Novel relationships among DNA methylation, histone modifications and gene expression in Ascobulus

José L. Barra, Allyson M. Holmes, Annie Grégoire, Jean-Luc Rossignol, Godeleine Faugeron

Novel relationships among DNA methylation, histone modifications and gene expression in *Ascobolus*

Running title: Gene silencing, DNA methylation, chromatin changes

Keywords: *Ascobolus*, chromatin, DNA methylation, gene silencing, histone modifications, nucleosomes

José L. Barra [1, 2, *], Allyson M. Holmes [1, 3], Annie Grégoire [1], Jean-Luc Rossignol [1] and Godeleine Faugeron [1]

[1] Institut Jacques Monod, UMR 7592 CNRS / Université Paris 7 / Université Paris 6, Tour 43, 2 Place Jussieu, 75251 Paris Cedex 05, France

[2] Present address: Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC), Departamento de Química Biológica, Facultad de Ciencias Químicas, Ciudad Universitaria, Universidad Nacional de Córdoba, Córdoba, Argentina

[3] Present address: Unité de Dynamique du Génome, URA 1644 CNRS / Institut Pasteur Département Structure et Dynamique des Génomes, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France

* To whom all correspondence and proofs should be sent:
José L. Barra, Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC), Departamento de Química Biológica, Facultad de Ciencias Químicas, Ciudad Universitaria, Universidad Nacional de Córdoba, Córdoba, Argentina
Phone: 54-351-4334168
Fax: 54-351-4334074
E-mail: jlbarra@mail.fcq.unc.edu.ar

Summary

By studying *Ascobolus* strains methylated in various portions of the native *met2* gene or of the *hph* transgene, we generalized our previous observation that methylation of the downstream portion of a gene promotes its stable silencing and triggers the production of truncated transcripts which rarely extend through the methylated region. In contrast, methylation of the promoter region does not promote efficient gene silencing. The chromatin state of *met2* methylated strains was investigated after partial micrococcal nuclease (MNase) digestion. We show that MNase sensitive sites present along the unmethylated regions are no longer observed along the methylated ones. These chromatin changes are not due to the absence of transcription. They are associated, in both *met2* and *hph*, with modifications of core histones corresponding, on the N terminus of histone H3, to an increase of dimethylation of lysine 9 and a decrease of dimethylation of lysine 4. Contrary to other organisms, these changes are independent of the transcriptional state of the genes, and furthermore, no decrease in acetylation of histone H4 is observed in silenced genes.

Introduction

Cytosine methylation is a major DNA modification, which plays an essential role in many organisms. It has been associated with genetic disorders (Egger et al., 2004), and mutations that reduce methylation levels result in embryonic lethality in mammals (Li et al., 1992), in various pleiotropic phenotypes in plants (Finnegan and Kovac, 2000) and interrupts the sexual cycle in the fungus *Ascobolus immersus* (Malagnac et al., 1997).

In mammals, genomic methylation occurs mostly in the context of CpG dinucleotides and affects about 70% of them. DNA methylation is known to act in transcription-mediated gene silencing. There are two general mechanisms by which CpG methylation is believed to repress transcription (reviewed in Bird, 2002). The first one involves modification of cytosines in the recognition sequence of DNA binding proteins, which in turn inhibits their binding to their cognate DNA sequences and thus denies access to regulatory regions. The second one, contrary to the first mechanism, involves proteins that specifically bind to methyl-CpG dinucleotides. Several methyl-CpG binding proteins have been identified and some have been shown to associate with histone deacetylases (Ng et al., 1999; Wade et al., 1999) or histone methyltransferases (Fujita et al., 2003; Fuks et al., 2003). Links between histone modifications and DNA methylation have been found in many organisms. In *Arabidopsis* and *Neurospora*, methylation of lysine 9 of histone H3 (H3-Lys9) is a prerequisite for DNA methylation (Tamaru and Selker, 2001; Jackson et al., 2002; Malagnac et al., 2002). In mammals, H3-Lys9 methylation appears to direct DNA methylation to pericentromeric heterochromatin (Lehnertz et al., 2003), and it has also been shown that DNA methylation can trigger H3-Lys9 methylation (Johnson et al., 2002; Soppe et al., 2002; Tariq et al., 2003).Although it is not yet clear what initiates the recruitment of the different epigenetic modifiers to their specific target sequences, it is generally assumed that DNA methylation represses gene expression by preventing transcription initiation (Bird and Wolffe, 1999).

Studies performed in two fungi point to the existence of another type of transcriptional effect of methylation, which could impair transcript elongation (Barry et al., 1993; Rountree and Selker, 1997). The genomes of these fungi are scanned for DNA sequences that are duplicated in cells entering the sexual phase (Rossignol and Faugeron, 1994). In *Neurospora*, duplicated copies are riddled with numerous C:G to T:A transitions by the RIP (Repeat-Induced Point mutation) process, and the sequences altered by RIP are typically methylated (Cambareri et al., 1989). In *Ascobolus*, the MIP (Methylation Induced Premeiotically) process, related to RIP, results in methylation of the duplicated copies without mutation (Rhounim et

al., 1992; Goyon et al., 1994). In both cases, methylation can involve all Cs, even those that do not belong to symmetrical motifs. In addition, methylation resulting from MIP is coextensive with the length of the duplication (Barry et al., 1993; Goyon et al., 1994). As a result of these processes, genes carried by duplications are silenced and the silencing persists even when a single copy has been inherited (Selker and Garrett, 1988; Rhounim et al., 1992). It was shown, using run-on nuclear assays, that methylation extending over the entire *am* and *mtr Neurospora* genes does not significantly inhibit the initiation of transcription, but affects transcript elongation (Rountree and Selker, 1997). In *Ascobolus*, the ability to direct in a predictable manner the *de novo* methylation of gene segments, allowed us to show that truncated transcripts were formed when methylation began downstream from the transcription start site of the *met2* gene (Barry et al., 1993). This gene, which encodes homoserine-O-transacetylase, is required for methionine biosynthesis (Goyon et al., 1988). The size of the truncated transcripts was the length expected if methylation were to block transcript elongation. When methylation spanned the promoter and the upstream part of the coding region, no transcripts were observed. This could be explained by methylation triggering a block of transcript elongation at the 5' end of the coding region. However, an effect of promoter methylation upon transcription initiation could not be excluded.

Using the chimeric foreign gene *hph*, which endows *Ascobolus* with hygromycin resistance, we extended our previous observation of methylation triggering transcript truncation, and we addressed the question whether methylation of the promoter region alone could also prevent transcription. By using both the native *met2* gene and the *hph* transgene, we have shown that methylation of the promoter regions has only a slight effect on gene silencing.

We have also compared the chromatin state of different methylated regions of *met2*, with that of the corresponding unmethylated regions. Chromatin changes, revealed by MNase footprinting analyses, were found in the methylated portion, and independently of the transcriptional state. Finally, we asked whether chromatin changes are associated with histone modifications. Methylation and acetylation of histones were monitored by chromatin immunoprecipitation analyses, using antibodies directed against methylated histone H3-lysine 4 and 9 and various isoforms of acetylated histone H4. DNA methylation and chromatin changes are found to be associated with histone H3-Lys9 methylation, but not with histone H4-hypoacetylation.

Results

Production of truncated transcripts in genes methylated in their downstream region

We first repeated some of the experiments reported in our previous work (Barry et al., 1993). Strains M-Dup1, M-Dup2 and M-Dup3, harboring the duplication of fragments 1M, 2M and 3M, respectively (Fig. 1) were crossed with the FB35 tester strain. One strain silenced for *met2* and having segregated away the duplicated fragment was isolated from each cross, i.e. MD1-1 from M-Dup1, MD2-1 from M-Dup2 and MD3-1 from M-Dup3. Southern hybridization of DNA digested with restriction enzymes sensitive to C methylation (Fig. 1C, D) showed that the duplicated region of *met2* from the Met^- derivatives was methylated, and that the methylation extent coincided with the duplication extent, as previously observed.

A semi-quantitative RT-PCR analysis (Fig. 2A) also confirmed previous observations. Indeed, when methylation covered the entire gene (MD1-1) or spanned the transcription start site, (MD3-1) no transcripts were found, as demonstrated by the absence of amplification products using the m1-m2 and m1-m3 primers. When methylation started around 700 bp downstream from the ATG translation start codon and extended downstream within the coding sequence (MD2-1), the m1-m2 RT-PCR product was present, although in lower amounts, and the m1-m3 product was almost totally absent. This confirmed the production of truncated transcripts, which very rarely extend through the methylated area, as expected if DNA methylation were to block transcript elongation.

To check whether this effect of methylation on transcription could be generalized, we created duplications of various portions of the *hph* gene in strain 9H2-3, which carries a functional chimeric *hph* construct conferring resistance to hygromycin. In strain H-Dup1, the duplication covers the downstream part of the coding sequence, beginning 250 bp from the ATG translation start codon and extending 1.7 kb downstream. In strain H-Dup2, used as a control, the duplication covers the entire chimeric construct (Fig. 1B). Strains H-Dup1 and H-Dup2 were crossed with a Hyg^S tester strain. In the progeny, silencing of *hph* would lead to 0 Hyg^R: 4 Hyg^S asci instead of the 2 Hyg^R: 2 Hyg^S segregation expected if *hph* were not silenced. Stable silencing was found in eight out of the 20 asci analyzed in the progeny of H-Dup1 and in nine out of 20 asci analyzed in the progeny of H-Dup2. DNA from the progeny of the two crosses was analyzed for methylation by Southern hybridization. The silenced Hyg^S progeny from both crosses always showed methylation (we analyzed four strains from H-Dup1 and nine from H-Dup2), whereas active Hyg^R progeny did not show any methylation (we analyzed six strains from H-Dup1 and eleven from H-Dup2). Moreover, the methylation

extended to the length of the duplication, as exemplified with derivatives HD1-1 and HD1-2 from H-Dup1 (Fig. 1G) and HD2-1 from H-Dup-2 (Fig. 1E-F).

Hph transcripts from the parental Hyg^R strain 9H2.3 and from the derivatives HD1-1, HD1-2 and HD2-1 were analyzed by RT-PCR (Fig. 3A). Both, h1-h2 and h1-h3 amplification products were obtained with the unmethylated control (WT) and were absent with the completely methylated strain HD2-1. In both HD1-1 and HD1-2 strains, the h1-h2 amplification product was present (although in lower amounts as compared to the positive control), showing that the upstream region of *hph* is transcribed. In contrast, the almost total absence of the h1-h3 amplification product indicates that these strains produce truncated transcripts, which very rarely extend beyond the location of the h3 primer. These results confirm the observations made with *met2*, suggesting that DNA methylation in *Ascobolus* efficiently silences genes by preventing the production of transcripts which span the methylated area.

Weak effect of promoter methylation upon gene silencing

To address the question whether methylation could also silence genes in *Ascobolus* by preventing transcription initiation, we constructed strain M-Dup4, which harbors an ectopic duplication of the 4M segment extending 1.6 kb upstream from the transcription start site and 30 bp downstream from it (Fig. 1A) and thus contains the *met2* promoter (Goyon et al., 1988). Dup-4 was crossed with a Met^- tester strain. In the progeny, silencing of *met2* should lead to 0 Met^+: 4 Met^- asci instead of the 2 Met^+: 2 Met^- segregation expected if *met2* were not silenced. Silencing was never found among the 70 asci analyzed. The methylation status of *met2* was analyzed by Southern hybridization in ten non-silenced Met^+ strains. Six of them displayed an unmethylated pattern similar to that of the wild-type strain, indicating that they had not been subjected to MIP (not shown). As exemplified with MD4-1 and MD4-2, the four other non-silenced strains displayed dense methylation in the region covered by the 4M segment (Fig.1C) but not in the downstream part of the gene (Fig. 1D). It appears therefore, that *met2* cannot be silenced by methylation of its promoter region.

Even though methylation of the promoter region of *met2* did not result in gene silencing, it could nevertheless affect transcription levels and/or alter the position of the transcription start site. In order to check the transcription level, we performed RT-PCR experiments with MD4-2 (Fig. 1C-D). Similar amounts of both m1-m2 and m1-m3 amplification products were obtained with the wild-type (WT) and the methylated MD4-2 strains (Fig. 2A). This indicates that methylation of the promoter region of the *met2* gene does not significantly affect the transcription level of this gene. To check whether methylation of the

promoter region could change the position of the transcription start site, we performed RT-PCR amplification of the 5' part of *met2* in strain MD4-2 with five pairs of primers, as indicated in Fig. 2B. As shown in Fig. 2C, amounts of RT-PCR products similar to those obtained with the wild-type control strain, were obtained using primers B-A, both located downstream from the transcription start site described for *met2* (Goyon et al., 1988). When the upper primer was located upstream from this site (C-A and D-A primers), RT-PCR products were also obtained with both strains, in similar amounts, although somewhat lower than that obtained with B-A primers, indicating the presence of a secondary transcription start site. Finally, no RT-PCR products were obtained with either strain when the upper primer was located more than 155 bp upstream from the major transcription start site (E-A and F-A primers). These results indicate that methylation of the promoter region of *met2* does not alter the position of the transcription start sites. They further support the conclusion that methylation of this 5' region does not affect the transcription of this gene.

In order to perform a similar analysis with the *hph* gene, we constructed the strain H-Dup3, which harbors an ectopic duplication of the 1.4-kb 3H segment (Fig.1B). H-Dup3 was crossed with a Hyg^S tester strain. Among 100 asci analyzed, two showed complete silencing and six showed partial silencing (in this case, the young mycelium grew poorly on the hygromycin-containing medium). However, partially as well as completely silenced *hph* strains reverted to Hyg^R within a few days when plated on media containing hygromycin, contrary to the silenced Hyg^S derivatives HD1 and HD2, which were stably silenced. In the H-Dup3 progeny, the region covered by the 3H segment was methylated in all of the completely or partially silenced strains, but also in four out of nine non-silenced strains issuing from distinct 2 Hyg^R:2 Hyg^S asci (Fig. 1E-F). Silenced and non-silenced strains showed similar methylation patterns. From these results, we estimate that more than 80% of the HD3 methylated derivatives were not silenced. Hence, contrary to *met2*, *hph* can be silenced by promoter methylation, albeit silencing occurs rarely and is not stably maintained.

Finally, we showed by primer extension analysis that, as found with *met2,* the methylation of *hph* in the 3H segment did not change the transcription start sites (Fig. 3B). The same three major transcription start sites (described in (Paluh et al., 1988)) were detected in the unmethylated control (WT), in the non-silenced (HD3-4) and in the partially silenced (HD3-2 and HD3-3) strains, although in reduced amounts in the latter strains. No product was obtained with the completely silenced HD3-1 strain.

We could rule out the possibility that silencing might result from a spreading of methylation, downstream from the transcription start site, hence affecting the transcript elongation process. Indeed, by using the bisulfite genomic sequencing method, we showed that

the methylation of the fully silenced HD3-1 strain does not extend beyond the transcription start site (Fig. 3C).

We further investigated by RT-PCR the effect of methylation on the level of transcription of *hph* (Fig. 3A). Both, h1-h2 and h1-h3 amplification products were obtained with the unmethylated control (WT), and not obtained with the fully methylated control (HD2-1). In agreement with the results of the primer extension analysis (Fig. 3B), similar amounts of both h1-h2 and h1-h3 RT-PCR products were obtained with the positive control and the methylated but non-silenced HD3-4 strain. Lower amounts were observed with the partially silenced HD3-2 and HD3-3 strains, while the completely silenced HD3-1 strain gave almost no product.

Even though the overall results demonstrate that methylation of the promoter region of the *hph* transgene can affect its transcription, it is striking that most of the methylated strains remain transcriptionally active and that none of them displays stable silencing.

Chromatin changes associated with DNA methylation

The observation that methylation starting in the coding sequence leads to the production of truncated transcripts prompted us to examine the effects of methylation on chromatin structure. For this analysis, we chose the *met2* gene, because it corresponds to a resident gene at its native location. We first compared, by using micrococcal nuclease (MNase) analysis, the *met2* chromatin state in the wild-type and in the silenced MD2-1 strains. As described above, this latter strain is methylated in the downstream portion of its coding sequence and produces truncated transcripts.

Comparisons of the hybridization patterns from chromatin prepared from protoplasts and naked DNA (Fig. 4A and B) indicate that the MNase digestion patterns do not result from a preferential DNA sequence-based MNase cleavage, except for band G. The wild-type chromatin gives rise to a series of discrete fragments corresponding to the distances between the MNase sensitive site(s) and the *Eco*RV site, as revealed by bands B-J in Fig. 4A. MNase digestion of the unmethylated portion of strain MD2-1 generates the same B-G fragments. In contrast, bands H and J, which correspond to sensitivity sites located in the methylated region (Fig. 1A), have disappeared. Probing the same blots for the unmethylated *masc2* gene (Goyon, 1998) showed identical banding patterns (Fig. 4C), and ethidium bromide staining of the same gels showed similar MNase digestion in both samples (Fig. 4D). We conclude that the differences in the banding patterns observed in Fig. 4A reflect a local chromatin change restricted to the methylated region.

Since the truncated transcripts found in MD2-1 rarely overlap with the methylated region, the changes in banding patterns could result from the transcriptionally inactive state of the corresponding methylated region, rather than from methylation itself. To investigate this possibility, we analyzed the chromatin of the same MD2-1 strain, together with strains MD1-1 and MD3-1 (Fig. 5). *Met2* transcripts are not detected in these two latter strains (Fig. 2A), which nevertheless differ in their methylation patterns (Fig. 1). In strain MD1-1, methylation extends beyond both ends of the gene, whereas in strain MD3-1, it is limited to its upstream portion. Hybridization patterns of MNase treated chromatin are shown in Fig. 5A. Control ethidium bromide staining patterns of MNase digestions are shown in Fig. 5B. MNase treated chromatin from strain MD2-1 shows, as observed previously (Fig. 4A), a disappearance of bands H and J. In the fully methylated strain MD1-1, most of the specific bands observed in the wild-type controls (WT1 and WT2) almost disappear (bands D, E, F, H and J). The decrease in intensity of bands H and J is similar to that observed in strain MD2-1. In contrast, the intensity of band C, which reflects a sensitivity site located at the border of the methylated region, increases considerably. Band G persists, but as mentioned above, it is most likely due to a preferential DNA sequence-based MNase cleavage, because it is present in naked DNA digestions and in all other samples, with approximately the same intensity (Figs. 4A, B and 5A). MNase-treated chromatin from strain MD3-1 displays a decrease in intensity of bands D and E in its short methylated region and, like the fully methylated strain MD1-1, an increase in intensity of band C at the upstream border of its methylated region. In its unmethylated region, the banding pattern is like that of the wild-type controls. Since the unmethylated coding region from strain MD3-1 is not transcribed, these results indicate that the loss of MNase sensitive sites observed in the silenced strains MD1-1 and MD2-1 does not result from transcriptional defects. Therefore, the changes in chromatin appear primarily associated with DNA methylation.

We also analyzed the chromatin of strain MD4-2, in which the 5’ region upstream from the transcription start site was methylated, yet without affecting *met2* transcription. Hybridization patterns of MNase-treated chromatin from this strain (Fig. 5A) showed a disappearance of band D, as with MD1-1 and MD3-1, but also of band C, in contrast with MD1-1 and MD3-1, which showed an increased intensity of this band. It is important to note that even though this region is methylated and the chromatin has been subsequently altered, the transcription of the gene is similar to that of the wild-type (Fig. 2A). In its unmethylated region, MNase-treated chromatin from strain MD4-2 displays the same banding pattern as does the wild-type.

These observations further support the conclusion that the changes observed in methylated chromatin do not result from changes in the transcription levels. Furthermore, the loss of the MNase sensitive sites is always observed on the whole length of the methylated regions. Site C, which displays an increased sensivity in strains MD1-1 and MD3-1, is located at the upstream border of their methylated regions.

Nucleosome analysis of chromatin associated with methylated DNA

To further investigate the chromatin state of the methylated region of the strains analyzed, we compared by Southern hybridization the nucleosomal arrays obtained after partial MNase digestion of the *met2* chromatin associated with DNA either methylated (MD1-1 and MD4-2) or unmethylated (WT). Two probes were used (Fig. 6A). Probe I corresponds to a 654 bp fragment located in the middle of the methylated region of strain MD1-1. In this strain, the methylated region extends about 2.3 kb upstream and 2.6 kb downstream from probe I. Since the nucleosomal repeat lengths in *Ascobolus* are approximately 174 ± 6 bp (J. L. Barra, unpublished), at least 13 nucleosomes can be present within the methylated region on either side of probe I. This region is unmethylated in strain MD4-2. The nucleosomal ladders revealed by probe I were similar for all three strains (Fig. 6B). Therefore, whether or not it is methylated, the probed region taken as a whole exhibits equal susceptibility to digestion by MNase. This suggests that the loss of MNase hypersensitive sites in the methylated coding region (Figs. 4A and 5A) more likely reflects a loss of the phasing of the nucleosome arrangement rather than a change in the chromatin state increasing its protection against MNase.

We reprobed the same Southern blots with probe II (Fig. 6C), which corresponds to a 306 bp fragment located in the middle of the methylated region of strain MD4-2 (Fig. 6A). This region extends over about 600 bp, equivalent to at least three nucleosomes, on each side of probe II. In strain MD1-1, the methylated region only extends downstream from the probe, over 5.2 kb. For the wild-type strain, hybridization with probe II compared to probe I reveals an increased amount of mono- and dinucleosomes, indicating that the upstream region encompassing the promoter is more sensitive to MNase than the coding region. However, for the MD1-1 and MD4-2 strains, no clear difference is seen between probe II and probe I. This leads us to two conclusions. Firstly, the methylation of the upstream region increases its protection against MNase, and secondly, the level of protection obtained in this manner is similar to that exhibited by the methylated or unmethylated coding region. In other words, two levels of susceptibility to MNase are observed. The unmethylated promoter region is the most sensitive, while the methylated promoter region as well as the coding region —independently

of its methylation status— exhibit lower sensitivity. The increased protection of the methylated promoter region may be paralleled with the presence of a smear between sites C and B in strains MD1-1, MD4-2 -and also MD3-1- (Fig. 5A). This smear may be explained if cutting by MNase is less efficient in the region lying immediately upstream from probe II in all of the tested strains, which share the feature of being methylated in the region of the *met2* promoter.

Acetylation and methylation of histones in chromatin associated with methylated DNA

In *Ascobolus*, one gene encodes histone H3, containing lysines at positions 4 and 9, and two genes encode histones H4, both containing the four conserved lysine residues able to be acetylated (J. L. Barra and L. Rhounim, unpublished). This justified an analysis of the level of acetylation and the level of methylation of histones in the altered chromatin associated with methylated DNA. For this analysis, we used two strains carrying the same reporter transgenic constructs containing the genes *met2* and *hph* (Fig. 7A). In these strains, either *hph* (strain FC75) or *met2* (strain KA7) have been densely methylated by MIP.

To compare the acetylation levels of histone H4 associated with methylated or unmethylated DNA, chromatin from strains FC75 and KA7 were immunoprecipitated using antibodies directed against either all four acetylated H4-lysines (tetraAc) or acetylated H4-lysine 8 (Lys8Ac) only. The DNA from genes *hph* and *met2* from each strain was then PCR-amplified. Fig. 7B shows that the levels of the PCR-products obtained with the methylated and the unmethylated genes in either strain after precipitation by tetraAc, as well as by Lys8Ac, is roughly similar, although methylated DNA seems to give a slight increase of amplification product, notably with the Lys8Ac antibody.

In another experiment, immunoprecipitation was made with antibodies directed against acetylated lysines at positions 16 (Lys16Ac) or 5 (Lys5Ac) of histone H4. DNA from genes *hph*, *met2* and *histone H1* was then PCR-amplified. The *histone H1* gene, which is constitutively transcribed, was used as an internal control. Analysis of the FC75 strain (Fig. 7C) shows that the methylated *hph* transgene, the unmethylated *met2* transgene and the unmethylated endogenous histone *H1* gene were similarly PCR amplified after chromatin precipitation using either antibody. The same conclusion can be derived from the analysis of the KA7 strain (Fig. 7D). Again, in the two strains, the methylated gene shows a slight increase of amplification, compared to the unmethylated gene and to the *histone H1* control. To verify that our study did address the acetylation state of histone H4, we performed Western analyses showing that antibodies directed against Lys8Ac, Lys16Ac and Lys5Ac efficiently and specifically bind to histone H4 (data not shown).

We conclude from these experiments that the methylated genes are not detectably hypoacetylated (and in fact might be slightly more acetylated) with regard to the transcribed unmethylated genes of the construct and to the constitutively transcribed *histone H1* gene. In addition, strains silenced for *met2* or for *hph* were treated with trichostatin A, a potent inhibitor of class I and II histone deacetylases. All attempts (with mycelium or protoplasts, grown in solid or liquid medium) were negative (J. L. Barra, unpublished). Trichostatin A was not able to derepress the silenced genes, although it significantly increased the overall level of acetylated histone H4 in the treated strains (J. L. Barra, D. Roche, K. Robbe, unpublished).

A comparison of the histone H3 methylation levels between the two reporter genes is shown in Fig. 7E and F. Analysis of the FC75 strain by PCR amplification, after chromatin immunoprecipitation using antibodies directed against the dimethylated H3-Lys4 (Fig. 7E) shows similar PCR amplification of the unmethylated *met2* and *histone H1* genes, and less PCR amplification of the methylated *hph* gene. Analysis of the KA7 strain using the same antibody (Fig. 7F) shows similar PCR amplification of the unmethylated *hph* and *histone H1* genes and less amplification of the methylated *met2* gene. This indicates that methylation of H3-Lys4 is preferentially associated with unmethylated expressed genes.

Opposite results were obtained after chromatin immunoprecipitation using antibodies directed against the dimethylated H3-Lys9. Analysis of the FC75 strain shows that the unmethylated *met2* and *histone H1* genes are poorly PCR amplified, compared to the methylated *hph* gene (Fig. 7E). Similarly, in strain KA7, the unmethylated *hph* and *histone H1* genes were poorly PCR amplified, compared to the methylated *met2* gene (Fig. 7F). This indicates that dimethylation of H3-Lys9 is preferentially associated with methylated silenced genes. In these experiments, PCR amplification was performed within the coding sequences. We then extended the analyses to the promoters of the two silenced genes (Fig. 7G) and found that dimethylation of H3-Lys9 was also associated with methylated promoters.

Because the two genes studied in strains FC75 and KA7 were both full-length methylated and completely silenced, we could not conclude whether the modifications of histone H3 methylation were correlated with gene silencing or with DNA methylation. To address this point, we analyzed strains whose methylation was restricted to their promoters (Fig. 8), using as controls, strains harboring the same genetic arrangement of the loci under investigation (Fig. 8A). For *met2*, MD4-2 was compared with FB14 and MD1-1, in which *met2* is either unmethylated and expressed, or full-length methylated and silenced. For *hph*, HD3-1 was compared with 9H2.3 and HD2-1, in which *hph* is either unmethylated and expressed, or full-length methylated and silenced. Interestingly, strains MD4-2 and HD3-1 differ in that *met2* is expressed in MD4-2, whereas *hph* is silenced in HD3-1. We analyzed the histone H3

methylation patterns in the promoters (Fig. 8B) and in the coding sequences (Fig. 8C) of the two genes. In the full-length methylated and silenced genes *met2* and *hph*, harbored by strains MD1-1 and HD2-1, respectively, dimethylation of H3-Lys9, but not dimethylation of H3-Lys4, was found along both the promoter regions and the coding sequences, consistent with the results obtained with KA7 and FC75. In the tester strains, genes *met2* (in MD4-2) and *hph* (in HD3-1) displayed, along their methylated promoter, the methylation of H3-Lys9 typical of silenced full-length methylated genes. In contrast, along their unmethylated coding sequences, they displayed the methylation of H3-Lys4 typical of unmethylated expressed genes. Since *hph* is silenced in HD3-1, while *met2* is expressed in MD4-2, we conclude that changes in histone H3 methylation, although they always appear to be associated with DNA methylation, are not correlated with gene silencing.

It is important to stress that a gene, in which DNA methylation is restricted to its promoter, can be normally expressed despite the fact that it displays both a modification of histone H3 methylation and a loss of MNase hypersensitive sites in the chromatin of its promoter.

Discussion

DNA methylation-associated transcript truncation as an efficient process for silencing genes

We observed that methylation of the promoter region of *hph* results in the silencing of this gene. However, this effect is not strong, since about 80% of the strains displaying methylation do not actually show a decrease of *hph* expression to a level conferring hygromycin sensitivity, and since the silenced state of the remaining 20% was never stably maintained. Moreover, methylation of the promoter of the *met2* gene never resulted in detectable silencing and affects neither the level nor the starts of transcription. In another study, methylation of the 4-kb segment encompassing the 5' region of the *b2* spore color gene, upstream from the translation start site, did not prevent gene expression among the thousands of individual cells observed (Colot and Rossignol, 1995). Therefore, although methylation of the promoter region might affect expression, this effect was infrequent, often partial and, when silencing occurred, it was never stably maintained.

The density of CpGs in the two promoter regions studied (more than four methyl-CpGs per 100 bp) was at least as strong as in mammalian CpG islands, in which methylation is associated with a strong inhibition of transcription (Antequera et al., 1990). Furthermore, methylation in *Ascobolus* also involves other Cs belonging to non-symmetrical motifs (Goyon et al., 1994), which are not usually methylated in mammals. This, together with the observation that dense methylation in the promoter of the resident *am* and *mtr Neurospora* genes does not prevent the initiation of transcription (Rountree and Selker, 1997), suggests that methylation in *Neurospora* and *Ascobolus* does not significantly affect the binding to the promoter region of the factors required for transcription.

In mammals, proteins binding to methylated DNA can mediate transcriptional repression at a distance (Cross et al., 1997; Nan et al., 1997). Such long-distance repression might be absent in fungi. The erratic effect of promoter methylation on the transcription of *hph* in *Ascobolus* might be possibly explained if the methylation of one (or some) specific C(s) were to prevent the binding of a specific transcription factor. Since methylation from one molecule to another suffers some heterogeneity (Goyon et al., 1994), only the strains in which a specific C would be methylated in most of the nuclei would be affected. The possibility that methylation in the promoter could trigger a chromatin change that would spread to the nearby transcription start region, resulting in an early inhibition of transcript elongation, is unlikely

because efficient silencing by MIP requires the methylation of at least 400 bp in the transcribed region (Goyon et al., 1996).

In contrast to the scarcity of gene silencing observed when promoters are methylated, efficient and stable silencing is the rule when methylation involves the transcribed part of the gene. Methylation of a downstream portion of this region is sufficient for efficient silencing. The silencing is then accompanied by the production of truncated transcripts. This latter effect, previously observed for *met2* (Barry et al., 1993), was generalized to the chimeric *hph* construct. This strongly suggests that the production of truncated transcripts reflects a general property of MIP in *Ascobolus* rather than some gene specific effect. Remarkably, as for *met2*, the sizes of the *hph* transcripts, as deduced from RT-PCR analyses, are consistent with the length expected if transcript elongation were blocked at the beginning of the methylated region. Although we cannot formally exclude that a post-transcriptional degradation of the transcripts in the region corresponding to the methylated portion of the DNA template might occur, an arrest in transcript elongation is the simplest hypothesis to account for the production of truncated transcripts. Similar observations reported on the *am* and *mtr Neurospora* genes favor the idea that the arrest of transcript elongation might be a general phenomenon in fungi (Rountree and Selker, 1997). Such an effect is not likely to take place in mammals. Indeed, in these organisms, almost all CpGs that do not belong to CpG islands are methylated, including those that are located within the coding sequences, within intronic DNA, or within dispersed repeats inserted into genes (Yoder et al., 1997). An effect of methylation on transcript elongation would impair transcription all along the genome, which is not observed. Indeed, in several imprinted loci, transcript elongation proceeds through silenced methylated domains (Li et al., 2004). In the rare cases where methylation of the coding sequence inhibits gene expression (Keshet et al., 1985; Graessmann et al., 1994), there is no indication that this inhibition could result from an arrest of transcript elongation.

DNA methylation-associated chromatin change

In vertebrates, chromatin is important in mediating transcriptional repression induced by methylation. Microinjection of methylated templates into the nuclei of mammalian cells or *Xenopus* oocytes showed that methylation could repress transcription only after chromatin formation (Buschhausen et al., 1987; Kass et al., 1997). In this work, we showed that methylation of the transcribed sequence is associated with a change in chromatin, independently of the transcriptional state. Strikingly, the change in chromatin starts at a position close to that where methylation starts and where transcript elongation is arrested. The coextensivity of chromatin change and methylation contrasts with the observation made on

plasmids injected in *Xenopus* oocyte nuclei (Kass et al., 1997). In this situation, the inactive chromatin structure resulting from methylation would spread to the unmethylated promoter region. This may reflect experimental differences, since we used the resident *met2* gene located at its native chromosomal position, which contrasts with plasmid constructs or it could also be dependent on the organism studied. Although the causal relationship between methylation and chromatin remodeling occurring after MIP is not yet known, our observations are better explained if the arrest of transcript elongation is mediated by a change in chromatin which could either impair the processivity of the RNA polymerase or prevent the binding of transcription factors required for elongation.

The chromatin changes associated with methylation in the promoter region do not seem to have important consequences upon transcription, at least for the *met2* gene that we studied, since both its level and the sites where it initiates, remained unchanged. More studies are required to understand the biological significance of this.

DNA methylation and core histone modifications

Methylated DNA in *Ascobolus* does not appear to be associated with hypoacetylated histone H4, and increasing the level of acetylated histone H4, using trichostatin A, did not lead to the derepression of silenced genes, in contrast to what occurs in other organisms. These results differ in particular from those obtained with *Neurospora,* where trichostatin A was able to derepress the methylated *hph* transgene (Selker, 1998). Recently, the analysis of the genome-wide acetylation profiles for eleven lysines in the four core histones of *Saccharomyces cerevisiae* revealed unique patterns of acetylation in promoters as well as in coding regions, and indicated that both hyper- and hypoacetylation of histones are correlated with gene activity (Kurdistani et al., 2004), clearly showing that the analysis of the relationship between transcription, DNA methylation and histone modifications is far from being completely understood.

We also showed that methylated DNA in *Ascobolus* was associated with dimethylated histone H3-Lys9 but not with dimethylated histone H3-Lys4, which preferentially bound unmethylated DNA. This result is consistent with the finding that in several organisms, H3-Lys9 and H3-Lys4 methylation marks heterochromatic regions and euchromatic regions, respectively (Litt et al., 2001; Noma et al., 2001). However, different histone methylation marks have been identified depending on the organisms. In *Neurospora,* trimethylated but not dimethylated histone H3-Lys9 is found associated with methylated DNA (Tamaru et al., 2003).

In *Arabidopsis*, dimethylated H3-Lys9 is a critical mark for DNA methylation and gene silencing (Jackson et al., 2004). The possibility in *Ascobolus* to direct methylation at short

DNA stretches such as promoter regions, allowed us to show that dimethylation of H3-Lys9 was tightly associated with DNA methylation, even in the absence of gene silencing. In this respect, H3-Lys9 methylation could be required for DNA methylation in *Ascobolus*. In *Neurospora*, tri-methylation of H3-Lys9, which is performed by the histone methyltransferase DIM-5, has been shown to be necessary for initiating DNA methylation (Tamaru et al., 2003). In both *Arabidopsis* and *Neurospora*, the loss of histone H3-Lys9 methylation often results in loss of DNA methylation, but not *vice-versa* (Jackson et al., 2002; Johnson et al., 2002; Tamaru et al., 2003).

The changes in chromatin structure and histone modifications associated with the arrest of transcript elongation in fungi could be different from those that inhibit transcription initiation in plants and mammals. Further studies are required to identify all the factors that participate in these changes and to determine which factors are similar or different to those acting in these organisms.

Biological meaning of the DNA methylation-associated transcriptional arrest

What could be the meaning of the difference in the effects of DNA methylation upon transcription in mammals and in fungi? In *Ascobolus* and *Neurospora*, the mechanisms of MIP and RIP lead to the specific methylation of naturally occurring DNA repeats which correspond principally to retroelements (Goyon et al., 1996; Selker et al., 2003). The arrest of transcript elongation may be more efficient in silencing parasitic sequences than the inhibition of transcription initiation. Indeed, it may prevent the copying of transposons from the promoter of a nearby gene. Moreover, it can efficiently prevent the transposition of LINE elements. Such elements are most often truncated in their 5' region, thus lacking the promoter. Therefore, only the full-length master copies can mediate transcription. The presence of truncated copies would be sufficient to trigger the methylation of the downstream part of the coding sequence of the full-length master copies, thus resulting in their silencing.

Methylation might also affect transcript elongation in other organisms such as plants. Plants differ from mammals and share similarities with *Ascobolus* and *Neurospora* in two respects (Martienssen and Colot, 2001). Firstly, they can display dense methylation, which is not restricted to CpGs. Secondly, methylation mainly involves repeated elements that tend to be clustered in intergenic regions, so that their silencing, if it consisted in a block of transcript elongation, would not impair the transcription of resident genes. Hohn *et al*. (Hohn et al., 1996) showed that methylation downstream from the transcription start site, can affect gene expression in plant protoplasts, but the effect upon transcript elongation was not investigated. Different mechanisms of homology-dependent gene silencing have been described in plants,

and some of them may show an association with methylation which is not detectable in the promoter region and heavily marked in the transcribed region of the silenced transgenes (Fagard and Vaucheret, 2000).

Experimental procedures

Strains, transformation procedures and media

In order to obtain *via* MIP various *Ascobolus* strains in which a defined genomic segment in either the *met2* gene or the *hph* transgene was methylated, we constructed a series of strains harboring an ectopic duplications of the chosen fragments. The construction of strains M-Dup1, M-Dup2, M-Dup3, harboring the ectopic duplication of the 5.7-kb *Hin*dIII 1M fragment, the 1.6-kb *Sph*I 2M fragment, and the 1.2-kb *Hin*dIII-*Nsi*I 3M fragment, respectively (Fig. 1) was previously described (Barry et al., 1993), in which they are named Dup1, Dup8 and Dup4, respectively. M-Dup4 was constructed *via* transformation of the wild-type recipient strain FB14 with plasmid pJL10. The genotype of M-Dup4 was then *met2*$^{+}$, *b2*$^{+}$, *rnd.1*$^{+}$, *[hph-4M]* in which 4M corresponds to the duplicated fragment of *met2* (Fig. 1). Strains H-Dup1 to H-Dup3 were obtained by transformation with plasmids pJL1 to pJL3, respectively, of strain 9H2.3 (*met2.Δ::amd*S, *b2*$^{+}$, *rnd1.2*, [*hph*]), which is deleted for *met2* and harbors one copy of the *hph* construct from plasmid pMP6. The genotype of strains H-Dup1 to H-Dup3 was then *met2.Δ::amd*S, *b2*$^{+}$, *rnd1.2*, *[hph]* [*met-H*] where H corresponds to fragments 1H to 3H (Fig. 1).

Tester strain FB35 (*met2.Δ::amd*S, *b2.Δ 1230*, *rnd1.2*), was used in sexual crosses with M-Dup strains and tester strain FB40 (*met2.Δ::amd*S, *b2.Δ 1230*, *rnd1*$^{+}$) was crossed with H-Dup strains. In these crosses, the tester strain and the Dup strain differed by their *b2* spore color gene and their *rnd1* spore shape gene. This allows one to distinguish, in octads issued from individual meiosis, each pair of ascospores corresponding to one of the four meiotic products. Furthermore, the *hph* marker associated with the M transgenic duplicated fragment and the *met2* marker associated with the H transgenic duplicated fragment allowed to easily characterize in the progeny the strains which had segregated away the duplicated fragment through meiotic segregation.

The HD3 strains that exhibited unstable silencing were maintained in a silenced state on media devoid of hygromycin.

Strains FC75 and KA7 harbor the *met2-b2-hph* insert methylated at *hph* (FC75) or *met2* (KA7) and are deleted for their resident *b2* and *met2* genes (Maloisel and Rossignol, 1998). The *met2* insert corresponds to the 3.7-kb *Hin*cII-*Sma*I fragment (Fig. 1A). The *hph* insert corresponds to the 3-kb *Hin*dIII fragment (Fig. 1B).

Standard transformation procedures and media were as described (Rhounim et al., 1994).

Plasmids

Plasmid pJL10 used to construct M-Dup4 resulted from integration into plasmid pMP6 of the 1.6-kb *Bam*HI-*Xba*I 4M fragment (Fig. 1A). Plasmid pMP6 (Malagnac et al., 1997) consists of a chimeric *hph* construct inserted into vector pUC18. This construct contains an *Eco*RI-*Kpn*I fragment, with the *Neurospora cpc-1* gene promoter region (Paluh et al., 1988) from which the two short unassigned open reading frames present downstream from the transcription start of the *cpc-1* gene were deleted, and a *Cla*I-*Sph*I fragment from plasmid pDH25 (Cullen et al., 1987), containing the coding sequence of the bacterial hygromycin B phosphotransferase (*hph*) gene and the transcription termination region of the *Aspergillus nidulans trpC* gene. The ability of the *cpc-1* promoter to drive *hph* expression in *Ascobolus* was verified by showing that the deletion of the promoter region, extending 70 bp upstream from the transcription start site, resulted in an almost total absence of transformants (not shown).

Plasmids pJL1 to pJL3 used to construct the H-Dup strains resulted from the insertion of fragments 1H to 3H (Fig. 1B) from the chimeric *hph* construct, respectively, into plasmid pGB20 (Goyon et al., 1996) which carries the *met2* gene from *Ascobolus* used as a selectable marker in transformation. Fragments 1H and 2H correspond to the 1.7-kb *Eco*RI-*Hin*dIII and 3-kb *Hin*dIII fragments from pMP6, respectively. The 1.4-kb 3H fragment was obtained by PCR amplification of pMP6, using the M13 reverse sequencing primer (New England Biolabs #S1201S) and a primer corresponding to the sequence located at positions 295-277 of the published nucleotide sequence (Paluh et al., 1988).

Isolation and manipulation of DNA, bisulfite genomic sequencing

DNA isolation and manipulations were as described (Malagnac et al., 1997). The bisulfite genomic sequencing procedure was performed as previously described (Goyon et al., 1994), except that the bisulfite treatment was done twice for each DNA sample in order to increase the efficiency of C to U conversion up to 98-100%. Under the conditions used, 5-methylcytosine remains unreactive. Because of the C to U conversion, strand specific PCR primers can be designed. The primers used for the *hph* PCR amplification of the treated DNA corresponded to positions 168-185 and 1097-1078 for the transcribed strand and positions 301-322 and 1176-1155 for the nontranscribed strand of the published nucleotide sequence (Paluh et al., 1988).

Isolation and manipulation of RNA for RT-PCR and primer extension analyses

Mycelia were grown two days in liquid medium. Similar amounts of total RNAs purified using either TRIzol Reagent (GIBCO/BRL) or the RNeasy kit (QIAGEN) were used for reverse transcriptase (RT)-PCR experiments performed either as previously described (Barry et al., 1993) or with the OneStep kit (QIAGEN). For *met2*, the m1, m2 and m3 primers used (Figs. 1A and 2A) corresponded to sequences located at positions 1754-1772, 2341-2323 and 2783-2765 of the published nucleotide sequence (GenBank accession number AY836153), respectively. Primers A, B, C, D, E and F (Fig. 2B and C) corresponded to sequences of *met2* located at positions 2364-2346, 1663-1679, 1587-1606, 1520-1538, 1453-1471 and 1373-1391 of the same sequence, respectively. The different combination of primers (A-B, A-C, A-D, A-E and A-F) gave equal amounts of product when used with genomic DNA (not shown). For *hph*, the h1 primer used (Figs. 1B and 3A) was located at positions 926-945 of the published nucleotide sequence (Paluh et al., 1988), and the h2 and h3 primers were located at positions 367-348 and 662-645 of the published nucleotide sequence (Cullen et al., 1987), respectively. The *hph* transcription start sites (Fig. 3B) were determined by primer extension experiments using the AMV Reverse Transcriptase Primer Extension System (Promega), a primer located at position 1003-974, according to the published nucleotide sequence (Paluh et al., 1988) and similar amounts of total RNAs.

For H1, the primer pairs used (Figs. 2A and 3A) corresponded to sequences located at positions 1116-1136 and 1820-1800, of the published nucleotide sequence (GenBank accession number AF190622).

Chromatin analysis

Protoplasts of the different strains were prepared as described (Faugeron et al., 1989). Samples of $2x10^7$ protoplasts were resuspended in 250 µl of permeabilisation buffer containing 15 mM Tris-HCl pH 7.5, 60 mM KCl, 15 mM NaCl, 5 mM MgCl2, 300 mM sucrose, 0.2% NP40, 5 mM CaCl2, and increasing amounts (0, 1.5, 4.5, 15 or 45 units) of freshly added micrococcal nuclease (MNase, Boehringer Mannheim). Samples were incubated 3 min at 25°C, and reactions were stopped by the addition of 250 µl of 50 mM Tris-HCl pH 7.5, 20 mM EDTA and 1% SDS. After extraction, nucleic acids were digested overnight with 15 units of *Eco*RV. For naked-DNA controls, samples of purified genomic DNA from $2x10^7$ protoplasts were resuspended in 250 µl of permeabilisation buffer, digested with MNase (0, 0.15, 0.45 and 1.5 units) and treated as described above. Digested DNA was size-separated on a 1.5% agarose gel. Southern blots were probed with a 253-bp random-primed ^{32}P-labeled fragment of *met2* located just upstream from the *Eco*RV site bordering the 3' end of the coding sequence of this

gene. This fragment was obtained by digestion with *Eco*RV of the PCR product amplified using primers corresponding to the sequences located at positions 3552-3566 and 4206-4189 of the published nucleotide sequence (GenBank accession number AY836153), followed by gel purification. Control hybridization was done by probing the unmethylated *masc2* gene with the random-primed ^{32}P-labeled 208-bp *Eco*RV-*Sac*I fragment of plasmid pCG92 (Goyon, 1998).

Nucleosome detection

Samples of 2x10^7 protoplasts were resuspended in 250 µl of permeabilisation buffer and treated with increasing amounts (0, 1.5, 4.5, 15 or 45 units) of freshly added micrococcal nuclease (MNase), as described above. Nucleic acids were extracted and size-separated on a 1.5% agarose gel. Southern blots were probed with *met2* probes I and II (Fig. 6A) obtained by PCR amplification using primers located at positions 3552-3566/4206-4189 and 698-715/988-1005 of the published nucleotide sequence (GenBank accession number AY836153), respectively. PCR products were gel purified and ^{32}P labeled by random priming.

Chromatin immunoprecipitation

In vivo cross-linking and chromatin immunoprecipitation were performed as previously described (Meluh and Broach, 1999), in which fungal cells were fixed with formaldehyde for 15 minutes at room temperature. Aliquots of sheared chromatin solution corresponding to 0.5 g of dry weight material, were incubated overnight at 4°C with anti-acetyl histone H4 antibody isoforms at a final concentration of 2 µg/ml (Upstate Biotechnologies, anti-acetyl-histone H4 (Lys5) cat #06-759, anti-acetyl-histone H4 (Lys8) cat #06-760, anti-acetyl-histone H4 (Lys12) cat #06-761, anti-acetyl-histone H4 (Ly16) cat #06-762) or with anti-dimethyl histone H3 antibody isoforms (Upstate Biotechnologies, anti-dimethyl-histone H3 (Lys 4) cat #07-030, anti-dimethyl-histone H3 (Lys 9) cat #07-212). Antibodies were precipitated using protein A-sepharose CL-4B beads (Amersham Pharmacia, cat #17-0780-01). A 1/50 fraction of the immunoprecipitated material with anti-acetyl H4, 1/20 of the immunoprecipitated material with anti-dimethyl H3 and 1/500 of the total input DNA were analyzed by PCR using primers designed within either the coding sequences or the promoters of *met2* and *hph* genes. For the coding sequences, primer pairs were located at positions 445-468 and 844-822 for *hph* (Cullen et al., 1987) and 3240-3263 and 3577-3554 for *met2* (GenBank accession number AY836153). For the promoters, primer pairs were located at positions 301-321 and 400-421 for *hph* (Paluh et al., 1988), and 1381-1400 and 1606-1587 for *met2* (GenBank accession number AY836153). Primer pairs used for the control *H1* gene were located at positions 1120-1143 and 1473-1450 (Barra et al., 2000). After an initial 5 min at 96°C, the DNA was amplified for 23-26 cycles of

96°C for 30 s, 53°C for 30 s, and 72°C for 1 min followed by extension of 7 min at 72°C . A fraction of the reactions was analyzed on agarose gels, and quantified using the NIH imagery software.

Acknowledgements

We thank Geneviève Almouzni for allowing us to verify in her laboratory the acetylation of histone H4 in strains treated with trichostatin A, Karine Robbe for help in construction of strain KA7, and Ariel Prunell and members of the laboratory for helpful discussions. This work was supported by grants from the Association pour la Recherche sur le Cancer (Contract 6200), the European Union (Contract BIO4-CT96-0253) and Fundación Antorchas (Argentina). J. L. B. was a recipient of a fellowship from the French Ministère des Affaires Etrangères followed by a fellowship from the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina. A.M.H was supported by the ARC and EMBO long-term fellowships.

Figure legends

Fig. 1. Restriction map and methylation analysis of *met2* and *hph*. (A) Gene *met2*. The black box indicates the coding sequence; the arrowed line shows the transcribed region; black inverted triangles indicate the position of the two introns; single-sided arrows (m1 to m3) show the positions and orientations of primers used in PCR and RT-PCR experiments. Partial map of the *Nde*II restriction fragments large enough to be detected in Southern hybridization is shown; fragment sizes are given in kilobase pairs (kb). 1M to 4M correspond to the segments duplicated in strains M-Dup1 to 4 and are used as probes in Southern analyses. (B) Gene *hph*. The coding sequence, transcribed region, primers used (h1 to h3), partial map of *Nde*II restriction fragments are indicated as in (A). 1H to 3H correspond to the segments duplicated in strains H-Dup1 to 3 and are used as probes in Southern analyses. In A and B, restriction enzymes are abbreviated as follows: B, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; Hd, *Hin*dIII; Hc, *Hin*cII; N, *Nsi*I; Sp, *Sph*I; S, *Sma*I; X, *Xba*I. (C, D) Southern hybridization analyses of derivatives MD1 to MD4 from strains M-Dup1 to 4; WT corresponds to FB14. (E-G) Southern hybridization analyses of derivatives HD1 to HD3 from strains H-Dup1 to 3; WT corresponds to 9H2.3. In (C-G) DNA digests were hybridized using the indicated probe. N and S correspond to *Nde*II and *Sau*3AI; both restriction enzymes cleave the sequence GATC, but *Sau*3AI does not cut it if the C is methylated. *Sau*3AI fragments larger than those expected from methylation of the duplicated portions of *hph* in strains HD2-1 (3.4 kb) and HD3-1 to 4 (2.5 kb) result from methylation of the vector sequences.

Fig. 2. RT-PCR analysis of *met2* transcription. Strains are as described in Fig. 1 and in the text. (A) m1-m2 and m1-m3 indicate the pairs of primers used in RT-PCR experiments (see Fig. 1). H1 corresponds to the RT-PCR products obtained for the *histone H1* gene, used as an internal control. C1 and C2 correspond to control PCR products obtained from the *met2* gene and its cDNA, respectively; M corresponds to a molecular size marker. (B) Schematic representation of the *met2* gene in the wild-type and MD4-2 strains. Black boxes and the arrowed line are as in Fig.1. The black inverted triangle indicates the position of the first intron. The white box filled with m's indicates the methylated region in the MD4-2 strain. The single-sided arrows (A, B, C, D, E and F) indicate the positions and orientations of the primers used in the RT-PCR experiments shown in panel C. (C) RT-PCR products obtained using different pairs of primers as indicated. The size and approximate position of the different RT-PCR products expected for the pair of primers used is indicated in base pairs (bp).

Fig. 3. Analysis of *hph* transcription. (A) RT-PCR analysis of *hph* transcription. Strains are described in Fig. 1 and in the text. h1-h2 and h1-h3 indicate the pairs of primers used (see Fig. 1). *H1* is an internal control (see Fig.2). C1 and C2 correspond to control PCR products obtained from the *hph* gene and its cDNA, respectively. M corresponds to a molecular size marker. (B) Primer extension analysis of *hph* transcription in HD3 derivatives having the promoter methylated (Fig.1). WT stands for the wild-type strain 9H2.3, used as a positive control (+). The fully methylated, stably silenced strain HD2-1 was used as a negative control (-).The HD3 derivatives studied were either silenced (S), partially silenced (PS) or non-silenced (NS). Horizontal arrows indicate the position of the three major primer extension products. (C) Distribution of cytosine methylation of individual DNA molecules derived from the transcribed (bottom) and the nontranscribed (top) DNA strands in the region spanning the transcription start site of *hph* in the silenced strain HD3-1. Each DNA molecule is represented by one horizontal symbol alignment. The region spanned by the duplicated DNA segment used to direct the methylation of this region is represented by an horizontal black line. Arrows show the transcription start sites. Squares symbolize C residues belonging to CpG dinucleotides and circles represent other Cs. C residues are indicated as open symbols when unmethylated and as filled symbols when methylated.

Fig. 4. Micrococcal nuclease analysis of *met2* chromatin in strain MD2-1. Protoplasts (A) and naked DNA (B) were incubated with increasing amounts of MNase (0, 1.5, 4.5, 15 and 45 units, and 0, 0.15, 0.45 and 1.5 units, respectively) and subjected to indirect end-labeling analysis. Samples were loaded on the same gel. The left-handed vertical box represents the *met2* gene, with the transcription start site (arrow), the coding sequence (gray box), the position of the *Eco*RV restriction site (EV) and the size markers in kilobase pairs (kb). The black vertical box indicates the probe used for hybridization. A-J indicates the nine major bands obtained with the wild-type strain FB14 (WT). Band A corresponds to the whole *Eco*RV fragment. The white vertical box, between the WT and MD2-1 panels, indicates the methylated region in strain MD2-1. Dots indicate the positions of the bands present in the wild-type control that changed in the methylated strain. (C) Hybridization of the blot shown in A with a probe corresponding to gene *masc2*. (D) Ethidium bromide staining of the gel used for hybridization shown in A.

Fig. 5. Comparative micrococcal nuclease analysis of *met2* chromatin in the four methylated strains. (A) Protoplasts from two cultures of the unmethylated strain FB14 (WT1 and WT2)

and from strains MD2-1, MD1-1, MD3-1 and MD4-2 were digested with increasing amounts of MNase (1.5, 4.5, 15 and 45 units) and treated as in Fig. 4. White vertical boxes, between panels, indicate the methylated regions in strains MD2-1, MD1-1, MD3-1 and MD4-2. All other indications are as in Fig. 4. (B) Ethidium bromide staining of the gels used in A.

Fig. 6. Analysis of the nucleosome content of *met2* chromatin in MD1-1 and MD4-2. (A) Schematic representation of the *met2* gene in the wild-type control and strains MD1-1 and MD4-2. See Figs. 1 and 2 for legends. C to J indicates the positions of the MNase sensitive sites of Figs. 4 and 5. Probes I and II used for hybridization are indicated. (B-D) Protoplasts were incubated with increasing amounts of MNase (0, 1.5, 4.5, 15 and 45 units) and subjected to Southern hybridization analysis using probe I (B) and probe II (C). (D) Ethidium bromide staining of the gels.

Fig. 7. PCR analysis of FC75 and KA7 chromatin immunoprecipitated using antibodies directed against various isoforms of histones H4 and H3. (A) Schematic representation of the *met2-b2-hph* transgenic locus in strains FC75 and KA7. White boxes represent the genes. The box filled with m's indicates methylation. (B) *met2* and *hph* PCR-products obtained after chromatin immunoprecipitation using antibodies directed against H4-acetylated lysines at positions 5, 8, 12 and 16 (TetraAc) or 8 (Lys8Ac). IN: PCR-products from input DNA. PI: PCR performed with the material precipitated with the pre-immune rabbit serum (negative control). (C-F) *met2*, *hph* and *histone H1* PCR-products obtained after chromatin immunoprecipitation using antibodies directed against H4-acetylated lysines at positions 16 (Lys16Ac) or 5 (Lys5Ac) (C, D) and against dimethylated H3-Lys4 (Lys4Met) and dimethylated H3-Lys9 (Lys9Met) (E, F). PI: negative control as in B; 1 and 2 correspond to two distinct samples. In B-F, PCR amplifications were performed within the *met2* and *hph* coding sequences (see Fig. 8A). (G) PCR-products obtained from the *met2* and *hph* promoters (see Fig. 8A) after chromatin immunoprecipitation performed as in E-F.

Fig. 8. Comparative analysis of the methylation patterns of histone H3 in the promoters and the coding sequences of genes *met2* from strain MD4-2 and *hph* from strain HD3-1. (A) Schematic representation of the *met2* and *hph* loci in the strains used in chromatin immunoprecipitation experiments. The white boxes filled with m's indicate the extent of DNA methylation. Dotted lines symbolize adjacent sequences in strains KA7 and FC75. Vertical hatched bars delineate the PCR amplified segments: prm and prh for the *met2* and *hph* promoters, respectively, and csm and csh for the *met2* and *hph* coding sequences, respectively.

(B-C) PCR products obtained after chromatin immunoprecipitation using antibodies directed against dimethylated H3-Lys4 (4) and dimethylated H3-Lys9 (9). FB14 and 9H2.3 are used as unmethylated controls for *met2* and *hph*, respectively, and MD1-1 and HD2-1 are used as full-length methylated and silenced controls for *met2* and *hph*, respectively. In B, prm and prh PCR amplifications within the promoters; in C, csm and csh PCR amplifications within the coding sequences. *H1* is used as a control as in Fig. 7.

References

Antequera, F., Boyes, J., and Bird, A. (1990) High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. *Cell* **62:** 503-514.

Barra, J.L., Rhounim, L., Rossignol, J.L., and Faugeron, G. (2000) Histone H1 is dispensable for methylation-associated gene silencing in *Ascobolus immersus* and essential for long life span. *Mol Cell Biol* **20:** 61-69.

Barry, C., Faugeron, G., and Rossignol, J.L. (1993) Methylation induced premeiotically in *Ascobolus*: coextension with DNA repeat lengths and effect on transcript elongation. *Proc Natl Acad Sci U S A* **90:** 4557-4561.

Bird, A. (2002) DNA methylation patterns and epigenetic memory *Genes Dev* **16**: 6-21.

Bird, A.P. and Wolffe, A.P. (1999) Methylation-induced repression--belts, braces, and chromatin. *Cell* **99:** 451-454.

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 U S A* **84:** 1177-1181.

Cambareri, E.B., Jensen, B.C., Schabtach, E., and Selker, E.U. (1989) Repeat-induced G-C to A-T mutations in Neurospora. *Science* **244:** 1571-1575.

Colot, V. and Rossignol, J.L. (1995) Isolation of the *Ascobolus immersus* spore color gene *b2* and study in single cells of gene silencing by methylation induced premeiotically. *Genetics* **141:** 1299-1314.

Cross, S.H., Meehan, R.R., Nan, X., and Bird, A. (1997) A component of the transcriptional repressor MeCP1 shares a motif with DNA methyltransferase and HRX proteins. *Nat Genet* **16:** 256-259.

Cullen, D., Leong, S.A., Wilson, L.J., and Henner, D.J. (1987) Transformation of *Aspergillus nidulans* with the hygromycin-resistance gene, *hph*. *Gene* **57:** 21-26.

Egger, G., Liang, G., Aparicio, A., and Jones, P.A. (2004) Epigenetics in human disease and prospects for epigenetic therapy. *Nature* **429:** 457-463.

Fagard, M., Vaucheret, H. (2000) (Trans)gene Silencing in Plants: How Many Mechanisms? *Annu Rev Plant Physiol Plant Mol Biol* **51**: 167-194.

Faugeron, G., Goyon, C., and Grégoire, A. (1989) Stable allele replacement and unstable non-homologous integration events during transformation of *Ascobolus immersus*. *Gene* **76:** 109-119.

Finnegan, E.J. and Kovac, K.A. (2000) Plant DNA methyltransferases. *Plant Mol Biol* **43:** 189-201.

Fujita, N., Watanabe, S., Ichimura, T., Tsuruzoe, S., Shinkai, Y., Tachibana, M., et al. (2003) Methyl-CpG binding domain 1 (MBD1) interacts with the Suv39h1-HP1 heterochromatic complex for DNA methylation-based transcriptional repression. *J Biol Chem* **278:** 24132-24138.

Fuks, F., Hurd, P.J., Wolf, D., Nan, X., Bird, A.P., and Kouzarides, T. (2003) The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *J Biol Chem* **278:** 4035-4040.

Goyon, C. (1998) Isolation and identification by sequence homology of a second putative C5-DNA-methyltransferase gene from *Ascobolus immersus*. *DNA Seq* **9:** 109-112.

Goyon, C., Barry, C., Grégoire, A., Faugeron, G., and Rossignol, J.L. (1996) Methylation of DNA repeats of decreasing sizes in *Ascobolus immersus*. *Mol Cell Biol* **16:** 3054-3065.

Goyon, C., Faugeron, G., and Rossignol, J.L. (1988) Molecular cloning and characterization of the met2 gene from *Ascobolus immersus*. *Gene* **63:** 297-308.

Goyon, C., Nogueira, T.I., and Faugeron, G. (1994) Perpetuation of cytosine methylation in *Ascobolus immersus* implies a novel type of maintenance methylase. *J Mol Biol* **240:** 42-51.

Goyon, C., Rossignol, J.L., and Faugeron, G. (1996) Native DNA repeats and methylation in *Ascobolus*. *Nucleic Acids Res* **24:** 3348-3356.

Graessmann, A., Sandberg, G., Guhl, E., and Graessmann, M. (1994) Methylation of single sites within the herpes simplex virus tk coding region and the simian virus 40 T-antigen intron causes gene inactivation. *Mol Cell Biol* **14:** 2004-2010.

Hohn, T., Corsten, S., Rieke, S., Muller, M., and Rothnie, H. (1996) Methylation of coding

region alone inhibits gene expression in plant protoplasts. *Proc Natl Acad Sci U S A* **93:** 8334-8339.

Jackson, J.P., Johnson, L., Jasencakova, Z., Zhang, X., PerezBurgos, L., Singh, P.B., et al. (2004) Dimethylation of histone H3 lysine 9 is a critical mark for DNA methylation and gene silencing in *Arabidopsis thaliana*. *Chromosoma* **112:** 308-315.

Jackson, J.P., Lindroth, A.M., Cao, X., and Jacobsen, S.E. (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. *Nature* **416:** 556-560.

Johnson, L., Cao, X., and Jacobsen, S. (2002) Interplay between two epigenetic marks. DNA methylation and histone H3 lysine 9 methylation. *Curr Biol* **12:** 1360-1367.

Kass, S.U., Landsberger, N., and Wolffe, A.P. (1997) DNA methylation directs a time-dependent repression of transcription initiation. *Curr Biol* **7:** 157-165.

Keshet, I., Yisraeli, J., and Cedar, H. (1985) Effect of regional DNA methylation on gene expression. *Proc Natl Acad Sci U S A* **82:** 2560-2564.

Kurdistani, S.K., Tavazoie, S., and Grunstein, M. (2004) Mapping global histone acetylation patterns to gene expression. *Cell* **117:** 721-733.

Lehnertz, B., Ueda, Y., Derijck, A.A., Braunschweig, U., Perez-Burgos, L., Kubicek, S., et al. (2003) Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. *Curr Biol* **13:** 1192-1200.

Li, E., Bestor, T.H., and Jaenisch, R. (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* **69:** 915-926.

Li, T., Vu, T.H., Ulaner, G.A., Yang, Y., Hu, J.F., and Hoffman, A.R. (2004) Activating and silencing histone modifications form independent allelic switch regions in the imprinted *Gnas* gene. *Hum Mol Genet* **13:** 741-750.

Litt, M.D., Simpson, M., Gaszner, M., Allis, C.D., and Felsenfeld, G. (2001) Correlation between histone lysine methylation and developmental changes at the chicken beta-globin locus. Science **293:** 2453-2455.

Malagnac, F., Bartee, L., and Bender, J. (2002) An *Arabidopsis* SET domain protein required for maintenance but not establishment of DNA methylation. *EMBO J* **21:** 6842-6852.

Malagnac, F., Wendel, B., Goyon, C., Faugeron, G., Zickler, D., Rossignol, J.L., et al. (1997) A gene essential for de novo methylation and development in *Ascobolus* reveals a novel type of eukaryotic DNA methyltransferase structure. *Cell* **91:** 281-290.

Maloisel, L. and Rossignol, J.L. (1998) Suppression of crossing-over by DNA methylation in *Ascobolus*. *Genes Dev* **12:** 1381-1389.

Martienssen, R.A. and Colot, V. (2001) DNA methylation and epigenetic inheritance in plants and filamentous fungi. *Science* **293:** 1070-1074.

Meluh, P.B. and Broach, J.R. (1999) Immunological analysis of yeast chromatin. *Methods Enzymol* **304:** 414-430.

Nan, X., Campoy, F.J., and Bird, A. (1997) MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. *Cell* **88:** 471-481.

Ng, H.H., Zhang, Y., Hendrich, B., Johnson, C.A., Turner, B.M., Erdjument-Bromage, H., et al. (1999) MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. *Nat Genet* **23:** 58-61.

Noma, K., Allis, C.D., and Grewal, S.I. (2001) Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. *Science* **293:** 1150-1155.

Paluh, J.L., Orbach, M.J., Legerton, T.L., and Yanofsky, C. (1988) The cross-pathway control gene of *Neurospora crassa*, *cpc-1*, encodes a protein similar to GCN4 of yeast and the DNA-binding domain of the oncogene v-jun-encoded protein. *Proc Natl Acad Sci U S A* **85:** 3728-3732.

Rhounim, L., Grégoire, A., Salama, S., and Faugeron, G. (1994) Clustering of multiple transgene integrations in highly-unstable *Ascobolus immersus* transformants. *Curr Genet* **26:** 344-351.

Rhounim, L., Rossignol, J.L., and Faugeron, G. (1992) Epimutation of repeated genes in *Ascobolus immersus*. *EMBO J* **11:** 4451-4457.

Rossignol, J.L. and Faugeron, G. (1994) Gene inactivation triggered by recognition between DNA repeats. *Experientia* **50:** 307-317.

Rountree, M.R. and Selker, E.U. (1997) DNA methylation inhibits elongation but not initiation

of transcription in *Neurospora crassa*. *Genes Dev* **11:** 2383-2395.

Selker, E.U. (1998) Trichostatin A causes selective loss of DNA methylation in *Neurospora*. *Proc Natl Acad Sci U S A* **95:** 9430-9435.

Selker, E.U. and Garrett, P.W. (1988) DNA sequence duplications trigger gene inactivation in *Neurospora crassa*. *Proc Natl Acad Sci U S A* **85:** 6870-6874.

Selker, E.U., Tountas, N.A., Cross, S.H., Margolin, B.S., Murphy, J.G., Bird, A.P., and Freitag, M. (2003) The methylated component of the *Neurospora crassa* genome. *Nature* **422:** 893-897.

Soppe, W.J., Jasencakova, Z., Houben, A., Kakutani, T., Meister, A., Huang, M.S., et al. (2002) DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in *Arabidopsis*. *EMBO J* **21:** 6549-6559.

Tamaru, H. and Selker, E.U. (2001) A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. *Nature* **414:** 277-283.

Tamaru, H., Zhang, X., McMillen, D., Singh, P.B., Nakayama, J., Grewal, S.I., et al. (2003) Trimethylated lysine 9 of histone H3 is a mark for DNA methylation in *Neurospora crassa*. *Nat Genet* **34:** 75-79.

Tariq, M., Saze, H., Probst, A.V., Lichota, J., Habu, Y., and Paszkowski, J. (2003) Erasure of CpG methylation in *Arabidopsis* alters patterns of histone H3 methylation in heterochromatin. *Proc Natl Acad Sci U S A* **100:** 8823-8827.

Wade, P.A., Gegonne, A., Jones, P.L., Ballestar, E., Aubry, F., and Wolffe, A.P. (1999) Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. *Nat Genet* **23:** 62-66.

Yoder, J.A., Walsh, C.P., and Bestor, T.H. (1997) Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet* **13:** 335-340.

FIGURE 1

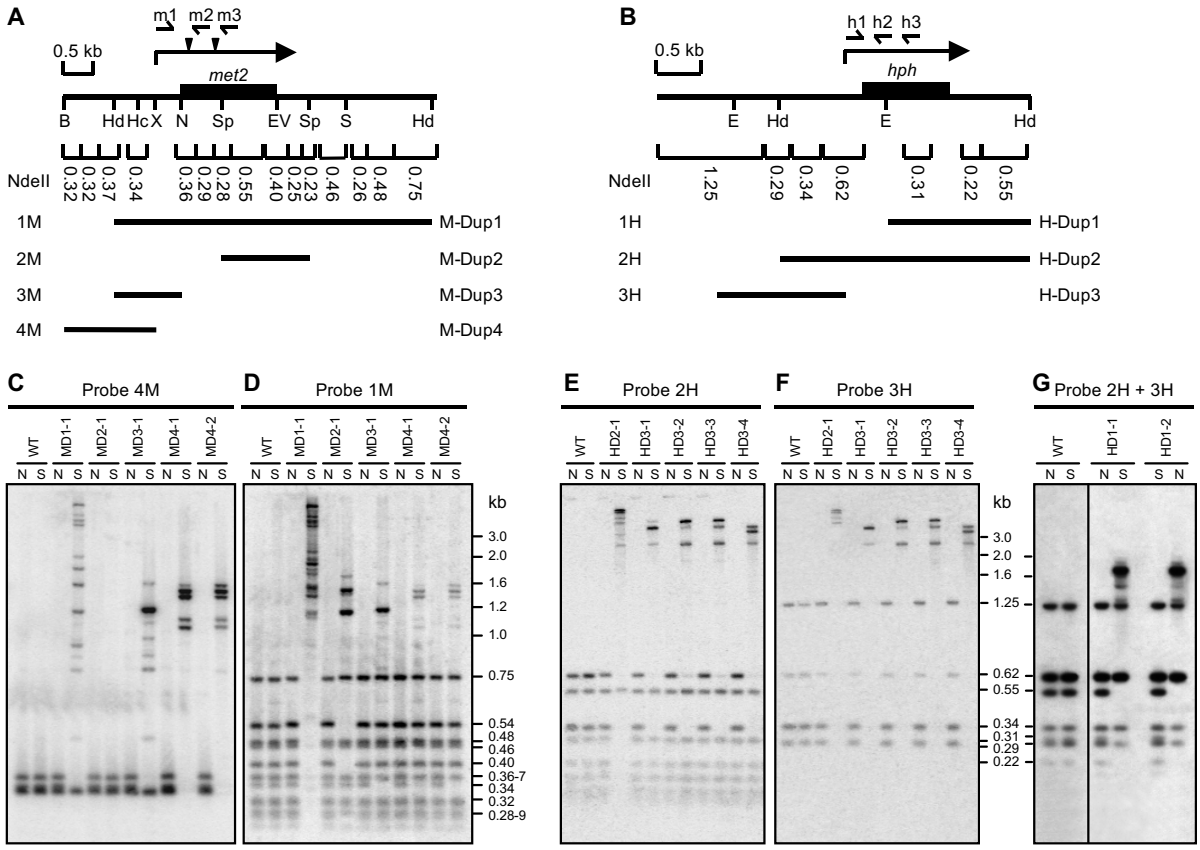

A
m1 m2 m3
0.5 kb
met2
B Hd Hc X N Sp EV Sp S Hd
NdeII
0.32 0.32 0.37 0.34 0.36 0.29 0.28 0.55 0.40 0.25 0.23 0.46 0.26 0.48 0.75
1M M-Dup1
2M M-Dup2
3M M-Dup3
4M M-Dup4
B
h1 h2 h3
0.5 kb
hph
E Hd E Hd
NdeII
1.25 0.29 0.34 0.62 0.31 0.22 0.55
1H H-Dup1
2H H-Dup2
3H H-Dup3
C Probe 4M
D Probe 1M
E Probe 2H
F Probe 3H
G Probe 2H + 3H
WT MD1-1 MD2-1 MD3-1 MD4-1 MD4-2
WT HD2-1 HD3-1 HD3-2 HD3-3 HD3-4
WT HD1-1 HD1-2
N S
kb
3.0 2.0 1.6 1.2 1.0 0.75 0.54 0.48 0.46 0.40 0.36-7 0.34 0.32 0.28-9
3.0 2.0 1.6 1.25 0.62 0.55 0.34 0.31 0.29 0.22

FIGURE 2

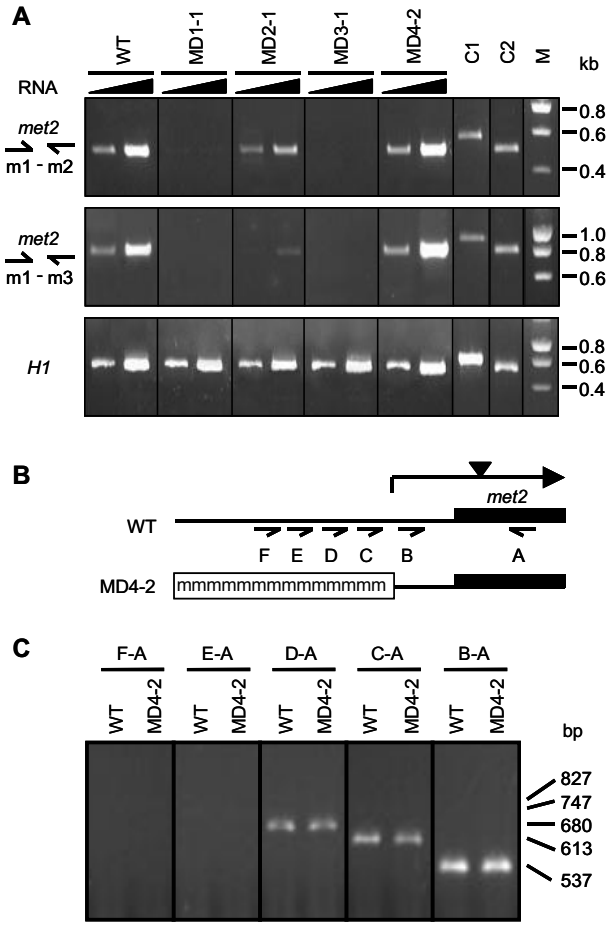

A
WT
MD1-1
MD2-1
MD3-1
MD4-2
C1
C2
M
kb
RNA
met2
m1 - m2
0.8
0.6
0.4
met2
m1 - m3
1.0
0.8
0.6
H1
0.8
0.6
0.4
B
met2
WT
F E D C B
A
MD4-2
mmmmmmmmmmmmmm
C
F-A
E-A
D-A
C-A
B-A
WT
MD4-2
bp
827
747
680
613
537

FIGURE 3

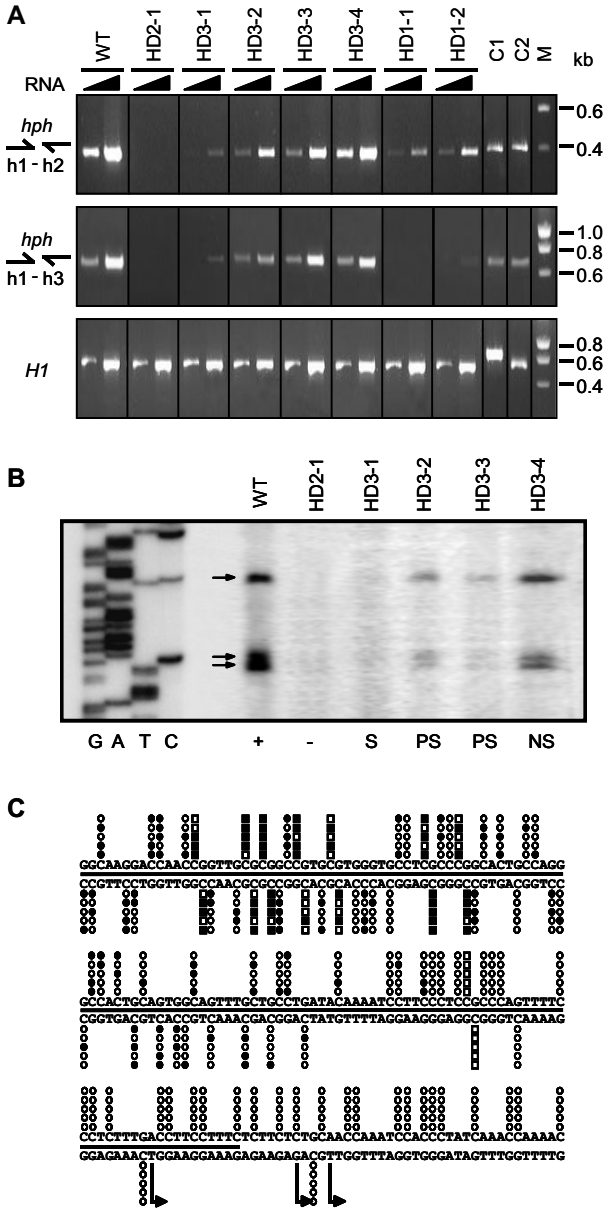

A
WT
HD2-1
HD3-1
HD3-2
HD3-3
HD3-4
HD1-1
HD1-2
C1
C2
M
kb
RNA
hph
h1 - h2
0.6
0.4
hph
h1 - h3
1.0
0.8
0.6
H1
0.8
0.6
0.4
B
WT
HD2-1
HD3-1
HD3-2
HD3-3
HD3-4
G A T C
+
-
S
PS
PS
NS
C

FIGURE 4

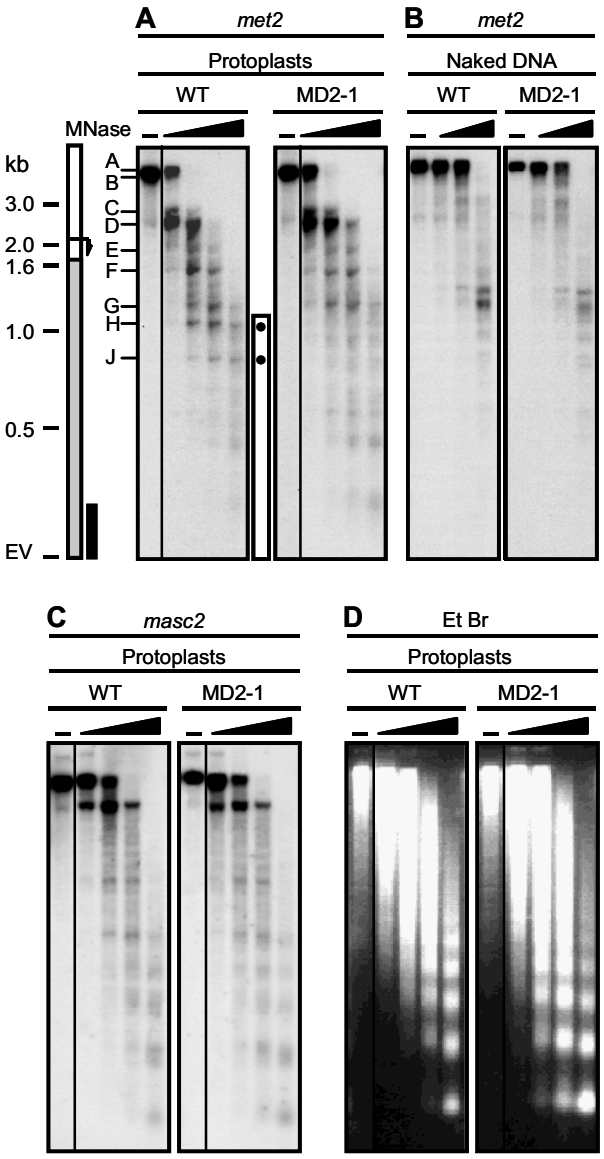

A
met2
Protoplasts
WT
MD2-1
MNase
kb
3.0
2.0
1.6
1.0
0.5
EV
A
B
C
D
E
F
G
H
J
B
met2
Naked DNA
WT
MD2-1
C
masc2
Protoplasts
WT
MD2-1
D
Et Br
Protoplasts
WT
MD2-1

FIGURE 5

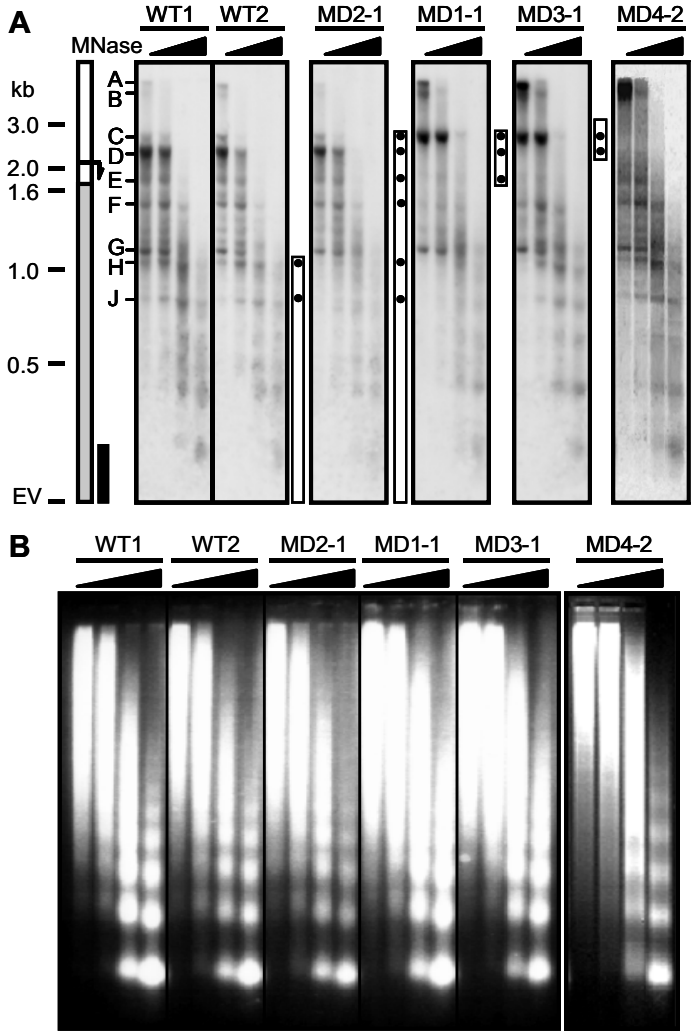

A
WT1
WT2
MD2-1
MD1-1
MD3-1
MD4-2
MNase
kb
3.0
2.0
1.6
1.0
0.5
EV
A
B
C
D
E
F
G
H
J
B
WT1
WT2
MD2-1
MD1-1
MD3-1
MD4-2

FIGURE 6

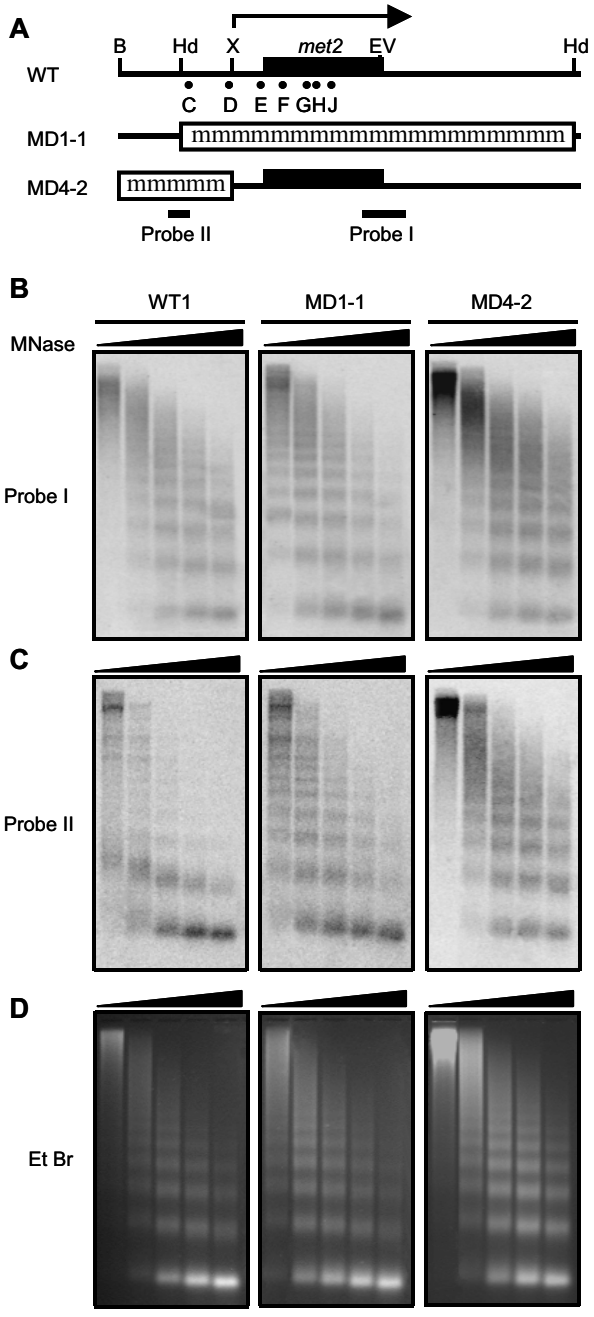

A
B
Hd
X
met2
EV
Hd
WT
C
D
E
F
GHJ
MD1-1
mmmmmmmmmmmmmmmmmmmm
MD4-2
mmmmm
Probe II
Probe I
B
WT1
MD1-1
MD4-2
MNase
Probe I
C
Probe II
D
Et Br

FIGURE 7

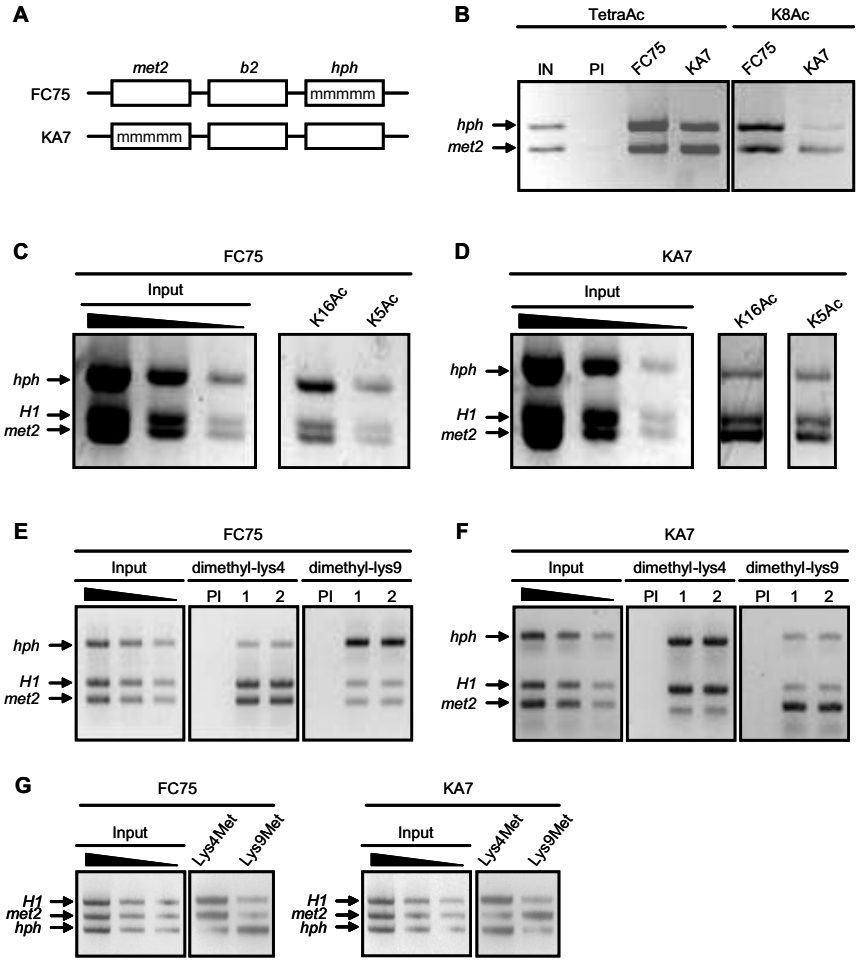
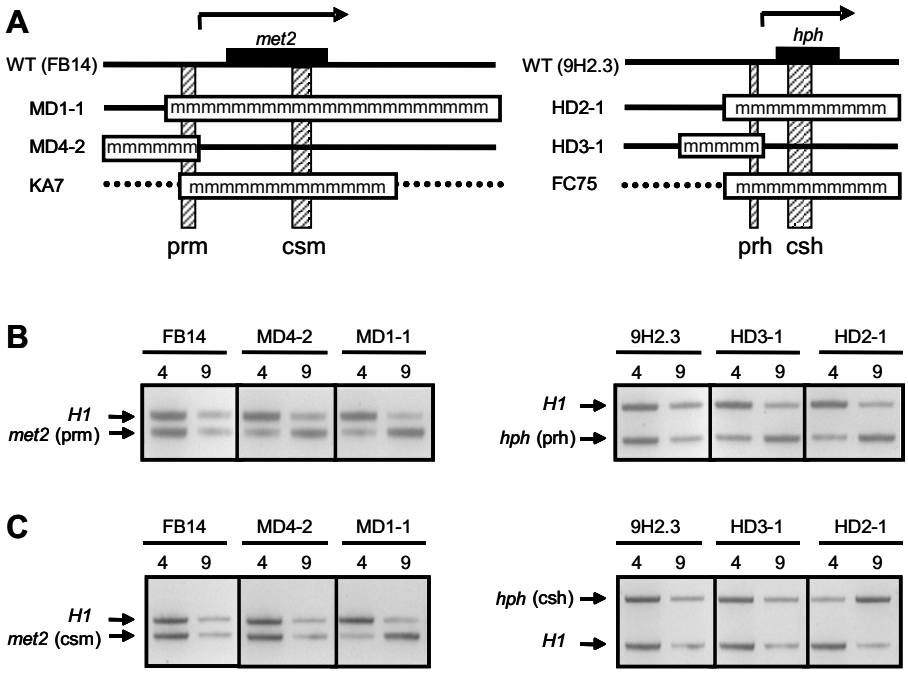

A
met2
b2
hph
FC75
mmmmm
KA7
mmmmm
B
TetraAc
K8Ac
IN
PI
FC75
KA7
FC75
KA7
hph
met2
C
FC75
Input
K16Ac
K5Ac
hph
H1
met2
D
KA7
Input
K16Ac
K5Ac
hph
H1
met2
E
FC75
Input
dimethyl-lys4
dimethyl-lys9
PI 1 2
PI 1 2
hph
H1
met2
F
KA7
Input
dimethyl-lys4
dimethyl-lys9
PI 1 2
PI 1 2
hph
H1
met2
G
FC75
Input
Lys4Met
Lys9Met
H1
met2
hph
KA7
Input
Lys4Met
Lys9Met
H1
met2
hph

FIGURE 8


A
met2
WT (FB14)
MD1-1
MD4-2
KA7
prm
csm
hph
WT (9H2.3)
HD2-1
HD3-1
FC75
prh
csh
B
FB14
MD4-2
MD1-1
4 9 4 9 4 9
H1
met2 (prm)
9H2.3
HD3-1
HD2-1
4 9 4 9 4 9
H1
hph (prh)
C
FB14
MD4-2
MD1-1
4 9 4 9 4 9
H1
met2 (csm)
9H2.3
HD3-1
HD2-1
4 9 4 9 4 9
hph (csh)
H1