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Nuclear Targeting of the Tegument Protein pp65 (UL83) of Human Cytomegalovirus: an Unusual Bipartite Nuclear Localization Signal Functions with Other Portions of the Protein To Mediate Its Efficient Nuclear Transport

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Large amounts of pp65 (UL83) of human cytomegalovirus are translocated to the cell nucleus during the first minutes after uptake of the tegument protein from infecting viral particles. Two stretches of basic amino acids which resembled nuclear localization signals (NLS) of both the simian virus 40 type and the bipartite type were found in the primary structure of pp65. Deletion of these sequences significantly impaired nuclear localization of the truncated proteins after transient expression. The results indicated that both elements contributed to the nuclear localization of the protein. When fused to the bacterial β-galactosidase, only one of the two basic elements was sufficient to mediate nuclear translocation. This element consisted of two clusters of basic amino acids (boxes C and D), which were separated by a short spacer sequence. In contrast to other bipartite NLS of animal cells, both basic boxes C and D functioned independently in nuclear transport, thus resembling simian virus 40-type NLS. Yet, complete translocation of β-galactosidase was only found in the bipartite configuration. When both boxes C and D were fused, thereby deleting the intervening sequences, the nuclear transport of β-galactosidase was reduced to levels seen with constructs in which only one of the boxes was present. Appropriate spacing, therefore, was important but not absolutely required. This was in contrast with results for other bipartite NLS, in which spacer deletions led to complete cytoplasmic retention. The presented results demonstrate that efficient nuclear transport of pp65 is mediated by one dominant NLS and additional targeting sequences. The major NLS of pp65 is an unusual signal sequence composed of two weak NLS which function together as one strong bipartite nuclear targeting signal.

The phosphorylated tegument pp65 (UL83) of human cytomegalovirus (HCMV), a betaherpesvirus, can be detected in up to 1% of the nuclei of polymorphonuclear leukocytes isolated from acutely infected patients (11). The presence of this protein in blood cells is independent of any detectable RNA synthesis from the gene encoding pp65 (12). This suggests that large quantities of the protein are brought into the cells via infecting viral particles and are translocated to the nucleus by the cellular transport machinery. Nuclear transport of tegument proteins has emerged as an important mechanism in the regulation of viral gene expression and replication. For the human immunodeficiency virus, it has been shown that the nucleophilic Gag protein can mediate the nuclear entrance of the viral preintegration complex of 300 Å (30 nm) in nondividing cells (4). In herpes simplex virus, the tegument protein VP16 is crucial for the transactivation of viral immediate-early promoters in the nucleus and for the initiation of lytic viral replication immediately after infection (for a review, see reference 27).

Depending on their size, proteins can enter the nucleus either via passive diffusion or by active transport through nuclear pores (13, 23). Active nuclear transport of proteins depends, in most cases, on the presence of short stretches of basic amino acids in the primary polypeptide structure. Although no single consensus has emerged for these nuclear localization signals (NLS), two classes have been defined (21, 32). One class, represented by the prototype sequence found in the T antigen of simian virus 40 (SV40), consists of one contiguous stretch of basic amino acids with a predominance of arginine and lysine residues (16). In addition, single proline residues have been frequently found close to or within the NLS. The second class, first described for *Xenopus laevis* nucleoplasmin, has a bipartite structure (25). Bipartite NLS have been defined as consisting of two short stretches of basic residues which are separated by a spacer region of 10 or more nonconserved residues (8, 18, 30, 34). Although they are occasionally similar in structure to the SV40 NLS, the single basic elements of bipartite NLS have a very limited function in nuclear import in animal cells (17, 25).

Sequence elements were found in the carboxy terminus of pp65 that resembled the structure of known NLS. One such element (RKTPRVTGGGAMAGASTSAGRKRK), termed motif A-B, consisted of two boxes of basic residues separated by a putative spacer element of 18 amino acids (aa). It thus matched the proposed consensus sequence of bipartite NLS. In addition to that, a contiguous stretch of basic amino acids (PKRRRHR) closely resembling the SV40 NLS (PKKKRKV) was located at the carboxy terminus of pp65. This sequence was followed by a second basic box (PKKHR), and they were separated by 13 nonbasic aa. The structure was termed motif C-D. This part of pp65 was consistent with the SV40 type as well as with the bipartite type structure of NLS.

In order to investigate which sequence elements were re-

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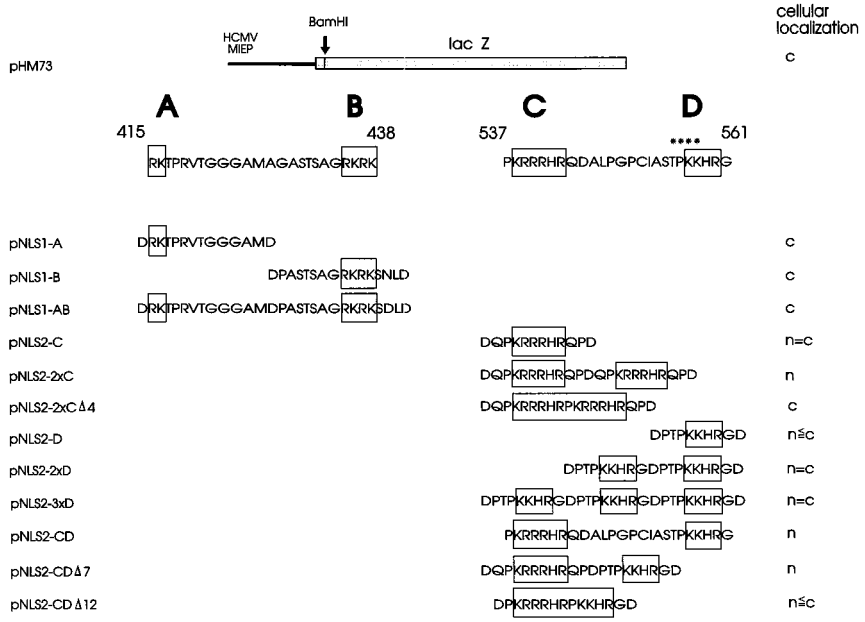

FIG. 1. Schematic representation of NLS–β-Gal fusion constructs. The β-Gal expression vector pHM73 and the *Bam*HI site used for cloning are given at the top of the figure. Below that, the sequences of the two NLS motifs (A-B and C-D) and the amino acids with respect to pp65 are shown. Asterisks indicate the location of a putative $p34^{cdc2}$ phosphorylation site in motif C-D. The structures of the different fusion constructs used for transfections are given in the central part of the figure. Boxes indicate the location of the basic clusters. The names of the plasmids are given at the left. The subcellular localizations, as evaluated on at least 100 different cells per transfection and plasmid in three different experiments, are depicted at the right. n, nuclear; c, cytoplasmic; n = c, n > c, n ≥ c, and n ≤ c indicate the relative intensities of the staining in the nuclear and cytoplasmic compartments. MIEP, major immediate-early promoter.

sponsible for the efficient nuclear accumulation of pp65 immediately after infection, deletion analyses were carried out. It became evident that both regions of pp65 containing the basic amino acid structures were involved but not equally active in nuclear transport and that additional nonconsensus NLS sequences may be present in the amino terminal part of pp65. Further analyses of the putative NLS regions revealed that only motif C-D but not motif A-B was sufficient to direct the cytoplasmic β-galactosidase (β-Gal) to the nucleus of transfected cells. Functional analysis of the two basic boxes C and D showed that the presence of both clusters, separated by a spacer sequence, was necessary for complete nuclear accumulation. In contrast to those of other bipartite NLS that have been analyzed (9, 17), both basic clusters could independently direct, though at reduced levels, the nuclear targeting of the β-Gal fusion protein. Therefore, the carboxy terminus of the viral tegument protein pp65 contains two structurally and functionally distinct bipartite nuclear targeting sequences which can direct nuclear uptake of large amounts of the polypeptide immediately after infection.

MATERIALS AND METHODS

Cells and virus. COS7 cells were grown in Dulbecco's modified Eagle's medium supplemented with glutamine, gentamicin, and 5% fetal calf serum. Human foreskin fibroblasts (HFF) were grown in modified Eagle's medium supplemented with glutamine, gentamicin, and 5% fetal calf serum. To investigate the uptake of pp65 from virus particles, HFF were seeded overnight in 10-cm-diameter plastic petri dishes at a rate of 1×10^6 cells per well. Fresh medium containing cycloheximide (200 μg/ml) was added 1 h prior to infection. Cells were infected with strain AD169 at a multiplicity of infection of 0.02 PFU per cell in the presence of cycloheximide. Infected cells were incubated at 37°C for 5, 10, 20, 30, or 60 min. After that, cells were washed with phosphate-buffered saline (PBS) and scraped off. Cell lysates were subsequently analyzed by Western blot (immunoblot).

The different HCMV particles in the supernatant of AD169-infected HFF were separated with a positive density-negative viscosity gradient system (15). Bands corresponding to the different forms of extracellular particles were collected by aspiration, pelleted, and resuspended in 0.04 M sodium phosphate buffer (pH 7.4). For indirect immunofluorescence analyses, HFF were seeded overnight on glass chamber slides and incubated with the purified virions (multiplicity of infection of 1.8 PFU per cell) and dense bodies for 30 min at 37°C in the presence of cycloheximide (100 μg/ml).

Expression plasmids. The construction of the deletion mutants was based on the eukaryotic expression plasmid pp65RSV containing the complete coding region for pp65 of HCMV, strain AD169 (5, 11, 28). Plasmid pp65TA, lacking coding sequences for aa 398 to 456, was constructed from pp65RSV. In-frame religation was achieved by cleavage of pp65RSV with *Afl*II and subsequent mung bean nuclease treatment. After digestion with *Tth*111I in a second step, the protruding ends were filled in with Klenow polymerase. The constructs pp65AX, lacking aa 456 to 561, and pp65NX, lacking aa 490 to 561, were constructed by deleting either an *Afl*II-*Xho*I fragment or an *Nae*I-*Xho*I fragment and religating the mung bean nuclease-digested ends. Plasmid pp65d, lacking aa 513 to 561 of pp65, was described elsewhere (11). For the cloning of pp65AN, which lacks aa 456 to 491, plasmid pp65RSV was digested with *Afl*II and *Nae*I. To ensure in-frame religation, two T residues were filled in at the *Afl*II site with Klenow polymerase, and this was followed by mung bean nuclease digestion to remove single-stranded ends. Deletion of a *Tth*111I-*Xho*I fragment and religation of the blunt ends after mung bean nuclease digestion resulted in the plasmid pp65TX, which lacks aa 398 to 561 of pp65. Plasmid pp65dTA, which lacks aa 398 to 465 and 513 to 561, was constructed from plasmid pp65d by digestion and ligation as described for pp65TA.

The eukaryotic expression vector pHM73 (kindly provided by T. Stamminger) used for the expression of β-Gal fusion proteins contained the bacterial *lacZ* gene lacking the first seven nonessential N-terminal codons. This construct was driven by the HCMV major immediate-early promoter enhancer. A single *Bam*HI site was inserted at the very 5′ end of the β-Gal gene, and this site was used for further cloning (Fig. 1). The DNA sequence generated by this encoded the amino acid sequence MG amino terminal to the sequences depicted in Fig. 1. Oligonucleotides (kindly provided by R. Vornhagen) were synthesized on an Applied Biosystems 381A synthesizer and purified with OPC Cartridges (Applied Biosystems, Weiterstadt, Germany) according to the recommendations of the supplier. pNLS1-AB 379-481 was constructed by PCR amplification (with pp65RSV as the template) of a fragment corresponding to aa 379 to 481. pNLS2-CD was constructed by PCR amplification of a region corresponding to aa 537 to 561. Constructs obtained by oligonucleotide cloning are depicted in Fig. 1. In-frame ligation of the different oligonucleotides and PCR fragments was verified by sequencing.

Transfections. COS7 cells were seeded overnight at a rate of 2.5×10^5 cells per well in six-well dishes containing microscope coverslips. The next evening, cells were transfected with 2 μg of DNA by the calcium phosphate coprecipita-

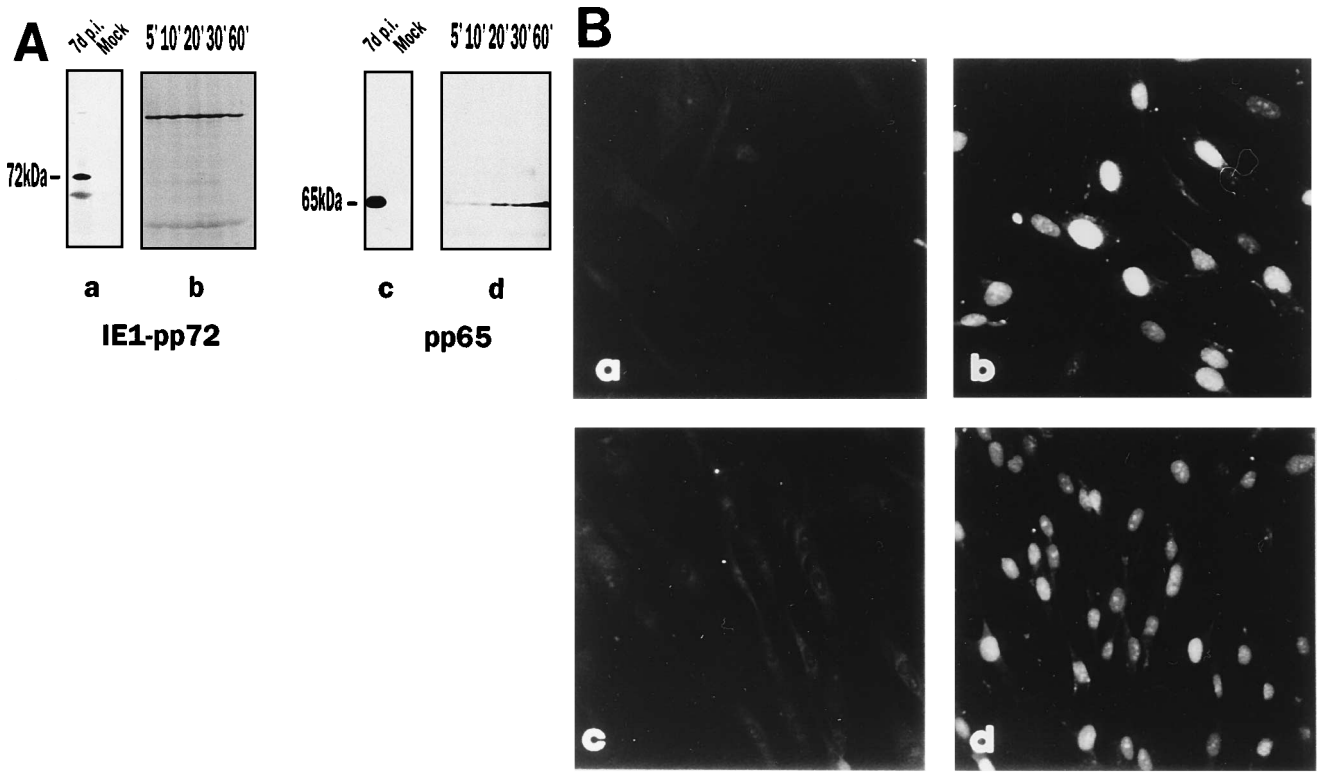

FIG. 2. Uptake and nuclear transport of pp65 after HCMV infection. (A) Immunoblot analysis of protein extracts from HFF infected with extracellular particles of HCMV. Cells were infected in the presence of cycloheximide at 37°C for the times indicated at the top of the figure. Panels a and b, immunoblot analysis with MAb p63-27 directed against the IE1-pp72 of HCMV; panels c and d, immunoblot analysis with MAb 28-77 directed against pp65. The locations of IE1-pp72 and pp65 are given to the left of the blots. Panels a and c represent controls. 7d p.i., HFF infected with HCMV for 7 days; Mock, uninfected HFF. Panels b and d were exposed for a prolonged period by comparison with the exposure periods for panels a and b. (B) Immunofluorescence analysis of the subcellular localization of pp65 after infection of HFF with purified dense bodies and virions. Cells were incubated with purified dense body particles (panels a and b) or virions (panels c and d) at 37°C for 30 min in the presence of cycloheximide (100 μg/ml). Staining was performed with antibodies against IE1-pp72 (panels a and c, MAb p63-27) and against the tegument protein pp65 (panels b and d, MAb MO58).

tion technique (1). The precipitate was removed after 14 h, and the cells were incubated for a further 24 h. The cellular distribution of the fusion proteins was analyzed by indirect immunofluorescence analysis.

Indirect immunofluorescence staining and Western blot analyses. Transfected cells grown on coverslips and infected HFF grown on chamber slides were washed in PBS and fixed in methanol at −20°C for 10 min. The slides were incubated for 30 min at 37°C with IE1-specific mouse monoclonal antibody (MAb) p63-27 (24), pp65-specific human MAb MO58 (22), or β-Gal-specific mouse MAb 87/55-60 (3). After three washes in PBS of 5 min each, the slides were incubated with fluorescein isothiocyanate-conjugated anti-human or anti-mouse immunoglobulin (Dako, Hamburg, Germany) at 37°C for 30 min. After three washes in PBS of 5 min each, immunofluorescence was evaluated with a Zeiss Axioskop microscope.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analyses were carried out essentially as described before (29), except that blocking and incubation with antibodies were carried out in PBS containing 0.1% Tween and 3 to 5% dry milk powder. For the detection of pp65, the MAb 28-77 was used (33). The blot was developed with the ECL chemiluminescent detection system (Amersham, Braunschweig, Germany) according to the recommendations of the manufacturer.

RESULTS

The tegument protein pp65 is rapidly transported to the nucleus after infection of cells with HCMV. The tegument protein pp65 is abundantly synthesized late after HCMV infection of permissive cells (7) and is subsequently packaged into progeny virus particles. In a previous study, we showed that pp65 is also present in cells immediately after infection (11). It was demonstrated that the presence of pp65 in these cells is attributable to uptake of the protein from infecting viral particles instead of to de novo synthesis. To investigate the kinetics of cellular uptake of this protein after infection, immunoblot analyses were carried out. Permissive HFF were infected with viral particles purified from cell culture supernatant and were incubated for 5, 10, 20, 30 or 60 min either in the presence or in the absence of the protein synthesis inhibitor cycloheximide. After different intervals, the cells were thoroughly washed and scraped off the culture vessels. Cell lysates were subjected to immunoblot analyses with a MAb directed against pp65. The same lysates were also tested with a MAb against the nonstructural immediate-early protein IE1-pp72 (UL123), which is not found in extracellular particles. Under these conditions, pp65 was already detectable after an incubation period of 5 min (Fig. 2A). The quantity of the protein increased over time. In contrast, no immediate-early protein could be detected in the controls.

To investigate whether the polypeptide was immediately transported to the nucleus, cells were infected with purified virions at a multiplicity of infection of 1.8 PFU per cell in the presence of cycloheximide; in addition, cells were also incubated with defective particles, commonly referred to as dense bodies. Dense bodies are enveloped structures synthesized by infected cells in vitro. The protein content of these particles is 90% pp65, while they lack viral DNA (26). Infection was stopped after 30 min, and the cellular location of pp65 was investigated by indirect immunofluorescence analysis. The tegument protein was exclusively detectable in the nucleus of cells

incubated with either dense bodies (Fig. 2B, panels a and b) or infectious virions (Fig. 2B, panels c and d). This was independent of the presence of cycloheximide during infection. No specific staining of the regulatory protein IE1-pp72 was detectable. In contrast to the pp65 gene, which belongs to the delayed early class, the gene encoding IE1-pp72 is among the first to be transcribed after infection. These data therefore indicated that cells can acquire pp65 from both infectious virions and dense bodies and that this protein is immediately and efficiently transported to the cell nucleus in the absence of viral gene expression.

Determination of the sequences necessary for nuclear transport of pp65. Infection experiments indicated that pp65 was very efficiently targeted to the nucleus of cells that were incubated with viral particles. Two regions were found in the amino acid sequence of pp65 which contained stretches of basic amino acids. Within each, two basic boxes (motifs A-B and C-D) were separated by spacer sequences of 18 and 13 aa, respectively (Fig. 3A). To analyze whether these sequences would function in nuclear import, different expression plasmids with deletions in the coding sequence for pp65 were constructed and transfected into COS7 cells (Fig. 3A). The localization of the wild-type and truncated pp65 was analyzed by indirect immunofluorescence with pp65-specific MAbs (Fig. 3B). As expected, transfection of plasmid pp65RSV containing the complete coding sequence for pp65 resulted in exclusively nuclear staining, verifying that nuclear transport of pp65 was independent of that of other viral proteins (Fig. 3B, panel a). Removal of the amino-terminal putative NLS (motif A-B) in clone pp65TA did not change the nuclear accumulation (Fig. 3B, panel b). However, deletion of different portions of the carboxy terminus of pp65, including motif C-D, led to an impairment of nuclear translocation (constructs pp65AX, pp65NX, and pp65d). The distributions of the recombinant proteins within transfected cells were identical according to a comparison of the different clones. One example is shown in Fig. 3B, panel c. This indicated that the sequence elements in the carboxy terminus of pp65 were necessary for complete nuclear accumulation of the protein.

To investigate whether the amino-terminal motif A-B was also involved in nuclear transport independent of motif C-D, constructs were made in which both elements were deleted. Transfection of the construct pp65TX, which coded for a truncated version of pp65 (aa 1 to 397) and lacked both putative NLS, resulted in partial cytoplasmic retention (Fig. 3A). Compared with that of the clones, in which only motif C-D was deleted, cytoplasmic retention with pp65TX was more pronounced. As the protein synthesized from this construct lacked more than 25% of wild-type pp65 and had an apparent molecular mass of about 40 to 45 kDa, passive diffusion, as reported for several other proteins of this size, could not totally be excluded. Therefore, clone pp65dTA was constructed (Fig. 3A). The protein made from this construct lacked both putative NLS but retained the intervening portions of pp65, resulting in an apparent molecular mass of about 60 kDa (data not shown). Transfection of this clone resulted in an enhanced cytoplasmic staining compared with that of the truncated protein of pp65TX (Fig. 3B, panel d). These results indicated that motif A-B was involved in nuclear localization. However, as the deletion of both A-B and C-D did not result in complete cytoplasmic retention, additional nonconsensus NLS amino terminal to A-B may contribute to the nuclear transport of pp65.

Basic clusters C and D function together as a bipartite NLS. To analyze which of the sequence elements found in pp65 would be sufficient to function as NLS, the different motifs were expressed in fusions with a cytoplasmic protein. For this, PCR fragments and oligonucleotides encoding various portions of the putative NLS were cloned in an eukaryotic expression vector encoding the β-Gal from *Escherichia coli*. The latter protein is a tetramer of 480 kDa which has been frequently used to analyze the NLS of nucleophilic proteins (18, 30, 34). The different constructs were transfected into COS

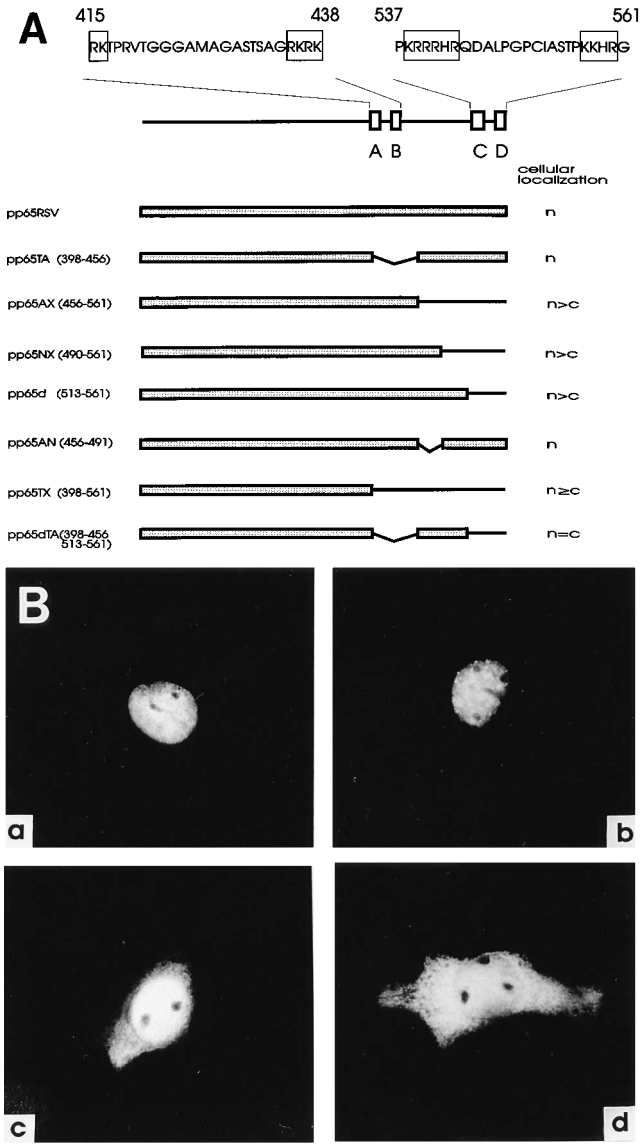

FIG. 3. Mapping of sequences involved in nuclear transport of pp65. (A) Structure and subcellular localization of mutated pp65 proteins. The sequences of the basic clusters A, B, C, and D with spacer sequences and their relative locations on pp65 are given at the top of the figure. Shaded boxes indicate the sequences contained in the different expression constructs. The amino acids, which were deleted in the constructs, are given to the left. The localizations in transfected COS7 cells are depicted to the right. n, nuclear; c, cytoplasmic; n = c, n > c, and n ≥ c indicate the relative intensities of the staining in the nuclear and cytoplasmic compartments. The distribution of the different proteins was assessed by comparing the stainings in at least 100 different cells per transfection. Experiments were repeated at least three times for each plasmid. (B) Immunofluorescence analysis of the subcellular localization of the deletion mutants of pp65. COS7 cells were transfected with constructs pp65RSV (panel a), pp65TA (panel b), pp65d (panel c), and pp65dTA (panel d) and stained with MAb MO58 directed against pp65. The sizes of the cells investigated were comparable, as verified by phase-contrast microscopy (data not shown).

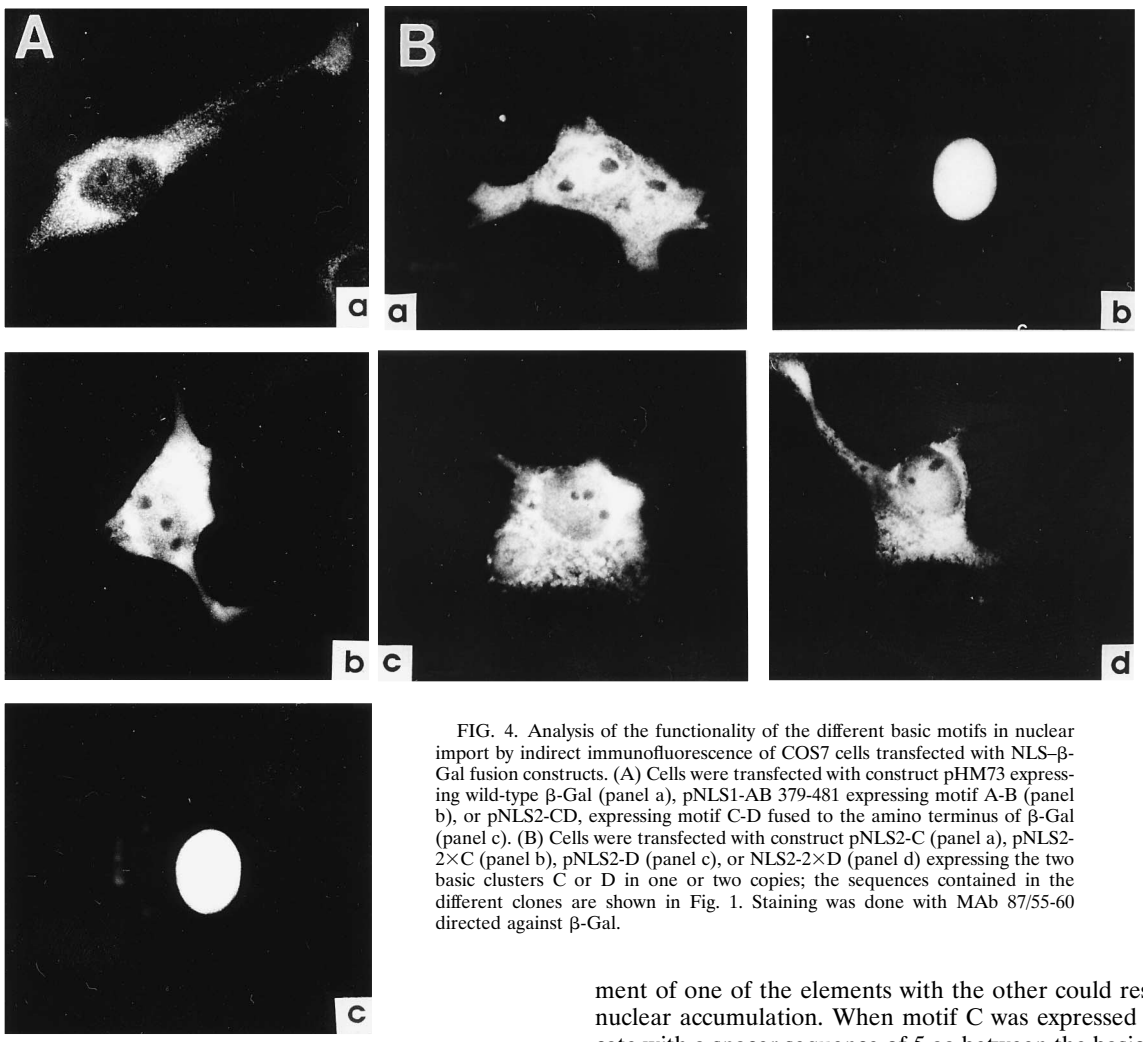

FIG. 4. Analysis of the functionality of the different basic motifs in nuclear import by indirect immunofluorescence of COS7 cells transfected with NLS–β-Gal fusion constructs. (A) Cells were transfected with construct pHM73 expressing wild-type β-Gal (panel a), pNLS1-AB 379-481 expressing motif A-B (panel b), or pNLS2-CD, expressing motif C-D fused to the amino terminus of β-Gal (panel c). (B) Cells were transfected with construct pNLS2-C (panel a), pNLS2-2×C (panel b), pNLS2-D (panel c), or NLS2-2×D (panel d) expressing the two basic clusters C or D in one or two copies; the sequences contained in the different clones are shown in Fig. 1. Staining was done with MAb 87/55-60 directed against β-Gal.

cells and analyzed by indirect immunofluorescence. Expression of the amino-terminal clusters A and B, alone or in combination, did not significantly change the cellular distribution of β-Gal (Fig. 1). Also, clone pNLS1-AB 379-481, encoding motif A-B and additional flanking sequences, was not significantly active in nuclear transport (Fig. 4A). In contrast, fusion of the carboxy-terminal element PKRRRHRQDALPGPCIASTPKKHR (motif C-D) to β-Gal in clone pNLS2-CD resulted in complete nuclear translocation of the protein (Fig. 4A), showing that a functional NLS was contained within this sequence. To further analyze the sequence elements which mediate nuclear transport, each of the basic portions was fused to the amino terminus of β-Gal. Both fusion proteins containing either one copy of C or D were translocated to the nucleus (Fig. 1 and Fig. 4B). In these experiments, a homogeneous staining of the cytoplasm and nucleus of transfected cells which was clearly distinct from the background nuclear staining seen with β-Gal alone was found (Fig. 4A). This indicated that each basic cluster could function independently in nuclear translocation. However, complete translocation was not seen with either of the two basic motifs. This indicated that the bipartite configuration was necessary for efficient accumulation of the protein.

As the primary structures of C and D were similar, we investigated in the next set of experiments whether replacement of one of the elements with the other could restore full nuclear accumulation. When motif C was expressed in duplicate with a spacer sequence of 5 aa between the basic clusters, complete nuclear translocation of the fusion protein was observed. When D was expressed in duplicate or in triplicate with an intervening sequence of 5 aa, no enhancement of nuclear translocation, as compared with translocation with the construct carrying only one copy, was seen (Fig. 1 and 4B).

These differences suggest that the ability of two copies of box C to mediate complete nuclear translocation of construct pNLS2-2×C (KR-8 aa-PKRRRHR) was not because of the generation of a match to the suggested consensus sequence for bipartite NLS; a similar match was also generated in pNLS2-3×D (KK-15 aa-PKKHR) without leading to an enhanced nuclear translocation. Therefore, although both C and D were comparably functional as single copies, they were not equivalent in their abilities to contribute to nuclear transport in a bipartite configuration.

Spacing requirements between basic boxes C and D. The spacer sequences found between the basic boxes in bipartite NLS described so far consist of 10 or more nonconserved amino acids (8). To elucidate the significance of the 13-aa spacer between boxes C and D of pp65 for nuclear import, β-Gal fusion constructs with reduced spacer sequences were made and transfected into COS cells. No alteration of the nuclear transport of the fusion protein compared with that of the wild type was observed after the spacer between C and D was reduced to 5 aa (pNLS2-CDΔ7). However, the sequence expressed in this clone (PKRRRHRQPDPTPKKHR) resem-

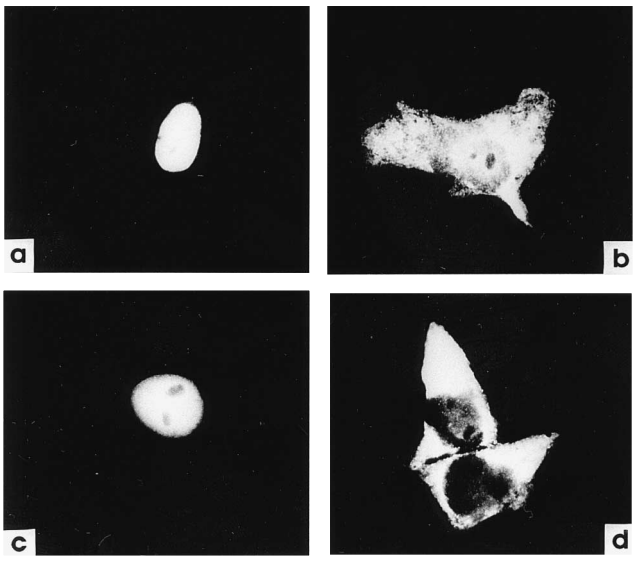

FIG. 5. Analysis of spacer requirements for nuclear import signals. COS7 cells were transfected with β-Gal fusion constructs pNLS2-CDΔ7 (panel a), pNLS2-CDΔ12 (panel b), pNLS2-2×C (panel c); or pNLS2-2×CΔ4 (panel d); stainings were done with MAb 87/55-60 directed against β-Gal. The sequences contained in the different constructs can be seen in Fig. 1.

bled the consensus for bipartite NLS (KR-10 aa-KKKK). Therefore, the residual 5 aa between C and D were deleted in construct pNLS2-CDΔ12 by otherwise retaining the original basic sequences. The protein made from this construct was significantly impaired in its nuclear import, leading to a cellular distribution comparable to that seen with either C or D alone (Fig. 4B and 5). This indicated that the spacer length was critical in this targeting sequence, which is in agreement with previous findings reported for bipartite NLS (25). In contrast to these reports, however, reduced levels of nuclear import were still possible with this construct.

To investigate whether the nuclear transport of a fusion protein containing C-D without a spacer sequence was due to our experimental conditions, control experiments were carried out. For this, a deleted version of construct pNLS2-2×C was generated. The primary sequence of pNLS2-2×C (PKRRRHRQPDQPKRRRHR) closely resembled that of clone pNLS2-CDΔ7, especially in that it matched the published consensus. Deletion of 4 aa between the two C boxes led to a construct (pNLS2-2×CΔ4) that was equivalent in structure to pNLS2-CDΔ12. In contrast to the latter, transfection of pNLS2-2×CΔ4 led to complete retention of the respective fusion protein after transfection. This result was in agreement with what had been published for bipartite NLS. Therefore, the NLS of pp65 is distinct from other bipartite NLS in that spacing requirements are less restrictive.

On the other hand, these results suggest that deletion of the spacer and not deletion of a putative $p34^{cdc2}$-kinase site overlapping with box D in construct pNLS2-CDΔ7 was responsible for the inhibition of complete nuclear translocation seen with pNLS2-CDΔ12 (Fig. 1 and 5). This is evidenced by the finding that although no such kinase site was present in construct pNLS2-2×C, deletion of the spacer in this plasmid to result in pNLS2-2×CΔ4 also led to the inhibition of nuclear translocation.

DISCUSSION

The tegument protein pp65 of HCMV was introduced into cells via infection with virions and dense bodies, and it subsequently accumulated in the nucleus. No cytoplasmic staining was observed at different time points immediately after infection in culture, indicating that effective cellular mechanisms were active in the nuclear translocation of large amounts of pp65. This effect is not restricted to cell culture experiments; independent of viral gene expression, pp65 can be detected in the nuclei of polymorphonuclear leukocytes during active infection in vivo (11, 12). Its rapid uptake and transport suggest that this protein serves as an important factor for the initial events of viral infection. Yet, little is known about the role of pp65 in the replication of HCMV. The finding that pp65 is associated with protein kinase activity (2, 31) implies that this polypeptide has some regulatory function in the activation of gene expression after infection (14). However, no transactivation has been demonstrated for pp65 so far (19, 28a). By deletional analysis, two sequence elements (motifs A-B and C-D) which contributed to the nuclear transport of pp65 were identified. Proteins lacking motif C-D were impaired in nuclear import, whereas all proteins containing this sequence were detected exclusively within the nucleus. This indicated that C-D functioned as an NLS. Yet, no complete cytoplasmic retention was observed when C-D was deleted, showing that additional sequence elements within pp65 were active in nuclear transport.

Successive deletion of sequences from the C terminus of pp65 in constructs pp65d, pp65NX, and pp65AX did not result in altered distribution, indicating that sequences between A-B and C-D did not contribute to the nuclear localization of pp65. Deletion beyond motif A-B (pp65TX) removed both A-B and C-D and led to only slightly enhanced cytoplasmic retention. Analysis of the protein made from pp65TX in immunoblots showed, however, that it had a relative mobility of about 40 to 45 kDa (data not shown). Proteins of that size may enter the nucleus via passive diffusion through nuclear pores (for a review, see reference 23). Thus, it could not be excluded that passive diffusion was responsible for the nuclear accumulation of the protein synthesized from pp65TX. Therefore, pp65dTA was constructed with the intervening sequences between A-B and C-D retained. The protein made from this plasmid showed an apparent molecular mass of about 60 kDa (data not shown) and thus should have been too large to diffuse through nuclear pores. When the localization of this protein was compared with the localization of the protein made from pp65d, an enhanced cytoplasmic retention was noted. This indicated that motif A-B contributed to the rapid nuclear accumulation of pp65, possibly influencing the kinetics of import. These effects were not caused by different steady-state levels of proteins made from pp65dTA and pp65d, as evidenced by immunoblot analyses with lysates from transfected COS cells (data not shown).

When both putative NLS were deleted, some nuclear targeting of the truncated protein could still be observed. Nuclear staining, however, was substantially reduced in comparison with that of the constructs in which one of the putative NLS was present. One reason for this residual nuclear accumulation could be that the sizes of the truncated proteins would allow passive diffusion. As discussed, this could account for the nuclear translocation of the protein made from pp65TX, but it was unlikely to be the reason for the nuclear accumulation of the pp65dTA protein. Therefore, additional portions of pp65 with no apparent homology to consensus sequences of NLS appear to function as weak targeting sequences. Extensive deletional analyses of aa 1 to 400 of pp65 will be required to

identify these sequences. In addition, it should be mentioned that proteins made from clones pp65RSV or pp65TA showed reduced steady-state levels when compared by immunoblot analyses with those synthesized after transfection of, e.g., clone pp65d or pp65TA (data not shown). Therefore, it cannot be excluded that part of the cytoplasmic retention seen with pp65d and pp65dTA when compared with that seen with the wild type may be attributable to the higher amount of these proteins within transfected cells.

In line with the deletion analyses, A-B and C-D showed differences in their abilities to target *E. coli* β-Gal into the nucleus. Motif A-B displayed no significant effect when expressed in fusion with β-Gal. The reason for this is not clear. It cannot be formally excluded that positional effects resulted in the failure of the fusion protein to translocate to the nucleus and that expressing the A-B motif at another site within β-Gal would have resulted in a different accumulation. However, fusion to the amino terminus of β-Gal was used in several studies to prove the sufficiency of a peptide sequence to function as a bona fide NLS (16, 20). Alternatively, it could be put forward that additional sequences outside motif A-B would be important for nuclear translocation mediated by this sequence. Flanking sequences immediately adjacent to A-B could not account for that, because a larger fragment containing A-B could not mediate nuclear translocation after being fused to β-Gal (pNLS1-AB 379-481). However, in a recent study, it was shown that sequences located distal to the NLS of the herpes simplex virus type 1 ICP8 could influence nuclear targeting (10). It therefore appears possible that in the case of pp65, distal protein structures and possibly phosphorylation at distant sites are critical for A-B to function as an NLS.

In contrast, a peptide sequence encompassing motif C-D could direct nuclear import, confirming that this sequence contained a bona fide NLS. The overall structure of this element consisting of basic boxes C and D and a putative spacer sequence of 13 aa appears to be remarkable in different respects. The amino-terminal box C (PKRRRHR) is almost identical in its basic structure with the NLS of SV40 (PKKKRKV). However, in contrast to the NLS of SV40, C alone could only partially target the β-Gal fusion protein to the nucleus. The *Xenopus* nuclear protein N1 has a bipartite NLS with an amino-terminal basic sequence (RKKKRK) which resembles both the SV40 NLS and C. Yet, this element was insufficient to independently direct nuclear import of a heterologous protein (17). Box C therefore appears to be functionally intermediate between the SV40 NLS and the amino-terminal basic clusters found in other bipartite NLS active in animal cells. In these bipartite targeting sequences, only two basic residues at the amino terminus have been shown to be required for the function of the whole NLS (25). Basic box C consisted of five basic residues interrupted by a histidine. Accurate mutational analysis is required to define the relevance of each of these residues in the bipartite structure; however, the independent ability of this sequence in targeting β-Gal to the nucleus suggests that the complete box C might be important for effective nuclear import in the bipartite configuration. The carboxy-terminal basic box D (PKKHR) was also able to direct nuclear transport when fused to β-Gal. In nucleoplasmin or N1, it has been shown that the respective basic elements in the NLS are unable to translocate a fused heterologous protein to the nucleus (9, 17). However, when multiple copies of the carboxy-terminal basic element of the nucleoplasmin NLS were conjugated to chicken serum albumin, limited transport was also observed (6).

Expression of two copies of C in a bipartite configuration led to the complete restoration of nuclear import, indicating that C could functionally replace D in the bipartite structure. It has to be noted that by duplicating C in this configuration, a peptide sequence (KR-8 aa-PKRRRHR) which resembled that of the consensus for bipartite NLS (KR-10 aa or more-KKKK) was generated. In contrast, expression of basic box D in duplicate or triplicate could not enhance nuclear import. This was so even though this sequence also matched the consensus for bipartite NLS. Therefore, C and D appeared to function differently in a bipartite configuration.

The pp65 NLS is somehow intermediate in function between the prototype SV40 NLS, which can function as a contiguous stretch of basic residues, and the typical bipartite NLS, represented by the nucleoplasmin-targeting sequence. It has been suggested that one or more receptor molecules interact with the basic structures in bipartite NLS (reference 32 and references therein). Our results suggest that (i) some independent receptor interaction with the isolated basic boxes C and D can occur, (ii) this interaction to some extent can result in nuclear translocation, and (iii) the receptor interaction with only one element is inefficient for nuclear targeting compared with the interaction in the bipartite configuration. One hypothesis which could explain these findings is that receptor molecules bind to bipartite NLS to form homo- or heterodimeric complexes which are stabilized by mutual interaction. Alternatively, one protein could contact the NLS at one basic site, and the receptor-NLS interaction could be stabilized by a second contact. Binding to a single basic element would be weak and thus result in limited nuclear transport.

In line with this are the results of our deletion analyses. Reduction of the spacer sequence in construct pNLS2-CDΔ7 resulted in the amino acid sequence PKRRRHRQPDPTPKKHR, which still conforms to the consensus set up by Robbins and colleagues (25). However, deletion of another 5 aa resulted in severe impairment of nuclear transport. If binding of two proteins, or possibly two portions of the same protein, was necessary for efficient nuclear translocation, deletion of the spacer might result in sterical hindrance or electrostatic repulse effects due to the accumulation of positively charged residues. In this view, the SV40 type of NLS would be, as suggested by Dingwall and Laskey (8), one extreme variant of targeting sequences in which the binding of one hypothetical receptor molecule would be thermodynamically stable enough to provide a substrate for the translocation machinery. Extensive analyses of this issue are required. The availability of considerable amounts of pp65 from extracellular dense bodies might provide a convenient system to isolate factors interacting with the different elements of this bipartite NLS.

In summary, this study shows that two NLS which are unequally important for efficient nuclear translocation of the protein across the nuclear membrane are present in the primary structure of the tegument protein pp65 of HCMV. One of these two NLS appears to be a novel variant of the bipartite targeting sequences of animal cells in that the basic elements could independently direct nuclear import of a fraction of the cytoplasmic β-Gal. Efficient translocation, however, was seen only in the bipartite configuration and when a minimal spacer sequence between the basic boxes was retained. This NLS can be used to independently study the interaction of cellular receptor molecules with the different elements of bipartite NLS.

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