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A nuclear protein that binds preferentially to methylated DNA *in vitro* may play a role in the inaccessibility of methylated CpGs in mammalian nuclei

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The effects of DNA methylation on gene expression and chromatin structure suggest the existence of a mechanism in the nucleus capable of distinguishing methylated and non-methylated sequences. We report the finding of a nuclear protein in several vertebrate tissues and cell lines that binds preferentially to methylated DNA *in vitro*. Its lack of sequence-specific requirements makes it potentially capable of binding to any methylated sequence in mammalian nuclei. An *in vivo* counterpart of these results is that methylated CpGs are inaccessible to nucleases within nuclei. In contrast, non-methylated CpG sites, located mainly at CpG islands, and restriction sites not containing this dinucleotide, are relatively accessible. The possibility that DNA methylation acts through binding to specific proteins that could alter chromatin structure is discussed.

Introduction

Methylation of DNA at the dinucleotide CpG is a phenomenon characteristic of many animal groups. The highest levels of m5C are found in vertebrates where about 60–90% of CpGs are methylated. Research done over the past ten years has led to a general consensus that correlates DNA methylation with repression of gene activity. This consensus derives mainly from the observation that many cellular genes are less methylated in expressing than in non-expressing tissues, though in most cases it is not easy to distinguish whether DNA methylation is a cause or a consequence of gene inactivation. For example, the CpG island-containing genes on the X chromosome become methylated following X inactivation in eutherian mammals (Monk 1986). The same happens to retroviral proviruses after infection of early mouse embryos (Jähner *et al.* 1982). In both cases there is evidence that the genes concerned become inactivated by mechanisms that act before their methylation (Lock *et al.* 1987; Gautsch & Wilson 1983).

Similar observations have been made in tissue culture. For example the inactivation of the γ-globin gene appears to occur before its *de novo* methylation, as does the suppression of the Mo-MLV genome in undifferentiated murine teratocarcinoma cells (Enver *et al.* 1988; Niwa *et al.* 1983). Therefore, although methylation is associated with gene inactivation, it may be a secondary event. The importance of DNA methylation in maintaining the inactive state is demonstrated by the fact that in some cases inactivation can be artificially reverted by 5-azacytidine, an inhibitor of DNA methylation. Genes on the inactive mouse or human X chromosomes can be reactivated by 5-azacytidine treatment, as can the mouse metallothionein gene (Mohandas *et al.* 1981; Compere & Palmiter 1981). It may not be a coincidence that

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methylation-mediated transcriptional repression is associated with CpG island-containing genes. These regions are not normally methylated except on the inactive X chromosome. Despite all of these studies, the mechanism by which DNA methylation mediates and maintains repression remains unknown.

Given the inhibitory effect of DNA methylation on transcription, it is not surprising that the promoters of many genes, particularly those expressed in all cell types, occur in non-methylated domains termed CpG islands (Bird *et al.* 1985). These are about 1 or 2 kilobases (kb) long and do not show the reduction in frequency of CpG suppression characteristic of highly methylated genomes. It has been suggested that CpG islands could act as gene markers in the nucleus. One of the advantages for the transcriptional machinery would be the discrimination of gene promoters from the rest of the genome. This type of model could explain why CpG islands have only been found in organisms with large genomes in which only a small fraction of DNA is ever transcribed, e.g. in vertebrates and higher plants (Bird 1987; Antequera & Bird 1988).

CpG methylation could interfere with transcription either directly, by preventing the binding of transcription factors, or indirectly, by binding to nuclear proteins that would render a particular site inaccessible to the factor. In the first case different results have been obtained for different factors. For example, two factors extracted from HeLa cells can neither bind to the adenovirus major late promoter nor stimulate transcription when the recognition sequence is methylated (Kovesdi *et al.* 1987; Watt & Molloy 1988 and this symposium). The Sp1 factor binds equally well to methylated and non-methylated sites and can stimulate transcription from both types of template (Harrington *et al.* 1988; Höller *et al.* 1988). In other cases the effect of methylation on transcription is not detected until the methylated sequences are assembled into chromatin (Buschhausen *et al.* 1987). It has been shown that sequences methylated *in vitro* adopt an inactive conformation when transfected into cells, inactive chromatin being defined by non-accessibility to nucleases (Keshet *et al.* 1986). These results suggest the existence of a mechanism that can distinguish between methylated and non-methylated DNA and directs the former into an inactive conformation.

How could the introduction of methyl groups in the DNA be transduced into conformational changes of chromatin? A possible model is that methylated DNA interacts specifically with certain nuclear proteins. These interactions could determine a different conformation for any particular sequence depending only on its methylation status. We are interested in the characterization of nuclear factors that could distinguish between methylated and non-methylated DNA. A nuclear protein that binds preferentially to a specific DNA sequence when it is methylated has been found in several tissues of animals with methylated genomes (Khan *et al.* 1988). It is, however, unlikely that a sequence-specific protein mediates a generalized methylated DNA-binding process. In the following sections we describe the identification of a nuclear protein that binds preferentially to methylated DNA in a sequence-independent manner. We also describe the specific protection of methylated CpGs against nucleases in intact nuclei (Meehan *et al.* 1989; Antequera *et al.* 1989).

Identification of a methylated-DNA binding protein in mammalian nuclei

At first we used a synthetic multimeric probe of approximately 130 base pairs (b.p.), CG11, which contains 20 *Hha* I and seven *Hpa* II sites that could be methylated with bacterial

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methyltransferases. End-labelled CG11 or fully methylated CG11, MeCG11, were used in band-shift assays in the presence of mouse-liver nuclear extract and non-methylated competitor DNA (figure 1). Only MeCG11 showed a complex of two discrete bands under the conditions used. It was necessary to use agarose gels because the complex failed to enter acrylamide gels (Meehan *et al.* 1989). Complexes of identical mobility were seen in mouse brain, spleen, kidney, rat liver and rabbit liver. They were also observed in several permanent cell lines of human and mouse origin.

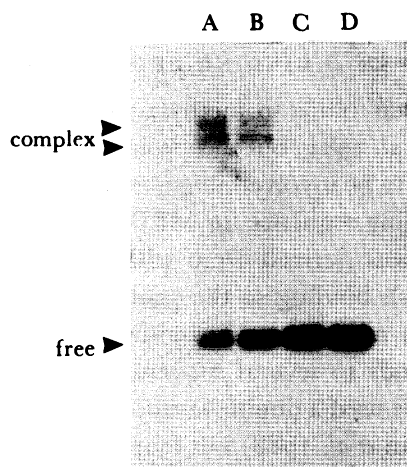

FIGURE 1. Presence of a protein that binds methylated DNA in mammalian nuclei. About 0.1 ng of labelled probe in either the methylated form (lanes A and B) or non-methylated form (lanes C and D) were incubated with 3 μg of mouse liver nuclear extract in the presence of 3 μg (lanes A and C) or 6 μg (lanes B and D) of non-methylated competitor DNA from *Micrococcus lysodeikticus* (75 % G+C rich). Complexes were resolved on 1.5 % agarose gels and dried onto DE81 filters before exposure to X-ray film.

Competition experiments with synthetic DNA molecules and DNA from organisms with methylated and non-methylated genomes demonstrated that the activity we were detecting bound to any sequence with 15 or more symmetrically methylated CpGs per substrate molecule, but otherwise it had no specific sequence requirements. The necessity for methylation was best demonstrated in competition experiments with mouse DNA and cloned whole library mouse cosmid DNA. The only overall difference between the two competitors is that one is methylated and the other is not. Only mouse DNA could effectively prevent the formation of the complex with MeCG11 (Meehan *et al.* 1989). On the basis of mass, the affinity of mouse DNA for the complex is approximately one two-hundredth of MeCG11, but if considered in terms of molar concentration of methyl-CpGs then mouse DNA (one MeCpG per 150 b.p.) is only sevenfold less efficient as a substrate for the complex compared to MeCG11 (one MeCpG per 5 b.p.). Hemi-methylated substrates or substrates that were methylated at non-CpG sites were not effective in forming the complex. We refer to the protein(s) responsible for this activity as 'methylated CpG binding protein' or MeCP.

Preliminary characterization of MeCP demonstrated that on gel filtration columns it runs as a large molecular mass complex with peaks at M_r 800000 and M_r 400000. However, in cross-linking experiments, a 120 kDa protein was found to specifically bind MeCG11. Binding could be prevented by preincubation with methylated DNA competitor suggesting that the 120 kDa protein is a component of the complex. This protein also co-purified with the band-shift

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activity through several columns, including DNA-affinity columns. The discrepancy in size between the gel-filtration and cross-linking experiments might be explained if MeCP has an unusual shape. If MeCP is rod-like, for instance, it would run anomalously relative to the size markers as the gel filtration columns were calibrated for globular size markers. The relative mobility of MeCP could not be reduced below M_r 400000 on gel filtration even in the presence of 4 M urea, which suggests that this is the size of the basic unit under these conditions.

MeCP is distinct from a previously identified sequence-specific methylated DNA binding protein, MDBP

A mammalian nuclear protein that binds to methylated DNA, termed MBDP, has been described in the literature (Huang *et al.* 1984) but this protein is highly sequence specific (Khan *et al.* 1988) and is therefore unlikely to be involved in general methylation-mediated inhibition of transcription. The consensus binding sequence for MBDP is 14 b.p. long and contains three MeCpGs. The original substrate was derived from pBR322. Point mutations within the consensus sequence reduce or abolish binding to the protein (Khan *et al.* 1988). If all three MeCpGs are mutated to TpG then no decrease in binding to MBDP is observed (Supakar *et al.* 1988). In contrast, MeCP binds to several sequences with no obvious relation to the consensus binding site for MBDP. We used a double-stranded oligonucleotide (W17/C17) that binds to MDBP as a monomer (Khan *et al.* 1988) but found that it was unable to bind MeCP unless ligated to form multimeric molecules of greater than 100 b.p. In this respect W17/C17 is typical of other methylated oligonucleotides that we tested in that it is dependent on a high density of MeCpG to bind in the bandshift assay (Meehan *et al.* 1989). These differences lead us to conclude that MeCP and MBDP are distinct activities.

Methylated sites are not accessible to nucleases in nuclei

The isoschizomers *Msp* I and *Hpa* II have been used widely in studies of DNA methylation because of their ability to cut differentially methylated sites. Both of them recognize the sequence CCGG but *Hpa* II cannot cut when the internal C in methylated whereas *Msp* I is insensitive to such modification. As a result, the restriction patterns they generate upon digestion of naked vertebrate DNA are very different. Surprisingly, when intact nuclei are digested with both enzymes the restriction pattern obtained after end-labelling of the fragments is identical. Each enzyme releases a nucleosomal ladder of DNA fragments. This ladder collapses if the end-labelled naked DNA fragments are digested with *Hpa* II before fractionation on the gel (figure 2). This suggests that the ladder is derived from CpG island chromatin because non-methylated *Hpa* II sites only occur at high frequency in this fraction of the genome. Chromatin outside CpG islands however, is extensively digested in a concentration dependent manner by a variety of restriction enzymes that do not include the dinucleotide CpG in their recognition sequence (Antequera *et al.* 1989), showing that non-island chromatin is not particularly resistant to nuclease digestion. These results suggest that *Msp* I is unable to cut methylated sites in nuclei.

To test whether the methyl group on its own is able to prevent *Msp* I from cutting in chromatin, we methylated non-methylated CCGG sites in nuclei with *Hpa* II methylase. After that, they remained equally sensitive to *Msp* I, suggesting that the presence of a methyl group

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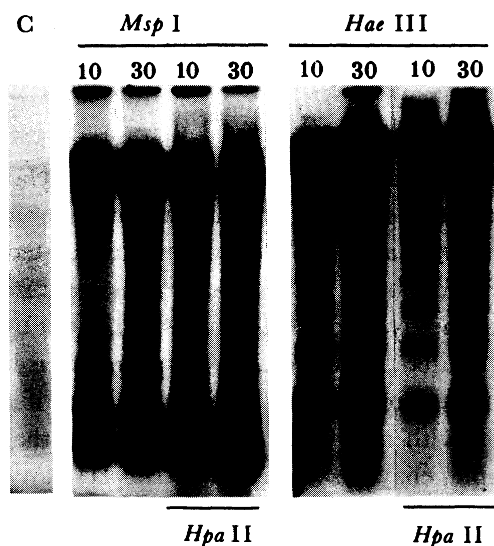

FIGURE 2. Digestion of mouse liver nuclei with *Msp* I and *Hae* III. Nuclei were incubated with no enzyme (lane C) or with 10 and 30 units of *Msp* I (CCGG) or *Hae* III (GGCC) respectively. DNA was extracted and end-labelled by filling in with the Klenow fragment of DNA polymerase I and radio-labelled (α-^{32}P) dCTP. One half of each sample was subsequently digested with *Hpa* II and all samples were resolved on 1.8% agarose gels. Gels were dried and exposed as in figure 1. Increasing the *Msp* I enzyme concentration does not lead to an increase in the released *Msp* I nucleosomal ladder, indicating that digestion is complete. In contrast, an increase in *Hae* III enzyme concentration does lead to an increase in the released *Hae* III nucleosomal ladder. The collapse of the *Msp* I but not of the *Hae* III nucleosomal ladders by *Hpa* II suggests that *Msp* I is cutting mainly at CpG islands whereas *Hae* III is cutting bulk methylated chromatin. Blockage of *Msp* I sites in bulk chromatin suggests that methylated sites are refractory to this enzyme in nuclei.

in its recognition sequence is not enough to prevent cutting. To assess if methylated CCGG sequences are representative of all methylated CpGs in the nucleus, we did some experiments with *Tth* (recognition sequence TCGA), another enzyme that is insensitive to cytosine methylation. To measure the accessibility of methylated *Tth* sites in the nuclei, it was necessary to adopt a somewhat different approach, as there is no sensitive isoschizomer available and *Tth* sites are not very abundant at CpG islands. Nuclei and naked DNA were digested with *Msp* I and *Tth* restriction enzymes and the resulting fragments were kinase labelled and enzymatically digested to single nucleotides. Quantitation of radioactive 5-methylcytosine and cytosine by two-dimensional thin-layer chromatography indicated the proportion of methylated CCGG and TCGA sites accessible to both endonucleases in nuclei. The comparison of these figures with those obtained for naked DNA showed that the relative availability of methylated sites in nuclei is about ten-times lower than in naked DNA. The fact that similar figures were obtained with both enzymes strongly suggests that blockage of methylated sites applies to most CpGs in the nucleus regardless of the sequence context that they are in (Antequera *et al.* 1989).

Analysis of specific sequences

It has been reported by several authors that methylated sites are not accessible to *Msp* I. Most studies have been done on CpG islands that are methylated on the inactive X chromosome (Wolf & Migeon 1985; Hansen *et al.* 1988). *Msp* I has been used widely for this purpose because of the abundance of sites at CpG islands and because of its insensitivity to

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methylation. In some cases, the protection against *Msp* I cutting has been attributed to the existence of inactive chromatin. We were interested in defining more precisely the nature of methylated CpG protection.

The strategy we followed was first to establish a methylation map by using *Msp* I and *Hpa* II on naked DNA and then to ask whether particular methylated sites are accessible to *Msp* I in nuclei. We have tested three loci: 16 kb of the human α-globin gene cluster; the HTF 9 locus of mouse (Lavia *et al.* 1987); and the CpG island associated with the hypoxanthine phosphoribosyl transferase gene on both active and inactive mouse X chromosomes (Lock *et al.* 1986). We have found that methylated CCGG sites are not cut by *Msp* I in nuclei, thereby confirming the whole-nuclei results (Antequera *et al.* 1989). We have also tested whether protection extended to sites in the vicinity of methylated CpGs, or if it was more or less limited to specific CpG sites. Digestion with non-CpG enzymes showed that their sites were at least partly cut in nuclei. For example, enzymes like *Alu* I or *Hae* III can extensively cut sequences in the inactive X chromosome and suggest that the blockage of methylated CpG sites is restricted to the site itself, possibly extending only some nucleotides away. This also confirms the pattern of digestion seen for bulk DNA in nuclei.

Methylated DNA-binding proteins and the function of DNA methylation

So far, we have discussed two independent phenomena: the existence of a protein that binds preferentially to methylated DNA and the protection of methylated CpGs in the nucleus. We do not have any direct evidence that MeCP is binding to methylated CpGs *in vivo*. Preliminary experiments however, suggest that the binding activity is much lower in mouse embryonal carcinoma PC13 cells than in normal tissues. Consistent with this observation, we have found that methylated CpG sites are at least twice as accessible.

The *in vitro* binding experiments discussed above show that MeCP has an affinity for clusters of methylated CpGs. Examples of such clusters that could be potential substrates *in vivo* has been found at methylated CpG islands in inactive X chromosomes (Lock *et al.* 1986; Wolf *et al.* 1984; Yen *et al.* 1984), the dispersed repetitive LINE elements (R. Meehan, unpublished results) and silenced retrovirus genomes (Jähner *et al.* 1982). In each of those cases, methylation is associated with repression of transcription. Only a small proportion of methylated CpGs are clustered in the genome, the majority being dispersed at an average density of about 1 per 150 b.p. of mouse DNA. Results from the competition experiments suggest that mouse DNA is sevenfold less efficient in its binding to MeCP as synthetic methyl-CpG-rich substrates. This suggests that a relatively large fraction of mouse DNA has the potential to bind MeCP. Whether or not MeCP has a function in complexing methylated CpGs in the nucleus, either on its own or by interacting with other proteins will not be definitively answered until MeCP itself has been purified.

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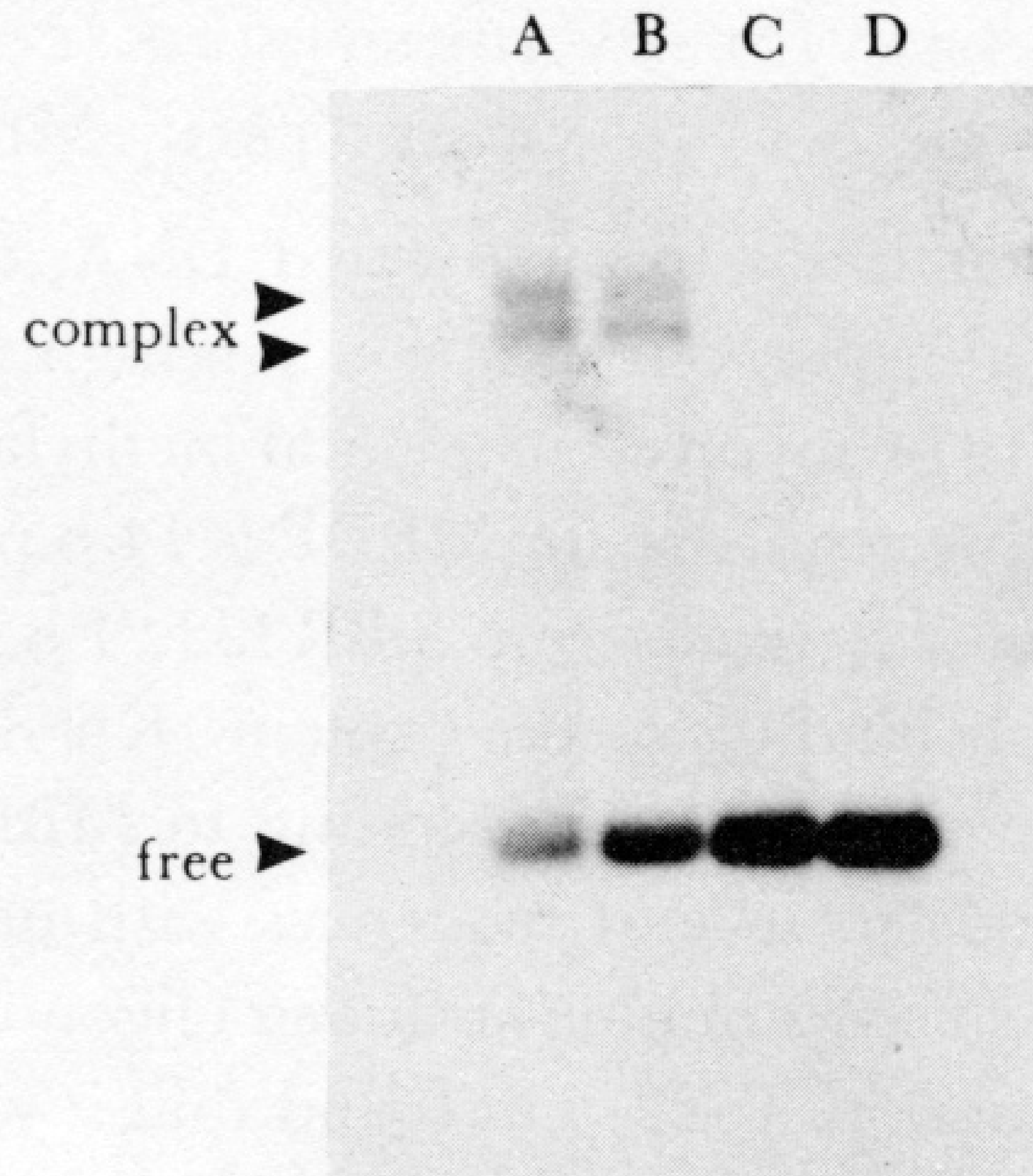

GURE 1. Presence of a protein that binds methylated DNA in mammalian nuclei. About 0.1 ng of labelled probe in either the methylated form (lanes A and B) or non-methylated form (lanes C and D) were incubated with 3 μg of mouse liver nuclear extract in the presence of 3 μg (lanes A and C) or 6 μg (lanes B and D) of non-methylated competitor DNA from *Micrococcus lysodeikticus* (75 % G + C rich). Complexes were resolved on 1.5 % agarose gels and dried onto DE81 filters before exposure to X-ray film.

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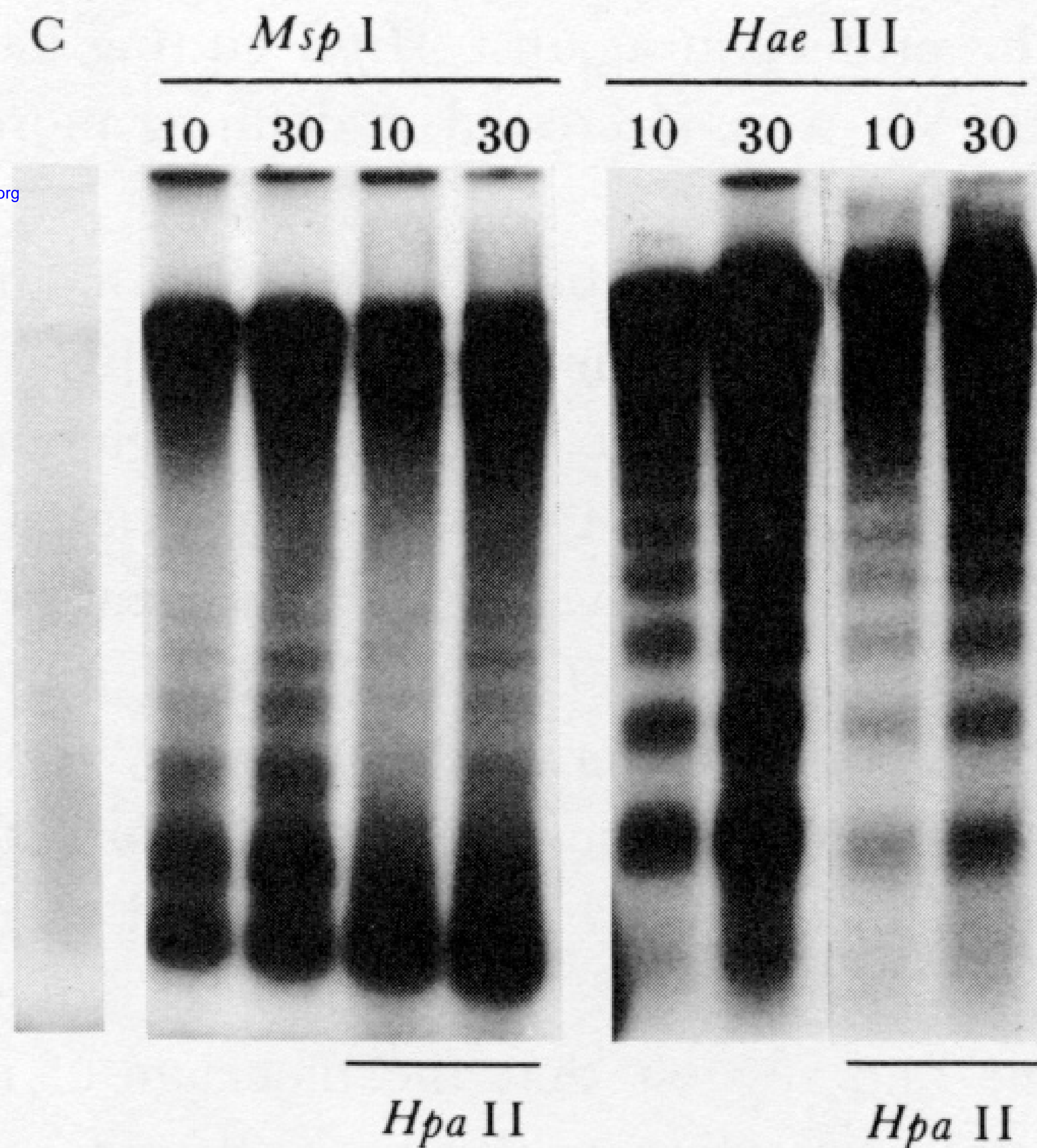

IGURE 2. Digestion of mouse liver nuclei with *Msp* I and *Hae* III. Nuclei were incubated with no enzyme (lane C) or with 10 and 30 units of *Msp* I (CCGG) or *Hae* III (GGCC) respectively. DNA was extracted and end-labelled by filling in with the Klenow fragment of DNA polymerase I and radio-labelled (α-^{32}P) dCTP. One half of each sample was subsequently digested with *Hpa* II and all samples were resolved on 1.8% agarose gels. Gels were dried and exposed as in figure 1. Increasing the *Msp* I enzyme concentration does not lead to an increase in the released *Msp* I nucleosomal ladder, indicating that digestion is complete. In contrast, an increase in *Hae* III enzyme concentration does lead to an increase in the released *Hae* III nucleosomal ladder. The collapse of the *Msp* I but not of the *Hae* III nucleosomal ladders by *Hpa* II suggests that *Msp* I is cutting mainly at CpG islands whereas *Hae* III is cutting bulk methylated chromatin. Blockage of *Msp* I sites in bulk chromatin suggests that methylated sites are refractory to this enzyme in nuclei.

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