Site-Directed Mutagenesis of Large Plasmids


ABSTRACT

A protocol combining recombination PCR and long-distance PCR is demonstrated to be highly accurate and rapid for site-directed mutagenesis of large (>10 kb) plasmids. Application of this protocol to the generation of mutant rabies virus glycoproteins expressed by the baculovirus/insect cell system illustrates the usefulness of this approach in facilitating structure-function relationships in this important eukaryotic expression system.

INTRODUCTION

Site-directed mutagenesis (SDM) of DNA is now commonly performed by methods using the polymerase chain reaction (PCR) technique. One popular and highly efficient procedure is recombination PCR (RPCR), which uses two mutagenic and nonmutagenic oligonucleotide pairs to generate two overlapping fragments of the entire plasmid under study. Recovery of the mutant clone is achieved after in vivo recombination between these PCR products (3,4). Although successful application of RPCR to mutagenesis of a plasmid of 7.1 kb was reported (8), in some circumstances, mutagenesis of inserts in larger plasmids (e.g., baculovirus transfer vectors >10 kb) would be desirable. However, this would require amplification of DNA target sequences of >5 kb and raises concerns over the introduction of spurious mutations due to the lack of proofreading activity associated with Taq DNA polymerase.

During our studies on the expression, in the baculovirus system of the glycoprotein (gp) of a wild-type (WT) rabies virus, we wished to examine the effect of altering particular structural motifs, especially N-glycosylation sites, of the protein. To facilitate production of several mutant versions of this protein, we investigated the feasibility of combining RPCR with the principles of long-distance PCR (1), in which efficient and accurate amplification is achieved using a mixture of thermostable DNA polymerases that combine high processivity and proofreading properties. This report investigates the feasibility of such an approach for SDM of large plasmids.

MATERIALS AND METHODS

Plasmid Construction

An expression cassette (RG) comprising the rabies gp open reading
frame downstream of a ribosome binding site and bounded by BamHI sites was constructed by reverse transcription (RT)-PCR of the G gene of street rabies virus [arctic fox strain (6); GenBank® Accession No. U11754]. This cassette was cloned into the BamHI site of baculovirus transfer vectors pAcYM1 and pAcMP3, which use the polyhedrin and basic protein promoters, respectively, to generate the plasmids pAcYM-RG and pAcMP-RG (see Figure 1).

Site-Directed Mutagenesis

Table 1 summarizes the primers used for clone characterization and mutagenesis at 3 sites within the G gene. Partially overlapping nonmutagenic PCR primers (BAC+/-) are directed to a site in the pUC portion of the transfer vector and generate PCR products with a 34-bp overlap. The mutagenic primer pairs were generally entirely complementary and generated PCR products overlapping by 28 bp (S3+/S3-), 34 bp (S1+/S1-, S1(a)+/S1- and S3(a)+/S3-) and 37 bp (S2+/S2-). For each trial, plasmid was digested separately with Scal for use with primer BAC+ and SstII (pAcMP-RG) or EagI (pAcYM-RG) for use with primer BAC-. Digests were terminated by heating (65°C for 10 min), and DNA was diluted to 25 ng/µL with 0.1× TE buffer (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). Using one mutagenic primer and one nonmutagenic primer, PCR was performed on each linearized plasmid by the Expand™ Long Template PCR System according to the supplier’s directions (Boehringer Mannheim Canada, Laval, QC, Canada). Reactions of 50 µL contained 50 mM Tris-HCl, pH 9.2, 16 mM ammonium sulphate, 1.75 mM MgCl₂, 0.35 mM each dNTP, 300 nM of each primer, 25 ng of plasmid template and 2.5 U of enzyme mixture (Taq and Pwo DNA polymerases). Thermal cycling was performed in a GeneAmp® PCR System 9600 (PE Applied Biosystems, Foster City, CA, USA) as follows: 2-min denaturation at 93°C, 10 cycles of 93°C for 10 s, 65°C for 30 s, 68°C for 4 min, followed by 15 cycles of the same profile with a 20-s increment in extension time/cycle. PCR product yield was confirmed by agarose gel electrophoresis before recovery by phenol-extraction and ethanol-precipitation. Final dried pellets were dissolved in 10 µL 0.1× TE buffer.

PCR products (1 µL) were transformed either together (for homologous

![Figure 1. Schematic of the two plasmids used for mutagenesis.](image-url)
recombination) or separately (for background evaluation) into *Escherichia coli* using MAX Efficiency DH5α™ Competent Cells according to the supplier (Life Technologies, Gaithersburg, MD, USA) with the following modifications. Only 50 µL of cells were used for each transformation and, after heat-treatment, 0.45 mL S.O.C. Medium (Life Technologies) was added. One half of the transformation solution was plated onto selective agar plates.

**Transformant Characterization**

Transformants were grown in selective media for mini-scale plasmid preparation using Wizard® Minipreps DNA Purification Systems (Promega, Madison, WI, USA). DNA sequencing was performed using fmol™ DNA Cycle Sequencing Systems (Promega) and 32P-labeled primers directed to the rabies G gene.

**Recombinant Baculovirus Generation and gp Expression**

All transfer vector constructs were used in homologous recombination, performed with BaculoGold® Linearized Baculovirus DNA (PharMingen, Mississauga, ONT, Canada), in Sf9 cells grown in TNM medium supplemented with 10% fetal bovine serum (FBS) by standard methods (7). Following a single round of plaque purification, DNA was prepared from small-scale baculovirus stocks as described previously (2), and recombinants were characterized by PCR (using primers BAC1-4) and sequencing.

For rabies gp expression, Hi-5 (*Trichoplusia ni*) cells, grown in Sf-900 II serum-free medium (SFM) (Life Technologies), were infected with recombinant baculovirus at a multiplicity of infection of 5. Tunicamycin (Sigma Chemical, St. Louis, MO, USA), where required, was added to growth medium at 5 µg/mL. Cells were harvested after 3 days and resuspended in disruption buffer [5.7 M urea, 2.8% sodium dodecyl sulfate (SDS), 1.8 M 2-mercaptoethanol]. These crude protein samples were subjected to electrophoresis through 10% polyacrylamide-SDS gels and transferred to nitrocellulose membrane. After washing in blocking buffer [5% skim milk powder, Tris-buffered saline (TBS), pH 8.0, 0.3% Tween® 20], the membrane was incubated with rabbit anti-rabies gp polyclonal antiserum for 2 h. Following three 5-min washes in TBS, the blot was incubated with goat anti-rabbit IgG alkaline phosphatase conjugate (Jackson Laboratories Bio/Can, Mississauga, ONT, Canada) for 2 h. After subsequent washing, protein bands were visualized by incubation with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate.
RESULTS

Mutations were directed to three sites within the RG cassette (see Figure 1): (i) S1, corresponding to amino acid (aa) 39 of mature gp, changes NXS to NXT and enhances N-glycosylation efficiency for the rabies ERA strain gp

(ii) S2 (aa 204/206), an additional N-linked glycosylation site, similar to that observed in other rabies strains, is incorporated; and (iii) S3 (aa 73), a ser to asn substitution, which does not alter N-glycosylation, is made. Table 2 summarizes the results of six separate trials in which the production of four distinct mutant clones was evaluated. For each trial, plasmid DNA was prepared from 12 or more transformants generated by homologous recombination and analyzed by BamHI digestion; all clones, with the exception of one from trial 1b, harbored a plasmid with restriction patterns consistent with the parental vector. Sequencing a fraction of these clones indicated that mutation rates approached 100% in most cases (Table 2).

In some trials, primers of different lengths were assessed for their mutagenesis efficiency. All primers generated the desired mutants with high efficiency except for the combination tested in experiment 4a. Replacement of the S3+ primer with S3(a)+ (having 6 additional bases at the 5′ end) in trial 4b apparently improved mutant recovery, perhaps by facilitating recombination due to the longer overlap (34 bp vs. 28 bp) between PCR products at the mutagenic site. The proofreading ability of the DNA polymerase mixture used will necessitate using primers retaining a certain minimal length 3′ terminal to the mutagenic base(s), thereby avoiding inadvertent repair of the mismatch; the shortest such sequence used here [9 bp in S1(a)+] was clearly sufficient for this purpose, and even shorter stretches might be successfully used.

To further evaluate sequence fidelity, a 250–300-base segment was sequenced for 48 mutant plasmids. In addition, the entire RG was sequenced for one clone of each of the four mutant genes generated. Of a total of 16 200 bases sequenced, only one inadvertent base change relative to the parental G gene sequence was found; a C to T substitution that would cause a conservative coding change (ala to val) close to S1. Plasmids pAcMP-RG and pAcYM-RG (WT G gene) and plasmids bearing the G gene mutants (pAcMP-RG-S1, pAcMP-RG-S2, pAcMP-RG-S1.2 and pAcYM-RG-S3) were used to generate recombinant baculoviruses (BAcMP-RG, BAcYM-RG etc). Rabies gp production by these recombinants was evaluated by Western blot (Figure 2). All cells infected with recombinant baculoviruses produced a protein of 58–65 kDa that cross-reacted with rabbit anti-rabies gp polyclonal anti-serum. This product was absent in control cells and cells infected with WT baculovirus. Treatment of infected cultures with tunicamycin, an inhibitor of N-linked glycosylation, reduced the size of all products to 58 kDa (data not shown).

DISCUSSION

This report details the feasibility of using principles of long-distance PCR coupled with RPCR for SDM of large plasmids. We have demonstrated the speed (similar to RPRC) and accuracy (<1 error per 16 000 bases) of this method by producing and characterizing four rabies G gene mutants. Expression of these mutated genes in the baculovirus/insect cell system yields proteins differing in size as predicted by the differential presence of N-glycosylation sites. The ability of tunicamycin to reduce all products to a common size supports the role of N-glycosylation in determining these size differences. This SDM method is attractive for any large plasmid, but given
the popularity and versatility of the insect/baculovirus protein expression system, the application described in this report is particularly relevant. By avoiding time-consuming subcloning of mutant genes into baculovirus transfer vectors without compromising the fidelity of the inserts themselves, combined with current rapid methods for generating recombinant baculoviruses through homologous recombination, this mutagenesis method will greatly facilitate structure-function studies in this expression system.

REFERENCES


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