GST Fusion Protein Expression Vector for In-Frame Cloning and Site-Directed Mutagenesis

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A novel glutathione *S*-transferase (GST) fusion protein expression vector, pGHX(-), was constructed to include a bacteriophage f1 origin of replication and a unique *Sal*I restriction site. The former enables the packaging of the vector into filamentous bacteriophage for the isolation of single-stranded (ss)DNA, while the latter was engineered to form part of the DNA that encodes the protease recognition site for factor Xa, downstream of the gene encoding GST (Figure 1). These modifications allow both site-directed mutagenesis and the subsequent expression of the encoded GST fusion protein using a single plasmid. This obviates the need for subcloning mutated foreign genes from a single-stranded vector into an expression plasmid and therefore avoids many time-consuming protocols such as DNA digestion, fragment isolation, ligation, transformation and screening. Importantly, when the *Sal*I site is used for blunt-end cloning, the amino acid sequence of the subsequently purified protein corresponds precisely and only to the encoding DNA of the cloned insert. No spurious N-terminal amino acids due to unavoidable cloning methodologies will be present in the purified protein product. This is particularly relevant to studies involving protein-protein interactions of purified binding domains and to studies of organelle protein targeting that involve N-terminal signal sequences (5).

GST fusion protein expression vectors have been used effectively for the high-level expression and subsequent purification of foreign gene products in *Escherichia coli* (4,6). Expression from one such vector, pGEX-3X (Pharmacia Biotech, Uppsala, Sweden), which we used as the precursor of pGHX(-), is under the control of the powerful *tac* promoter.

A 535-bp *Eco*RI/*Bam*HI fragment containing the f1 origin of replication (9) was isolated from plasmid pUC-f1 (Pharmacia Biotech), treated with Klenow enzyme (Boehringer Mannheim GmbH, Mannheim, Germany) and cloned into the *Aat*II site of pGEX-3X (after blunt-ending with T4 DNA polymerase; Pharmacia Biotech). Its orientation was determined by *Eco*RI digestion, and a clone containing the minus (-) orientation was selected for site-directed mutagenesis of the cloning site. ssDNA was isolated after superinfection with bacteriophage M13-VCS (Stratagene, La Jolla, CA, USA). About twice as much pGHX(-) ssDNA as M13VCS ssDNA was produced upon superinfection. The oligonucleotide (5′-ATCGAAGGTCGACGGATC-CCCGGG-3′) was then used to introduce a unique *Sal*I site (mutagenic nucleotides are underlined) at position 928 in the sequence (Figure 1) using the Sculptor™ *In Vitro* Mutagenesis System (Amersham International plc, Little Chalfont, Bucks, UK). Plasmids containing the *Sal*I restriction site were selected and the mutation and integrity of the fusion junction confirmed by dideoxy sequencing.

Although protocols exist for the mutagenesis of double-stranded DNA templates (3), they are comparatively expensive and time-consuming and require multiple oligonucleotides. The extremely high frequency of mutation attainable (7) and the requirement for only one mutagenic oligonucleotide have sustained the appeal for methods of site-directed mutagenesis using ssDNA templates. We deliberately selected the minus orientation of the f1 origin to make oligonucleotide design and subsequent analysis of dideoxy sequencing results as simple as possible. The non-coding strand of the inserted gene will be packaged and isolated as ssDNA. Therefore, DNA sequences obtained from the use of pGHX(-) ssDNA template and any oligonucleotides designed for use with pGHX(-) must be equivalent to the coding strand of the inserted gene.

We introduced the unique *Sal*I site expressly for the purpose of cloning polymerase chain reaction (PCR) products corresponding precisely and only to a protein coding region. Thus, when pGHX(-) is digested with *Sal*I and blunt-ended by treatment with Klenow enzyme, the resulting 3′ end of the vector precisely matches the end of the factor Xa recognition sequence (Figure 1). Factor Xa will cleave GST from fusion proteins to release the foreign protein encoded by the PCR product starting at the very first nucleotide of the inserted DNA. Some strategies using other vectors (including pGEX-3X) or restriction sites incorporated into PCR primers (to enable unidirectional cloning) invariably produce proteins after cleavage that contain exogenous amino acids at their N terminus not found in the authentic protein (8).

We have verified the performance of pGHX(-) using the manganese superoxide dismutase (MnSOD) gene of *E. coli*. PCR primers were designed to amplify the MnSOD gene from *E. coli* genomic DNA. The 5′ primer sequence corresponded to the coding region beginning from the second codon because an ATG start codon is not required when cloning into pGHX(-). Standard PCR conditions were followed except for the following important modifica-

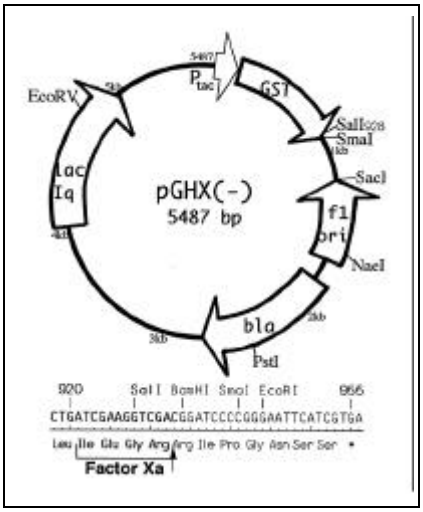

Figure 1. Plasmid map and cloning site of pGHX(-). The GST DNA coding and amino acid sequence and the factor Xa protease recognition site are shown below the plasmid map. Genes, the *tac* promoter (P_{tac}) and the f1 origin of ssDNA replication (f1 ori) are shown as arrows in their correct orientation. Only the *Sal*I site is intended for cloning after blunt-ending. The vector DNA sequence will end in-frame with the GST gene at nucleotide position A932 (*Sal*I site ending GTC-GA-3′), precisely at the end of the DNA encoding the factor Xa protease recognition sequence (an arrow indicates the cleavage site, the C-terminal side of Arg225). Although the *Sma*I site is also unique, the *Eco*RI and *Bam*HI sites are not. Not all unique restriction sites are shown.

tions: *(i)* omission of a post-cycling extension step (typically at 72°C for 10 min), *(ii)* addition of 2 U Klenow enzyme directly to the PCR tubes in situ and incubation at 30°C for 30 min and *(iii)* purification of PCR products by spin filtration. These steps significantly enhance blunt-end cloning efficiency and enable the routine blunt-end cloning of PCR products into a variety of other vectors. The ability to simultaneously clone the same PCR product into non-fusion protein expression vectors such as pTrc99A (Pharmacia Biotech; prepared by *Nco*I digestion and Klenow treatment) is a great advantage (8). The first step increases cloning efficiency by reducing the propensity of *Taq* DNA polymerase to act as a terminal transferase (1), while step 2 ensures complete extension of unfinished reaction products. The effectiveness of Klenow treatment can be readily evaluated as a band-sharpening effect on the PCR product in agarose gels. Although many DNA polymerases have been shown to exhibit a terminal transferase activity (2), treatment with Klenow enzyme under the conditions described (step 2) improves blunt-end cloning efficiency from less than 10% (8 experiments) to greater than 40% (11 experiments, some >70%).

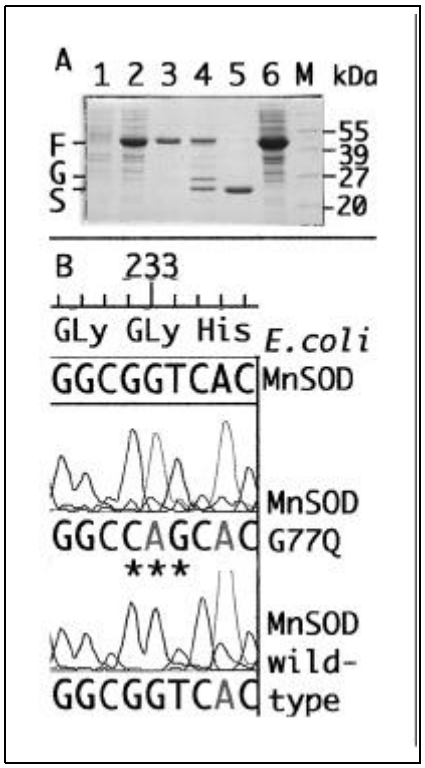

Figure 2. Expression, purification and mutagenesis of pGHX-MnSOD. (A) Coomassie® blue-stained SDS-PAGE. The pGHX-MnSOD construct was transformed into *E. coli* OX326A (Δ*sodA*, Δ*sodB*) and grown in 50 mL rich medium. Expression of GST fusion protein induced by the addition of 10 mM isopropyl β-D-thiogalactopyranoside (IPTG) when the cell density reached an optical density $(OD)_{600}$ of 0.4. After overnight growth, samples were saved and subsequently analyzed (lane 2, 5 µL). This compares with similarly grown *E. coli* without the construct (lane 1, 5 µL). After passage through a French pressure cell, soluble proteins were subjected to glutathione affinity chromatography, and the GST::MnSOD fusion protein (F, 49 kDa) was purified (lane 3), cleaved with factor Xa (lane 4) to release GST (G, 26 kDa) and re-chromatographed (lane 5) to purify MnSOD (S, 23 kDa). Expression from pGHX-MnSOD(G77Q) is shown in lane 6. Molecular weight markers are shown in kDa in lane M. (B) Electropherogram of pGHX-MnSOD ssDNA wild-type (lower panel) and G77Q mutant (upper panel). The sequence of *E. coli* MnSOD is shown above. Codon G77 corresponds to nucleotides 232–234, all of which were mutated using a pGHX-MnSOD ssDNA template (asterisks). Dideoxy sequences were obtained using a primer specific for the (-) strand of the vector PGEXPLUS (5′-GTTTG-GTGGTGGCGACCATCCT-3′).

Expression, purification and mutagenesis using a pGHX-MnSOD construct are illustrated in Figure 2. Analysis of expression and purification was followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2A). The sequence of the purified MnSOD protein (Figure 2A, lane 5) corresponds precisely to the native MnSOD sequence produced by *E. coli*. Mutagenesis of an entire codon (G77Q) using a 27-mer oligonucleotide and ssDNA template prepared from the pGHX-MnSOD clone was confirmed by dideoxy sequencing (Figure 2B), which also confirmed that no other mutations were present in the MnSOD gene or flanking regions. The mutant protein was subsequently expressed without any need for further subcloning (Figure 2A, lane 6). Furthermore, the subsequent introduction of further mutations into the MnSOD gene is greatly simplified because ssDNA template for the site-directed mutagenesis reaction can be obtained simply upon superinfection of cultures harboring the pGHX-MnSOD(G77Q) mutant plasmid.

This vector should prove useful for any studies that require the production of authentic proteins or protein domains. pGHX(-) can be obtained directly from the authors upon request.

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