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A Direct Transposon Insertion Tool for Modification and Functional Analysis of Viral Genomes

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Advances in DNA transposition technology have recently generated efficient tools for various types of functional genetic analyses. We demonstrate here the power of the bacteriophage Mu-derived in vitro DNA transposition system for modification and functional characterization of a complete bacterial virus genome. The linear double-stranded DNA genome of *Escherichia coli* bacteriophage PRD1 was studied by insertion mutagenesis with reporter mini-Mu transposons that were integrated in vitro into isolated genomic DNA. After introduction into bacterial cells by electroporation, recombinant transposon-containing virus clones were identified by autoradiography or visual blue-white screening employing α-complementation of *E. coli* β-galactosidase. Additionally, a modified transposon with engineered *Not*I sites at both ends was used to introduce novel restriction sites into the phage genome. Analysis of the transposon integration sites in the genomes of viable recombinant phage generated a functional map, collectively indicating genes and genomic regions essential and nonessential for virus propagation. Moreover, promoterless transposons defined the direction of transcription within several insert-tolerant genomic regions. These strategies for the analysis of viral genomes are of a general nature and therefore may be applied to functional genomics studies in all prokaryotic and eukaryotic cell viruses.

Methods based on DNA transposition offer versatile tools for genetic analysis of a variety of biological processes (12, 34). The most efficient strategies are typically based on essentially random insertions of mobile DNA elements into the genomes of organisms ranging from viruses and bacteria to higher eukaryotes. Classical examples include transposon Tn*3* and Tn*10* insertions into the genomes of bacteriophage M13 and λ, respectively (36, 49), as well as insertions of the transposing bacteriophage Mu genome into the chromosome of *Escherichia coli* for gene and genome analysis (20) and genome-wide transposon tagging with the P element in *Drosophila* (50). Currently, advances in DNA transposition technology are rapidly generating ever more sophisticated systems for the analysis of single genes, gene networks, and entire genomes (14). In particular, methods based on in vitro reactions employing purified components and involving a variety of mobile elements have been remarkably effective (for recent examples, see references 13, 21, 23, 24, 25, 28, 32, 38, and 59).

To date, the use of in vivo DNA transposition-based insertion tools to study many types of viruses has been difficult or impossible due to incompatibility between the virus host and the transposon delivery system. Optimally, both the viral nucleic acid replication machinery and the expressed transposition machinery should be operative within a single compatible cell. For example, this situation may be achieved with certain bacterial viruses that replicate in host cells that, in turn, contain inducible or naturally active transposons (36, 49). Another approach to transposition mutagenesis of viral genomes is based on prior cloning of the viral nucleic acid in an infective form within a DNA vehicle (e.g., plasmid, cosmid, PAC, or BAC) that is compatible for replication in *E. coli*. Such an in vivo strategy has been used to identify genes important for virus propagation in herpesviruses (15, 57) and cytomegalovirus (63). However, cloned infectious virus genomes are typically not available for most virus species and isolates and therefore in vivo transposition methods are not applicable in these cases. Consequently, it would be desirable to develop a general in vitro insertion system that would allow direct integration of transposon DNA into viral genomes.

Bacteriophage Mu is one of the best-characterized mobile genetic elements and the first for which an in vitro DNA transposition reaction was established (44). The Mu-based in vitro reaction has since been considerably simplified, and the macromolecular components that are sufficient for efficient in vitro DNA transposition into intermolecular targets include the transposon DNA, the target DNA, and the MuA transposase protein (22, 31). The reaction does not exhibit stringent target specificity requirements (17, 30, 31, 32, 45), and consequently it enables the transfer of custom-designed transposons into essentially random positions along the target DNA. The in vitro reaction faithfully reproduces the accurate 5-bp target site duplication (30, 32) that is a hallmark of Mu transposition (1, 33). The system has proven effective in functional analysis of a DNA region (31), DNA sequence determination (17, 30), and generation of mouse gene-targeting constructs (60).

Bacteriophage PRD1 (family *Tectivirida*) is a broad-host-range virus that infects, among others, *Salmonella enterica* and *E. coli* (3, 46) and has striking structural and functional similarities to human adenoviruses (11). PRD1 exhibits distinctive features such as covalently linked terminal proteins at the 5′ ends of the virus genome and a membrane located inside the

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TABLE 1. Bacteria, phage, plasmids, and transposons used in this study

Strain, phage, plasmid or transposon	Genotype, phenotype, or description	Source or reference
Bacteria		
E. coli K-12		
DH5α	F$^-$ ϕ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17* (r_k^-, m_k^+) *supE44 thi-1 gyrA96 relA1 phoA*	Life Technologies
DK8[a]	F$^-$ Δ(*srl-recA*)*306 lacZya536*(*lacZ*(Am)) *rpsL*	37
S. enterica serovar Typhimurium LT2(pLM2)	PRD1 host	43
Phage PRD1	Wild type	46
Plasmids[b]		
pUC19	*bla lacZ′*	61
pSupF-Mu	*bla* SupF-Mu	31
RP1	*bla kan tet*	29
pLM2	*kan bla*(Am) *tet*(Am)	42
pLacZ′-Mu	*bla* LacZ′-Mu	This study
pLacZ′-Mu(NotI)	*bla* LacZ′-Mu(NotI)	This study
Transposons		
SupF-Mu	*supF*, Mu wild-type R ends	31
LacZ′-Mu	*lacZ′*, Mu wild-type R ends	This study
LacZ′-Mu(NotI)	*lacZ′, Not*I sites on transposon ends	This study

[a] DK8(pLM2) was used for detection of the *supF* supression phenotype.
[b] Broad-host-range plasmids RP1 and pLM2 encode the receptor for PRD1.

viral capsid (for a review, see reference 4). The linear, 14,927-bp double-stranded PRD1 DNA genome has been sequenced (10; see GenBank accession no. M69077 and reference 9 for a revised edition), and its near total lack of useful restriction sites makes modification of this genome problematic. Additionally, the terminal proteins, essential for initiation of phage replication (5, 6, 55), effectively prohibit the cloning of the infective phage genome into a standard cloning vector (plasmid, PAC, or BAC). However, in principle, PRD1 is suitable for transposition-based functional analysis because electroporation of the virus genome into a host cell is sufficient to initiate productive infection (40). Furthermore, the organization of the PRD1 genome at the transcriptional level has been established (26), providing comparative data for functional analysis.

We utilized in vitro DNA transposition technology with custom-designed mini-Mu transposons to generate a number of bacteriophage PRD1 mutants that collectively not only identified several genomic regions that are tolerant of transposon insertions but also revealed genes or putative genes (represented by open reading frames, ORFs) that are unnecessary for virus propagation. New information about several PRD1-encoded proteins was also gained through evaluation of C-terminal deletions caused by transposon insertions. In addition, promoterless reporter transposons not only defined the direction and activity of transcription in various genomic locations but also indicated the apparent lack of transcriptional activity in certain genome regions. Furthermore, we used end-modified transposons to introduce new restriction sites that will enable easy modification of the phage genome in the future. The strategy we describe is of a general nature and thus is applicable to a variety of other prokaryotic and eukaryotic cell viruses.

MATERIALS AND METHODS

Proteins, reagents, DNA techniques, and primers. Mu transposase (MuA), proteinase K, Dynazyme II DNA polymerase (used for PCR), and calf intestinal alkaline phospatase were obtained from Finnzymes, Espoo, Finland. T4 polynucleotide kinase and restriction endonucleases were from New England Biolabs. Bovine serum albumin and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Sigma; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was from Invitrogen; and Triton X-100 was from Roche. [γ-^{32}P]ATP (3,000 Ci/mmol) and [α-^{32}P]dCTP (600 Ci/mmol) were from Amersham. Standard DNA techniques were performed as described previously (52). Enzymes were used as recommended by the suppliers. Plasmid DNA was prepared using plasmid purification kits from Qiagen.

Primers used were HSP19 (5′-CCGCTGTAAAGTGTTACGTTG), HSP20 (5′-CGAAAGACCGCGGTCCAGCTG), HSP69 (5′-GAGAGATCTGCGGCC GCGCACGAAAAACGCGAAAGC), HSP98 (5′-GGCAGATCTATGCGG CATCAGAGC), HSP99 (5′-AGCAGATCTCAATTTCACACAGGAAAC), HSP210 (5′-CAGGCATGCAAGCTTGGCGT), and HSP211 (5′-ATTTCAC ACCGCATATGGTGCACT).

Bacterial strains, phages, plasmids, and transposons. The bacteria, phages, plasmids, and mini-Mu transposons used in this study are listed in Table 1. The bacteria were grown at 37°C in Luria-Bertani (LB) broth or on LB agar plates (52). Phage PRD1 was grown on *S. enterica* serovar Typhimurium LT2(pLM2). For the selection and maintenance of plasmids, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 10 μg/ml; tetracycline, 12.5 μg/ml; and chloramphenicol, 5 μg/ml. The SupF-Mu transposon and its respective pUC19-derived carrier plasmid (called pSupF-Mu below) have been described previously (31). The LacZ′-Mu transposon-carrying plasmid was generated by initially amplifying a *lacZ′*-containing PCR fragment with the primer pair HSP98 and HSP99, using pUC19 as a template. The fragment was digested with *Bgl*II and cloned into a *Bam*HI-digested pSupF-Mu vector backbone to generate plasmid pLacZ′-Mu. The LacZ′-Mu(NotI) transposon-carrying plasmid was generated by targeted mutagenesis. Initially, a PCR fragment was amplified with the single mutagenic primer HSP69 (hybridizing to each transposon end), using pLacZ′-Mu as a template. The fragment was digested with *Bgl*II and cloned into a *Bgl*II-digested pSupF-Mu vector backbone to generate plasmid pLacZ′-Mu(NotI). Linear transposons were isolated from their carrier plasmids by *Bgl*II digestion and purified by anion-exchange chromatography as described previously (31). When required, the transposon 5′ ends were dephosphorylated with calf intestinal alkaline phosphatase after which the DNA was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Labeled transposon

DNA was then purified by gel filtration through a Bio-Gel P30 column (Bio-Rad). For plaque hybridization, transposon probes were labeled with [α-^{32}P]dCTP by using random hexanucleotide primers (Random Primed; Roche). Probes were purified on a Nick G50 column (Pharmacia).

Phage purification and isolation of infectious genome. For isolation of infectious genome, phage PRD1 was grown on *S. enterica* serovar Typhimurium LT2(pLM2), purified in sucrose gradients as described previously (7), and resuspended in 20 mM Tris (pH 7.4). Native genome (i.e., phage DNA with covalently 5′-linked proteins) was isolated from the purified phage particles without pronase treatment as described previously (41). Isolated DNA was resuspended in water and stored at −20°C. The microcentrifuge tubes used for ethanol precipitation were treated with 2% dimethyldichlorosilane in 1,1,1-trichloroethane (LK Bromma) to reduce the adherence of 5′-genome-linked proteins on tube walls. For analytical procedures, viruses were grown and genomes were isolated essentially as described previously (54), except that 100 μg of proteinase K per ml was used instead of pronase. The isolated phage DNA was further purified using columns provided in a QIAprep Spin Miniprep kit (Qiagen) as specified by the manufacturer.

In vitro transposition reaction and electroporation into host cells. The standard in vitro transposition reaction mixture (25 μl) contained 5.0 pmol of transposon donor, 0.2 pmol of PRD1 target DNA, 32.5 pmol (2.65 μg) of MuA transposase, 25 mM Tris-HCl (pH 8.0), 100 μg of bovine serum albumin per ml, 15% (wt/vol) glycerol, 0.05% (wt/vol) Triton X-100, 0.12 M NaCl, and 10 mM $MgCl_2$. The reaction was carried out at 30°C for the times indicated. For electroporation, the transposition reaction was stopped by the addition of 25 μl of 1% sodium dodecyl sulfate and incubation at 25°C for 30 min, followed by dilution with 50 μl of water. Aliquots (1 μl) were electroporated into 40 μl of *E. coli* K-12 strain DH5α(RP1) electrocompetent cells that were prepared as described previously (2). Electroporation was carried out using 2-mm electrode spacing cuvettes and the Bio-Rad Genepulser II with the following settings: capacitance, 25 μF; voltage, 2.5 kV; and resistance, 200 Ω.

Screening and sequencing of insertion mutants. Mutant phage clones were identified by plaque hybridization on Hybond N+ membranes (Amersham) using α-^{32}P-labeled transposons as probes. Alternatively, *lacZ*′-containing mutants were detected by blue-white screening using 1.33 mM IPTG and 0.027% X-Gal in the LB soft-agar overlay on the plates. Transposon insertion sites were determined by sequencing with transposon-specific primers HSP19 and HSP20 for SupF-Mu and HSP210 and HSP211 for *lacZ*′-containing transposons. DNA-sequencing reactions were performed using a BigDye terminator cycle-sequencing kit (Applied Biosystems), and the products were analyzed on an ABI 377 XL automated sequencer (Applied Biosystems). Sequences were matched to the PRD1 sequence by using Gap4 from the Staden Package (58).

RESULTS

Experimental outline. We designed artificial mini-Mu transposons that were initially integrated into the phage PRD1 genome by in vitro DNA transposition (Fig. 1). The insertionally mutagenized genomes were then electroporated into *E. coli* cells, and the resultant mutant phages were identified by plaque hybridization using radioactive transposon probes or by blue-white screening based on α-complementation of truncated *lacZ* gene products of *E. coli*. DNA was isolated from mutant phages, and the transposon insertion sites were determined initially by restriction analysis and subsequently by sequencing the exact site. Finally, the results were compiled as functional maps of the PRD1 genome.

Artificial transposons. The mini-Mu transposons used in this study (Fig. 2A) contained a 50-bp segment of wild-type or slightly modified (containing a *Not*I site) Mu R-end DNA including the critical MuA binding sites R1 and R2 (56) as an inverted repeat within each end (Fig. 2B). The transposon SupF-Mu contained the *supF* amber suppressor gene of *E. coli* between the Mu ends. Similarly, a *lacZ*′ gene segment coding for the α-fragment of *E. coli* β-galactosidase was included in the transposons LacZ′-Mu and LacZ′-Mu(NotI). The transposons were used in linear precut form, a configuration that ensures efficient in vitro assembly of stable transpososomes (22, 31, 56).

Transposition reaction. In the standard in vitro transposition reaction (31), mini-Mu transposons are integrated essentially randomly into plasmid targets, generating a gapped transposition intermediate that can be accurately repaired by bacterial host machinery on introduction into compatible cells (e.g., *E. coli*). Analogously, initial mini-Mu transposon integrations into nonessential regions of the PRD1 dsDNA genome in vitro are expected to yield a viable virus progeny via repair of the transposition intermediate following electroporation into the host cells. We began to investigate this possibility by first analyzing products of the in vitro transposition reaction with radioactively labeled supF-Mu transposon as the donor DNA and phage PRD1 native genome as the target. Restriction analysis and subsequent agarose gel electrophoresis/autoradiography of the reaction products indicated that transposons readily integrated into the virus genome with no bias toward any particular genomic loci (data not shown). The results were consistent with previous target site selection data obtained both qualitatively and quantitatively for Mu in vitro transposition (17, 30, 31, 32), demonstrating essentially random integration into a longer segment of DNA.

Native PRD1 genomes can be readily introduced into *E. coli* host cells by electroporation, where they initiate productive virus propagation (40). On the other hand, transposon insertion into most regions of the PRD1 genome is expected to yield nonviable viruses. Accordingly, we set up a dual assay system to indirectly detect transposon insertions into the PRD1 genome (Fig. 3). The disappearance of transposon DNA over time, an indication of transposon integration, was monitored by agarose gel electrophoresis (Fig. 3A), and electroporation of reaction products into *E. coli* cells detected viable viruses that survived the transposition reaction (Fig. 3B). The effects of transposon end modification (*Not*I site included) were also studied in this experiment.

The gel analysis (Fig. 3A) revealed that most of the transposon DNA disappeared within 80 min with either LacZ′-Mu or LacZ′-Mu(NotI) transposon substrates, indicating efficient integration of transposon DNA into the PRD1 genome. While the latter reaction progressed somewhat more slowly, most of the transposon DNA was eventually consumed in the reaction. For both substrates, the electroporation analysis (Fig. 3B) indicated a gradual decrease over time in virus plaque-forming capacity which eventually fell ~100-fold by the final time point (80 min). Also in this assay, LacZ′-Mu(NotI) exhibited slower reaction kinetics compared with LacZ′-Mu, consistent with the gel assay results. Collectively, these results indicated that at least a fraction of the in vitro-integrated transposons inactivated viruses by insertion into essential gene regions. Nevertheless, it was expected that some of the transposons would be inserted into nonessential regions. We identified such phage clones by plaque hybridization using transposon DNA as a probe (Fig. 1; Table 2). For this analysis, we used samples from the 15-min time point of the previous experiment because, at that time point, virus viable counts had decreased to 10% of the original level, yet electroporation still yielded a reasonable number of plaques for screening purposes (Fig. 3B). LacZ′-Mu and LacZ′-Mu(NotI) generated clones that hybridized to the transposon DNA at a rate of 10 and 4%, respectively. These

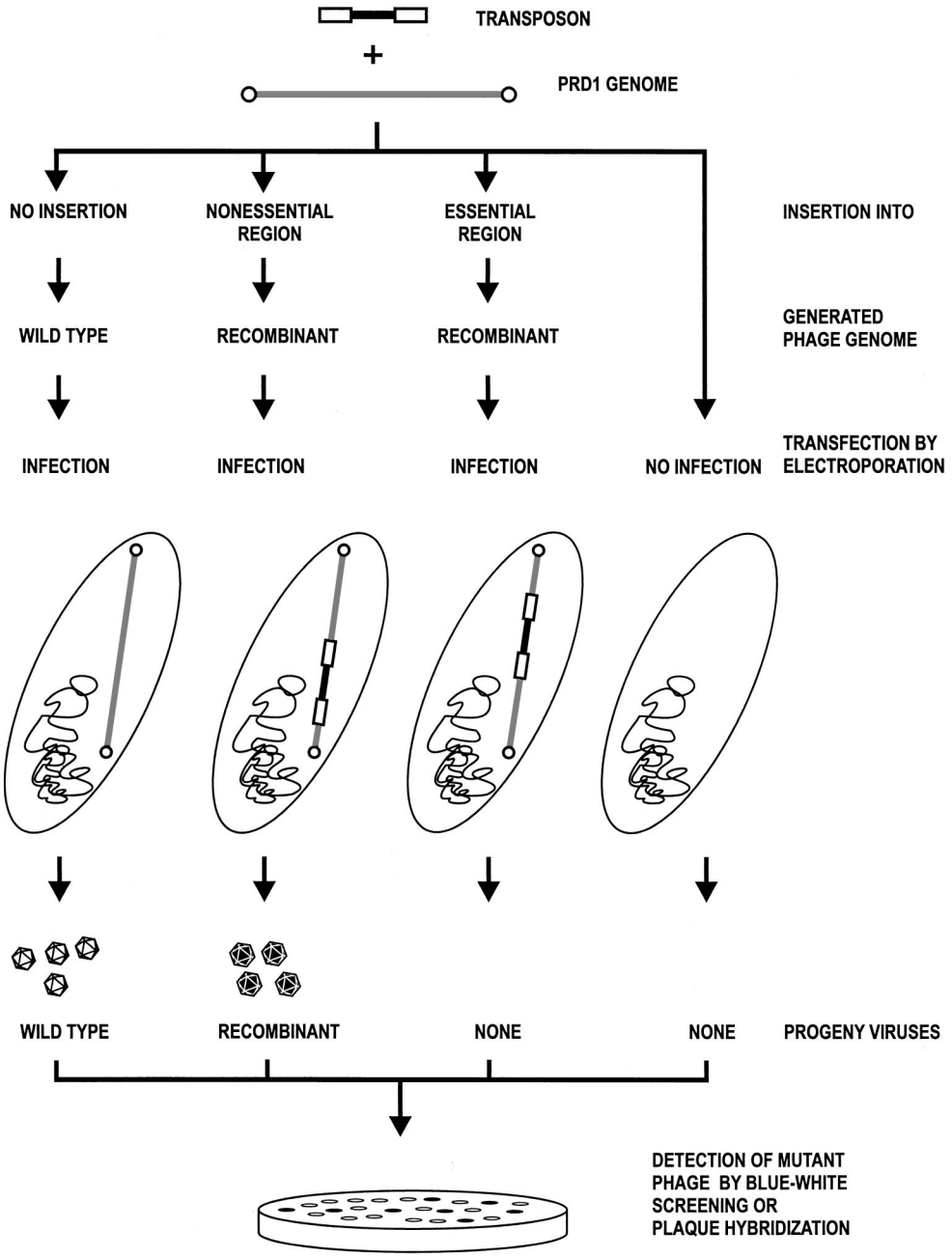

FIG. 1. Overview of the insertion mutagenesis strategy used (see Results for details). In vitro DNA transposition reaction with custom-designed mini-Mu transposon as a donor DNA and bacteriophage PRD1 native genome as a target DNA generates recombinant PRD1 viruses as indicated. Recombinant clones are detected among wild-type clones by using blue-white screening or plaque hybridization. The rectangles in the transposon DNA ends indicate 50 bp of Mu R-end DNA (56). The circles in the PRD1 genome ends indicate covalently attached terminal proteins (5, 6).

results indicated that transposon DNA could be integrated into regions of the PRD1 genome that were not important for virus propagation, thus resulting in viable virus progeny. Similar results were obtained when SupF-Mu transposon was used in the experiments (data not shown).

The assay system we utilized also allowed the detection of α-complementation of β-galactosidase on indicator plates (blue-white screening [see Materials and Methods]). Accordingly, promoterless *lacZ′*-containing transposons yielded blue plaques if integrated into the genome in the proper orientation under control of an operational phage promoter. Around 30 to 40% of all the insertion mutant clones exhibited β-galactosidase activity, manifested as blue plaques (Table 2). These results indicated that a sizeable fraction of the insertionally mutagenized viruses expressed the additional *lacZ′* gene residing within the transposon.

Verification of integration by restriction analysis. A transposon integration into the PRD1 genome increases the ge-

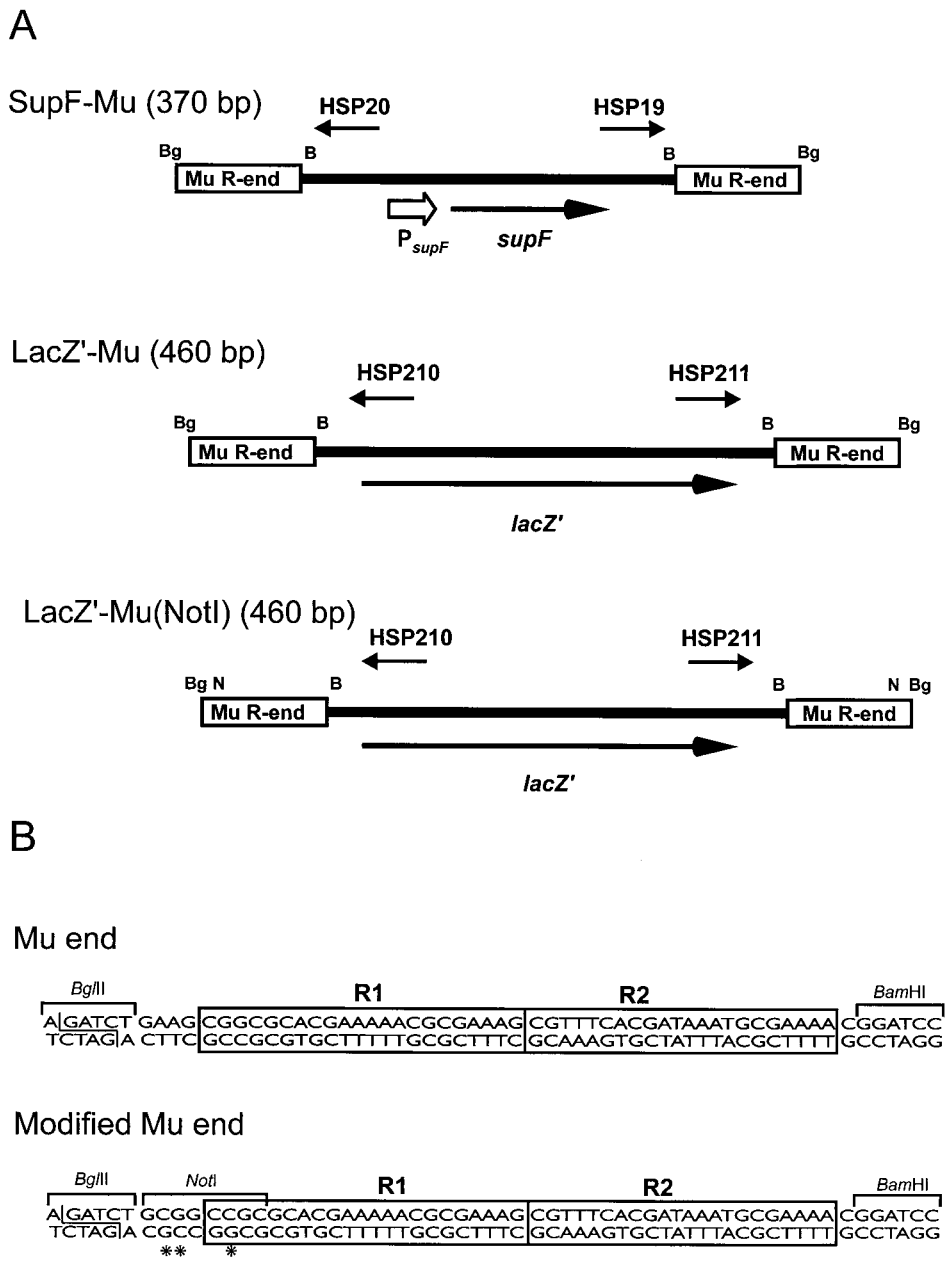

FIG. 2. (A) Mini-Mu transposons. Linear mini-Mu transposons used in this study are defined as segments of DNA that contain 50 bp of Mu R-end DNA as inverted repeats at each end (31, 56). Long arrows indicate marker genes (*supF* and *lacZ'*). P_{supF} denotes the native *E. coli* promoter driving *supF* expression. Short arrows indicate the binding sites of the primers used for DNA sequencing. B, Bg, and N indicate the restriction sites *Bam*HI, *Bgl*II, and *Not*I, respectively. (B) Wild-type and modified (containing a *Not*I site) Mu ends. The *Bgl*II cleavages used for isolation of the transposons from their carrier plasmids (31) are indicated by kinked lines. R1 and R2 (boxed sequences) denote MuA binding sites (56). Asterisks indicate the end modifications that generated the *Not*I restriction sites.

nome size by the unit length of that transposon. Thus, the region into which a transposon becomes inserted in each clone can be detected by genome restriction analysis and a mobility shift of a particular restriction fragment. Therefore we plaque purified a number of insert-containing phage clones by two rounds of successive plaque isolation. Genomic DNA from each clone was then subjected to *Bgl*I restriction analysis (Fig. 4). As expected, each purified clone contained stably integrated DNA, and most of the clones exhibited restriction enzyme digestion patterns consistent with a single transposon insertion: 90 of 101 integrations of SupF-Mu, 12 of 16 integrations of LacZ′-Mu, and 43 of 51 integrations of LacZ′-Mu-(NotI). The remainder of the clones exhibited somewhat more complex restriction patterns, indicating additional genome rearrangements, but these clones were not studied further (see Discussion).

Determination of mini-Mu transposon integration sites. Transposon integration sites within the PRD1 genome would be expected to reveal nonessential genes or genomic regions (Fig. 5). We therefore used DNA sequencing to determine the

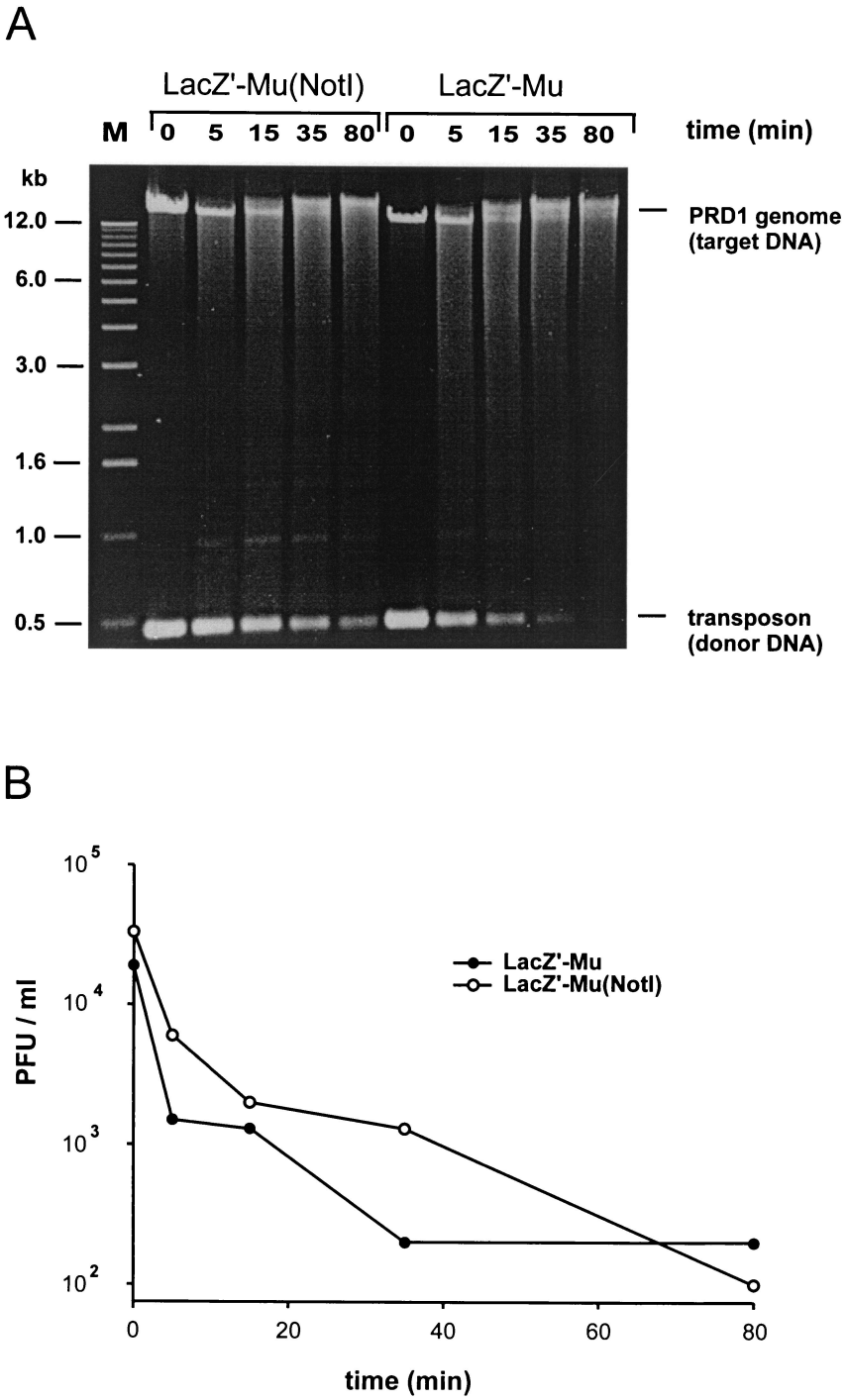

FIG. 3. Transposition reaction kinetics. The *lacZ′*-containing transposons (indicated at the top) were used as the donor in a transposition reaction with phage PRD1 genome as the target. Reaction products were analyzed by agarose gel electrophoresis and by electroporation into *E. coli* DH5α(RP1) cells. (A) Agarose gel electrophoresis. Disappearance of the transposon DNA fragment over time is indicative of transposon DNA integration (31). (B) Electroporation. PFU indicate phage survival over the course of the transposition reaction.

exact integration sites for 85 SupF-Mu (Fig. 6) and 45 LacZ′-Mu or LacZ′-Mu(NotI) (Fig. 7) transposons. All these clones contained a single transposon, consistent with the above restriction analysis, and each transposon was flanked by a 5-bp target site duplication, thus confirming that integrations were generated by DNA transposition chemistry.

In general, integration sites were not distributed evenly along the genome sequence, most probably reflecting an uneven distribution of essential and nonessential genomic regions. In particular, the ends of the genome contained sizeable insertion-tolerant zones, as indicated by multiple transposon insertions. However, only a few integrated transposons were

TABLE 2. Generation of insertion mutants[a]

Transposon	Total no. of plaques	Total no. of mutants	Proportion of insertion mutants (%)	No. of:		Proportion of blue plaques (%) for:	
				Colorless insertion mutant plaques	Blue insertion mutant plaques	Total plaques	Mutant plaques
LacZ′-Mu	897	91	10	54	37	4	40
LacZ′-Mu(NotI)	931	41	4.4	28	13	14	31

[a] Plaques were analyzed following electroporation (15-min reaction time).

detected in the central genome region. These data are in accordance with the location and tight packaging of the known essential genes in the PRD1 genome (26).

The terminal genome regions flanking the region bordered by genes *VIII* and *XII* sustained a number of insertions, consistent with the noncoding nature of these sequences (10, 26). Several of these insertions occurred in sequences comprising the 110-bp inverted terminal repeats (53). Some transposons were detected in intergenic regions, including an insertion between genes *XI* and *XVI* as well as several insertions between genes *XIX* and *XII*. Although most of the known genes and candidate genes (ORFs) were intolerant of insertions, a number of insertions were found within known and putative coding regions. Some of the ORFs (u, v, and gene *XIX*) appeared to tolerate insertions remarkably well, with confirmed integration sites spanning the entire ORF. Some ORFs, most notably genes *VIII*, *XII*, and *XVII*, as well as ORF t, sustained insertions only in distal regions that code for the C- termini of the encoded proteins (or putative proteins).

Blue-white screening for the insertion of *lacZ′*-containing transposons enables an analysis of gene expression and the direction of transcription in different genomic regions. Each of the LacZ′-Mu and LacZ′-Mu(NotI) transposons that generated blue virus plaques was inserted in an orientation that enabled *lacZ′* expression from the known upstream promoters, indicating that the reporter transposon analysis system that was utilized was efficient in locating regions of active gene expression. Most of the colorless plaques were generated in either of two ways, by the insertion of transposons in an opposite relative orientation with respect to the known direction of transcription in a given genomic region or by insertions into the extreme right end of the genome upstream of the first leftward-reading promoter P'_{E130}. In four cases, colorless plaques were generated by a transposon insertion in the correct relative orientation in relation to the supposed direction of local transcription (three in ORF u, and one in ORF q [see Discussion]).

DISCUSSION

Our work demonstrates an efficient strategy for the modification and functional characterization of the complete genome of bacterial virus PRD1. The method relies on direct in vitro

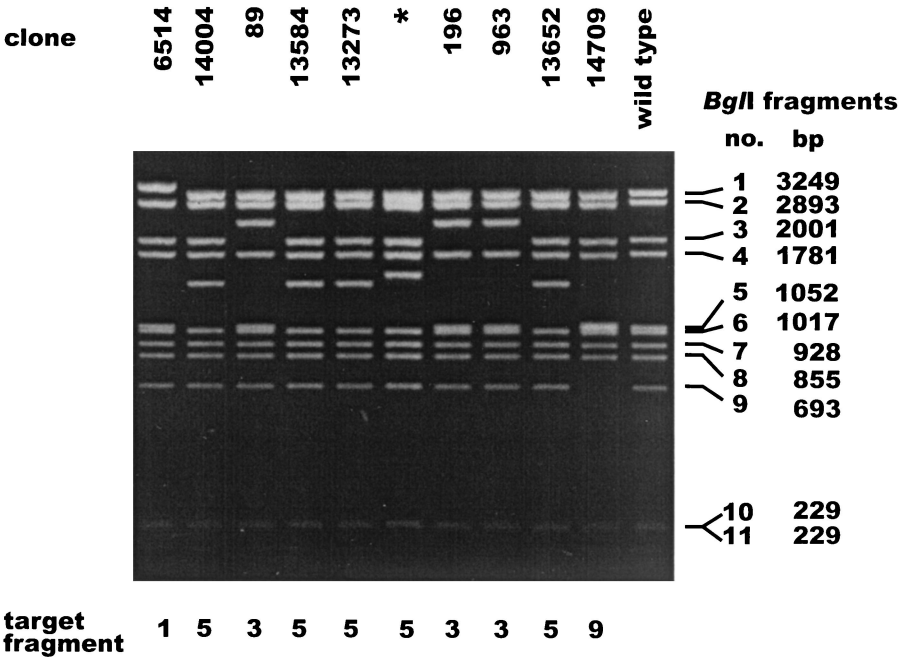

FIG. 4. A representative assortment of phage clone genomes (mutagenized with SupF-Mu) analyzed by *Bgl*I restriction digestion and agarose gel electrophoresis. Integration of a single SupF-Mu into a given restriction fragment adds 371 bp to the size of the fragment. The clone indicated by an asterisk represents a more complex genome rearrangement (see Discussion). The clone numbering scheme is indicated in Fig. 6.

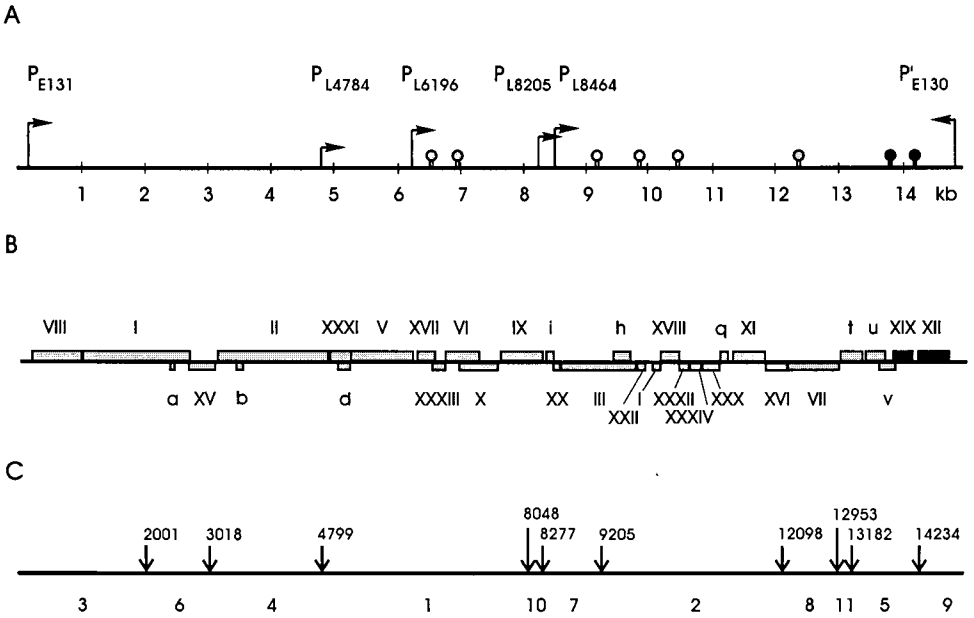

FIG. 5. Functional organization of the PRD1 genome (26). The figure is slightly modified to accommodate updated information (9; P. Rydman, J. Bamford, and D. Bamford, unpublished data). (A) Promoters (arrows) and terminators (stalked balls). (B) Known genes (roman numerals) and ORFs (lowercase letters). (C) *Bgl*I restriction sites (arrows) and their coordinates. The numbering (1 to 11) refers to the restriction fragments generated (see Fig. 4).

integration of mini-Mu transposon DNA into the target viral genomic DNA and does not require prior cloning of the genome. In principle, the strategy may be applied directly to any viral double-stranded DNA (dsDNA) genome, linear or circular, provided that means are available for introduction of the genome into host cells for productive infection. Cloned versions of infectious virus genomes (e.g., within plasmids, PACs, or BACs) can also be used as targets, effectively extending the scope of the method to single-stranded (ssDNA) and RNA viruses. The strategy has now been applied successfully also for direct insertional mutagenesis of the linear dsDNA genome of the T3-type *Yersinia enterocolitica* phage ΦYeO3-12 (S. Kiljunen, H. Vilen, H. Savilahti, and M. Skurnik, unpublished data); and an alternate version of the strategy, involving the transposon ends only and generating short (15-bp) insertions, has been used to modify the RNA genomes of human immunodeficiency virus (39) and *Potato virus A* (35).

The two different marker genes used in our studies, *supF* and *lacZ′*, were chosen because of their small size and potential usefulness in gene expression analysis. Our initial goal with respect to the *supF* marker gene was to suppress a chromosomal *E. coli lacZ* amber mutation residing in the *E. coli* genome, thereby allowing blue-white screening for the identification of transposon insertions. While a control experiment (involving the *supF* gene in an introduced plasmid) indicated a suppression phenotype for the *lacZ*(Am) mutation strain (blue bacterial colony), we were unable to detect the suppression phenotype (blue plaques) with any of the SupF-Mu insertion mutant viruses (data not shown). The basis of this observation is unclear but may involve the shutdown of host gene expression by phage during infection or inadequate timing in the expression and maturation of the critical components required for gene expression. Nonetheless, the 85 SupF-Mu transposon insertions that were mapped provided an informative distribution pattern regarding the functional organization of the PRD1 genome. Another set of 45 insertion sites was mapped with the two *lacZ′*-containing transposons. In this case, additional functional data were provided by the transposon-encoded *lacZ′* marker gene, which was expressed by many of the virus clones as revealed by blue color formation on indicator plates. The intensity of the color varied among the virus clones, evidently reflecting differences in gene expression levels, but this aspect was not studied further.

Transposon ends were modified to introduce new *Not*I restriction sites into the PRD1 genome. Because each clone was unique with respect to the site of this sequence modification, a collection of such mutant genomes is expected to be a valuable resource for further manipulation of the PRD1 genome by conventional restriction and ligation-based techniques (8, 54). Relative to wild-type MuR ends, the transposons containing *Not*I-modified ends were somewhat less efficient in the assembly of functional transposition complexes. However, under the defined reaction conditions used in this study, this deficiency was not prohibitive and could be overcome by extending the reaction time (Fig. 3).

The in vitro mini-Mu transposition-based strategy proved to be a quick and effective way of producing phage mutants, and most (86%) of the insertionally mutagenized genomes contained a single inserted transposon. The remainder of the clones exhibited more complex genome rearrangements, largely consistent with scenarios involving initial insertion by two transposons and recombination between the homologous regions of those transposons. This supposition is in accordance with the expectation that, statistically, a fraction of the ge-

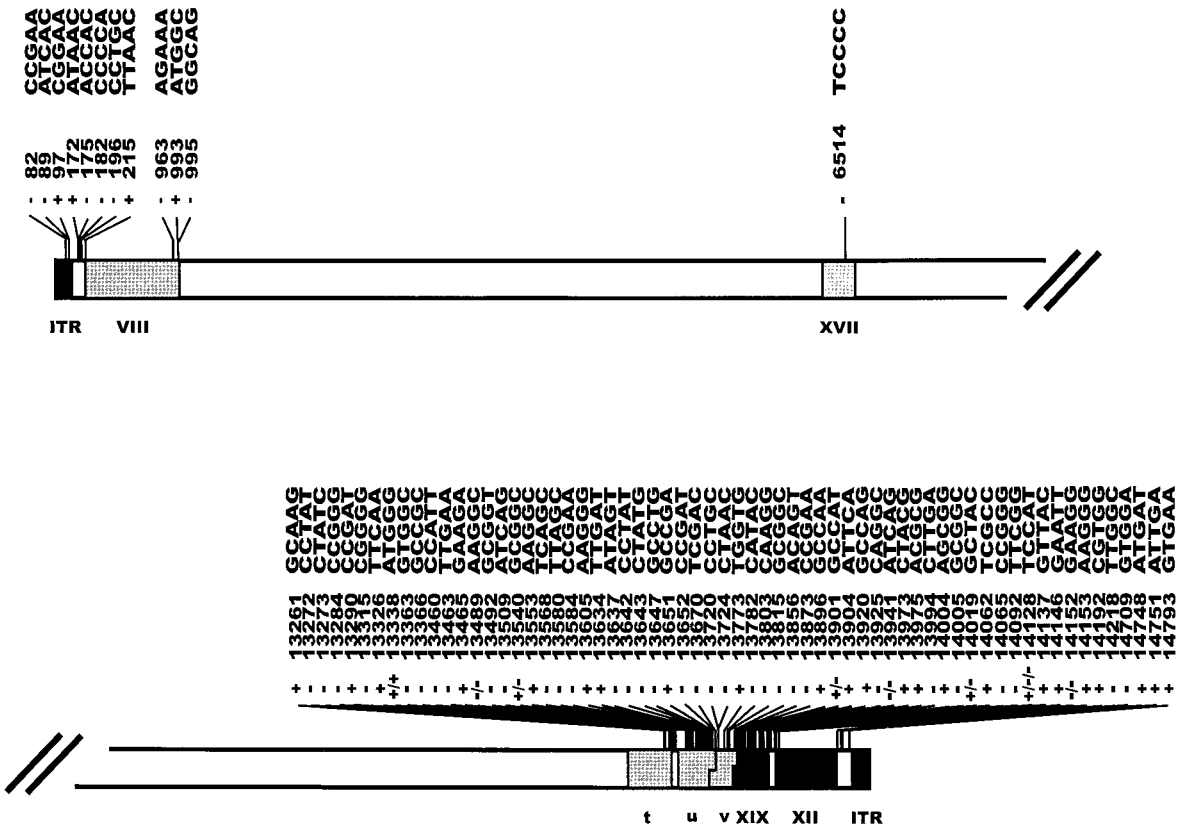

FIG. 6. SupF-Mu integrations into the PRD1 genome. The numbers indicate the integration sites and refer to the first base pair of the 5-bp duplicated sequence (shown in each case after the corresponding number). Note that the mutant sequence differs from the wild-type sequence only after the duplicated sequence (i.e., 5 bp after the number). The orientation of the inserted transposon is indicated by + (orientation of the marker gene from left to right compared to the genome sequence) or − (opposite direction).

nomes should contain two or more transposons following the transposition reaction. Importantly, only a few clone types appeared multiple times in our mutant collection (Fig. 6 and 7), indicating that the original distribution of insertion sites was relatively random, a result consistent with our earlier data on Mu target site selection (30, 31, 32).

The present study reveals new aspects of PRD1 biology and confirms previous data on PRD1 genome organization (9, 10, 26). Our data establish the feasibility of integrating a Mu-derived artificial transposon into the PRD1 genome with no evident effects on the phage life cycle. First, the results demonstrate that within the context of the PRD1 genome, the gapped and partially single-stranded transposition DNA intermediate can be accurately repaired within host cells following electroporation. However, the intriguing question still exists of whether the repair machinery is host or phage encoded or possibly a combination of the two. Second, the results indicate that at least ~460 bp of additional DNA can be introduced into the PRD1 genome without affecting the phage viability, indicating that the available space in the wild-type phage particle is not fully occupied by the wild-type genome. Similar results were obtained by Bamford and Bamford (8), who managed to insert an extra 393 bp of *lacZ* DNA into two selected loci within the PRD1 genome by using a restriction-and-ligation strategy or by using in vivo homologous recombination between an engineered plasmid and the phage genome.

In principle, transposon insertions can directly inactivate viruses by introducing a stop codon into the ORF of an essential gene. Similarly, virus propagation may be suppressed or abrogated if the control of genome replication or phage gene expression is critically disturbed. Thus, a compilation of transposon integration sites will indicate nonessential regions in the viral genome. In total, 113 (87%) of 130 integration sites were mapped near the right end of the PRD1 genome between map coordinates 13261 and 14838. In 11 cases the integration site was in the far left end of the genome, between coordinates 82 and 215, and only 6 integration sites were detected between these two regions. These results are logical, given that the gene density within the PRD1 genome is high, with only very short noncoding regions between genes (10, 26), and thus many of the insertions may inactivate genes directly. However, another explanation, involving critical effects on transcription levels, is also compatible with these results. In this regard, the promoter-containing SupF-Mu transposon may be more destructive to the genome than are the promoterless *lacZ′* transposons.

Integration sites closest to the genome ends left the extreme 86 bp (left) and 94 bp (right) unaltered. Evidently, sequences that are important for viral DNA replication in these regions (i.e., bp 1 to 20 [62]) do not tolerate insertions. The functions of the region encompassing the approximate coordinates of bp 20 to 80 in the left inverted terminal repeat (ITR) and its equivalent in the right end are not clear. However, a sequence

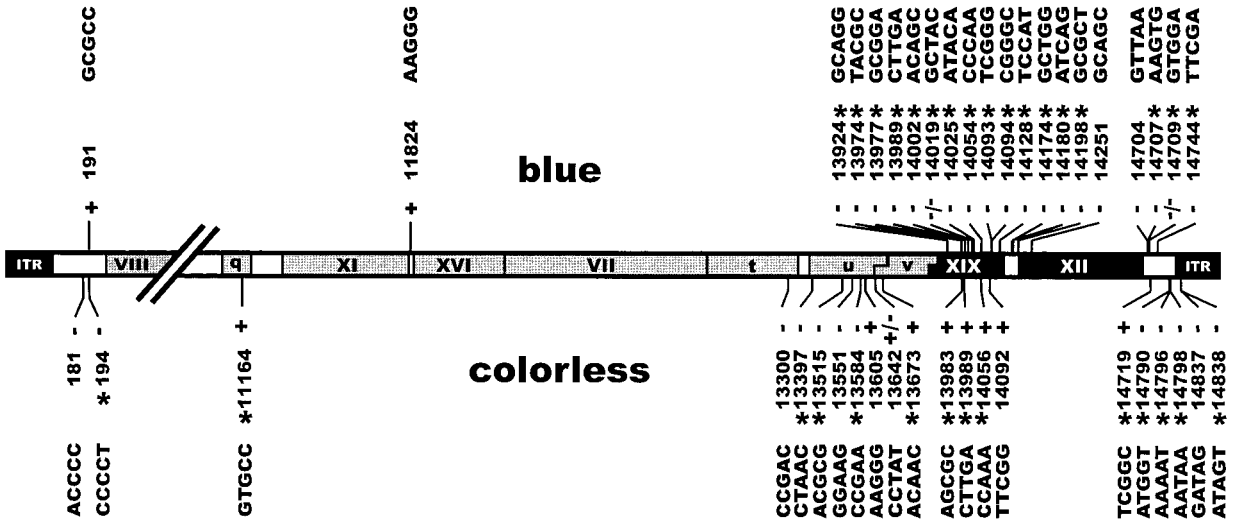

FIG. 7. LacZ′-Mu and LacZ′-Mu(NotI) integrations into the PRD1 genome. Transposon integration sites in phage clones that generated blue and colorless plaques in the blue-white screen are indicated above and below the depicted genome, respectively. Numbering of the integration sites and orientation of the transposons are as in Fig. 6. The LacZ′-Mu(NotI) integration sites are labeled with an asterisk to distinguish them from those of LacZ′-Mu.

comparison between five closely related PRD1-type viruses (53) suggests the existence of a system that maintains the identical nature of the genome ends, and this system may be operational within or close to this region. In addition, experimental evidence for the existence of this putative genome end-monitoring system has been obtained through analysis of chimeric PRD1-type viruses (54). Accordingly, insertions into this ITR region would have generated virus clones with unstable genomes that would probably not have been included in our mutant collection. The stable integrations within the ITR region encompassing the approximate coordinates of bp 80 to 110 establish that 110-bp ITRs are not necessary for PRD1 viability and that an 86-bp ITR is sufficient.

According to our analysis, the known PRD1 genes and ORFs can be divided into three classes: (i) those that do not tolerate transposon insertions, (ii) those that tolerate insertions but apparently only in restricted locations, and (iii) those that tolerate transposon insertions throughout their entire length.

The insertion-nontolerant class of genes and ORFs contains most of the known PRD1 genes (Fig. 5), including those encoding the phage DNA polymerase (gene *I* [43, 55]), major structural capsid protein (gene *III* [7, 16, 43]), proteins forming the vertex complex (genes *II*, *V*, and *XXXI* [8, 19, 27, 43, 51]), and many of the proteins associated with the viral membrane (9). While expression level disturbances cannot be ruled out as an explanation for the transposon intolerance of these genes, the most likely scenario is direct interference of essential function by introduction of a stop codon into the protein-coding region.

The class of genes that tolerates some transposon insertions, but not along the entire reading frame, includes genes *VIII* (genome terminal protein [5, 6, 43]), *XII* (ssDNA binding protein [43, 48]), and *XVII* (nonstructural assembly factor [18, 43]). While the products of these phage genes appear to be essential for phage plaque-forming capability, the domains that tolerated C-terminal deletions caused by transposon insertions are not. In all of these cases, a truncated but functional gene product was apparently expressed (Fig. 8). ORF t also appears to belong to this class since insertions were detected only in its distal region, hinting that it is an authentic protein-expressing gene. The existence and function of its gene product have been elusive, although very recent findings indicate that the gene product is indeed expressed and involved in host cell lysis (P. Rydman and D. Bamford, unpublished data). ORF q acquired only one transposon insertion close to the distal end of the reading frame, and thus it is not possible to offer a reasonable prediction about its expression and requirement for phage viability.

In the third class, gene *XIX* (ssDNA binding protein [43, 47]) tolerated transposons throughout its coding region, consistent with its nonessential nature. Also, ORFs u and v acquired transposons throughout their coding regions. Hence, their putative gene products, if expressed, are nonessential for phage viability. Three of the *lacZ′*-containing transposons located within ORF u were properly oriented for *lacZ′* expression (transcription from left to right). However, as indicated by the presence of colorless plaques, they failed to produce detectable levels of the *lacZ′* gene product. Therefore, the expression of ORF u and the downstream ORF v is very weak at best but most likely nonexistent.

We did not detect gene expression activity from the transposon residing in ORF q, although in principle the direction of local transcription should have allowed detection. Furthermore, expression levels of the reporter within the transposon should have been detectable since a downstream insertion of the *lacZ′* gene (between genes *XI* and *XVI*) generated a blue plaque. A possible explanation for the lack of color formation in this case is that the 5′ end of LacZ′-Mu formed a recombinant joint with the target region around bp 11164, thus producing an mRNA with an unfavorable secondary structure that prevented *lacZ′* translation. This possibility is reinforced by the

gene VIII (780 bp, 259 aa)

wt …AAKKKYKRRQKRGYGSKGV*
995 …AAKKKYKRRQKRGYGS**EAAHEKRESVSR***
993 …AAKKKYKRRQKRGYG*
963 …AAKKK*

gene XVII (261 bp, 86 aa)

wt …DISPAEKPDNQPELTGITFEGDNNDQ*
6514 …DISP**EAAHEKRESVSR***

ORF q (111 bp, 36 aa)

wt …ILCQWFKRISNGA*
11164 …ILC**LRPRTKNAKAFHDKCENGSQFHTGNSYDHDYAKLA CLQVDSRGSPGTELEFTGRRFTTS***

ORF t (351 bp, 117 aa)

wt …VQSKIAPINQPGPIDSDNDKPGRTFND*
13326 …VQSKIAPINQPGPIDSDNDKPGRTFN**EAAHEKRESVSR***
13315 …VQSKIAPINQPGPIDSDNDKPG*
13300 …VQSKIAPINQPGPIDSD*
13290 …VQSKIAPINQPGPI**EAAHEKRESVSR***
13284 …VQSKIAPINQPG**EAAHEKRESVSR***
13273 …VQSKIAPI*
13272 …VQSKIAPI**EAAHEKRESVSR***
13261 …VQSK*

gene XII (483 bp, 160 aa)

wt …AAAEAKPAAKAKAKAEA*
14218 …AAAEAKPAAKAKA**NEAAHEKRESVSR***
14251 …AAAEA**AHEKRESVSR***

FIG. 8. Predicted translation products of C termini from selected genes and ORFs after transposon insertion. In each case, the wild-type (wt) sequence is shown above the mutant sequence(s). Asterisks indicate translation stop signals. Boldface letters denote amino acid sequence not encoded by the wild-type sequence. aa, amino acids.

fact that a stable RNA hairpin can indeed be drawn from the prospective RNA produced from such a joint sequence (not shown).

Conclusions. We described the use of several mini-Mu transposons for the facile discrimination of essential and nonessential regions and genes in the PRD1 genome. In addition, our analysis yielded critical new information about PRD1-encoded proteins and, in several cases, indicated to what extent C-terminal deletions of essential proteins could be tolerated. Furthermore, the expression analysis allowed an assessment of PRD1 genome functional organization and verified sequence-based predictions (10) and functional analyses (26) regarding the direction of transcription in many gene regions. In some regions the analysis yielded new information concerning gene expression, thus indicating that the system may be used to probe active gene expression of uncharacterized viral genomes. In general, the system should also be useful for generating new restriction sites within virus genomes and determining the extent to which additional DNA can be packaged in virus particles. The Mu in vitro DNA transposition-based strategy combines an essentially random transposon integration profile with a biological selection scheme that allows straightforward identification of potential genome modification sites. Such information is extremely valuable when virus genomes are modified for vector purposes.

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