Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA

(transformation/insertional mutagenesis/gene cloning)

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ABSTRACT Introduction of restriction enzyme along with linearized plasmid results in integration of plasmid DNA at genomic restriction sites in a high proportion of the resulting transformants. We have found that electroporating *Bam*HI or *Eco*RI together with *pyr5-6* plasmids cut with the same enzyme stimulates the efficiency of transformation in *Dictyostelium discoideum* more than 20-fold over the rate seen when plasmid DNA alone is introduced. Restriction enzyme-mediated integration generates insertions into genomic restriction sites in an apparently random manner, some of which cause mutations. About 1 in 400 of the *Dictyostelium* transformants displayed arrested or aberrant development. The integrated plasmid, along with flanking genomic DNA, was excised from some of these mutants, cloned in *Escherichia coli*, and used to transform other *Dictyostelium* cells. Homologous recombination within the flanking sequences resulted in the same phenotypes displayed by the original mutants, directly demonstrating that the affected genes were responsible for the specific morphological phenotypes. This method of insertional mutagenesis should be useful for tagging, and subsequent cloning, of many developmentally important genes that can be identified by their mutant phenotypes.

Mutations affecting many different developmental processes have been isolated in *Dictyostelium discoideum* following chemical mutagenesis (1–3). Mutant phenotypes include alterations in cell–cell adhesion, cellular motility, and chemotaxis of the amoebae, as well as aberrations in slug and fruiting-body formation (4). Many of the mutated genes have been mapped to particular linkage groups by parasexual genetics (5, 6), but in only a few cases has it been possible to isolate the affected genes. Cloning by functional complementation with plasmid-borne genomic libraries has not been successful for developmental genes where direct selections cannot be applied. Transposon tagging has led to the isolation of developmental genes in other systems, including *Myxococcus* (7), *Drosophila* (8), and *Caenorhabditis* (9, 10), but depends on the ability to mobilize transposable elements, which has not been possible in *Dictyostelium*. On the other hand, procedures for the isolation of strains that have integrated bacterial plasmids carrying selectable markers are well developed in *Dictyostelium* (11–13). If the plasmids were to integrate into broadly distributed sites throughout the genome, insertions might tag many different genes and allow their rapid isolation.

Schiestl and Petes (14) recently found that introducing the restriction enzyme *Bam*HI together with a *Bam*HI-cut DNA fragment increased the number of transformed yeast cells and showed that the DNA fragment was often integrated into *Bam*HI sites of the host genome. This surprising result led us to explore restriction enzyme-mediated integration (REMI)

in *Dictyostelium*. We have found that introducing any one of several different restriction enzymes along with linearized plasmid DNA stimulates the efficiency of transformation significantly, as long as the plasmid ends match those of the digested recognition site of the accompanying enzyme. The plasmids integrated into the appropriate restriction sites >70% of the time in the host strains that we used. We found no apparent preference for sequences flanking the restriction sites, indicating that integration may be otherwise quite random. We describe a simple method of insertional mutagenesis in *Dictyostelium* that uses REMI to tag genes based on their mutant phenotypes.

MATERIALS AND METHODS

Strains and Nomenclature. Strains were maintained on SM plates growing in association with *Klebsiella aerogenes* and were grown in liquid HL-5 medium (15), or HL-5 supplemented with uracil (20 μ g/ml) in the case of *pyr5-6* mutant strains. The parental strain, AX4 (16), is a clonal isolate of AX3 (17). Insertion sites were named for the strain in which they were originally isolated, using IS as a prefix. Thus the insertion isolated in AK108 was named IS108 and simply labels a particular position in the *Dictyostelium* genome.

DNA Mapping. Restriction maps of plasmids and *Dictyostelium* genomic DNA were constructed by standard DNA manipulations and Southern blot analysis. Southern blot analysis was performed by alkaline transfer of DNA in agarose gels to Magna NT (Micron Separations, Westboro, MA) nylon membrane as described by Vollrath *et al.* (18). Blots were hybridized as described (19) and probed with DNA fragments labeled with [α - 32 P]dCTP by random-primed synthesis (20).

Plasmid Construction. The *Dictyostelium* integrating vectors DIV1 and DIV2 were constructed by ligating the 3.8-kilobase-pair (kb) *pyr5-6*-containing *Cla* I fragment from pDU3B1 (21) into the *Acc* I site of pGEM-3 (Promega). The structure of DIV1 is shown in Fig. 1A, and DIV2 is the same but contains the *pyr5-6* fragment in the opposite orientation. DIV6 was constructed by ligating the *Pst* I–*Sac* I *pyr5-6* fragment from DIV1 into *Pst* I/*Sac* I-digested pGEM-5Zf(+) (Promega).

A 6.7-kb *Bgl* II fragment that includes the *pyr5-6* gene was subcloned from yeast artificial chromosome (YAC) clone 188. Intact YAC188 was separated from the endogenous yeast chromosomes as described (22), isolated as a gel slice, and digested *in situ* with *Bgl* II. The YAC188 *Bgl* II fragments were then electroeluted onto DE81 paper (Whatman), eluted from the paper with 1 M NaCl, purified, and cloned into the *Bam*HI site of pGEM-3 by standard cloning techniques (19). Plasmid p188.50 was identified from this sublibrary by colony blot hybridization with a 32 P-labeled *pyr5-6* gene probe isolated from pDU3B1 (21) and was confirmed by Southern blot

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Abbreviations: REMI, restriction enzyme-mediated integration; YAC, yeast artificial chromosome; FOA, 5-fluoroorotic acid.

FIG. 1. Structure of the *pyr5-6* locus in the *Dictyostelium* integrating vector DIV1, the wild-type strain AX4, and the deletion strain HL330. (A) DIV1 is a bacterial plasmid (thin curved line) carrying a 3.5-kb *pyr5-6* fragment. (Note that the bacterial DNA is not drawn to scale.) (B) A map of the 6.7-kb region of the *Dictyostelium* genome surrounding the *pyr5-6* locus is shown in AX4. The *Bgl* II fragment was used to construct a *pyr5-6* deletion fragment in the plasmid pΔPYR (see *Materials and Methods*). The deletion fragment was introduced into AX4, and HL330 was isolated after selection for FOA resistance. Restriction sites: B, *Bam*HI; Bg, *Bgl* II; C, *Cla* I; E, *Eco*RI; K, *Kpn* I; P, *Pvu* II.

analysis to contain a 6.7-kb fragment surrounding the gene with a restriction map that matched the map obtained from the AX4 genome (see the AX4 map in Fig. 1A). pΔPYR is analogous to p188.50 except that the central 3.3-kb *Cla* I–*Kpn* I *pyr5-6* fragment is absent. It was constructed by ligating the 1.7-kb *Hind*III–*Cla* I fragment (upstream of *pyr5-6*) of p188.50 into *Hind*III/*Acc* I-digested pGEM-3 and then ligating the 1.7-kb *Kpn* I fragment (downstream of *pyr5-6*) of p188.50 into this vector's *Kpn* I site. A 3.4-kb *Hind*III–*Eco*RI fragment was purified from pΔPYR and used to transform AX4 to 5-fluoroorotic acid (FOA) resistance (ref. 13; see below). The *pyr5-6* deletion strain HL330 was isolated by screening the *Dictyostelium* transformants by Southern blot analysis for the absence of the central 2.0-kb *Kpn* I fragment of p188.50 (see Fig. 1).

Transformation Conditions. Transformation conditions were modified from Howard *et al.* (23). Strains were grown to $1\text{--}2 \times 10^6$ cells per ml in HL-5 medium or HL-5 plus uracil, and the cells were pelleted in a conical 50-ml tissue culture tube at 1000 rpm in a clinical centrifuge. The medium was decanted and as much residual medium as possible was aspirated from the walls of the tube prior to suspension of the cells at 10^7 per ml in ice-cold electroporation buffer. At this point, cells, glass tubes, and cuvettes were kept on ice. Plasmid DNA was used either undigested or linearized with a particular restriction enzyme and purified by phenol extraction and ethanol precipitation prior to use for transformation. Aliquots (0.8 ml) of the cell suspension were mixed with 40 μ g of DNA in a glass test tube and immediately exposed to 2.25 kV/cm in a 0.4-cm-gap electroporation cuvette in a Bio-Rad Gene Pulser set at 0.9 kV and 3 μ F. Time constants ranged from 0.6 to 1.1 msec for all experiments. For REMI transformation, 100–200 units of restriction enzyme was mixed with the cell/DNA mixture and electroporation was carried out immediately. For *Sau*3A1 REMI, *Bam*HI-digested vector was used. Restriction enzymes were obtained from BRL. Cells were plated in standard Petri dishes immediately following electroporation at $1\text{--}2 \times 10^5$ per ml in FM medium (24), or HL-5 containing FOA (100 μ g/ml) and uracil (20 μ g/ml) (13), and were incubated at room temperature. Transformation frequencies for all experiments ranged from 5×10^{-6} to 8×10^{-5} . For FOA selection, the medium was changed every 4–6 days and clones appeared in

2–3 weeks, after which the transformants were cloned by plating them in association with *K. aerogenes* on SM plates (15). For the uracil prototrophic selection in FM medium, the FM was changed after 6–8 days, and transformants were cloned on SM plates after 12–14 days.

Mutant Screening. Single *Dictyostelium* cells grow into colonies on SM plates in a lawn of *K. aerogenes*. When the bacterial food source is exhausted in the center of such colonies, the population of cells begins to develop into many distinct multicellular aggregates, each of which continue through development to form mature fruiting bodies. To screen for mutants, transformants were plated on SM plates with bacteria in pools of about 100 transformants. Colonies that displayed aberrant developmental morphology were picked for further analysis. Since these colonies came from pools of 100 transformants, particular mutants made up about 1% of the total population of the ≈ 1000 clones that were screened for each pool. Only one strain of each distinct phenotype was saved from each pool. Since a mutant was recovered only about once in every three pools, it is unlikely that two distinct mutants with the same phenotype were present in any of the pools. Thus, the frequency of obtaining mutants was extrapolated from the number of mutant phenotypes recovered on the SM plates and the number of transformants in the original selection plates.

Genomic Cloning. To clone integrated plasmids from *Dictyostelium* into *Escherichia coli*, 0.5 μ g of genomic DNA from the insertion strain was digested with a restriction enzyme that does not cut the integrating vector and that was previously determined by Southern blot analysis to produce a vector-containing fragment <15 kb in size. The DNA was purified, dissolved in 10 μ l of sterile water, then brought up to 0.5 ml in ligase buffer (19). Ten units of T4 DNA ligase (BRL) was added and incubated for >12 hr at 12–15°C, after which the ligation products were precipitated with ethanol, dissolved in 40 μ l of sterile water; 2–6 μ l was used to electroporate SURE *E. coli* cells (Stratagene) with a Bio-Rad Gene Pulser according to the manufacturer's suggested protocol.

RESULTS

Deletion of the *pyr5-6* Gene. Strains carrying mutations or having small deletions in the *pyr5-6* gene do not grow in minimal medium (FM) in the absence of uracil (13). Transformants of these strains can be selected for uracil prototrophy (*ura*⁺) after electroporation of supercoiled plasmids containing the *pyr5-6* gene. The *pyr5-6* gene was chosen as the selectable marker in these studies because integration of a single copy of the gene on a supercoiled plasmid is known to complement *pyr5-6* mutants with high efficiency (13). However, attempts to obtain insertional mutants by transformation with supercoiled *pyr5-6*-based plasmids such as DIV1 (Fig. 1A) were unsuccessful because >99% of the transformants resulted from one of two events: homologous integration at the mutant *pyr5-6* locus, or apparent restoration of the *pyr5-6* gene function without plasmid integration (unpublished results).

Table 1. REMI of plasmid DNA

Conditions for electroporation of plasmid DIV2 into HL330	Stable transformants per 10^6 cells	
	Exp. 1	Exp. 2
<i>Bam</i> HI-digested plasmid		
No enzyme added	0.25	0.6
<i>Bam</i> HI added (REMI)	13	14
<i>Eco</i> RI-digested plasmid		
No enzyme added	0.75	1.0
<i>Eco</i> RI added (REMI)	25	62

FIG. 2. REMI of plasmid DNA into the *Dictyostelium* genome. Samples of genomic DNA from 10 randomly chosen HL330 transformants generated by *Bam*HI REMI of DIV2 plasmid were digested with *Bam*HI, transferred to nylon membrane, and hybridized with 32 P-labeled DIV2 (see *Materials and Methods*). Most of the transformants display hybridization to a 6.7-kb *Bam*HI fragment, the same size as the DIV2 fragment.

In an attempt to increase the proportion of transformants with integration events at locations other than the *pyr5-6* locus, a host strain was constructed that lacked nearly all of the genomic region homologous to the *pyr5-6* plasmids. Approximately 1.7 kb of DNA from each side of the *pyr5-6* gene were fused together and used to transform cells of strain AX4 (Fig. 1B). Transformants were selected for resistance to FOA. One of the transformants, strain HL330, was found to carry only about 130 base pairs homologous to sequences in the DIV1 and DIV2 *pyr5-6* plasmids.

Transformants can be readily isolated from strain HL330 following selection in medium lacking uracil (see below). The great majority of such transformants grow at the same rate as wild-type strains and develop normally. Therefore, we could score for morphological aberrations at any developmental stage that might result from integration of plasmid DNA into a gene of interest.

REMI. Schiestl and Petes (14) have described the integration of linear DNA into the yeast genome mediated by the restriction enzyme *Bam*HI. The process was found to be efficient in yeast and led to the proposal that REMI would be useful in other systems. We have tested the effect of electroporating cells of strain HL330 with either *Bam*HI or *Eco*RI along with the *pyr5-6* plasmid DIV2 cut with the same enzyme (Table 1). Without adding enzyme we recovered transformants at frequencies of about 5×10^{-7} , whereas with added enzyme we recovered transformants at 20- to 60-fold higher frequencies (Table 1). As a control, we electroporated heat-denatured *Bam*HI with DIV2 plasmid cut with this enzyme and found no stimulation of the rate of transformation (data not shown). Thus, active restriction enzymes appear to stimulate the frequency of integration of linear DNA into *Dictyostelium* cells more than they do in yeast.

Evidence that the plasmid integrated into the host at one of its homologous restriction sites would be the clean excision of the 6.7-kb DIV2 plasmid DNA upon digestion of genomic DNA isolated from the REMI transformants with the appropriate restriction enzyme. An example of this analysis (Fig. 2) shows the results of probing a Southern blot of *Bam*HI-

Table 3. Enzyme requirements for REMI

Electroporation conditions	Stable transformants per 10^6 cells
No enzyme added	0.33
REMI with <i>Bam</i> HI	9.0
REMI with <i>Eco</i> RI	0.37
REMI with <i>Sau</i> 3A1	12

*Bam*HI-digested plasmid DIV6 was electroporated into HL330.

digested DNA isolated from 10 transformants generated by electroporation of plasmid together with *Bam*HI. The blot was probed with vector-specific sequences and showed that 9 out of 10 of these transformants had integrated the plasmid at a *Bam*HI site. A total of 48 *Bam*HI REMI transformants have been analyzed this way, and 35 were found to give the 6.7-kb DIV2 fragment upon digestion with *Bam*HI. Similar experiments were carried out with 14 transformants generated with *Eco*RI REMI, and 10 of these were found to give the 6.7-kb DIV2 fragment upon digestion with *Eco*RI. Thus, REMI resulted in plasmid integration at the homologous restriction sites for these enzymes in >70% of the transformants.

Digesting genomic DNA isolated from transformants with enzymes not used for the REMI transformation should generate diverse-sized fragments recognized by the plasmid-specific probe if the integration sites are dispersed throughout the genome. As shown in Table 2, digestion of DNA from 10 *Bam*HI REMI transformants with three restriction enzymes gave many different-sized fragments. Moreover, comparison of the sizes of the fragments obtained from each transformant indicated that no two of these 10 transformants had a plasmid integrated into the same site.

Schiestl and Petes (14) have suggested that introduction of restriction enzymes cuts the host genome at one or more of its recognition sites and that the plasmid is ligated there in a manner dependent on its compatible ends. If this suggestion is true, the enzyme added for REMI must generate DNA ends that are the same as the ends of the plasmid being introduced. We tested this by using various enzymes with *Bam*HI-cut plasmid in REMI transformation (Table 3). While the homologous enzyme, *Bam*HI, stimulated transformation 27-fold in this experiment, the heterologous enzyme *Eco*RI did not stimulate the rate of transformation above the level seen when no enzyme was added to the electroporation buffer. It was of interest that *Sau*3A1 stimulated the rate of transformation as much or more than *Bam*HI, considering that these two enzymes generate the same 5' overhang (GATC). These results indicate that the ends of the incoming plasmid DNA must match the recognition site of the restriction enzyme for REMI to take place, and argue in favor of the simple ligation repair model.

Tagging Developmental Genes in *Dictyostelium*. Large-scale transformations were carried out to assess the feasibility of using REMI to obtain developmental mutants in *Dictyostelium*. Integrating vectors were digested with *Eco*RI or *Bam*HI and used to transform cells by electroporation in the presence of *Eco*RI or *Bam*HI. *Sau*3A1 was also used with *Bam*HI-

Table 2. Analysis of independent insertion sites

Enzyme	Fragment size, kb									
	1	2	3	4	5	6	7	8	9	10
<i>Bgl</i> II	13	12	16	18	11	17	13	11	10	13
<i>Cla</i> I	15	14	9.0	19	14	16	7.0	11	7.8	11
<i>Eco</i> RI	16	14	9.5	18	13	12	9.0	7.0	15	7.5

Sizes of the genomic fragments that include the integrated vector are shown. DNA from each of 10 *Bam*HI REMI transformants was digested with restriction enzymes that do not cut within the vector (*Bgl* II, *Cla* I) or that cut only once in the vector (*Eco*RI) and analyzed by Southern blot hybridization with a vector-specific probe (see *Materials and Methods*). Each transformant contained a single hybridizing band for each enzyme digestion.

digested vectors. The transformants generated were screened for morphological defects as they grew and developed clonally on bacterial plates. A particular example of this is outlined in Fig. 3A. Of a total of about 6000 primary transformants, 16 were found to arrest development at specific stages or have aberrant terminal morphology. Four mutants were aggregation-deficient, 4 aggregated partially, 2 formed tight aggregates but failed to progress to apical tip formation, and 6 had aberrant terminal fruiting-body morphology, such as small fruiting bodies. The electroporation of cells in the presence of *Bam*HI or *Eco*RI in the absence of

A. REMI

B. Homologous Recombination

FIG. 3. Strategy for tagging and cloning developmental genes in *Dictyostelium* by REMI of plasmid DNA. (A) An outline for REMI mutagenesis is shown in which plasmid integration into genomic *Bam*HI restriction sites occurs without extensive homology. The specific case of mutant AK120 is used as an example. The integrated plasmids can be cloned from the genome by digesting the mutant's genomic DNA with a restriction enzyme that does not cut the vector and leaves some genomic DNA flanking the insertion site attached to the integrated plasmid. The fragments generated are then circularized by ligation and transformed into *E. coli*. (B) Confirmation that an integrated plasmid caused the observed phenotype is accomplished by introduction of the linearized clone into a test strain in which homologous recombination of the plasmid at the original genomic site predominates. Complete correspondence between the integration at the original site and the mutant phenotype indicates that the insertion caused the mutation.

Table 4. Reintroduction of cloned insertions by homologous recombination

Plasmid DNA electroporated into HL330	Percent mutant transformants
p108Cla	93
p108Bgl	94
p111Bgl	95
p120Cla	98
p120Bgl	97
p127Cla	25

Each plasmid was cloned from the insertional mutant strain indicated by the plasmid number, using the enzyme abbreviated in the plasmid name (*Cla* I or *Bgl* II). Each plasmid was digested with the enzyme used in its release prior to transformation. Linearized plasmids were introduced into the host cells without added restriction enzymes. In each case, several hundred transformants were screened.

DNA, in each case, produced no mutants in >2500 clones analyzed and did not affect cell viability compared with the electroporation of cells in the presence of heat-inactivated enzyme or no enzyme (data not shown).

Proof that insertional events were the cause of the observed phenotypes was obtained by reintroducing disrupted insertion sites back into the parental strain as outlined in Fig. 3. To do this, integrated plasmid was recovered along with various amounts of flanking DNA from the mutant strains. Genomic DNA was digested with enzymes that do not cut within the plasmid and cloned in *E. coli*. When such clones were linearized and reintroduced into wild-type cells, it was hoped that homologous recombination would result in replacement of the endogenous gene with the disrupted copy in a high proportion of transformants (Fig. 3B). This was carried out for four different mutant strains, AK108, AK111, AK120, and AK127. In each case the mutant phenotype of the original insertion was recovered in a substantial percentage of the transformants (Table 4). These results demonstrate that the insertions carried in these four strains each disrupt a developmentally required genetic element.

The region surrounding the insertion site was mapped in several of the resulting transformants. Each of the transformants that showed the mutant phenotype had the same genomic structure as the original mutant insertion strain, and the region was unaltered in the few wild-type transformants recovered from the same experiment. The plasmid appears to have integrated at a separate site in those transformants that developed normally. Results of a *Bgl* II digestion of a wild-type (AK224) and a mutant (AK225) strain isolated after transformation with plasmid generated from strain AK108 are shown in Fig. 4. The wild-type transformant shows the endogenous 3.0-kb fragment as well as a new band (>12 kb), whereas the mutant transformant has only the 9.7-kb fragment seen in the original mutant strain AK108, in which the 6.7-kb DIV2 plasmid integrated into a *Sau*3A1 site. Nine additional mutant and nine additional wild-type transformants from this experiment were analyzed in the same way. Each mutant displayed only a 9.7-kb *Bgl* II band as seen in the original AK108 mutant, and each of the wild-type transformants had the wild-type 3-kb *Bgl* II fragment and a new *Bgl* II band corresponding to the transforming fragment at a different genomic location. Similar results were obtained for mutants AK111, AK120, and AK127.

DISCUSSION

We have demonstrated that restriction enzymes added during electroporation of linearized plasmids stimulate the efficiency of transformation at least 20-fold in *Dictyostelium*. Moreover, REMI resulted in the integration at restriction

FIG. 4. Transfer of the IS108 insertion into HL330 causes a defect in aggregation during development. (A) Genomic maps of IS108 as found in AK108 and the parent HL330 are shown. Data were obtained from restriction mapping of the plasmids p108Cla and p108Bgl, as well as from Southern blot analysis of AK108 and HL330. Note that there are only 200 base pairs of DNA between IS108 and the *Bgl* II site on the left, and 320 base pairs between IS108 and the *Cla* I site on the right. Restriction sites are defined in the legend to Fig. 1 (S, *Sau*3A1). (B) Southern analysis of the strains shown in A and two secondary transformants generated by transforming *Cla* I-linearized p108Cla (cloned with *Cla* I from AK108) into HL330. AK224 is developmentally wild type (*agda*⁺), and AK225 is aggregation-deficient (*agda*⁻). Genomic restriction fragments generated with *Bgl* II were separated by agarose gel electrophoresis, blotted to a nylon filter, and hybridized with the 1-kb *Cla* I fragment surrounding IS108 as a probe.

sites determined by the added enzyme in >70% of the transformants. It is not likely that restriction enzymes stimulate transformation by simply producing nonspecific recombinogenic double-strand breaks, since *Eco*RI did not stimulate the integration of *Bam*HI-linearized plasmids. Rather, restriction enzymes probably initiate cycles of digestion and ligation at their recognition sites in the genome, and linear plasmids occasionally interrupt proper repair by integrating into the site as a consequence of having homologous ends. Such a mechanism would account for our observation that *Sau*3A1 stimulates integration of *Bam*HI-linearized plasmids as well as *Bam*HI itself, since the overhangs left by these enzymes are identical.

We have used REMI to tag several different developmental genes that were recognized by the phenotypic consequences of suffering an insertion. In several cases, the mutant phenotypes were directly shown to result from the insertion event by characterizing newly transformed cells that had acquired the disrupted gene by homologous recombination. These transformants showed the same developmental phenotype found in the insertional mutant from which the disrupted gene was recovered. A wide range of phenotypes were found even among transformants of a single experiment, suggesting that unrelated genes were disrupted. Mapping and sequence analyses of the DNA flanking the insertion sites confirmed that the insertions tagged independent sites. The

sequences at the insertion sites were found to conform to the predicted restriction sites but showed no homology to sequences at ends of the integrated plasmid in the neighboring bases. Open reading frames were found for several hundred bases on each side of the insertion sites, and probes from these sequences hybridized to unique bands on Northern blots of RNA samples taken during development (unpublished observations). These results strengthen the conclusion that the plasmids disrupted developmental genes when they integrated into the genome.

Both the sequence data and the variety of developmental phenotypes generated by *Bam*HI-mediated integration suggest that insertions occur at many *Bam*HI sites throughout the genome. Whether or not there are preferences for certain sites over others will be known only when several thousand insertion strains have been analyzed in detail, since there are about that many *Bam*HI sites in the *Dictyostelium* genome. Even if only a subset of the *Bam*HI sites are readily tagged, use of other restriction enzymes such as *Eco*RI or *Sau*3A1 will mediate integration at an independent set of sites. Preliminary results from using *Cla* I and *Bgl* II in REMI experiments suggest that each of these enzymes works as well as *Bam*HI and so increases the number of targets for mutational tagging of genes. Insertional mutagenesis should be able to identify any gene that is not present in functionally redundant copies and is not essential for viability. All the selections and screens previously devised for obtaining mutants by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (4) should be applicable to REMI mutagenesis.

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