are difficult to see because of a lack of contrast.

To further enhance the detection of chromogranin A bands, the blot was wetted and blocked as above and then incubated with an affinity-purified rabbit antiserum to bovine chromogranin A, followed by HRP-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA, USA). The blot was developed with luminol as described above. Figure 2C shows that a strong chromogranin A band was obtained by re-probing this membrane. The original antibodies were most likely bound to the dried blot but were at least partially competitively displaced by the fresh antibodies during re-probing.

The results presented here show that immunoblots can be reused after extended storage. In both cases presented, the blots contained relatively large amounts of the target proteins (clathrin and chromogranin A, respectively). Thus, re-probing should be especially useful in situations where good antibodies were not originally available, but it can also be used to confirm earlier data that have faded upon storage. In the latter case, some image enhancement may also be possible by computer scanning of the faded blot.

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Universal Template Plasmid for Introduction of the Triple-HA Epitope Sequence into Cloned Genes

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Epitope tagging has been widely used for analysis of gene products. Subcloning the genes of interest into an epitope encoding segment of expression plasmids is commonly used for the epitope tagging experiments. However, by this approach, the epitope-tagged genes are expressed under different conditions (i.e., promoters, terminators etc. provided from the particular expression plasmid) from the authentic gene and frequently result in the different functionalities and cellular localization from the authentic products. For in vivo analyses, the epitope-tagged gene should be placed between its own promoter and terminator sequences. Conventional protocols for constructing such modified gene fragments include multiple steps: (i) introduction of a restriction site at the tagging site by site-

directed mutagenesis; (ii) construction of a tag fragment with compatible restriction sites at both ends by direct DNA synthesis or excision from plasmids; and (iii) ligation of the tag to the target gene (3). It is also possible to introduce epitopes into target genes by one-step mutagenesis using the Kunkel protocol (4). However, this approach requires a long oligonucleotide covering sequences for annealing and the epitope-coding region (i.e., over 110 bp for the triple hemagglutinin [HA] tag). It is still difficult to prepare such long oligonucleotides, even using current advanced DNA synthesis technologies.

We developed a simpler method for creating the epitope-tagged versions of the genes by applying the "sticky feet"-directed mutagenesis strategy (2). This method allows efficient insertion of the epitope encoding sequence to any locations of the genes of interest. We constructed a plasmid, pSK-3xHA, encoding triple repeats of 9 amino acid residues (YPYDVPDYA) derived from the HA of human influenza virus (Figure 1A). The triplicate HA tags are reported to provide greater sensitivity than the single one (3). We describe introduction of the 3x-HA tag into the N terminal of the PEP12 product (1) of *Saccharomyces cerevisiae* as an example. A pair of oligonucleotides, 5'-ACATAATTGTGTTGAGATGTACCCATATGACGTTCCA-3' (homologous sequence to the 5'-untranslated region and the initial Met codon of PEP12 was underlined) and 5'-CCACCAAA-AAATTCGTCTTCCGAAGCGTAGTCAGGTACGTCGTAA-3' (the underlined is the antisense strand sequence for PEP12 N-terminal encoding region), was synthesized, and the former was phosphorylated by treatment with T₄ polynucleotide kinase in the presence of ATP. The oligonucleotides were used for polymerase chain reaction (PCR) amplification (10 cycles) on pSK-3xHA. The PCR product of approximately 120 bp was purified by agarose gel electrophoresis on a 1.5% SeaPlaque® gel (FMC BioProducts, Rockland, ME, USA). The PCR product (approximately 50 ng) was mixed in annealing mixture (10 µL) containing 0.2 µg of single-stranded DNA produced from a PEP12 gene in pBlue-script® II KS (Stratagene, La Jolla, CA,

USA) (pBS-PEP12) by *dut ung* strain CJ236, 0.5 mM dNTP, 1× PCR buffer (Perkin-Elmer Japan, Chiba, Japan) and 2.5 U of AmpliTaq® DNA Polymerase (Perkin-Elmer Japan). Annealing reaction was done by incubating the mixture at 93°C for 2 min, 67°C for 30 s and 37°C for 1 min, with about 1 min to drop between each temperature. Then the annealing mixture was added to 10 µL of extension/ligation mixture containing 40 mM Tris-HCl, pH 7.5, 20 mM MgCl₂, 30 mM NaCl, 5 mM dithiothreitol (DTT), 0.5 mM ATP, 1 U of Sequenase™ enzyme (Amersham Japan, Tokyo, Japan) and 3 U of T4 DNA ligase, and incubated at room temperature for 30 min. DNA was precipitated by ethanol and dissolved into 10 µL of TE buffer (Tris-HCl, pH 8.0, 1 mM EDTA) and introduced into *dut*⁺

ung⁺ strain XL1-Blue® (Stratagene). Miniprep DNAs were prepared from the Ap^R colonies and analyzed by restriction enzymes. An *Mlu*I site was included in the epitope coding sequence of pSK-3xHA to facilitate the ease of screening for successful mutagenized clones. If the epitope coding sequence was generated, the *Mlu*I site was introduced while the unmodified pBS-PEP12 did not have the *Mlu*I site. In this particular experiment, we obtained 3 clones having the *Mlu*I site among 24 Ap^R. By nucleotide sequencing, these 3 clones were shown to have the HA sequence at the correct position without any accidental mutations in the PEP12 coding region. The ratio of mutagenized/original constructs seems to be lower than with the conventional Kunkel method, presumably because of the

Figure 1. (A) Structure of pSK-3xHA. A set of oligonucleotides (HA-F: 5'-GGGTACCATATG-ACGTTCCAGACTACGGTATCCGTACGACGTTCCGGAT-3' and HA-R: 5'-AACTGCAGCGTAGTCAGGTACGTCGTAAGGGTAAGCGTAATCCGGAACGTCG-3'; *Kpn*I and *Pst*I recognition sequences are underlined) was synthesized and amplified by PCR as primer dimer. The PCR product was digested with *Kpn*I and *Pst*I and introduced into the multiple cloning site of pBluescript II SK(+) to generate pSK-3xHA. In the epitope encoding sequence, the *Mlu*I recognition site was included. The primer design was assisted with the PrimerSelect module of the Lasergene® package (DNA Star, London, England, UK). (B) Preparation of mutagenic primer and site-directed mutagenesis by the "sticky-feet" strategy. A pair of primers, S and A, is to be used for the PCR amplification with pSK-3xHA as a template. The primers S and A should have 18 and 21 bases of homologous sequences (anchors: see text) to pSK-3xHA, respectively, and homologous sequences to the gene to be modified (usually 18–20 bases). The length of the anchors to pSK-3xHA should not be changed without particular reasons to avoid primer-dimer formation and misannealing. Either S or A, depending on whether the sense or antisense strand of the target gene is to be rescued from CJ239, was phosphorylated before the PCR amplification. The PCR amplification on pSK-3xHA with the primers S and A produced an approximately 150-bp product. This product can be separated from the unincorporated primers S and A by gel electrophoresis. Then the purified product serves as a mutagenic primer in the Kunkel mutagenesis protocol (4).

Benchmarks

existence of an abundance of competitive oligonucleotides (the opposite strand created during the PCR) in the annealing-extension reaction. Nevertheless, we obtained at least three, usually over a half-dozen candidates within two dozen Ap^R colonies in several different epitope tagging experiments. When the modified DNA fragment encoding 3xHA-Pep12p was introduced into yeast cells, a protein of 38–40 kDa was recognized by the anti-HA monoclonal antibody (12CA5; Boehringer Mannheim GmbH, Mannheim, Germany) on the immunoblot of the total cell lysate (data not shown). The 3xHA epitope adds 3.4 kDa to the molecular mass of Pep12p (35–36 kDa) (1). We concluded that the 38–40-kDa protein is HA-tagged Pep12p.

We applied the “sticky-feet” mutagenesis protocol (2) to epitope tagging using the plasmid pSK-3xHA. Similar techniques can be applied to other epi-

tope tags including c-myc, multiple histidines etc. Our limited experience on the PCR-based protocol or the Kunkel mutagenesis protocol (4) indicates that the latter introduces fewer accidental mutations. This protocol describes minimum exposure (usually only 10 cycles) of target sequences to *Taq* DNA polymerase, thus reducing the chance of misincorporation. This feature provides another advantage for the epitope tagging experiment.

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