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Set of Vectors for the Expression of Histidine-Tagged Proteins in Vaccinia Virus Recombinants

BioTechniques 30:524-529 (March 2001)

ABSTRACT

Vaccinia virus expression vectors are widely used to direct the expression of proteins in eukaryotic cells. Here, we describe a new set of plasmid vectors designed for the expression of histidine-tagged proteins in the vaccinia system. To facilitate the rapid isolation of virus recombinants, the plasmids contain a viral gene (F13L) that serves as an efficient selection marker based on virus plaque phenotype. Histidine codons and restriction sites derived from pET-16b bacterial expression plasmid were included, thus facilitating the transfer of genes between E. coli and vaccinia expression plasmids. Plasmids in which the gene is placed downstream of either a strong vaccinia virus or a T7 promoter were constructed, allowing for constitutive or conditional expression, respectively, of the foreign protein.

INTRODUCTION

Since the development of the vaccinia virus vector technology, this system has been exploited as a mammalian expression vector for a large number of proteins. In addition to their use as immunizing agents, the proteins expressed from recombinant vaccinia viruses are often used to perform functional studies.

A selection system based on virus plaque formation allows for the positive selection of vaccinia virus recombinants (2). The system takes advantage of the nonplaquing phenotype of a mutant vaccinia virus (F13L deletion mutant, called vRB12). Selection relies on the reintroduction of gene F13L in the viral genome together with the foreign gene. While wild-type vaccinia virus plaques are visible in less than 48 h, the vRB12 deletion mutant requires a 5–7-day incubation to form tiny plaques. There-

fore, plaque isolation under the standard 2-day vaccinia plaque assay is sufficient for recombinant virus selection.

Polyhistidine-tagged proteins have been expressed in both prokaryotic and eukaryotic systems, including vaccinia virus (8,9). The advantages of histidine tagging are that the protein can be easily purified by immobilized metal affinity chromatography and detected using antihistidine tag-specific antibodies.

In this report, we describe the development of cloning vectors for the expression of histidine-tagged proteins in the vaccinia system, using either vaccinia or T7 promoters and incorporating the F13L vaccinia selection system. Thus, genes can be expressed under the control of a synthetic early/late vaccinia promoter to ensure constitutive expression of the protein during infection. Alternatively, if genes are cloned downstream of the T7 promoter, expression of the corresponding protein is achieved by co-infection with a vaccinia recombinant expressing T7 RNA polymerase.

MATERIALS AND METHODS

Cells, Virus, and Plasmids

BSC-1 and CV1 cells were obtained from ATCC (Manassas, VA, USA) and grown in Eagle's minimum essential medium (EMEM) supplemented with 5% fetal bovine serum (FBS). Virus vRB12 is a nonplaquing vaccinia virus mutant derived from the Western Reserve (WR) strain in which 93% of the F13L gene coding sequence is deleted (1). Recombinant vaccinia virus vTF7.3 (7) and plasmid pSC11 were obtained from Dr. B. Moss (National Institutes of Health, Bethesda, MD, USA). Virus infections were carried out according to standard procedures for vaccinia viruses (5). Plasmid pRB21 (2), derived from the pGEM-7 ZF(-) (Promega, Madison, WI, USA), contains an intact and functional F13L and flanking sequences. Plasmid pET-16b (Novagen, Madison, WI, USA) contains the bacteriophage T7 promoter and carries an N-terminal histidine-tagged sequence followed by a factor Xa site. Plasmid pSC11 contains the *E. coli* LacZ gene (3).

Plasmid Construction

Plasmid pTIH was constructed by modifying the promoter/polylinker region in plasmid pRB21. First, a fragment containing the T7 Φ 10 promoter and the Lac operator was amplified by PCR from plasmid pET-16b using primers 5'-ATCGAGATGTCCGCCGAAA-3' (*SalI* site underlined) and 5'-TATTTCTCGAGGGGAATTGTGAGCCGCTCACA-3' (*XhoI* site underlined), digested with *SalI* and *XhoI*, and inserted into the *XhoI* site of plasmid pRB21 to construct plasmid pT. Then, a fragment containing a foot and mouth disease virus-enhanced internal ribosome entry site (IRES) sequence was amplified by PCR from plasmid pIR (11) using primers 5'-CAGCGTGCCGTCGACCTCAGCAGGTT-3' (*SalI* site underlined) and 5'-ACCGGAATTC~~CAAGTTCGAGCTCAG~~-3' (*EcoRI* site underlined), digested with *SalI* and *EcoRI*, and inserted between the *XhoI*-*EcoRI* sites of plasmid pT to construct pTI. Finally, a fragment containing the histidine tag sequence and T7 terminator was amplified by PCR from pET-16b (Novagen) using primers 5'-TTTAAGAATTCGATATACCATGGCCAT-CAT-3' (*EcoRI* and *NcoI* site underlined) and 5'-TCCTTTCTAGAAAACCCCTCAAGACC-3' (*BglII* site underlined), digested with *EcoRI* and *BglII*, and inserted between the *EcoRI*-*BamHI* sites of plasmid pTI to construct pTIH. DNA sequencing confirmed the promoter and relevant cloning sites in plasmid pTIH. Surprisingly, we found that the final *BglII* site had been preserved during cloning.

pVH was constructed by replacing the T7 promoter/lac operator/IRES portion in pTIH by a vaccinia early/late synthetic promoter (4). This was achieved by cloning an *Acc65I*/*EcoRI* fragment containing most of the coding sequence of gene F13L and the synthetic vaccinia virus early/late promoter from plasmid pRB21 into the *Acc65I*/*EcoRI* sites of plasmid pTIH.

For expression of histidine-tagged β -galactosidase (β -gal), the coding sequence of the *LacZ* gene was inserted in frame into the *BamHI* site of pTIH and pVH. For this, a *BamHI* fragment containing the *LacZ* gene was excised from plasmid pSC11 and transferred

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Figure 1. Plasmids pTIH and pVH. Schematic diagram showing relevant features of pTIH and pVH. Relative locations of T7 promoter (T7), T7 terminator (T7t), internal ribosome entry site (IRES), ribosome binding site (rbs), vaccinia virus early/late promoter (VV), and unique restriction sites are shown. In the lower section, the sequences around the translation initiation and the location of restriction sites useful for the insertion of foreign genes are shown. The cleavage site for the protease factor Xa is indicated. Note that the polyhistidine tag and factor Xa cleavage sequence is positioned at the N terminus of open reading frames cloned into the NdeI, XhoI, or BamHI sites.

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into the *Ban*HI sites of plasmids pTIH and pVH to generate pTIH-LacZ and pVH-LacZ, respectively.

Isolation of Recombinant Viruses

Virus manipulations were carried out using standard protocols (5). To generate virus recombinants, CV1 cells were infected with vaccinia virus vRB12 and then transfected with pTIH-LacZ or pVH-LacZ using lipoTAXI transfection reagent (Stratagene, La Jolla, CA, USA). Normal plaque forming viruses were selected by plaquing the progeny under an agarose overlay. After a 2-day incubation, large plaques were selected. After a total of three rounds of plaquing, virus stocks were grown and titrated. The resulting recombinant viruses were designated vTIH-LacZ and vVH-LacZ, respectively.

Expression of β -Gal

Monolayers of BSC-1 cells in 2-cm² wells were either co-infected with vTIH-LacZ and vTF7.3 or infected with vVH-LacZ. After 24 h, cells were harvested and disrupted in 100 μ L 1% Nonidet[®] P-40 (NP40), 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA. After clarification by low speed centrifugation (10 min, 12000 $\times$ g), the β -gal activity in the extracts was determined by O-nitrophenyl β -D-galactopyranoside (ONPG) assay as described previously (10) in parallel with defined amounts of purified β -gal (Sigma, St. Louis, MO, USA).

Purification of Recombinant Protein

Confluent monolayers of BSC-1 cells were co-infected with vTIH-LacZ and vTF7.3 viruses or infected with vVH-LacZ. After 72 h, the cells were collected by centrifugation, resuspended in 10 mL of 10 mM imidazole, 20 mM phosphate, 0.5 M NaCl, pH 7.4, and then subjected to three rounds of freeze-thawing followed by sonication. Histidine-tagged β -gal was purified using a Ni²⁺ affinity chromatography column (Amersham Pharmacia Biotech, Uppsala, Sweden). For this, cell extracts were loaded onto 1-mL Ni²⁺ columns and washed with 10 mM imidazole, 20 mM phosphate, 0.5 M NaCl,

pH 7.4. Bound protein was finally eluted with 500 mM imidazole, 20 mM phosphate, 0.5 M NaCl, pH 7.4. Fractions eluted from the column were analyzed by SDS-PAGE and Coomassie[®] Brilliant Blue staining.

RESULTS AND DISCUSSION

Plasmids pTIH and pVH (Figure 1) contain a N-terminal tag sequence including 10 histidine codons, a factor Xa cleavable sequence, and cloning sites derived from the bacterial expression plasmid pET16b. In addition, they include the vaccinia virus F13L gene for virus selection and flanking virus sequences to mediate recombination in the vaccinia genome. pVH is designed for constitutive expression of the protein, since the foreign gene is placed downstream of a synthetic early/late vaccinia promoter. pTIH contains a T7 promoter together with a sequence to generate a hairpin loop structure at the 5' end of the T7 transcript and an IRES element. Hairpin loop structure based on lac operator has been shown to enhance stability (12), and IRES elements enhance the translatability (6) of T7

transcripts in the vaccinia system.

To demonstrate the usefulness of the system, we cloned the *E. coli* LacZ gene into pVH and pTIH plasmids in frame with the histidine tag. The resulting plasmids were used to generate recombinant viruses vVH-LacZ and vTIH-LacZ, respectively. As expected, infection with vVH-LacZ produced constitutive expression of β -gal, whereas expression from vTIH-LacZ was dependent on co-infection with a T7-RNA polymerase-expressing virus (Figure 2A). In both cases, β -gal production was similar and amounted to 1–2 mg β -gal/10⁸ cells in 24 h.

To analyze the functionality of the histidine tag for purification, two roller bottle cultures were infected with vVH-LacZ or co-infected with vTIH-LacZ and vTF7.3. As in the previous experiment, levels of β -gal production by vVH-LacZ and vTIH-LacZ were similar. Cell lysates were prepared and subjected to affinity chromatography using a Ni²⁺ resin. Results obtained from the vVH-LacZ infection are shown in Figure 2B. After elution of unbound material, retained protein was eluted with a buffer containing 500 mM imidazole. A single protein band

Figure 2. Expression and purification of β -gal. (A) β -gal expression. BSC-1 cells were mock infected (M) or infected with the vaccinia viruses indicated and lysed at 24 h postinfection. The amount of β -gal in the cell lysates was estimated by enzymatic assay. (B) β -gal purification. Infected cell lysates containing 10 his-tagged β -gal were subjected to Ni²⁺ affinity chromatography. Proteins were visualized by Coomassie staining. Lane 1, cell lysate; lane 2, unbound material; lane 3, wash with 10 mM imidazole; lane 4, elution with buffer containing 500 mM imidazole.

of the expected size for β -gal was detected in the eluted samples (Figure 2B). About 2 mg purified protein, estimated by the β -gal assay, was obtained from two roller bottle cultures (1600-cm² culture or 2.5×10^8 cells).

The expression system presented here combine a method for easy isolation by positive selection of vaccinia recombinants expressing a foreign protein with a purification system based on the incorporation of histidine residues in the expressed protein. Since the tag sequence and cloning sites are derived from bacterial expression plasmid pET-16b, transfer between bacterial and vaccinia expression vectors is straightforward. Finally, the use of T7-driven expression should facilitate the expression of genes whose constitutive expression prevents the isolation of vaccinia recombinants.

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I.G. and M.M.L. contributed equally to this work. We thank Alí Alejo and Julio Coll for critical reading of the manuscript. Work was supported by grant no. PB98-0046 from Dirección General de Investigación Científica y Técnica. Address correspondence to Dr. Rafael Blasco, Departamento de Mejora genética y biotecnología, INIA, Ctra. La Coruña km 7, E-28040 Madrid, Spain. e-mail: blasco@inia.es

Received 19 July 2000; accepted 22 September 2000.

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