of a biotinylated probe to RNA when hydrolysis and removal of endogenous proteins are not performed (4).

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Mapping of Nuclear Localization Signals by Simultaneous Fusion to Green Fluorescent Protein and to β-Galactosidase

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Mapping of nuclear localization signals (NLSs) is an important topic for the investigation of nuclear protein function. The classical approach is to determine the relative position of a NLS by means of deletion mutagenesis and then to confirm its functionality by constructing fusions to cytosolic proteins like chloramphenicol acetyl transferase (e.g., Reference 12), β-galactosidase (β-gal; e.g., Reference 8) or, as reported recently, to green fluorescent protein (GFP) (e.g., Reference 6). The NLS activity of a polypeptide is proven when the fusion proteins are directed to the nucleus. The GFP of the jelly fish Aequorea victoria is a small protein of 238 amino acids that exhibits a bright green fluorescence upon illumination with blue light. Because of its small molecular weight of 30 kDa, GFP is suitable for use as a fluorescent tag to investigate mechanisms of intracellular trafficking (for a review see Reference 4). However, this low molecular weight is disadvantageous for the mapping of NLS sequences because GFP alone or small protein fragments fused to GFP can diffuse passively into the nucleus even in the absence of a NLS.
Benchmarks

To avoid this passive diffusion, we created two vectors, termed pHM829 and pHM830, that are suitable for the expression of protein fragments or total proteins simultaneously fused to GFP and to β-gal (see Figure 1). The GFP part functions as a fluorescent tag that can be detected both in living and in paraformaldehyde-fixed cells. The β-gal part increases the molecular weight of the fusion proteins, thus preventing passive diffusion of smaller fusion proteins into the nucleus. In addition, it allows the use of alternative detection methods either by means of histochemical staining or by immunodetection of β-gal with commercially available monoclonal antibodies (MAbs). Although a simultaneous detection of GFP-fluorescence and β-gal activity by histochemical staining is not possible because of the strong absorbance of the GFP fluorescence by the indigo product of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), immunodetection of β-gal using a MAb against β-gal (Boehringer Mannheim GmbH, Mannheim, Germany) together with a TRITC-conjugated secondary antibody (Dianova GmbH, Hamburg, Germany) facilitates co-localization of GFP and β-gal within the same cell. Even biochemically inactive fusion proteins might be detected by using MAbs directed against β-gal. Because full-length proteins can also be tagged with GFP and β-gal, both deletion analysis and the test for functionality of a supposed NLS can be performed within the same vector. This circumvents the requirement of specific antibodies for immunodetection of the respective protein. Whereas in plasmid pHM829, DNA fragments can be fused to the 3′ end of β-gal and to the 5′ end of GFP, it is the opposite in pHM 830. Therefore, cloning into both vectors in parallel could additionally reveal whether the NLS activity of a protein fragment depends on its relative position within a protein.

We tested the functionality of both vectors and found that after cloning into these vectors, entire proteins or fragments of polypeptides comprising NLS activity had accumulated in the nucleus. This was shown for the strong NLSs of nucleoplasmin and the large-T antigen of simian virus 40 (SV40) and for the full-length protein pUL84 of human cytomegalovirus (HCMV). In addition, nuclear localization could also be observed for the NLS of the infected cell protein 8 (ICP8) of herpes simplex virus type 1 (HSV1) and the NLS-2 of the assembly protein precursor of the simian cytomegalovirus (SCMV); both of which are supposed to be rather weak NLSs (9,10). On the other hand, in the absence of a NLS, the fusion proteins were retained efficiently in the cytosol. Therefore, it can be concluded that pHM829 and pHM830 are generally applicable vectors for the simple and reliable mapping of NLS sequences.

All DNA manipulations during construction of the following plasmids were performed using standard methods (1). To create the NLS test vector pHM829, a modified form of GFP termed GFP-H (adapted to mammalian codon usage; Reference 13), was amplified by polymerase chain reaction (PCR) using plasmid pTR-UF5 (kindly provided by N. Muzyczka, University of Florida, Gainesville, FL, USA) as a template and then inserted into the multiple cloning site (MCS) of plasmid pcDNA3 (Invitrogen BV, Groningen, The Netherlands) using HindIII and XhoI sites. The PCR primers for this procedure introduced additional restriction sites for ClaI, XhoI and Asp718 upstream and an EcoRI site downstream of GFP-H, while the XhoI site of pcDNA3 was destroyed. Afterwards, the HindIII and XhoI sites of this construct were used for the insertion of β-gal linked to the 5′ end of the GFP-H sequence by subcloning the β-gal PCR product that was generated using pSV-β-Galactosidase Control Vector (Promega, Mannheim, Germany) as a template and primers that introduced the two additional unique restriction sites between β-gal and GFP-H, namely NheI and XhoI. The resulting plasmid was called pHM760. Plasmid pHM829 was then created by insertion of a double-stranded synthetic oligonucleotide into the NheI and the XhoI sites of pHM760. By this procedure, the origi-
nal restriction sites for *Nhe*I and *Xba*I were destroyed, and a MCS was introduced that is comprised of several unique restriction sites (*Nhe*I, *Afl*II, *Sac*I, *Pml*I and *Xba*I) and is flanked by short sequences coding for spacer peptides, namely pro-gly-pro-ala upstream of the MCS and gly-pro-gly-pro downstream of the MCS. The NLS test vector pHM830 was created in an analogous manner by sequential PCR amplification and cloning of the coding sequences for GFP-H and β-gal into plasmid pcDNA3 (resulting in plasmid pHM761) followed by insertion of an identical MCS. Afterwards, we constructed control plasmids that contained: (i) coding sequences for the nucleoplasmin NLS, (ii) NLS of the SV40 large-T antigen, (iii) NLS of the ICP8 of HSV1 and (iv) NLS-2 of the assembly protein precursor of SCMV. These

![Figure 2. Expression of plasmid pHM830 and various fusion constructs in HeLa cells. HeLa cells were transfected either with the NLS test vector pHM830 (Panels a–d) or constructs containing the nucleoplasmin NLS (Panels e–h), the SV40 large-T antigen NLS (Panels i, k–m), the herpes simplex virus ICP8 NLS (Panels n–q), the NLS-2 of the assembly protein precursor of simian CMV (Panels r–u) within plasmid pHM830 or a construct containing the ORF UL84 of HCMV within plasmid pHM761 (Panels v–y). For Panels a–c, e–g, i, k, l, n–p, r–t, v, w and x, HeLa cells were fixed with 3.7% paraformaldehyde in phosphate-buffered saline followed by permeabilization and subsequent immunodetection of β-gal by using a MAb against β-gal and a TRITC-conjugated secondary antibody. In addition, cells were counterstained with DAPI to visualize the nuclei. Panels a, e, i, n, r and v show the GFP fluorescence; Panels b, f, k, o, s and w show the corresponding TRITC fluorescence obtained after immunodetection of β-gal, whereas the corresponding DAPI fluorescence can be seen in Panels c, g, l, p, t and x. For Panels d, h, m, q, u and y, β-gal in transfected HeLa cells was detected histochemically in a separate reaction.](image)
regions of DNA were inserted as synthetic oligonucleotides into the MCS of pHM829 and pHM830. Second, the open reading frame (ORF) UL84 of HCMV, which encodes a putative nuclear protein, was amplified by PCR using plasmid pcDNAUL84 (3) as a template and primers with XbaI sites. This PCR product was then cloned into the XbaI site of plasmids pHM760 and pHM761 (identical in structure to pHM829 and pHM830, respectively, but without a MCS).

To assess the intracellular localization of the encoded fusion proteins, HeLa cells were transfected by the calcium phosphate co-precipitation procedure using BES (1). Living cells were then analyzed for GFP expression by fluorescence microscopy. Alternatively, the cells were fixed for detection of GFP fluorescence (Figure 2, Panels a, e, i, n, r, v), and stained with a MAb against β-gal and a TRITC-conjugated secondary antibody (Figure 2, Panels b, f, k, o, s, w) followed by counterstaining with 4',6-diamidino-2-phenylindole (DAPI) to visualize the nuclei (Figure 2, Panels c, g, l, p, t, x). In addition, transfected cells were fixed for subsequent histochemical staining of β-gal activity (Figure 2, Panels d, h, m, q, u, y) (2). As anticipated, the fusion of GFP to either the C- or N-terminus of β-gal in the absence of a NLS resulted in a clear retention of the fluorescence signal in the cytoplasm (Figure 2, Panels a–e; fluorescence patterns for GFP, β-gal and DAPI after transfection of plasmid pHM830, respectively). Insertion of either the nucleoplasmin NLS (KRPAATKKAGQKK; Reference 11), which is an example of a bipartite NLS, or the SV40 large-T antigen NLS (PKKKRKV; Reference 7), represents a typical monopartite nuclear localization sequence. J. Biol. Chem. 269:615-623.

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Benchmarks