*Journal of General Virology* (2002), **83**, 1783–1790. Printed in Great Britain

# Regulated nuclear targeting of cauliflower mosaic virus

Aletta Karsies,[1] Thomas Merkle,[2] Boris Szurek,[3] Ulla Bonas,[3] Thomas Hohn[1] and Denis Leclerc[4]

[1] Friedrich Miescher Institute, PO Box 2543,CH-4002 Basel, Switzerland

[2] Institut für Biologie II, Zellbiologie, Universität Freiburg, 79104 Freiburg, Germany

[3] Institute of Genetics, Martin-Luther University, 06120 Halle, Germany

[4] Centre de Recherche en Infectiologie, Université Laval, Ste-Foy, Qc, G1V 4G2, Canada

**The mature cauliflower mosaic virus (CaMV) capsid protein (CP), if expressed in the absence of other viral proteins, is transported into the plant cell nucleus by the action of a nuclear localization signal (NLS) close to the N terminus. In contrast, virus particles do not enter the nucleus, but dock at the nuclear membrane, a process inhibited by anti-NLS antibodies or by GTP$\gamma$S, and apparently mediated by interaction of CP with host importin $\alpha$. The very acidic N-terminal extension of the viral CP precursor inhibits nuclear targeting of the protein and hence the precursor is localized in the cytoplasm. We hypothesize that this provides a control mechanism which ensures that the CP precursor is used for virus assembly in the cytoplasm and that only mature virus particles reach the nuclear pore.**

## Introduction

Many viruses replicate or transcribe their genome in the nucleus of their hosts, requiring a mechanism by which the genome can enter the nucleus. Generally, the nuclear membrane acts as a barrier to virus entry, and specific viral proteins are required to mediate transport of the viral genome into the nucleus (Whittaker & Helenius, 1998). Most retroviruses circumvent the transport problem by establishing infection during mitosis, when nuclear membranes are broken down (Roe *et al.*, 1993). However other retroviruses, e.g. human immunodeficiency virus type 1 (HIV-1), and pararetroviruses, i.e. animal hepadnaviruses and plant caulimoviruses, can infect resting cells, inferring active transport of the viral genome across the nuclear membrane.

The type member of caulimoviruses is cauliflower mosaic virus (CaMV; Rothnie *et al.*, 1994). A pool of CaMV particles containing open circular DNA is established in the cytoplasm of an infected plant cell via aphid transmission, cell-to-cell transport and *de novo* synthesis. Another pool of viral genomes, in the order of 10–100 copies of minichromosomes comprising supercoiled circular viral DNA and host histones, accumulates in the nucleus. From these minichromosomes, viral RNAs are produced and transported to the cytoplasm for translation, packaging and reverse transcription. Viral proteins together with virus particles accumulate in inclusion bodies composed of the product of CaMV ORF VI. Virus particles are then transported to other cells, taken up by aphids or used to re-infect the nucleus (see Fig. 5).

**Author for correspondence:** Thomas Hohn.
Fax +41 61 6973976. e-mail hohn@fmi.ch

The CaMV virion is an icosahedron, with a diameter of 54 nm, composed of 420 subunits of the viral capsid protein (CP) (Cheng *et al.*, 1992). CP exists in three major forms, called p44, p39 and p37, that are processed by removal of the acidic N and C termini of the CP precursor. In p44, the first 76 amino acids (aa) and about 40 aa from the C terminus are removed by the viral aspartic proteinase (Torruella *et al.*, 1989; A. Karsies and others, unpublished). The N termini of p39 and p37 are blocked, precluding exact mapping by protein sequencing (Martinez-Izquierdo & Hohn, 1987). Mature CPs are glycosylated (Du Plessis & Smith, 1981), and p44 (but not p39 or p37) is phosphorylated (Martinez-Izquierdo & Hohn, 1987).

The CaMV CP might be responsible for the nuclear transport of viral DNA since it harbours nucleic acid binding domains close to its C terminus and, close to its N terminus, a simian virus-40 (SV40)-like nuclear localization signal (NLS) that is conserved throughout the caulimovirus family (Leclerc *et al.*, 1999). The NLS is responsible for nuclear import of the mature CP outwith the viral context. In mature viruses, the NLS is exposed at the surface of the virions (Leclerc *et al.*, 1999).

Here we show that purified virions do not enter the nucleus, but dock to the nuclear membrane. This interaction is inhibited by anti-NLS antibodies or by GTP$\gamma$S, an inhibitor of

nuclear import. The regular pattern of interaction suggests that specific receptors on the nuclear membrane are involved, probably associated with the nuclear pores. Moreover, CP interacts specifically with importin α, indicating that virus docking is mediated by this import receptor.

## Methods

■ **Plasmids.** The different CaMV CP constructs were cloned downstream of the haemagglutinin (Ha11) epitope in pTAGNLS as described (Leclerc *et al.*, 1999). Clones starting at codon 77 have a fused methionine initiation codon. The three Glu → Ala mutations in positions 83, 84 and 85 to generate p(1–489)*, p(1–332)* and p(77–332)* (* represents the EEE83AAA designation for the mutant sequence) were introduced using the QuickChange site-directed mutagenesis kit (Stratagene) using the complementary oligonucleotide pair 5′ GCAATAGGAGAAACATCTGCAGCAGCTAGCGATTCAGGAGAAGAACCTG 3′ and 5′ CAGGTTCTTCTCCTGAATCGCTAGCTGCTGCAGATGTTTCTCCTATTGC 3′.

■ **Plants and viruses.** *Brassica rapa* plants (turnip 'Just Right') were grown at 22 °C with a 16 h photoperiod as described (Blanc *et al.*, 1993). The Strasbourg strain of CaMV cloned in the *Sal*I site of pBR322 (pCa37) was used for the infection of 4-week-old turnip plants. Virus was purified as described (Lebeurier *et al.*, 1980).

■ **Plant protoplasts and protein analysis by indirect immunofluorescence.** Culture conditions for *Orychophragmus violaceus* suspension cultures, protoplast preparation and transfection have been described previously (Fütterer *et al.*, 1989, 1990). *Nicotiana plumbaginifolia* mesophyll protoplasts ($3 \times 10^5$) were transfected with 20 μg of plasmid using the polyethylene glycol method (Goodall *et al.*, 1990). *B. rapa* protoplasts were prepared according to the same protocol with modifications as described (Leclerc *et al.*, 1999). Optimal expression of protein was found to occur 8 h after transfection. Protoplasts were collected and treated for indirect immunofluorescence (Mieszczak *et al.*, 1992).

The rabbit polyclonal anti-Ha11 antiserum was obtained from Babco (Richmond, CA, USA), and the goat anti-rabbit antibody conjugated with FITC from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). The anti-Ha11 antiserum was chosen for this purpose because it gives very low background fluorescence. The samples were examined under oil using a Leitz microscope equipped with a Leitz Fluotar 40 × objective and epifluorescence filters or with a confocal Leica DMIRBE microscope using a Leitz 40 × objective and Leica Scanware.

■ **Preparation of nuclei, permeabilized cells and nuclear import assays.** Permeabilized protoplasts of *Nicotiana tabacum* BY-2 cells and nuclei from *O. violaceus* and *B. rapa* were isolated as described (Merkle *et al.*, 1996). Purified CaMV virions (Blanc *et al.*, 1993) were labelled with a fluorescent dye (CY3.5; Amersham) following the manufacturer's protocol. *B. rapa* plants were inoculated with the labelled viruses to verify their infectivity. After 30 min of labelling, viruses were infectious; however, after labelling for 1 h infectivity dropped significantly. Therefore, only preparations labelled for 30 min were used. DAPI (1 μg/ml) or SYTO 13 (1 μg/ml; Amersham) was used to stain nuclear DNA. Nuclear import assays were performed with permeabilized cells or isolated nuclei in a total volume of 30 μl containing about $10^5$ nuclei, 0·2 mM GTP and 50 ng of fluorescently labelled virus particles. Incubation was for 20 min at room temperature. To increase contrast in confocal microscopy, the samples were washed with 0·5 ml of import buffer (20 mM HEPES, 100 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT and 1% BSA).

Antibody inhibition tests were performed by pre-treating the labelled virions with anti-NLS immunoglobulin G (NLS-IgG) (Leclerc *et al.*, 1999).

■ **Extraction of proteins, SDS–PAGE and immunoblotting.** Proteins were extracted from transfected protoplasts with phenol and precipitated by methanol (Hurkman & Tanaka, 1986). Proteins were separated by SDS–PAGE (Schagger & von Jagow, 1987) and electroblotted overnight to nitrocellulose membranes at 30 V (constant voltage). The membrane was treated and probed with anti-CaMV CP precursor IgG (Himmelbach *et al.*, 1996). These antibodies were used for Western blotting because they gave a stronger signal than the anti-tag antibody.

■ **Yeast two-hybrid system.** Interaction between CP and importin α was shown with the DupLEX-A yeast two-hybrid system (Origene Technologies, Rockville, MD, USA). CP fragments 125–332 and 77–332, containing an Ha11 tag at the N terminus (Leclerc *et al.*, 1999), were cloned in-frame with the *lexA* gene into the *Bam*HI–*Sal*I restriction sites of the pEG202 yeast expression plasmid. The pJG4-5 galactose-inducible yeast plasmid was used to express importin α from *Capsicum annuum* (Szurek *et al.*, 2001), fused in-frame to the B42 activation domain (AD). Yeast strain EGY48 containing the pSH18-34 high sensitivity *lacZ* reporter plasmid was used. The interaction was quantified in liquid assay using chlorophenol red β-D-galactopyranoside (CPRG; Roche Biochemicals) as described in the Clontech yeast protocols handbook.

## Results

To analyse nuclear targeting mediated by the NLS of the CaMV CP and its regulation at different stages of the replication cycle, we analysed the intracellular localization of precursor and mature forms of the CP in transfected protoplasts, and of whole virus particles in permeabilized cells. Proteins were localized by immunofluorescence using the Ha11 tag. Whole virus particles were localized by virtue of their labelling with a fluorescent dye.

### The acidic N-terminal domain of the CP precursor inhibits nuclear targeting

CaMV p44 starts with ORF IV aa 77 and ends around aa 454 (Martinez-Izquierdo & Hohn, 1987). CP derivatives starting at aa 77 and ending with aa 480, 411 or 332 were all localized in the nucleus after expression, confirming earlier results (Leclerc *et al.*, 1999). This localization is dependent on an NLS motif close to the N terminus (Fig. 1A); mutation of this motif abolished nuclear localization. On the other hand, nuclear localization is independent of the basic C-terminal region and the Zn-finger (Leclerc *et al.*, 1999).

Analysis of constructs expressing full-length CP precursor [p(1–489); Fig. 1A], or the C-terminal truncated proteins [p(1–362) and p(1–265)], is complicated by the presence of protein instability determinants at the N terminus and, for the full-length precursor, at the C terminus also (Karsies *et al.*, 2001). However, mutation of a cluster of acidic amino acids to

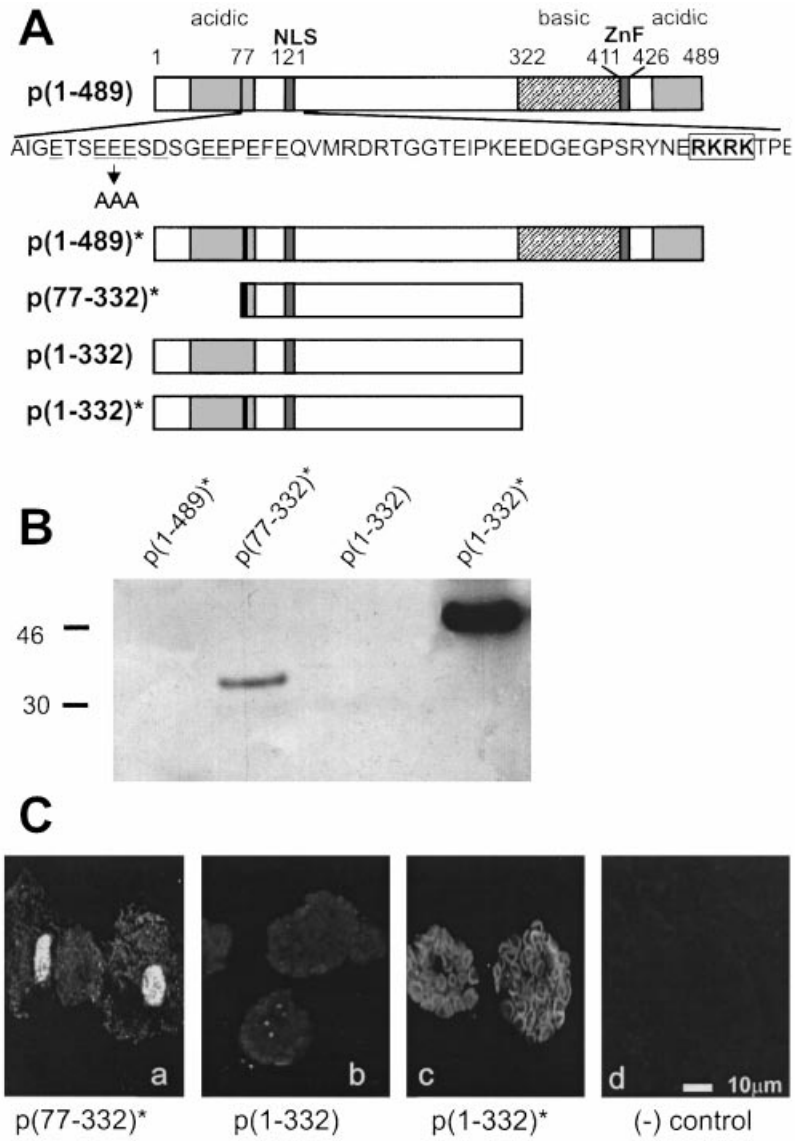


Fig. 1. Transport of CP derivatives into the nucleus. (A) Schematic representation of CaMV CP precursor and derivatives. The light grey regions are rich in acidic amino acids and the striped region is rich in basic amino acids. The central white region is believed to be involved in protein–protein interactions and virus assembly. The dark grey bars represent the NLS and the Zn-finger motifs, and a black bar the EEE84AAA mutation. The amino acid sequence of the N terminus of p44, one of the major processed forms of the CP present in the purified virus, is shown below the scheme. The mutation of glutamic acids 83, 84 and 85 to alanine, increasing the stability of the CP precursor, is indicated. (B) Detection of CP derivatives, by Western blotting using anti-CP-precursor antibodies, in extracts of *N. plumbaginifolia* protoplasts transfected with different forms of CaMV CP. (C) Indirect immunofluorescence of *N. plumbaginifolia* protoplasts transfected with different forms of CaMV CP. (a) p(77–332)* with the three Glu residues at positions 83, 84 and 85 mutated to Ala residues. (b) p(1–332) containing wt sequence. (c) p(1–332)* with the same mutation as in (a). (d) Negative control, protoplasts transfected with the empty vector.

yield p(1–332)*, p(77–332)*, etc. (* represents the EEE83AAA exchange) in the N-terminal region stabilizes certain constructs sufficiently to allow detection by Western blotting or immunofluorescence (Fig. 1). Note that the Western signal produced by p(77–332)* is considerably weaker than that of p(1–332)* because the antibody recognizes the acidic N-terminal domain of the latter protein more efficiently. This stabilizing mutation was not sufficient to allow detection of the full-length precursor protein [p(1–489)*], because the acidic C terminus also constitutes an instability element (Karsies *et al.*, 2001).

Indirect immunofluorescence revealed that the protein derived from p(1–332)* remained in the cytoplasm, where it appeared as ring-like structures, probably because of displacement by, or a non-specific interaction with, the chloroplasts (Fig. 1C). In contrast, the protein derived from p(77–332)* (Fig. 1C) or p(77–332) (not shown) was found in

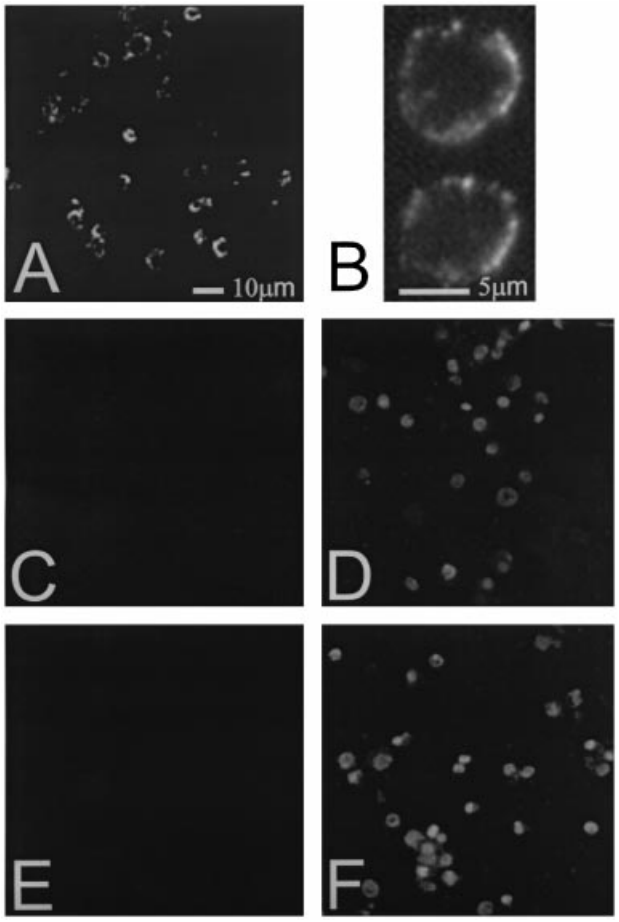


Fig. 2. CaMV particles dock to the nuclear membrane in permeabilized *N. tabacum* BY-2 protoplasts. Labelled virus particles were incubated with permeabilized cells for 20 min before washing with the import buffer. (A, B) Nuclei stained with fluorescently labelled CaMV particles (A) and an enlarged view of two nuclei (B). (C, D) Same as panel (A) except that the labelled virus particles were pre-incubated for 20 min at room temperature with the antibody raised against the NLS of CP (C). DNA staining of same field with syto13 (D). (E, F) Same as panel (A) except that the permeabilized cells were pre-treated with 5 mM GTP$\gamma$S for 5 min before adding the labelled virus to the reaction (E). DNA staining of the same field with syto13 (F).

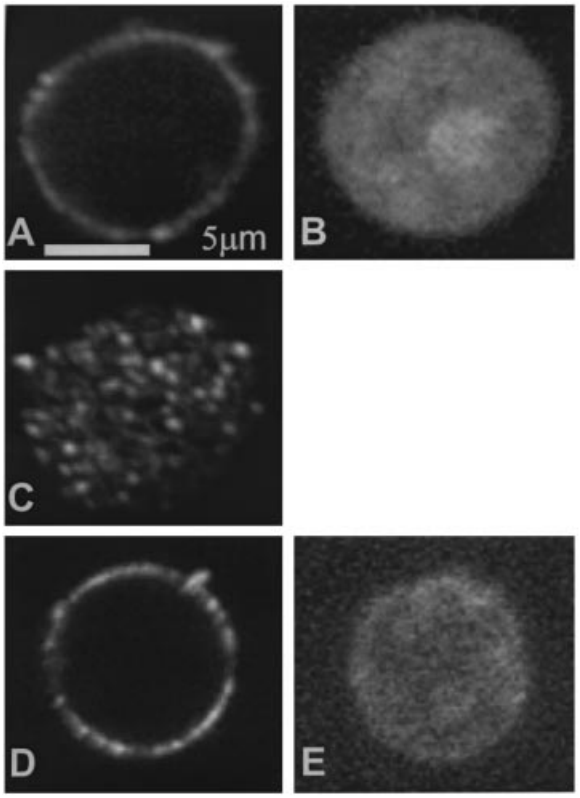


Fig. 3. CaMV particles dock to the nuclear membrane of isolated nuclei. Labelled virus particles were incubated with nuclei for 20 min before washing with import buffer. (A) Fluorescently labelled CaMV particles observed on the surface of an *O. violaceus* nucleus. (B) DNA staining of the same field with syto13. (C) Scanning of the surface of the nucleus shown in A. (D) Fluorescently labelled CaMV particles observed on the surface of a *B. rapa* nucleus. (E) DNA staining of the same field with syto13.

the nucleus. This indicates that the N-terminal region of the CP precursor inhibits nuclear targeting by the CP-NLS.

### CaMV particles dock to the nuclear membrane via the NLS of their CP

Mature CaMV virions were isolated and tested for their intracellular localization. For this purpose, permeabilized *N. tabacum* BY-2 protoplasts (Merkle *et al.*, 1996) were incubated with labelled virions and analysed. BY-2 cells were disrupted with 0·01% Triton X-100 by passing the cells several times through a 1 ml syringe. The cells were collected by low-speed centrifugation and washed twice with the nuclear import buffer. The resulting permeabilized cells were incubated with the fluorescent CaMV particles, washed once with the nuclear import buffer and mounted on a glass slide. The fluorescence was localized to the nucleus and confocal scanning microscopy revealed that virions were actually located at the surface of the nuclear membrane (Fig. 2A, B), rather than being in the interior of the nucleus. Possibly the size of the virions precluded their migration through the nuclear pore. The interaction was inhibited by the addition of anti-NLS antibodies (Fig. 2C), but not by pre-immune serum (not shown), indicating that the NLS located at the surface of the virion is responsible for the interaction. Inhibition of virion–nucleus interaction was also observed when GTP$\gamma$S, an efficient inhibitor of nuclear import (Merkle *et al.*, 1996), was included in the reaction mixture (Fig. 2E).

Tobacco is not a natural host of CaMV. We therefore repeated the experiments described above using isolated nuclei (Merkle *et al.*, 1996) from cells of *O. violaceus* (Fig. 3A–C) and *B. rapa* (Fig. 3D, E), which are host plants for CaMV. Again we observed an interaction between the labelled virus and the surface of the nuclear membrane (Fig. 3A). In this case, the fluorescence appeared as discrete foci when the surface of the nucleus was scanned with the confocal microscope (Fig. 3C).

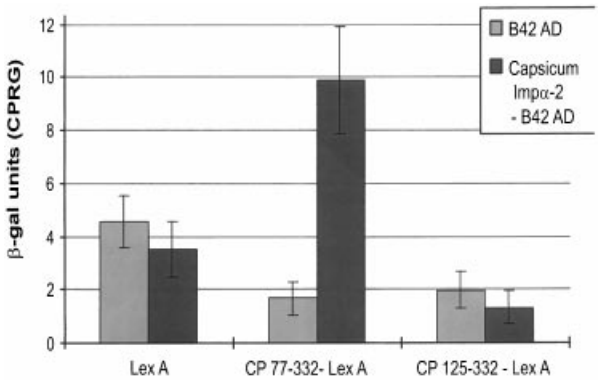


Fig. 4. CaMV CP interacts with importin α. Two fragments of CP were individually fused to the LexA DNA-binding protein, and cotransformed in yeast with *Capsicum annuum* importin α fused to the B42 activation domain. The strength of the interaction was measured by liquid assay using CPRG as a substrate.

Based on this observation we suggest that the labelled virions interact with the membrane at specific sites, probably the nuclear pores. In future studies we aim to confirm this hypothesis by electron microscopy.

### Interaction of the CP with importin α

Since importin α is involved in most cases of nuclear transport (Whittaker & Helenius, 1998), we used the LexA two-hybrid system to test for a specific interaction between CP and plant importin α. Interactions were quantified using a β-galactosidase liquid assay (Fig. 4). CP fragments p(77–332) and p(125–332) could be used for this experiment. However, p(1–332) could not be used, since it showed non-specific interaction with the B42 activation domain. Results showed that p(77–332) interacts with importin α in this system whereas p(125–332), which lacks the NLS sequence, does not. Although further studies will be needed to address the role of importin α, this result correlates interaction between the CP and importin α with CP nuclear localization.

## Discussion

Isolated mature CaMV CP is transported into the nucleus via an NLS close to its N terminus (Leclerc *et al.*, 1999). Here we show that this NLS also functions in the context of assembled virions. However, in this case the (fluorescently labelled) particles bind to the nuclear membrane and remain there. The NLS-mediated specific binding of the virus to the nuclear membrane can be interpreted as being mediated by importin α, a protein involved in nuclear import of NLS-containing proteins, which in plants is found mainly at the nuclear membrane surface (Smith *et al.*, 1997). Support for this interpretation is given by the weak, but specific, interaction of the CP with a plant importin α in the LexA two-hybrid system (Fig. 4). This interaction may be stronger for the virus particle, since it is built from 420 capsid subunits and hence presents 420 NLSs. In contrast to animal cells, importin α is closely associated with the nuclear pore of plant cells (Merkle *et al.*, 1996). This is the reason, as shown by Merkle *et al.* (1996), that there is no requirement for cytosolic components for the interaction of the virus with the cellular membrane. The inhibition of binding of the virus to the nuclear membrane by GTPγS could be the result of the inactivation of RAN, a GTP-binding protein involved in nuclear import, since this analogue of GTP is known to maintain RAN in a GTP-bound form that cannot be hydrolysed (Dickmanns *et al.*, 1996). As a consequence, the interaction of the virion with the surface of the nucleus is inhibited because the RAN-GTP form dissociates the importin αβ heterodimer by displacing α from an overlapping binding site on β (Moroianu *et al.*, 1996).

The interaction of the virus with the nuclear membrane is not uniform and discrete foci can be seen at the surface of the membrane (Fig. 3C). This discontinuity of the signal indicates that the virus interacts with discrete structural units of the nuclear membrane that are probably the nuclear pores. Very similar images were obtained for constituents of the nuclear pores (e.g. Nup84, Nup49p or Nup133p) (Bastos *et al.*, 1997; Belgareh & Doye, 1997; Bucci & Wente, 1997). Importin α associated with an NLS-containing protein has been shown to interact strongly with the cytoplasmic filaments of the nuclear pore (Panté & Aebi, 1996). These filaments could thus represent the specific sites of the interaction between virus and nucleus.

CaMV now joins a collection of different DNA viruses (Izaurralde *et al.*, 1999), e.g. papovaviruses (Yamada & Kasamatsu, 1993), geminiviruses (Kunik *et al.*, 1998) and hepadnaviruses (Kann *et al.*, 1997), that target their genomic DNA to the nucleus via an NLS located on the CP. In contrast, most retroviruses do not need an NLS, since they are restricted to dividing cells (Roe *et al.*, 1993). Exceptions are the lentiviruses; their capsids disassemble prior to nuclear targeting releasing a preintegration complex (PICs). Several constituents of the PIC [integrase (Gallay *et al.*, 1997), Vpr (Freed *et al.*, 1997) and perhaps matrix protein (Gallay *et al.*, 1995)] have been implicated in nuclear targeting.

It is still unclear how CaMV DNA enters the nucleus. In our assay the bulk of CP remains at the surface of the nuclear membrane and hence we consider it unlikely that intact CaMV particles pass into the interior of the nucleus as has been suggested for SV40 (Yamada & Kasamatsu, 1993). Although we cannot exclude the possibility that an essential element that would mediate this process is missing, the addition of cytoplasmic extracts to our *in vitro* assay did not change the interaction of the labelled virus with the nucleus (data not shown). In any case, entry of the complete capsid into the nucleus would have to involve major distortions of the particle. With a diameter of more than 50 nm, the capsid is much larger than the size limit for NLS-coated gold particles (26 nm; Feldherr *et al.*, 1984), and also still larger than cargo-receptor–gold complexes (39 nm; Panté & Kann, 2002), which

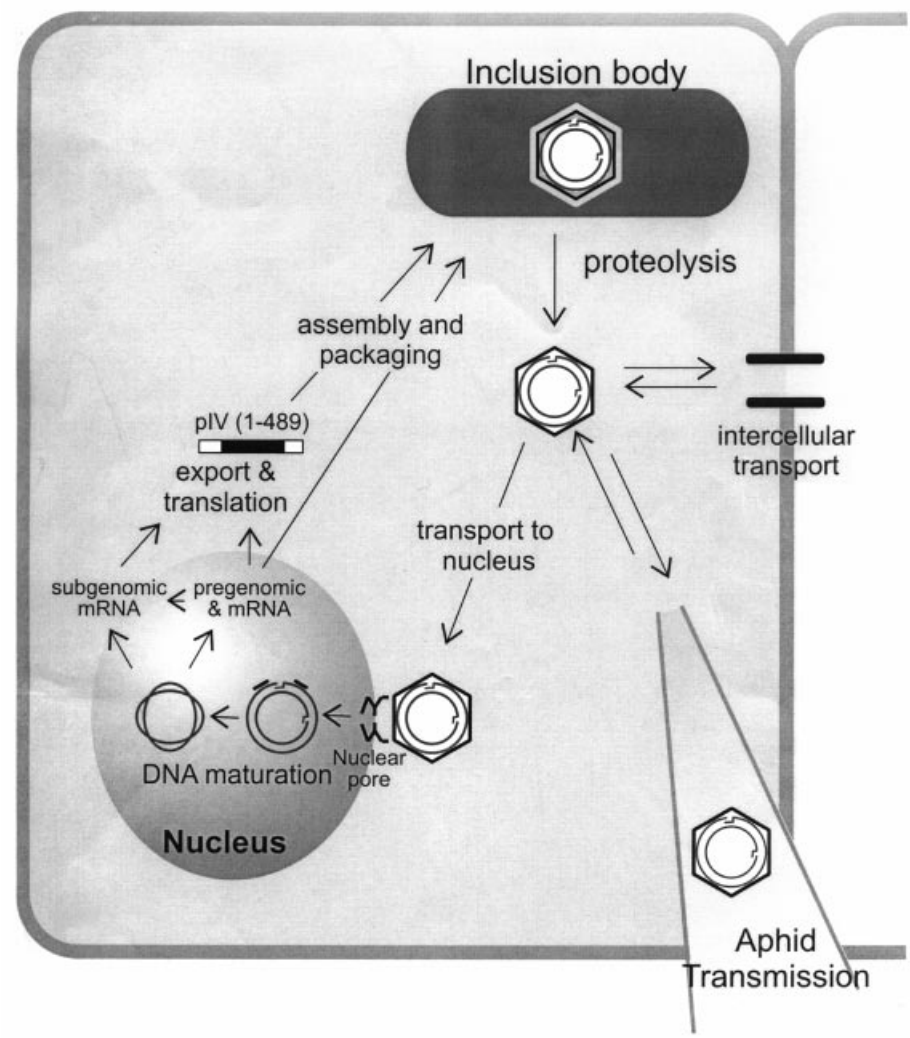


Fig. 5. The CaMV replication cycle. See text (Introduction and Discussion) for details.

can enter through a nuclear pore. More likely, after docking of the virion to the nuclear pore, the capsids would fully or partially disassemble and the DNA would be released into the nucleus, as has been suggested for hepatitis B virus (HBV) (Kann *et al*., 1997, 1999) and herpes simplex virus (Izaurralde *et al*., 1999). In CaMV, transport through the pore could then be mediated by a few CP subunits or by another protein such as reverse transcriptase. In HBV, not only the CP but also the reverse transcriptase carries an NLS (Kann *et al*., 1997).

The CaMV CP precursor is very unstable in plant cells, probably because the N and C termini removed during maturation contain sequences conferring instability (Karsies *et al*., 2001). However, removal of the acidic C-terminal region and mutation of three adjacent glutamic acids within the acidic N-terminal region created a more stable CP precursor variant, p(1–332)*. The acidic N terminus of this variant appears to be a *cis*-active inhibitor of nuclear targeting. The fact that CP precursor protein is excluded from the nucleus despite the presence of a NLS may point to an interesting regulation of intracellular localization: the NLS might be masked by the acidic N terminus of the precursor protein in order to retain it in the cytoplasm to interact with RNA, form previrions and allow reverse transcription. This would assure that only mature, DNA-containing, virions are transported to the nucleus.

It could be that the N-terminal extension of the CP precursor competes with the NLS by binding to a cytoplasmic receptor, e.g. to mediate assembly. In HIV-1, for instance, myristylation of the matrix domain directs the Gag precursor protein to the plasma membrane, where assembly and budding occur (Gelderblom *et al*., 1989). Alternatively, the N-terminal polypeptide could mask the NLS directly by modifying the protein structure. A masking of the NLS in immature capsid (core) protein was suggested for HBV. In this case, however, and in contrast to CaMV (Leclerc *et al*., 1999), activation of the NLS occurred upon phosphorylation of a specific site (Kann *et al*., 1999). We hypothesize that for both viruses, HBV and CaMV, regulation of the CP NLS is important since it ensures virus assembly in the cytoplasm and minimizes the damage

that CP could cause in the nucleus. Interestingly, the CaMV CP is highly toxic to *E. coli* cells, where the DNA replication- and RNA production-machineries are not shielded by a nuclear membrane (Fütterer *et al*., 1988).

To refine the model of the virus replication cycle we propose that one or more of the NLSs located on the CP molecules interact with the nuclear pore (Fig. 5). Subsequently, the virus disassembles and the DNA passes through the pore, either as such or complexed with some CP molecules and/or other proteins, but not with the complete capsid. Once inside the nucleus, the viral genome is repaired and the now supercoiled form associates with histones to form a mini-chromosome, which is transcribed. The acidic N-terminal region of the CP precursor (1–489) inhibits nuclear targeting and therefore this form of protein remains in the cytoplasm, where it is readily degraded (Karsies *et al*., 2001). Gradually, as viral infection progresses, inclusion bodies appear that protect the CP precursor from degradation and in which virus particles accumulate. During or after assembly and packaging, the viral protease will remove the first 76 amino acids from the CP precursor, and this proteolysis will expose the NLSs at the surface of the virions, allowing their transport to the nucleus, interaction with the nuclear pore and DNA release into the nucleus to increase the number of nuclear viral minichromosomes. There are indications that the C-terminal acidic region of the CP precursor also has masking functions. In this case the nucleic acid-binding motif of the CP precursor is affected (Guerra-Peraza *et al*., 2000).

The mature virus particles can also remain in the inclusion body, be transported to a neighbouring cell via plasmodesmata or be captured by a feeding aphid that will transmit the virus to another plant. The mechanism of partition between these fates remains an open question.

We thank M. Müller for preparation of protoplasts. We also thank B. Hohn and H. Rothnie for critical reading of this manuscript and for discussions. This work was supported by the Novartis Research Foundation.

## References

**Bastos, R., Ribas de Pouplana, L., Enarson, M., Bodoor, K. & Burke, B. (1997).** Nup84, a novel nucleoporin that is associated with CAN/Nup214 on the cytoplasmic face of the nuclear pore complex. *Journal of Cell Biology* **137**, 989–1000.

**Belgareh, N. & Doye, V. (1997).** Dynamics of nuclear pore distribution in nucleoporin mutant yeast cells. *Journal of Cell Biology* **136**, 747–759.

**Blanc, S., Cerutti, M., Usmany, M., Vlak, J. M. & Hull, R. (1993).** Biological activity of cauliflower mosaic virus aphid transmission factor expressed in a heterologous system. *Virology* **192**, 643–650.

**Bucci, M. & Wente, S. R. (1997).** In vivo dynamics of nuclear pore complexes in yeast. *Journal of Cell Biology* **136**, 1185–1199.

**Cheng, R. H., Olson, N. H. & Baker, T. S. (1992).** Cauliflower mosaic virus: a 420 subunit (T = 7), multilayer structure. *Virology* **186**, 655–668.

**Dickmanns, A., Bischoff, F. R., Marshallsay, C., Luhrmann, R., Ponstingl, H. & Fanning, E. (1996).** The thermolability of nuclear protein import in tsBN2 cells is suppressed by microinjected Ran-GTP or Ran-GDP, but not by RanQ69L or RanT24N. *Journal of Cell Science* **109**, 1449–1457.

**Du Plessis, D. H. & Smith, P. (1981).** Glycosylation of the cauliflower mosaic virus capsid polypeptide. *Virology* **109**, 403–408.

**Feldherr, C. M., Kallenbach, E. & Schultz, N. (1984).** Movement of a karyophilic protein through the nuclear pores of oocytes. *Journal of Cell Biology* **99**, 2216–2222.

**Freed, E. O., Englund, G., Maldarelli, F. & Martin, M. A. (1997).** Phosphorylation of residue 131 of HIV-1 matrix is not required for macrophage infection. *Cell* **88**, 171–173.

**Fütterer, J., Gordon, K., Pfeiffer, P. & Hohn, T. (1988).** The instability of a recombinant plasmid, caused by a prokaryotic-like promoter within the eukaryotic insert, can be alleviated by expression of antisense RNA. *Gene* **67**, 141–145.

**Fütterer, J., Gordon, K., Pfeiffer, P., Sanfaçon, H., Pisan, B., Bonneville, J. M. & Hohn, T. (1989).** Differential inhibition of downstream gene expression by the CaMV 35S RNA leader. *Virus Genes* **3**, 45–55.

**Fütterer, J., Bonneville, J. M., Gordon, K., DeTapia, M., Karlsson, S. & Hohn, T. (1990).** Expression from polycistronic cauliflower mosaic virus pregenomic RNA. In *Posttranscriptional Control of Gene Expression*, pp. 349–357. Edited by J. E. G. McCarthy & M. F. Tuite. Berlin: Springer.

**Gallay, P., Swingler, S., Song, J., Bushman, F. & Trono, D. (1995).** HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase. *Cell* **83**, 569–576.

**Gallay, P., Hope, T., Chin, D. & Trono, D. (1997).** HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proceedings of the National Academy of Sciences, USA* **94**, 9825–9830.

**Gelderblom, H. R., Özel, M. & Pauli, G. (1989).** Morphogenesis and morphology of HIV structure-function relations. *Archives of Virology* **106**, 1–13.

**Goodall, G. J., Wiebauer, K. & Filipowicz, W. (1990).** Analysis of pre-mRNA processing in transfected plant protoplasts. *Methods in Enzymology* **181**, 148–161.

**Guerra-Peraza, O., de Tapia, M., Hohn, T. & Hemmings-Mieszczak, M. (2000).** Interaction of the cauliflower mosaic virus coat protein with the pregenomic RNA leader. *Journal of Virology* **74**, 2067–2072.

**Himmelbach, A., Chapdelaine, Y. & Hohn, T. (1996).** Interaction between cauliflower mosaic virus inclusion body protein and capsid protein: implications for viral assembly. *Virology* **217**, 147–157.

**Hurkman, W. J. & Tanaka, C. K. (1986).** Solubilization of membrane proteins for analysis by two-dimensional gel electrophoresis. *Plant Physiology* **81**, 802–806.

**Izaurralde, E., Kann, M., Panté, N., Sodeik, B. & Hohn, T. (1999).** Viruses, microorganisms and scientists meet the nuclear pore. *EMBO Journal* **18**, 289–296.

**Kann, M., Bischof, A. & Gerlich, W. H. (1997).** In vitro model for the nuclear transport of the hepadnavirus genome. *Journal of Virology* **71**, 1310–1316.

**Kann, M., Sodeik, B., Vlachou, A., Gerlich, W. H. & Helenius, A. (1999).** Phosphorylation-dependent binding of hepatitis B virus core particles to the nuclear pore complex. *Journal of Cell Biology* **145**, 45–55.

**Karsies, A., Hohn, T. & Leclerc, D. (2001).** Degradation signals within both terminal domains of the cauliflower mosaic virus capsid protein precursor. *Plant Journal* **27**, 335–343.

**Kunik, T., Palanichelvam, K., Czosnek, H., Citovsky, V. & Gafni, Y. (1998).** Nuclear import of the capsid protein of tomato yellow leaf curl virus (TYLCV) in plant and insect cells. *Plant Journal* **13**, 393–399.

**Lebeurier, G., Hirth, L., Hohn, T. & Hohn, B. (1980).** Infectivities of native and cloned DNA of cauliflower mosaic virus. *Gene* **12**, 139–146.

**Leclerc, D., Chapdelaine, Y. & Hohn, T. (1999).** Nuclear targeting of the cauliflower mosaic virus coat protein. *Journal of Virology* **73**, 553–560.

**Martinez-Izquierdo, J. & Hohn, T. (1987).** Cauliflower mosaic virus coat protein is phosphorylated in vitro by a virion associated protein kinase. *Proceedings of the National Academy of Sciences, USA* **84**, 1824–1828.

**Merkle, T., Leclerc, D., Marshallsay, C. & Nagy, F. (1996).** A plant in vitro system for the nuclear import of proteins. *Plant Journal* **10**, 1177–1186.

**Mieszczak, M., Klahre, U., Levy, J. H., Goodall, G. J. & Filipowicz, W. (1992).** Multiple plant RNA binding proteins identified by PCR: expression of cDNAs encoding RNA binding proteins targeted to chloroplasts in *Nicotiana plumbaginifolia*. *Molecular and General Genetics* **234**, 390–400.

**Moroianu, J., Blobel, G. & Radu, A. (1996).** Nuclear protein import: Ran-GTP dissociates the karyopherin alphabeta heterodimer by displacing alpha from an overlapping binding site on beta. *Proceedings of the National Academy of Sciences, USA* **93**, 7059–7062.

**Panté, N. & Aebi, U. (1996).** Sequential binding of import ligands to distinct nucleopore regions during their nuclear import. *Science* **273**, 1729–1732.

**Panté, N. & Kann, M. (2002).** The nuclear pore complex is able to transport macromolecules with diameters of about 39 nm. *Molecular Biology of the Cell* 10·1091/mbc.01-06-0308.

**Roe, T.-Y., Reynolds, T. C., Yu, G. & Brown, P. O. (1993).** Integration of murine leukemia virus DNA depends on mitosis. *EMBO Journal* **12**, 2099–2108.

**Rothnie, H. M., Chapdelaine, Y. & Hohn, T. (1994).** Pararetroviruses and retroviruses: a comparative review of viral structure and gene expression strategies. *Advanced Virus Research* **44**, 1–67.

**Schagger, H. & von Jagow, G. (1987).** Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry* **166**, 368–379.

**Smith, H. M., Hicks, G. R. & Raikhel, N. V. (1997).** Importin alpha from *Arabidopsis thaliana* is a nuclear import receptor that recognizes three classes of import signals. *Plant Physiology* **114**, 411–417.

**Szurek, B., Marois, E., Bonas, U. & Van den Ackerveken, G. (2001).** Eukaryotic features of the *Xanthomonas* type III effector AvrBs3: protein domains involved in transcriptional activation and the interaction with nuclear import receptors from pepper. *Plant Journal* **26**, 1–16.

**Torruella, M., Gordon, K. & Hohn, T. (1989).** Cauliflower mosaic virus produces an aspartic proteinase to cleave its polyproteins. *EMBO Journal* **8**, 2819–2825.

**Whittaker, G. R. & Helenius, A. (1998).** Nuclear import and export of viruses and virus genomes. *Virology* **246**, 1–23.

**Yamada, M. & Kasamatsu, H. (1993).** Role of nuclear pore complexes in SV40 nuclear targeting. *Journal of Virology* **67**, 119–130.

Received 5 February 2002; Accepted 15 March 2002