

Vectors to Target Protein Domains to Different Cellular Compartments

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ABSTRACT

The pcDNA3 mammalian expression vector uses the cytomegalovirus (CMV) promoter to express proteins cloned into an adjacent polylinker. We have modified this vector to create three different targeting constructs: Go to Plasma Membrane (pGTM), Go to Nucleus (pGTN) and Go to Nucleus, Activate Transcription (pGTNAT). pGTM expresses a protein as a fusion to a myristic acid attachment signal, which targets proteins to the cell membranes. pGTN expresses an inserted protein as a fusion to a nuclear localization sequence (NLS), targeting it to the nucleus. pGTNAT incorporates an NLS (directing proteins to the nuclear compartment), an acid blob (a transcriptional activation domain) and a hemagglutinin epitope tag, creating a 107-amino acid fusion domain. We then cloned green fluorescent protein (GFP) into the three novel vectors and pcDNA3, and we transfected HeLa cells to test the new targeting constructs. Immunofluorescence analysis showed that the GFP protein localizes to the nucleus when over-expressed in pGTN and pGTNAT, localizes to the plasma membrane and perinuclear membrane when in pGTM and is ubiquitous through the cell in pcDNA3. We anticipate that these new vectors will prove very useful in future expression studies to examine the function of particular proteins when they are localized to specific cellular compartments.

INTRODUCTION

One of the approaches commonly followed in characterizing a novel protein is to over-express the intact protein, or protein truncations, from an expression vector and then establish interactions between particular domains of this protein and its cellular partners. One problem with such an approach is that the dissection of a protein

into constituent domains may deprive some domains of particular signals that formerly directed the full-length protein to specific subcellular compartments. With the protein domains removed from their normal cellular compartment, and hence sequestered from their cellular partners, it becomes problematic to assess any interaction, even between molecules that normally associate with high affinity. To overcome this localization problem, we have modified the widely used mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA, USA) to create three different targeting vectors that direct proteins or their domains to particular cellular compartments.

pcDNA3 utilizes a cytomegalovirus (CMV) promoter in conjunction with the bovine growth hormone (BGH) polyadenylation transcription termination signal sequences to allow high-level mRNA expression of inserted genes. It also encompasses a neomycin-resistance gene (neo^R) to allow the selection of stable mammalian cell lines. Here we describe three novel versions of pcDNA3: Go to Membrane (pGTM), Go to Nucleus (pGTN) and Go to Nucleus, Activate Transcription (pGTNAT). pGTM expresses a protein as a fusion to a myristic acid attachment signal (MAS) (8), which targets proteins to the cell periphery. pGTN expresses an inserted protein as a fusion to a nuclear localization sequence (NLS) targeting it to the nucleus. pGTNAT incorporates an NLS as with pGTN, but also includes an acid blob (AB) (a transcriptional activation domain) and a hemagglutinin epitope tag; it is suitable for use in mammalian two-hybrid strategies (10).

As a test of function, we cloned green fluorescent protein (GFP) of the jelly fish *Aequorea victoria* (2,7) into the three new vectors and the parent vector pcDNA3, and transfected HeLa cells. Immunofluorescence analysis showed that the GFP localizes to the nucleus when over-expressed in pGTN and pGTNAT, localizes to the cell plasma membrane and perinuclear membrane when in pGTM and is ubiquitous throughout the cell in pcDNA3. We anticipate these vectors will facilitate study of protein interactions at specific subcellular locations.

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MATERIALS AND METHODS

Preparation of pGTM

To construct pGTM, we designed two primers (EG436 and EG437). When annealed, these primers possessed the sequences in Table 1, which encodes the MAS (8) MGSSKSK with overhangs complementary to *Hind*III (5') and *Eco*RI (3'). The annealed oligonucleotides were gel-purified and ligated into *Hind*III-*Eco*RI-restricted pcDNA3 and transformed into *E. coli*. The MAS sequence was confirmed by automated sequencing. Figure 1A shows the domain structure and cloning sites of pGTM.

Preparation of pGTNAT

To construct pGTNAT, pJG4-5 (4) was used as a source of targeting and transcriptional activation. pJG4-5 contains a 320-bp fusion cassette incorporating an NLS from simian virus 40 (SV40) (5), an AB that activates transcription (6) and a hemagglutinin tag (HA-tag). The gel-purified fusion cassette was ligated into *Hind*III-*Eco*RI-restricted pcDNA3 and transformed into *E. coli*. The sequence of the fusion cassette insert was confirmed by automated sequencing. Figure 1B shows the domain structure and cloning sites of pGTNAT.

Table 1. EG436 and EG437 Primer Sequences

5'-AG CTT ATG GGG AGT AGC AAG AGC AAG G-3'
3'-A TAC CCC TCA TCG TTC TCG TTC CTT AA-5'
M G S S K S K

Preparation of pGTN

To construct pGTN, we designed two primers (EG377 and EG398) that possess the sequence 5'-CTGGCTTATCGAAATTAAT-3' and 5'-GGGGAATTCACCAGCTACGTTTC-3', encoding the SV40 large T derived NLS (PP-KKKRKVA) flanked by *Hind*III and *Eco*RI restriction sites. We performed a polymerase chain reaction (PCR) using pGTNAT as template. The amplified product was gel-purified and ligated into restricted *Sma*I pBluescript® (Stratagene, La Jolla, CA, USA), from which the NLS was then excised and ligated into *Hind*III-*Eco*RI-restricted pcDNA3. The sequence of the NLS was confirmed by automated sequencing. Figure 1C shows the domain structure and cloning sites of pGTN.

Cloning the GFP into the Targeting Vectors and pcDNA3

To test the new vectors, GFP was used as a reporter. The complete GFP coding sequence was excised from pUC19 using an *Xba*I-*Apa*I digestion.

The purified product was inserted into pGTM, pGTN, pGTNAT and pcDNA3 similarly cleaved with *Xba*I and *Apa*I as cloning sites. Ligations were transformed into DH5α™ cells (Life Technologies, Gaithersburg, MD, USA), then plasmids were isolated using a Midi Prep Kit (Qiagen, Chatsworth, CA, USA). DNA sequencing was performed for each vector to check for the insertion of GFP in-frame.

Transfection and Immunofluorescence Analysis

Cells were split 24 h before transfection onto cover slips. Transfection of cells was carried with LIPOFECTAMINE™ Reagent (Life Technologies) using 1 μg of the plasmid and 6 μL of LIPOFECTAMINE in 200 μL OPTI-MEM® I Reduced Serum Media (Life Technologies). Cells were incubated for 6 h with the transfection mixture and then supplemented with 2 mL Dulbecco's modified Eagle medium (DMEM). Cells were then incubated for an additional 12 h before immunofluorescence analysis; coverslips were washed twice with 1× phosphate-buffered saline (PBS) then fixed with 3.5% paraformaldehyde for 7 min. Following that, cells were washed with 1× KB (0.01 M Tris, pH 7.5, 0.15 M NaCl and 0.1% bovine serum albumin [BSA]) for 5 min and then were observed microscopically using a 40× oil-immersion objective.

RESULTS AND DISCUSSION

Transfection of HeLa cells with GFP in the three targeting vectors and the parent plasmid pcDNA3 followed by immunofluorescence was used to test the function of the three novel targeting vectors.

Because the CMV promoter used in pcDNA3 is so potent, proteins are expressed at high levels intracellularly. With standard incubation post-transfec-

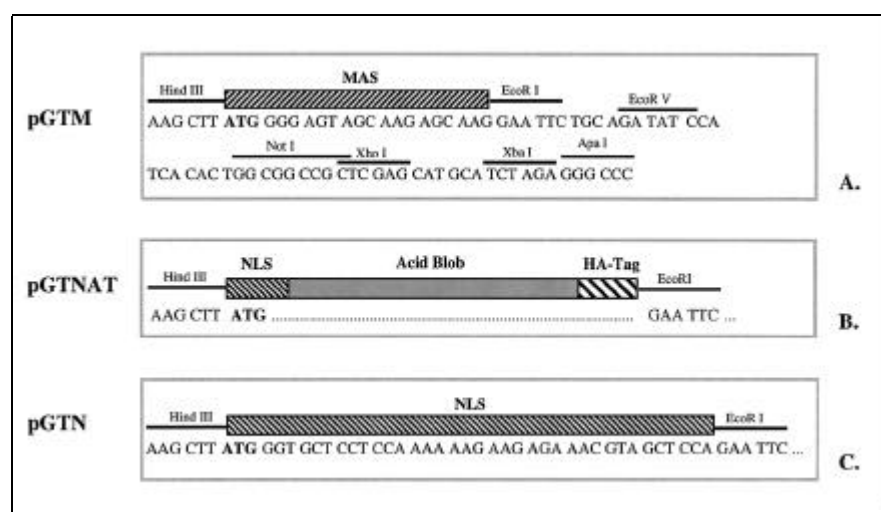


Figure 1. (A) Polylinker region of pGTM showing the reading frame of the MAS in relation to available restriction sites. (B) Polylinker region of pGTNAT with the reading frame of the NLS, AB and HA-Tag. (C) Polylinker region of pGTN with the coding frame of the NLS shown relative to the cloning sites.

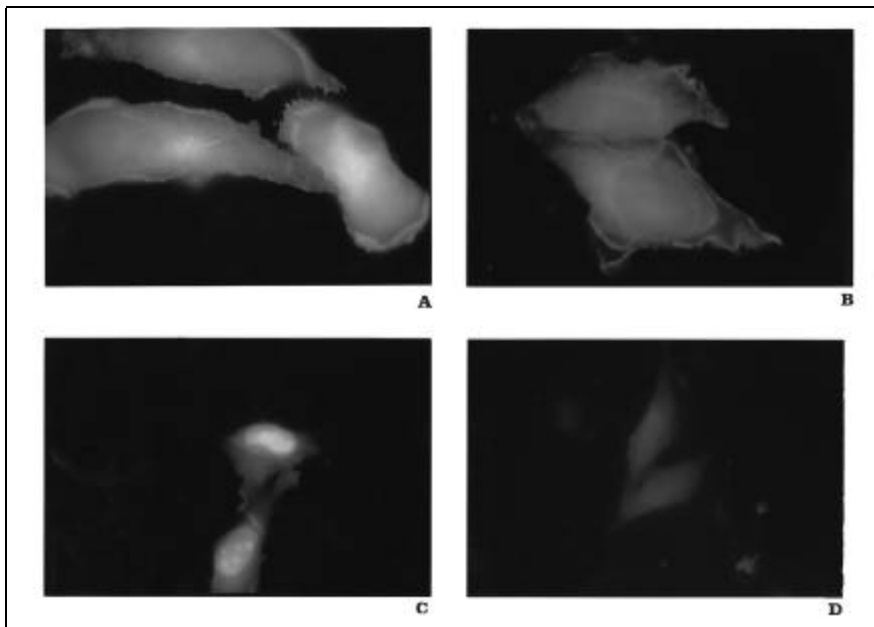


Figure 2. HeLa cells seen under fluorescence microscope using a 40× oil-immersion objective after 14-h recovery post-transfection. With pGTM-GFP, cells show fluorescence at the cell membrane site (A and B); pGTN-GFP cells show heavy nuclear fluorescence (C); and pcDNA3-GFP cells show weaker staining ubiquitous throughout the cell (D).

tion (24–48 h), GFP expressed from the parental and targeting vectors is abundant ubiquitously throughout the cell, with obvious artifactual aggregates of proteins. This high over-expression is linked to substantial cellular toxicity, as we noted lysed or abnormally shaped cells on plates at those latter times. In contrast, we determined that at earlier time points following transfection (12–15 h), protein levels were lower, and little if any toxicity was noted.

When a plasmid over-expressing GFP from pGTM was transfected into HeLa cells, immunofluorescence analysis at an optimal period post-transfection (ca. 14 h) showed a bright fluorescence concentrated at the cell membranes (Figure 2, A and B). Bright fluorescence was observed both at the cell periphery, reflecting localization to the plasma membrane and also at the nuclear envelope. Cytoplasmic staining was relatively low in comparison.

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Transfection of pGTN (Figure 2C) or pGTNAT (not shown) into HeLa cells resulted in intense nuclear staining, with a considerable reduction in general cytoplasmic staining (Figure 2C). In contrast, following transfection of GFP expressed from the parent vector pcDNA3, the protein was found to be ubiquitous throughout the cell, with no specific localization to any subcellular compartment (Figure 2D). In summary, these data indicate that GFP localizes to the cell membranes when expressed from pGTM, to the nucleus when expressed from pGTN or pGTNAT and is ubiquitous in the cell when expressed from pcDNA3.

A major problem in studying proteins and protein domains is the localization of the truncated domains to subcellular compartments that do not match the normal location of the protein. This is generally addressed by over-expressing proteins to high levels, so that some population reaches the intended location; the disadvantage of this approach is that high-level expression can have cellular toxicity and may lead to nonspecific interactions. We have overcome this problem by constructing three mammalian targeting vectors (pGTM, pGTN and pGTNAT) to direct the expressed protein to the cell membranes or to the nucleus. We note that in addition to their function in targeting proteins, some other aspects make these vectors useful in mammalian cells studies. pGTNAT in particular might be useful in mammalian two-hybrid applications (10).

In addition, the testing of the novel vectors resulted in three independent constructs: pGTM-GFP, pGTN-GFP and pGTNAT-GFP, which incorporate a reporter gene for protein expression, GFP, under the control of the CMV promoter. These can be used as protein localization markers facilitating analysis of subcellular compartments by immunofluorescence. GFP presents some advantages over other markers (such as β -galactosidase and luciferase) in that it does not require the addition of exogenous substrates or cofactors, but is detected by simply irradiating the cell with near UV or blue light, making it very easy to monitor protein localization within the cell.

Finally, we anticipate that other

mammalian vectors will be constructed to direct proteins and protein domains to other locations in the cell by the use of different targeting signals. Candidates for such signals might be mitochondrial (1), focal adhesion targeting (9), Golgi complex targeting (3) and others. This approach might also be modified to include the use of weaker or inducible promoters to lessen the toxic effects of over-expression as well as to allow a tighter control of compartment localization.

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