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Nuclear localization of budgerigar fledgling disease virus capsid protein VP2 is conferred by residues 308–317

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The capsid protein VP2 of budgerigar fledgling disease virus (BFDV) contains two sequences (residues 309–315 and 334–340) which are homologous to the prototypic nuclear localization sequence (NLS) of the simian virus 40 T-antigen. Using recombinant potential NLS-β-galactosidase fusion proteins we identified amino acid residues 308–317 (VPKRKRKLPT) to be the NLS of BFDV capsid proteins VP2 and VP3. Microfluorometry studies show that the BFDV-VP2 signal is considerably more efficient in nuclear transport kinetics, than the NLS of SV40-VP2, corresponding to amino acid residues 317–326 (PNKKKRKLSR).

Nuclear localization sequence; β-Galactosidase fusion protein; Budgerigar fledgling disease virus

1. INTRODUCTION

Budgerigar fledgling disease virus (BFDV) and Simian virus 40 (SV40) are both members of the polyoma virus subgroup of the papova virus family. The icosahedral capsid structure of these viruses consists of structural proteins VP1, VP2 and VP3 [1]. Among these VP2 and VP3 are closely related, since VP3 is identical to the C-terminal 234 amino acids of the VP2 protein's 352 residues, in SV40. The structure of BFDV viral proteins is very similar in general, but shows two clusters of basic amino acid residues (BFDV-VP2 residues 309–315 and 328–340, respectively), instead of one in SV40 which is conserved in all polyoma viruses [2]. In the case of SV40-VP2, this region is necessary and sufficient for nuclear localization [3–5] and as a nuclear localization signal (NLS) it shows considerable similarity to the prototype NLS of SV40 T-antigen [6,7]. The structure of NLSs and potential mechanisms of their function have recently been reviewed by Roberts [8], Silver [9] and Garcia-Bustos et al. [10]. In order to determine the NLS of BFDV-VP2 and to compare it with the NLS of SV40-VP2, we constructed recombinant proteins in which the two presumptive NLS segments of BFDV-VP2 and the single one of SV40-VP2 were inserted into the amino terminus of *E. coli* β-galactosidase.

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2. MATERIALS AND METHODS

2.1. *Construction and expression of NLS-*lacZ *hybrids*

To create N-terminal NLS-β-galactosidase fusion genes, appropriate oligonucleotides were synthetized on an Applied Biosystems Synthetizer (Weiterstadt, Germany) and inserted in an *Kpn*I–*Hin*dIII partially digested and gel purified vector fragment of pPR24 [12]. Hybrid *lacZ* genes were characterized by restriction analysis and expressed under p_{tac} promoter control in the presence of 0.5 mM IPTG (Boehringer Mannheim), overnight at 37°C. Expressed fusion proteins (see Fig. 1) showed β-galactosidase activity, at molecular masses of 117 kDa monomer.

2.2. *Protein purification*

Purification of the fusion proteins was as described previously [11–13] via affinity chromatography on *p*-amino-benzyl-β-D-galactosidase-sepharose (Sigma, Deisenhofen, Germany). Fusion proteins were concentrated in the presence of PBS or injection buffer (48 mM K_2HPO_4, 14 mM NaH_2PO_4, 45 mM KH_2PO_4, pH 7.2) on centricon 30 (Amicon, Witten, Germany). Protein concentration was between 0.5–6.5 mg/ml.

2.3. *Mammalian cells and microinjection*

Rat hepatoma tissue culture cells (HTC) and Vero cells used for the microinjection experiments were cultured in DMEM medium in the presence of 10% fetal calf serum. In the case of HTC, polykaryons were formed by treatment of confluent monolayers with polyethylene glycol (PEG; Boehringer Mannheim), which has the advantage that the cells are not able subsequently to undergo mitosis [14].

2.4. *Histochemical assay of β-galactosidase and 5-IAF labelling*

For determining the subcellular localization of injected fusion proteins a previously described histochemical assay [12,13,15] was used. This assay makes use of the β-galactosidase activity of the hybrid proteins. After microinjection, culture dishes with microinjected cells were kept in the incubator for different times, before they were fixed and labeled with X-gal. During an incubation period over 6–16 h —depending on β-galactosidase activity — the blue colour developed at various time points and its subcellular location could be analysed by microscopy (see Fig. 2). For microfluorimetric studies the fusion

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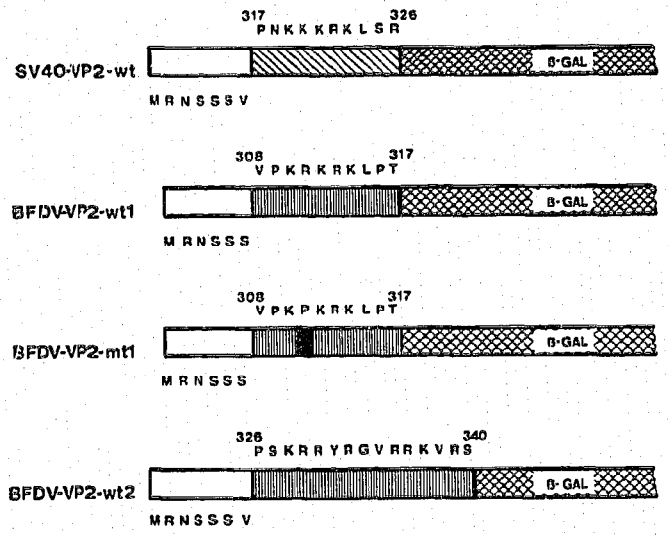

Fig. 1. Scheme of the fusion proteins constructed in the present study. The N- and C-terminal amino acid residues of BFDV-VP2 ▥ and SV40-VP2 ▩ are indicated by numbers. Empty boxes correspond to linker regions. β-Gal ▨; β-galactosidase moiety (amino acid residues 6–1023). The black bar indicates an amino acid exchange (R →P) for amino acid residue 311 of BFDV-VP2.

proteins were labeled with 5-iodoacetamidofluorescein (5-IAF; Molecular Probes, Eugene, USA) [12], and the distribution of proteins was determined after microinjection by microfluorimetry [12,16,17] at various time points.

3. RESULTS AND DISCUSSION

In kinetic determinations of the subcellular localization of fusion proteins (Fig. 1) by the X-gal assay, SV40-VP2-wt was completely accumulated in the nucleus 15 h after injection (Fig. 2A), whereas BFDV-VP2-wt1 was already accumulated in the nucleus after 3 h (Fig. 2C). In contrast to the wild type proximal sequence of BFDV-VP2, both a mutant variant thereof ($R^{311} \rightarrow P$; BFDV-VP2-mt1) and a fusion protein containing the distal sequence of basic amino acid residues instead of the 308–317 sequence (BFDV-VP2-wt2) resulted in cytoplasmic localization of the respective proteins even 13 or 10 h after microinjection, respectively (Fig. 2B,D).

Nuclear accumulation kinetics were studied by scoring microinjected Vero cells for a fusion protein distribution either as cytoplasmic, approximately equal cytoplasmic and nuclear, predominantly nuclear, or completely nuclear localization of the fusion proteins after various incubation times (Fig. 3). The results show clearly that BFDV-VP2-wt1 was completely nuclear localized within 3 h ($t_{1/2} \simeq 1.5$ h, Fig. 3B) whereas SV40-VP2-wt nuclear accumulation was maximal at 15 h (Fig. 3A). No nuclear accumulation of BFDV-VP2-wt2 was observed up to 16 h (Fig. 3C).

For more detailed studies of nuclear transport kinetics with increased time resolution, fluorescence labelled fusion proteins were employed. Twenty-six minutes after cytoplasmic microinjection in HTC polykaryons the nucleocytoplasmic fluorescence ratio $F_{n/c}$ was 1.45

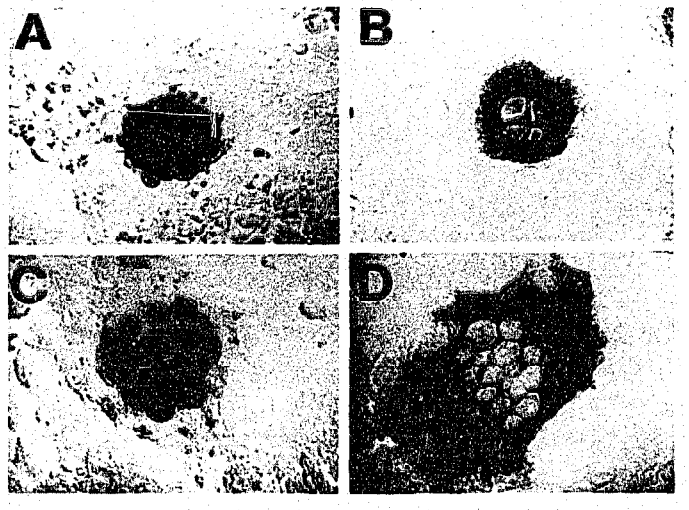

Fig. 2. Subcellular localization of BFDV-VP2 and SV40-VP2 fusion proteins determined by a histochemical assay (X-gal method). Fusion proteins were microinjected into the cytoplasm of HTC polykaryons at 37°C. The cells were fixed after different times. Subcellular location was determined by transmission microscopy. (A) SV40 VP2-wt 15 h, (B) BFDV-VP2-mt1 13 h, (C) BFDV-VP2-wt1 3 h, and (D) BFDV-VP2-wt2 10 h after microinjection, respectively.

± 0.15 (mean ± SD, $n = 4$) for BFDV-VP2-wt1. In contrast, BFDV-VP2-mt1 showed only a value of $F_{n/c} = 0.44 \pm 0.07$ (mean ± SD, $n = 6$) The $F_{n/c}$ value for SV40-VP2 was 0.46 ± 0.09 (mean ± SD, $n = 5$). No differences were observed between HTC and Vero cells. In summary, our data indicate, that amino acid residues 308–317 (VPKRKRKLPT) of BFDV are responsible for nuclear localization of fusion proteins BFDV-VP2 and BFDV-VP3 at higher rates than the SV40-VP2 signal sequence. The crucial role of arginine 311 of BFDV-VP2-wt for nuclear transport was demonstrated through exchange of arginine-311 by proline, which completely abolished nuclear transport of the hybrid protein. The second cluster of basic residues in BFDV-VP2 corresponding to amino acid residues 326–340 (PSKRRYRGVRRKVRS), however, was not able to induce nuclear transport of β-galactosidase fusion proteins. Amino acid residues 308–315 of BFDV-VP2 are very similar to residues 317–324 of SV40-VP2 and show the NLS consensus sequence motif X-X-K-K/R-X-K/R discussed in a review by Roberts [8]. Roberts et al. [3] as well as Wychowski et al. [4] have shown that SV40-VP2 sequences, harboring the chosen amino acid residues in this study for the construct SV40 VP2-wt are able to target cytoplasmic proteins poliovirus VP1 and pyruvate kinase respectively into the nucleus. Surprisingly, transport into the nucleus of BFDV-VP2-wt1 was much faster than that of a fusion protein carrying the NLS of SV40-VP2 317-PNKKKRKLSR-326. In a previous study [13] we have shown that the nuclear transport kinetics of the NLS of SV 40 T-antigen is enhanced by a casein kinase II site flanking the NLS. Within the flanking sequences of the NLS of SV40-VP2 there is no

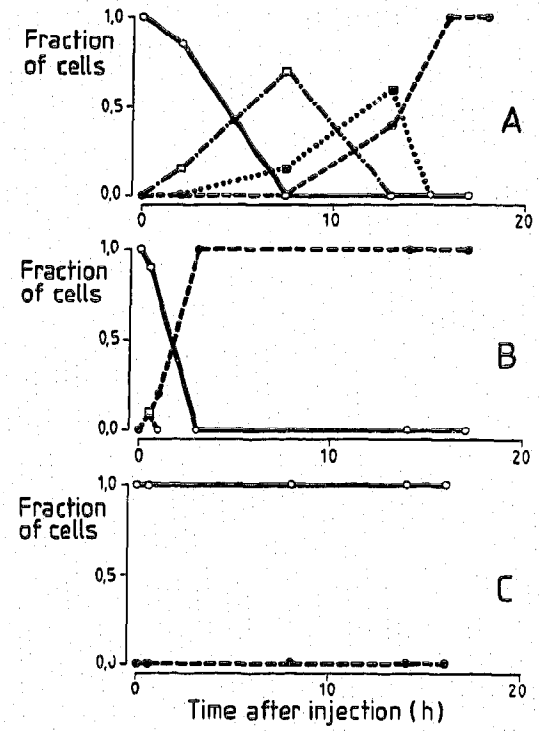

Fig. 3. Kinetics of nuclear accumulation. After microinjection of fusion proteins into the cytoplasm, cells (Vero) were kept in the incubator at 37°C for the times indicated and then fixed and subjected to the X-gal assay. 45–100 cells for each construct were scored according to cytoplasmic localization (○—○), approximately equal distribution in cytoplasm and nucleus (□·–·□), pre-dominantly nuclear localization (▣·····▣), or completely nuclear localization (●----●). (A) SV40-VP2-wt, (B) BFDV-VP2-wt1, (C) BFDV-VP2-wt2.

putative phosphorylation site for casein kinase II, able to increase the transport rate. In contrast such a putative site is present in BFDV-VP2 (residue 235- SFFE-238) although further away and absent from the fusion proteins used in this study. Therefore differences observed in the rates of translocation have to be due to deviations in the two signal sequences and their immediate vicinities, and the BFDV-VP2 signal may be somewhat closer to an ideal nuclear location signal than the SV40-VP2 sequence.

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