

## Characterization of a nuclear localization signal of canine parvovirus capsid proteins

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We investigated the abilities of synthetic peptides mimicking the potential nuclear localization signal of canine parvovirus (CPV) capsid proteins to translocate a carrier protein to the nucleus following microinjection into the cytoplasm of A72 cells. Possible nuclear localization sequences were chosen for synthesis from CPV capsid protein sequences (VP1, VP2) on the basis of the presence of clustered basic residues, which is a common theme in most of the previously identified targeting peptides. Nuclear targeting activity was found within the N-terminal residues 4–13 (PAKRARRGYK) of the VP1 capsid protein. While replacement of Arg10 with glycine did not affect the activity, replacement of Lys6, Arg7, or Arg9 with glycine abolished it. The targeting activity was found to reside in a cluster of basic residues, Lys5, Arg7, and Arg9. Nuclear import was saturated by excess of unlabelled peptide conjugates (showing that it was a receptor-mediated process). Transport into the nucleus was an energy-dependent and temperature-dependent process actively mediated by the nuclear pores and inhibited by wheat germ agglutinin.

**Keywords:** nuclear location signal; canine parvovirus; synthetic peptide; microinjection.

Canine parvovirus (CPV) is a nonenveloped DNA virus of the autonomous Parvoviridae family that causes enteritis and myocarditis in canidae (Cotmore and Tattersall, 1987; Reed et al., 1988). CPV contains three structural proteins in the mature virion (VP1–VP3). The early phase of the CPV life cycle involves a series of sequential events starting with the attachment of a virion to receptors on the cell surface (Basak et al., 1994). Viruses are then internalized by a pH-dependent endocytic pathway (Basak and Turner, 1992; Marsh and Helenius, 1989). At present, the mechanism involved in the release of the virus from acidified endosomes is still unknown. Upon penetration of the virus from endosomes into cytoplasm, the viral DNA and associated proteins are transported to the nucleus, where viral transcription and replication occur. It has been suggested that the uncoating process and release of viral DNA takes place mostly in the nucleus, and incoming coat proteins are believed to be involved in the initiation of viral gene expression (Cotmore and Tattersall, 1987). For the assembly of new virions, viral proteins enter the nucleus. At present, relatively little is known about the nuclear targeting signal of parvoviral proteins. The bipartite nuclear localization signal (NLS) of the minute virus of mice (MVM) has been established (Nüesch and Tattersall, 1993).

In this study, we investigated the NLS of CPV capsid proteins. We studied the abilities of six synthetic peptides from CPV capsid protein sequences (VP1, VP2) to localize a carrier protein

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**Abbreviations.** CPV, canine parvovirus; MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester; MVM, minute virus of mice; NLS, nuclear localization signal, SV40, simian virus 40; WGA, wheat germ agglutinin.

(BSA) to the nucleus, following microinjection into the cytoplasm of the A72 cell, and nuclear transport was evaluated by immunofluorescence at various times after microinjection. Peptides were chosen on the basis of the presence of clustered basic residues. The results demonstrated that the N-terminal residues 4–13 of VP1 can function as a nuclear localization signal. To define further the identified potential NLS region of VP1, we synthesized a set of mutated peptides by changing one or two basic amino acids with glycine to scan the significance of specific amino acid residues for nuclear localization. To determine whether the nuclear import is a receptor-mediated process, i.e. if it can be saturated by excess of peptide conjugates, we coincubated 10-fold or 100-fold excess of unlabelled peptide conjugates with labelled peptide conjugates and monitored the nuclear import. We also investigated the nuclear transport of specific peptide conjugates in energy-depleted or chilled cells, and lectin inhibition of nuclear transport.

## MATERIALS AND METHODS

**Peptide synthesis and peptide-protein conjugation.** The peptides were synthesized by Fmoc chemistry on a simultaneous multiple peptide synthesizer (SMPS 350, Zinsser Analytic, Frankfurt, Germany). The purity of the peptides was analyzed by reverse-phase HPLC (System Gold, Beckman Instruments Inc., Fullerton CA, USA). A C-terminal cysteine residue was added to the peptides for coupling. Additional glycines were used as spacers in the shortest sequences. Peptides were conjugated to BSA (Sigma) with a heterobifunctional cross-linking reagent *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Sigma) by a procedure similar to that described previously (Lanford et al., 1986; Clever and Kasamatsu, 1991). Briefly, BSA (6.75 mg) was resuspended in 0.25 ml 50 mM sodium phosphate, pH 7.0, to which 0.5 mg MBS dissolved in dimethylformamide (10 mg/

ml) was slowly added. The reaction was allowed to proceed for 30 min at room temperature with slow stirring, and unreacted MBS was removed by gel filtration on a Sephadex G25 column (Pharmacia Biotech). Approximately 1.5 mg activated BSA was mixed with 3 mg peptide in 0.6 ml 50 mM sodium phosphate, pH 7.0, and they were allowed to react for 3–5 h at room temperature with slow stirring. Unconjugated peptide was removed by dialysis in 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4 (NaCl/P<sub>i</sub>) overnight at 4°C. The conjugates were examined by SDS/PAGE and Coomassie brilliant blue staining (Sigma) to ensure that satisfactory coupling ratios were obtained (Lanford et al., 1986). Proteins were analyzed by SDS/PAGE as described (Lanford et al., 1986). To label dialyzed peptide conjugates with fluorescent rhodamine B isothiocyanate (TRITC) (Sigma), the volume of conjugates was adjusted to 1 ml with 1 M NaHCO<sub>3</sub>. To this solution, 2 mM rhodamine B isothiocyanate (dissolved in acetone) was added in three aliquots at 5-min intervals with occasional mixing. Finally, there was a five-fold molar excess of rhodamine over BSA. The incubation was continued for 30 min at room temperature with mixing, and the reaction was quenched with 1 mM glycine, pH 8.5. Unbound rhodamine was removed by gel filtration on a Sephadex G25 column preequilibrated with 50 mM sodium phosphate, pH 7.0. The BSA-peptide conjugates were concentrated by spinning in Fugisep microconcentrators (Intersep) and stored at 1 mg/ml at –70°C. Labeled peptide-BSA conjugates were spun at 15 000 g for 15 min prior to microinjection.

**Cells and (cytoplasmic and nuclear) cell microinjection.** A72, a canine fibroma cell line (Binn et al., 1980), was grown (37°C, 5% CO<sub>2</sub>) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco). For the microinjection, cell cultures were inoculated with 1 × 10<sup>4</sup> cells/cm<sup>2</sup>, and cells were grown for two days on round microgrid coverslips (diameter 12 mm, 175 µm grid size, Eppendorf). Microinjections were performed with an Eppendorf 5246 microinjector and an Eppendorf 5171 micromanipulator 5171, the latter being mounted on an IMT-2 inverted microscope (Olympus Optical Co.). Capillaries for injection were prepared from glass tubing (GC 120 F-15, Clark Electromedical Instruments) using a model P 97 capillary puller from Sutter Instruments. During the microinjection, the cells were covered with 4 ml cell culture medium. We used constant air pressure (100–200 hPa) for the microinjection. The synthetic peptide conjugates were microinjected into the cytoplasm at a concentration of 1–1.4 mg/ml in 50 mM sodium phosphate, pH 7.0. Approximately 100 cells/coverslip were injected, and all experiments were performed at least twice. Following microinjection, the medium was removed and replaced with 2.5 ml new medium. The cover slips were washed four times by dipping after an appropriate cultivation time at 37°C in NaCl/P<sub>i</sub>, pH 7.4. Finally, the cells were fixed in methanol (–20°C) for 6 min and mounted on glass slides with glycerol containing 10% (by vol.) NaCl/P<sub>i</sub> and 1 mg/ml *p*-phenylenediamine. The cells were viewed through a fluorescence microscope and samples were stored at –20°C.

**Wheat germ agglutinin inhibition of nuclear translocation.** To assess the effect of wheat germ agglutinin (WGA) on binding to the envelope, cells were microinjected with peptide-linked rhodamine-BSA together with 0.4 mg/ml WGA (Sigma). The samples were incubated for 60 min at 37°C and processed for microscopy as described above.

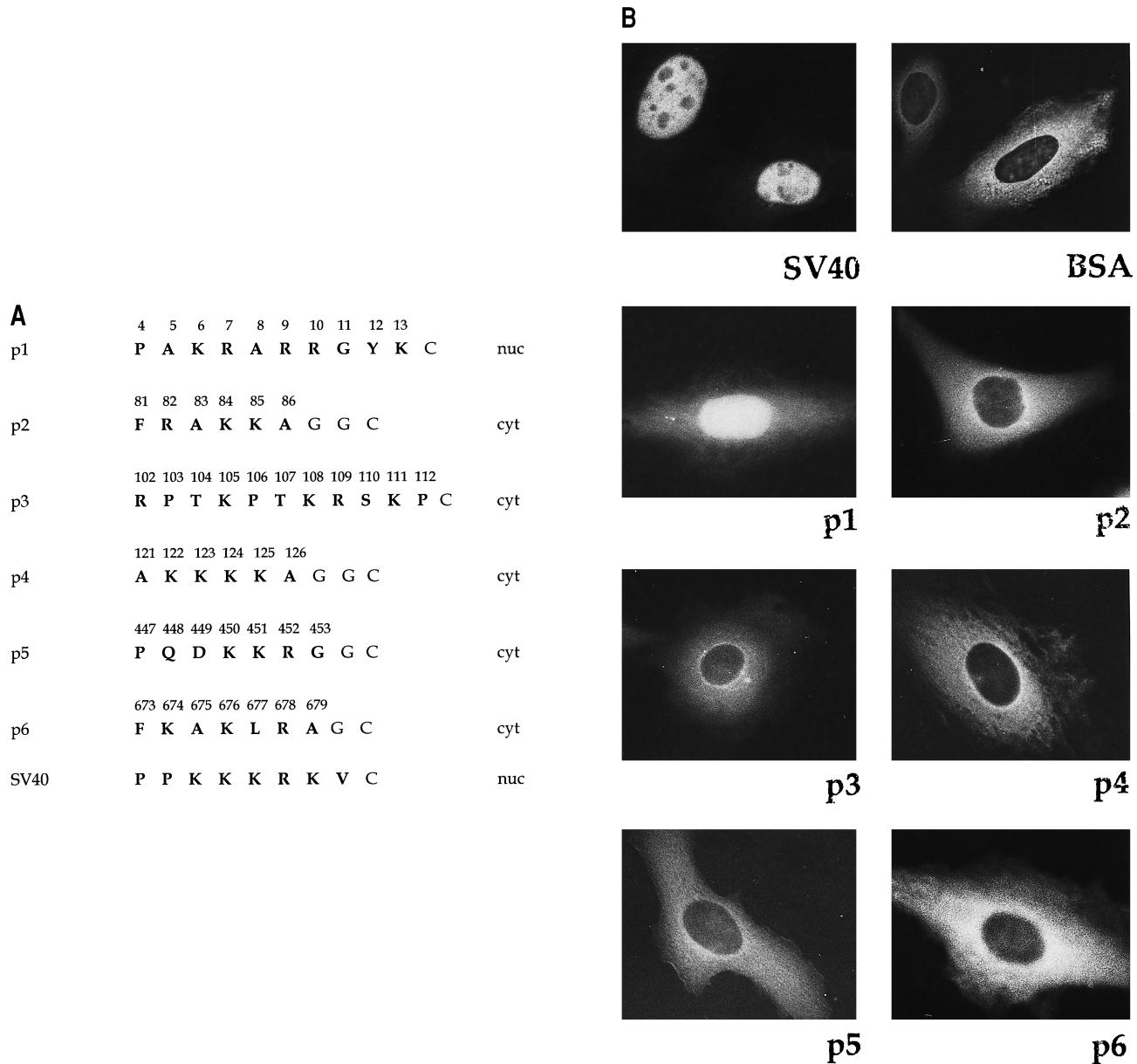
**ATP and the temperature dependence of nuclear translocation.** To test the requirements for ATP, cells were microinjected following a 30-min preincubation and in the presence of Hank's balanced salt solution with 1 µM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (uncoupler of oxidative phosphorylation) and 6 mM 2-deoxyglucose (inhibitor of glycol-

ysis) at 37°C (Breeuwer and Goldfarb, 1990). Following microinjection the cells were further incubated in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and 2-deoxyglucose for 30 min at 37°C before processing for microscopy as described above. To test if the effect of ATP depletion was reversible, a proportion of the microinjected cells were washed with normal medium and incubated in that medium for 30 min prior to fixation and mounting. Chilling studies were performed by cooling the cells on ice 30 min before and 30 min following injection (Breeuwer and Goldfarb, 1990; Richardson et al., 1988). The cells were fixed and mounted as described above. To determine whether the inhibition of nuclear accumulation caused by the chilling treatment was reversible, the temperature of the cells was raised to 37°C for 30 min prior to processing for microscopy.

## RESULTS

**Peptide-mediated nuclear transport.** To test if CPV capsid protein sequences (VP1, VP2) contain a potential NLS, we synthesized six peptides whose amino acid sequence was derived from capsid protein areas containing clustered basic residues (Fig. 1A). The abilities of peptides to target BSA to the nucleus were assessed following microinjection of the rhodamine-conjugated peptide-BSA conjugates into the cytoplasm of A72 cells. One of the sequences (p1), comprising the N-terminal residues 4–13, was able to efficiently target BSA to the nucleus at 60 min postinjection (Fig. 1). Five additional sequences of capsid proteins were tested and residues 81–86 (p2), residues 102–112 (p3), residues 121–126 (p4), residues 447–453 (p5), and residues 673–679 (p6) were found to be ineffective at targeting BSA to the nucleus at 60 min (Fig. 1) or 90 min postinjection (data not shown). The simian virus 40 (SV40) peptide, based on an SV40 large-T antigen sequence (Kalderon et al., 1984a), which was used as a positive control, was highly effective at targeting BSA to the nucleus at 60 min postinjection (Fig. 1). However, peptide p1 which was found to be effective at nuclear targeting, was slightly less effective than the SV40 peptide since a small amount of cytoplasmic staining was observed with this conjugate at 60 min postinjection (Fig. 1B) and only a small difference in cytoplasmic staining was observed between 60 min and 90 min. The carrier protein (BSA) alone did not readily diffuse into cell nuclei, as rhodamine-conjugated BSA was found to remain in the cytoplasm of A72 cells after injection for 60 min (Fig. 1B) and 90 min (data not shown).

We also tested the effect of substituting glycine for basic amino acid on p1 peptide-induced nuclear transport. Eight peptides harboring mutated sequences of p1, an effective nuclear targeting signal, were synthesized and the subcellular localization of the peptide conjugates was examined 60 min (Fig. 2) and 90 min (data not shown) following microinjection. To determine first whether multiple replacements of basic amino acid residues have an effect on nuclear targeting, double replacements were introduced among residues 6, 7, 9, and 10 (Fig. 2A). The peptides so obtained were not capable of nuclear transport (Fig. 2B). When single replacements of basic amino acids were introduced into a cluster of Lys6, Arg7, Arg9, and Arg10 (Fig. 2A), we found that sequence p1h with glycine as the replacement to Arg10 was the only mutated sequence that successfully targeted a carrier protein to the nucleus (Fig. 2B). While replacement of Lys6 (p1e), Arg7 (p1f), or Arg9 (p1g) with glycine induced cytoplasmic distribution of peptide-BSA conjugates, perinuclear accumulation of conjugates was also observed (Fig. 2B). The targeting activity was found to reside in a cluster of basic residues, the cluster of Lys6, Arg7, and Arg9.



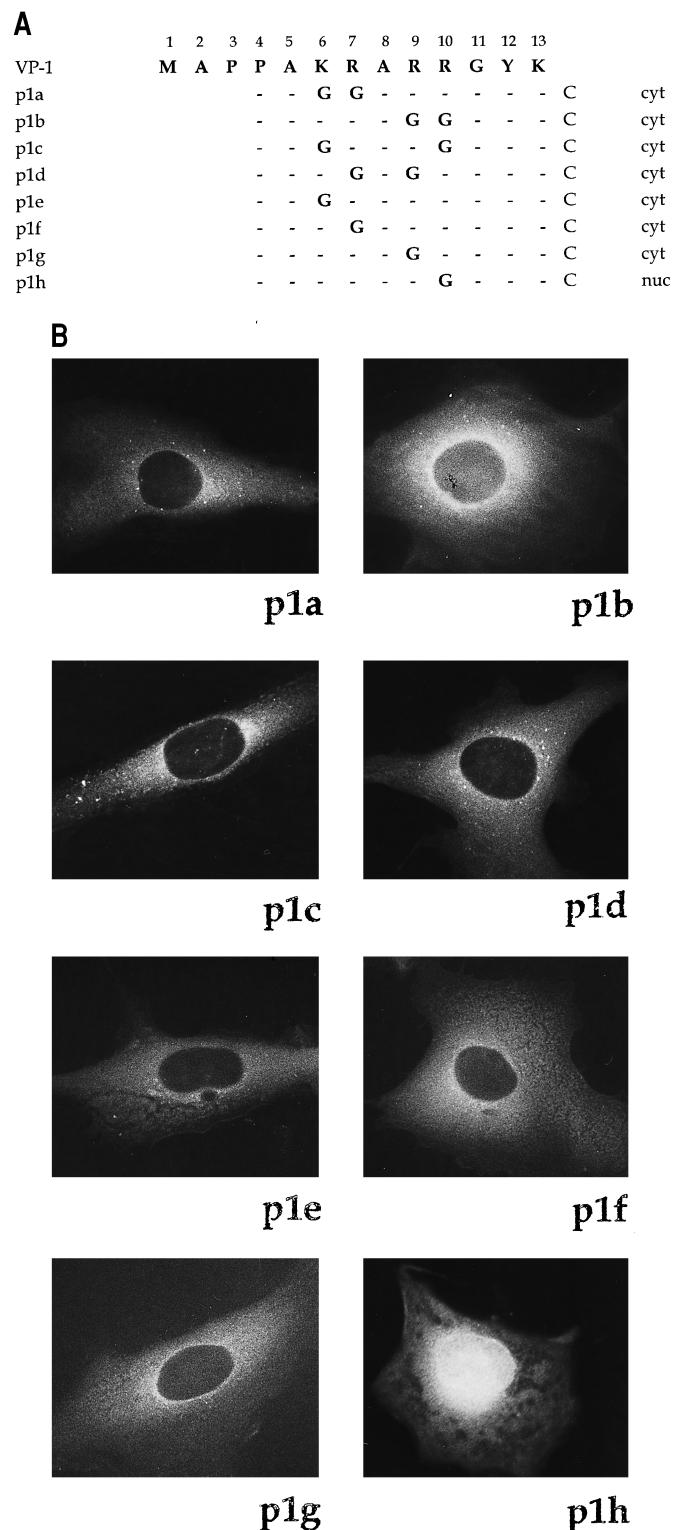
**Fig. 1. Amino acid sequences of synthetic peptides homologous to the basic, arginine-rich and lysine-rich sequences found in coat proteins of CPV, and the cellular localization of microinjected carrier protein (BSA) conjugated to synthetic peptide.** (A) The cysteine and glycine residues at the C-terminal are additional residues not contained in the native coat protein sequence. Cysteine residues were added for chemical cross-linking and glycine residues for lengthening, respectively. Peptides are named on the left and the subcellular localization of each peptide conjugate is summarized on the right. Nuc, nuclear localization; cyt, cytoplasmic localization. (B) Immunofluorescence photographs of cells microinjected with rhodamine-conjugated BSA (BSA), SV40-peptide-BSA (SV40), and CPV coat-peptides (p1–p6). Cells were harvested 1 h after microinjection.

To determine whether the nuclear import can be saturated by excess of peptide conjugates, we coinjected the fluorescently labelled p1-BSA conjugates with an excess of unlabelled p1-BSA. Upon coinjection of tenfold excess of unlabelled p1-BSA conjugates with labelled p1-BSA conjugates, the nuclear fluorescence decreased markedly while 100-fold excess inhibited nuclear accumulation totally (data not shown). As a control, labelled SV40-BSA was coinjected with an excess of unlabelled p1-BSA. Coinjection with tenfold excess of unlabelled p1-BSA conjugates resulted in slightly decreased nuclear fluorescence and 100-fold excess inhibited nuclear fluorescence markedly but not completely (data not shown).

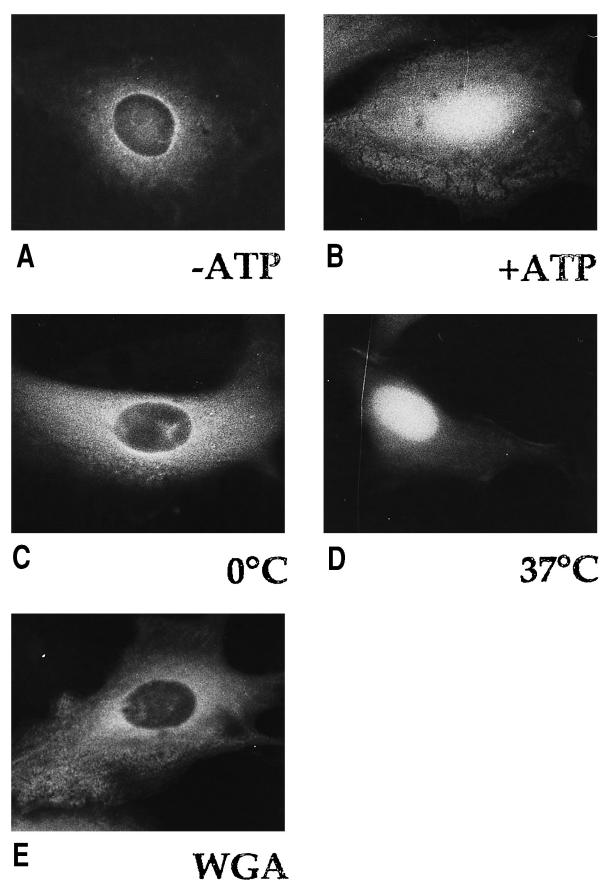
To determine whether peptide conjugates with the nuclear targeting activity (p1, p1h) were able to leave the nucleus, we

monitored the nuclear export of conjugates microinjected into A72 cell nuclei. No nuclear export of p1 or p1h peptide conjugates was observed within 15 min, 30 min, and 3 h after microinjection into A72 cell nucleus (data not shown). Peptide conjugates introduced directly into the nucleus were retained by the nucleus within the time frame of the experiment.

**ATP dependence of nuclear translocation.** The cellular distribution of microinjected p1, p1h, and SV40 BSA conjugates, was studied in the presence of inhibitors of ATP production. Some of the rhodamine-labelled p1-BSA (Fig. 3) concentrated in a thin rim around the periphery of the nucleus whereas others were located diffusely throughout the cytoplasm following microinjection in cells depleted of ATP. Similar results were obtained



**Fig. 2.** Amino acid sequences modified from the nuclear translocation signal of CPV VP-1 coat protein and cellular localization of microinjected carrier protein (BSA) conjugated to synthetic peptide. (A) The N-terminal residues of CPV coat protein VP-1 are shown in single-letter code and, for modified peptides only, amino acid alterations are indicated by letters in place of a corresponding modification point. Modified peptides are named on the left and the subcellular localization of each peptide-conjugate is summarized on the right. Nuc, nuclear localization; cyt, cytoplasmic localization. Cysteine residues were added for chemical cross-linking and glycine residues for lengthening, respectively. (B) Immunofluorescence photographs of cells microinjected with modified peptide conjugates (p1a–p1h) and harvested 1 h after microinjection.



**Fig. 3.** ATP dependence and temperature dependence of nuclear envelope binding, and effect of wheat germ agglutinin (WGA) on nuclear transport. Fluorescent micrographs showing the distribution of microinjected p1-BSA conjugate after incubation at low temperature (C) or at 37°C (D).

with p1h-BSA and SV40-BSA. The narrow perinuclear band was seen most clearly when low concentrations of NLS conjugates were injected. When the injected, energy-depleted cells were returned to a medium lacking deoxyglucose and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and allowed to recover for 30 min, p1-BSA (Fig. 3) subsequently accumulated in the nuclei, thereby indicating the reversible nature of inhibition. The nuclear accumulation of p1h-BSA and SV40-BSA could also be demonstrated after removal of the inhibitors of ATP production.

**Temperature dependence of nuclear translocation.** We investigated whether the nuclear transport of p1-, p1h-, and SV40-BSA conjugates was inhibited by low temperature. p1-BSA (Fig. 3), p1h-BSA, and SV40-BSA microinjected into chilled cells were not observed to reach the nucleus, but instead were localized around the nucleus in a narrow band or in the cytoplasm, thereby demonstrating transport arrest. When the microinjected, chilled cells were warmed and allowed to recover for 30 min, p1-BSA (Fig. 3), p1h-BSA, and SV40-BSA accumulated in the nucleus, thereby indicating the reversibility of the inhibition. In a manner similar to that observed with the ATP-depleted cells, the thin rim around the perinuclear region was seen most clearly when low concentrations of NLS conjugates were injected.

**Effect of wheat germ agglutinin on nuclear transport.** When injected together with p1-BSA (Fig. 3), p1h-BSA, and SV40-

BSA conjugates, WGA blocked the import of the conjugates into the nucleus. However, it did not prevent the accumulation of conjugates at the nuclear envelope, resulting in rim staining in some of the injected cells. In most of injected cells, the cytoplasmic staining was noticeable, and the narrow rim staining was obtained clearly in only a small proportion of injected cells.

## DISCUSSION

Proteins are targeted to the cell nucleus by nuclear localization signals that allow selective entry through the nuclear pore complex (Dingwall and Laskey, 1986; Roberts, 1989; Görlich and Mattaj, 1996; Görlich, 1997; Nigg, 1997). The nuclear localization signals (NLSs) can be classified into three categories: prototypic NLSs, consisting of short stretches of basic amino acids resembling the single basic domain of the SV40 large-T antigen (Goldfarb et al., 1986; Kalderon et al., 1984b; Lanford and Butel, 1984); the double basic (bipartite) domain, resembling *Xenopus laevis* nucleoplasmmin NLS (Robbins et al., 1991; Dingwall and Laskey, 1991); and the types of NLSs without any classical basic stretches (Michaud and Goldfarb, 1991; Fischer et al., 1991; Kambach and Mattaj, 1992; Siomi and Dreyfuss, 1995; Wang et al., 1997; Weighardt et al., 1995; Michael et al., 1997; Nigg, 1997). Small proteins appear to diffuse freely across the nuclear membrane, but a selective mechanism regulates the entry of larger proteins (Dingwall, 1985; Lanford et al., 1986). Transport of most nuclear proteins through the nuclear pore complex has been shown to require energy and to be temperature dependent. Nuclear accumulation is therefore considered not to be a process based on simple diffusion through the pores and intranuclear binding, but instead mediated actively by the nuclear pores (Breeuwer and Goldfarb, 1990; Newmeyer and Forbes, 1988; Richardson et al., 1988). Because CPV virions are assembled in the nucleus of host cells, the virion proteins synthesized in the cytoplasm must be translocated into the nucleus. Nuclear translocation might also play a role in the entry phase of CPV infection.

We used a microinjection approach to identify within CPV VP1/2 a sequence that functions as NLS. We found that the peptide PAKRARRGYK, corresponding to the N-terminal residues 4–13 of the capsid protein VP1, was able to target a carrier protein to the nucleus. Although the amino acid sequence of VP1 carries several clusters of basic residues, the one represented by p1 appears to have specificity since none of the other five peptides tested were able to induce nuclear translocation of BSA. It has previously been shown that the alteration of one basic amino acid of SV40 large-T NLS to a non-basic amino acid (Kalderon et al., 1984a) prevents the normal nuclear accumulation. By using mutated synthetic peptides, we demonstrated that, in particular, lysine at position 6 and two arginines at positions 7 and 9, are essential for nuclear targeting activity of the VP1 p1 peptide, since the substitution of glycine for any of these amino acids prevents nuclear translocation.

The nuclear import of p1-BSA was saturable as already demonstrated for canonical NLSs (Goldfarb et al., 1986). The p1-BSA conjugate was able to significantly inhibit nuclear uptake of SV40-BSA, suggesting that both conjugates were imported by the same pathway.

In the experimental system used, the nuclear translocation induced by p1 had several characteristics of a facilitated, active signal-dependent nuclear import (Breeuwer and Goldfarb, 1990; Richardson et al., 1988). The entry of peptide conjugates into the nucleus through nuclear pores via passive diffusion could be excluded because of the size of the conjugates. In the control experiments, rhodamine-labelled BSA alone did not enter the

nucleus. Furthermore, many of the conjugates tested did not enter the nucleus, thereby excluding the possibility that the conjugation process itself would have caused changes in the carrier protein rendering it susceptible to nuclear translocation.

The nuclear translocation of p1-BSA was reversibly arrested in energy-depleted or chilled cells. Under these conditions, binding of the conjugate to the nuclear membrane could be demonstrated. Thus the p1-BSA conjugate behaved like a typical protein having an active NLS (Richardson et al., 1988). The translocation by p1 was also inhibited by WGA, a lectin that has been shown to inhibit the accumulation of nuclear proteins by blocking the translocation step of the transport (Adam and Adam, 1994; Finlay et al., 1987), probably by interacting with O-glycosylated nucleoporins (Finlay et al., 1987). WGA has been reported not to affect binding of the transported protein to nuclear pores (Finlay et al., 1987; Moore and Blobel, 1992; Newmeyer and Forbes, 1988). Thus the dissection of the nuclear transport process into binding and translocation phases (Richardson et al., 1988) was demonstrated in the present work.

At present, the mechanisms of the nuclear targeting and nuclear entry of capsid proteins before virion assembly are poorly understood. Studies with the autonomous parvovirus MVM have shown the virus to be capable of productive infection only when two capsid proteins, VP1 and VP2 are present (Tullis et al., 1993). According to crystallographic studies, CPV has a disordered N-terminal portion of VP1 not required for coat assembly (Tsao et al., 1991). It may be assumed that the N-terminus of VP1 is also accessible in the free VP1 protein and hence may be a good candidate for a region carrying an active NLS. It is also suggested that VP1 is required for the transport of MVM to the nucleus (Tullis et al., 1993). It is not known whether MVM enters the nucleus as an intact virion or partially disassembled DNA-protein complex (Tullis et al., 1993).

In summary, the results reported here describe the identified potential of NLS in the N-terminal region of the VP1 capsid protein that is alone sufficient to localize the carrier protein to the nucleus. A cluster of basic residues (Lys6, Arg7, and Arg9) is important for localization activity.

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