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Characterization of the nuclear localization signal of the hepatitis delta virus antigen

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Abstract

The delta antigen (HDAg) is the only protein encoded by the hepatitis delta virus (HDV) RNA genome. The HDAg contains an RNA binding domain, a dimerization domain, and a nuclear localization signal (NLS). The nuclear import of HDV RNPs is thought to be one of the first tasks of the HDAg during the HDV replication cycle. Using c-*myc*–PK fusions with several regions of the HDAg in transfection assays in Huh7 cells, we found that the HDAg NLS consists of a single stretch of 10 amino acids, EGAPPAKRAR, located in positions 66–75. Deletion and mutation analysis of this region showed that both the acidic glutamic acid residue at position 66 and the basic arginine residue at position 75 are essential for promoting nuclear import.

Keywords: Hepatitis D virus; Delta antigen; Nuclear localization signal

Introduction

The hepatitis delta virus (HDV) is the causative agent of one of the most severe forms of viral hepatitis. The HDV coinfects or superinfects hepatocytes already infected with the hepatitis B virus (HBV) resulting in an increased risk of cirrhosis or fulminant hepatitis (Govindarajan et al., 1984; Jacobson et al., 1985). The clinical association between the HDV and the HBV is due to the fact that the outer envelope of the HDV consists of HBV surface antigens (HBsAgs) which are necessary for virus packaging and propagation of infection (Smedile et al., 1994). The HDV genome consists of a 1.7 kb negative and circular ssRNA molecule in which a single ORF was identified (reviewed by Taylor, 2003). Transcription from this ORF involves the redirection of cellular DNA-directed RNA polymerase II, and results in the production of a 0.8 kb mRNA molecule and subsequent synthesis of the only virus protein, the delta antigen (HDAg) (Macnaughton et al., 2002; Moradela and Taylor, 2001). Two forms of this protein (S-HDAg, p24 and L-HDAg, p27) are derived as a consequence of an editing mechanism that occurs in the RNA antigenome (Casey and Gerin, 1995). As a result, a stop codon UAG is transformed into a tryptophan codon UGG extending the ORF by 19 additional amino acids. Although both S-HDAg and L-HDAg share most of the amino acid sequence and functional domains, they seem to play quite opposite roles during HDV replication. S-HDAg is thought to stimulate replication (Yamaguchi et al., 2001; Cheng et al., 2003; Huang and Wu, 1998) and L-HDAg, which is isoprenylated at the cysteine residue located four amino acids from the C terminus, was shown to trans inhibit replication and to be essential for virus packaging (Chang et al., 1991; Glenn et al., 1992).

Specific domains were identified in both S-HDAg and L-HDAg: an RNA binding domain, a dimerization domain, and a nuclear localization signal (NLS) (see Taylor, 2003; Cunha et al., 2003). The RNA binding domain consists of two arginine-rich stretches located between amino acids 97–107 and 136–146, respectively. The dimerization domain is located between amino acids 12 and 60. Concerning the NLS, two reports were previously published describing its putative location on the HDAg. Chang et al. (1992) used several constructs consisting of

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different portions of the HDAg amino acid sequence fused to β galactosidase to show that two functional NLSs are present in the delta antigen. The first NLS corresponded to aa 35–44 and the second was assigned to a bipartite sequence located between aa 67 and 88. At the same time, in an attempt to identify the minimal amino acid sequence in the HDAg capable to promote nuclear import of fusion proteins, Xia et al. (1992) used several portions of the HDAg fused to α-globin in a similar approach. The authors identified the same bipartite amino sequence (aa 67–88) as a functional NLS. However, it was additionally shown that the first NLS identified by Chang et al. (1992), corresponding to aa 35–44, was not functional in that system.

The active transport of macromolecules between the nucleus and the cytoplasm is an energy dependent process that requires the involvement of receptor proteins, named importins or exportins, that recognize specific amino acid sequences in the cargos to promote transport through the nuclear pores (reviewed by Stewart, 2007; Pemberton and Paschal, 2005). The NLS present in the SV40 T antigen was the first to be identified and it consists of a single stretch of seven basic amino acids (Kalderon et al., 1984a, 1984b). Typically, the first NLSs identified, now known as classical NLSs, consisted of a single or bipartite sequence of basic amino acids (Dingwall and Laskey, 1991). However, further identification of several different NLSs led to the conclusion that nuclear import of proteins, namely hnRNPs (Izaurralde and Adam, 1998), can be promoted by other signals distinct from classical NLS sequences.

In this work, we made use of pyruvate kinase reporter system (Michael et al., 1997) to identify and characterize the minimal amino acid sequence present in the delta antigen, capable of promoting nuclear import.

Results

The first 58 amino acids of the delta antigen do not contain a NLS

In this work, we utilized a well-characterized c-*myc*–PK-based reporter system to identify and characterize the HDAg NLS (Michael et al., 1997). The plasmid pcDNA1–c-*myc*–PK containing the DNA sequence coding for the hnRNP K NLS (a kind gift of Dr. Beatriz Fontoura, University of Texas, Southwestern Medical Center at Dallas) was used as positive control in parallel transfections throughout this work. Negative controls consisted of the plasmid pcDNA1–c-*myc*–PK devoid of the hnRNP K NLS. After transfection, the c-*myc*–PK fusion protein is localized exclusively in the cytoplasm (data not shown).

We first decided to investigate the presence of a functional NLS in aa 1–33 of the HDAg. To do this, we cloned a 262 bp cDNA fragment encoding for amino acids 34–116 in the plasmid pcDNA1–c-*myc*–PK. This plasmid was used to transiently transfect Huh7 cells. After 24 h, the intracellular localization of the corresponding fusion proteins was determined by immunofluorescence and confocal microscopy.

The c-*myc*–PK–δ34–116 fusion protein was confined to the nucleus thus allowing to conclude that the first 33 amino acids of the HDAg are not necessary to promote the nuclear import of c-*myc*–PK (Fig. 1A). To determine if a functional NLS is present in the first 58 amino acids of HDAg, namely in amino acids 35–44, we generated c-*myc*–PK–δ58–116 and c-*myc*–PK–δ13–55 fusion proteins, respectively. These constructs were used to transfect Huh7 cells and the subcellular localization of the fusion proteins was determined. We observed that the c-*myc*–PK–δ58–116 fusion protein was confined to the nucleus (Fig. 1B). In contrast, the c-*myc*–PK–δ13–55 fusion protein was localized exclusively in the cytoplasm (Fig. 1C). Taken together, these results indicate that the first 58 amino acids of the HDAg are not able to promote the nuclear import of the c-*myc*–PK fusion constructs, and thus they do not contain a functional NLS.

The HDAg NLS is located between amino acids 66 and 88

After determining that the amino acids 1–58 do not contain a functional NLS, we proceeded to the analysis of a different region of the HDAg comprising amino acids 58–116, which include the second putative NLS. To do this, we first constructed plasmid pcDNA1–c-*myc*–PK–δ13–195Δ68–91 which contains the cDNA sequence coding for amino acids

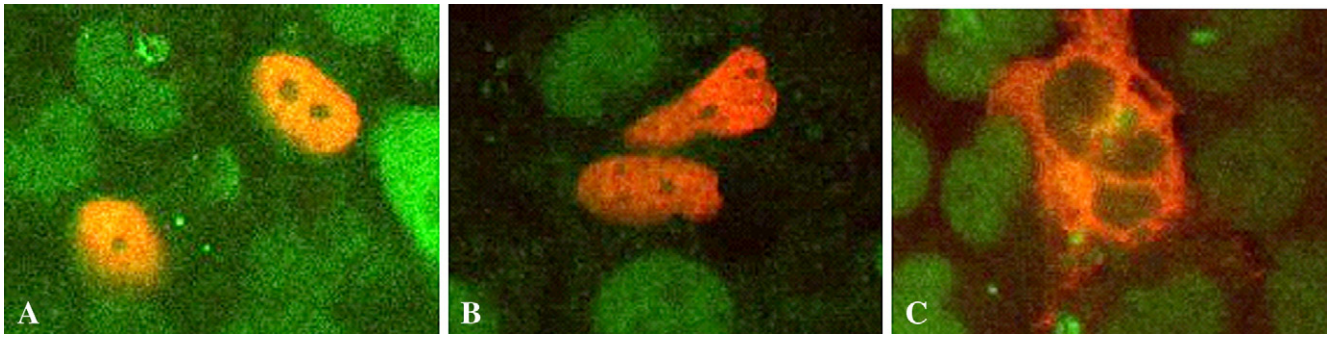

Fig. 1. Subcellular localization of the c-*myc*–PK fusion proteins containing different portions of the HDAg. Huh7 cells were transfected with plasmids encoding for the c-*myc*–PK–δ34–116 protein, c-*myc*–PK–δ58–116, and c-*myc*–PK–δ13–55 fusion proteins (A–C, respectively). One day after transfection, the cells were fixed with formaldehyde, permeabilized with Triton X-100, and incubated with a mouse anti-c-*myc* antibody (red) to detect the fusion constructs, and a rabbit anti-lamin A/C antibody to detect the nuclei (green).

13–195 devoid amino acids 68–91. This plasmid was used to transfect Huh7 cells and the intracellular distribution of the fusion proteins was determined by immunofluorescence and confocal microscopy. Fig. 2A shows the exclusively cytoplasmic localization of the fusion protein. This result, together with that obtained with the pcDNA1–c-*myc*–PK–58–116 (see Fig. 1B), indicates that an NLS must be localized between amino acids 58 and 116 and that at least some amino acids in residues 68–91 are necessary for its function.

Previous results (Chang et al., 1992; Xia et al., 1992) pointed to the existence of a functional NLS in the HDAg comprising amino acids 67–88. We decided to confirm this result by generating c-*myc*–PK–δ67–88 fusion proteins, followed by transfection of Huh7 cells with the obtained plasmid construct. Our results, however, could not confirm the presence of this putative NLS since the fusion protein was detected exclusively in the cytoplasm of transfected cells (Fig. 2B). Taking into account this observation, we decided to perform a comprehensive analysis of this region. First, we cloned the HDAg cDNA sequence corresponding to amino acids 58–88 in the pcDNA1–c-*myc*–PK vector. After transfection, the intracellular distribution of the fusion protein was determined by immunofluorescence and confocal microscopy. We observed that the c-*myc*–PK–δ58–88 fusion protein was localized exclusively in the nucleus allowing to conclude that a functional NLS must be present in this region of the HDAg (Fig. 2C).

After determining that an NLS is present between amino acids 58 and 88 of the HDAg and that amino acids 67–88 are not sufficient to promote nuclear import of c-*myc*–PK fusion constructs, we proceeded to the analysis of the HDAg amino acid sequence located upstream of amino acid 67. In order to rule out the possibility that an NLS is present between amino acids 58 and 67, we constructed c-*myc*–PK–δ58–67 fusion proteins. Immunofluorescence analysis of the localization of this protein in Huh7 cells, showed an exclusively cytoplasmic pattern (Fig. 3A). Following this result, we decided to generate a series of constructs consisting of the c-*myc*–PK protein fused to HDAg amino acids 61–88, 64–88, 65–88, and 66–88 (c-*myc*–PK–δ61–88, c-*myc*–PK–δ64–88, c-*myc*–PK–δ65–88, and c-*myc*–PK–δ66–88, respectively). All fusion proteins were found in the nucleus of Huh7-transfected cells (Figs. 3B–E). These results, together with the observation that the c-*myc*–PK–δ67–88 fusion protein is detected exclusively in the cytoplasm of transfected cells, indicate that the HDAg NLS is localized between amino acids 66 and 88.

The HDAg NLS is not bipartite

Having established that the HDAg NLS is located between aa 66 and 88, and that the E66 residue is crucial for its function, we decided to investigate the importance of the last amino acid residues corresponding to the second part of the putative bipartite NLS sequence (PRKR). To do this, we constructed c-*myc*–PK proteins fused to amino acids 64–88 of the HDAg where the three last downstream basic residues (RKR) were substituted by neutral alanine residues (c-*myc*–PK–δ64–85AAA). Upon transfection, the intracellular localization of the fusion proteins was determined by immunofluorescence and confocal microscopy. All fusion proteins were found to be localized in the nucleus of Huh7 cells indicating that the last three amino acid residues of the putative bipartite NLS are not necessary for its function (Fig. 4A). The next approach was designed in order to further investigate the possible role of each of the two regions of the putative bipartite NLS. This was performed by constructing c-*myc*–PK–δ66–75 and c-*myc*–PK–δ76–88 fusion proteins. After transfection, the c-*myc*–PK–δ66–75 fusion protein was detected exclusively in the nucleus of Huh7 cells (Fig. 4B). In contrast, the c-*myc*–PK–δ76–88 fusion protein could be observed only in the cytoplasm (Fig. 4C). These results, show that the second downstream half of the putative HDAg NLS, comprising amino acids 76–88, is not necessary for function, and is not able to promote the nuclear import of the c-*myc*–PK fusion protein. Accordingly, the HDAg NLS must be present between amino acids 66 and 75.

The HDAg NLS consists of amino acids 66 to 75

After establishing that the HDAg NLS is comprised between amino acid residues 66 and 75, we decided to determine the

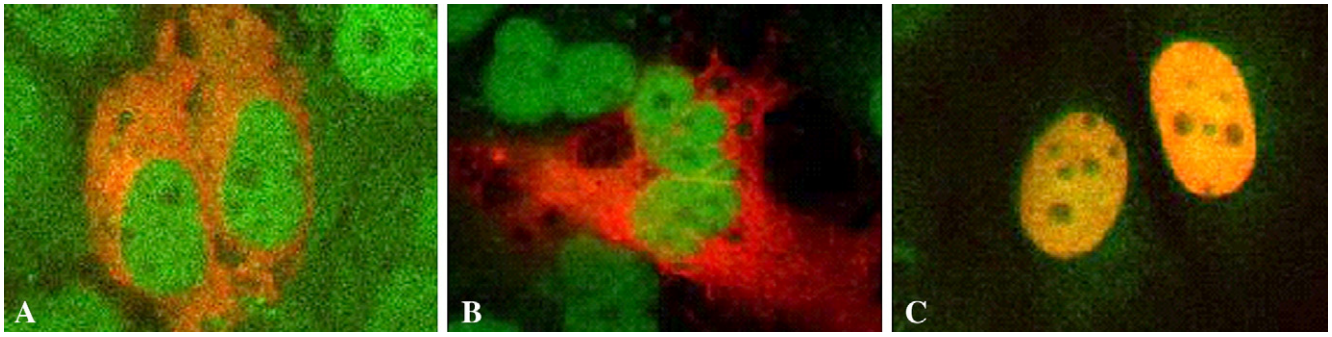

Fig. 2. Intracellular distribution of the c-*myc*–PK fusion proteins containing different portions of the HDAg. Huh7 cells were transfected with plasmids encoding for the c-*myc*–PK–δ13–195Δ68–91, c-*myc*–PK–δ67–88, c-*myc*–PK–δ58–88 fusion proteins (A–C, respectively). Cells were transfected for 24 h, fixed with formaldehyde, and permeabilized with Triton X-100. Detection of the fusion constructs was performed using a mouse anti-c-*myc* antibody (red). Nuclei were stained using a rabbit anti-lamin A/C antibody (green).

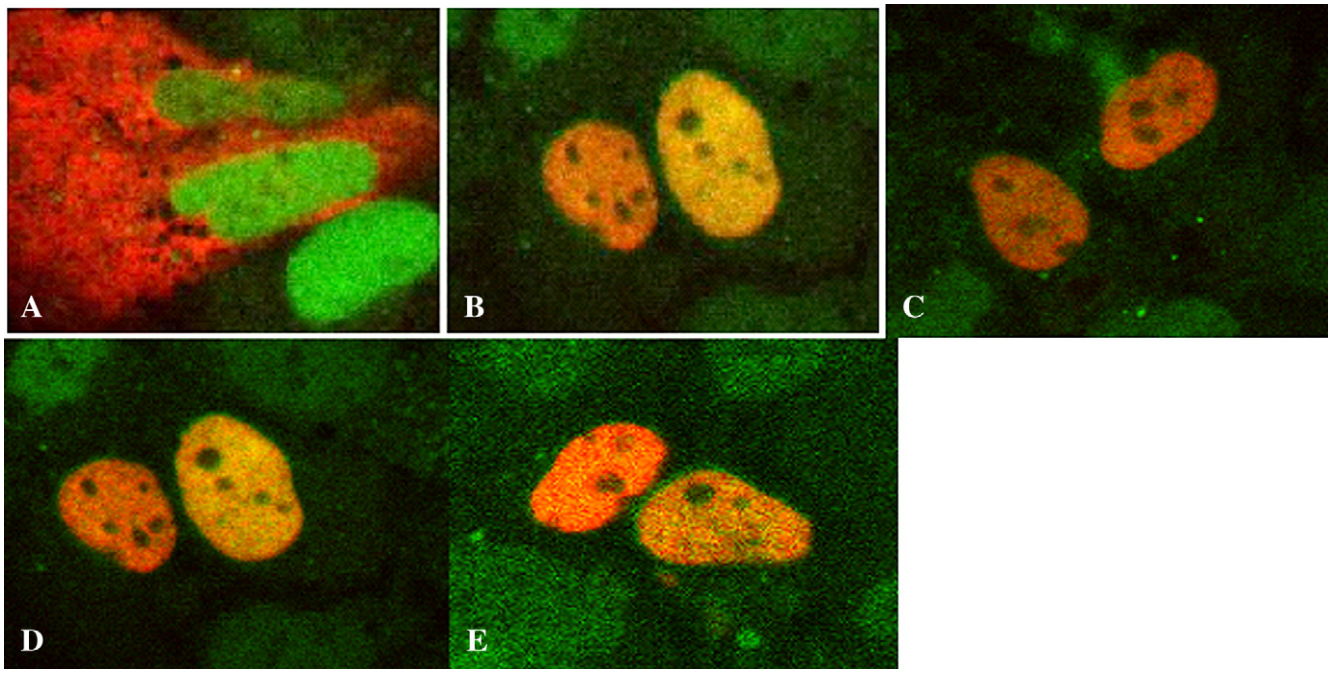

Fig. 3. Localization of the c-*myc*–PK fusion proteins containing different portions of the HDAg. Huh7 cells were transfected with plasmids coding for c-*myc*–PK–δ58–67, c-*myc*–PK–δ61–88, c-*myc*–PK–δ64–88, c-*myc*–PK–δ65–88, and c-*myc*–PK–δ66–88 (A–E, respectively). Cells were transfected for 24 h, fixed with formaldehyde, and permeabilized with Triton X-100. Detection of the fusion constructs was performed using a mouse anti-c-*myc* antibody (red). A rabbit anti-lamin A/C antibody was used to stain the nuclei (green).

minimal amino acid sequence capable of promoting protein import to the nucleus. To do this we generated c-*myc*–δPK–66–74 fusion constructs, where the R75 amino acid residue was deleted. This construct was used to transfect Huh7 cells and the fusion protein was detected by immunofluorescence. The observed cytoplasmic localization of the protein construct (Fig. 5A) shows that the R75 residue is necessary to promote nuclear import.

The amino acid 66 in the HDAg corresponds to a glutamic acid residue. In order to evaluate its importance for NLS function, we generated c-*myc*–PK–δ66–75 constructs where the E66 residue was changed to alanine (c-*myc*–PK–δE66A–75). Immunofluorescence analysis of transfected cells showed that, in this case, the fusion protein could be detected both in the nucleus or the cytoplasm of transfected cells (Fig. 5B, arrow) pointing to a partial impairment of NLS function due to the E66A amino acid substitution.

After determining that the minimal amino acid sequence in the HDAg capable of promoting the nuclear import of c-*myc*–PK fusion constructs consists of residues 66–75, we finally decided to investigate if this sequence can also function as a NLS in the delta antigen. To do this, we first made use of plasmids pEGFP–HDAg and pEGFP–HDAgΔNLS. These plasmids were used to transfect Huh7 cells and the subcellular localization of the corresponding fusion proteins was observed under a confocal microscope. The obtained results

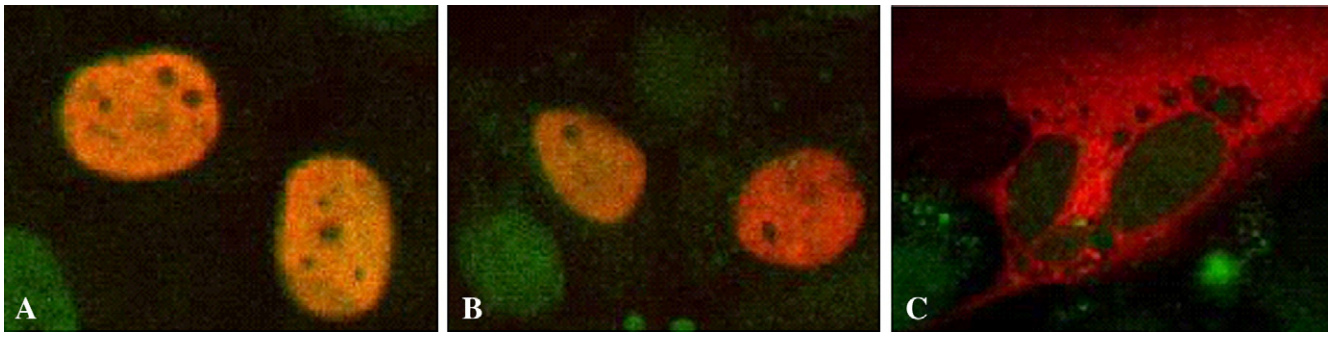

Fig. 4. Subcellular localization of the fusion proteins containing different portions of the HDAg. Huh7 cells were transfected with plasmids encoding for c-*myc*–PK–δ64–85AAA, c-*myc*–PK–δ66–75, c-*myc*–PK–δ76–88 (A–C, respectively). Cells were transfected for 24 h, fixed with formaldehyde, permeabilized with Triton X-100. Detection of the fusion constructs was performed using a mouse anti-c-*myc* antibody (red). A rabbit anti-lamin A/C antibody was used to stain the nuclei (green).

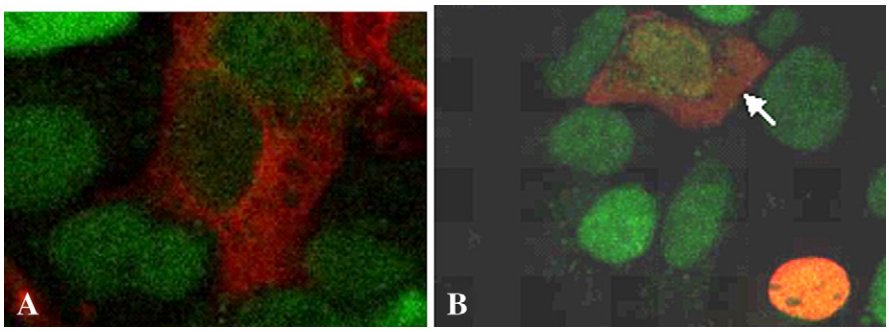

Fig. 5. Intracellular distribution of the c-*myc*–PK fusion proteins containing different portions of the HDAg. Huh7 cells were transfected with plasmids encoding for c-*myc*–δPK–66–74 and c-*myc*–PK–δE66A–75 (A and B, respectively). Cells were transfected for 24 h, fixed with formaldehyde, permeabilized with Triton X-100. Detection of the fusion constructs was performed using a mouse anti-c-*myc* antibody (red). Nuclei were stained using a rabbit anti-lamin A/C antibody (green).

show that the pEGFP–HDAg fusion protein was detectable only in the nucleus of transfected Huh7 cells whereas the pEGFP–HDAgΔNLS was found exclusively in the cytoplasm (Fig. 6).

Although these results indicate that amino acid residues 66–75 are necessary and sufficient to promote the nuclear import of HDAg, we could not rule out a possible involvement, in the nuclear import, of the flanking sequences in the junction regions of the fusion proteins. To investigate this hypothesis we, constructed plasmids pCI–δAg, pCI–δAgΔNLS, and pCI–δAgE66A which contain the full-length cDNA sequence of HDAg, the full-length HDAg cDNA devoid amino acid 66–75 coding sequences, and the full-length HDAg cDNA where the Glu^{66} GAG codon was changed to a GCA Ala codon. After transfection, the intracellular localization of the native wt HDAg and of the corresponding mutant proteins was determined by immunofluorescence. The obtained results showed that the HDAgΔNLS construct was confined to the cytoplasm indicating that the amino acid residues 66–75 comprise the HDAg NLS (Fig. 7C). Moreover, a single E66A amino acid substitution impaired the nuclear import of the native HDAg. In this case, the mutant HDAg could be detected both in the nucleus and the cytoplasm of transfected cells (Fig. 7E, arrow).

Finally, transfection of human HeLa cells and mouse 3T3 fibroblasts with the same constructs showed identical results indicating that the NLS is functional independently of the cell type (data not shown).

Taken together, our results show that the HDAg bears a single NLS which is not bipartite and consists of a single stretch of 10 amino acid residues, EGAPPAKRAR, located in positions 66–75.

Discussion

In this work, we identified and characterized the minimal amino acid sequence in the HDAg capable of promoting nuclear import. The strategy was based on the pcDNA–c-*myc*–PK eukaryotic expression vector, a widely used system to identify NLSs (Michael et al., 1997; Enninga et al., 2003), in which several cDNA sequences coding for different regions of the HDAg were cloned. The obtained constructs were used to transiently transfect Huh7 cells and the intracellular distribution of the fusion proteins was determined by indirect immunofluorescence and confocal microscopy.

Two reports aimed to identify the HDAg NLS have been previously published (Chang et al., 1992; Xia et al., 1992).

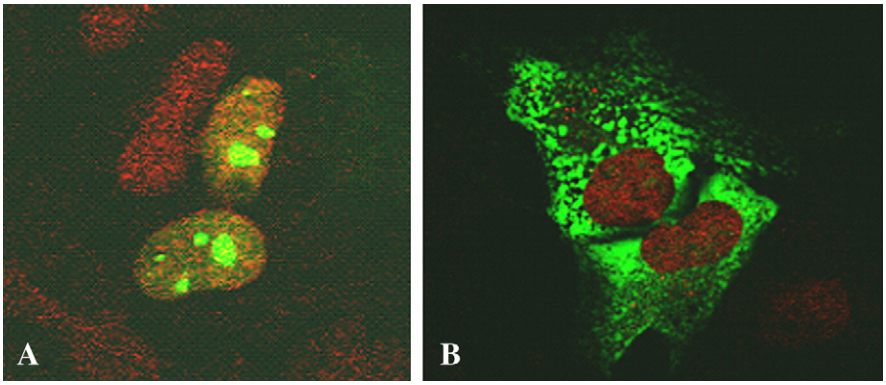

Fig. 6. Subcellular localization of the EGFP–HDAg and EGFP–HDAgΔNLS fusion proteins. Huh7 cells were transfected with plasmids pEGFP–HDAg and pEGFP–HDAgΔNLS (A and B, respectively). Cells were transfected for 16 h, fixed with formaldehyde, and permeabilized with Triton X-100. Nuclei were stained using a rabbit anti-lamin A/C antibody (red).

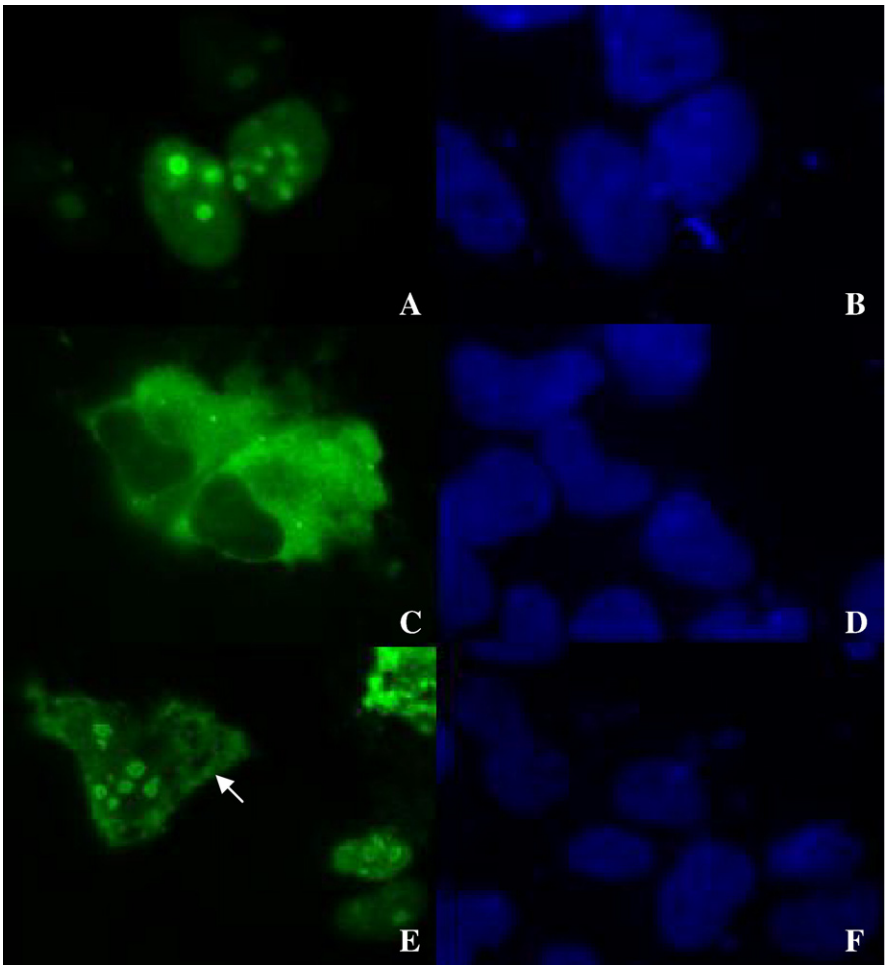

Fig. 7. Localization of HDAg, HDAgΔNLS, and HDAgE66A constructs in Huh7 cells. Cells were transfected with plasmids pCI–δAg (A, B), pCI–δAgΔNLS (C, D), and pCI–δAgE66A (E, F), respectively. After fixation with formaldehyde and permeabilization with Triton X-100, the HDAg was detected with a specific rabbit polyclonal antibody B3 (green). Nuclei were stained with DAPI.

Using β-galactosidase fusion constructs, Chang et al. (1992) reported the existence of two functional NLSs in the HDAg, one of them comprising amino acids 35–44. However, Xia et al. (1992) used α-globin fusion constructs and found that this amino acid sequence was not sufficient to promote nuclear import of fusion constructs. Both groups agreed that a functional NLS consisting of a bipartite sequence located between amino acids 67 and 88 is present in the HDAg. The identified NLS (67GAPPAKRAR....PRKR88) is rich in basic amino acids and is similar to classical NLSs.

In order to characterize the two putative HDAg NLSs, we performed a comprehensive analysis of the amino acid sequence making use of c-*myc*–PK fusion constructs. The obtained results, concerning the subcellular localization of the different fusion constructs used in this work, are summarized in Table 3.

First, we tried to confirm the presence of a NLS between amino acid residues 35 and 44 of the HDAg as described by Chang et al. (1992). We were not able to identify an NLS in the first 58 amino acids of the HDAg, namely in the region comprising residues 35–44. Strikingly, when we tried to confirm that an NLS is present in amino acids 67–88, the corresponding c-*myc*–PK fusion protein was detected exclusively in the cytoplasm of transfected cells. Further analysis allowed us to conclude that the additional presence of the amino acid residue E66 is sufficient to promote nuclear import and thus is crucial for NLS function in our system. In an attempt to analyse in detail the HDAg NLS and determine the minimal amino acid sequence capable of promoting import we generated several c-*myc*–PK fusion proteins that included different parts of the previously described bipartite HDAg NLS comprising amino acids 67–88. Upon transfection, the intracellular localization of the proteins was analysed. We found that the second part of the putative HDAg NLS – PRKR – is not necessary for its function. Mutational analysis of amino acid E66 and deletion of amino acid R75 showed that both the acidic glutamic acid and the basic arginine residues are necessary for NLS function.

The discrepancy between our results and those of Xia et al. (1992) and Chang et al. (1992) could be due to conformational constraints related to the different systems of fusion protein constructs. However, using a GFP-based expression

vector containing the full-length HDAg cDNA, we confirmed our findings indicating that the single stretch of 10 amino acid residues in positions 66–75 (EGAPPAKRAR) is necessary and sufficient to promote nuclear import of the HDAg. Since we still could exclude a possible involvement of the flanking sequences in the junction regions of the fusion proteins in nuclear targeting, we additionally performed deletion and mutational analysis of the native HDAg. Our results clearly show that the native HDAg devoid of amino acid residues 66–75 is located exclusively in the cytoplasm. This indicates that this 10-aa stretch is necessary for nuclear import and that other putative, previously described, NLS sequences are not sufficient to target the native HDAg to the nucleus. Moreover, a single amino acid substitution E66A in the whole HDAg impaired nuclear import confirming the importance of this acidic residue for NLS function.

Lee et al. (1998) strikingly reported that L-HDAg deletion mutants L-d65/75 and L-d50/75, which do not contain amino acid residues 66–75 and 50–75, respectively, displayed different localizations in COS7 cells upon transfection. The L-d65/75 deletion mutant was found both in the cytoplasm and in the nucleolus of transfected cells whereas the L-50/75 L-HDAg mutant localized exclusively in the nucleoplasm. The nuclear localization of the L-50/75 HDAg mutant could imply the existence of a second NLS in the delta antigen. However, the L-65/75 mutant was not specifically targeted to the nucleus and rather seemed to passively diffuse through nuclear pores. More recently, Tan et al. (2004) showed that the Ser-123 residue in the L-HDAg modulates the targeting of the protein to the nucleolus, nuclear SC-35-containing speckles, and the cytoplasm. Taken together, these data do not support the existence of a second NLS in the HDAg, in accordance with Xia et al. (1992) and our observations.

It has been previously speculated that the import of HDV RNPs to the nucleus could involve a cell-type-specific pathway (Tavanez et al., 2002). This could also account for the different findings concerning the precise location of the HDAg NLS. We tested this hypothesis using human HeLa cells and mouse 3T3 fibroblasts, and found that the NLS identified in this work is functional independent of the cell type.

It is thought that one of the first functions of the HDAgs in the HDV replication cycle is the import of virus RNPs to the nucleus where replication takes place (Chou et al., 1998). The precise characterization of the HDAg NLS represents an important step towards the identification of the cellular receptors involved in the import pathway.

Materials and methods

Cell culture and transfection

Huh7 cells were cultured as monolayers in RPMI 1640 medium supplemented with 10% fetal calf serum. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

For transfection the cells were grown on 10×10 mm^2 glass coverslips. A day after being plated, cells were transfected using FuGene 6 transfection reagent (Roche) according to the manufacturer's instructions. Approximately 1 μg of DNA was used per assay and the cells were analyzed by immunofluorescence 16–24 h posttransfection.

Construction of HDAg deletion mutants

The eukaryotic expression vectors pcDNA1–c-*myc*–PK (Michael et al., 1997), pCI–*neo* (Promega), and pEGFP–C1 (Clontech) were used in this study.

The c-*myc*–tagged pyruvate–kinase–HDAg fusion proteins, containing amino acids 13 to 50, 34 to 116, 58 to 116, 88 to 116, 58 to 88 and 61 to 88, respectively, of the HDAg, were generated by PCR using the corresponding primers listed in Table 1, and full-length S-HDAg cDNA as template. The

Table 1
Synthetic primers used to generate specific HDV cDNA fragments by PCR

HDAg amino acids	Oligonucleotide sequence	
	Forward primer	Reverse primer
34–116 [a]	5′ TTT CAA TTG GAC CTC CGG AAG ACA AAG AAG 3′	5′ TTT GTC GAC GCC GAT AGC TGC TTC TTC TTG 3′
58–116 [a]	5′ TTT CAA TTG CTC GGA AAG AAG GAT AAG GAT GG 3′	5′ TTT GTC GAC GCC GAT AGC TGC TTC TTC TTG 3′
13–67 [a]	5′ TTT CAA TTG AGA GAA GAG ATC CTC GAG CAG TG 3′	5′ TTT TCT AGA CCC CTC TCC ATC CCT TAT CCT TC 3′
92–195 [a]	5′ TTT TCT AGA GGA GGA TTC ACC GAC AAG GA 3′	5′ TTT GTC GAC GGA AAT CCC TGG TTT CCC CT 3′
13–50 [a]	5′ TTT CAA TTG AGA GAA GAG ATC CTC GAG CAG TG 3	5′ TTT GTC GAC CCA GGG ATT TTC GTC CTC TAT C 3′
88–116 [a]	5′ TTT CAA TTG CCT CTC AGG GGA GGA TTC AC 3′	5′ TTT GTC GAC GCC GAT AGC TGC TTC TTC TTG 3′
58–88 [a]	5′ TTT CAA TTG CTC GGA AAG AAG GAT AAG GAT GG 3′	5′ TTT GTC GAC CCT CTT CCG AGG TCC GGA GT 3′
61–88 [a]	5′ TTT CAA TTG AAG GAT GGA GAG GGG 3	5′ TTT GTC GAC CCT CTT CCG AGG TCC GGA GT 3′
1–65 [b]	5′ TTAT AAG CTT CG ATG AGC CGG TCC GAG TCG 3′	5′ TTT TCT AGA TCC ATC CTT ATC CTT CTT TCC G 3′
75–195 [b]	5′ TTT TCT AGA CGA ACG GAC CAG ATG GAG G 3′	5′ TTAT GTC GAC CTA TGG AAA TCC CTG GTT TCC C 3′
1–195 [c]	5′ TTT TCT AGA ATG AGC CGG TCC GAG TCG 3′	5′ TTT TCT AGA CTA TGG AAA TCC CTG GTT TCC C 3′
1–65 [c]	5′ TTT TCT AGA ATG AGC CGG TCC GAG TCG 3′	5′ TTT GTC GAC TCC ATC CTT ATC CTT CTT TCC G 3′
75–195 [c]	5′ TTT GTC GAC CGA ACG GAC CAG ATG GAG G 3′	5′ TTT TCT AGA CTA TGG AAA TCC CTG GTT TCC C 3′

[a] Fragment cloned in pcDNA1–c-*myc*–PK.
[b] Fragment cloned in pEGFP–C1.
[c] Fragment cloned in pCI–*neo*.

amplification products were cloned into the *Eco*RI and *Xho*I sites of pcDNA1–c-*myc*–PK.

To generate c-*myc*–PK fusion constructs containing amino acids 58 to 67, 64 to 88, 65 to 88, 66 to 88 66–75, 76 to 88 and 66 to 74 of the HDAg, respectively, specific complementary oligonucleotides were synthesized (see Table 2; Metabion, Germany). Annealing of complementary oligonucleotides was performed *in vitro* by disolving in annealing buffer (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA), and heating at 95 °C for 5 min. followed by slow cooling at room temperature. The resulting annealed double-stranded oligonucleotides were subsequently cloned into the *Eco*RI and *Xho*I sites of pcDNA1–c-*myc*–PK.

For the construction of pcDNA1–c-*myc*–PK–13–195Δ68–91, two DNA fragments coding for amino acids 13 to 67 and 92 to 195 of the HDAg, respectively, were generated by PCR using the primers listed in Table 1. After amplification, fragments were digested with *Xba*I, and ligated using T4 DNA ligase (Fermentas) before being cloned into the *Eco*RI and *Xho*I cloning sites of pcDNA1–c-*myc*–PK.

To generate the EGFP–HDAgΔNLS and HDAgΔNLS constructs, DNA fragments coding for amino acids 1 to 65 and 75 to 195 of the HDAg were generated by PCR using the corresponding primers listed in Table 1. The DNA fragments for EGFP–HDAgΔNLS were digested with *Xba*I before being ligated whereas the fragments for HDAgΔNLS were digested with *Sal*I and ligated. The resulting fragments were cloned into the *Hind*III and *Sal*I cloning sites of pEGFP–C1 or into the *Xba*I site of pCI–*neo*.

All digestion reactions were monitored by electrophoresis in 1% agarose gels. The obtained constructs were used to transform *E. coli* MC1061/P3 cells (Invitrogen). The correct insertion of the fragments was first confirmed by digestion with the *Ban*II, *Apa*I, *Eco*RI or *Apo*I restriction enzymes, followed by DNA sequencing (STAB Vida, Portugal).

Construction of HDAg NLS mutants

To construct NLS point mutants, complementary synthetic oligonucleotides (see Table 2) were designed in order to code for HDAg portions with specific residues replaced by alanine: 86A87A88A (amino acids 64 to 88 of HDAg with Arg^{86}, Lys^{87} and Arg^{88} changed), 86A88A (amino acids 64 to 88 with Arg^{86} and Arg^{88} both changed), K87A (amino acids 64 to 88 with residue Lys^{87} replaced), E66A-75 (amino acids 66 to 75 with residue Glu^{66} replaced by alanine residue). *In vitro* annealing of complementary oligonucleotides was performed by heating at 95 °C followed by slow cooling as described before. The obtained double stranded DNA fragments were cloned into the *Eco*RI and *Xho*I sites of pcDNA1–c-*myc*–PK.

The HDAg mutant HDAgE66A (HDAg with the Glu^{66} residue replaced by an alanine residue) was obtained by inserting the synthetic oligonucleotide 57–75 (see Table 2) into the *Eco*RI and *Apa*I sites of the HDAg coding sequence. The construct was cloned into the *Xba*I cloning site of pCI–*neo*.

Table 2
Synthetic oligonucleotides used to generate specific HDV cDNA fragments

HDAg amino acids	Oligonucleotide sequence
58–67	5′ AA TTG CTC GGA AAG AAG GAT AAG GAT GGA GAG GGG G 3′
58–67 [a]	5′ TC GAC CCC CTC TCC ATC CTT ATC CTT CTT TCC GAG C 3′
64–88	5′ AA TTG GAT GGA GAG GGG GCT CCC CCC GCG AAG AGG GCC CGA ACG GAC CAG ATG GAG GTA GAC TCC GGA CCT CGG AAG AGG G 3′
64–88 [a]	5′ TC GAC CCT CTT CCG AGG TCC GGA GTC TAC CTC CAT CTG GTC CGT TCG GGC CCT CTT CGC GGG GGG AGC CCC CTC TCC ATC C 3′
65–88	5′ AA TTG GGA GAG GGG GCT CCC CCC GCG AAG AGG GCC CGA ACG GAC CAG ATG GAG GTAGAC TCC GGA CCT CGG AAG AGG G 3′
65–88 [a]	5′ TC GAC CCT CTT CCG AGG TCC GGA GTC TAC CTC CAT CTG GTC GGT TCG GGC CCT CTTT CGC GGG GGG AGC CCC CTC TCC C 3′
66–88	5′ AA TTG GAG GGG GCT CCC CCC GCG AAG AGG GCC CGA ACG GAC CAG ATC GAG GTA GAC TCC GGA CCT CGG AAG AGG G 3′
66–88 [a]	5′ TC GAC CCT CTT CCG AGG TCC GGA GTC TAC CTC CAT CTG GTC CGT TCG GGC CCT CTT CGC GGG GGG AGC CCC CTC C 3′
86A87A88A	5′ AA TTG GAT GGA GAG GGG GCT CCC CCC GCG AAG AGG GCC CGA ACG GAC CAG ATG GAG GTA GAC TCC GGA CCT GCG GCA GCA G 3′
86A87A88A [a]	5′ TC GAC TGC TGC CGC AGG TCC GGA GTC TAC CTC CAT CTG GTC CGT TCG GGC CCT CTT CGC GGG GGG AGC CCC CTC TCC ATC C 3′
86A88A	5′ AA TTG GAT GGA GAG GGG GCT CCC CCC GCG AAG AGG GCC CGA ACG GAC CAG ATG GAG GTA GAC TCC GGA CCT
86A88A [a]	5′ TC GAC TGC CTT CGC AGG TCC GGA GTC TAC CTC CAT CTG GTC CGT TCG GGC CCT CTT CGC GGG GGG AGC CCC CTC TCC ATC C 3′
K87A	5′ AA TTG GAT GGA GAG GGG GCT CCC CCC GCG AAG AGG GCC CGA ACG GAC CAG ATG GAG GTA GAC TCC GGA CCT
K87A [a]	5′ TC GAC CCT TGC CCG AGG TCC GGA GTC TAC CTC CAT CTG GTC CGT TCG GGC CCT CTT CGC GGG GGG AGC CCC CTC TCC ATC C 3′
66–75	5′ AA TTG GAG GGG GCT CCC CCC GCG AAG AGG GCC CGA G 3′
66–75 [a]	5′ TC GAC TCG GGC CCT CTT CGC GGG AGC CCC CTC C 3′
E66A-75	5′ AA TTG GCA GGG GCT CCC CCC GCG AAG AGG GCC CGA G 3′
E66A-75 [a]	5′ TC GAC TCG GGC CCT CTT CGC GGG GGG AGC CCC TGC C 3′
76–88	5′ AA TTG ACG GAC CAG ATG GAG GTA GAC TCC GGA CCT CGG AAG AGG G 3′
76–88 [a]	5′ TC GAC CCT CTT CCG AAG TCC GGA GTC TAC CTC CAT CTG GTC CGT C 3′
66–74	5′ AA TTG GAG GGG GCT CCC CCC GCG AAG AGG GCC G 3′
66–74 [a]	5′ TC GAC GGC CCT CTT CGC GGG AGC CCC CTC C 3′
57–75	5′ AA TTC TCG GAA AGA AGG ATA AGG ATG GAG CAG GGG CTC CCC CCG CGA AGA GGG C 3′
57–75 [a]	5′ C TCT TCG CGG GGG GAG CCC CTG CTC CAT CCT TAT CCT TCT TTC CGA G 3′

[a] Sequences complementary to HDV cDNA.

Table 3
Schematic representation of the c-*myc*–PK–HDAg fusion constructs used in this study and its localization in Huh7 cells after transfection

HDAg a.a.		Cellular localization
34-116	LGK KDKDGEGAPP AKRARTDQME VDSGPRKR	N
58-116	^{58}LGK KDKDGEGAPP AKRARTDQME VDSGPRKR	N
13-55		C
	LGK KDKDGEG	C
58-88	^{58}LGK KDKDGEGAPP AKRARTDQME VDSGPRKR^{88}	N
89-116		C
58-67	^{58}LGK KDKDGEG^{67}	C
61-88	^{61}KDKDGEGAPP AKRARTDQME VDSGPRKR^{88}	N
64-88	^{64}DGEGAPP AKRARTDQME VDSGPRKR^{88}	N
65-88	^{65}GEGAPP AKRARTDQME VDSGPRKR^{88}	N
66-88	^{66}EGAPP AKRARTDQME VDSGPRKR^{88}	N
86A87A88A	^{64}DGEGAPP AKRARTDQME VDSGP**AAA**^{88}	N
86A88A	^{64}DGEGAPP AKRARTDQME VDSGP**A**K**A**^{88}	N
K87A	^{64}DGEGAPP AKRARTDQME VDSGPR**A**R^{88}	N
66-75	^{66}EGAPP AKRAR^{75}	N
E66A-75	^{66}**A**GAPP AKRAR^{75}	C/N
66-74	^{66}EGAPP AKRA^{74}	C

N – nucleus; C – cytoplasm.

Immunofluorescence

For indirect immunofluorescence, cells grown on coverslips were rinsed with PBS, fixed with 3.7% paraformaldehyde in PBS, for 10 min at room temperature, and permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. Cells were then washed with PBS containing 0.05% Tween 20 (PBS–Tw) and incubated with primary antibodies for 1 h at room temperature. After washing, with PBS–Tw, cells were further incubated for 1 h with appropriate secondary antibodies. In some experiments, nuclei were stained with 0.5 μg/mL DAPI, for 5 min.

After washing, the coverslips were mounted in Vecta Shield (Vector Laboratories, UK) and sealed with nail polish.

Fluorescent-labeled samples were analyzed under a laser scanning microscope Zeiss LSM 510 equipped with an Argon Ion laser (488 nm) to excite FITC fluorescence and a Helium–Neon laser (543 nm) to excite Texas Red fluorescence. The equipment was calibrated using multicolor fluorescent beads (Molecular Probes, USA) and a dual-band filter that allows simultaneous visualization of green and red fluorescence. In some experiments, a Zeiss Axiovert 200M fluorescence microscope was used.

Antibodies

The following primary antibodies were used in this study: mouse monoclonal antibody 9E10 (BD Biosciences) which recognizes the c-*myc* tag, rabbit polyclonal antibodies anti-delta antigens (B3; Saldanha et al., 1990) and rabbit polyclonal antibody anti-lamin A/C (BD Biosciences).

Anti-mouse IgG and anti-rabbit IgG conjugated either with FITC or Texas Red (Jackson ImmunoResearch Laboratories, USA) were used as secondary antibodies.

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