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A rapid, small scale method for characterization of plasmid insertions in the *Dictyostelium* genome

C. Barth, D. J. Fraser and P. R. Fisher*

School of Microbiology, La Trobe University, Bundoora 3083, Melbourne, Australia

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ABSTRACT

A rapid, simple method for characterization of plasmid insertions in the *Dictyostelium discoideum* genome was developed. It is based on the capability of linear plasmid multimers in the insertions to recircularize efficiently in *Escherichia coli* cells. This recombinational recircularization of plasmid multimers provides a highly sensitive and reliable tool for determining whether individual *Dictyostelium* transformants resulted from restriction enzyme-mediated integration (REMI) or from recombinational integration of plasmid (RIP). The method also reveals any rearrangements in RIP insertions and provides an estimate of the vector copy number in any particular transformant.

Insertional mutagenesis and gene tagging in *Dictyostelium discoideum* are based on either restriction enzyme-mediated integration (REMI) or recombinational integration of plasmid (RIP) into the genome (1,2). Within a single insertion site generated via RIP, the vector copy number varies from a few to several hundred tandemly duplicated copies (2–4), whereas successful REMI transformation results in the insertion of only a single copy of the transforming vector (1,5). Recently, we found that when *Escherichia coli* cells are transformed with *Dictyostelium* genomic DNA (gDNA) containing tandemly duplicated plasmid DNA, the plasmid multimers recircularize *in vivo* with high efficiency and without deletions. The recircularization process involves homologous recombination between the tandemly duplicated vector copies present in such insertions, thereby regenerating exactly the circular monomeric form of the original transformation vector (6). Plasmid monomers, by contrast, recircularize inefficiently and inexactly due to the lack of extended regions of homology that can direct the recombination. Since REMI transformants contain only a single vector copy, *E.coli* transformation with gDNA isolated from them is expected to be less efficient than transformation with gDNA isolated from RIP transformants. However, a significant proportion of presumed REMI transformants can contain plasmid insertions that have arisen from RIP as a consequence of partial digestion of plasmid DNA prior to transformation and/or preinsertional recircularization events. This results in the formation of multicopy plasmid insertions (1,7). As the efficient recovery of disrupted genes relies on the insertion of only a single plasmid into an appropriate restriction site, putative REMI mutants must be screened via a laborious process involving the preparation and digestion of gDNA, gel electrophoresis and subsequent Southern blot analysis. Here we report a rapid, small scale method for characterizing plasmid insertions in the *D.discoideum* genome, that allows the efficient identification of REMI transformants. It also allows the estimation of the vector copy number present in the genomes of RIP transformants.

Relying on the high efficiency of *E.coli* electrotransformation, we were able to simplify existing procedures for gDNA preparation (2,8) in terms of time and labour, allowing the screening of large numbers of *Dictyostelium* transformants. The transformants were cultured in 1.5 ml of HL-5 medium up to a density of 0.5–1.0 × 10^7 cells/ml. The cells were harvested by centrifugation (1–3 s, at 15 800 *g* in an Eppendorf centrifuge), washed once in water and then resuspended in 300 µl TES buffer (10 mM Tris, pH 8.0; 1 mM EDTA; 0.1% SDS; 30 µg/ml RNase). After cell lysis by freezing (10 min, –70°C) and thawing (on ice), 30 µg of Proteinase K was added and the lysate was incubated for 1 h at 37°C. It was then extracted with phenol/chloroform/isoamylalcohol (25:24:1 v/v/v) and ethanol precipitated. The recovered gDNA (always ~300 ng) was resuspended in 10 µl of water prior to electroporation (9) into *E.coli* DH5α cells (estimated electrocompetence: 5 × 10^7/µg pUC19 DNA).

Using this method we examined plasmid insertions in 43 *Dictyostelium* RIP transformants and in 30 putative REMI transformants. All DH5α transformations with gDNA of the RIP transformants resulted in high colony numbers (15–300). In contrast to this, transformation with gDNA of 19 REMI transformants failed to give any colonies. In a further 11 cases colony numbers similar to those obtained in *E.coli* transformations with gDNA of RIP transformants were obtained, suggesting that these presumptive REMI transformants actually contained more than one plasmid copy per insertion. We confirmed this by Southern blot analysis of gDNAs of 10 RIP and 10 putative REMI transformants, the latter including five whose transformation into *E.coli* resulted in high colony numbers (Fig. 1). The gDNA was digested with *Bam*HI, separated by pulsed field gel electrophoresis (PFGE) and blotted onto nylon membrane prior to screening for plasmid DNA. *Bam*HI cuts once in the *Dictyostelium* transformation vector, releasing a plasmid fragment of identical size to the original transformation vector only if more than one vector copy

*To whom correspondence should be addressed. Tel: +61 3 9479 2496; Fax: +61 3 9479 1222; Email: fisher@latrobe.edu.au

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

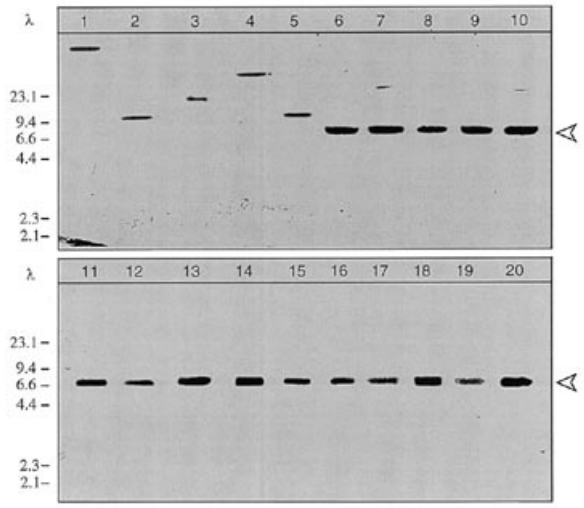

Figure 1. Composition of plasmid insertions in the genome of *Dictyostelium* transformants. The transformation vector used in all experiments was pPROF160, containing a neomycin and a blasticidin resistance cassette under the control of Actin15 promoters. *Bam*HI-digested gDNA of REMI and RIP transformants was separated by PFGE (10 h, 4 s switching time, 200 V, 14°C), blotted according to standard protocols and screened for plasmid DNA using a DIG-labeled probe (Boehringer Mannheim, Germany). Lanes 1–5, gDNA of REMI transformants that did not yield any *E.coli* colonies; lanes 6–10, gDNA of REMI transformants that transformed *E.coli* efficiently; lanes 11–20, gDNA of RIP transformants. *Hin*dIII-digested DNA size standards (λ) and the size of the linearized transformation vector (shown by the arrow; 6.7 kb) are indicated.

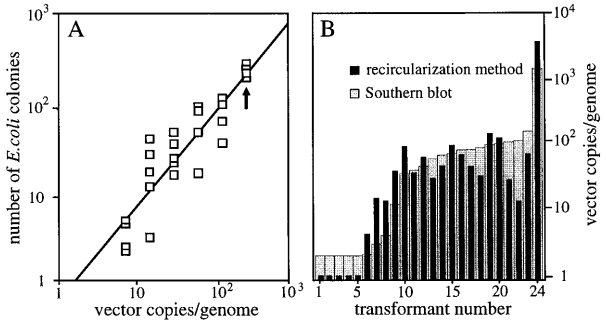

Figure 2. (**A**) Correlation between vector copy number per *Dictyostelium* genome and *E.coli* colony numbers obtained. DH5α cells were transformed with *Dictyostelium* gDNA (HPF274, 100 vector copies per genome) after serial dilution with gDNA of an untransformed wildtype strain (AX2). A total of 1 µg of gDNA was used in all cases except those transformations indicated by an arrow, in which 2 µg of HPF274 gDNA was used to simulate a transformation involving 200 copies per genome. The regression line was fitted by the least squares method. (**B**) Comparison of vector copy number per *Dictyostelium* genome as estimated from Southern blots (stippled bars) and the electroporation method (solid bars). Data is shown for 24 individual transformants arranged in ascending order according to the Southern blot estimates. Transformants 1–5 had confirmed, single copy REMI insertions and did not yield any *E.coli* colonies.

is present in the insertion. In *Bam*HI-digested gDNA of the REMI transformants whose DNA transformation did not yield any *E.coli* colonies, a band similar in size to the linearized transformation vector was not detected, excluding a multicopy arrangement of the vector in these transformants (Fig. 1, lanes 1–5). In contrast to this, *Bam*HI digestion of gDNA of the five putative REMI transformants whose gDNA transformed *E.coli* with high efficiency, released a band identical in size to the linearized transformation vector (Fig. 1, lanes 6–10). This indicates that the plasmid insertions of these transformants did not contain single plasmid molecules as desired, but instead contained multiple, tandemly duplicated plasmid copies. Similar results were obtained with *Bam*HI-digested gDNA of all RIP transformants (Fig. 1, lanes 11–20). It is probable that those transformants isolated by the REMI method which are found to contain plasmid multimers arise by recircularization in *Dictyostelium* and subsequent RIP rather than by REMI (7). Thus, transformation of *E.coli* with *Dictyostelium* gDNA is a reliable tool for distinguishing multicopy from single copy plasmid insertions and thereby allows efficient screening for real REMI transformants.

The method also allows rapid characterization of the multimeric insertions found in RIP transformants. Recombinational recircularization faithfully reveals the existence of rearrangements or deletions within such insertions because of the exactness of recombinational recircularization in *E.coli* (6). Estimation of the vector copy number per *Dictyostelium* genome is also readily performed because of the correlation we observed between the vector copy number in the *Dictyostelium* genome and the number of *E.coli* transformants obtained after electrotransformation with *Dictyostelium* gDNA (Fig. 2A). It is therefore possible to estimate the vector copy number in any *Dictyostelium* transformant simply by comparing the relative number of the colonies obtained after electroporating *E.coli* with similar amounts of gDNA of this transformant and a strain containing a known vector copy number in its genome. We confirmed the usefulness of this method by transforming *E.coli* with gDNA of 25 *Dictyostelium* transformants, one (HPF261) known to contain 35 vector copies (6) and the others containing an unknown copy number. Comparison of the colony numbers obtained led to copy number estimates that agreed well with the estimates based on Southern blot analysis (Fig. 2B). Thus, the method presented here allows a rapid screening for REMI transformants or, in overexpression studies, for RIP transformants containing large insertions without rearrangements.

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REFERENCES

1 Kuspa,A. and Loomis,W.F. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 8803–8807.
2 Nellen,W., Silan,C. and Firtel,R.A. (1984) *Mol. Cell. Biol.*, **4**, 2890–2898.
3 Knecht,D.A., Cohen,S.M., Loomis,W.F. and Lodish,H.F. (1986) *Mol. Cell. Biol.*, **6**, 3973–3983.
4 Hildebrandt,M. and Nellen,W. (1991) *Biochem. Biophys. Res. Commun.*, **181**, 884–888.
5 Morio,T., Adachi,H., Sutoh,K., Yanagisawa,K. and Tanaka,Y. (1995) *J. Plant Res.*, **108**, 111–114.
6 Barth,C., Wilczynska,Z., Pontes,L., Fraser,D.J. and Fisher,P.R. (1996) *Plasmid*, **36**, 86–94.
7 Katz,K.S. and Ratner,D.I. (1988) *Mol. Cell. Biol.*, **8**, 2779–2786.
8 Hughes,J.E. and Welker,D.L. (1988) *Nucleic Acids Res.*, **16**, 2338.
9 Dower,W.J., Miller,J.F. and Ragsdale,C.W. (1988) *Nucleic Acids Res.*, **16**, 6127–6145.