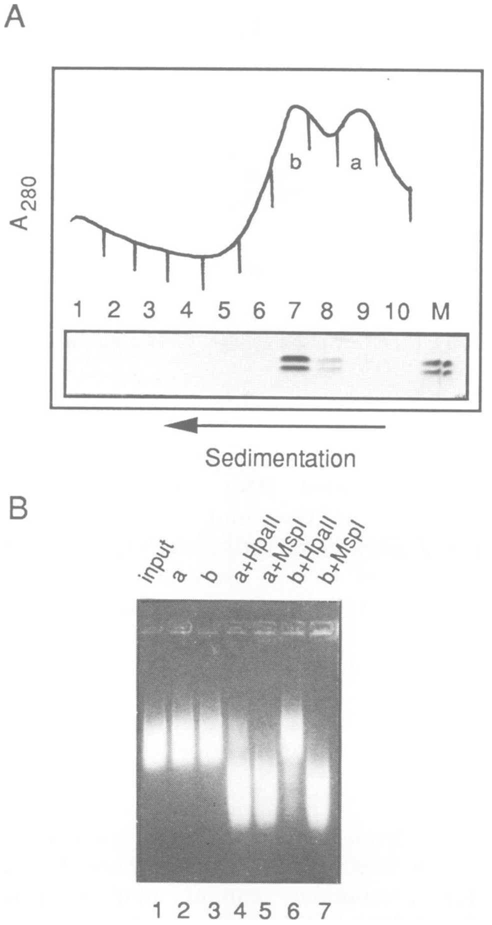

Fig. 1. Histone H1 preferentially forms complexes with methylated DNA in a competition experiment. (**A**) Sucrose gradient analysis of complexes between chicken H1 and sea urchin sperm ~420 bp DNA, containing equimolar amounts of methylated and non-methylated fragments, formed at a protein:DNA input ratio of 40% (w/w) in 1 mM Na phosphate, pH 7.4, 40 mM NaCl, 0.2 mM EDTA. Fractions across the gradient (1–10, bottom to top) were analysed for protein content in an SDS–18% polyacrylamide gel (lanes 1–10), shown beneath the gradient. M, 1 μg of chicken H1 as marker. (**B**) Analysis (2% agarose–TAE gel) of the DNA in the two peaks from the sucrose gradient. Lane 1, input DNA; lanes 2 and 3, DNA from peaks a (fraction 9) and b (fraction 7), respectively; lanes 4 and 5, DNA from peak a after digestion with *Hpa*II and *Msp*I respectively; lanes 6 and 7, DNA from peak b after digestion with *Hpa*II and *Msp*I.

Cooperative binding of H1 depends on the methylation level of the DNA

Since we had shown previously that cooperative binding of H1 is ionic strength dependent (Clark and Thomas, 1986) (using DNA from chicken erythrocytes, which was therefore methylated), we now compared binding to methylated and unmethylated DNA at three different ionic strengths (30, 35 and 45 mM NaCl) by sedimentation analysis; we also studied two levels of methylation (high and low). The DNA (~420 bp) was again from the unmethylated fraction of the sea urchin genome; it was methylated *in vitro* either at every CpG dinucleotide with *Sss*I methylase or only at the internal cytosine of -GCGC- sequences with *Hha*I methylase, or it was mock methylated by omission of *S*-adenosylmethionine from the methylation reaction.

H1–DNA complexes were formed at input ratios (H1:DNA) of 40% (w/w) in buffer (1 mM Na phosphate, pH 7.4) containing 30, 35 or 45 mM NaCl, and analysed in sucrose gradients containing the same buffer and NaCl concentrations (Figure 2); the protein content of the peaks was determined by SDS–PAGE (not shown). At the highest ionic strength (45 mM) all three DNA samples showed two peaks: a slower sedimenting peak of free DNA (or, for the non-methylated DNA, of low H1 content) and a faster sedimenting peak of H1-complexed DNA, indicative of cooperative binding of H1 (Clark and Thomas, 1986, 1988). At 35 mM NaCl, cooperative binding no longer occurred to the unmethylated DNA, and was somewhat reduced for the moderately (*Hha*I-) methylated DNA. Only at 30 mM NaCl was there a significant reduction in cooperativity for the heavily (*Sss*I-) methylated DNA and at this ionic strength both the moderately methylated and the unmethylated DNA showed a single sedimenting peak, which contained distributively bound H1. H1 therefore binds cooperatively to both unmethylated and methylated

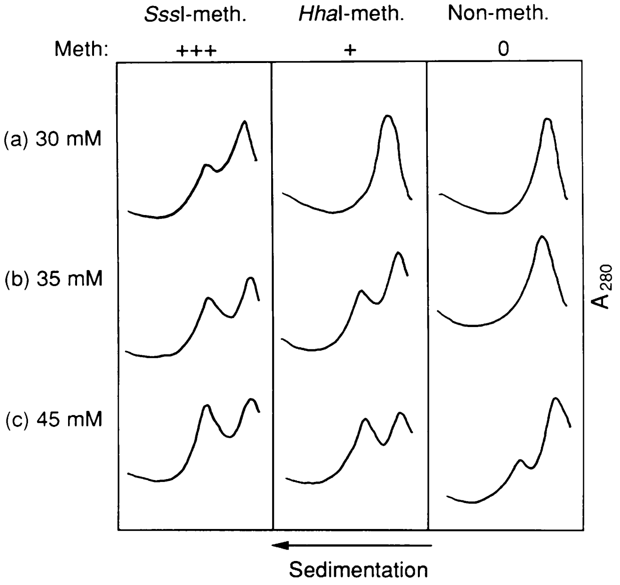

Fig. 2. The onset of cooperative binding of H1 to DNA is ionic strength dependent and is correlated positively with the density of methylation. Sucrose gradient analysis of complexes (40% w/w input ratio) formed at (a) 30 mM; (b) 35 mM; or (c) 45 mM NaCl, using chicken H1 and non-methylated sea urchin sperm DNA (~420 bp) which was methylated with either *Sss*I methylase or *Hha*I methylase, or mock methylated (= non-methylated).

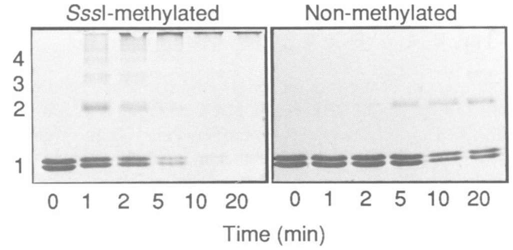

Fig. 3. H1 molecules are bound in a closely juxtaposed array on methylated but not non-methylated DNA. Time course of cross-linking of chicken H1 bound to *Sss*I-methylated or non-methylated DNA with dithio*bis*(succinimidyl propionate). Complexes were formed in 10 mM triethanolamine–HCl, pH 7.5, 0.2 mM EDTA, 30 mM NaCl, at an input ratio of 40% (w/w) H1:DNA using ~420 bp DNA, and cross-linked without fractionation. The cross-linked proteins were analysed in an SDS–18% polyacrylamide gel which was stained with Coomassie Blue.

DNA, but the degree of cooperativity is correlated positively with the extent of methylation.

Chemical cross-linking supported the conclusions drawn from the sedimentation analysis. Dithio*bis*(succinimidyl propionate) treatment of unfractionated H1–DNA mixtures containing *Sss*I-methylated or unmethylated DNA, and identical with those loaded on to the sucrose gradients at 30 mM NaCl (see Figure 2), showed that at this salt concentration only complexes formed with *Sss*I-methylated DNA gave oligomers of H1 (Figure 3). This indicated close juxtaposition of protein molecules due to cooperative binding; at the longer times of cross-linking large cross-linked oligomers were excluded from the gel. The non-methylated complexes gave only cross-linked dimers and some trimers, consistent with dispersive binding of H1.

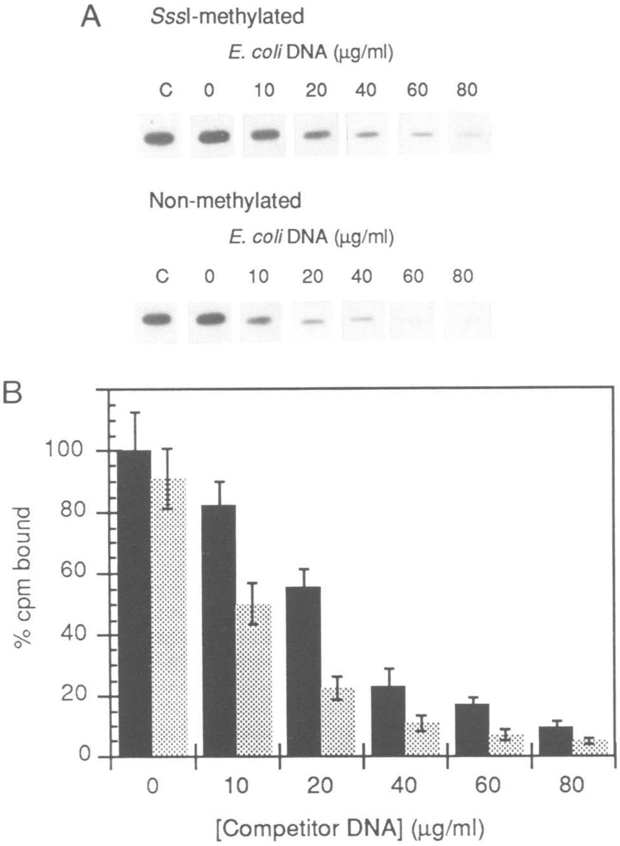

Fig. 4. Southwestern slot-blots show that histone H1 has a higher affinity for methylated DNA. (**A**) Autoradiogram; each slot contains 0.5 µg of chicken H1 immobilized on a nitrocellulose membrane and probed with ^{32}P-end-labelled CG11 (135 bp; 27 CpGs), either *Sss*I methylated or non-methylated, at the concentration of *E.coli* competitor DNA indicated. Slots C: H1 loading controls for each set of slots, probed with unmethylated CG11 in the absence of competitor. (**B**) The data from four independent experiments, each with four replicate slots for each competitor DNA concentration, were quantitated using a Molecular Dynamics PhosphorImager. Black, methylated probe; stippled, non-methylated.

To compare the intrinsic affinity of H1 for methylated and non-methylated DNA, we used a 'Southwestern' slot-blot DNA binding assay, in which the H1 was immobilized and presumably unable to bind cooperatively. The DNA was a 135 bp fragment (CG11) with 27 CpG dinucleotides (Meehan *et al.*, 1989). In the absence of competitor, *Sss*I methylation of the DNA caused only a slight increase in binding (Figure 4A and B). Increasing amounts of competitor DNA progressively displaced both the methylated and unmethylated DNA, but a slightly higher affinity of the methylated DNA for the immobilized H1 was evident throughout. Although a small difference, it could well be important in promoting the nucleation step in the formation of the cooperative complexes resolved in sucrose gradients (see Discussion).

Binding of the globular domain of H1 to methylated DNA

The isolated globular domain of H1, excised by tryptic digestion, shares with the parent molecule the property of cooperative binding to DNA through the formation of 'tramline' complexes at low ionic strength (~10 mM) (Thomas *et al.*, 1992), presumably as a consequence of its two DNA binding sites (Ramakrishnan *et al.*, 1993).

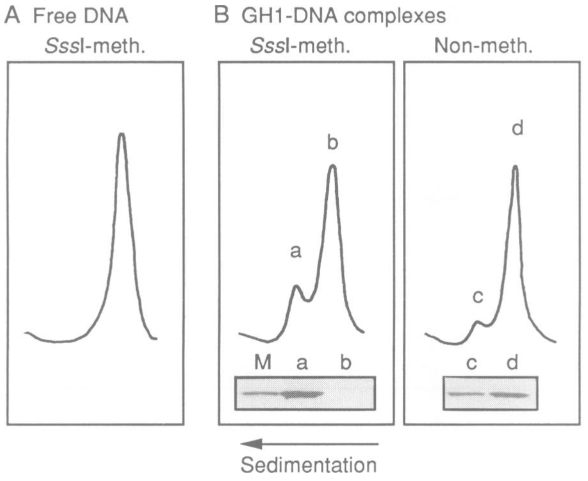

Fig. 5. The globular domain of H1 binds more cooperatively to methylated DNA. Sucrose gradient analysis of (**A**) free methylated DNA and (**B**) GH1–DNA complexes formed at 20% (w/w) input ratio with *Sss*I-methylated or non-methylated ~420 bp sea urchin sperm DNA. 5–30% (w/v) sucrose gradients were centrifuged at 30 000 r.p.m. for 16.5 h. The peaks (a–d) in (B) were analysed for protein content in an SDS–18% polyacrylamide gel shown beneath the gradient. M, 1 μg chicken H1 globular domain as a marker.

Sucrose gradient analysis showed that binding of the globular domain, like that of intact H1, was also promoted by *Sss*I methylation of the DNA (Figure 5).

A preference of H1 and its globular domain for methylated DNA is also evident in gel electrophoretic assays

Having demonstrated a preference of H1 for bulk methylated DNA, we tested a defined DNA fragment (118 bp; sequence of one strand shown in Materials and methods) that was amenable to study using a gel electrophoretic assay, and asked whether methylation of a single *Hpa*II site by *Hpa*II methylase (giving a symmetrically methylated fragment containing a single methyl-CpG pair) was sufficient for H1 to exhibit the preference. This assay was also convenient for comparison of different H1 species.

When 100 ng of chicken H1 was mixed with 5 ng of radiolabelled 118 bp probe, methylated or not, and 100 ng of much longer unlabelled, sheared *E.coli* genomic (unmethylated) DNA (average length ~800 bp) at an ionic strength that would allow cooperative binding (see Materials and methods), essentially all the H1 and DNA were present in H1–DNA complexes; these contained mainly long DNA but also the shorter probe molecules which remained in the wells of the gel (Figure 6A, lanes 1 and 8). When the amount of *E.coli* DNA was increased from 100 to 160 ng, it displaced all the probe, methylated or unmethylated, which then migrated as free DNA (lanes 7 and 14). The behaviour of methylated and unmethylated DNA between these two points was determined in a titration with the amount of competitor increased in small increments (lanes 2–6 and 9–13). More competitor (~30%) was needed to displace the methylated probe completely from the large complexes in the wells (160 ng, versus 120 ng for the unmethylated probe) (Figure 6A, compare lane 14 with lane 3). Thus the presence of a single methyl-CpG pair appeared to enhance the stability of H1–DNA complexes (see Discussion).

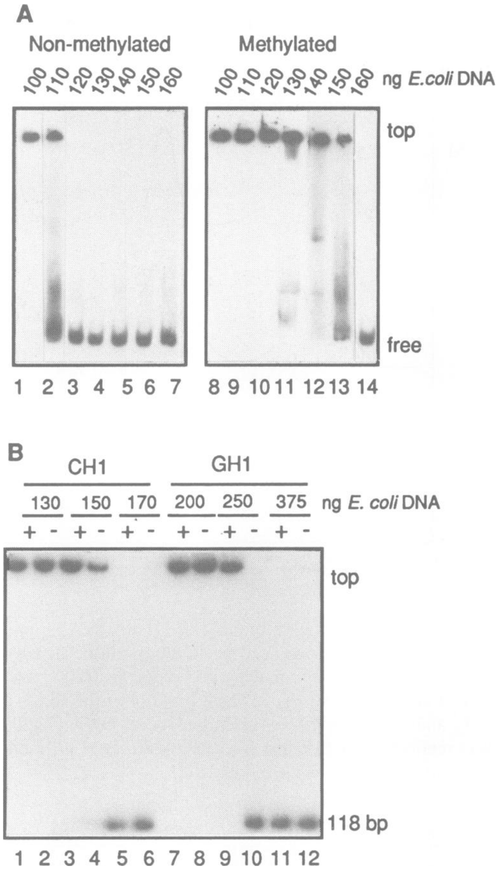

Fig. 6. Differences in the binding of H1 and its isolated globular domain to 118 bp unique sequence DNA containing a single methyl-CpG pair. (**A**) Complexes were formed between 100 ng of chicken H1, 5 ng of ^{32}P-labelled 118 bp DNA, either methylated or non-methylated, and 100–160 ng of *E.coli* unlabelled DNA (average length ~800 bp). The products were analysed in a 5% polyacrylamide–TBE gel and the gel autoradiographed. (**B**) Complexes of 100 ng of the globular domain (GH1) or C-terminal tail (CH1) of H1 with methylated (+) or non-methylated (–) 118 bp DNA were formed and analysed as in (A). This gel summarizes the results of a titration as in (A) and shows only (lowest to highest) the maximum amount of *E.coli* DNA that allows binding of both probes as complexes in the wells, the minimum amount that gives complete displacement from the wells of the unmethylated probe only, and the minimum amount needed to abolish completely the binding of both probes. [Note that the amounts of *E.coli* DNA needed here are not directly comparable with those in (A) because the molar protein–DNA input is higher for GH1 than for H1, for the same weight input.]

The preference of the globular domain for methylated DNA was also tested in this assay (Figure 6B). The gel summarizes the results of a titration as in Figure 6A and shows only (lowest to highest, in three pairs of lanes) the maximum amount of *E.coli* DNA that allows binding of both probes as complexes in the wells, the minimum amount that gives complete displacement from the wells of the unmethylated probe only, and the minimum amount needed to abolish binding of both probes completely. The results show that, as with the whole molecule, complexes formed with GH1 and methylated DNA needed more of the longer *E.coli* DNA (50% more) for disruption than those formed with non-methylated DNA. With the C-terminal fragment (CH1) there was no such discrimination.

Various H1s and H1 variants were also tested in the gel electrophoretic assay with increasing amounts of *E.coli*

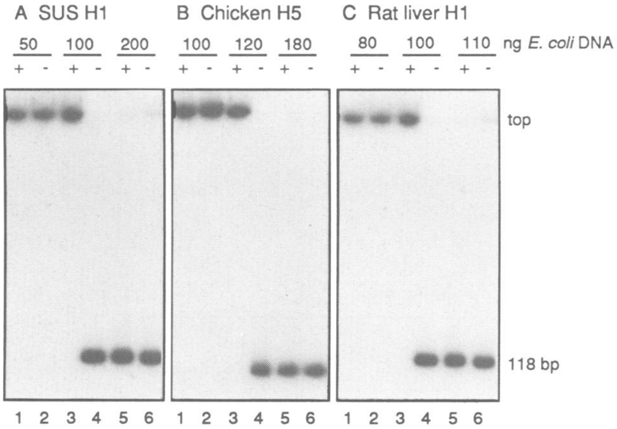

Fig. 7. Different preferences of different linker histones for complex formation with singly methylated 118 bp DNA. (**A**) Sea urchin sperm (SUS) H1; (**B**) chicken H5; (**C**) rat liver H1. Linker histone (100 ng) was mixed with 5 ng of ^{32}P-radiolabelled methylated (+) or non-methylated (-) 118 bp DNA and different amounts of unlabelled *E.coli* competitor DNA. The three competitor values shown were chosen to summarize the results of a titration (not shown) as described in the legend to Figure 6. The products were analysed in a 5% polyacrylamide–TBE gel and the gel autoradiographed.

DNA. Figure 7 shows the results for selected competitor amounts (based on preliminary titrations, as described above), indicating the minimum amount needed to displace completely the methylated or non-methylated probe from the large complexes in the wells. Sea urchin sperm H1, chicken erythrocyte H5 and rat liver H1 all showed a preference for methylated DNA, in the order: sea urchin sperm H1>H5>chicken H1>rat liver H1; 100, 50, 30 and 10% excesses of competitor DNA, respectively, were needed to disrupt the methylated complex relative to the non-methylated (Figure 7). The transition between the probe being bound in a complex and running as free DNA was very sharp; in the case of rat liver H1, a 10% increase in the amount of competitor DNA was sufficient to abolish the binding to the non-methylated probe. A plausible explanation is preferential and cooperative binding of H1 to the longer competitor DNA due to the formation of more stable (tramline) complexes.

The preference of H1 for the methylated 118mer with a single methyl-CpG pair (relative to the unmethylated 118mer) was also apparent in sedimentation experiments (Figure 8A). The *Hpa*II-methylated fragment (Figure 8A, right hand panel) gave the fast sedimenting peak indicative of cooperative binding even at 35 mM NaCl, whereas the non-methylated fragment showed appreciable complex only at 45 mM NaCl (Figure 8A, left hand panel). Somewhat surprisingly, the multiply methylated 118mer (Figure 8A, centre) showed intermediate behaviour, and there was appreciable complex at 40 mM NaCl. (Perhaps multiply methylated complexes are less, rather than more, stable/well defined than their counterparts with a single methyl-CpG pair because multiple nucleation points for the formation of cooperative complexes lead to less uniquely defined complexes.) The magnitude of the preference for H1 binding was less than with the longer (~420 bp) bulk DNA under the same conditions (Figure 2), presumably because of the greater contribution of end effects in the shorter fragment, which may have been sufficient to destabilize the complexes completely in the gel assay. Despite the enhanced preference for multiply methylated bulk DNA shown in the sedimentation experiments and the increase in affinity of H1 for multiply methylated DNA detected in the Southwestern assay, the gel electrophoretic method failed to show a difference between the same 118mer when multiply methylated and non-methylated (Figure 8B), for reasons that are not clear.

In order to determine whether the roughly central location of the single methyl-CpG pair at the *Hpa*II site in the 118mer was relevant to the clear cut effect in the gel assay, an alternative methylation site (*Hha*I) was introduced (by PCR-mutagenesis) 8 bp from the left hand end of the fragment (see Materials and methods). Methylation at this site led to no detectable preference in H1 binding using the gel assay [Figure 8C; compare the *Hha*I panel with the non-methylated (NM) panel], in striking contrast to the result when the methylation site was approximately centrally located (Figure 8C, *Hpa*II panel; Figure 6A). A possible explanation might be that if the first H1 to bind is recruited to an approximately centrally located methylated site, the initial ternary complex (one H1 and two duplexes), might be a particularly good substrate for cooperative binding of H1 in either direction to give fully formed, stable, tramline complexes. Recruitment of H1 to the extreme end of a DNA molecule, as with the *Hha*I-methylated fragment, would be expected to be more prone to end effects and less favourable for cooperative H1 binding.

Methylated MspI sites in both H1 and GH1–DNA complexes show enhanced protection against digestion

We have asked whether binding of H1 to methylated DNA protects *Msp*I cleavage sites from digestion. Complexes were formed between chicken H1 and *Bam*HI-linearized ~3 kb plasmid DNA which had been either mock methylated or completely methylated with *Sss*I methylase. Methylated and unmethylated complexes were equally sensitive to digestion by *Nla*III which has no CpG in its recognition site (Figure 9C). In contrast, the methylated DNA complexes were digested more slowly by *Msp*I than the unmethylated (Figure 9B). At the high enzyme to DNA ratio used, this is a true feature of the H1–DNA complexes rather than a reflection of a preference of *Msp*I for cutting non-methylated (versus methylated) naked DNA, which is evident under conditions of much more limited digestion than those shown in Figure 9A where the methylated and unmethylated DNA are cut at essentially identical rates (Waalwijk and Flavell, 1978; and data not shown). In contrast to the results from similar experiments with mouse liver H1 and plasmid DNA (Higurashi and Cole, 1991), the protection of *Msp*I sites in the H1-methylated DNA complex appears to be general (Figure 9B); the limit digest is identical for methylated and unmethylated DNA.

Complexes of the globular domain of H1 with methylated and unmethylated DNA were similarly probed with restriction enzymes. GH1 again exhibited the properties of the whole molecule (Figure 9D and E), as in the gel retardation and sedimentation experiments. In contrast, the C-terminal tail produced by chymotryptic digestion of H1 did not confer any protection on the methylated DNA (Figure 9F).

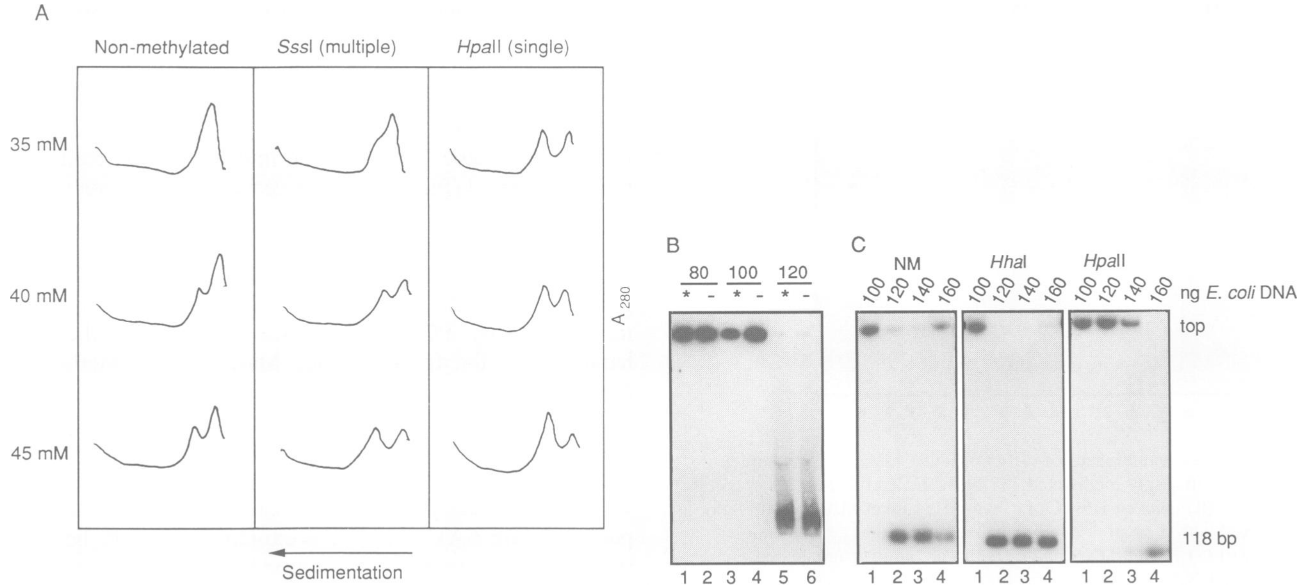

Fig. 8. (**A**) A preference for the methylated 118 bp DNA containing single or multiple methyl-CpG pairs, shown in a sedimentation assay. Sucrose gradient analysis of complexes (40% w/w input ratio) formed at 30 mM, 35 mM or 45 mM NaCl as indicated, using chicken H1 and DNA which was either non-methylated, *Hpa*II methylated (single methyl-CpG pair) or multiply (*Sss*I) methylated. (**B**) Apparent lack of preference for a multiply methylated fragment shown by a gel electrophoretic assay. Chicken H1 (100 ng) was mixed with 5 ng of ^{32}P-radiolabelled multiply *Sss*I-methylated (*) or non-methylated (–) 118 bp DNA with different amounts of *E.coli* competitor. The products were analysed in a 5% polyacrylamide–TBE gel and the gel autoradiographed. As in Figures 6B and 7, the competitor values shown summarize the results of a titration. (**C**) A roughly centrally placed methylated site, but not a more distally located site, leads to preferential binding of H1. The complexes were formed and analysed as above using the same radiolabelled 118 bp DNA but modified to introduce a single *Hha*I site 8 bp from one end of the molecule; either this site or the roughly central one was methylated using *Hha*I or *Hpa*II, respectively.

Discussion

It seemed an attractive possibility that methylation of DNA in a particular chromatin region might be reflected directly in stabilization of chromatin higher order structure, through alteration of the interactions of an intrinsic chromatin protein, for example H1. H1 seals two turns of DNA around the nucleosome and stabilizes higher order structure, possibly through H1–H1 interactions since H1 molecules are in close proximity. As reported here, various lines of evidence suggest that H1 shows preferential and enhanced binding to methylated DNA. It seems likely, therefore, that this may be at least one component in the repression of transcription from methylated (relative to unmethylated) transfected DNA through formation of an inert chromatin structure (see Introduction).

H1 binds preferentially to methylated DNA

Earlier work had shown that the ionic strength-dependent cooperative binding of H1 to DNA probably occurs through recruitment of successive H1 molecules to an initial ternary complex comprising two parallel duplexes bound to two DNA binding sites on H1 (Clark and Thomas, 1986, 1988), which are probably on the globular domain (Thomas *et al.*, 1992; Ramakrishnan *et al.*, 1993). By analysis in sucrose gradients we have shown that, under conditions that favour cooperative binding of H1, there is a clear preference of H1 for methylated over unmethylated DNA in a competition experiment. The onset of cooperativity occurs at a lower salt concentration when the DNA is methylated and particularly if it is heavily methylated. Southwestern slot-blots suggest that the intrinsic affinity of H1 for DNA is also enhanced by methylation. This may be important in initiating the formation of the tramline complexes observed in sucrose gradients, perhaps by facilitating or stabilizing the formation of the presumed initial ternary complex. Selective protection of methylated *Msp*I sites against restriction endonuclease digestion would be consistent with an enhanced and direct interaction of H1, and of the isolated globular domain (but not of the isolated C-terminal tail), with -CMeCGG-, although altered interaction of the enzyme with DNA due to subtle, local, H1-induced, methylation-dependent conformational changes cannot be ruled out.

A gel assay, in which a radiolabelled oligonucleotide probe (118mer) was excluded from H1–DNA complexes by the presence of an excess of much longer competitor DNA, demonstrated a preference of H1 for a probe containing a single methyl-CpG pair relative to unmethylated; the latter was excluded from complexes with less competitor (Figure 6). Similar results in a gel assay were obtained with another oligonucleotide containing a single methyl-CpG pair (data not shown) and also suggested in the work of others (Jost and Hofsteenge, 1992). A preference for the methylated 118mer was also demonstrated in the quite different sedimentation assay with no competitor. The preference in the gel assay appeared to be dependent on the roughly central location of the methyl-CpG pair, since the effect was lost when a distal *Hha*I site engineered into the fragment was methylated instead of the central (*Hpa*II) site. A likely explanation, as discussed above (Results), is that a central methylation site leads to a stable ternary complex of H1 and two duplexes primed for further cooperative H1 binding. Whatever the explanation, the roughly centrally methylated 118 bp fragment is suitable for conveniently demonstrating in a gel the

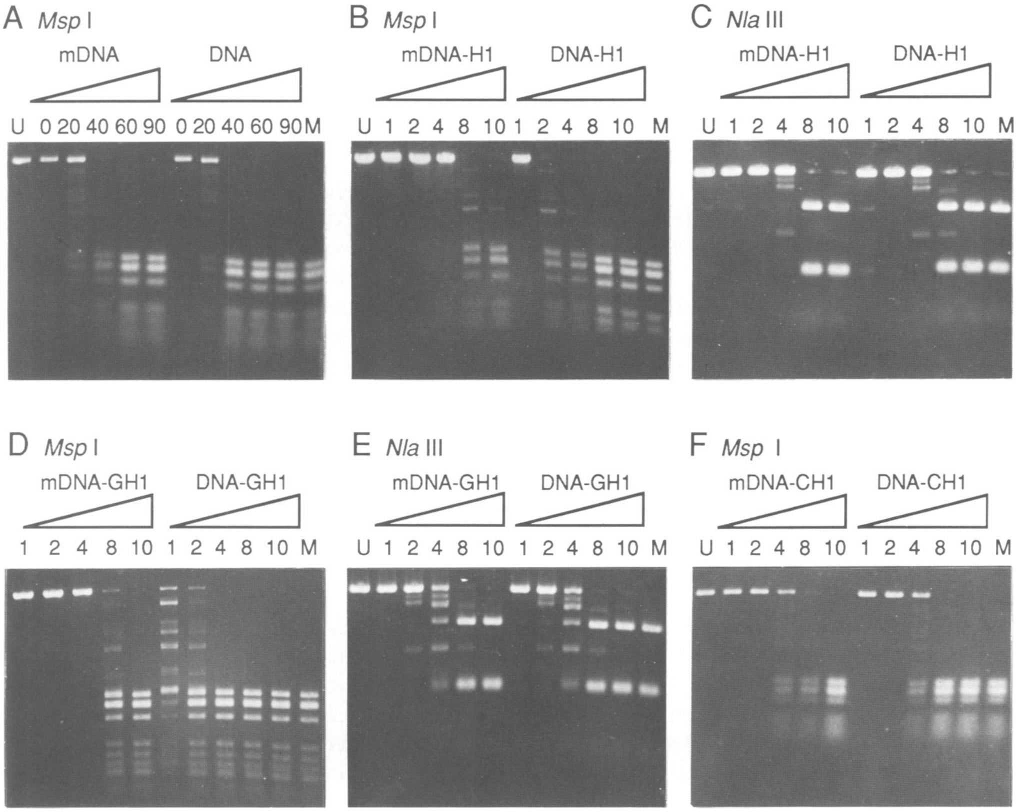

Fig. 9. Time courses of restriction endonuclease digestion of methylated and non-methylated DNA which was either naked (**A**) or complexed with H1 (**B** and **C**), GH1 (**D** and **E**) or CH1 (**F**). (A) Digestion of 0.5 μg of methylated (mDNA) or non-methylated (DNA) with 50 U of *Msp*I for various times (indicated in seconds). Complexes were formed by mixing H1 and linearized ~3 kb plasmid DNA (see Materials and methods), multiply methylated with *Sss*I methylase or mock methylated, at an input ratio of 60% (w/w) H1:DNA, 15% (w/w) GH1:DNA or 21% (w/w) CH1:DNA. Complexes containing 0.5 μg of DNA were digested with 50 U of *Msp*I (B, D or F) or *Nla*III (C or E) for various times (indicated in minutes) and the fragments resolved in 1.5% agarose–TAE gels. U, undigested DNA (methylated in A, B and F, and unmethylated in the other panels); M, plasmid DNA (0.5 μg) digested to completion.

unambiguous preference of H1 for DNA containing a single methyl-CpG pair that is shown to exist in solution, as demonstrated in the sedimentation assay.

The gel assay using the parent 118mer with the internal methylation site showed that different H1s and H1 variants differed in their preferences for methylated DNA. The preference was greater for sea urchin sperm H1 and chicken erythrocyte H5, which are associated with transcriptionally inert chromatins, than for H1, in the order spH1>H5>H1. Chromatins containing spH1 and H5 also have an enhanced stability *in vitro*, in the same order, compared with rat liver chromatin, as demonstrated by hydrodynamic methods (Bates *et al.*, 1981; Thomas *et al.*, 1986). If methylation of the DNA were to stabilize the binding of these extreme tissue-specific H1 variants to chromatin, the combination of methylation and special variant would then act synergistically in repression of the genome. Somatic H1 contains six or seven subtypes, less extreme than the variants H5 and spH1, but it is possible that these too may differ in their recognition of methylated DNA and perhaps stabilization of methylated and non-methylated chromatin.

The discrimination in favour of methylated DNA shown by H1 occurs also for the isolated globular domain, GH1, which like H1 binds cooperatively to DNA, forming 'tramlines' (Thomas *et al.*, 1992). GH1 is also able to discriminate between methylated and unmethylated DNA in the assay with the 118 bp probe with a single internal methyl-CpG pair and competitor DNA as judged by gel electrophoresis, as well as between unmethylated and multiply methylated DNA as judged by sedimentation analysis. Since GH5 (and GH1, which is highly homologous) probably interacts with DNA at one of its DNA binding sites through interaction of helix III with the major groove (Ramakrishnan *et al.*, 1993), this interaction could, in principle, sense directly the presence of the methyl groups of the methyl-CpG pair in the groove. If the globular domain binds to the entering and exiting duplexes (but see Hayes *et al.*, 1994), which the two duplexes in the tramline complexes probably mimic, methylation at the 'boundaries' of the chromatosome (but perhaps not, for example, within the body of the nucleosome core) might affect the interaction of H1 with chromatin. It is probably unwise to speculate on the details of such an effect by extrapolating from H1–DNA complexes, but qualitatively it seems very likely that features of H1–DNA binding in the complexes, including the effect of DNA methylation, will persist in chromatin.

Relation to other work

Previous work using a variety of defined DNA sequences and H1s is somewhat contradictory on the question of whether or not H1 has a preference for methylated DNA. Some studies, like this one, do find a preference. Gel retardation assays showed that a repressor of the vitellogenin gene (MDBP-2), immunologically related to H1, had a preference for oligonucleotides longer than 30 bp containing a single methyl-CpG pair (Jost and Hofsteenge, 1992). MDBP-2, from which two tryptic peptides matched globular domain sequences from H1, was evidently not H1 itself; the apparent M_r in SDS–polyacrylamide gels was ~21 kDa, rather than ~30 kDa characteristic of H1 which migrates anomalously slowly in SDS gels, and might be H1 lacking the N-terminal tail, or possibly truncated in its C-terminal tail. Secondly, filter binding assays demonstrated a preference of (acid-extracted) calf thymus H1 for multiply methylated plasmid DNA with inhibition of transcription (Levine *et al.*, 1993).

Band shift assays with a 271 bp fragment containing the *Xenopus borealis* 5S rRNA gene showed no preference for (multiply) methylated DNA (Nightingale and Wolffe, 1995) and we are likewise unable to demonstrate a preference in band shift assays with a multiply methylated unique sequence probe (possible reasons discussed above), although a clear preference exists and is readily apparent in sedimentation assays. It was suggested (Nightingale and Wolffe, 1995) that the preference of H1 for methylated DNA found in previous studies (Jost and Hofsteenge, 1992; Levine *et al.*, 1993) may have been sequence-dependent. However, by using the quite different approach of sedimentation analysis in sucrose gradients, with bulk DNA representative of a large region of the sea urchin genome, we have avoided any artefacts due to sequence specificity in H1 binding. The complexes isolated in the gradients are demonstrably soluble and there is no need for competitor DNA. From these experiments (as well as from the band shifts with the singly methylated 118mer), we conclude that H1 does show a preference for methylated DNA under conditions where it can bind cooperatively. In addition, Southwestern slot-blot assays also show some intrinsic preference of H1 for methylated sequences.

Previous experiments showed that 5-methylcytosine preferentially accumulates in H1-containing mononucleosomes produced by micrococcal nuclease digestion of nuclei, leading to the suggestion that H1 binds with a higher affinity to methylated mononucleosomes *in vivo* (Ball *et al.*, 1983). In apparent contradiction, the binding of H1 to reconstituted chromatosomes containing the *X.borealis* 5S rRNA gene has been shown recently to be insensitive to methylation of all the CpGs in the DNA (Nightingale and Wolffe, 1995). However, the two results are not necessarily contradictory since the positions of the methylated sites relative to the chromatosome boundaries may be crucial if H1 interacts directly in chromatin with methyl-CpG, as it appears to do in H1–DNA complexes; studies of reconstituted chromatosomes with a strong positioning sequence for the core histone octamer may thus lead to different conclusions from those on bulk native chromatosomes. The preferential association of H1 with bulk methylated nucleosomes (Ball *et al.*, 1983) may reflect their later release by micrococcal nuclease digestion, possibly as a result of a more stable chromatin structure; as a result they are less 'trimmed' by exonuclease activity and more likely to retain their H1. The enhanced stability of methylated chromatin could be due, for example, to more stable H1 interactions, or to the binding of some accessory protein such as MeCP2 which may be lost during release of mononucleosomes, even if untrimmed (Meehan *et al.*, 1992).

Since it is now clear that the formation of H1–DNA complexes is sensitive to DNA methylation, the effect of methylation on H1 binding in chromatin should be studied at the level of higher order structure, with oligonucleosomes long enough to fold up into 30 nm filaments where there may be stabilizing axial H1–H1 interactions, possibly but not necessarily cooperative. However, the availability of material for direct comparison of the effect of H1 *per se* on methylated and non-methylated chromatin is problematic: methylated chromatin formed in chromatin assembly extracts (e.g. from *Xenopus*) may be compromised by the presence of MeCPs; and in vertebrate genomes non-methylated chromatin occurs in blocks of only 1–2 kb (as CpG islands; Bird, 1986) giving material which is too short for *in vitro* folding studies. Ideally chromatin would be appropriately reconstituted from purified components, but in the absence of good reconstitution methods for this purpose we are also exploring the possibility of using chromatin from organisms such as sea urchin, which contains sizeable interspersed blocks of methylated and non-methylated DNA (Bird *et al.*, 1979). We have already exploited this property to obtain the non-methylated dinucleosomal DNA used here.

If the effect of methylation on H1 binding to DNA is reflected in its binding in chromatin, as we have argued seems likely, there appears to be synergy between the intrinsic effect of H1 on chromatin folding on the one hand, and DNA methylation on the other, in the stabilization of chromatin higher order structure. This may be modulated further by methylcytosine binding proteins such as MeCP2 (Lewis *et al.*, 1992). H1 may in general organize higher order structure in a metastable state. The structure may be loosened by loss of some of the H1 to give transcriptionally competent chromatin (Kamakaka and Thomas, 1990, and references therein), or stabilized by stronger interactions in methylated chromatin in repressed regions. CpG islands, which are both unmethylated and H1-depleted (Tazi and Bird, 1990) would be in a more open chromatin structure, distinct from the more highly packaged and transcriptionally less accessible surrounding regions.

Materials and methods

Linker histones and domains

The linker histones H1 and H5 were extracted from chicken erythrocyte nuclei with 0.65 M NaCl and separated by cation-exchange chromatography (Clark and Thomas, 1986). H1 was extracted from rat liver nuclei (Noll *et al.*, 1975) with 0.55 M NaCl and purified similarly. Sea urchin sperm H1 was isolated from mature *Echinus esculentus* sperm nuclei (Clark *et al.*, 1988). The globular domain of chicken erythrocyte H1 (residues ~22–102) was prepared by tryptic digestion of H1 (Thomas *et al.*, 1992), and the C-terminal fragment (residues 106–217) by chymotryptic digestion (Clark *et al.*, 1988).

DNA fragments

Non-methylated sea urchin sperm DNA. Non-methylated DNA (~420 bp) was isolated from dinucleosomes generated by micrococcal nuclease digestion of the non-methylated chromatin fraction preferentially released by digestion of sea urchin sperm nuclei. In the sea urchin, the genome occurs in methylated and non-methylated blocks (Bird *et al.*, 1979).

Echinus esculentus sperm nuclei (Thomas *et al.*, 1986) (50 A_{260} units of nuclei in 500 µl of 50 mM Tris–HCl, pH 7.5, 25 mM sucrose, 1 mM $CaCl_2$) were digested with micrococcal nuclease (17 U/ml nuclei) on ice for 20 min, and the digestion was terminated by the addition of

EDTA to 2 mM. After 30 min on ice to allow nuclear lysis, the sample was dialysed against 5 mM triethanolamine–HCl, pH 7.5, 1 mM EDTA, 0.25 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C for 4 h and then fractionated in 15 ml linear sucrose gradients [5–30% (w/v) containing the same buffer and with a 2 ml 50% (w/v) sucrose cushion] which were centrifuged at 15 000 r.p.m. for 16 h at 4°C in a Beckman SW28 rotor.

The single chromatin peak (which was shown by digestion of the DNA with *Hpa*II and *Msp*I to be unmethylated) was pooled and concentrated by dialysis against solid sucrose and then against 50 mM Tris–HCl, pH 7.5, 1 mM $CaCl_2$, at 4°C. The chromatin was then further digested at $A_{260} = 20$ with micrococcal nuclease (50 U/ml) at 37°C for 5 min and the reaction stopped by chilling on ice and the addition of EGTA to 2 mM. One ml of the digested chromatin was then loaded on to a 35 ml linear sucrose gradient (composition as above) and centrifuged as above but at 27 000 r.p.m. DNA was extracted from the dinucleosomal peak and had a weight-average size of ~420 bp (nucleosomal repeat length of *E.esculentus* sperm, ~240 bp; Hill *et al.*, 1991). It was methylated at CpG dinucleotides as required using *Sss*I or *Hha*I methylases (New England Biolabs) with twice the amounts of enzyme and cofactor (*S*-adenosylmethionine) recommended by the supplier; mock methylations contained everything except *S*-adenosylmethionine.

DNA fragment for Southwestern assays. The fragment was an HPLC-purified 135 bp fragment (CG11) with 27 CpG dinucleotides (Meehan *et al.*, 1989).

DNA fragments for gel retardation assays. The parent 118mer was an HPLC-purified *Bam*HI–*Xba*I restriction fragment of pBend2 (Kim *et al.*, 1989) with a single *Hpa*II site (underlined) that could be methylated with *Hpa*II methylase and the sequence (only one strand shown): 5′-CTAGAGTCGACACGCGTAGATCTGCTAGCATCGATCCATGGACTAGTCTCCAGTTTAAGATATCCAGCCTGCCCGGGAGGCCTTCGCGAAATATTGGTACCCCATGGAATCGAGGATC-3′.

A modified 118mer produced by PCR-mutagenesis (as recommended by Stratagene) differed from the parent 118mer towards the 5′ end of the strand shown, where the sequence was changed to 5′-CTAGAGGCGCC... to produce a single *Hha*I site (underlined) that could be methylated using *Hha*I methylase.

The 118 bp and 135 bp DNA fragments were radiolabelled using Klenow polymerase in the presence of [α-^{32}P]dATP and freed of unincorporated label using a spun G50 Sephadex column (Sambrook *et al.*, 1989). Mock methylation, or methylation of the single *Hpa*II site, was carried out as detailed above. The extent of methylation was checked by digestion with *Hpa*II and electrophoresis in a 6% polyacrylamide gel run in 0.5× TBE (TBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA).

Sedimentation analysis of H1–DNA and GH1–DNA complexes

H1–DNA complexes were formed in siliconized tubes in 1 mM Na phosphate, pH 7.4, 0.2 mM EDTA, 0.25 mM PMSF and various concentrations of NaCl essentially as described previously (Clark and Thomas, 1986). H1 was added last, from a solution at 1 mg/ml, to give a final H1:DNA ratio of 40% (w/w); the final DNA concentration was 30 μg/ml. The mixtures (1 ml) were incubated for 45 min at room temperature and then analysed in 5–20% (w/v) linear sucrose gradients (17 ml) containing the same buffer and salt concentration as the incubation mixture and centrifuged at 24 000 r.p.m. for 2 h in a Beckman SW28 rotor. The gradients were fractionated and monitored at 280 nm.

GH1 complexes were formed as previously described (Thomas *et al.*, 1992), essentially as for H1 but in 5 mM triethanolamine–HCl, pH 7.5, 5 mM NaCl, 0.1 mM EDTA, 0.25 mM PMSF, at a protein:DNA input ratio of 20% (w/w). The complexes were then analysed, together with free DNA, in 5–30% (w/v) linear sucrose gradients (17 ml) containing the same buffer and centrifuged at 30 000 r.p.m. for 16.5 h in a Beckman SW28 rotor.

Chemical cross-linking of H1 in H1–DNA complexes

Complexes of H1 and DNA formed as described above were treated with 0.2 mg/ml dithio*bis*(succinimidyl propionate) [Pierce; stock solution (50 mg/ml) freshly prepared in dry dimethylformamide] for various times up to 20 min. The samples were then precipitated with 25% (w/v) trichloroacetic acid and the products analysed in an SDS–18% polyacrylamide gel which was then fixed and stained with Coomassie Blue R250 (Thomas and Kornberg, 1978).

Slot-blot Southwestern assays

Chicken H1 (0.5 μg) in 100 μl of binding buffer (20 mM HEPES, 40 mM NaCl, 3 mM $MgCl_2$, 10 mM 2-mercaptoethanol, pH 7.9) was applied in each of a series of slots to nitrocellulose membrane (Amersham, Hybond-C) by vacuum filtration using a BRL slot-blot apparatus. The protein was denatured in guanidinium chloride and renatured by dilution in binding buffer (Vinson *et al.*, 1988), both at 23°C, and the membrane was then blocked in 4% (w/v) non-fat dried milk in the same buffer and washed in this buffer containing 0.1% (v/v) Triton X-100 (Miskimins *et al.*, 1985). The membrane was then cut into two sets of strips, each strip containing four slots to be treated together; one set of slots was probed with ^{32}P-labelled CG11 (135 bp; Meehan *et al.*, 1989), which had been *Sss*I methylated at all 27 CpGs, and the other with the same DNA unmethylated. Probing was carried out for 1 h at room temperature in binding buffer containing 0.1% (v/v) Triton X-100 with different concentrations (0–80 μg/ml) of sheared *E.coli* DNA for each strip within a set. The strips were washed in binding buffer containing 0.01% Triton X-100 and dried. The bound radioactivity was visualized by autoradiography and quantitated (four independent determinations) using a Molecular Dynamics PhosphorImager using ImageQuant software.

Gel electrophoretic assays

Histone H1 (100 ng) from various sources was incubated at room temperature for 45 min with 5 ng of ^{32}P-labelled 118mer probe and various amounts of unlabelled sheared *E.coli* DNA as indicated, in a final volume of 20 μl containing 5% (w/v) Ficoll, 50 mM HEPES, pH 7.6, 50 mM KCl. The samples, to which bromophenol blue was added, were analysed at 4°C in 5% polyacrylamide–0.25× TBE gels run at 15 mA until the dye front had just left the gel. Gels were then dried under vacuum on to Whatman 3MM paper and exposed to pre-flashed Fuji RX film at −80°C with two intensifying screens.

Restriction endonuclease protection assays

Sixty μg of H1 and 100 μg of *Sss*I-methylated or non-methylated DNA [*Bam*HI-linearized plasmid DNA (pTZ18R with an 118 bp insertion of the *Bam*HI–*Xba*I fragment of pBend2, see above, between the *Bam*HI and *Xba*I sites)] were mixed in 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 2 mM $MgCl_2$, 2 mM dithiothreitol and incubated for 45 min at room temperature. After centrifugation in a microfuge to remove any aggregates, aliquots containing 2.5 μg of DNA (determined spectrophotometrically) were digested with 50 U of *Msp*I or *Nla*III at 37°C and 0.5 μg samples removed at various times. Digestion was stopped by the addition of EDTA to 5 mM and the DNA fragments extracted with phenol/chloroform (1:1, v/v), ethanol precipitated and analysed in a 1.5% agarose–TAE gel (TAE: 40 mM Tris acetate–10 mM EDTA).

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References

Ball,D.J., Gross,D.S. and Garrard,W.T. (1983) 5-methylcytosine is localised in nucleosomes that contain histone H1. *Proc. Natl Acad. Sci. USA*, **80**, 5490–5494.

Bates,D.L., Butler,P.J.G., Pearson,E.C. and Thomas,J.O. (1981) Stability of the higher-order structure of chicken erythrocyte chromatin in solution. *Eur. J. Biochem.*, **119**, 469–476.

Bird,A.P. (1986) CpG-rich islands and the function of DNA methylation. *Nature*, **321**, 209–213.

Bird,A.P., Taggart,M.H. and Smith,B.A. (1979) Methylated and unmethylated DNA compartments in the sea urchin genome. *Cell*, **17**, 889–901.

Buschhausen,G., Wittig,B., Graessmann,M. and Graessmann,A. (1987) Chromatin structure is required to block transcription of the methylated herpes simplex virus thymidine kinase gene. *Proc. Natl Acad. Sci. USA*, **84**, 1177–1181.

Cedar,H. (1988) DNA methylation and gene activity. *Cell*, **53**, 3–4.

Cerf,C., Lippens,G., Ramakrishnan,V., Muyldermans,S., Segers,A., Wyns,L., Wodak,S.J. and Hallenga,K. (1994) Homo- and heteronuclear two-dimensional NMR studies of the globular domain of histone H1: full assignment, tertiary structure, and comparison with the globular domain of H5. *Biochemistry*, **33**, 11079–11086.

Clark,D.J. and Thomas,J.O. (1986) Salt-dependent co-operative interaction of histone H1 with linear DNA. *J. Mol. Biol.*, **187**, 569–580.

Clark,D.J. and Thomas,J.O. (1988) Differences in the binding of H1 variants to DNA: cooperativity and linker-length related distribution. *Eur. J. Biochem.*, **178**, 225–233.

Clark,D.J., Hill,C.S., Martin,S.R. and Thomas,J.O. (1988) α-Helix in the carboxy-terminal domains of histones H1 and H5. *EMBO J.*, **7**, 69–75.

Draves,P.H., Lowary,P.T. and Widom,J. (1992) Co-operative binding of the globular domain of histone H5 to DNA. *J. Mol. Biol.*, **225**, 1105–1121.

Hayes,J.J., Pruss,D. and Wolffe,A.P. (1994) Contacts of the globular domain of histone H5 and core histones with DNA in a 'chromatosome'. *Proc. Natl Acad. Sci. USA*, **91**, 7817–7821.

Higurashi,M. and Cole,R.D. (1991) The combination of DNA methylation and H1 histone binding inhibits the action of a restriction nuclease on plasmid DNA. *J. Biol. Chem.*, **266**, 8619–8625.

Hill,C.S., Rimmer,J.M., Green,B.N., Finch,J.T. and Thomas,J.O. (1991) Histone–DNA interactions and their modulation by phosphorylation of Ser-Pro-X-Lys/Arg- motifs. *EMBO J.*, **10**, 1939–1948.

Johnson,C.A., Goddard,J.P. and Adams,R.L.P. (1995) The effect of histone H1 and DNA methylation on transcription. *Biochem. J.*, **305**, 791–798.

Jost,J.-P. and Hofsteenge,J. (1992) The repressor MDBP-2 is a member of the histone H1 family that binds preferentially *in vitro* and *in vivo* to methylated nonspecific DNA sequences. *Proc. Natl Acad. Sci. USA*, **89**, 9499–9503.

Kamakaka,R.T. and Thomas,J.O. (1990) Chromatin structure of transcriptionally competent and repressed genes. *EMBO J.*, **9**, 3997–4006.

Keshet,I., Lieman-Hurwitz,J. and Cedar,H. (1986) DNA methylation affects the formation of active chromatin. *Cell*, **44**, 535–543.

Kim,J., Zweib,C., Wu,C. and Adhya,S. (1989) Bending of DNA by gene-regulatory proteins—construction and use of a DNA bending vector. *Gene*, **85**, 15–23.

Levine,A., Yeivin,A., Ben-Asher,E., Aloni,Y. and Razin,A. (1993) Histone H1-mediated inhibition of transcription initiation of methylated templates *in vitro*. *J. Biol. Chem.*, **268**, 21754–21759.

Lewis,J.D., Meehan,R.R., Henzel,W.J., Maurer-Fogy,I., Jeppesen,P., Klein,F. and Bird,A. (1992) Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. *Cell*, **69**, 905–914.

Meehan,R.R., Lewis,J.D., McKay,S., Kleiner,E.L. and Bird,A.P. (1989) Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. *Cell*, **58**, 499–507.

Meehan,R.R., Lewis,J.D. and Bird,A.P. (1992) Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. *Nucleic Acids Res.*, **20**, 5085–5092.

Miskimins,W.K., Roberts,M.P., McClelland,A. and Ruddle,F.H. (1985) Use of a protein-blotting procedure to identify nuclear proteins that recognize the promoter regions of the transferrin receptor gene. *Proc. Natl Acad. Sci. USA*, **82**, 6741–6744.

Nightingale,K. and Wolffe,A.P. (1995) Methylation at CpG sequences does not influence histone H1 binding to a nucleosome including *Xenopus borealis* 5S rRNA gene. *J. Biol. Chem.*, **270**, 4197–4200.

Noll,M., Thomas,J.O. and Kornberg,R.D. (1975) Preparation of native chromatin and damage caused by shearing, *Science*, **187**, 1203–1206.

Ramakrishnan,V., Finch,J.T., Graziano,V., Lee,P.L. and Sweet,R.M. (1993) Crystal structure of the globular domain of histone H5 and its implications for nucleosome binding. *Nature*, **362**, 219–223.

Renz,M. (1975) Preferential and cooperative binding of histone 1 to chromosomal mammalian DNA. *Proc. Natl Acad. Sci. USA*, **72**, 733–736.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Supakar,P.C., Weist,D., Zhang,D., Inamdar,N., Zhang,X.-Y., Khan,R., Ehrlich,K.C. and Ehrlich,M. (1988) Methylated DNA-binding protein is present in various mammalian cell types. *Nucleic Acids Res.*, **16**, 8029–8044.

Tazi,J. and Bird,A. (1990) Alternative chromatin structure at CpG islands. *Cell*, **60**, 909–920.

Thoma,F., Koller,T. and Klug,A. (1979) Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructure of chromatin. *J. Cell Biol.*, **83**, 403–427.

Thomas,J.O. and Kornberg,R.D. (1978) A study of histone–histone associations by chemical cross-linking. *Methods Cell Biol.*, **18**, 429–440.

Thomas,J.O., Rees,C. and Butler,P.J.G. (1986) Salt-induced folding of sea urchin sperm chromatin. *Eur. J. Biochem.*, **154**, 343–348.

Thomas,J.O., Rees,C. and Finch,J.T. (1992) Cooperative binding of the globular domains of histones H1 and H5 to DNA. *Nucleic Acids Res.*, **20**, 187–194.

Vinson,C.R., LaMarco,K.L., Johnson,P.F., Landschulz,W.H. and McKnight,S.L. (1988) *In situ* detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. *Genes Dev.*, **2**, 801–806.

Waalwijk,C. and Flavell,R.A. (1978) *Msp*I an isoschizomer of *Hpa*II which cleaves both nonmethylated and methylated *Hpa*II sites. *Nucleic Acids Res.*, **5**, 3231–3236.

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A recent paper [Campoy,F.J., Meehan,R.R., McKay,S., Nixon,J. and Bird,A. (1995) *J. Biol. Chem.*, **270**, 26473–26481] concludes that the binding of H1 to DNA is indifferent to CpG methylation, based mainly on gel retardation assays with multiply methylated probes, which may be problematic, as discussed above. A nitrocellulose filter binding assay did show a small preference for methylated DNA. An experiment to test the effect of methylation on H1 binding to reconstituted chromatin was inconclusive because of the low H1 content of the chromatin.