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DNA Methylation and Specific Protein-DNA Interactions

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DNA methylation and specific protein–DNA interactions

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The effect of site-specific CpG methylation on the binding of a HeLa cell transcription factor (MLTF) has been studied. Methylation at a central site within the binding sequence for the factor is found to strongly inhibit binding of MLTF and to inhibit MLTF-dependent transcription *in vitro*. Methylation of a CpG site only six bases away has no demonstrable effect on binding. When the central CpG is methylated on one strand only, binding of MLTF is partially inhibited. The effects of methylation on the binding of MLTF and on the binding of a limited number of other proteins to DNA demonstrate that methylation in some cases exerts highly specific effects on gene expression. Site-specific demethylation has previously been seen to be associated with the promoter and upstream regions of genes, which suggests that sequence-specific DNA binding proteins that interact with promoters may be involved in the demethylation process. Specific demethylation of a human metallothionein 2A promoter-chloramphenicol acetyl transferase gene construct in mouse L-cells demonstrates that the promoter region sequences of this gene are sufficient to programme their own demethylation.

Introduction

A substantial body of evidence indicates that methylation of CpG dinucleotides in vertebrate DNA is involved in the regulation of gene expression (for reviews see Doerfler (1983); Holliday (1987); Cedar (1988). The pattern of methylation of different CpG sites within many genes studied has been shown to be tissue specific, that is, to undergo specific developmental changes. It has been found generally, but not universally, that lack of methylation of a gene correlates with its expression in a particular cell type. It is well established that methylation can cause an inhibition of transcription (see below), but it is still not clear how and to what extent methylation is used to control gene expression during development and whether changes in methylation of genes are involved as primary switches in their expression or only as a means of stably maintaining established states.

The most direct evidence that methylation can cause inhibition of expression comes from experiments where DNA is methylated *in vitro* and assayed for activity after introduction back into cells. In a number of systems methylation has been shown to repress transcription. Transfection experiments have also demonstrated that the natural methylation of the inactive X chromosome inhibited expression of X-linked genes. It has also been established that critical methylation sites are normally located in the promoter region at the 5′ end of genes studied, whereas methylation within transcribed sequences is generally of much less effect (Busslinger *et al.* 1983; Kruczek & Doerfler 1983; Langner *et al.* 1984; Keshet *et al.* 1985; Murray & Grosveld 1987). This shows that methylation acts primarily by blocking initiation rather than elongation of transcription. Inhibition could arise indirectly, as has been suggested, through the preferential packaging of methylated regions of DNA into inactive chromatin (Keshet *et al.*

1986; Murray & Grosveld 1987). Indirect inhibition of a methylated thymidine kinase gene has been shown by its delayed inactivation following microinjection; inhibition is immediate, however, if the DNA is pre-formed into chromatin before injection (Buschhausen *et al.* 1985, 1987). For most of the examples where DNA has been methylated using restriction methyltransferases, it is likely that inhibition is mediated through this mechanism. It has also recently been shown that methylation can inhibit transcription by directly blocking the binding of positively acting transcription factors (Kovesdi *et al.* 1987; Watt & Molloy 1988).

This paper considers the role of site-specific CpG methylation in the regulation of gene expression and the possible function of sequence-specific DNA binding proteins or transcription factors in mediating changes in methylation patterns.

Methylation inhibits binding of the transcription factor MLTF

The transcription factor isolated from HeLa cells was initially identified because it is required for optimal transcription from the adenovirus major late promoter (AdMLP). MLTF has been shown to bind to the promoters of the mouse metallothionein I and rat γ-fibrinogen genes and to activate transcription *in vitro*. Bindings sites for MLTF are also found in promoters of a number of other cellular genes, including the human growth hormone gene and N-*ras* oncogene (see Watt & Molloy (1988) for references). Because two CpG sites are contained in

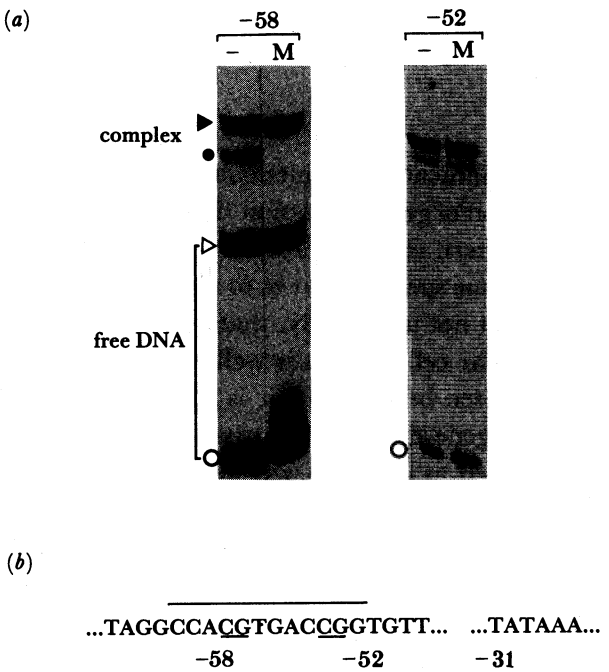

Figure 1. Binding of MLTF to methylated DNAs. (*a*) DNAs were incubated with MLTF-containing fractions and free DNA separated from DNA–MLTF complexes by gel electrophoresis (for experimental details see Watt & Molloy (1988)). Free DNA of the test fragment (○) and a longer control fragment (▷) are indicated, as are complexes of MLTF with the test fragments (●) and the control fragment (▶). The test DNA was either methylated (M) at the site shown above the tracks or was unmethylated (−). The control, unmethylated DNA was co-incubated with the test DNA in the left pair of tracks. For the right pair of tracks a partially purified fraction containing MLTF was used, whereas for the left pair of tracks it had been further purified by sequence-specific DNA affinity chromatography. (*b*) Sequence of the AdMLP with the binding site for MLTF overlined and the −58 and −52 CpG sites underlined. (Figure drawn in part from Watt & Molloy (1988), with permission of Genes & Development.)

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the region of the AdMLP, which is protected from DNase I when MLTF is bound, it was interesting to examine the effects of methylation at these sites on the binding of MLTF (see figure 1).

One of these CpG sites (−52 site) is contained within an *Hpa*II recognition sequence and so could readily be studied by using DNA fragments methylated specifically at the site using *Hpa*II methylase. The other site (−58 site) could not be specifically enzymatically methylated, so overlapping oligonucleotides were synthesized with specific incorporation of 5-methylcytosine when required. These were end-filled to generate 100 base pair (b.p.) DNA fragments, which were then used to study MLTF binding. Binding of MLTF to DNA was assayed by using a gel retardation assay in which MLTF–DNA complexes that have a slower mobility through the acrylamide gel are separated from free DNA. In some experiments a control unmethylated DNA fragment of different length was co-incubated with the test fragments and complexes that formed on the two DNAs can be readily distinguished (figures 1 & 2). Methylation of DNA at the −58 site was seen to strongly inhibit binding of MLTF (figure 1) and also transcription from the AdMLP *in vitro* (Watt & Molloy 1988). In contrast, methylation at the −52 had no apparent effect on binding (figure 1), consistent with the lack of effect of *Hpa*II methylation on transcription previously observed (Jove *et al.* 1984). The major features of the binding of MLTF to methylated DNAs (Watt & Molloy 1988) can be summarized as follows.

1. the effect of CpG methylation is highly site-specific and is essentially like a point mutation within the binding site.

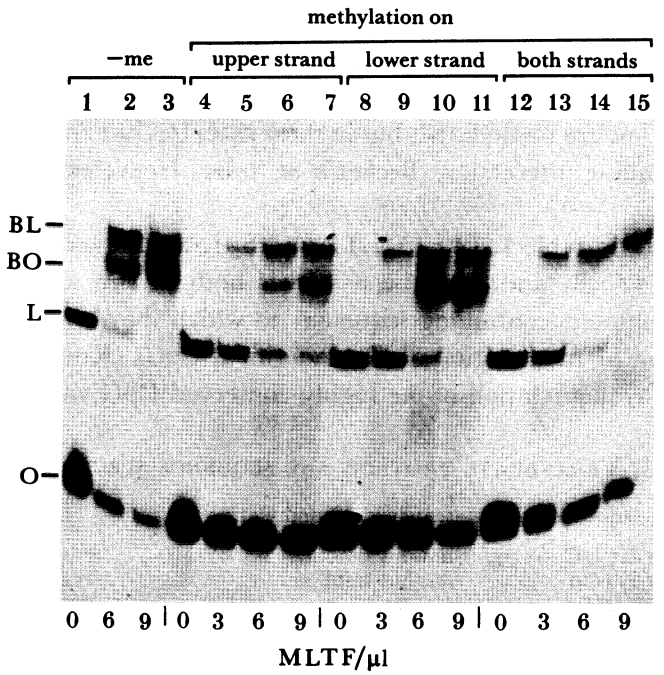

FIGURE 2. Binding of MLTF to hemi-methylated DNAs. Double-stranded oligomer DNAs (O) methylated on the upper or lower strand only, on both strands or on neither strand were co-incubated with increasing amounts of MLTF in the presence of a control unmethylated DNA (L) and free DNA resolved from the two MLTF-DNA complexes (BO and BL) by gel electrophoresis. Methylation on the oligomer DNA was none, −me lanes 1–3, upper strand, lanes 4–7, lower strand, lanes 8–11, and both strands, 12–15. (Figure from Watt & Molloy (1988) with permission of Genes & Development.)

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2. Methylation decreases the equilibrium level of binding of MLTF, having a major effect on the dissociation rate of the MLTF–DNA complex.

3. Methylation may exert its effect on binding directly, with the methyl groups which sit in the major groove of the DNA helix interfering with specific base recognition contacts. Alternatively, as methylation alters the local helix geometry, local alteration of DNA structure may reduce binding affinity.

4. DNA methylated on only one strand binds MLTF but with reduced affinity compared with unmethylated DNA (figure 2). Possible implications of this will be considered later.

Methylation of the central CpG of the MLTF binding site within the promoter of a cellular gene would thus be expected to directly inhibit transcription. We are currently examining whether methylation of the MLTF site within the promoters of the human growth hormone gene and the N-*ras* oncogene is correlated with their expression.

Effects of cytosine methylation on binding to DNA of other proteins

Post-replicative methylation of cytosine at the 5′ position occurs widely in both prokaryotes and eukaryotes. As the methyl group sits in the major groove of the DNA it provides an obvious feature which could be used potentially either to enhance specific protein binding or to interfere with specific protein–DNA interactions. Methylation of CpG dinucleotides has also been shown to influence DNA structure and can increase the propensity for alternating purine–pyrimidine tracts to adopt a left-handed helical configuration (Behe & Felsenfeld 1981) as well as causing local alterations in helix geometry (Fox 1986; Watt & Molloy 1988).

In higher eukaryotes the interaction of CpG methylated DNA with specific proteins has only been studied in a limited number of cases. Table 1 lists vertebrate proteins that bind in a sequence-specific manner to DNA and for which the effect of methylation on binding has been studied. Additional proteins have been shown to bind to DNA when CpG sites within their DNase 1 footprint regions are methylated, but in these cases it is not known whether these bases are involved in the binding interaction (Becker *et al.* 1987; Hoeveler & Doerfler 1987; Saluz *et al.* 1988).

Three of the proteins in table 1, E2F, MLTF and Sp1, are sequence-specific transcription factors found in HeLa cells and have been shown both *in vivo* and *in vitro* to stimulate transcription from promoters containing sequences to which they bind. Binding of both E2F and MLTF is inhibited by methylation of the underlined CpGs (Kovesdi *et al.* 1987; Watt & Molloy, 1988), whereas methylation of the highly conserved CpG within the binding site for

Table 1. Vertebrate proteins and sequence-specific binding

protein	binding sequence[a]	consequence of methylation	reference
E2F	TTTCGCGC	inhibits binding	Kovesdi *et al.* (1987)
MLTF	CCACGTGA	inhibits binding	Watt & Molloy (1988)
Sp1	GGGCGG	no effect	Ben-Hattar *et al.* (1988)
			Harrington *et al.* (1988)
			Höller *et al.* (1988)
TAT factor	..GCGC..	inhibits binding	Becker *et al.* (1987)
rooster factor	AAGCGATA	required for binding	Saluz *et al.* (1988)
MDBP	..CGRCG..	required for binding	Wang *et al.* (1986)

[a] For TAT factor and rooster factors sequences shown are central within the footprint regions, whereas for MDBP the sequence is the core of the binding site but a wider region is involved.

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Sp1 does not directly block factor binding or transcription (Ben-Hattar *et al.* 1988; Harrington *et al.* 1988; Höller *et al.* 1988). Binding of a protein to a site about 1000 b.p. upstream of the initiation site of the rat tyrosine amino transferase (TAT) gene is also inhibited by CpG methylation within its binding site (Becker *et al.* 1987). Although this protein has not been shown to be involved in regulation of the gene, demethylation of its binding site correlates with the onset of transcription. Two proteins have been identified that require methylation of CpG sites for binding. The function of one of these, MDBP, is not known (Supakar *et al.* 1988), whereas the other, 'rooster factor', binds to a site immediately 3′ to the transcription initiation site of the avian vitellogenin gene (Saluz *et al.* 1988). This factor is found in liver cells of roosters but not hens and its binding thus correlates with the inactive state of the gene. Another protein, NHP4, which binds to the same sequence region, is unaffected by methylation and is distributed widely in cells both expressing and not expressing the vitellogenin gene. A further protein which requires CpG methylation for DNA binding has recently been identified by Bird *et al.* (this symposium). These examples illustrate the effect of CpG methylation is specific to each protein–DNA interaction and may be neutral, inhibitory or required for binding. So far, the examples in which a specific effect of methylation has been seen are consistent with the generally observed repression caused by methylation. Indeed, it would perhaps be surprising to find methylation stimulating the binding of specific positive regulatory factors in direct competition with the apparently sequence-independent packing of methylated DNA into inactive chromatin. It is also clear that studies of gene methylation *in vivo* that utilize restriction enzymes to probe the status of methylation may fail to analyse critical binding sites.

The question of whether the effects of methylation on binding of specific proteins are important control points in developmental gene regulation remains an open one. For both the TAT factor and the 'rooster factor', demethylation of the specific site correlates with gene expression; expression of the 'rooster factor' itself appears to be developmentally regulated, whereas TAT factor, which is inhibited from binding by methylation, is present in both expressing and non-expressing cells. Many transcription factors bind to sequences that lack CpG sites, but others, such as Sp1, bind whether the site is methylated or not, so that their binding can only be indirectly affected by methylation. If CpG methylation is used as a specific developmental switch it is likely to involve interaction with sequence-specific DNA binding proteins as discussed below.

Site-specific demethylation

The mechanisms by which changes in methylation patterns occur during development are very poorly understood. It had long been realized that demethylation could occur passively through a failure to methylate hemi-methylated sites after DNA replication. However, it has also been shown that demethylation can occur without DNA replication. In the chick vitellogenin system, demethylation occurs in a site- and strand-specific manner (Saluz *et al.* 1986). In murine erythroleukaemia cells, induction to differentiate is followed by extensive genome-wide demethylation and then by remethylation as an apparently early part of the reprogramming of the cells for terminal differentiation (Razin *et al.* 1988).

Whether occurring through an active or a passive process, the final site-specificity of DNA demethylation is probably dependent on the action of sequence-specific DNA binding proteins. For the chick vitellogenin gene, demethylation takes place in the vicinity of sites occupied by

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sequence-specific DNA binding proteins, the oestradiol receptor and glucocorticoid receptor (Saluz *et al.* 1988). Two systems have been described in which DNA methylated *in vitro* undergoes demethylation following transfection into cells, the actin gene into myoblast cells (Yisraeli *et al.* 1986) or the dihydrofolate reductase (DHFR) gene into CHO cells (Shimada *et al.* 1987); for further examples see Cedar *et al.* (this symposium). In both cases, specific demethylation events that correlate with active expression of the genes are localized at the promoter regions. This suggests that transcription factors themselves, or related DNA-binding proteins that bind to the promoters, may be involved in the process.

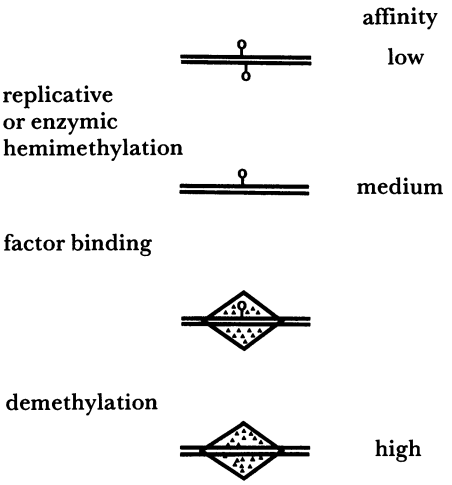

FIGURE 3. A possible scheme for transcription factor-mediated demethylation. (♀) 5-methyl cytosines; (▲-diamond) transcription factor.

A possible way in which transcription factors could be involved in a developmentally programmed demethylation process is suggested by the binding characteristics of MLTF to methylated and hemi-methylated DNAs (figure 3). The initial methylated state of the MLTF site has a very low affinity for the factor; after each round of replication the site is present in the cell transiently in a hemi-methylated state. Alternatively, hemi-methylation could occur through an active process. This represents a site of intermediate affinity and, dependent on its level within the cell, MLTF could occupy the site and hence preclude action of the methylase enzyme. After a further round of replication, or via an active process, the site would become fully demethylated and therefore a heritable high affinity site. This effect at a specific methylation site would be analogous to the 'locking' or re-enforcement mechanism proposed for the more general role of methylation on maintaining gene regions in an inactive chromatin configuration, but envisages the transcription factors themselves as mediators in the unmasking of their own sites. It has already been proposed that Sp1, which is insensitive to methylation, may function to hold its binding sites in an unmethylated state (Höller *et al.* 1988). In this case, and for other transcription factors that are insensitive to CpG methylation, competition between binding of factors and packaging of DNA into inactive chromatin may determine whether the promoter region will be active and/or demethylated.

We have recently found that the promoter of the human metallothionein 2A gene (MET2A) is specifically demethylated in mouse L cells. The promoter of four genes, the MET2A gene, Herpes virus thymidine kinase (TK) gene, a chick feather keratin gene and the AdMLP, were

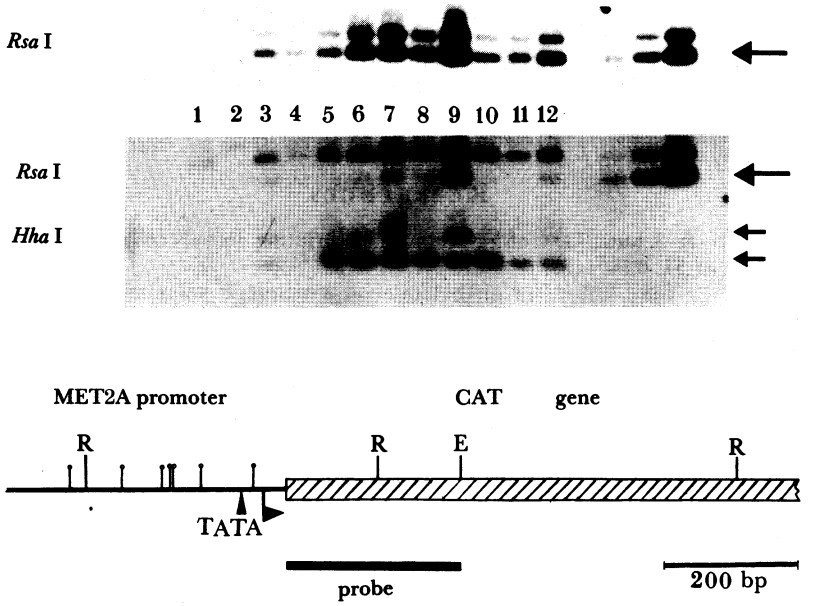

FIGURE 4. Demethylation of MET2ACAT gene promoter. Shown are Southern blots of genomic DNA from 12 independent colonies isolated after transfection of *Hha*I-methylated MET2ACAT gene into mouse L cells, along with quantitation standards of *Hha*I-methylated plasmid pMET2ACAT in the right-hand four tracks. DNA was cut with *Rsa*I (top) or *Rsa*I plus *Hha*I (lower). At the bottom is a map of the promoter-gene region showing the *Rsa*I (R) and *Eco*RI (E) sites, sites of *Hha*I-methylation (↑) and the TATA box and transcription start site (↳); a bar indicates the region used as a probe. In the autoradiographs the longer *Rsa*I fragment that contains no *Hha*I sites is that from within the CAT gene (540 b.p.). The lower band (large arrow) covers the promoter and 5′ end of the CAT gene; bands indicated by smaller arrows are fragments extending from the *Rsa*I site in the CAT gene to the nearest and second *Hha*I sites in the promoter.

linked to the chloramphenicol acetyl transferase (CAT) reporter gene and methylated *in vitro* using *Hpa*II and/or *Hha*I methylases. After co-transfection with a plasmid carrying a selectable marker, a number of individual colonies for each construct were isolated and CAT activity measured. In contrast to the other constructs the MET2ACAT colonies were found to be expressing CAT levels similar to those seen for colonies derived from transfection with unmethylated DNA. On examination of the DNA from 12 separate colonies containing from less than 1 to more than 100 gene copies per haploid genome it was seen that all colonies had undergone extensive demethylation in the promoter region (figure 4). The demethylation was extensive, but not always complete; there is a tendency for more of the integrated copies to remain fully or partially methylated in those colonies carrying a larger number of genes. This is suggestive that the capacity to demethylate can be saturated. By contrast, and in agreement with previous experiments in which methylated genes have been transfected into mouse L-cells (see, for example, Kruczek & Doerfler (1983); Keshet *et al.* (1985); Murray & Grosveld (1987)), methylation was stably maintained on the integrated DNA of the other promoter constructs.

As these constructs differ only in the inserted promoter region, this shows that the promoter region from the MET2A gene (530 b.p.) can programme its own demethylation in mouse L-cells. The promoter itself is CG-rich and has characteristics of a small CpG island and in this respect is similar to the DHFR gene studied by Shimada *et al.* (1987). The promoter region of the MET2A gene has been extensively characterized and the binding sites determined for a

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number of *trans*-acting factors that are required for its optimal or controlled expression (Imagawa *et al.* 1987; Mitchell *et al.* 1987). Hence, this provides an opportunity to determine the sequence regions required for promoter demethylation and to compare them with those contributing directly to transcriptional activity.

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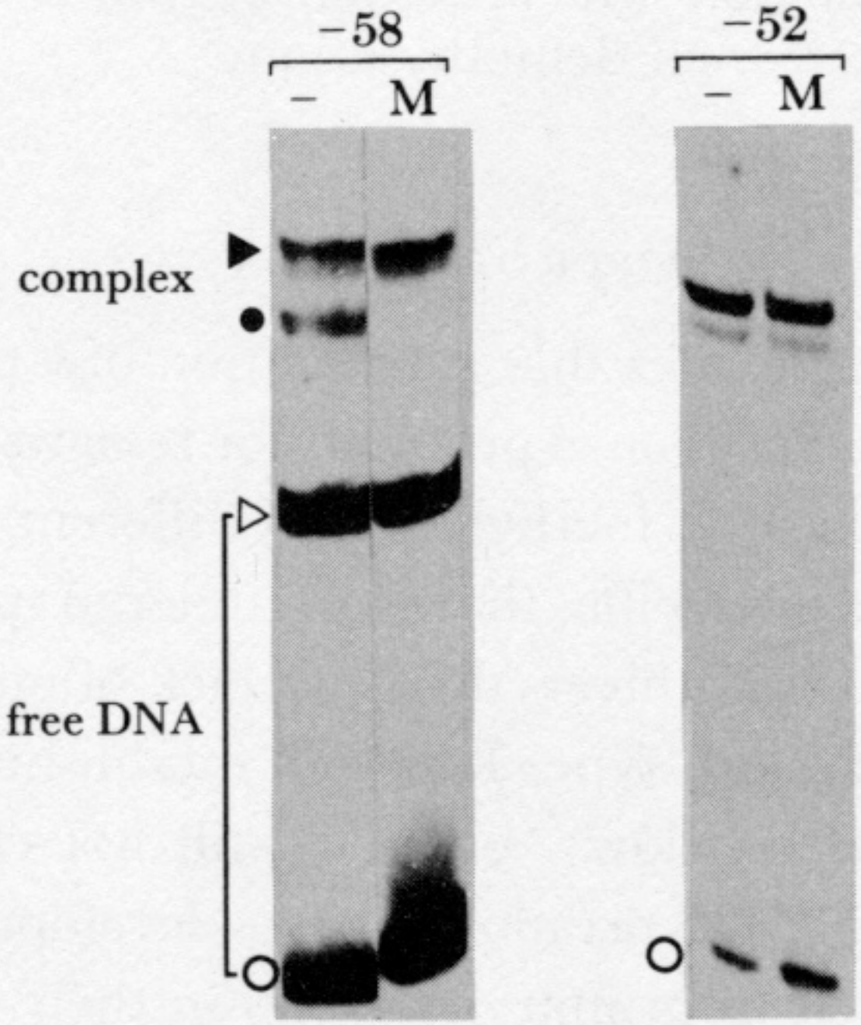
(*a*)



(*b*)

...TAGGCCACGTGACCGGTGTT... ...TATAAA...

−58 −52 −31

IGURE 1. Binding of MLTF to methylated DNAs. (*a*) DNAs were incubated with MLTF-containing fractions and free DNA separated from DNA–MLTF complexes by gel electrophoresis (for experimental details see Watt & Molloy (1988)). Free DNA of the test fragment (○) and a longer control fragment (▷) are indicated, as are complexes of MLTF with the test fragments (●) and the control fragment (▶). The test DNA was either methylated (M) at the site shown above the tracks or was unmethylated (−). The control, unmethylated DNA was co-incubated with the test DNA in the left pair of tracks. For the right pair of tracks a partially purified fraction containing MLTF was used, whereas for the left pair of tracks it had been further purified by sequence-specific DNA affinity chromatography. (*b*) Sequence of the AdMLP with the binding site for MLTF overlined and the −58 and −52 CpG sites underlined. (Figure drawn in part from Watt & Molloy (1988), with permission of Genes & Development.)

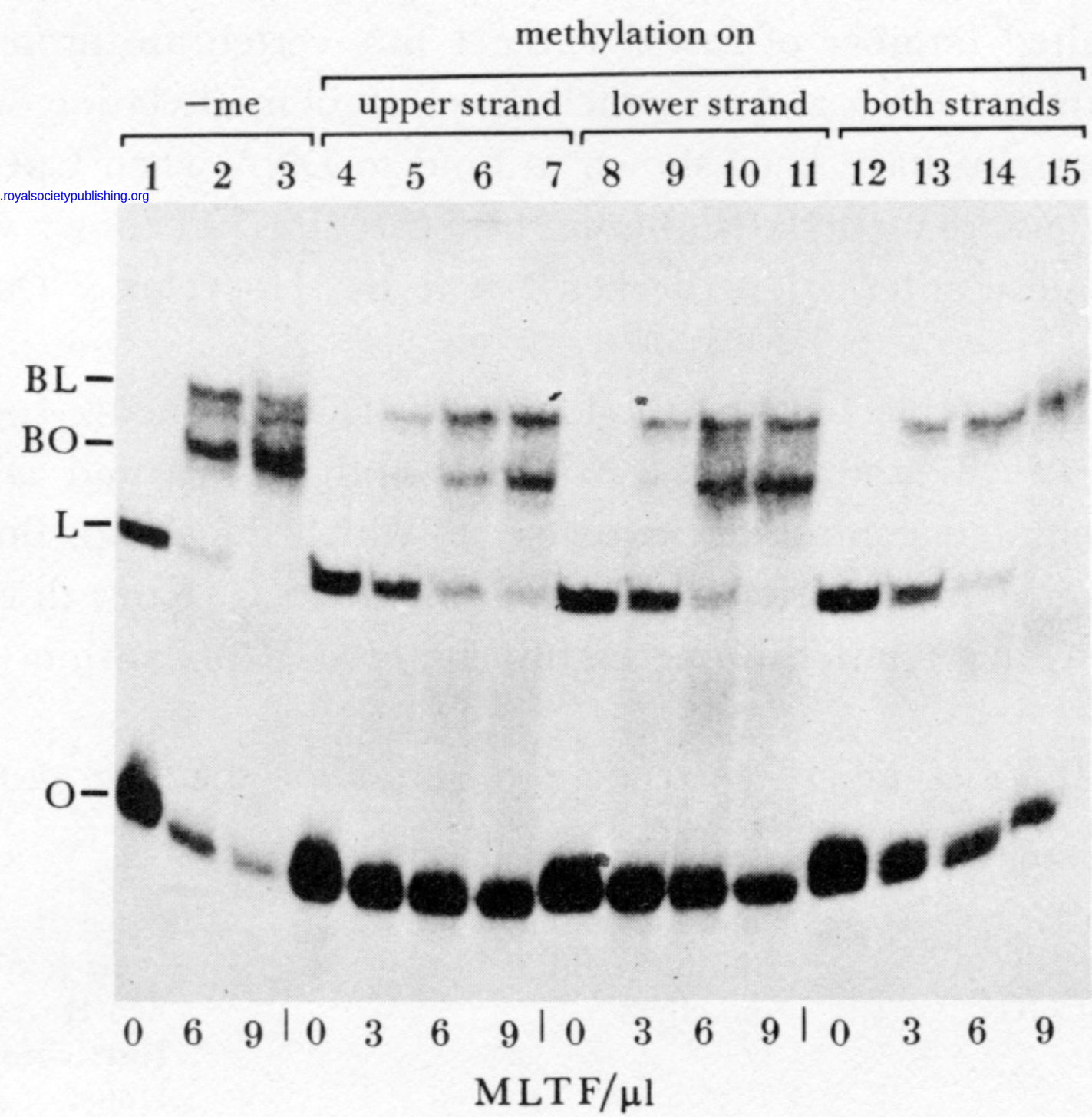


FIGURE 2. Binding of MLTF to hemi-methylated DNAs. Double-stranded oligomer DNAs (O) methylated on the upper or lower strand only, on both strands or on neither strand were co-incubated with increasing amounts of MLTF in the presence of a control unmethylated DNA (L) and free DNA resolved from the two MLTF-DNA complexes (BO and BL) by gel electrophoresis. Methylation on the oligomer DNA was none, −me lanes 1–3, upper strand, lanes 4–7, lower strand, lanes 8–11, and both strands, 12–15. (Figure from Watt & Molloy (1988) with permission of Genes & Development.)

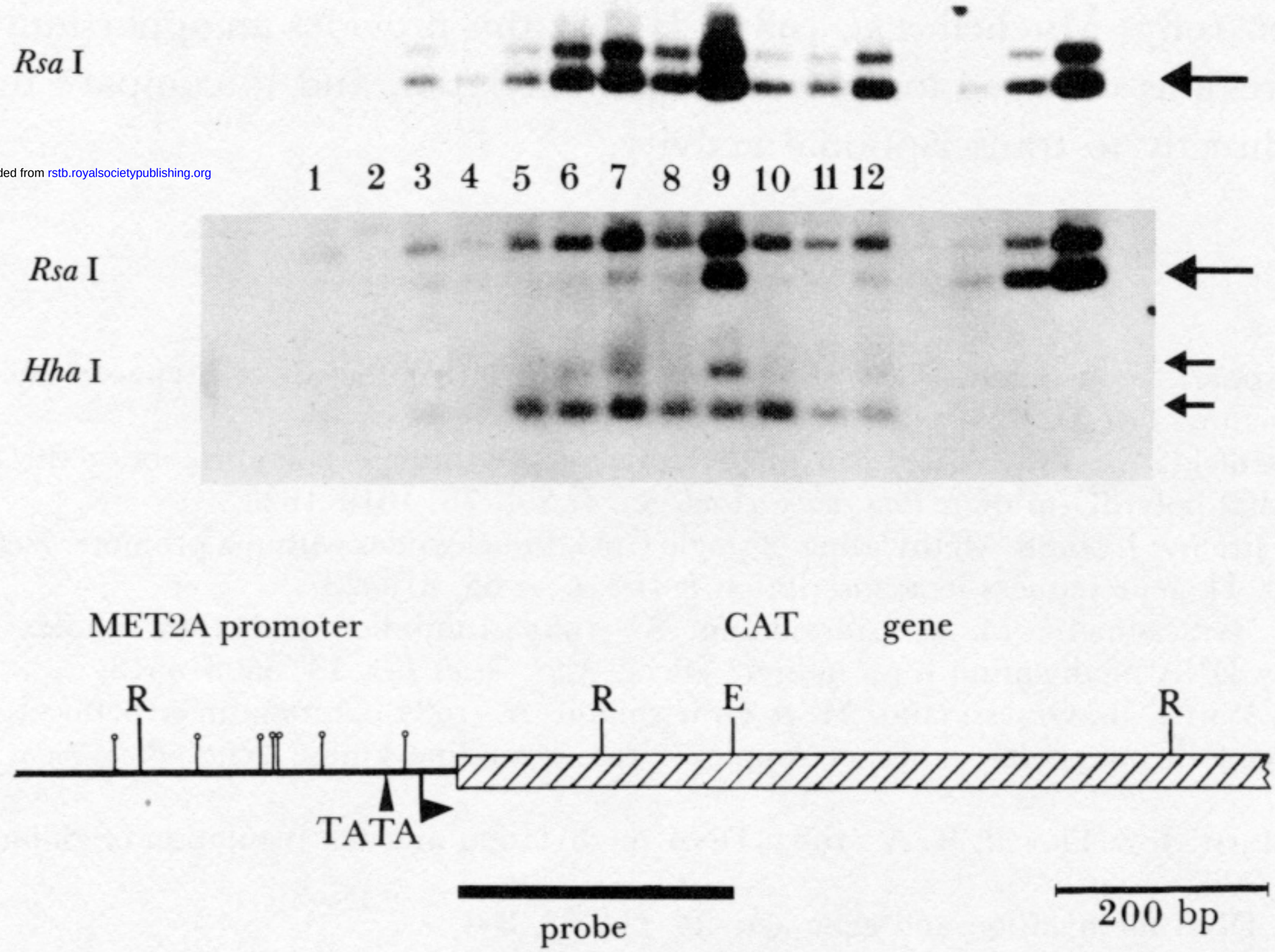


FIGURE 4. Demethylation of MET2ACAT gene promoter. Shown are Southern blots of genomic DNA from 12 independent colonies isolated after transfection of *Hha*I-methylated MET2ACAT gene into mouse L cells, along with quantitation standards of *Hha*I-methylated plasmid pMET2ACAT in the right-hand four tracks. DNA was cut with *Rsa*I (top) or *Rsa*I plus *Hha*I (lower). At the bottom is a map of the promoter-gene region showing the *Rsa*I (R) and *Eco*RI (E) sites, sites of *Hha*I-methylation (↑) and the TATA box and transcription start site (↳); a bar indicates the region used as a probe. In the autoradiographs the longer *Rsa*I fragment that contains no *Hha*I sites is that from within the CAT gene (540 b.p.). The lower band (large arrow) covers the promoter and 5′ end of the CAT gene; bands indicated by smaller arrows are fragments extending from the *Rsa*I site in the CAT gene to the nearest and second *Hha*I sites in the promoter.