Genome Defense and DNA Methylation in *Neurospora*

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The continuity of life is attributed to the faithful copying of DNA. It is therefore intriguing that some organisms chemically modify their DNA in ways that can lead to heritable changes. Soon after the discovery that a fraction of cytosines are methylated in DNA of some eukaryotes—including vertebrates, plants, and some fungi—evidence began to accumulate that DNA methylation is inherently mutagenic. For example, in mammals, where methylation is almost exclusively found at cytosines immediately preceding guanines (“CpG” dinucleotides), it was noticed that methylated chromosomal regions are typically unexpectedly poor in CpGs and unexpectedly rich in TpGs and CpAs, apparently as a result of C to T mutations at sites subject to methylation (Bird 1980). It was assumed that this reflected the fact that 5-methyl-cytosine, like cytosine, is somewhat unstable, spontaneously decomposing to 5-methyl-uridine (thymine) and that G:T mismatches are repaired less efficiently than G:U mismatches. Discovery of repeat-induced point mutation (RIP) in the filamentous fungus *Neurospora crassa* raised the possibility that DNA methyltransferases and similar enzymes may be directly responsible for such mutations (Selker 1990b; Freitag et al. 2002). What positive functions does DNA methylation serve? Although the control and function of DNA methylation are both still hotly debated, it is becoming increasingly clear that DNA methylation is one of several mechanisms that defends the eukaryotic genomes from mischievous DNA. *Neurospora* provided an early, and exceptionally clear, illustration of this and it continues to provide a convenient model to elucidate DNA methylation and other epigenetic processes.

THE METHYLATION LANDSCAPE

Although *Neurospora* was initially reported to have no methylated DNA (Antequera et al. 1984), observations in the 1980s revealed cytosine methylation in this model eukaryote. After methods became established to transform *Neurospora*, it was noticed that some transforming sequences are subject to de novo methylation, especially when they are repeated (Bull and Wootton 1984; Selker et al. 1987a; Orbach et al. 1988). At the same time, natural patches of methylation were found associated with several 5S rRNA pseudogenes (Selker and Stevens 1985; Margolin et al. 1998). No methylation has been reported

in any of the hundreds of protein-coding genes that have been studied. Indeed, as discussed below, recent analyses suggest that most DNA methylation is associated with relics of transposons. We now know that ~1.5% of the cytosines in the DNA of *N. crassa* are methylated (Russell et al. 1987; Foss et al. 1993).

The first methylated region characterized in detail is the 1.6-kb zeta-eta (ζ - η) region (Selker and Stevens 1985; Selker et al. 1993b). This consists of a diverged tandem duplication of a 0.8-kb segment of DNA, including a 5S rRNA gene. Comparison of this region with the corresponding chromosomal region of strains lacking the duplication led to the idea that repeated sequences can somehow induce DNA methylation (Selker et al. 1985, 1987a; Grayburn and Selker 1989) and ultimately led to the discovery of the genome defense system that we named RIP (Selker et al. 1987b; Selker and Garrett 1988; Cambareri et al. 1989). Both the ζ - η region and the psi-63 (ψ 63) region, the second methylated region discovered in *Neurospora* (Metzenberg et al. 1985; Foss et al. 1993; Miao et al. 1994; Margolin et al. 1998), are products of RIP.

THE PROTOTYPICAL GENOME DEFENSE SYSTEM: RIP

RIP detects duplicated sequences in the haploid genomes of special dikaryotic cells resulting from fertilization and then riddles both copies of the duplicated sequence with polarized transition mutations; C:G pairs are replaced with T:A pairs (Cambareri et al. 1989). RIP has a clear sequence preference (Cs preceding As are mutated most frequently), making it straightforward to recognize sequences that have been mutated by RIP (Margolin et al. 1998). In a single passage through the sexual cycle, up to ~30% of the G:C pairs in duplicated sequences can be changed to A:T pairs (Cambareri et al. 1991). After RIP, remaining cytosines are generally methylated, including those not in symmetrical sequences (e.g., CpGs) (Selker et al. 1993a). The methylation does not depend on the continued presence of sequence duplications and is normally stable through numerous rounds of DNA replication (Selker and Garrett 1988; Singer et al. 1995). DNA molecules show different patterns and levels of modification (Selker and Stevens 1985; Selker et al. 1993a). This heterogeneous methylation can extend beyond the mu-

tated region and even beyond the edge of the segment that was originally duplicated (Cambareri et al. 1989; Selker et al. 1993a; Irelan and Selker 1997; Miao et al. 2000).

Results of genome-wide analyses of DNA methylation suggest that the vast majority of methylated residues are in relics of RIP (Galagan et al. 2003; Selker et al. 2003). Indeed, the only methylation not known to have resulted from RIP is that in the tandemly arranged rDNA (Perkins et al. 1986). A survey of methylated *Neurospora* sequences isolated by affinity chromatography using the methyl-binding domain (MBD) of MeCP2 revealed clear evidence of RIP in 47 of 51 sequenced fragments (Selker et al. 2003). Analysis of these sequences revealed significant similarities with a variety of transposable elements identified in other organisms, including both retrotransposons (e.g., lolligag, Tad, Tcen, and DAB1) and DNA-type transposons (e.g., Dodo1, Dodo2, Dodo3, listless, dPunt, Punt3, and Nogo) (Selker et al. 2003). Similar information came from large-scale genome sequencing projects. About 10% of the genome is composed of repetitive DNA and most of this is identifiable as transposon-like sequences that have been riddled with mutations by RIP (Galagan et al. 2003). Not a single intact transposable element was identifiable, consistent with failures to detect transpositions in standard *Neurospora* strains (Kinsey and Helber 1989). We conclude that RIP is an efficient genome defense mechanism. Does this mean that *Neurospora* cannot generate gene families by gene duplications? Nearly 20% of *N. crassa* genes are found in multigene families, but, interestingly, nearly all of the paralogs are sufficiently divergent and/or short that they should be invisible to RIP (Galagan et al. 2003). Thus, although *Neurospora* has gene families, RIP is presumably an obstacle to evolution of new genes through gene duplication (Galagan and Selker 2004).

DE NOVO AND MAINTENANCE METHYLATION IN *NEUROSPORA*

Laboratory experiments revealed that most products of RIP, but not their unmutated counterparts, are methylated (Selker et al. 1987a, 1993a; Cambareri et al. 1991; Singer et al. 1995; Miao et al. 2000). Similarly, predicted products of RIP identified in the genome based on their sequence composition are generally methylated (Galagan et al. 2003; Selker et al. 2003). In principle, such methylation could reflect signals for de novo methylation that work in vegetative cells. Alternatively, the mutated sequences may be methylated because of propagation of methylation established earlier (e.g., during RIP) and they could lack the capacity to trigger methylation de novo in vegetative cells. These possibilities were tested by determining whether a given sequence can induce methylation after it is stripped of its methylation by molecular cloning or by drug treatment (Singer et al. 1995). Such experiments revealed that, in general, sequences altered by RIP can trigger de novo methylation in vegetative cells. Most sequences mutated by RIP become remethylated both at their original genomic location and at arbitrary chromosomal positions (Selker et al.

1987a). Some products of RIP with relatively few mutations, however, do not normally become remethylated at their original site, suggesting that the observed methylation represents propagation of methylation established earlier (Singer et al. 1995). Although surprising, considering that this methylation is heterogeneous and is not limited to symmetrical sites, the capacity of *Neurospora* to perform such “maintenance methylation” on some sequences was experimentally demonstrated (Selker et al. 2002).

cis-ACTING SIGNALS THAT CONTROL DNA METHYLATION

As mentioned above, some transforming sequences are subject to de novo methylation without going through the sexual cycle (Bull and Wootton 1984; Selker et al. 1987a, 1993a; Orbach et al. 1988; Selker 1990a; Pandit and Russo 1992; Romano and Macino 1992). We have found that sequences differ in their susceptibility to methylation in vegetative cells. Native, unmutated sequences are not susceptible, while many foreign sequences with resemblance to sequences mutated by RIP are (M. Freitag and E. Selker, unpubl.). This methylation appears loosely correlated with the copy number of transforming DNA. Although single-copy sequences are less frequently methylated than multicopy sequences (Pandit and Russo 1992; Romano and Macino 1992; Selker et al. 1993b), multicopy sequences are not always methylated (Selker and Garrett 1988).

Observations on the specificity of methylation in *Neurospora* led to the “collapsed chromatin model,” in which DNA methylation is the fate of sequences that are inert (i.e., completely inactive [Selker 1990a]). One prediction of the model was that short sequences should not have the ability to trigger methylation, for example, when inserted into an active gene. To explore this and other possibilities, we developed efficient gene-targeting systems that allowed us to test the methylation potential of single copies of sequences integrated precisely and without extraneous sequences at a common chromosomal position (at the *am* locus on LG VR [Miao et al. 1994] or the *his-3* locus on LG IR; Margolin et al. 1997). Using these systems, we demonstrated that mutations per se (e.g., numerous A:T to G:C mutations) do not trigger methylation and that products of RIP, such as the ζ - η region, effectively contain multiple, additive methylation signals, whose effects can spread hundreds of base pairs into flanking sequences. Fragments of the ζ - η region as short as 171 bp can trigger methylation (Selker et al. 1993b; Miao et al. 2000). We found that mutation density per se does not determine whether sequences become methylated and that neither A:T-richness nor high densities of TpA dinucleotides, typical attributes of methylated sequences in *Neurospora*, are essential features of methylation signals. Nevertheless, both A:T-richness and high densities of TpA dinucleotides appear to promote methylation in *Neurospora*. These and other findings from “transplanting” small fragments of genes, pieces of DNA mutated by RIP, and synthetic sequences led us to con-

clude that methylated sequences do not simply reflect the absence of signals that prevent methylation; they apparently contain positive signals that trigger methylation (Miao et al. 2000).

To better define the nature of these signals, we developed a more sensitive assay to test the capacity of short (25–100-bp) synthetic oligonucleotides to trigger methylation at a specific locus (Tamaru and Selker 2003). Our system used a *his-3* targeting vector carrying a 100-bp ζ - η segment surrounded by a lightly RIP-mutated allele of the *am* gene. We demonstrated that this mosaic construct does not trigger methylation itself at *his-3*, but does provide a sensitive context to assess the potential of various sequences to induce methylation. A variety of random sequences consisting of only A and T residues triggered methylation of nearby cytosines. Introduction of G:C pairs into the A:T-rich sequences was strongly inhibitory and both As and Ts were found to be required on the same strand to trigger significant methylation. Nevertheless, neither TpA nor ApT dinucleotides were essential. Tests of 20-, 25-, 40-, and 80-mer fragments of the most potent sequence, (TAAA)_n, showed that longer tracts of this sequence act as stronger signals. It seems possible that an unidentified “A:T-hook”-type protein mediates methylation in *Neurospora*. Consistent with this possibility, we found that Distamycin A, an analog of the A:T-hook motif, interferes with de novo methylation in *Neurospora* (Tamaru and Selker 2003). Nevertheless, some of our findings do not support this hypothesis. In particular, we found that sequences with only two bp A:T tracts (e.g., (CTA)_n) can induce methylation. In addition, using A:T-hook protein HMG-I (kindly provided by R. Reeves, Washington State University, Pullman), we showed that the sequence preference for HMG-I binding does not simply correlate with the sequence preferences for de novo methylation. Additional work will be required to determine how relics of RIP, and similar degenerate sequences, are recognized to trigger methylation in vegetative cells of *Neurospora*.

FORWARD GENETICS APPROACH TO ELUCIDATE CONTROL AND FUNCTION OF DNA METHYLATION

Neurospora is well suited to identify components of the methylation machinery by genetic approaches that do not rest on prior knowledge or preconceptions. We have successfully used several approaches to identify mutants defective in methylation (*dim*), but have not yet saturated the *Neurospora* genome for such mutations. DNA methylation can silence genes in *Neurospora* (Rountree and Selker 1997), allowing for the direct selection of mutations that affect DNA methylation. Nevertheless, most known *dim* mutants have been identified in other ways, such as in “brute force” screens using Southern hybridization to identify mutants that affect methylation (Foss et al. 1993, 1995). Two mutants, *dim-2* and *dim-5*, abolish all detectable DNA methylation. Mapping and complementation studies revealed that the *dim-2* gene encodes a DNA methyltransferase (DMTase) (Kouzminova

and Selker 2001), while *dim-5* encodes a histone methyltransferase (HMTase) (Tamaru and Selker 2001).

Biochemical characterization of DIM-2 should be interesting. This predicted 1454-amino-acid protein is responsible for all detected DNA methylation in *Neurospora*, including methylation in a variety of sequence contexts. Mutations preventing all methylation have not been described in other eukaryotes, which may reflect the fact that DNA methylation results from multiple DMTases in most organisms that have been characterized and that DNA methylation is essential in some organisms. Although the DIM-2 carboxy-terminal domain includes all the well conserved motifs characteristic of DMTases, its amino-terminal domain shows no marked similarity to previously described proteins (Kouzminova and Selker 2001).

CONTROL OF DNA METHYLATION BY HISTONE METHYLATION

The discovery that *dim-5* encodes a HMTase provided the first indication that DNA methylation is regulated by histone methylation, at least in some organisms. Biochemical work showed that DIM-5 is specific for lysine 9 of histone H3 and that it efficiently trimethylates this residue in vitro and in vivo, unlike all previously characterized HMTases (Tamaru and Selker 2001; Tamaru et al. 2003). In order to determine whether histone H3 is the critical substrate for DIM-5 in vivo, we transformed a *Dim*⁺ *Neurospora* strain with engineered histone H3 genes carrying a mutation at lysine 9 that would preclude methylation. Amino acid substitutions at lysine 9 dramatically reduced DNA methylation, implicating histone H3 as the critical substrate for DNA methylation (Tamaru and Selker 2001). Evidence that some DNA methylation in *Arabidopsis* is also controlled by histone methylation came soon thereafter (Jackson et al. 2002; Malagnac et al. 2002). Interestingly, the critical mark in *Arabidopsis* appears to be di-, rather than tri-, methyl-lysine 9 (Jackson et al. 2004). Most recently, Jenuwein and colleagues have provided evidence in mice that trimethylation of lysine 9 of histone H3 directs DNA methylation to major satellite repeats at pericentric heterochromatin (Lehnertz et al. 2003). The extent to which DNA methylation is controlled by histones in eukaryotes is not yet known.

SEARCH FOR OTHER COMPONENTS OF DNA METHYLATION PATHWAY

In principle, forward genetics should reveal all components of the DNA methylation pathway except for those that are essential or redundant. However, use of reverse genetics and biochemical approaches can facilitate the identification of components in the methylation process. For example, evidence from other systems that a heterochromatin protein, HP1 (Eissenberg and Elgin 2000), binds methylated lysine 9 of histone H3 (Bannister et al. 2001; Jacobs et al. 2001; Lachner et al. 2001), led us to search for an HP1 homolog in *Neurospora* and to test its possible involvement in DNA methylation by disrupting

the HP1 gene (*hpo*) by RIP. We found that HP1 is indeed essential for DNA methylation in *Neurospora*, implying that this protein directs DIM-2 to DNA associated with chromatin in which histone H3 is methylated at lysine 9 (Freitag et al. 2004a).

Clues that the RNAi machinery may be involved in DNA methylation (Wassenegger et al. 1994; Aufsatz et al. 2002; Chan et al. 2004) prompted us to test the components of the *Neurospora* RNAi machinery, including apparent RNA-dependent RNA polymerases, dicers, argonautes, and RecQ helicase homologs that had been identified by genetic or bioinformatics approaches (Galagan et al. 2003). We found no evidence that any of the RNAi machinery is involved in initiation or maintenance of DNA methylation (Freitag et al. 2004b). In contrast, mutational analyses of histone deacetylase genes of *Neurospora* revealed that at least one of these genes is involved in DNA methylation in *Neurospora* (K. Smith et al., unpubl.), consistent with the observation that the histone deacetylase inhibitor Trichostatin A reduces DNA methylation in some chromosomal regions of this organism (Selker 1998).

CONCLUSIONS

Why control DNA methylation through histone methylation? In the last few years it has become increasingly clear that histones are more than structural proteins; they are informational molecules (Jenuwein and Allis 2001). Particular combinations of posttranslational modifications of histones, including phosphorylation, methylation, acetylation, ubiquitination, and ADP ribosylation, can influence the function of the associated DNA and the modifications can depend on the presence or absence of other modifications. For example, methylation of lysine 9 on histone H3 is inhibited by phosphorylation of serine 10 and methylation of lysine 4, which are associated with active sequences (Rea et al. 2000; E. Berge et al., unpubl.) and there are indications that histone acetylation also influences methylation of lysine 9 (Selker 1998; Nakayama et al. 2001; K. Smith et al., unpubl.). Thus histones are well suited to integrate information bearing on the DNA that they are associated with.

Neurospora provided the first example of a genome defense system, RIP, and has more recently revealed two additional genetic mechanisms related to RNA interference (RNAi) mechanisms discovered in plants and animals that should also resist change in the genome. The first, "quelling," is specific to the vegetative phase of the *Neurospora* life cycle and is responsible for destroying RNA homologous to aberrant RNAs, for example, resulting from expression of tandem arrays of transgenes (Romano and Macino 1992; Catalanotto et al. 2002). The second, meiotic silencing by unpaired DNA (MSUD) (Aramayo and Metzberg 1996; Shiu et al. 2001), scans paired homologs in meiosis for unpaired sequences and then destroys RNA matching unpaired DNA (Shiu and Metzberg 2002; Lee et al. 2004). DNA methylation is commonly associated with both sequences mutated by RIP and with transgenes that trigger quelling, but it is not

required for either process (Cogoni et al. 1996; Kouzminova and Selker 2001). Similarly, the RNAi processes are not required for DNA methylation. Thus, DNA methylation may be considered a fourth genome defense system, although there are undoubtedly additional connections between *Neurospora*'s genome defense systems that are yet to be elucidated.

It should be interesting to discover similarities and differences between the *Neurospora* genome defense systems and those in other organisms. For example, we need to better characterize how DNA methylation controls transposable elements in *Neurospora* and mammals. In mammals, DNA methylation appears to block initiation of transcription, but not transcription elongation (Jones 1999); while in *Neurospora*, DNA methylation does not appear to inhibit transcription initiation, but nonetheless prevents accumulation of transcripts (Rountree and Selker 1997). Results of in vivo labeling experiments in *Neurospora* and nuclear run-on assays suggest that methylation interferes with elongation, but it remains possible that methylation also triggers RNA degradation (e.g., by an RNAi pathway). Another difference between *Neurospora* and mammals pertains to how methylation is perceived. Whereas mammals possess a variety of protein complexes that specifically recognize methylated DNA and recruit chromatin modification factors (Bird and Wolffe 1999), *Neurospora* lacks MBD proteins, but preliminary investigations suggest that *Neurospora* uses different proteins to recognize methylated sequences (G. Kothe et al., unpubl.). Finally, until we have a better understanding about how different organisms "decide" which sequences to methylate, the possible generality of mechanisms involved in the function and control of DNA methylation will not be clear.

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