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Monitoring and Purification of Proteins Using Bovine Papillomavirus E2 Epitope Tags

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ABSTRACT

We describe here the use of two newly mapped bovine papillomavirus type 1 (BPV-1) E2 protein epitopes as tags. We

constructed several vector plasmids for overexpression as well as for moderate expression of single- or double-tagged proteins in either *Escherichia coli* or eukaryotic cells. The new tags were fused to several proteins, and the activity of the tagged proteins was tested in different assays. The tags were shown not to interfere with the function of these proteins in vivo and in vitro. Interaction of the monoclonal antibodies 3F12 and 1E2 with their respective epitopes was specific and had high affinity in a variety of conditions. We have demonstrated that the 3F12 antibody-epitope interaction tolerates high salt concentrations up to 2 M. This permits immunoprecipitation and immunopurification of the tagged proteins in high-salt buffers and reduction of the nonspecific binding of the contaminating proteins. We also provide a protocol for DNA binding and DNase I footprinting assays using the tagged, resin-bound DNA-binding proteins. The BPV-1 E2-derived tags can be recommended as useful tools for detection and purification of proteins.

INTRODUCTION

Epitope tagging is a recombinant DNA technique by which a protein is made immunoreactive to a preexisting antibody. This technique simplifies detection, characterization, purification and in vivo localization of proteins and has become a standard method of molecular genetics (3). However, some tags are not useful in certain applications due to high background binding. In some cases, affinity purification and immunoprecipitation of a tagged protein is problematic due to co-precipitation of contaminating proteins.

Here, we describe the use of two recently mapped bovine papillomavirus type 1 (BPV-1) E2 protein epitopes as tags. We constructed several vector plasmids for over-expression as well as for moderate-level expression of either single- or double-tagged proteins in *Escherichia coli* and eukaryotic cells. The new tags were fused to functionally different proteins: a bacterial transcriptional activator, XylS, that aggregates and becomes nonfunctional at high levels of expression, several mutants of the tumor suppressor protein p53 for overexpression in *E. coli* and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for

expression in eukaryotic cells. Hereby, we report the use of BPV-1 E2-derived tags and respective monoclonal antibodies for the identification of proteins on Western blots, in the immunofluorescence staining of cells and DNA band-shift assay. In addition, we describe an application of the resin-bound tagged protein in DNA binding and DNase I footprinting assays.

MATERIALS AND METHODS

Plasmid Constructions

For the construction of pBR-3F12 and pBR-1E2, we inserted the coding sequence of XylS with the N-terminally fused influenza virus hemagglutinin (HA) epitope (2) from the pET11c-based parent plasmid pETSN117 (4) between the *NheI* and *BamHI* sites of pBR322. The subcloned fragment contained in addition to a ribosome binding site, an extra *NdeI* site and a start codon preceding the tag sequence. The resultant plasmid pBRSN 117 contains *XbaI* and *BamHI* sites for cloning of a recombinant coding sequence (4). Then, the coding sequence of the HA epitope between *NdeI* and *XbaI* sites was replaced with double-stranded synthetic oligonucleotides encoding peptides GVSSTSSDFRDR and TTGHYSVRD, recognized by anti-BPV-1 E2 monoclonal antibodies 3F12 and 1E2, respectively (7).

For the construction of pBR-NC, we amplified *xylS* sequence by PCR using the 3'-end primer containing a coding sequence for the peptide TSSDFRDR. The peptide recognized by 3F12 Mab was fused in frame to the C-terminus of XylS and flanked by *KpnI* and *BamHI* sites. The resultant PCR fragment was cloned into pBR-1E2 generating the expression plasmid with cloning sites *XbaI* and *KpnI*.

pET-3F12 was generated by cloning the *BamHI/NdeI* fragment from pBR-3F12-*xylS* into the corresponding sites in pET-11c. Then, the *XylS* gene was removed by cleavage with *XbaI* and *BamHI* and replaced with coding sequences for different mutant p53 proteins bearing *XbaI* and *BglIII* sites at the ends.

For the construction of pCG-3F12, double-stranded synthetic oligonu-

Table 1. Main Characteristics of Expression Vectors

Plasmid	Promoter	Tag location	Tag sequence
pBR-1E2	<i>Ptet</i>	N-terminus	<p>M* <u>T T G H Y S V R D S</u> R...</p> <p>5'-CAT <u>ATG</u> ACA ACA GGG CAT TAC TCT GTA AGA GAT <u>TCT AGA GGA TCC</u> -3'</p> <p>NdeI XbaI BamHI</p>
pBR-3F12	<i>Ptet</i>	N-terminus	<p>M* <u>G V S S T S S D F R D R S</u> R...</p> <p>5'-CAT <u>ATG</u> GGT GTC TCA TCC ACC TCT TCT GAT TTT AGA GAT CGC <u>TCT ACA GGA TCC</u> -3'</p> <p>NdeI XbaI BamHI</p>
pBR-NC	<i>Ptet</i>	N-terminus 1E2 C-terminus 3F12	<p>M* <u>T T G H Y S V R D S</u> R...</p> <p>5'-CAT <u>ATG</u> ACA ACA GGG CAT TAC TCT GTA AGA GAT <u>TCT AGA</u>-3'...</p> <p>NdeI XbaI</p> <p>...G <u>T S S D F R D R</u> STOP</p> <p>...5'-GGT <u>ACC</u> TCT TCT GAT TTT AGA GAT CGC TGA <u>GGA TCC</u>-3'</p> <p>KpnI BamHI</p>
pET-3F12	T7	N-terminus	<p>M* <u>G V S S T S S D F R D R S</u> R...</p> <p>5'-CAT <u>ATG</u> GGT GTC TCA TCC ACC TCT TCT GAT TTT AGA GAT CGC <u>TCT AGA</u>-3'</p> <p>NdeI XbaI</p>
pCG-3F12	CMV IE	N-terminus	<p>M* <u>G V S S T S S D F R D R</u>...</p> <p>5'-TCT <u>AGA</u> ATG GGT GTC TCA <u>AGT ACT</u> TCT TCT GAT TTT AGA GAT CGC...</p> <p>XbaI ScaI</p> <p>...G S K L P G L Q G T R S</p> <p>...GGA TCC <u>AAG CTT CCC</u> GGG <u>CTG CAG</u> GGT ACC AGA TCT-3'</p> <p>BamHI HindIII SmaI PstI KpnI BglII</p>

In amino acid sequences, the epitopes are underlined and the initiator methionine codons are marked with asterisks
 In nucleotide sequences, cleaving sites for restriction endonucleases are underlined.
 Restriction enzymes which cut once per plasmid are marked in bold. pET-3F12 contains an additional XbaI site which is sensitive to *dam* methylation and is not cleaved when the plasmid is isolated from *dam*⁺ strains

cleotide encoding 3F12 epitope and incorporating multicloning sites was inserted between the *Xba*I and *Bgl*III sites of pCG (9). Plasmid pCG-3F12-GAPDH contains rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inserted between *Bam*HI and *Bgl*III sites of pCG-3F12. Plasmid pCG-3F12-GAPDH-NLS contains nuclear localization signal of human p53 (amino acids 305–327) fused to the C-terminus of the GAPDH protein.

Immunoaffinity Binding of 3F12-XylS

pBR-3F12-*xylS* bearing *E. coli* DH5 α cells were grown in LB medium

supplemented with 100 μ g/mL ampicillin at 20°C to an A₆₀₀ of approximately 1.0. Cells were harvested, washed with TBS and resuspended in 1/10 volume of high-salt lysis buffer containing 100 mM Tris-HCl, pH7.5, 1.5 M NaCl and 5 mM EDTA, 20% (wt/vol) glycerol. Cell suspension was frozen in liquid nitrogen and stored at -70°C.

For the preparation of crude extracts, cells were thawed and dithiothreitol (DTT; 10 mM), PMSF (100 μ g/mL), aprotinin (1 μ g/mL), and CHAPS (0.2%) were added. Cells were incubated with lysozyme (0.5 mg/mL) on ice for 20 min and disrupted by sonication. The lysate was clarified by centrifugation at 40 000 $\times$ *g* at 4°C for 30 min.

Affinity beads were prepared by coupling 3F12 anti-BPV E2 monoclonal antibody to divinylsulfon-activated Toyopearl® HW65 TSK-gel (TOSOH, Tokyo, Japan). The 3F12-TSK affinity beads were incubated with crude lysate at 4°C for 1 h with end-over-end agitation. Beads were washed extensively with high-salt lysis buffer on glass filter. These 3F12-XylS beads were stored at -20°C in approximately 10 gel volumes of storage buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM MgCl₂, 80 μ M EDTA, 80 μ M DTT, 50% (vol/vol) glycerol, 100 μ g/mL PMSF and 1 μ g/mL aprotinin. These beads were used for DNA binding and “footprinting” assays.

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DNA Precipitation Assay

End-labeled restriction fragments of pUPM190 *HpaII/HinI* digest and 50 μ L of the suspension of 3F12-XylS beads were mixed with 0.3 mL of DNA binding buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM EDTA, 10% (wt/vol) sucrose, 0.1% CHAPS, 700 μ g/mL BSA, 1 μ g/mL aprotinin and 1 mM *meta*-toluate. The mixture was incubated for 30 min at room temperature with end-over-end agitation. Beads were washed 3 $\times$ with 1 mL of DNA binding buffer and incubated with Proteinase K (50 μ g/mL) at 37°C for 30 min in stop solution containing 200 mM NaCl, 5 mM EDTA and 1% SDS. The retained DNA was released by phenol-extraction, ethanol-precipitated, and identified in 5% PAGE under non-denaturing conditions.

DNase I Footprinting

The *Om*-containing *EcoRI/XhoI* fragment of pUPM190 was end-labeled at *XhoI* site in the lower strand with Klenow fragment. The labeled probe was incubated with 50 μ L of the suspension of 3F12-XylS beads in DNA binding buffer as for the DNA precipitation. The beads were washed 3 $\times$ and suspended in 100 μ L of DNase I buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM MgCl₂, 1 mM CaCl₂, 100 μ M DTT, 100 μ M EDTA, 100 μ g/mL BSA and 2 μ g/mL sonicated salmon-sperm DNA. DNase I was added to concentrations of 0.6–3.0 μ g/mL, and the reaction mixture was incubated for 1 min at 30°C. The reaction was stopped by 200 μ L of stop solution containing 600 mM NaCl, 120 mM EDTA, 3% SDS and 150 μ g/mL dextran, and the beads were treated with Proteinase K (50 μ g/mL) at 37°C for 30 min. DNA was extracted from the beads with phenol/chloroform, precipitated with ethanol and analyzed on sequencing gel. DNase I cleavage of the unbound template was carried out in the same buffer at DNase I concentrations of 30–300 ng/mL.

Gel-Shift Assay

Gel-shift assay was performed as previously described (1). We used the

crude lysate preparations of p53 mutants. The double-stranded p53 con oligo (5'-GAT CCG GAC ATG CCC GGG CAT GTC CGG ATC-3') was used as a probe. Protein-DNA complexes were separated from the unbound DNA on 5% PAGE (55:1).

Immunodetection of Proteins

For Western blot analysis, cells expressing the tagged proteins were lysed in SDS sample buffer, total protein extracts were separated on 12% SDS-PAGE and electroblotted onto nitrocellulose membrane filters. Blots were blocked with 1% non-fat dry milk for 1 h, incubated with anti-BPV E2 1E2, 3F12 or anti-p53 pAb 240 monoclonal antibodies, followed by incubation with anti-mouse IgG alkaline phosphatase-conjugated secondary antibody.

For immunofluorescence staining, Saos-2 cells were transfected with plasmids expressing 3F12-GAPDH-NLS or 3F12-GAPDH proteins. Cells were grown on microscopy cover glasses and fixed 24 h after transfection with methanol at -20°C. Proteins were detected with 3F12 and anti-mouse Ig antibody conjugated with FITC as a primary and secondary antibody, respectively.

RESULTS AND DISCUSSION

Moderate Level Bacterial Expression of Epitope-Tagged Proteins

The XylS protein, a transcriptional activator from the TOL plasmid pWWO of the soil bacterium *Pseudomonas putida*, like some other AraC/XylS family transcription factors, does not tolerate high level of over-expression and is prone to aggregation both inside the cell as well as in the solution, in the course of purification (4,5). We expressed XylS and several truncated variants of the protein at a near to native level in *E. coli* and tested their physiological activities in vivo. For that, we constructed vectors for the moderate level of expression of epitope-tagged fusion proteins. The vectors were based on pBR322, and the *tet* promoter of this plasmid was used for expression of recombinant proteins. The vectors pBR-3F12 and pBR-1E2 were constructed for the expression of pro-

teins with N-terminally fused peptides GVSSTSSDFRDR and TTGHYSVRD, recognized by anti-BPV E2 monoclonal antibodies 3F12 and 1E2, respectively (7). Both vectors contain *XbaI* and *BamHI* sites for cloning of the recombinant sequence. We also constructed the vector pBR-NC for the expression of proteins with different epitope tags in both N- and C-termini. The N-terminal tag was TTGHYSVRD as in pBR-1E2 and the C-terminal tag was TSSDFRDR, a shorter version of the epitope recognized by 3F12 Mab. The cloning sites for a coding sequence in pBR-NC are *XbaI* and *KpnI* (Table 1).

We transformed *E. coli* DH5 α with plasmids expressing the tagged versions of XylS and analyzed the expression of the tagged XylS proteins by Western blotting. The 3F12 antibody recognized both 3F12-XylS and NC-XylS proteins, while the 1E2 antibody recognized 1E2-XylS and NC-XylS proteins as single bands on the Western blot (Figure 1A, lanes 1–6). No cross-reaction with cellular proteins was observed. However, when 3F12 Mab was used for the detection of the double-tagged NC-XylS protein, a much weaker signal was detected when compared with the 1E2 Mab signal. (Figure 1A, lanes 4 and 6). This could be explained by the use of the shorter version of the 3F12-specific epitope in the double-tagged protein. We studied the effect of these tags on the activity of XylS protein in *E. coli* strain CC118*Pm-lacZ*, which carries a chromosomal copy of the XylS responsive *Pm* promoter fused to the *lacZ* gene (6). The tags had no effect on the transcriptional activation by XylS (data not shown).

Study of Specific DNA Binding by the Matrix-Attached Epitope-Tagged Protein

XylS is a DNA-binding protein, which specifically binds to the *Om* operator sequence and activates the responsive *Pm* promoter. However, suitable conditions to study the soluble XylS in vitro have not been found, as the protein tends to aggregate. To demonstrate the site-specific DNA binding of epitope-tagged XylS in vitro, we used immunobound 3F12-XylS protein that was attached to the TSK

beads through the N-terminal tag and 3F12 antibody. Western blot analysis showed that high salt concentrations, up to 2 M NaCl, do not hinder the interaction of 3F12 MAb with the specific epitope (data not shown). Therefore, to avoid aggregation of XylS and coimmunoprecipitation of contaminating proteins, the crude lysate was prepared in a high-salt lysis buffer, containing 1.5 M NaCl, and 3F12-XylS was bound to the affinity resin carrying 3F12 Mab by a single-step, batchwise procedure.

The DNA binding assay was carried out by mixing the 3F12-XylS beads with a mixture of end-labeled restriction fragments of the *Om*-containing plasmid pUPM190 (4). After several washes, the bound DNA was released and identified by gel electrophoresis, using the input mixture of fragments as a marker. Figure 2A shows that only a

single *Om*-containing fragment of the pUPM190 digest was retained on the beads, and binding of any other fragment could not be detected.

Further, we analyzed the interaction of the immobilized 3F12-XylS protein with *Om* by DNase I footprinting. The specific complexes of the *Om*-containing DNA fragment and TSK-bound 3F12-XylS were prepared identically as for DNA precipitation and were treated with DNaseI. After the cleavage, DNA was extracted from the beads and analyzed on a sequencing gel. As a control, the unbound template was cleaved at lower concentrations of DNaseI to obtain the equal rate of cleavage. Figure 2B shows that 3F12-XylS protects a 44 bp area on the lower strand and four DNase I hypersensitive sites occur within the protected region. We obtained an identical DNaseI foot-

Figure 1. Detection of the tagged proteins. (A) Western blot analysis of the tagged proteins. Total protein extracts, separated by 12% SDS-PAGE and electroblotted, were analyzed using 1E2 (lanes 1–3), 3F12 (lanes 4–9 and 13–15) or anti-p53 pAb 240 (lanes 10–12) primary antibodies and alkaline phosphatase-conjugated secondary antibody. Lanes 1–6: *E. coli* DH5 α cells producing tagged XylS protein, bearing pBR-3F12 (lanes 1 and 4), pBR-1E2 (lanes 2 and 5) and pBR-NC (lanes 3 and 6) derived expression plasmids. Lanes 7–12: *E. coli* BL21 (DE3) cells bearing pET-3F12 derived expression plasmids, producing 3F12-tagged p53 variants Δ N39 Δ C362 (lanes 7 and 10), Δ N39 Δ C362trp248 (lanes 8 and 11) and Δ N61 Δ C362 (lanes 9 and 12). Lanes 13 and 14: Saos-2 cells transfected with pCG-3F12 derived expression plasmids producing 3F12-tagged GAPDH (lane 13) and GAPDH-NLS (lane 14). Saos-2 cells expressing untagged p53 were used for a negative control (lane 15). (B and C) Subcellular localization of 3F12-GAPDH and 3F12-GAPDH-NLS proteins. Saos-2 cells were transfected with pCG-3F12-GAPDH (B) or pCG-3F12-GAPDH-NLS (C) and tagged proteins were detected with immunofluorescence analysis.

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print earlier with HA-epitope tagged XylS, using similar technical approach (4). DNA precipitation and DNase I footprinting with a resin-bound epitope-tagged protein (10) are the methods of choice for proteins that are prone to aggregation and are difficult to purify.

High Level Bacterial Expression of Epitope-Tagged Proteins

For high-level bacterial expression of N-terminally tagged proteins, we constructed the vector pET-3F12, a derivative of pET-11c that contains the tag-encoding sequence and the cloning sites identical to pBR-3F12 (Table 1). The coding sequences for mutant p53 proteins were cloned into the vector. Mutant proteins Δ N39 Δ C362 and Δ N61 Δ C362 had their transactivation and regulatory parts deleted, but maintained the ability to bind DNA, while the mutant protein Δ N39 Δ C362trp248 had lost its DNA-

binding ability due to the point mutation in its DNA-binding domain (8). Plasmids, generated for the T7 promoter-directed expression of 3F12-tagged p53 fusion proteins were transformed into *E. coli* strain BL21 (DE3).

Expression of the tagged p53 proteins was monitored by Western blot analysis. The tag-specific 3F12 antibodies and p53-specific pAb240 antibodies were used for the detection of the proteins. Using this vector system resulted in enormous overexpression of the protein detected with 3F12 Mab as well as with pAb240 antibodies (Figure 1A, lanes 7–12).

The DNA-binding activity of the tagged p53 proteins was studied in a band-shift assay (Figure 2C). The p53 mutant Δ N39 Δ C362trp248, carrying a point mutation in its DNA-binding domain, was used as a negative control. The mutants Δ N39 Δ C362 and Δ N61 Δ C362 are functional in DNA binding

and produced a shifted band. To show that the produced complex really contains p53, the protein-DNA complexes were supershifted with the tag-specific monoclonal antibody 3F12. This way, epitope tags can be used to verify specificity of the shifted complex in a DNA band-shift assay without purification of the protein.

Expression of Epitope-Tagged Proteins in Eukaryotic Cells

For eukaryotic expression, two coding sequences of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were cloned into pCG-3F12 plasmid (Table 1). The first had 3F12 epitope fused in frame to GAPDH amino acids 2–333 (pCG-3F12-GAPDH) and the second also contained a nuclear localization signal of p53 (amino acids 305–327) fused to the C-terminus of the GAPDH protein (pCG-3F12-GAP-

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Figure 2. Use of the tagged proteins in DNA binding assays. (A) DNA precipitation by 3F12-tagged XylS. Radiolabeled *HpaII/HinfI* digest of the *Om*-containing plasmid pUPM190 was incubated with 3F12-beads containing 3F12-XylS. Unbound DNA was removed by washing. Bound DNA was extracted from the beads and analysed on non-denaturing TBE/PAGE (5%), using the fragment mixture as a marker (lane 1). Only a single fragment, which contained the XylS binding site *Om*, was retained on the beads (lane 2). (B) DNase I footprinting by resin-bound 3F12-tagged XylS. *XhoI/EcoRI* *Om* containing fragment from pUPM190 was end-labeled in the lower strand at 3' terminus and incubated with 3F12-beads containing 3F12-XylS. Unbound DNA was removed by washing. Both free and protein-bound templates were subjected to DNase I cleavage. Lane 1, G-specific DNA sequence marker; lane 2, DNase I digest of the unbound DNA fragment; lane 3, DNase I digest of the fragment bound to 3F12-XylS. Brackets indicate the region protected from the DNase I cleavage by the DNA-bound XylS. (C) Band-shift assay of the 3F12-tagged p53 proteins. The DNA-binding activity of the p53 proteins was studied by separating the protein-DNA complexes from the unbound DNA on 5% PAGE (55:1). Crude *E. coli* lysates containing p53 mutants $\Delta\Delta C362$ and $\Delta\Delta C362$ trp248 produce a shifted band (lanes 1 and 4). Supershift with tag-specific monoclonal antibody 3F12 was used to show that the protein-DNA complexes contain p53 (lanes 2 and 5). The $\Delta\Delta C362$ trp248 p53 protein carrying a point mutation in its DNA-binding domain was used as a negative control (lane 3).

DH-NLS).

Saos-2 cells were transfected with 1 μ g of expression plasmids and analysed 24 h after transfection. Expression and localization of the proteins were determined by Western blotting and immunofluorescence analysis, respectively. The 3F12 antibody recognized both proteins as single bands on the Western blot (Figure 1A, lanes 13 and 14) and no cross-reaction with cellular proteins was observed (Figure 1A, lane 15). Immunofluorescence staining of transfected cells with 3F12 antibody indicated that both proteins were localized in the appropriate compartment of the cell: The 3F12-GAPDH in the cytoplasm and 3F12-GAPDH-NLS in the nucleus (Figure 1, B and C). These results indicate that 3F12 epitope-tag can be used for the detection and determination of the localization of proteins expressed in eukaryotic cells.

Advantages of the BPV E2-Derived Epitope Tags

We analyzed the expression of proteins tagged with the BPV E2-derived epitopes in *E. coli* and eukaryotic cells. Detection of the tagged proteins both on immunoblots and by immunofluorescence staining of cells indicates low background activity, sensitivity and good signal-to noise ratio of the used epitope-antibody combinations. We did not observe any cross-reaction with cellular proteins. Because of the high specificity of the epitope-antibody interaction, our tagging system is especially useful for the studies of protein localization in the cells. In addition, we have shown that interaction of 3F12 MAb with the specific epitope is not hindered by high salt concentrations. That allows us to immunoprecipitate and immunopurify the tagged proteins in high-salt conditions and to avoid coimmunoprecipitation of contaminating proteins as well as to avoid aggregation of the protein of interest in the course of purification.

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