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Methylation of an α-foetoprotein gene intragenic site modulates gene activity

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ABSTRACT

By comparing the methylation pattern of MspI/HpaII sites in the 5′ region of the mouse α-foetoprotein (AFP) gene of different cells (hepatoma cells, foetal and adult liver, fibroblasts), we found a correlation between gene expression and unmethylation of a site located in the first intron of the gene. Other sites did not show this correlation. In tranfection experiments of unmethylated and methylated AFP-CAT chimeric constructions, we then showed that methylation of the intronic site negatively modulates expression of CAT activity. We also found that a DNA segment centered on this site binds nuclear proteins; however methylation did not affect protein binding.

INTRODUCTION

A growing body of evidence suggests that DNA methylation may constitute a general mechanism for gene repression (for reviews, see 1–2). At first, methylation pattern studies have established a clear cut correlation between DNA undermethylation and gene activity; indeed, genes are usually less methylated in the expressing state than in the silent state (3–6). More convincing arguments that DNA methylation at CpG sites is effectively implicated in gene repression were brought by gene transfer experiments showing that *in vitro* methylated constructions were usually less transcriptionaly active than their unmethylated homologues (7–12). However, very few reports have combined the analysis of the methylation state of specific sites *in vivo*, with gene transfer experiments of *in vitro* methylated constructions (9,10). The mechanisms by which DNA methylation inhibits gene transcription are still unknown, but it was shown that DNA modification may interfere with the binding of *trans*-acting positive regulatory factors to their recognition sequence (direct inhibition) (13–16), or may stimulate the binding of a methyl-CpG binding protein which inhibits transcription (indirect inhibition) (17). DNA methylation may also operate at the level of chromatin structure conformation (18–20).

Alpha-foetoprotein (AFP) is the major protein in the serum of mammalian foetus. The AFP gene is expressed in a tissue-specific manner and is developmentaly regulated (22–23). AFP is produced in foetal hepatocytes, and its synthesis is shut off after birth, so that normal adult liver contains AFP mRNA levels that are 10^{-3}- to 10^{-4}-fold lower than those found in foetal liver. Expression of the AFP gene is essentially controlled at the level of transcription (24–26), and this transcriptional control is dependent on the action of *trans*-acting regulatory molecules, as first suggested by the results of somatic cell hybridization experiments and then shown by DNA-mediated cell transfection experiments of cloned AFP sequences (27–29). However, some evidence suggests that *cis*-acting mechanisms might also participate in the regulation of AFP gene expression: indeed, expression of the AFP gene in some somatic cell hybrids is determined by whether the gene is derived from an expressing or a non-expressing parental cell (30).

In this work, we adressed the question of the possible role of DNA methylation in the control of the AFP gene expression. In particular, we were interested in determining whether cessation of AFP expression during liver development is associated with *de novo* methylation of the gene, and whether such possible methylation events might play a functional role in the maintenance of the silent state of the AFP gene in adult hepatocytes.

The intragenic region of the AFP gene was previously found to be generally hypomethylated in AFP-producing mouse or rat hepatoma cells, and in mouse foetal liver, relative to mouse adult liver (31–34). As regards the 5′ region of the gene, two studies (35,36) of MspI/HpaII sites (CCGG) of the rat AFP gene showed that the level of demethylation of one site located in the first exon (position +3) is very well correlated with the level of gene activity, both in hepatoma cell lines and in normal liver (methylation at this site increases after the gene has become inactive during liver maturation; ref. 36). The methylation level at other sites in the 5′ flanking region (36) or in the intragenic region (35) did not show such a good correlation with gene inactivity.

In this report, the MspI/HpaII methylation pattern of the 5′ region of the mouse AFP gene was first analyzed in expressing and non-expressing cell types (hepatoma cells, feotal liver, adult liver, fibroblasts), and then, a functional analysis, based on transfections of *in vitro* methylated constructions was carried out. An excellent correlation was found between AFP gene expression and hypomethylation of a single site (M_0), located in the first intron of the gene. *In vitro* methylation at this site reduces the

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expression of the transfected constructions. However, methylation of this site does not prevent the binding of nuclear proteins detected in band-shift experiments.

MATERIALS AND METHODS

Biological material

Normal mouse fibroblasts were cultured from dissected foetus skin. Foetal livers were obtained from C57L mice at days 17 to 19 of gestation. Adult livers were obtained from 4 month-old C57L mice. BWTG3 and HepG2 are mouse and human AFP-producing hepatoma cells, respectively (30,37). Hela is a human carcinoma cell line (38). All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum.

DNA extraction and Southern blot analysis

High molecular weight DNA was isolated according to the method of Gross-Bellard et al. (39). Restriction enzymes were used in the buffers recommended by the supplier; incubations were for at least 3 hours, with 10 units of enzyme/μgr of DNA. Completeness of the digestion was checked by parallel digestion of genomic DNA mixed with phage lambda DNA. Electrophoretic separation of restriction fragments on agarose gels and DNA transfer were done according to published procedures (40).

Probes and hybridization

The DNA probes were isolated from mouse AFP genomic fragments, cloned in phage lambda, and recloned in pBR322. The restriction map of the 5′ region of the mouse AFP gene was established using published data (28,41) and by simple and double digestions (see Fig. 1A). Probes (a) and (b) were extracted from low melting-point agarose after digestion with adequate restriction enzymes. Probe (a) is an EcoRI-BamHI fragment, cut out from a genomic clone limited at its 5′end by an EcoRI site artefactually created during the cloning procedures (this site is thus not indicated on the map shown in Fig. 1A). Probe (b) was isolated after PvuII and HindIII digestion of pHAF-CAT (42). The probes were labelled ($2-4\times108$ cpm/ug) by nick-translation, using a Radiochemical Center (Amersham) kit.

Plasmids and constructions

Plasmids were constructed and purified using standard protocols. The construction of the pHAF-CAT and pHAF-I_{del}-CAT plasmids was described earlier (42). The pHAF-M_0-CAT plasmid was derived from pHAF-I_{del}-CAT as follows. On the one hand, pHAF-I_{del}-CAT was opened by PstI partial digestion at position +1.1 kb (5′ end of the second intron). This fragment was blunted and recut with SacI (position +280), generating two fragments of 0.9 and 5.5 kb. The latter fragment was isolated. On the other hand, pHAF-I_{del}-CAT was digested with MboII and a 690 bp fragment containing the site M_0 was isolated, blunted and cut with SacI, generating two fragments of 560 and 130 bp. The intronic 130 bp fragment that contains the site M_0 was isolated, and ligated to the 5.5 kb fragment previously described. The pHAF-M_1-CAT plasmid was also derived from the pHAF-I_{del}-CAT by generating a 146 bp deletion containing the site M_0: pHAF-I_{del}-CAT was opened by SacI and partially digested with MboII (cleavage at position +426). The resulting 6.2 kb fragment was isolated, blunted and ligated.

In vitro methylation

20 μg of plasmid were incubated with 30 units of HpaII methylase for 2 hours at 37°C in a buffer containing 50 mM Tris HCl pH 7.5, 10 mM EDTA, 5mM β-mercaptoethanol and 80 μM S-adenosyl-methionine (freshly prepared). Reaction was stopped by incubating the sample 20 min at 65°C and then extracting with phenol-chloroform and then with chloroform. After ethanol precipitation, completion of methylation was tested by MspI/HpaII restriction.

Transfections

Transfections and CAT assays were done as descried earlier (42), except that 5 μg of plasmid DNA were used, instead of 10 μg.

Nuclear extracts and mobility shift assays

Nuclei were isolated as described (43) by resuspending the cells in 10 ml lysis buffer (0.3 M sucrose, 10mM Hepes KOH pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1mM EGTA, 0.5mM DTT, O.5 mM PMSF and 0.5% NP40) (44) and centrifuged for 4 min at 3000 rpm in a HB4 rotor. The pellet was resuspended in 10 ml lysis buffer without NP40 and centrifuged trough a 3.5 ml sucrose cushion (30% sucrose, 10mM Hepes KOH pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.1mM EGTA, 0.5mM DTT, O.5 mM PMSF) for 15 min at 3000 rpm. The pellet was isolated and nuclear extracts were prepared as described previously (45). Mobility shift assays were performed as described previously (44). The probe was a 224 bp SfanI-MboII fragment (position +202 to position +426) isolated from the first intron of the AFP gene and cloned in the SmaI site of the pUC12 polylinker. This fragment was excised from pUC12 by EcoRI and BamHI restriction and radioactivelly labelled by partial filling of recessed 3′ termini. 1 μg of nuclear extracts were incubated for 20 min at room tempature with increasing concentrations of pUC12 competitor DNA in a buffer containing 10mM Hepes pH 7.9, 25mM Kcl, 10% glycerol, 2mM MgCl2, 0.2 mM EDTA, 0.5mM DTT. After addition of 1 ng of labelled probe (3000−5000 cpm) and incubation for another 20 min at room temrature, the samples were separated in a low ionic strengh 5% polyacrylamide gel containing 1 mM EDTA, 3.3mM sodium acetate and 6.7 mM Tris HCl pH 7.5.

RESULTS

The methylation state of the 5′ region of the mouse AFP gene was studied in different cell types, using the classical approach (3), namely by comparing the Southern blot patterns obtained after digestion of the DNAs with the two isochizomers, MspI (that cuts the CCGG sequence irrespectively of the inner cytosine methylation) and HpaII (that cuts CCGG but does not cut CmCGG). We first completed the restriction map in the 5′ region of the mouse AFP gene (41). This analysis was done for an 8 kb AFP genomic clone derived from EcoRI-digested DNA of BWTG3 mouse hepatoma cells (of C57L origin) (42), and spanning about 5.5 kb upstream the transcription start. The following restriction sites were mapped: EcoRI, BamHI, HindIII, MspI/HpaII, and PvuII, as shown in Fig. 1A. Four MspI/HpaII sites were studied in this region: two sites (M_2 and M_{-1}) are located relatively far upstream the transcription start, one site (M_0) is located in the first intron (see the sequence in ref. 28), and the fourth site, M_{+1} is in the beginning of the second exon (46). Mouse genomic DNAs of foetal or adult liver, of hepatoma

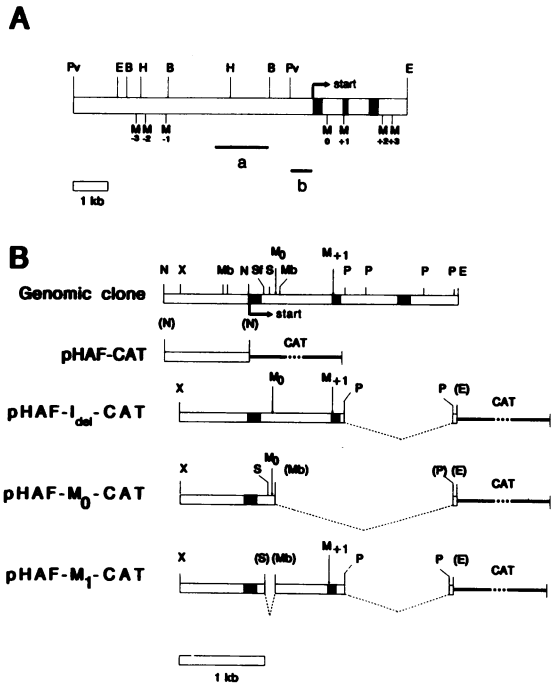

Fig. 1. A: Restriction map of the 5′ end of the mouse AFP gene and its flanking sequence. The arrow indicates the position of the cap site; the black boxes represent the first three exons. The probes used were the (a) and (b) fragments. The symbols used to denote restriction sites are: B=BamHI, E=EcoRI, H=HindIII, M=MspI (the MspI sites are numbered from −3 to +3), Pv=PvuII. **B**: General scheme showing the restriction map of the mouse AFP gene 5′ region relevant to the constructions used, and the structure of these constructions, made by inserting AFP fragments upstream the bacterial CAT gene (interrupted black line). Dotted lines in the intragenic region of the chimeric constructions correspond to deletions. The symbols used to denote restriction sites are: N=NlaIV; X= XbaI; Mb=MboII (second MboII site at position +426); Sf=SfanI (position +202; other SfanI sites are not shown); S=SacI (position +280); P=PstI, E=EcoRI, M_0 (position +373) and M_{+1} (in the second exon)=MspI sites; symbols in parentheses represent restriction sites destroyed in the corresponding construction.

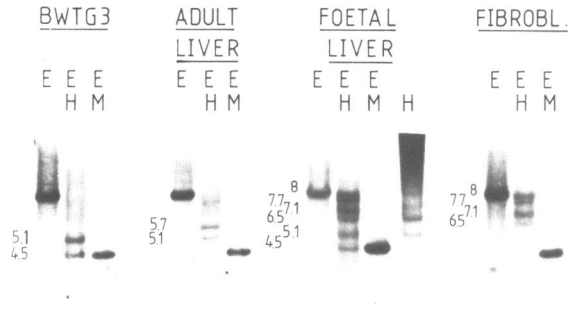

Fig. 2. Southern blot analysis of the DNA methylation levels at the 5′ region of the mouse (C57L) AFP gene in hepatoma cells (BWTG3), adult liver, foetal liver and fibroblasts. The DNAs were cleaved with EcoRI (E) and either HpaII (H) or MspI (M), except the foetal liver sample labelled H, that was treated with HpaII only. The probe used was the fragment (a) (see Fig. 1). The size of the bands is indicated in kilobases.

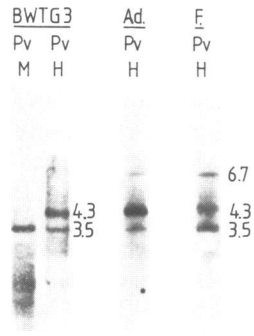

Fig. 3. Methylation state of the MspI site −1 (M_{-1}) in mouse hepatoma cells (BWTG3), adult liver (Ad.) and foetal liver (F.). The DNAs were digested with PvuII (Pv) and either MspI (M) or HpaII (H) (adult and foetal liver DNA samples treated with PvuII and MspI gave, like BWTG3 DNA, a single band at 3.5 kb). The probe used was probe (a), like in Fig. 2.

cells and of fibroblasts were first analyzed by Southern blot analysis, after simple or double digestion with the above restriction enzymes, and using different probes. Identical patterns were obtained (Figs 2, 3 and 4, and other data not shown), confirming that the 5′ region of the mouse AFP gene is not grossly modified during development or in the BWTG3 hepatoma cells and that, as expected, polymorphism is not detected in these different C57L DNA samples (including the MspI sites).

Expressing cells (hepatoma, foetal liver) are characterized by the presence of DNA molecules unmethylated at both sites M_{-1} and M_0

The methylation state of the sites M_{-1} and M_0 in the four studied mouse DNAs (foetal and adult liver, hepatoma cells, fibroblasts) was first examined, using probe (a), which corresponds to a segment located between these two sites (Fig. 1A). As expected (see map in Fig. 1A), a 4.5 kb restriction fragment was revealed by the Southern blot analysis, after digestion of the different DNA samples with MspI (with or without EcoRI, see Fig. 2). Digestion with HpaII (and EcoRI) yielded more complex patterns, each of which was specific to one of the four cell preparations tested. The 4.5 kb fragment was generated in the hepatoma cell and in the foetal liver DNAs, but not in the adult liver or fibroblast DNAs. A band at about 5.1 kb was common to the three hepatic DNAs (and was the only band accompanying the 4.5 kb band in the case of the hepatoma cell DNA). The adult liver DNA (and this DNA only) showed a band at 5. 7 kb. Finally, bands at about 6.5, 7.1, 7.7 and 8 kb were detected in the foetal liver DNA, in the fibroblast DNA and, to a lesser extent in the adult liver DNA. These different patterns were obtained reproducibly, with different DNA samples; the results were not modified by increasing the quantity of HpaII used in the digestion; finally, completeness of the digestion was checked by parallel digestion of genomic DNA with phage lambda DNA.

These results are interpreted as follows. (i) The 4.5 kb band generated by HpaII digestion of hepatoma and foetal liver DNAs indicates that expressing cells are characterized by the presence of DNA molecules simultaneously demethylated at sites M_{-1} and M_0. (ii) The 5.1 kb band, found in the three hepatic samples, can be generated in two ways (see Fig. 1): by unmethylation of the sites M_{-2} and M_0 (in combination with methylation of M_{-1}) or by unmethylation of the sites M_{-1} and M_{+1} (in combination with methylation of M_0). (iii) The 5.7 kb band present in HpaII-restricted DNA from adult liver must be derived from DNA molecules simultaneously methylated at sites M_{-1} and M_0, but unmethylated at the two flanking sites, M_{-2}

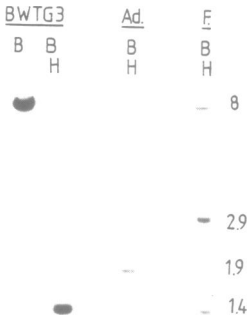

Fig. 4. Methylation state of MspI site 0 (M_0) in mouse hepatoma cells (BWTG3), adult liver (Ad.) and foetal liver (F.). The DNAs were digested with BamHI (B) alone, or with HpaII (H). The probe used was probe (b) (see Fig. 1A). In the foetal liver DNA sample, the 2.9 kb fragment results very likely from BamHI cleavage (5′ end) combined with HpaII cleavage at the site M_{+2} (3′end) (the sites M_0 and M_{+1} being methylated), whereas the 8 kb fragment corresponds to a BamHI-BamHI fragment extending to a BamHI site located in the sixth exon of the gene, 5.1 kb downstream the site M_{+3} (not shown in Fig. 1, see 40) and containing methylated sites at positions M_0, M_{+1}, M_{+2} and M_{+3}.

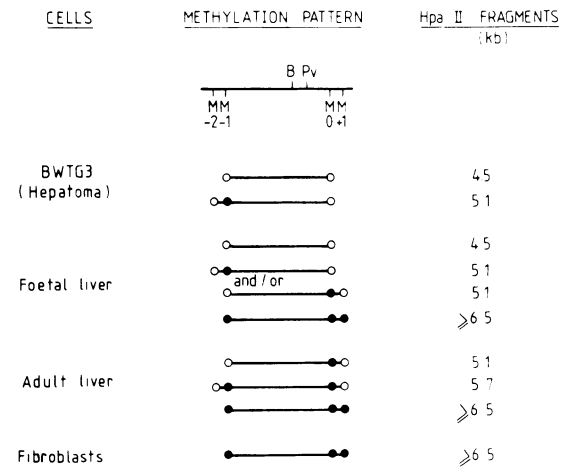

Fig. 5. Summary of the methylation patterns identified in the mouse AFP gene in the mouse hepatoma cells BWTG3, the foetal liver, the adult liver and fibroblasts. Closed and open circles represent methylated or unmethylated cytosines, respectively, at the different MspI/HpaII sites (from −2 to +1). B and Pv are BamHI and PvuII restriction sites, respectively (see Fig. 1A). The size (in kilobases) of the HpaII fragments, visible in Fig. 2, and corresponding to each of the methylation patterns is indicated.

and M_{+1}. (iv) The high molecular weight bands present in the foetal liver, adult liver and fibroblast DNAs, reveal that these DNAs contain molecules methylated at several contiguous sites, including sites M_{-1} and M_0. The 8 kb band is due to two EcoRI cleavages and thus reflects methylation of DNA molecules at all MspI/HpaII sites located between these EcoRI sites. The 7.1 and 7.7 kb bands are formed only when the HpaII-digested DNA is also restricted with EcoRI (compare, in Fig. 2, the foetal liver sample digested with EcoRI + HpaII with that digested with HpaII alone) and thus reflect demethylation at a single MspI/HpaII site, located at one of the ends of EcoRI-EcoRI segment. These results thus indicate that the foetal liver contains several classes of methylation pattern: DNA molecules simultaneously demethylated at sites M_{-1} and M_0 (like in the hepatoma cells) and DNA molecules simultaneously methylated at these sites, and at other sites (like in fibroblasts). The foetal liver thus combines the methylation patterns existing in the hepatoma and fibroblast HpaII-digested DNAs. Since this organ contains a significant fraction of non-parenchymal cells, like erythropoietic cells, which are not expressing the AFP gene, it is not surprising that a fraction of foetal liver DNA molecules exhibit the same methylation pattern as that of fibroblast DNA.

Hepatoma and normal liver DNAs contain molecules methylated at site M_{-1} but unmethylated at site M_{-2}

As mentionned above, and as shown in Fig. 1A, the MspI/HpaII restriction map of the C57L mouse AFP gene is such that methylation in this region can generate a 5.1 kb restriction fragment in two ways: by unmethylation of the sites M_{-2} and M_0, in combination with methylation of M_{-1}, or by unmethylation of the sites M_{-1} and M_{+1}, in combination with methylation of M_0. In order to allow a choice between these two possibilities, we first determined whether the site M_{-1} is methylated in the hepatic cells, by submitting the three hepatic DNAs to double restriction with HpaII and PvuII. In the case of methylation at the site M_{-1}, probe (a) should reveal a 4.3 kb fragment, generated by HpaII cleavage at the site M_{-2} and PvuII cleavage at the 3′ end, whereas in the case of unmethylation at this site, the probe should detect a 3.5 kb fragment, resulting from HpaII cleavage at the unmethylated site M_{-1} and PvuII cleavage at the 3′ end. Fig. 3 shows that both fragments were obtained with the three hepatic DNAs. We can thus conclude that in the hepatic DNAs (hepatoma, foetal liver, adult liver), the site M_{-1} is partially methylated, and that a methylated site M_{-1} can be associated with an unmethylated site M_{-2}. Fig. 3 also shows that a labelled band is observed at about 6.7 kb, in the foetal liver DNA and, to a lesser extent in the adult liver DNA (but not in the hepatoma cell DNA). This band corresponds to a PvuII-PvuII restriction fragment containing the methylated sites M_{-3}, M_{-2} and M_{-1}. In normal liver, a third pattern of methylation is thus found, which combines methylation at these three sites.

The results discussed above, illustrated by Fig. 2 and 3, show that in hepatoma cells, the two bands (4.5 and 5.1 kb) generated by HpaII digestion are derived from DNA molecules unmethylated at site M_0 and either unmethylated or methylated at site M_{-1} (4.5 and 5.1 kb HpaII band, respectively). Is each of these two types of DNA molecules expressing the AFP gene? The answer to this question is very likely to be positive. Indeed, we isolated some clonal hybrid lines, derived from the fusion of BWTG3 hepatoma cells with fibroblasts, that are actively producing AFP, and whose DNA exhibits either the 4.5 kb or the 5.1 kb HpaII band (not shown). Methylation at the site M_{-1} thus appears compatible with gene expression (in association with an unmethylated site M_0).

The site M_0 is largely unmethylated in hepatoma DNA, partially methylated in foetal liver DNA and largely methylated in adult liver DNA, where the site M_{+1} is at least partially unmethylated

The methylation state of the site M_0 was then further studied, using the probe (b) (Fig. 1A), after double digestion with HpaII and BamHI. Unmethylation at this site is expected to yield a BamHI-HpaII fragment of 1.4 kb, whereas methylation is expected to yield a 1.9 kb fragment (HpaII cleavage at the unmethylated site M_{+1}). As illustrated in Fig. 4, adult liver DNA showed a 1.9 kb band, whereas hepatoma cell DNA and

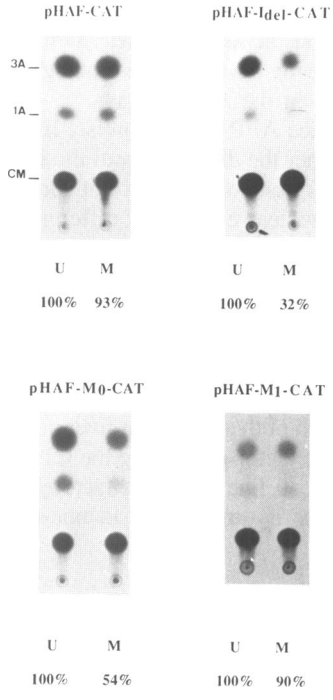

Fig. 6. CAT activities detected by the different constructions represented in Fig. 1B, transfected in the AFP-producing hepatoma cells HepG2, relative to the unmethylated plasmid. A comparison between the CAT activities directed by the different plasmids is shown in table 1. U=unmethylated plasmid; M=methylated plasmid; CM=chloramphenicol; 1A=1-acetate-chloramphenicol; 3A=3-acetate-chloramphenicol.

foetal liver DNA showed a 1.4 kb band (foetal liver DNA also showed larger bands, due to methylation at several contiguous sites). We thus conclude that: (i) in the adult liver DNA, the site M_0 is extensively methylated (next to an unmethylated site M_{+1}); (ii) in the hepatoma cell DNA, the site M_0 is essentially unmethylated ; (iii) in the foetal liver DNA, the situation is intermediate: methylation of site M_0 is partial (and the molecules methylated at this site are, at least in some cases, unmethylated at the next site, M_{+1}). As shown previously (Fig. 3), methylation at the site M_{-1} is also partial in the foetal liver DNA preparation. The fact that methylation is partial at the two adjacent sites, M_{-1} and M_0 did not allow us to determine whether the 5.1 kb HpaII band detected with probe (a) (Fig. 2) in the foetal liver DNA is due to cleavages at sites M_{-2} and M_0 (like in hepatoma cells DNA) or at sites M_1 and M_{+1} (like in adult liver DNA), or both.

Fig. 5 summarizes our results. The main observations drawn from this study on the methylation of the 5′ end of the mouse AFP gene and its upstream region are the following ones: (1) the fibroblast DNA is highly methylated in this region; (2) the hepatoma DNA is less methylated than the other DNAs tested and in this DNA, the site M_0 is largely unmethylated; (3) the adult liver DNA is less methylated than the foetal liver DNA, but site M_0 shows an inverse evolution, being extensively methylated in the adult organ and only partially methylated in the foetal liver; (4) the only good correlation observed between unmethylation of a MspI/HpaII site located in the AFP gene 5′ region and gene expression is found at site M_0, located in the first intron of the gene, at position +373 (lack of correlation is found at the other three sites examined: site M_2 is partially unmethylated in the adult liver; site M_{-1} is partially methylated in hepatoma cells, and partially unmethylated in the adult liver; site M_{+1} is partially unmethylated in the adult liver).

Table 1. CAT activities directed by the unmethylated and methylated plasmids.

Plasmids	CAT activity (%)	
	unmethylated plasmid	methylated plasmid
pHAF-CAT	100	94
pHAF-I_{del}-CAT	100	32
pHAF-M_0-CAT	69	37
pHAF-M_1-CAT	26	24

100%=5 pmoles acetylated-CM/μg protein.

Functional analysis: methylation at site M_0 modulates gene expression

In order to test the significance of the correlation observed at site M_0 between methylation and gene inactivity, we carried out a functional analysis. This approach was based on DNA transfection experiments of *in vitro* methylated chimeric conctructions containing cloned AFP sequences (including intragenic sequences) linked to the bacterial reporter gene CAT. The first construction tested, pHAF-I_{del}-CAT (see Fig. 1B), was chosen because it was previously shown to be expressed at a high level when transfected in the human AFP-producing hepatoma cell line HepG2 (42). This construction contains an AFP fragment (position −800 to +2400) that includes the proximal enhancer (42,47), the promoter (28,29) and also the sites M_0 and M_{+1}. The plasmid was methylated *in vitro* by HpaII methylase and completion of methylation was verified by MspI/HpaII restriction. Either methylated or not, it was transfected by calcium phosphate precipitation in HepG2 cells, and 48 h later, CAT activity was measured. Our results (Fig. 6; Table 1) show that methylation causes a reproducible reduction in the level of CAT synthesis. To verify that this result was not due, either to the trivial methylation of MspI sites located in the vector sequence of the construction, or to a loss of transfectability of the modified plasmid, we performed a control experiment using a construction (pHAF-CAT, see Fig. 1B) which differs from pHAF-I_{del}-CAT by the fact that it only contains a very short segment of the AFP intragenic sequence (position +1 to +36) and thus lacks the sites M_0 and M_{+1}. Transient transfection experiments showed that the methylated and unmethylated pHAF-CAT plasmids directed the same level of CAT synthesis. It can thus be concluded that the reduction in CAT activity observed with the methylated pHAF-I_{del}-CAT plasmid, when compared to its unmethylated homologue, is indeed due to methylation of the sites M_0 and/or of M_{+1}.

Additional transfections were done to determine whether the reduction of CAT activity observed with the methylated pHAF-I_{del}-CAT plasmid was due to modification at the site M_0 or M_{+1}, or alternatively, whether methylation at both sites could have a cooperative effect. To do so, two new plasmids were constructed, each containing only one of the two sites (pHAF-M_0-CAT and pHAF-M_1-CAT, see Fig. 1B). These two plasmids were methylated and transfected as described previously. The results presented in Fig. 6 show that the methylated pHAF-M_0-CAT plasmid directs a level of CAT activity that is lower than its unmethylated homologue, while the level of CAT synthesis of the pHAF-M_1-CAT construction was not affected by DNA

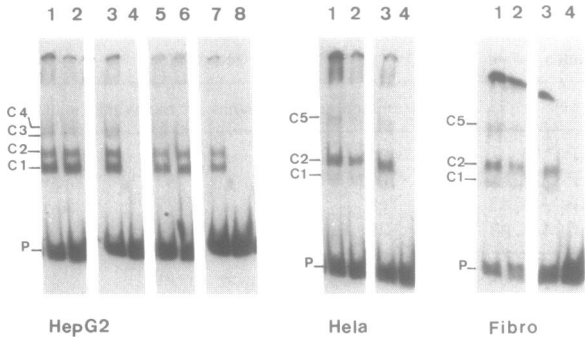

Fig. 7. Electrophoretic mobility shift assays performed with nuclear extracts from HepG2 hepatoma cells (left panel), HeLa cells (middle panel), and human fibroblasts (right panel). Lanes 1 and 2: unmethylated SfMb fragment (position +202 to +426) as labelled probe incubated nuclear proteins and 0.2 μg (1) or 0.5 μg (2) of pUC12 DNA; lane 3=lane 2; lane 4=same as lanes 2 and 3, plus a 20 fold molar excess of unlabelled probe; lanes 5 and 6: methylated SfMb fragment as labelled probe, incubated with HepG2 nuclear proteins and 0.2 μg (5) or 0.5 μg (6) of pUC12 DNA; lane 7=lane 1; lane 8: same as lanes 7 and 1, plus a 20 fold molar excess of methylated unlabelled probe. P=free probe; C1 to C5=shifted complexes.

modification. These data suggest that the inhibitory effect of DNA methylation on the expression of the pHAF-Idel-CAT plasmid was principaly due to modification at the site M_0. They are thus in agreement with the observation that methylation of the site M_0, but not of the site M_{+1}, is correlated with gene inactivity.

The results summarized in table 1 also show that the unmethylated pHAF-M_1-CAT construction is expressed at a lower level than the other three constructions used in this work. The pHAF-M_1-CAT plasmid differs from the pHAF-I_{del}-CAT by a 146 bp deletion that includes the site M_0, and it thus seems that this short intronic region could contain a positive *cis*-acting regulatory element of the AFP gene. In addition, the unmethylated pHAF-M_1-CAT plasmid and the methylated pHAF-I_{del}-CAT exhibit similar levels of CAT activity as if DNA methylation at the site M_0 was sufficient to obliterate the positive effect of this putaitve positive element.

Nuclear protein binding to the first intron segment containing the methylation site M_0

Since it was previously shown that DNA methylation may inhibit gene activity by preventing the binding of *trans*-acting regulatory factors to their target sequence, we investigated this possibility in the case of the AFP site M_0, by performing band shift assays on a DNA fragment centered on the site M_0 (SfMb fragment = a 224 bp SfanI-MboII fragment, from position +202 to position +426). In the first experiment, this radioactively labelled fragment was incubated with 1 μg of nuclear extracts from the AFP-producing hepatoma cells HepG2 and increasing concentrations of pUC12 DNA, chosen as a non- specific competitor DNA. Two major (C1 and C2) and two minor (C3 and C4) complexes, appearing as two doublets, were detected (Fig. 7, left panel, lanes 1,2). The formation of these complexes was specific: they disappeared totally by competition with a 20-fold molar excess of unlabelled probe (Fig. 7, left panel, lanes 3,4). The same experiment was then repeated using *in vitro* methylated SfMb fragment. As shown in Fig. 7 (left panel, lanes 5,6), DNA methylation at the site M_0 did not inhibit the formation of any of these complexes. In additon, competition by the methylated or unmethylated probe gave identical results (Fig. 7, left panel, lanes 3,4 and 7,8). These results suggest that DNA methylation at the site M_0 does not reduce gene expression by directly peventing the binding of one of the nuclear proteins detected in the above assays.

In addition, since AFP expression is a liver-specific trait, we determined whether the distribution of the factors binding to the SfMb fragment is tissue-specific or not. Band shift assays were repeated, using nuclear extracts of different origins (Hela cells and human fibroblasts). Fig. 7 (middle and right panels) shows that in the two cell types, the C1 and C2 complexes are also present. However, the C1 complex is much less abundant than in the hepatoma cell extracts (relative to the C2 complex). In addition, the C3/C4 doublet was not detectable, whereas a new minor band (or doublet) (C5) of lower electrophoretic mobility, was present in each of these two non-liver nuclear extracts. These data show that the factor generating the C1 complex is enriched in the hepatoma cell line, and that factor(s) generating the C3 and C4 complexes is (are) absent in non-liver cells.

DISCUSSION

This work shows that hypomethylation of a MspI site (M_0) located in the first intron of the mouse AFP gene is correlated with gene activity: the site M_0 is unmethylated or hypomethylated in AFP-producing cells (hepatoma cells and foetal liver), and hypermethylated in non-expressing cells (adult liver and fibroblasts; extensive methylation of the whole region was observed in fibroblasts). These results are in agreement with data obtained by others in the 5′ region of the rat gene, where an excellent correlation was found between gene expression and hypomethylation of a MspI site located at position +3, i.e. in the first exon of the gene (35,36). Cessation of AFP gene expression during liver development is thus associated with *de novo* methylation in the intragenic region. We have also shown that *in vitro* methylation at the site M_0 specifically reduces the activity of the AFP promoter when transfected in AFP-expressing cells, and that a DNA fragment containing this site binds nuclear factors.

It has been shown in different cases, including the AFP gene (36,48−51), that methylation occurs only after the gene has become inactive, suggesting that *de novo* methylation generally is a secondary event in genetic repression. However, our results on the effect of *in vitro* methylation on the activity of transfected plasmids suggest that DNA modification at this site plays a functional role in AFP gene regulation. The simplest interpretation is that DNA modification at the site M_0 constitutes a mechanism implicated in the maintenance of the inactive state of the AFP gene in adult hepatocytes. This *cis*-acting negative mechanism might participate in the imprinting which seems to be imposed on the adult hepatocyte AFP gene, as suggested by cell fusion experiments showing that active and inactive AFP genes coexist in somatic cell hybrids formed between AFP-producing hepatoma cells and normal adult hepatocytes (30).

How could DNA methylation inhibit gene transcription? DNA methylation was shown in some cases to repress gene transcription by preventing the binding of *trans*-regulatory factors to their recognition sequence (13−16). Our results suggest that this is not the case here. Indeed, while band shift experiments revealed the binding of different factors to the DNA region flanking the site M_0, DNA methylation at this site did not prevent the formation of any of these complexes. However, it is possible that our experiments did not detect a factor whose

binding might be affected by DNA methylation. Alternatively, DNA methylation might indirectly modify the structure or the interactions of factors bound to the methylated region.

The data presented in this study suggest that the M_0 site containing 146 bp fragment (SacI−MboII fragment, deleted in pHAF-M_1-CAT) behaves as a positive regulatory element, when the M_0 site is unmethylated. Indeed, the pHAF-M_1-CAT plasmid, which lacks this segment, is expressed at a lower level than the pHAF-I_{del}-CAT construction, which contains this segment (Fig. 1B). In this respect, it is striking that this 146 bp fragment contains a B1 element (a family of mouse repetitive sequences) which, when transfected in HepG2 cells, was shown to be transcribed, in the antisense direction (52,53). RNA polymerase III transcribed repetitive sequences may act as *cis*-acting regulatory elements of RNA polymerase II transcribed genes, as was previously shown in a few cases (54,55). The lower CAT activity directed by the pHAF-M_1-CAT construction might be due to the loss this repetitive sequence. Strikingly, the pHAF-I_{del}-CAT construction, when methylated on the site M_0 is expressed at lower level, as if the positive effect exerted by the 146 bp fragment was abrogated. The M_0 site being located in the promoter of this B1 element, methylation of this site might down-regulate B1 element transcription, and, indirectly, activity of the AFP promoter. However, the 146 bp fragment also contains several other potential regulatory sequences (AP1-like, HNF3-like site, HNF4 and CEBP-like sites)(56−59) which might be targets of transcriptional factors.

Finally, the first intron of the AFP gene contains, upstream the 146 bp segment, the hexanucleotide TGTCCT, which resembles the 3′ end of the consensus sequence of hormone responsive elements (HRE) (TGTTCT and TGACCT for the glucocorticoïd and estradiol response elements, GRE and ERE, respectively) (60,61). A TGTCCT element is also found in the promoter region of the AFP gene and is known to be involved in hormonal controls (26,47 and references therein). Like this upstream element, the intronic HRE might be a target of steroid hormone receptors, and in particular of glucocorticoids.

It is intriguing that several potential binding sites for various transcriptional factors are present in the first intron of the AFP gene. Together with our results, these observations suggest that the first intron of the AFP gene might participate in the control of gene activity.

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