Poetry of *b1* Paramutation: *cis*- and *trans*-Chromatin Communication

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The term paramutation was coined by Alexander Brink in the 1950s to describe a meiotically heritable change in the expression of one allele when it was combined with another specific allele. In Brink's classic work he proposed that chromosomes possess a paragenetic function in addition to their genetic one (Brink 1960). Brink distinguished mutations, which arise as discrete events in undirected fashion, are stably transmitted, and are very rarely reversible, from "paramutations," which occur in directed fashion under specific conditions and are transmitted, but are often unstable, progressively changing back to the original phenotype. Brink was using the term "paramutation" in its broadest sense, equivalent to the term "epimutation" commonly used today. In addition to the classic examples of interactions between alleles, paramutation-like interactions have also been described between homologous transgenes or between transgenes and homologous endogenous genes at allelic or nonallelic positions in a number of species, including plants, fungi, animals, and humans. Thus, a more general definition of paramutation would be "*trans* interactions between homologous sequences that set up distinct epigenetic states that are heritable through meiosis."

In all the systems described to date, only specific alleles undergo paramutation. Alleles sensitive to altered expression are termed paramutable, and alleles inducing the change, paramutagenic. Following paramutation, previously sensitive alleles are termed paramutant (or paramutated). Most alleles do not participate in paramutation; these alleles are referred to as either neutral or nonparamutagenic. Two assays are routinely used to monitor paramutation: (1) the ability of a paramutagenic allele to cause a heritable change in the paramutable allele (referred to as establishment of paramutation) and (2) the heritable alteration of the paramutant allele into a paramutagenic allele. In a few examples, such as the *b1* system described in this review, these two phenotypes always occur simultaneously and completely. In contrast, at most other loci, the extent of paramutagenicity acquired by a paramutant allele depends on the circumstances of the cross, if it occurs at all (for summary, see Chandler and Stam 2004).

The degree of penetrance with which paramutation occurs and the subsequent stability of the altered state can vary among different phenomena. The most common situation is illustrated by the classic system studied by

Brink, *r1*, in which the paramutant state, although heritable, is frequently unstable. In contrast, the other classic maize system studied by Coe, *b1*, shows 100% penetrance with the paramutated allele always becoming fully paramutagenic and the resulting paramutagenic state being extremely stable. A summary of all known paramutation phenomena and a comparison of their penetrance and stability has recently been published (Chandler and Stam 2004).

The most thoroughly analyzed examples of paramutation are in maize, involving four different genes that encode transcription factors that activate the colored flavonoid pigment biosynthetic pathways (for review, see Chandler et al. 2000, 2002). The fact that the amount of pigment observed reflects the levels of the regulatory proteins (Patterson et al. 1993; Hollick et al. 2000) and that even subtle changes in expression (two- to fourfold) are visually distinguishable likely explains why paramutation was discovered at these genes.

This review summarizes key experiments involving the *b1* locus in maize, including experiments that have revealed the key sequences required for paramutation. The nature of these sequences combined with the isolation of mutations that prevent paramutation suggest models to explain both the *cis*- and *trans*-communication.

b1 PARAMUTATION PHENOTYPES

Expression of the *b1* gene, which encodes a transcription factor required to activate the anthocyanin biosynthetic pathway (Chandler et al. 1989), results in purple pigment. The two alleles involved in *b1* paramutation, *B'* and *B-I*, are expressed in the seedling and most of the mature plant tissues. The highly expressed paramutable *B-I* allele is unstable, it spontaneously gives rise to *B'* at frequencies between ~0.1% and 10% (Coe 1966). In contrast to *B-I*, the *B'* allele is extremely stable, no changes to *B-I* have been observed with more than 100,000 plants examined (Coe 1966; Patterson and Chandler 1995; V. Chandler, unpubl.). When *B'* and *B-I* are crossed together, paramutation always occurs; *B-I* is always changed into *B'* (Fig. 1). The chromosome that enters the cross carrying *B-I* exits the cross carrying *B'*; thus, the presence of *B'* always changes the unstable *B-I* allele into *B'*. The new *B'* allele, symbolized as *B'** is just as paramutagenic as the parental *B'*, as demonstrated in Figure 1.

Figure 1. Paramutation at *b1*. Paramutable *B-I* (dark purple plant) is always changed to *B'* (light purple plant) in *B'/B-I* heterozygotes. Newly paramutated *B'*, *B'**, is indistinguishable from *B'*; it always paramutates naïve *B-I* in subsequent crosses.

Most *b1* alleles do not participate in paramutation. The assay to test whether an allele participates in paramutation is diagrammed in Figure 2, with the results typically seen with *b1* alleles.

Paramutation Causes Reduced Transcription But Does Not Require a Functional *b1* Protein of *B-I*

Once the *b* gene was cloned it became possible to address the molecular basis for altered pigment in *B'* and *B-I*. Comparison of *b* mRNA levels and relative transcription rates in nuclei isolated from *B'* and *B-I* plants (Patterson et al. 1993) indicate that *B'* has a 10–20-fold reduction in transcription relative to *B-I*. No change in the size or 5' ends of the mRNA was observed. The proteins encoded by the *B'* and *B-I* alleles are equally capable of

Figure 2. Paramutation test result with neutral alleles. Neutral alleles can be recessive or dominant to *B'* with respect to pigment levels. The *B-N* example shown here is more pigmented than *B'* and is dominant such that the F1 looks like the *B-N* allele. The test of whether the allele can become paramutagenic is to cross the F1 plant with *B-I* as shown. If the allele is neutral, it does not become paramutagenic and cannot paramutate *B-I*, which will segregate in 50% of the progeny as shown.

Figure 3. Paramutation test result with paramutagenic alleles. The paramutagenic allele shown is *b-v*, which has a small deletion in the *b*-coding region causing a frameshift and no functional B protein to be made. This allele becomes paramutagenic when crossed to *B'*, which is demonstrated by crossing the F1 plant to *B-I*; all the progeny in the next generation are *B'* because both *b-v* and *B'* are paramutagenic, symbolized by the '*'.

activating the anthocyanin pathway (Patterson et al. 1993). Extensive examination of DNA methylation differences between *B'* and *B-I* failed to show any differences in DNA methylation in the promoter proximal or coding regions (Patterson et al. 1993).

To date, all alleles that participate in *b1* paramutation are transcribed, but some, such as *b-v*, are nonfunctional. The *b-v* allele contains a frameshift and does not produce a protein capable of activating the anthocyanin pathway (M. Beaudet et al., unpubl.). When crossed to *B'*, the *b-v* allele becomes *b'-v* (Coe 1966), capable of paramutating *B-I* (Fig. 3). This result demonstrates that the B protein is not required for paramutation and this experiment illustrates how an allele that is not pigmented can be monitored for paramutation activity. Because the *b-v* allele results in a slightly different transcript, it was also used to demonstrate that reduced transcription is observed at both alleles in *B'/B-I* plants (G. Patterson and V. Chandler, unpubl.).

GENETIC DISSECTION OF *b1* PARAMUTATION

The unusual features of *b1* paramutation, i.e., its full penetrance and extreme stability, enabled a genetic dissection, which has delimited the key sequences required for paramutation and identified multiple genes that, when mutant, prevent paramutation.

Identification of the Key Sequences Required for *b1* Paramutation

Crosses with neutral alleles demonstrated that the ability to participate in paramutation is linked to *B'* and *B-I* (Coe 1966; Patterson and Chandler 1995). Intergenic and intragenic recombination mapping indicated that sequences required for paramutation are tightly linked to *b*, and located in the upstream 5'-flanking region (Patterson

and Chandler 1995). Restriction mapping (~100 sites) within the coding and flanking regions of *B-I* and *B'* detected no differences within ~15 kb including the 4.5-kb transcribed region (Patterson et al. 1993). Our recent fine-structure recombination mapping experiments demonstrated that the sequences required for both paramutation and high transcription (enhancer activity) are within a 6-kb region located 100 kb upstream of the *B-I* transcription start site (Stam et al. 2002a,b). Within this region *B'* and *B-I* have seven tandem repeats of an 853-bp sequence otherwise unique in the genome; neutral alleles have one. Examination of recombinant alleles with different numbers of tandem repeats (one, three, and five) demonstrated that the repeats are required for both paramutation and enhancer function (Stam et al. 2002a). Experiments are in progress to determine if the repeats are sufficient for paramutation and enhancer activities.

The 6-kb region is identical in *B-I* and *B'*, demonstrating that epigenetic mechanisms mediate the stable silencing associated with *B'* paramutation. The tandem repeat sequences are differentially methylated in *B-I* relative to *B'*, but the change in repeat methylation follows establishment of the *B'* epigenetic state (Stam et al. 2002a). In the repeats, *B-I* has a more open chromatin structure relative to *B'* and DNaseI hypersensitivity differences developmentally precede transcription at *b*, suggesting the repeat chromatin structure could be the heritable imprint distinguishing the two transcription states (Stam et al. 2002a).

Mutations That Prevent *b1* Paramutation

Forward genetic screens using the *b1* paramutation system identified three genes required for paramutation (Dorweiler et al. 2000; V. Chandler, unpubl.). The phenotypes of each mutation in the presence of *B'* are illustrated in Figure 4. The full penetrance of paramutation within the F_1 and transmission of only *B'* to the F_2 generation have enabled screens at both the F_1 and F_2 generations for mutations that affect paramutation. All F_1 progeny should be lightly pigmented because paramutation occurs with 100% penetrance; thus rare dark F_1 plants are candidates for dominant mutations that prevent paramutation and rare families, segregating one-quarter dark plants in the F_2 generation, are candidates for carrying a recessive mutation that increases the expression of the *B'* allele. The first mutation isolated was *mop1-1* (*mediator of paramutation1*), which was isolated by crossing *B-I* plants that contained active *Mutator* transposable ele-

ments to *B'* plants, self-pollinating the resulting F_1 plants and screening for recessive mutations that resulted in dark purple seedlings (Dorweiler et al. 2000).

Two other mutations were isolated from a chemical mutagenesis experiment: EMS-mutagenized *B'* pollen was crossed onto ears of *B-I* plants to generate F_1 *B'/B-I* plants. The F_1 plants were screened for rare dark plants; one dark plant was identified. Subsequent crosses demonstrated that the mutation, designated *mop2-1*, was dominant and it prevented the establishment of paramutation (K. Kubo et al., unpubl.). Further crosses also revealed that when the *mop2-1* allele is homozygous it causes a modest increase in the level of pigment that accumulates in *B'* plants, suggesting it is in fact semidominant (J. Dorweiler and V. Chandler, unpubl.).

All other F_1 plants were light, and these were self-pollinated to generate F_2 progeny to screen for recessive mutations. Screens of F_2 progeny for segregation of darkly pigmented seedlings and plants identified a second recessive mutation, *mop3-1* (J. Dorweiler et al., unpubl.). Subsequent experiments demonstrated that both *mop1-1* and *mop3-1* increased the transcription of *B'*, making plants homozygous for the mutations look like *B-I*, but the mutations did not heritably change *B'* into *B-I* (Dorweiler et al. 2000; J. Dorweiler and V. Chandler, unpubl.). When *B' mop1-1* or *B' mop3-1* homozygotes are outcrossed to a recessive *b* allele, all of the resulting progeny have the *B'* phenotype, indicating that the mutations must be present to increase *B'* pigment levels and the *B'* allele was not heritably changed to *B-I*. Importantly, like *mop2-1*, both *mop1-1* and *mop3-1* prevent the establishment of paramutation. Examples of crosses illustrating how prevention of paramutation is examined can be found in Dorweiler et al. (2000).

A similar screen was done using paramutation at the *pl1* gene, which encodes a myb-related transcription factor required for pigment in seedlings and many mature plant tissues. The initial screens also identified another allele of *mop1* (*mop1-2*) and two additional genes, referred to as *rmr1* (*required for maintenance of repression1*) and *rmr2* (Hollick and Chandler 2001). Subsequent experiments have demonstrated that mutations in all five of these genes affect paramutation at both *pl1* and *b1* (Dorweiler et al. 2000; V. Chandler et al., unpubl.). In addition, the *mop1* mutation has also been shown to prevent paramutation at the *r1* locus (Dorweiler et al. 2000), which is intriguing as *r1* paramutation appears quite distinct from *b1* and *pl1* paramutation (for discussion, see Chandler et al. 2000; Chandler and Stam 2004). The effect of the other mutations on *r1* paramutation is under investigation.

In addition to affecting paramutation at the three maize loci, the *mop1* gene is also required for maintaining the extensive DNA methylation associated with silencing of *Mutator* transposons (Lisch et al. 2002). However, *mop1-1* is distinct from the *ddm1* mutation, which affects transposon DNA methylation and silencing in *Arabidopsis* (Miura et al. 2001; Singer et al. 2001), as *mop1-1* does not have a global effect on the methylation of repeated sequences such as centromeres and rDNA (Dorweiler et al.

Figure 4. Phenotypes of *trans*-acting mutants affecting paramutation. Plants that are homozygous for *B'* and for each of the three mutations are shown. Plants that are homozygous for *B'* and heterozygous for each of the mutations look like *B'* (not shown).

2000). Intriguingly, within the first generation of introducing homozygous recessive *mop1* mutant alleles, *Mutator* methylation is decreased, but it takes several more generations in the presence of the *mop1* mutation before transposition of the element is reactivated (Lisch et al. 2002). In unpublished studies, all five paramutation mutations have been shown to reactivate some, but not all, of the transcriptionally silent transgenes tested (V. Chandler, unpubl.).

Potential Function of *mop* Genes

In previous publications we have speculated that the *mop* and *rmr* mutations are involved in some aspect of chromatin remodeling (Dorweiler et al. 2000; Hollick et al. 2000). The molecular nature of the *mop* or *rmr* genes is not yet known, as cloning is still underway. However, a candidate gene approach has eliminated approximately 140 genes that were theoretical candidates for *mop1* and *mop2*, based on their role in chromatin and epigenetic silencing in other species. Using molecular markers, the *mop1* and *mop2* genes have been mapped to a 3-cM and 1-cM interval in maize, respectively (Y. Cai et al., unpubl.). The map positions of these two genes have been compared to those of a number of maize candidates for genes involved in chromatin and epigenetic silencing. The candidate genes that have been excluded based on their map position include many genes highly homologous to genes previously shown to be involved in chromatin and epigenetic gene regulation: the 6 known DNA methyltransferase genes in maize (Cao et al. 2000; Papa et al. 2001), the 2 maize genes homologous to *ddm1* (Jeddeloh et al. 1998), 13 genes homologous to other putative *snf2*-related chromatin remodeling components (Verbsky and Richards 2001), the maize gene most homologous to *mom1* (Amedeo et al. 2000), 25 genes homologous to putative histone methyl transferases (Springer et al. 2003), and 8 and 7 genes homologous to histone acetyltransferases and histone deacetyltransferases, respectively (Pandey et al. 2002; data generated by K. Cone, University of Missouri and posted on The Plant Chromatin Database; www.chromDB.org). Experiments are in progress to map an additional approximately 100 maize genes.

MODELS FOR *b1* PARAMUTATION

Models for *b1* paramutation need to account for the following features. The high expressing *B-I* allele can be spontaneously silenced, while *trans*-silencing occurs at a much higher frequency than the spontaneous changes. Both spontaneous and directed paramutation are established in somatic cells and the states are heritably transmitted through mitosis and meiosis. The sequences required for paramutation are also required for the high transcription state associated with *B-I*. Tandem repeats are required for *b1* paramutation.

The observation that *mop1* affects multiple epigenetic phenomena suggests that paramutation is likely to share mechanistic components with those phenomena, i.e., transposon and transgene silencing. It is tempting to hy-

pothesize a role for RNA interference (RNAi) in paramutation, as RNAi has been shown to contribute to both transposon and transcriptional transgene silencing in several species (Ketting et al. 1999; Tabara et al. 1999; Pal-Bhadra et al. 2002; Lippman et al. 2003), features shared with *mop1*. It is also likely that that multiple mechanisms are involved in different examples of paramutation, as *mop1* does not affect all aspects of paramutation at each locus. There is precedent for multiple mechanisms mediating an epigenetic phenomenon, as recent studies of transposon reactivation in *Arabidopsis thaliana* revealed distinct pathways are involved in transposon silencing (Lippman et al. 2003).

Below separate models are discussed for the *cis* interactions (long-distance enhancement of *B-I* expression and the spontaneous change from *B-I* to *B'*), and the *trans* communication that occurs between *B'* and *B-I*.

Model for *cis*-interactions

Paradigms for the long-distance *cis*-interactions between the tandem repeats and the *b1* promoter proximal region derive from studies on the globin loci and imprinted genes in mammals, particularly results from the *Igf2* and *H19* loci (Chandler et al. 2002). Figure 5A presents our favorite model, "boundary element gone awry."

Another possibility is that protein factors that bind to the *b1* enhancer sequences in the *B-I* state mediate sequestration into compartments compatible with expression. When paramutation occurs those proteins would be unable to bind to the sequences when they are in the *B'* state, resulting in an altered chromatin state that localizes the adjacent gene to a nonpermission chromatin environment and reduced expression. The repeats associated with *b1* may then be able to communicate with other homologous sequences *in trans*, either through pairing or *trans*-RNA mechanisms, causing those sequences to localize to a nonpermissive environment for transcription as well. Further dissection of the *b1* sequences required for enhancer and paramutation activities using transgenic approaches should address whether paramutation and enhancer activities are distinct or overlap.

Model for *trans*-interactions

Two major models for *trans*-communication are RNA-mediated chromatin changes and direct interactions between chromatin complexes (Fig. 5B). Our current understanding of *b1* paramutation is consistent with either or both models being involved in establishment or maintenance of *b1* paramutation. A more detailed discussion of these models is in Chandler and Stam (2004).

Pairing Model. There are several examples of studies from *Drosophila* and yeast in which tandem repeats can mediate physical interactions *in trans*. Tandem repeat arrays of *Drosophila* transgenes can mediate silencing in a copy-number-dependent way, demonstrating long-range interactions with other sequences *in cis* and *in trans* (Henikoff 1998). In yeast, tandem repeat arrays of the

Figure 5. Model for *b1* paramutation. (A) Model for *cis*-communication by the tandem repeats. Two genes exist within the 120-kb region diagrammed, the *b1* gene (purple box) and an unknown gene identified as an expressed sequence tag (yellow box); the direction of transcription is indicated by the arrows under the boxes. The region containing the sequences required for paramutation is indicated in red: the neutral allele (*B-n*), which only contains a single copy of the sequences that are tandemly repeated in *B'* and *B-I*, is symbolized by a small rectangle; the large rectangle and diamond in *B'* and *B-I* symbolize distinct chromatin structures associated with the tandem repeats. The enhancer is indicated as a green oval; the exact location of the enhancer has not been determined. The neutral allele and the repeat region in the *B'* allele are both postulated to form a functional boundary element (symbolized by a brown rectangle) that prevents the enhancer from communicating with the *b* gene located 100-kb downstream. Instead the enhancer is envisioned to communicate with a different gene located upstream (green arrows, communication; red arrows, no communication). In the *B-I* allele the boundary element is postulated not to form because of differences in chromatin structure and/or distinct proteins bound. The vertical black arrows symbolize the DNaseI hypersensitive sites seen in *B-I*. The different sized and colored ovals indicate distinct protein complexes bound to the different alleles. (B) *trans*-communication could occur through direct interactions between the repeats (pairing model) or via a RNA-based communication (*trans*-RNA model); see the text for discussion of these models.

lacO or *tetO* operators associate in nonmeiotic cells irrespective of their genomic location, with associations occurring with similar frequencies between allelic and ectopic arrays (Aragon-Alcaide and Strunnikov 2000; Fuchs et al. 2002).

One frequent test for the involvement of pairing is to ask if translocations, which should disrupt pairing, disrupt the phenomenon under investigation. Several different translocations have been examined for both *b1* and *r1* paramutation, and none to date disrupt paramutation (for review, see Coe 1966; Brink 1973; Chandler et al. 2000). However, in all examples cited the translocation break-points were not adjacent to the loci, leaving open the possibility that pairing could still occur. In addition, once the epigenetic state is established it is heritable in the absence of the inducing allele; thus, brief physical interactions rather than stable synapsis may be all that is required to establish paramutation.

One possibility is that arrays of repeat sequences increase the local concentration of bound proteins, and the higher concentration confers distinct chromatin proper-

ties that can mediate pairing of homologous sequences or association of regions with similar heterochromatic properties (Sage and Csink 2003). Such pairing can result in subnuclear localization to heterochromatin domains (Csink and Henikoff 1996; Dernburg et al. 1996). Pairing would then enable the transfer of chromatin complexes from one sequence to another, similar to what has been hypothesized for the communication between complexes at enhancers and RNA polymerase initiation sites resulting in transvection (Morris et al. 1999).

Another possibility is that pairing is mediated by proteins with properties similar to the Polycomb complex (PcG), which binds to Polycomb response elements (PREs). In *Drosophila*, silenced states initiated at PREs can affect the expression of genes over considerable distances (Orlando and Paro 1993), and PRE-containing transposons inserted on different chromosomes can physically interact, resulting in enhanced silencing (Sigrist and Pirrotta 1997). Biochemical studies have also shown that PcG complexes can mediate template bridging; proteins bound to one polynucleosome template can recruit a second template from solution (Lavigne et al. 2004).

trans-RNA Model. There are examples of transgene arrays that mediate transcriptional (for review, see Vaucheret and Fagard 2001) and posttranscriptional silencing (for review, see Vaucheret et al. 2001; Matzke et al. 2002) through production of double-stranded RNA homologous to the promoter or transcribed regions, which then triggers RNA interference (RNAi). In many of the reported instances the transgene arrays contain inverted repeats; thus it is easy to envision how dsRNA is produced. However, there are also examples of tandemly repeated transgenes that are efficiently silenced in a variety of species (Assaad et al. 1993; Cogoni et al. 1996; Ketting and Plasterk 2000; Mourrain et al. 2000; Ma and Mitra 2002; Pal-Bhadra et al. 2004). Tandem repeats are involved in maintaining the proper chromatin structure necessary for centromere function (Dawe 2003), and in *Schizosaccharomyces pombe* components of the RNAi machinery have been shown to process transcripts derived from these repeats and mediate the formation and maintenance of silent chromatin, essential for centromere function (Volpe et al. 2002, 2003). The RNAi machinery is also required for heterochromatin formation and silencing of tandemly repeated transgenes in *Drosophila* (Pal-Bhadra et al. 2004).

Results from studies of the *b1* system demonstrated that seven or five copies of the tandem repeat were highly paramutagenic, while one copy was completely inert (neutral allele). Interestingly, an allele with three copies of the repeat was paramutagenic, but unstable and less penetrant (Stam et al. 2002a). These results suggest a cellular mechanism for sensing the number of repeats. A model for efficient maintenance of silencing within the tandem repeats at centromeres invokes that siRNAs are regenerated efficiently from tandem array transcripts by RNA-dependent RNA polymerase (RdRP) and Dicer, while siRNAs from single-copy sequences or dispersed repeats would eventually become depleted through sub-

sequent rounds of priming by RdRP with further downstream primers (Martienssen 2003). Thus, one speculation is that an increased number of tandem repeats results in a higher abundance of siRNAs, required to stably maintain the silencing associated with paramutation.

A second possibility is that longer RNAs are mediating *trans* interactions that induce chromatin structural changes. X-inactivation in mammals is mediated through a *cis*-acting RNA, *Xist*, which confers a chromatin-based mechanism of inactivation on adjacent sequences (for review, see Chow and Brown 2003). *Xist* action is repressed by the antisense gene, *Tsix*, whose full-length RNA product is complementary to *Xist* RNA in mice (Shibata and Lee 2003). Thus, a model for *b1* paramutation is that expression of noncoding RNAs (sense and/or antisense) could mediate altered chromatin formation, and the RNAs could function both *in cis* and *in trans*. It is also possible both mechanisms are employed; the chromatin states established *in cis* through an RNA mechanism might be communicated to the other allele via pairing interactions.

POTENTIAL CONSEQUENCES OF PARAMUTATION

Numerous organisms employ mechanisms for sensing and counting homologous sequences. Paramutation may represent a defense against invasive transposons and viruses, postulated from studies of transgene silencing (Matzke et al. 2002). Another possibility is that paramutation represents the aberrant function of machinery involved in identifying and maintaining chromatin boundaries between genes and nearby repetitive sequences. Independent of the function of paramutation, there are significant evolutionary implications, such as providing an adaptive mechanism for transferring favorable expression states to progeny and a mechanism for establishing functional homozygosity in polyploids. Paramutation-like phenomena may also contribute to the low penetrance and other aspects of non-Mendelian inheritance frequently observed for genes involved in complex human diseases and the segregation of quantitative characters. It is also possible that paramutation contributes to inbreeding depression and hybrid vigor. These possibilities are discussed further in Chandler and Stam (2004).

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