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Site-directed Mutagenesis of Herpesvirus Glycoprotein Phosphorylation Sites by Recombination Polymerase Chain Reaction

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The recombination polymerase chain reaction (PCR) was adapted for site-directed mutagenesis of herpesvirus glycoprotein phosphorylation sites in a large vector of 7.1 kb. Four complementary oligonucleotide primers were prepared to generate two linear fragments having homologous ends. These fragments were combined and used to transform the *Escherichia coli* cells. Recombination in vivo of the linear products effected the generation of circular plasmids with the desired mutations. Of the 10 clones that were screened and sequenced, all 10 had the expected mutations in two threonine sequences. This technique, which does not require the use of restriction endonuclease to subclone the mutated fragments into the vector, provides a simple and efficient approach to site-specific mutagenesis and can be used to mutate sequences in large plasmid vectors.

RESULTS AND DISCUSSION

Recently, a very rapid and simplified method called recombination PCR (RPCR) was introduced that permits the site-directed mutagenesis of DNA and the generation of recombinant constructs by the recombination in vivo of PCR-generated linear products.(1,2) Because the initial experiments were performed in the pUC19 vector (2686 bp), we were uncertain whether RPCR would be equally efficacious in a much larger construct. In this report, we describe the application of RPCR to mutate a herpesvirus glycoprotein gene in a 7.1-kb construct. The gene product, called varicella-zoster virus (VZV) glycoprotein gpI, is of interest because it contains a putative phosphorylation domain in its cytoplasmic tail.(3) Two threonine residues, in particular, appear to be phosphorylated in a reaction catalyzed by mammalian casein kinase II.(4) To study this novel modification of herpesviral glycoproteins further, we have taken advantage of the RPCR technique to mutate simultaneously two nonadjacent threonine sequences in the gpI gene.

The herpesvirus gene construct used in the mutagenesis experiments consisted of a 2.4-kb DNA fragment containing VZV glycoprotein I (gene 68) target sequence cloned into the plasmid pCMV5 which is 4.7 kb.(5,6) The template was linearized with the restriction endonucleases *Eag*I or *Sma*I. Oligonucleotide primers were prepared on an Applied Biosystems Synthesizer and were purified by column chromatography at the University of Iowa DNA Facility. The sequences of the mutagenic oligonucleotides were: primer 1, 5′-TCTgCaGATgCGGAAGAAGAGTTTGGTAACGCG-3′, primer 2, 5′-TTCCGcATCtGcAGATTCCGAGTCCTC-3′ (the nucleotide changes are in lowercase characters); primer 3, 5′-AACAGCGGTAAGATCCTTGAG-3′; primer 4, 5′-AAACTCTCAAGGATCTTAC-3′. The oligonucleotides were designed so that there were two sets of complementary pairs.

Mutagenesis by RPCR involves the generation of DNA fragments with homologous ends, that when combined and cotransfected into cells will undergo in vivo recombination and generate circular products. The VZV gpI gene (115,808 bp to 117,676 bp) has two threonine sequences beginning at 117,593 bp and 117,599 bp.(5) The basic strategy for site-directed mutagenesis by RPCR is shown in Figure 1, with a linearized template. In addition to the conversion of two threonines to alanine residues, we made one further substitution at location 117,595 bp to introduce a new restriction enzyme site. Primers 1 and 2, which contained the mismatch, were the mutating primers. Their 5′ ends overlapped by 15 bp. Primer 3 (748–768 bp, sense strand) and primer 4 (755–773 bp, anti-sense strand) were within the ampicillin resistance gene in the vector. Their 3′ ends overlapped by 14 bp, resulting in homologous ends that were 21 bp long. In this RPCR, primers 1 plus 4 and primers 2 plus 3 were used in pairs to generate linear products (Fig. 1).

PCR reactions were carried out with *Taq* polymerase in a Perkin–Elmer Cetus Thermal Cycler. Amplification of DNA fragments from the plasmid template was achieved by adding 1 ng of template DNA, 200 μM of each dNTP, 25 pmoles of each primer, and 1.25 units of *Taq* polymerase in a total volume of 50 μl of *Taq* polymerase buffer. These samples were overlaid with 50 μl of mineral oil. Reactants underwent 25 cycles of denaturation

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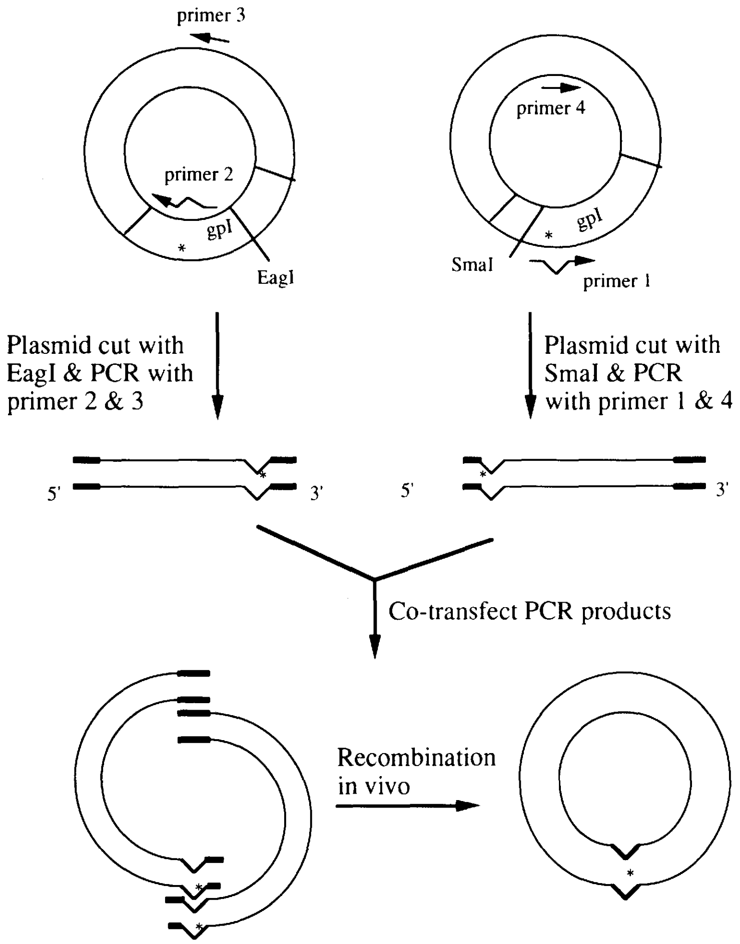

FIGURE 1 Schematic representation of the RPCR method for mutating two threonines in pCMV5-gpI construct (7.1 kb) without a gel-purification step. The asterisk designates the mutagenesis site. Primer 1 is complementary to primer 2. Primer 3 is complementary to primer 4. The thick lines represent the homologous ends.

(94°C, 30 sec), annealing (50°C, 30 sec), and extension (72°C, 2 min), followed by a final extension (72°C, 7 min). Two linear products were generated, which were 3.8 kb and 3.3 kb, respectively. A 2.5-μl amount of each product was combined and used to transform directly the Max-competent DH5α *E. coli* cells (BRL, Life Technologies, Gaithersburg, MD), resulting in 170 colonies.

The mutant was distinguished from the parental wild-type plasmid by virtue of the introduction of a *Pst*I site in the mutant sequence. When the linearized templates were used for PCR reaction, 10 clones were analyzed and all contained the expected mutation by this criterion. The clones that tested positive by restriction endonuclease digestion were analyzed further by double-stranded sequencing with two primers that flanked the mutated region of 538 bp. Ten clones were sequenced and all had the desired mutations. Sequence data demonstrating the mutation in comparison with wild type are shown in Figure 2. The two threonines in the wild type were changed to alanines (ACG to GCA, ACG to GCG). Of more than 3100 nucleotides inspected, no errors were uncovered.

The use of RPCR for site-directed mutagenesis represents a major technical improvement over prior methods. Both the standard methods as well as overlap extension methods involve a restriction endonuclease digestion step to subclone the mutated sequences into a vector.(7) RPCR allows for amplification as well as mutation of the DNA target sequence in the vector. Since RPCR totally eliminates the enzymatic step, it simplifies the mutagenesis process and reduces the time and effort required to generate site-directed mutations. When linearized templates were used, the mutation efficiency was virtually 100%. Linearization of the template prior to each PCR reaction simplifies the whole procedure and has several advantages: (1) it increases the amplification efficiency of PCR reaction, (2) it eliminates the gel purification step, and (3) it eliminates supercoiled templates, thereby reducing the background colonies containing the original templates.(1) In addition, we designed the primers in such a way that two of them are within the ampicillin-resistance gene. Thus, we can make additional mutations in the VZV gpI gene by the RPCR meth-

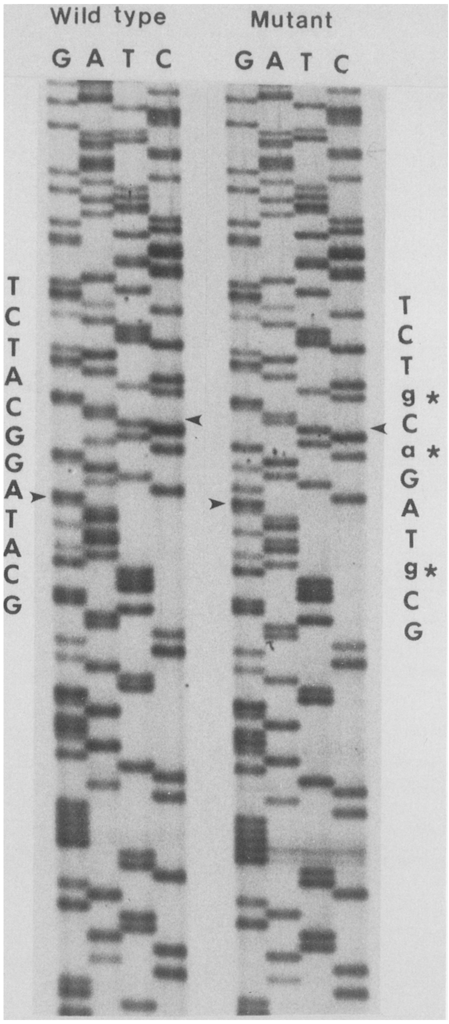

FIGURE 2 DNA sequence of the threonine mutant generated by RPCR. Only the region of interest is shown. (*) Altered nucleotides; (arrowheads) the region of the lettered nucleotides.

od, but require only one new pair of mutating primers for each mutation.

These studies with site-directed mutagenesis will help delineate the domains of the cytoplasmic tail of this viral cell surface glycoprotein(4) and should prove useful for similar studies in other systems.

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