Construction of multiple-epitope tag sequence by PCR for sensitive Western blot analysis

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ABSTRACT

Epitope tagging is a powerful technique to characterize a recombinantly expressed protein encoded by cDNA without the purification of the protein and the immunization of animals. In some cases, however, the expression of a tagged protein is too low to analyze by Western blot. We have developed a simple method to generate tandem repetitive nucleotide sequence by PCR, which allows us to label a protein of interest with a multiple-epitope tag. When five myc epitopes were attached to vaccinia virus protein CrmA, its signal was multiplied 5.8 times in Western blot analysis, compared with that of one epitope-tagged CrmA.

Once new cDNA is cloned, an antibody against the encoded protein is important to analyze a recombinantly expressed protein in bacteria and mammalian cells. Epitope tagging (1) dispenses with a labor-intensive and time-consuming procedure such as the forced expression, the purification and the immunization of a recombinant protein. By adjoining an epitope peptide to a protein of interest using gene manipulation, a commercially available antibody to the epitope substitutes for an antibody to a recombinant protein. But the amount of expressed tagged protein is too low for analysis in some cases. We have tackled the construction of a multiple-epitope tag by polymerase chain reaction (PCR), because it is reasonable to assume that a multiple-epitope tag composed of an epitope sequence by anti-epitope antibody.

Complementary oligonucleotide primers of myc tag (2), primer 5′ and 3′, were designed so as to hybridize to each other at the 3′ end of 12 bases and encode two units of epitope peptide sequence using the codons observed most frequently in Xenopus laevis (Fig. 1A). Aliquots (0.8 nmol) of each primer were mixed, heated at 65°C for 2 min, and phosphorylated by 40 U of T4 polynucleotide kinase (Takara, Japan) at 37°C for 1 h in 40 µl reaction mixture containing 50 mM Tris–HCl pH 8.0, 10 mM MgCl2, 10 mM 2-mercaptoethanol and 0.5 mM ATP. After incubation the enzyme was inactivated at boiling temperature for 3 min and the reaction mixture was cooled to room temperature. PCR reactions were performed using 0.4, 0.8 or 1.6 µM of each primer in a final volume of 100 µl containing 4 mM MgCl2 and 5 U of Stoffel fragment of Taq DNA polymerase I (Perkin Elmer) which has neither 5′–3′ nor 3′–5′ exonuclease activity (3). Amplification was carried out starting from room temperature for 30 cycles with 30 s denaturation at 93°C, 30 s annealing at 60°C and 30 s extension at 72°C. Cycling was terminated by 15 min incubation at 72°C. PCR products were electrophoresed on a 4% polyacrylamide gel. The ethidium bromide-stained gel showed that the crossing-over between PCR products during the cycling reaction created multiple-epitope tags (Fig. 1B) and the smaller the amount of primers used in the PCR reaction, the greater the size of the main products (Fig. 2A). Furthermore, more enzyme elaborated the longer multiple-epitope tag (Fig. 2B), indicating that the amount of Taq DNA polymerase is a rate-limiting factor in this PCR reaction. The DNA sample loaded in lane 2 of Figure 2A was purified with Wizard PCR prep DNA purification resin (Promega, USA) to remove primers, inserted into a Smal site of pBluescript (Stratagene, USA), and sequenced. Among 14 ends of seven independent clones, only one end had 9 nt deleted and all others were intact. However, when other Taq DNA polymerases with exonuclease activity were used in PCR amplification instead of the Stoffel fragment, deletions, averaging 11.4 nt, occurred at all 10 ends of five clones.

In order to determine whether a multiple-epitope tag enhances the sensitivity of detection in Western blot analysis, the insertion of a PCR product into CrmA (4) expression vector was undertaken. To introduce a Smal site in the crmA gene, this gene was amplified using both 5′ crmA primer (5′-GGGAGATCCGCTAGCTGGGATATCTCTCACGAGAAATG-3′) which is composed of an EcoRI site, the consensus Kozak sequence (5), a Smal site and 18 bases encoding amino acids 2–7 of CrmA (4), and 5′ crmA primer (5′-GGGGGCGGCCTTTAACATAGGTGTCCGAGAG-3′) which contains a NotI site, a stop codon and 19 bases complementary to the final six amino acids of CrmA. The PCR product was digested with EcoRI and NotI and ligated to the expression vector, pcDNA1/Amp (Invitrogen, USA). A DNA fragment encoding a tag with five epitopes was purified electrophoretically (Fig. 2A, lane 2), and inserted into a Smal site of the crmA gene in the expression vector (pcrmA-5 tag). As a control, a DNA fragment encoding CrmA labelled with one myc tag was created by PCR using the 3′ crmA primer and the modified 5′ crmA primer which has a DNA sequence of myc tag instead of a Smal site (5′-GGGAGATCCGCTAGCTGGGATATCTCTCACGAGAAATG-3′) and ligated into the same vector in the similar way (pcrmA-1 tag).

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Figure 1. (A) Design of two primers to construct a multiple-epitope tag sequence by PCR. DNA and amino acid sequences of an myc epitope are boxed. Arrows indicate the 5' to 3' direction. (B) Schematic representation of strategy for the construction of a multiple-epitope tag. Black and grey arrows represent the primers used in the first cycle and the oligonucleotides synthesized by PCR, respectively. Boxes mean myc epitopes.

Figure 2. Analysis of PCR products using primer 5' and 3'. (A) Amplification was carried out using 0.4 (lane 1), 0.8 (lane 2) or 1.6 µM (lane 3) of each primer. (B) PCR reaction was performed by 5 (lane 1), 10 (lane 2) or 20 U (lane 3) of the Stoffel fragment in 100 µl reaction mixture. Lane M contains a DNA size marker (100 bp ladder). Arrowheads represent the positions of dimer, trimer, tetramer and pentamer.

Myc tag and crmA DNA sequences in these clones were determined to confirm neither deletion nor mutation in them. In vitro transcription by T7 RNA polymerase and translation in rabbit reticulocyte lysate using pcrmA-1 tag and -5 tag showed the similar amounts of 40 and 45 kDa proteins (Fig. 3A), respectively. However, Western blot analysis by anti-myc antibody revealed that CrmA tagged by five epitopes was labelled 5.8 times more intensively than the protein with one epitope based on the measurement using ARGUS-50 (HAMAMATSU, Japan) and standardization by the internal control, β-galactosidase (Fig. 3B and C).

The simple method described above will not only enable us to construct tandem repetitive sequence of DNA, RNA and protein easily, but also contribute to the molecular analysis of the interplay between DNA (RNA or protein) and protein by multiplying the recognition sequence.

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REFERENCES