function of the human protein will help our understanding of silencing in yeast as well as in other organisms.

Gasser: You described the phenotype of mating-type silencing in the presence of the human gene, but since we know that dosage is important and you are looking at this in a *sir2* null, it is possible that the human gene restores Sir2p levels to a level that is insufficient to repress at telomeres. It appears that Sir2p is more limiting at telomeres than at mating-type loci.

Pillus: In order to address whether there might be competition between the loci, Joyce Sherman deleted the silent mating-type loci and then repeated the experiments I described. She did not see a shift in protein function towards the telomeres, suggesting that there is no competition. I should add that this chimeric construct also promotes rDNA silencing.

Barlow: Is there a parallel system to mating-type switching in humans?

Pillus: It's a fascinating idea but I don't know of any supporting evidence.

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Epigenetic mechanisms in the regulation of the maize *Suppressor–mutator* transposon

Ramesh Raina, Michael Schläppi* and Nina Fedoroff[1]

*Biology Department and Biotechnology Institute, The Pennsylvania State University, University Park, PA 16802 and *Department of Biology, Marquette University, Milwaukee, WI 53201, USA*

Abstract. Transcription and transposition of the maize *Suppressor–mutator* (*Spm*) transposon are epigenetically controlled. Methylation of specific element sequences prevents transcription and transposition in a heritable manner. Reactivation and demethylation occur in the presence of an active element, implying the existence of an element-encoded epigenetic activator. The methylation target sequences are the 0.2 kb promoter and an 0.35 kb GC-rich downstream sequence. Two *Spm*-encoded proteins, TnpA and TnpD, participate in transposition. In addition, TnpA has positive and negative regulatory activities. TnpA represses and activates the unmethylated and methylated *Spm* promoters, respectively, and it participates in the transient and heritable demethylation of the promoter and GC-rich region. There is evidence that TnpA-mediated repressor and epigenetic activator functions occur by different molecular mechanisms.

1998 Epigenetics. Wiley, Chichester (Novartis Foundation Symposium 214) p 133–143

Half a century ago, Barbara McClintock reported that certain genetic elements, now called transposons, move from one chromosomal site to another. Although it is clear that transposons are both ancient and abundant, their role in organismal evolution remains enigmatic. Insertion of transposons into genes or regulatory sequences generally disrupts gene function, but it can also reprogram gene expression (reviewed in Fedoroff 1989a). Transposons cause chromosome breaks that stimulate illegitimate recombination, rearrangements and gene conversion. Chromosomes of many organisms are laden with dozens, hundreds or thousands of copies of related transposable element sequences. Transposons fall into groups of related sequences, termed element families, which are further subcategorized

[1]This chapter was presented at the symposium by Nina Fedoroff.

based on their transposition mechanisms and relatedness to viruses. Mobilization of just one family of elements can cause structural alterations at many chromosomal sites simultaneously. Genetic changes, large and small, are inevitable sequelae of transposable element activity. Transposable elements of all kinds outreplicate the genome, accumulating over time. The puzzle is not how transposons survive in evolution, but how organisms survive their transposable elements and, perhaps, even accrue evolutionary benefits.

The emerging answer is that there are many molecular devices for modulating transposition (Howe & Berg 1989). These include layers of *cis*- and *trans*-acting mechanisms that function at transcriptional, translational and post-translational levels. This chapter focuses on the epigenetic regulatory system that governs expression of the maize *Suppressor–mutator* (*Spm*) transposon. *Spm* expression and transposition are regulated by a complex mechanism that has some of the characteristics of the familiar reversible regulatory mechanisms of prokaryotes and eukaryotes, but also has components resembling the more stable changes in gene expression associated with paramutation in plants and imprinting in animals (Brink 1973, Lyon 1993). Although the underlying molecular mechanisms are presently best understood for the *Spm* element, it is likely that other maize transposons are regulated similarly (Fedoroff & Chandler 1994).

Transposition and transcription of the *Spm* element are controlled negatively by both genetic and epigenetic mechanisms. The element itself encodes an autoregulatory protein that promotes the reactivation of an epigenetically silent, transpositionally disabled element and represses transcription of the active element's promoter. The existence of both epigenetic and autoregulatory mechanisms is already evident in McClintock's early genetic work on *Spm*. The *Spm* element's control system is currently among the most thoroughly studied epigenetic regulatory mechanisms. It is a paradigm uniquely accessible to both genetic and molecular analysis because it controls genes that are not essential for survival of the organism. But there is a growing body of evidence that similar mechanisms are central to plant development and physiological adaptations to changing environmental conditions (Finnegan et al 1993, 1996, Ronemus et al 1996, Sorensen et al 1996).

Discovery of epigenetic regulation

McClintock observed that a *Spm* element could change or 'mutate' to one of a number of different forms in which it was either silent or showed a pattern of alternation between active and inactive phases during development (McClintock 1957, 1958, 1959, 1961, 1962, 1965, 1971). She recognized different developmental patterns of element expression and understood that a given pattern is heritable, yet capable of changing to still different heritable expression patterns. She also

reported that elements could remain silent for multiple plant generations, returning to an active form at a low frequency, and observed that an inactive element could be activated by the presence of an active element (McClintock 1971). The results of her initial studies leading to the discovery of transposable elements suggested to McClintock that transposable elements are normal genomic constituents maintained in a deeply inactive, but genetically competent state (McClintock 1946). She later showed that previously undetectable transposable elements could be converted to an active form under circumstances of extensive chromosome breakage (McClintock 1950a,b, 1951). These early observations suggested the existence of an epigenetic mechanism which maintains plant transposons in a silent, but genetically intact form.

Suppressor–mutator structure and expression

The *Spm* element is 8.4 kb in length and superficially simple in organization (Fig. 1a). Only a single transcription unit has been identified so far and it occupies most of the element's length (Pereira et al 1986, Masson et al 1987, 1989). The few hundred base pairs outside of the transcription unit consist of sequences essential for both transcription and transposition of the element. The ends comprise inverted repeats of a 13 bp sequence, CACTACAAGAAAA. These

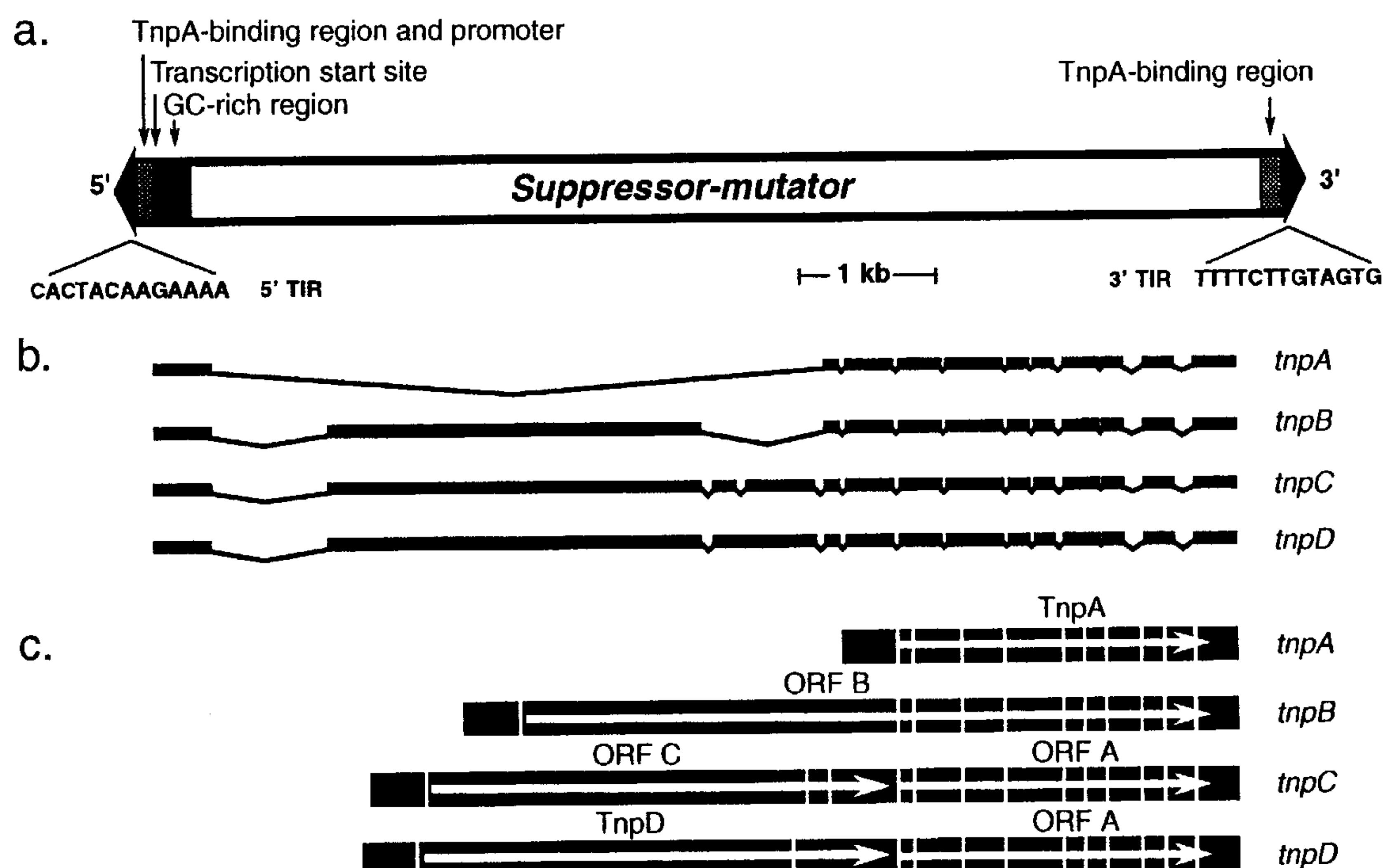

FIG. 1. A diagrammatic representation of the *Suppressor–mutator* element's structure (a), transcripts (b) and open reading frames (ORFs) (c). Black boxes in parts b and c represent exons. TIR, terminal inverted repeat.

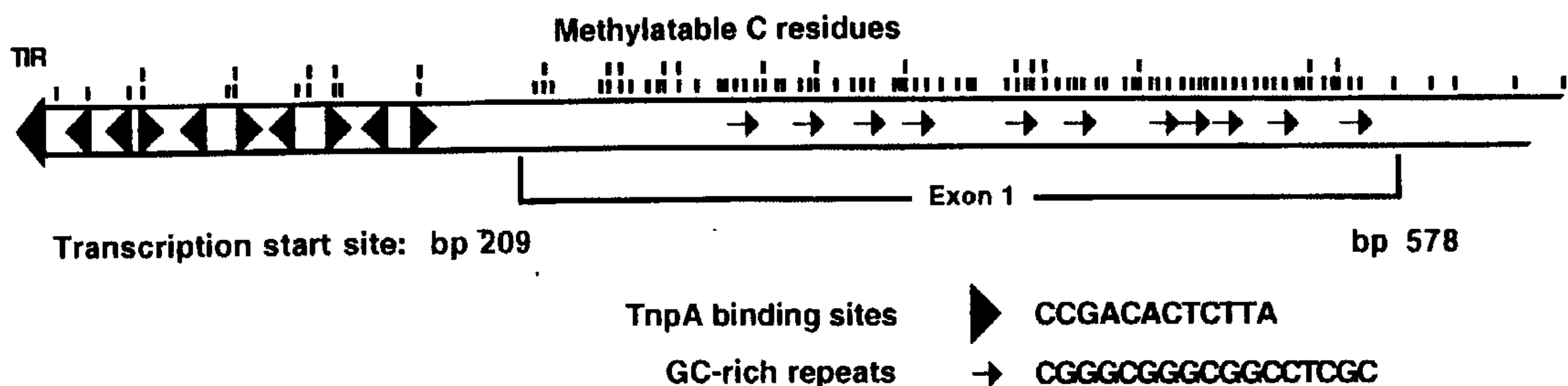

FIG. 2. The 5′ end of the *Suppressor–mutator* (*Spm*) transposon. The diagram shows the repetitive sequences and methylatable C residues in the promoter and the GC-rich downstream sequence of *Spm*. The 5′ terminal 0.2 kb contains nine copies, some in each orientation, of TnpA binding sequences. The GC-rich first exon contains 11 direct repeats of a 17 bp GC-rich sequence. Arrows indicate the position and orientation of the repeats. TIR, terminal inverted repeat.

short terminal inverted repeats (TIRs) demarcate the segment of DNA that transposes. Between the TIRs and the transcription unit at both element ends, there are several hundred base pairs designated the 'subterminal repetitive regions'. Each contains multiple copies of a 12 bp sequence having 75% or more identity with the consensus sequence CCGACACTCTTA (Fig. 2). These are binding sites for an element-encoded protein, TnpA (Pereira et al 1986, Masson et al 1987, Gierl et al 1988). The element's promoter is contained within the first 0.2 kb at the element's 5′ end and is co-extensive with the subterminal repetitive region (Raina et al 1993).

The element's single primary transcript is alternatively spliced, yielding mRNA sequences with different open reading frames (ORFs), some with extensive overlaps (Fig. 1b). Four large transcripts generated by different patterns of splicing have been identified and designated *tnpA*, *tnpB*, *tnpC* and *tnpD*, in order of increasing size (Masson et al 1989). The *tnpA* transcript is the shortest and most abundant by a factor of 50–100. Two of the transcripts contain a single ORF, while the others contain two ORFs (Fig. 1c). Each transcript has one ORF that is unique to it, and it is this coding sequence that has been given the transcript's name. Only the TnpA and TnpD proteins have been assigned functions: both are necessary for transposition (Masson & Fedoroff 1989, Frey et al 1990).

In addition, TnpA is both a positive and a negative regulator of the *Spm* promoter (Gierl et al 1988, Schläppi et al 1993, 1994). Although the precise function of TnpD is not known, the results of recent experiments reveal that TnpD binds to both free and DNA-bound TnpA (R. Raina, M. Schläppi & N. Fedoroff, unpublished results 1996). There is evidence that TnpD stabilizes binding of TnpA to DNA (R. Raina, M. Schläppi, B. Karunanandaa & N. Fedoroff, unpublished results 1996). Much remains to be learned about the

manner in which TnpA and TnpD interact with each other and with the *Spm* element to promote transposition.

The molecular basis of epigenetic inactivation

Studies have been done on the molecular differences between genetically silent and active *Spm* elements (Banks et al 1988). The sequence just downstream from the transcription start site of the element is rich in GC base pairs (Fig. 2) and contains many sequences in which the C residues can be modified by methylation (Masson et al 1987, Banks et al 1988). Analysis of DNA from plants with active and inactive elements using methylation-sensitive restriction enzymes showed that both active and inactive elements are extensively methylated, although the adjacent sequences at the insertion site studied are not (Banks et al 1988). Active elements differ from inactive ones by the absence of methylation in a restricted region of about 0.6 kb surrounding the transcription start site (Fig. 2). Active elements are unmethylated in the 0.2 kb promoter region and inactive elements are methylated. Methylation is variable in the GC-rich region just downstream from the transcription start site and the extent of C methylation in the GC-rich region shows a positive correlation with the heritability of the epigenetically inactive state. Extensively methylated elements are transcriptionally silent, do not transpose autonomously and are mobilized at a low frequency by an active element, indicating that methylation inhibits both transcription and transposition (Banks et al 1988). However, maintenance of an inactive element in the presence of an active one results in both its transient and heritable reactivation, albeit over a period of several plant generations (Fedoroff 1989b). This observation implies the existence of an element-encoded epigenetic activator.

A deeper understanding of *Spm* inactivation and reactivation has been achieved through studies in a transgenic tobacco system devoid of the many background *Spm* elements present in maize. *Spm* is reversibly inactivated in tobacco, just as it is in maize, and inactivation is accompanied by preferential methylation of the element's 5′ end (Schläppi et al 1993). Introduction of the *tnp*A cDNA, but not of the transposon's three other coding sequences, results in the reactivation of an inactive element (Schläppi et al 1993). Hence, TnpA is the element-encoded epigenetic activator. TnpA-mediated *Spm* activation is accompanied by a decrease in 5′ terminal methylation (Schläppi et al 1993). Using *Spm* promoter–firefly luciferase gene fusions, it was found that the promoter is rapidly inactivated and methylated in transformed tobacco cells, but only if the GC-rich downstream region is present (Schläppi et al 1994). If the element's promoter lacks the GC-rich downstream region, the luciferase gene continues to be expressed. Thus the GC-rich downstream region is required for inactivation and methylation of the element's promoter.

TnpA is both a repressor and an activator

Luciferase reporter gene assays have been used to study the effect of TnpA on both an active, unmethylated *Spm* promoter and an inactive, methylated *Spm* promoter. TnpA represses the active, unmethylated *Spm* promoter and activates the inactive, methylated promoter (Schläppi et al 1994). Moreover, promoter reactivation is accompanied by a decrease in promoter methylation. TnpA has both a DNA-binding and a dimerization domain (Trentmann et al 1993). Using domain-swapping experiments in an *in planta* double-hybrid system, it was shown that TnpA functions only as a repressor, although it can be converted to an efficient transcriptional activator of the *Spm* promoter by addition of a viral transcription activation domain (Schläppi et al 1996). Thus, TnpA is a repressor and does not appear to be a conventional transcriptional activator, implying that its participation in the activation and demethylation of the methylated *Spm* promoter occurs by a different, as yet unknown mechanism.

Summary and conclusions

McClintock's early genetic studies revealed that the *Spm* transposon is subject to epigenetic inactivation that can be reversed in the presence of an active *Spm* (McClintock 1957, 1958, 1959, 1961, 1962, 1971). Subsequent molecular studies in maize showed that negative epigenetic regulation is associated with methylation of the *Spm* element's promoter and the adjacent, downstream GC-rich sequence, and that element reactivation in the presence of an active *Spm* is accompanied by demethylation (Banks et al 1988). Studies in transgenic plants revealed that the promoter is the methylation target sequence and that rapid methylation depends on the presence of the GC-rich sequence (Schläppi et al 1993, 1994). The promoter, which is inactive when methylated, is reactivated and demethylated in the presence of the element-encoded protein, TnpA (Schläppi et al 1994). However, studies to date suggest that TnpA is exclusively a transcriptional repressor, although it can be converted to an transcriptional activator by addition of a transcription activation domain (Schläppi et al 1996).

TnpA binds to methylated *Spm* DNA, albeit less strongly than to unmethylated DNA (A. Elhofy, R. Raina & N. Fedoroff, unpublished results 1996). However, it is not likely that its ability to facilitate *Spm* promoter activation and demethylation is the result of preferential binding to unmethylated binding sites, since sequences in the GC-rich downstream region that do not bind DNA also become demethylated in its presence (Schläppi et al 1994). The observation that TnpA's C-terminus is required for promoter activation and demethylation, but not its repressor activity, suggests that the former involves interaction of TnpA with an additional protein or proteins (Schläppi et al 1994). A novel, active demethylation

enzyme has recently been detected in mammalian cells, and it has been suggested that demethylation of specific sequences may occur through its interaction with sequence-specific binding proteins (Weiss et al 1996). The ability of TnpA to promote demethylation of sequences downstream from its binding sites suggests that it may play such a targeting role for a non-sequence-specific demethylase.

Acknowledgements

This work was supported by the National Institutes of Health MERIT award GM4296.

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DISCUSSION

Wolffe: What is known about the mechanism of demethylation? Is the idea that demethylation is a consequence of TnpA moving along from the site of nucleation, that there's a threshold effect, such that there is a certain limiting concentration of TnpA, that the complex which moves down the GC-rich element is nucleated and that it is this element which is sensitive to methylation status?

Fedoroff: The short answer is that we don't know. We have tried unsuccessfully to set up heterologous systems to study them. However, we do know that TnpA binds to methylated DNA with reduced affinity, so our current hypothesis is that TnpA tells a demethylase where to demethylate. It is not the case that only methylatable sites in binding sequences are affected: sequences immediately outside the binding sites become demethylated, as well as those in the GC-rich region several hundred base pairs away. It is possible that TnpA binds with a reduced affinity and changes the conformation (or attracts other proteins) such

that the probability of DNA demethylation, in any given replication cycle, decreases fractionally. We are now setting up *in vitro* experiments to determine whether direct demethylation is involved.

Meyer: The binding region for TnpA has several DNA elements that could give rise to secondary structures. Is anything known about its structure *in vivo*, or whether methylated DNA regions are more likely to form secondary structures? Also, does TnpA binding influence the secondary structure of that region?

Fedoroff: We wouldn't even know how to do those kinds of experiments *in vivo* because at the moment we can't even get a footprint. However, we do know that there are 15 binding sites at the 3′ end, and if four or five of these are deleted transposition frequency is not affected, whereas if nine are deleted the transposition frequency decreases dramatically. In addition, if the GC-rich region at the 5′ end is deleted then the transposition frequency also decreases dramatically.

Meyer: Has anybody substituted individual bases which change the sequence but not the ability to form a secondary structure?

Fedoroff: We don't even know yet whether a secondary structure forms.

Flavell: Could you tell us more about what happens in different cell types. Do methylation changes occur in some cell types at a greater frequency than in others? And what do the observations about kernel scoring of these activities have to say about the frequency of events that are taking place in other kinds of cells?

Fedoroff: Kernel development commences with a double fertilization event. Therefore, the endosperm has the same genetic constitution as the zygote except that it has two copies of the maternal genome, i.e. it's triploid. It is as if the kernel represents a genetic window into the next generation. When you see a genetic change in the kernel, this often reflects a parallel genetic change in the genome (the controls are siblings that don't show those changes in the kernels). We have measured the extent of methylation in different tissue types in plants that have a greater tendency for the element to be transmitted in a genetically active form via tillers. Tillers are not different tissue types, but they are developmentally distinct side-shoots of the main stalk of a corn plant. We found that the probability that an element is transmitted in an inactive form is highest for pollen and lowest for female gametes produced on tillers, and this is reflected in the level of methylation of the element.

Wolffe: Does this imply that there has been a developmental change?

Fedoroff: It implies that there are developmental differences. Sometimes the distinction between male and female breaks down: ears are produced on the tassels of progressive tillers, so that there are fertile male and female gametes produced at a single site. In such cases the male/female repression also breaks down, which is probably due to a general methylation that is also working on other genes.

Jaenisch: Is this phenomenon affected when you change the overall methylation level?

Fedoroff: This has been tried using 5-azacytidine, but this compound is so mutagenic in corn that we don't know what to conclude. McClintock originally used plants in which chromosome breakage was occurring, so presumably SOS systems are activated in these plants (reviewed in Fedoroff 1983). However, people have subsequently used X-irradiation, γ-irradiation and culturing cells—processes that alter the methylation of sequences and are mutagenic. This is the origin of somaclonal variation, some of which may be epigenetic, but some of which is genetic. There are no simple answers to your question.

Jaenisch: Have the antisense methyltransferase approaches been successful?

Finnegan: These experiments are not possible to perform because there isn't enough homology between the methyltransferases to be effective as antisense agents in different species.

Fedoroff: We are getting closer because we've introduced *Suppressor–mutator* (*Spm*) promoter-driven luciferase constructs into *Arabidopsis* and we will soon be able to make the relevant crosses to methylase antisense lines. However, we don't yet know whether *Arabidopsis* turns the *Spm* promoter off. We did not see any luciferase activity in the first set of experiments, but in the course of discovering that the GC-rich sequence is an internal silencer, we created constructs that had enhancers upstream. We are now going to use these in an effort to obtain plants with detectable luciferase levels.

Wolffe: I would like to ask a question about this unusual GC-rich region. Is it a typical CpG island?

Fedoroff: Unlike a mammalian CpG island, which is a region of normal CG to GC ratio, the GC-rich sequence downstream of the *Spm* transcription start site is 80% G + C.

Wolffe: Is this region a particularly useful imprinting mark for the *Spm* element? Is it sequence specific, is it distance dependent and is a certain density of CpG required for the element to be functional?

Fedoroff: We have not done experiments to address this. I do know that others have assessed plant genomes for GC-rich stretches, and transposable elements are particularly visible in such analyses (Gardiner-Garden & Frommer 1992).

Wolffe: Is this true for all transposable elements in maize?

Fedoroff: All the elements that are well characterized—*Mu*, *Ac* and *Spm*—have extremely GC-rich sequences, and they are all inactivated by methylation. Inactivation is probably the mechanism by which transposable elements survive for long periods in plant genomes. This is why McClintock was able to activate them, and why all the mutagenic treatments and demethylation treatments activate them. The connection between chromosome breakage and demethylation is not clear.

Laird: Can you expand on the connection between transposition and reactivation?

Fedoroff: If an element is extremely methylated it is not transcribed. Even if the source of transposase is in the form of an active *trans*-acting element, the frequency of transposition of a highly methylated element is low.

Laird: Is transposition occurring in addition to reactivation? And in this context what is the role of TnpA?

Fedoroff: TnpA is required for transposition and reactivation.

Laird: Is it possible to separate those two events?

Fedoroff: Reactivation can occur without transposition.

Martienssen: I thought you said that the reporter gene constructs only have one end of the *Spm* element. As two ends are required for transposition, the reporter could not transpose.

Fedoroff: No, that is not true. Reactivation is generally measured by the ability of the element to inactivate another gene with an inserted defective element elsewhere; I think that's what you referred to as the reporter. Suppression is probably related to the binding of TnpA to the multiple sites at element ends, which inhibits expression of either the reporter gene or the *Spm* element itself. These processes can be separated. In kernels uniform suppression of the reporter gene occurs in all cells, and then there is a transposition event in a subset of cells. Conversely, if you set up a genetic cross in which you can see both inactivation and transposition late in development, then the frequency of inactivation is about the same as the frequency of transposition. To explain this, we hypothesize that once the transposition complexes are formed, they can either fall apart—in which case the element is probably remethylated, so you could see the expression of a reporter gene that was previously suppressed by it—or they will transpose. The probability that it will become inactive and methylated appears to be higher after transposition. When I was looking for elements that were inactive, the vast majority turned out to be transposed elements.

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