Genetic analysis in fungi using restriction-enzyme-mediated integration

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Restriction-enzyme-mediated integration (REMI), a method for generating nonhomologous integration of transforming DNA into the chromosomes of eukaryotic cells, has been used for insertion mutagenesis and other genetic studies in diverse organisms. Insertion mutations generated by REMI have facilitated the genetic dissection of developmental pathways in *Dictyostelium discoidium* and the isolation of virulence factors in several plant pathogenic fungi. Recent work indicates that REMI occurs by nonhomologous end joining.

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Abbreviations

NHEJ	nonhomologous end-joining
PSS	protruding single strand
REMI	restriction-enzyme-mediated integration
RFLP	restriction fragment length polymorphism

Introduction

Insertion mutagenesis has many attractive features; most importantly, the mutation of interest is physically marked, providing a facile means to isolate and analyze DNA flanking the site of the insertion. Transposons have been exploited to create insertion mutations with great success in many bacterial and a few eukaryotic systems. Unfortunately, transposons are not uniformly found in fungi, especially laboratory strains, and those found have not demonstrated characteristics, such as stability and random integration, required to prove useful for genetic analysis [1–3]. Therefore, approaches utilizing ectopic (nonhomologous) integration of DNA fragments have been used as a means of insertion mutagenesis in fungal systems. In the majority of fungi, ectopic integration of transforming DNA is much more frequent than homologous, targeted integration [4]. A method for generating nonhomologous integration events, termed restriction-enzyme-mediated integration (REMI) [5], has been successfully used for mutagenesis in a variety of organisms.

REMI was first demonstrated in *Saccharomyces cerevisiae*, an organism in which homologous integration is extremely efficient [5]. Transforming with nonhomologous DNA linearized by digestion with restriction enzyme *Bam*HI, Schiestl and Petes [5] demonstrated that inclusion of *Bam*HI in the transformation mix promoted the integration of the transforming DNA into *Bam*HI sites in the *S. cerevisiae* genome. A simple interpretation of this integration event is as follows: first, during transformation, the restriction enzyme enters the nucleus along with the linearized transforming DNA whose ends have been generated by the added enzyme; second, the restriction enzyme digests the host chromosome at its specific recognition sites; and third, the complementary ends of the chromosomal DNA and the transforming fragment are ligated *in vivo* to generate a nonhomologous integration event.

In this review, we discuss the possible mechanism(s) of REMI, which can be thought of as a form of nonhomologous end-joining (NHEJ) [6], and the use of REMI as a genetic tool, primarily in fungi.

The mechanism of REMI

Additional work in *S. cerevisiae* by Manivasakam and Schiestl [7•] has greatly increased our understanding of the mechanism(s) of integration events generated by REMI. To define more clearly the role played by the restriction enzyme, the authors demonstrated that *Bam*HI–E77K, a derivative of *Bam*HI that retains the ability to bind DNA but cleaves DNA at a 1000-fold lower rate, failed to enhance integration. These results demonstrated that the endonucleolytic activity of the added restriction enzyme was required for the process.

In addition to *Bam*HI (which gives rise to 5′ protruding single strands or 5′ PSSs), *Bgl*II and *Kpn*I (which produce 5′ and 3′ PSSs, respectively) also mediated REMI in *S. cerevisiae* [7•]. The success of these transformation events depended on linearization of the transforming plasmid because no integrations were seen with undigested plasmids lacking recognition sites for these restriction enzymes. If the undigested plasmid contained a recognition site for the enzyme, a low frequency of integration was seen, suggesting that digestion of both plasmid DNA and host genome during the transformation process is less efficient. Analysis of integration events produced by *Bgl*II and *Kpn*I indicated that, as with *Bam*HI, a high percentage of the integrations generated by REMI were conservative integrations (i.e. integrations into the appropriate restriction site with conservation of the site and without plasmid rearrangement). The restriction enzymes *Asp*I718, *Eco*RI, *Sal*I, and *Hin*DIII, which produce 5′ PSSs, did not increase integration efficiency. Additionally, enzymes tested that produced blunt-ended DNA fragments, in this

case *Hpa*I, *Msc*I, and *Sma*I, were unable to stimulate integration. Although *Eco*RI did not stimulate integration, transformation of *Eco*RI digested plasmid DNA with added *Eco*RI led to conservative integration at *Eco*RI sites in three out of ten transformants. In contrast, without the addition of *Eco*RI no transformants of twenty tested contained conservative integrations within *Eco*RI sites.

Generally, experiments involving REMI utilize the same enzyme to generate the ends of the transforming fragment and to stimulate integration and the majority of the integrations are conservative. This does not, however, have to be the case; Manivasakam and Schiestl [7•] demonstrated that *Bam*HI, *Bgl*II, and *Kpn*I can also stimulate integration when the transforming DNA fragment is generated by restriction with different enzymes. *Bam*HI, *Bgl*II, and *Kpn*I were able to stimulate integration of DNA fragments with 5′ or 3′ PSSs but not with blunt ended DNA fragments. These events require that the PSSs contain microhomology of at least two base pairs.

Various combinations of enzymes and the resulting insertion sites were analyzed, and possible mechanisms of integration were suggested. Integrations that involved the use of restriction enzymes that generate compatible ends, such as *Bam*HI and *Bgl*II (5′ GATC), resulted in hybrid junctions, and, mechanistically, are equivalent to conservative insertions. When *Bgl*II and *Sal*I, which generate the noncomplementary 5′ PSSs GATC, and TCGA, were used the junctions appeared to be generated by annealing of the two terminal bases, gap filling, and ligation. More complex events were also observed such as when *Asp*I was the restriction enzyme used to generate the ends of the transforming DNA fragment and *Kpn*I was the resistriction enzyme added during the transformation. *Asp*I and *Kpn*I are restriction endonucleases that share the same recognition site but generate different single stranded ends. *Asp*I produces a 5′ PSS whereas *Kpn*I generates the same sequence as a 3′ PSS. Analysis of chromosomal insertion sites indicated that 27% of the junctions were *Kpn*I sites and could be generated as follows: the 5′ PSS (*Asp*I) of the transforming DNA fragment is filled in *in vivo* and converted to a 3′ PSS by a host 5′-3′ exonuclease, these ends are complementary to and anneal with the chromosomal ends generated by the added restriction enodnuclease *Kpn*I and ligation occurs. When the 5′ PSS ends of the transforming DNA fragment generated by *Asp*I were filled in to create blunt ends prior to transformation (i.e. *in vitro*), the percentage of junctions that were *Kpn*I sites increased to 50%.

As described above, the variety of mechanisms underlying integration by REMI are essentially NHEJ events [6]. The *RAD50* gene product is required for the NHEJ repair pathway [8,9], and was previously shown to be required for REMI [10]. The *S. cerevisiae HDF1* gene product, a homolog of mammalian Ku70 which is required for repair of chromosomal double strand breaks, is involved in NHEJ [9]. It has also been shown that integration by illegitimate recombination or REMI is greatly reduced in an *hdf1* strain [7•]. Thus, the factors involved in NHEJ events are also required for integrations generated by REMI.

REMI as a method for insertion mutagenesis

Kuspa and Loomis [11] first exploited REMI to generate tagged insertion mutations in *Dictyostelium discoidium*. Introduction of various restriction enzymes with transforming plasmid DNA whose free ends matched the accompanying enzyme stimulated transformation 20–60 fold. Greater than 70% of the integration events were conservative integrations. Additionally, ten independent integration events were shown to have occurred in different sites as each yielded unique chromosomal junction fragments, suggesting that integration was occurring randomly. Furthermore, the phenotype of a mutant strain was shown to be caused by the REMI insertion by recovery of the transforming plasmid plus flanking sequences and reconstitution of the insertion and phenotype by homologous recombination. In *D. discoidium*, the method of REMI has been used extensively for saturating mutagenesis and suppressor analysis to isolate genes involved in processes such as development and cytokinesis (for example see [12–15]).

Building on the work of Schiestl and Petes [5] and the successful use of REMI as a tool for genetic analysis in *D. discoidium*, researchers studying a variety of fungi have begun to utilize REMI as a means to mutagenize fungal genomes (see Table 1) [16–20].

REMI has been used to generate tagged mutations in the Ascomycete *Cochliobolus heterostrophus*, a pathogen of corn [16]. Lu *et al.* [16] used a linearized nonhomologous plasmid and the restriction enzyme *Hin*DIII to isolate two independent integrations at different *Hin*DIII sites in the expansive *Tox1* locus. Few transformants were recovered in the absence of *Hin*DIII, and addition of *Hin*DIII stimulated transformation 20-fold. Thus, in contrast to *S. cerevisiae* [7•], use of the restriction enzyme *Hin*DIII was successful for REMI in *C. heterostrophus*. As in *S. cerevisiae* [7•], circular plasmid was a poor substrate for integration in *C. heterostrophus*.

Bölker *et al.* [17] used REMI to tag pathogenicity genes in the basidiomycete *Ustilago maydis*, the causative agent of corn smut disease. Adding *Bam*HI to a transformation mix containing undigested, nonhomologous plasmid DNA yielded approximately 50% conservative integrations; additional integrations occurred at *Bam*HI sites but without restoration of the recognition site. Successful use of a circular plasmid for REMI contrasts with the results described above for *S. cerevisiae* [7•] and *C. heterostrophus* [16]. Generally, stimulation of integration upon addition of restriction enzyme has been thought to be the hallmark of the REMI process. In the case of *U. maydis*, however, *Bam*HI did not enhance

Table 1

Examples of genetic analysis in fungi using REMI.

Organism	Form of transforming DNA	Homology of transforming DNA	Restriction enzyme	Stimulation of transformation	Type of integration	References
Candida albicans (imperfect)	Linear	Some homology to genome	*Bam*HI	~17 fold	>95% conservative integrations all appear random	[19,23]
Cochliobolus heterosporus (ascomycete)	Linear	No homology to genome	*Hin*DIII	~20 fold	Two *tox*⁻ mutants analyzed were conservative integrations at independent *Hin*DIII sites	[16]
Coprinus cinereus (basidiomycete)	Circular or linear	Homology to genome	*Bam*HI, *Eco*RI, *Pst*I	~7 fold	32–67% conservative integrations all appear random	[20]
Magnaporthe grisea (ascomycete)	Linear	No homology to genome	*Bam*HI, *Bgl*II, *Hin*DIII, *Eco*RV	≤10 fold	28–72% conservative integrations all appear random	[18,21]
Saccharomyces cerevisiae (hemi-ascomycete)	Linear	No homology to genome	*Bam*HI, *Bgl*II, *Kpn*I (for conservative REMIs)	~7 fold	80–90% conservative integrations all appear random	[5,7•]
Ustilago maydis (basidiomycete)	Circular	No homology to genome	*Bam*HI	None	~50% conservative integrations	[17]

integration. Rather, the inclusion of *Bam*HI during the transformation changed the type of integration event seen. Transformation without the enzyme yielded primarily tandem integration events. When *Bam*HI was present, transformation yielded primarily (90%) single integration events. Analysis of two pathogenicity mutants recovered after REMI demonstrated that the integrations conferred the mutant phenotypes [17].

The ascomycete *Magnaporthe grisea* is the causative agent of rice blast disease [18,21]. Shi *et al.* [18] used linear nonhomologous DNA and a variety of restriction enzymes to transform this fungus by REMI. *Bam*HI, *Bgl*II, *Hin*DIII and *Eco*RV stimulated integration 10-fold, 10-fold, 2-fold, and less than 2-fold, respectively. The optimal enzyme concentration for enhanced transformation varied with the enzyme chosen for REMI; for example, *Bam*HI required one-tenth the amount of enzyme as *Hin*DIII to achieve optimal stimulation. Although only negligible stimulation of integration was seen with EcoRV, 42% of integrations were conservative integrations that retained the *Eco*RV site. In terms of NHEJ, the mechanism whereby blunt-ended transforming fragments and chromosomal ends are brought into juxtaposition for ligation is not obvious. It is not simply a matter of an exonuclease creating regions of microhomology that can be trimmed, filled in, and ligated, because the *Eco*RV site is frequently retained. Possibly, the added restriction enzyme not only creates double strand breaks but participates in bringing recombination partners together. Alternatively, in contrast to *S. cerevisiae*, *M. grisea* may possess an 'alignment protein' as has been postulated to operate in *Xenopus* oocyte extracts where most combinations of PSS and blunt ends are joined [6,22]. When enzymes that do not produce compatible ends were used in *M. grisea* (e.g. a *Bam*HI linearized fragment and *Apa*I as the added enzyme) integration was not stimulated and was possibly depressed. Using the restriction enzyme *Bam*HI to stimulate integration, the authors [21] recovered five mutants out of 600 transformants screened. In all cases, the mutant phenotype cosegregated with hygromycin B resistance (the selective marker used for this transformation) in ascospore progeny, indicating that the insertion mutation conferred the phenotype.

Granado *et al.* [20] recently undertook a systematic analysis of REMI in the basidiomycete *Coprinus cinereus*. Using a variety of restriction enzymes (*Bam*HI, *Eco*RI, or *Pst*I) integration was stimulated up to seven-fold at optimal enzyme concentration and, as seen with *M. grisea* [18], optimal enzyme concentration varied with the enzyme chosen. *Eco*RI was ineffective in stimulating DNA integration in *S. cerevisiae* [7•] but was effective in *C. cinereus*. Both circular or linear nonhomologous plasmids integrated with similar efficiency and, as with *U. maydis* [17], addition of restriction enzyme affected the type of integration seen in *C. cinereus*. In transformations without added enzyme, 72% of transformants had multiple integrations. In contrast, when *Bam*HI was included during transformation 67% of transformants analyzed had single integrations.

In the opportunistic fungal pathogen *Candida albicans*, addition of the restriction enzyme *Bam*HI to a transformation mix containing a plasmid linearized with *Bam*HI stimulated transformation approximately 17-fold, and greater than 95% of the integrations were conservative integrations into random *Bam*HI sites [19]. Sequence analysis of integration events demonstrated that some in-

tegrations had occurred into *Bam*HI sites within structural genes ([19]; C Higgins, CA Kumamoto, unpublished data).

Decreased viability and increased mutation rates associated with REMI

Electroporation of various restriction enzymes into mammalian cells results in both decreased viability and increased chromosomal aberrations [23,24]. In *S. cerevisiae*, overexpression of *Eco*RI is toxic [25]. Manivasakam and Schiestl [7•] did not observe a decrease in viability after transformation of *S. cerevisiae* in the presence of restriction enzyme. These experiments, however, did not address the possibility that only a small fraction of the cells take up the restriction enzyme and transforming DNA. In *C. albicans*, increasing amounts of *Bam*HI decreased the number of transformants recovered after homologous transformation with a selectable marker, suggesting the restriction enzyme decreases viability of those cells that take up macromolecules (AD Giusani and CA Kumamoto, unpublished data). In *M. grisea* and *C. cinereus* transformation rates decreased with higher enzyme concentrations [18,20]. It is possible that transformation with high concentration of restrictions enzymes produces genetic damage as well as integration events.

Additional applications of REMI to genetic analysis

REMI–RFLP mapping in *D. discoidium*

Kuspa and Loomis extended the use of REMI as a genetic tool in *D. discoidium* by utilizing REMI insertions for genome mapping [26,27]. Single, random integrations of a plasmid carrying a rare restriction site (in this case an *Apa*I site) were generated by REMI at *Bam*HI sites throughout the genome. Thus, each strain contained a distinct *Apa*I site whose genomic location was mapped. Mapping of these unique *Apa*I sites acquired through REMI events with respect to other rare restriction sites, chromosome ends, and flanking genes allowed the authors to generate a high resolution physical map of the *D. discoidium* genome. This approach is readily applicable to any fungus in which one can recover single, random integration events.

Promoter trapping

Another technique, developed for use in the slime 'molds' *Dictyostelium* and *Polysphondylium*, involved the use of REMI to integrate a promoterless reporter gene construct (*lacZ* or green fluorescent protein, respectively) randomly and stably throughout the genome [28,29]. Transformants were then screened to isolate promoters that were expressed under a particular set of conditions, in these cases during fruiting body development.

Dominant genetic approach

Brown *et al.* [30] demonstrated that genetic constructs could be transformed into a *C. albicans* strain by REMI, allowing stable maintenance of the construct without rearrangement. A plasmid containing the regulated promoter of the *C. albicans MAL2* gene fused to the *C. albicans URA3* coding sequence was transformed by REMI into the *C. albicans* genome. All transformants tested demonstrated regulated *URA3* expression. Additionally, greater than 96% of colonies analyzed after growth under nonselective conditions retained the construct. This system could be exploited for the isolation of genes on the basis of the dominant expression of a phenotype in *C. albicans*.

Complementation analysis

Because insertions generated by REMI are often single, stable integration events, functional analysis of various alleles of a gene of interest by complementation using constructs integrated by REMI would be feasible. Isolation of plasmids that complement mutations of interest using a library of plasmids introduced by REMI would also have advantages. Single copy integration would reduce the potential for isolating suppressing clones and permit easy recovery of the complementing DNA. This approach circumvents the lack of stable plasmids in most fungi and the inherent instability, amplification, and multiple integrations seen with ectopic integration in many fungi.

Conclusions

The technique of REMI has been exploited in a variety of fungi ranging from hemi-acomycetes and acomycetes to basidiomycetes. Although optimization and choice of restriction enzyme for REMI in various fungi is somewhat empirical, in most cases, development of REMI has involved addition of restriction enzyme and the appropriate REMI vector to preexisting transformation protocols. The use of REMI as means to undertake genetic analysis in fungi provides several advantages: the creation of random insertion mutations that are physically tagged; the stimulation of transformation frequency several fold; and the creation of single genomic insertions that are stable and unrearranged. In addition to insertion mutagenesis, REMI is also being utilized in new ways such as for RFLP mapping, promoter trapping, and dominant genetics; and this list of uses will undoubtedly grow. Transformation methods for human fungal pathogens including *Histoplasma capsulatum*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *Blastomyces dermatitidis*, have been developed, allowing introduction of DNA and, in some cases, homologous recombination [31–37]. REMI will likely be a useful additional tool for genetic analysis in these interesting and important organisms.

Note added in proof

After submission of this review, S'ånchez and coworkers reported the successful use of REMI as a genetic tool in *Aspergillus nidulans* [38•].

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This is a significant paper in that it changes the way in which REMI has heretofore been perceived. Most discussions of the REMI process focused on the stimulation of transformation and mechanistically described the process as essentially a ligation reaction. This work shows that REMI is a type of nonhomologous end joining and that the stimulation of integration varies from none to quite high depending upon variables such as the degree of processing required for the end joining process. Additionally, because the entire sequence of the *S. cerevisiae* genome is known, within this paper are data addressing randomness of integration with respect to chromosomes, open reading frames, and untranscribed regions and mutagenic lesions such as deletions associated with the integration event.

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This is the first report of the successful use of REMI as a genetic tool in *Aspergillus nidulans*.