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Nuclear Localization Signals, but Not Putative Leucine Zipper Motifs, Are Essential for Nuclear Transport of Hepatitis Delta Antigen

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Hepatitis delta antigen (HDAg) is the only known protein of hepatitis delta virus and was previously shown to localize in the nucleoplasm of infected liver cells. In this study, nuclear localization signals of HDAg were defined by expressing various domains of the antigen in both hepatic and nonhepatic cells as β-galactosidase fusion proteins. A cytochemical staining assay demonstrated that a domain from amino acid residues 35 to 88 of HDAg was able to facilitate transport to the nucleus of the originally cytoplasm-localized protein β-galactosidase. Two nuclear localization signals, NLS1 and NLS2, which are similar to those of simian virus 40 T antigen and polyomavirus T antigen, respectively, were identified. Either NLS1 or NLS2 alone was sufficient for the nuclear transport of HDAg. However, a fusion protein (N65Z) containing β-galactosidase and the N-terminal 65 amino acids of HDAg, containing NLS1, was localized exclusively in the cytoplasm and perinuclear region. A possible hydrophobic subdomain between amino acid residues 50 and 65 may block the function of NLS1. Nevertheless, N65Z could enter the nuclei of transfected cells when it was coexpressed with full-length HDAg. Entry into the nucleus may be mediated by the coiled-coil structure rather than the putative leucine zipper motif located between amino acid residues 35 and 65. The existence of two independent nuclear localization signals may ensure the proper functioning of HDAg in the multiplication of delta virus in the nucleus. In addition, two putative casein kinase II sites (SRSE-5 and SREE-126) that may be important in controlling the rate of nuclear transport were found in HDAg.

Hepatitis delta virus (HDV) possesses an unusual single-stranded circular RNA genome (17) and is a satellite of hepatitis B virus (33, 34). HDV RNA consists of approximately 1.7 kb (9, 23, 24, 43) and is a ribozyme that can undergo self-cleavage and self-ligation (19, 26, 38, 39, 45–48). Hepatitis delta antigen (HDAg) is the only known protein of HDV and is encoded from the antigenomic strand of the viral RNA (2–4, 23, 34, 43). Therefore, HDV is a negative-stranded RNA virus. Although mechanisms for the pathogenesis of HDV are still unclear, HDAg appears to be associated with severe chronic hepatitis and fulminant hepatitis (1, 11, 14). There are two forms of HDAg in the liver or serum of delta hepatitis patients (2, 3, 28, 44, 51). The antigens are highly basic and have molecular sizes corresponding to 195- and 214-amino-acid species. The large HDAg possesses an extra 19-amino-acid domain at the C terminus and is derived from the small HDAg as the consequence of a specific point mutation (22, 49). It has been shown that the small HDAg can facilitate HDV RNA replication (18), whereas the large HDAg acts as a negative regulator (5). However, the detailed molecular mechanisms involved in HDV replication are not clear.

We previously expressed HDAg in a monkey kidney cell line and demonstrated that HDAg is a nuclear phosphoprotein with an associated RNA binding activity (4). Further studies showed that the binding is mediated via the middle one-third domain of HDAg and is specific for HDV RNA (21). The interaction also occurs within HDV particles (21). Therefore, the middle domain of HDAg is thought to play a central role in the replication of HDV RNA through an RNA-protein interaction. Further definition of the functional domains of HDAg may help us learn the biological significance of its binding to HDV RNA and its nuclear localization. This should be a key step toward an understanding of the mechanisms involved in the replication of HDV RNA.

Here we have undertaken a study to characterize the nuclear localization signals of HDAg. The expression of HDAg–β-galactosidase fusion proteins and cytochemical staining revealed that HDAg contains two independent nuclear localization signals (NLS1 and NLS2) rather than a bipartite signal (50). Although NLS1 alone is sufficient for nuclear transport, an additional hydrophobic subdomain present in the N65Z fusion protein can override the transport. Results from site-directed mutagenesis and cotransfection experiments suggest that the coiled-coil structure rather than the putative leucine zipper motif is responsible for forming dimers or multimers between N65Z and HDAg in vivo. This oligomerization, in turn, brings about the transport of N65Z from the cytoplasm to the nucleus.

MATERIALS AND METHODS

Primers. Synthetic oligonucleotides used as primers in amplification reactions in this study are listed in Table 1.

Construction of HDAg–β-galactosidase fusion plasmids. (i) Plasmids pN88Z and pN163Z. The *Escherichia coli lacZ* gene, encoding amino acid residues 8 and higher of the β-galactosidase protein, which still retains the enzymatic activity for degrading the substrate, X-Gal (5-bromo-4-chloro-3-indolyl-β-galactoside), was isolated from pMC1871 (Pharmacia) following double digestion with *Sma*I and *Pst*I

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TABLE 1. Synthetic primers used in the generation of HDV-specific DNA fragments[a]

Primer	Oligonucleotide sequence
4	1474 TTC gTG ATT gTC gTC TTT AAC gTC CGG A 1501
5*	1501 TCC GGA cGT TAA AGA cGA cAA TCA cGA A 1474
11	1407 TCC ATC CTT ATC CTT CTT TCC G 1428
12	1452 CCA GGG ATT GTC TTC CTC TAG T 1473
67*	1501 TCC GGA AGg TAA AGA AGA AAA TCA AGA AAg TA 1470
68*	1273 ACA AGA GGA AGC AGg TAT CGT C 1252
87	1288 CTT CGT CGG TGA TCC TGC CTC T 1309
88*	1663 CCA GCA GTC TCC TCT TTA CAG A 1642
101	1542 CCA CTG CTC GAG GAT GTC TTC CCT 1565
117	720 CCG GCT GGG CAA CAT TCC GAG G 741

[a] Sequences were either homologous or complementary (*) to the nucleotides of HDV genomic RNA and are numbered as described by Makino et al. (23). Lowercase letters indicate mutated nucleotides that were created for the generation of site-specific mutations in HDAg–β-galactosidase fusion proteins.

restriction endonucleases. For construction of pN88Z (Fig. 1), the *Sma*I-*Pst*I fragment of *lacZ* was blunt ended with T4 DNA polymerase before ligation to the unique *Stu*I site of plasmid pECE-d-BE (4). Recombinant plasmid pN88Z encodes a fusion protein, N88Z, representing the first 88 amino acids of HDAg at the N terminus fused to the 8th amino acid of β-galactosidase at the C terminus. For construction of pN163Z, the blunt-ended *Sma*I-*Pst*I fragment of *lacZ* was inserted into the unique *Sma*I site of pECE-d-BE. Recombinant plasmid pN163Z encodes the first 163 amino acids of HDAg fused to the 8th amino acid of β-galactosidase.

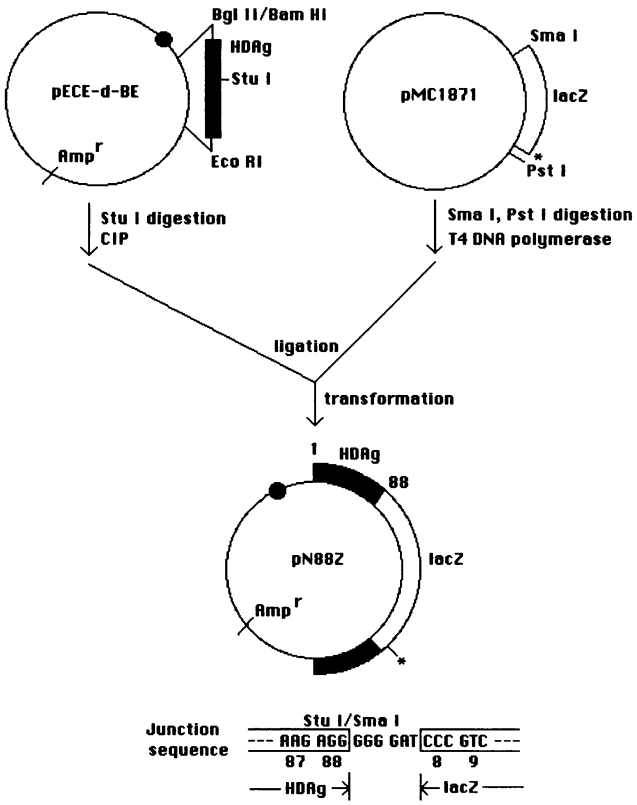

FIG. 1. Schematic diagram of the construction of HDAg–β-galactosidase fusion plasmid pN88Z. Plasmid pN88Z contains sequences corresponding to the first 88 amino acids of HDAg and a full-length coding sequence, except for the first 7 amino acid residues of β-galactosidase (*lacZ*). Solid boxes represent the HDAg coding region. Open boxes represent the *lacZ* sequences. Positions for the stop codon of β-galactosidase are indicated by asterisks. Closed circles represent the replication origin of SV40. Sequences at the junction of HDAg and *lacZ* are shown. CIP, alkaline phosphatase from calf intestine.

(ii) Plasmids pN65Z and pN50Z. A 257-bp DNA fragment that represents the HDV sequence from nucleotides (nt) 1407 to 1663 was generated by a polymerase chain reaction (PCR) as described by Saiki et al. (35). In brief, a reaction mixture containing PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, primers 88 and 11, 1 ng of pECE-d-BE, and *Taq* DNA polymerase (Promega) was subjected to 30 cycles of amplification. Each cycle consisted of 94°C for 1 min, 40°C for 1.5 min, and 72°C for 2 min. For construction of plasmid pN65Z, the 257-bp DNA fragment was subcloned into modified pN163Z (pN163Z that had been digested with *Xho*I-*Sma*I and blunt ended with T4 DNA polymerase). pN65Z encodes a fusion protein, N65Z, corresponding to the first 65 amino acids of HDAg fused to the 8th amino acid of β-galactosidase. A similar approach was taken to construct plasmid pN50Z, except that the synthetic oligonucleotides used in the PCR were primers 88 and 12 and a 212-bp PCR fragment representing the HDV sequence from nt 1452 to 1663 was generated. The resulting plasmid, pN50Z, encodes a fusion protein, N50Z, consisting of the first 50 amino acids of HDAg fused to the 8th amino acid of β-galactosidase.

(iii) Plasmid pN35Z. For construction of pN35Z, a 75-bp DNA fragment was isolated from plasmid pECE-d-BE following treatment with *Mro*I and the Klenow fragment of DNA polymerase I before *Sac*II digestion. The 75-bp DNA fragment was then subcloned into modified pN163Z from which the 459-bp *Sac*II-*Sma*I DNA fragment had been deleted. Recombinant plasmid pN35Z encodes a fusion protein, N35Z, representing the first 35 amino acids of HDAg fused to the 8th amino acid of β-galactosidase.

(iv) Plasmid pN20Z. For construction of pN20Z, a 122-bp DNA fragment was generated by PCR as described for pN65Z, except that the synthetic oligonucleotides used were primers 88 and 101. A 30-bp DNA fragment that represents the HDV sequence from nt 1542 to 1571 (*Sac*II site) was obtained following digestion of the 122-bp PCR product with *Sac*II and gel purification. The 30-bp DNA fragment was then subcloned into modified pN163Z from which the 459-bp *Sac*II-*Sma*I DNA fragment had been deleted. Recombinant plasmid pN20Z encodes a fusion protein, N20Z, representing the first 20 amino acids of HDAg fused to the 8th amino acid of β-galactosidase.

(v) **Plasmids pN163Z(MS) and pN88Z(M).** For construction of pN163Z(MS), a 162-bp *Mro*I-*Stu*I fragment was removed from pN163Z and self-ligated following a blunt-end reaction with T4 DNA polymerase. pN163Z(MS) encodes an HDAg–β-galactosidase fusion protein, N163Z(MS), containing the N-terminal 163 amino acids of HDAg with an internal deletion from residues 35 to 88. For construction of pN88Z(M), a 120-bp *Mro*I fragment that represents amino acid residues 35 to 74 of HDAg was deleted from pN88Z. Therefore, plasmid pN88Z(M) encodes an HDAg–β-galactosidase fusion protein, N88Z(M), that represents the N-terminal 34 amino acids and the 75th to 88th amino acids of HDAg fused to β-galactosidase.

(vi) **Plasmid pN88Z*.** For construction of pN88Z*, site-directed mutagenesis was accomplished by the procedure of Higuchi et al. (13) with primers containing specific mismatches in amplification reactions. In brief, two pairs of synthetic oligonucleotides were used to prime DNA synthesis from pECE-d-BE (4): primers 87 and 5 and primers 88 and 4, which is complementary to primer 5. Resultant DNAs from each of the two reactions were then mixed, denatured, and reassembled, and an additional PCR was performed in the presence of primers 87 and 88. The additional PCR generated a 376-bp product representing nt 1288 to 1663 of HDAg with specific mutations (T to g mutations at nt 1477, 1483, 1486, and 1495). This product was then digested with *Sac*II-*Stu*I, and the resulting 234-bp *Sac*II-*Stu*I fragment was used to replace the cognate fragment present in pN88Z. This step created a plasmid, designated pN88Z*, that encodes fusion protein N88Z*, containing the N-terminal 88 amino acid residues of HDAg, except for the changes from lysine to threonine at residues 36, 39, 40, and 42. The specific mutations in pN88Z* and other leucine zipper mutants used in this study were confirmed by sequencing of the double-stranded plasmid DNA with the Sequenase system (US Biochemicals).

Construction of HDAg expression plasmids with leucine zipper mutations. (i) Plasmid pECE-d-SM(N). For construction of pECE-d-SM(N), a two-step amplification procedure was carried out. In brief, in the first step, the reaction mixture consisted of PCR buffer, primers 67 and 87, 1 ng of pECE-d-BE, and *Taq* DNA polymerase. A DNA fragment of 214 bp was generated after 30 cycles of amplification. One-tenth of the amplified mixture was then subjected to a second amplification, in which the 214-bp DNA fragment was used as the first primer and an additional primer, 88, was added as the second primer. The resulting PCR product, a 376-bp DNA fragment, represents the HDV sequence from nt 1288 to 1663 with mutations at positions 1472 and 1493. This fragment was further digested with *Sac*II-*Stu*I to generate a 234-bp DNA fragment. After gel purification, the 234-bp DNA fragment was subcloned into modified pECE-d-SM (50) from which the 234-bp *Sac*II-*Stu*I DNA fragment had been deleted. Recombinant plasmid pECE-d-SM(N) encodes a small HDAg mutated at two leucine residues (Leu-37 to Val and Leu-44 to Val), resulting in a disruption of the putative leucine zipper motif at the N terminus of HDAg (20).

(ii) **Plasmid pECE-d-SM(M).** The strategy used for the construction of pECE-d-SM(M) was similar to that used for the construction of pECE-d-SM(N), except that the synthetic oligonucleotides used in the first PCR were primers 117 and 68 and the additional primer used in the second PCR was primer 5. The resulting PCR product, a 782-bp DNA fragment that represents the HDV sequence from nt 720 to 1501, was subjected to digestion with *Stu*I-*Sal*I to generate a 372-bp DNA fragment. After gel purification, the 372-bp DNA fragment was subcloned into modified pECE-d-SM from which the 372-bp *Stu*I-*Sal*I DNA fragment had been deleted. The resultant recombinant plasmid, pECE-d-SM(M), encodes a small HDAg mutated at a leucine residue (Leu-115 to Val), resulting in a disruption of the putative leucine zipper motif in the middle region of HDAg.

(iii) **Plasmid pECE-d-SM(MN).** For construction of pECE-d-SM(MN), the *Sac*II-*Stu*I fragment of pECE-d-SM(N) was subcloned into pECE-d-SM(M) by replacement of its cognate fragment. Recombinant plasmid pECE-d-SM(MN) represents a small HDAg in which both of the putative leucine zipper motifs have been mutated.

Cell lines and DNA transfection. A monkey kidney cell line, COS7, and a human hepatoma cell line, Alexander cells, were used in this study. They were routinely grown at 37°C in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum as described previously (4). DNA transfection was performed with cationic liposomes as described by the manufacturer (BRL). In brief, dishes (35 mm) of rapidly dividing cells at a density near confluency were washed twice with Opti-MEM I reduced serum medium (GIBCO). Plasmid DNA at a concentration of 1 μg/0.5 ml of Opti-MEM I medium was mixed vigorously with lipofectin (10 μg/0.5 ml of Opti-MEM I medium), and the mixture was added to the cells. The transfected cells were incubated at 37°C for 6 h before a change of fresh complete medium. At 24 h following transfection, cytochemical staining was carried out to analyze the subcellular localization of HDAg appearing as β-galactosidase fusion proteins.

Cytochemical staining. The staining of transfected cells with X-Gal was done as described previously (37) but with modifications. In brief, transfected cells at 24 h posttransfection were washed with phosphate-buffered saline and then fixed with a solution containing 2% (vol/vol) formaldehyde and 0.2% glutaraldehyde for 5 min at room temperature. Following an additional wash with phosphate-buffered saline, the fixed cells were incubated at 37°C for 1 to 4 h with 1 ml of the substrate, X-Gal (1 mg/ml), in a buffer containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 2 mM $MgCl_2$. Subcellular compartments in which the HDAg–β-galactosidase fusion proteins were expressed would be stained blue and identified under a light microscope.

RESULTS

The N-terminal one-third of HDAg mediates the nuclear localization of its fusion proteins in transfected nonhepatic as well as hepatic cells. We previously showed that HDAg is a nuclear phosphoprotein (4). A sequence comparison with known nuclear localization signals identified two possible nuclear localization signals in the N-terminal one-third of HDAg that are similar to those of simian virus 40 (SV40) large T antigen and polyomavirus large T antigen (15, 29). To functionally define the nuclear localization signals of HDAg, we first constructed plasmid pN88Z, which contains an HDAg–β-galactosidase fusion gene driven by the SV40 promoter and is capable of expression in eukaryotic systems. Procedures for the construction of pN88Z are illustrated in Fig. 1. This plasmid encodes a fusion protein representing the first 88 amino acids of HDAg at the N terminus fused to the 8th amino acid of β-galactosidase at the C terminus. Twenty-four hours following transfection of pN88Z into nonhepatic COS7 cells, cytochemical staining was carried out. The results demonstrated that HDAg fusion

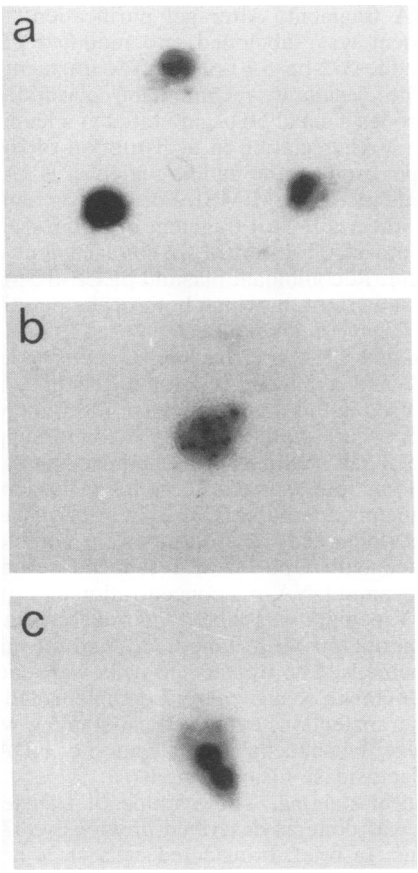

FIG. 2. Subcellular localization of HDAg–β-galactosidase fusion protein N88Z. Either COS7 cells (a and b) or Alexander cells (c) were transfected with plasmid pN88Z by the procedures described in the text. Twenty-four hours after the transfection, the cells were fixed and the fusion protein expressed was detected by cytochemical staining of β-galactosidase.

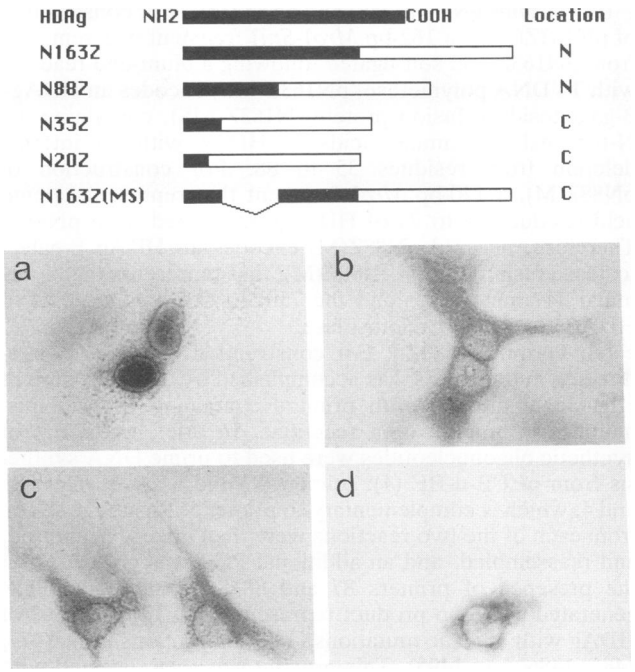

FIG. 3. Structure and subcellular localization of HDAg–β-galactosidase fusion proteins N163Z, N35Z, N20Z, and N163Z(MS) in transfected COS7 cells. (Top) Structure of HDAg–β-galactosidase fusion proteins. The fusion proteins contain either the first 163 amino acids of HDAg (N163Z) or a portion of this sequence fused to the 8th amino acid of β-galactosidase. Closed boxes represent the HDAg sequences, and open boxes represent the β-galactosidase sequences. The wavy line indicates a deletion in the HDAg sequence. N and C, nuclear and cytoplasmic locations, respectively. (Bottom) Subcellular distribution of HDAg–β-galactosidase fusion proteins N163Z (a), N35Z (b), N20Z (c), and N163(MS) (d) in transfected COS7 cells.

protein N88Z was localized exclusively in the nucleus. It was homogeneously distributed (Fig. 2a) or formed granules in the nuclei (Fig. 2b), indicating that the N-terminal 88 amino acids of HDAg indeed contain nuclear localization signals that drive originally cytoplasm-localized β-galactosidase into the nucleus. Using a phase-contrast microscope, we further confirmed that staining of the granules was restricted to the nucleoplasm rather than the nucleolus (unpublished observation).

Since HDAg was shown to be localized in the nuclei of infected livers (32), we further examined the distribution of HDAg fusion protein N88Z in transfected Alexander cells, a human hepatoma cell line that constitutively expresses the surface antigen of hepatitis B virus. Cytochemical staining showed that N88Z was localized in the nuclei of Alexander cells (Fig. 2c), as in transfected COS7 cells. In addition, this nuclear distribution was observed in both COS7 cells (Fig. 3a) and Alexander cells (data not shown) for fusion protein N163Z, which includes the N-terminal 163 amino acids of HDAg.

For determination of the minimal sequences required for the nuclear localization of HDAg, fusion plasmids with various deletions in the antigen sequence were constructed and expressed in COS7 cells. The β-galactosidase fusion proteins with either 35 or 20 N-terminal amino acids of HDAg were localized exclusively in the cytoplasm of transfected cells (Fig. 3b or c, respectively). These results indicate that sequences within the domain from amino acid residues 35 to 88 of HDAg are required for its transport to the nucleus. This hypothesis was further confirmed from the observation that an HDAg–β-galactosidase fusion protein, N163Z(MS), representing the N-terminal 163 amino acids but without amino acids 35 to 88 of HDAg, was localized exclusively in the cytoplasm of transfected cells (Fig. 3d). Moreover, the addition to N163Z(MS) of the C terminus up to amino acid 212 of HDAg did not change its cytoplasm localization (data not shown). Taken together, these results indicate that nuclear localization signals are present only in the N-terminal one-third of HDAg. None was identified in the middle and C-terminal one-third of HDAg.

Two nuclear localization signals are present in HDAg. Further studies with other mutant constructs of the N terminus of HDAg indicated that amino acid residues 35 to 50 of HDAg possess a nuclear localization signal (NLS1), because N50Z, containing the N-terminal 50 amino acids of HDAg, was expressed in the nuclei of transfected cells (Fig. 4a). Interestingly, fusion protein N88Z*, which has been mutated at lysine residues (lysine 36, 39, 40, and 42) to threonine within this domain, retained its nucleus-localizing ability (Fig. 4b). This result raised the possibility that a second nuclear localization signal (NLS2) exists in the

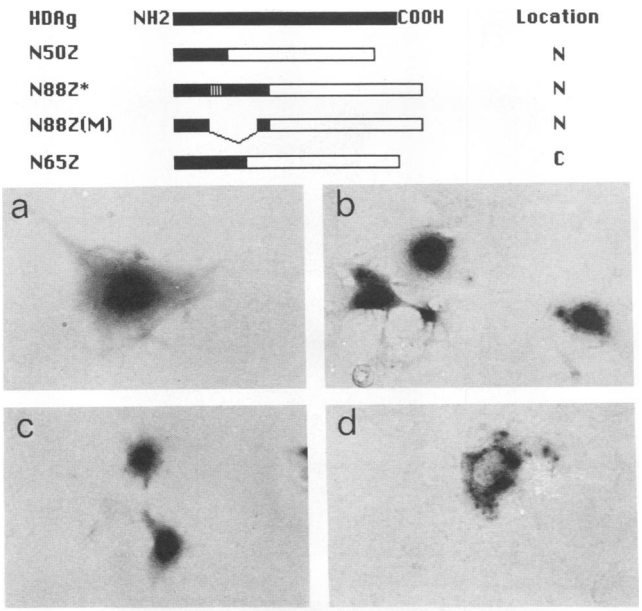

FIG. 4. Structure and subcellular localization of HDAg–β-galactosidase fusion proteins with deletions or point mutations in the N-terminal domain of HDAg. (Top) Structure of fusion proteins N50Z, N88Z*, N88Z(M), and N65Z. Symbols are as described in the legend to Fig. 3. Specific point mutations in N88Z* are also indicated. (Bottom) Subcellular distribution of HDAg–β-galactosidase fusion proteins N50Z (a), N88Z* (b), N88Z(M) (c), and N65Z (d) in transfected COS7 cells.

domain from amino acid residues 51 to 88 of HDAg. A study was performed with plasmid pN88Z(M) to test this hypothesis. pN88Z(M) could encode a fusion protein, N88Z(M), containing the N-terminal 34 amino acids and amino acids 75 to 88 of HDAg fused to β-galactosidase. The predominant nuclear distribution of N88Z(M) (Fig. 4c) provides evidence that the domain from amino acids 75 to 88 is important for nuclear transport.

The results agree with the finding from a sequence comparison that there are two possible nuclear localization signals in the N-terminal one-third of HDAg. One of them, NLS1, contains highly basic amino acid residues (RKLKK KIKKL-44) and is highly homologous (homology indicated by boldface type) to those of SV40 large T antigen (PP**KKK** RKV-132; 15) and the glucocorticoid receptor (YRKCLQAG MNLEA**RKTKKKIK**GIQQATA-524; 27). The other nuclear localization signal, NLS2, contains proline and basic amino acid residues (PRKRP-89) and is similar to the nuclear localization signal of polyomavirus T antigen (VS**RKRP** RP-196; 29).

A possible hydrophobic subdomain of HDAg can override NLS1. The N-terminal 50 amino acid residues of HDAg are able to drive, but not efficiently, β-galactosidase to the nucleus (Fig. 4a). To determine whether any extra sequences are needed for efficient nuclear transport, we constructed a plasmid, pN65Z, that encodes a fusion protein, N65Z, containing the N-terminal 65 amino acids of HDAg fused to β-galactosidase. To our surprise, following transfection of pN65Z into COS7 cells, fusion protein N65Z was observed to be distributed in the cytoplasm and perinuclear region with an apparently granular pattern (Fig. 4d). The staining pattern was similar in all COS7 cells and was also consistent with that of Alexander cells transfected with pN65Z (unpublished observation). Since fusion protein N65Z contains NLS1 and was originally expected to be nucleus localized, we further examined its structural characteristics. From a hydropathy profile, a hydrophobic subdomain (PWLGNI KGIIG) located from amino acid residues 49 to 59 was predicted. The role of this hydrophobic domain is not clear. However, in the absence of NLS2, the hydrophobic domain seems to override NLS1 in the nuclear transport of HDAg, as suggested for a variant form of SV40 T antigen (41).

Are putative leucine zipper motifs essential for the nuclear transport of HDAg? There are two putative leucine zipper motifs in HDAg; they are located near the N terminus and the middle portion. Moreover, NLS1 overlaps the putative N-terminal motif. It was of interest to determine whether the putative leucine zipper motifs are involved in controlling the nuclear transport of HDAg. As shown in Fig. 4c, when COS7 cells were transfected with plasmid pN88Z(M), which contains neither of the putative leucine zipper motifs, the expressed N88Z(M) fusion protein was localized predominantly in the nucleus. This result indicates that the putative leucine zipper motifs are not essential for the nuclear transport of HDAg. Nevertheless, we have found that HDAg forms dimers or oligomers in vitro (unpublished data). Further studies were performed to investigate whether the nuclear transport of HDAg can be mediated by oligomerization in vivo.

COS7 cells were cotransfected with pN65Z and pECE-d-BE, encoding a large HDAg, or with pN65Z and pECE-d-SM, encoding a small HDAg. Interestingly, cytochemical staining showed that both small (Fig. 5a) and large (Fig. 5b) HDAgs could facilitate the transport of the N65Z fusion protein from the cytoplasm to the nucleus. However, neither of the HDAgs could facilitate nuclear transport of N35Z, which consists of the N-terminal 35 amino acids of HDAg (Fig. 5c and data not shown). These results suggest that a subdomain from amino acid residues 35 to 65 mediates complex formation between truncated HDAg and wild-type HDAg. This complex, in turn, facilitates the nuclear transport of the originally cytoplasm-localized N65Z fusion protein.

For testing whether the putative leucine zipper motifs are required for the control of complex formation, fusion plasmids that encode small HDAgs mutated at the putative leucine zipper motifs of the N terminus, the middle portion, or both were constructed and designated pECE-d-SM(N), pECE-d-SM(M), and pECE-d-SM(MN), respectively. Cotransfection experiments were carried out with pN65Z and each of the mutant plasmids. None of the mutations affected the function of HDAg in supporting the nuclear transport of the originally cytoplasm-localized fusion protein N65Z (Fig. 6). This result further suggests that a cryptic signal other than the putative leucine zipper motifs of HDAg is involved in mediating complex formation between HDAgs as a mechanism of facilitating the nuclear transport of otherwise cytoplasm-localized proteins.

DISCUSSION

We previously showed that HDAg is a nuclear phosphoprotein with RNA binding activity (4). The RNA binding domain is localized in the middle one-third of HDAg (21). In this report, the nuclear localization of HDAg was demonstrated in both monkey kidney cells and human hepatoma cells by cytochemical staining of HDAg–β-galactosidase fusion proteins. We showed that the nuclear transport of HDAg can be mediated by either of the two nuclear local-

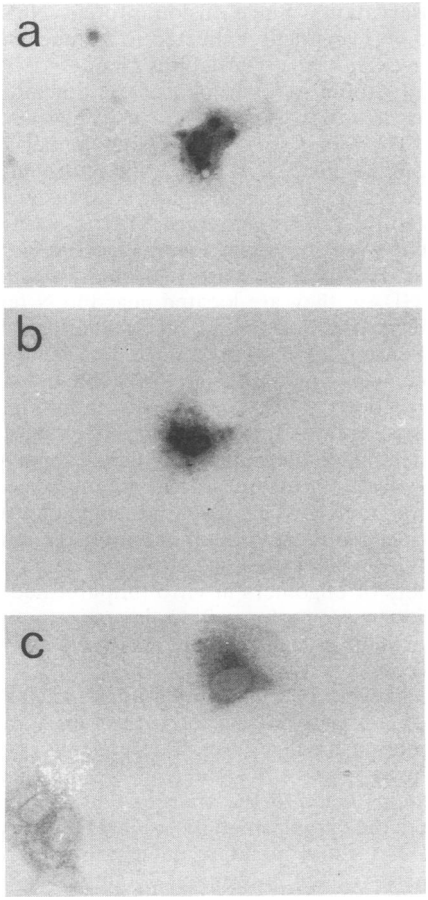

FIG. 5. Subcellular localization of HDAg–β-galactosidase fusion proteins N65Z and N35Z coexpressed with a full-length HDAg. COS7 cells were cotransfected with an HDAg–β-galactosidase fusion plasmid and a plasmid that encodes a full-length HDAg of either a small form (plasmid pECE-d-SM) or a large form (plasmid pECE-d-BE). The results of a cytochemical staining assay are shown. (a) N65Z and small HDAg. (b) N65Z and large HDAg. (c) N35Z and large HDAg.

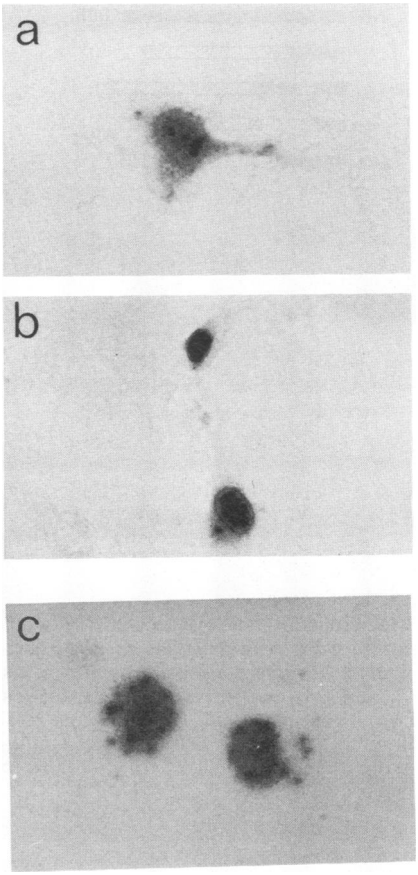

FIG. 6. Subcellular localization of HDAg–β-galactosidase fusion protein N65Z when it was coexpressed with small HDAgs. COS7 cells were cotransfected with plasmid pN65Z and a plasmid encoding a small HDAg mutated at the putative leucine zipper motifs of the N terminus [pECE-d-SM(N)] (a), the middle portion [pECE-d-SM(M)] (b), or both the N terminus and the middle portion [pECE-d-SM(MN)] (c).

ization signals, NLS1 and NLS2, that are located in the N-terminal one-third of HDAg. Therefore, RNA binding and nuclear localization are distinct and separable functions of HDAg involved in the multiplication of HDV.

The amino acid sequences of NLS1 are highly conserved among five HDV isolates (6, 7, 23, 36, 43). NLS1 shares similarity with NL1 of the glucocorticoid receptor and the nuclear localization signal of SV40 T antigen at the sequences RKXKKKIK-42 and KKKXK-42, respectively (15, 27; Fig. 7). We showed that the N-terminal 50 amino acids, containing NLS1 of HDAg, were capable of directing nuclear targeting when they were fused to β-galactosidase (Fig. 4a). However, Xia et al. recently reported that the domain from amino acid residues 32 to 48 of HDAg is not functional for nuclear transport when it is fused to α-globin (50). Therefore, the N-terminal flanking sequences near the 32nd amino acid of HDAg may be required for nuclear transport. Alternatively, the discrepancy in these observations may be due to different global structures of the fusion proteins. It is possible that NLS1 is exposed on the surface of HDAg–β-galactosidase molecules and can be recognized by cellular machinery for nuclear transport. Furthermore, HDAg–β-galactosidase fusion protein N65Z, which contains the N-terminal 65 amino acid residues of HDAg, failed to accumulate in the nucleus (Fig. 4d). This result may have been due to the presence of a hydrophobic subdomain (PWLGNIKGIIG-59) that overrides NLS1 independently of a protein context, as in the case of an SV40 large T antigen variant (41).

The RKRP-89 sequence in NLS2 of HDAg is conserved perfectly among various HDV isolates and is identical to part of the sequence of the nuclear localization signal (VSR KRPRP-196) of polyomavirus T antigen (29; Fig. 7). Our results indicate that amino acid residues 75 to 88 are functional for the nuclear transport of HDAg–β-galactosidase fusion protein N88Z(M), even though slight staining was observed in the cytoplasm (Fig. 4c). The inefficient nuclear transport may have been due to the lack of a proline residue at position 89 of HDAg. Alternatively, NLS1, which is absent from N88Z(M), may be essential for efficient nuclear transport. In the presence of two nuclear localization signals, a fusion protein such as N88Z could get into the nucleus efficiently (Fig. 2). This nuclear localization may ensure the proper functioning of HDAg involved in the multiplication of HDV. Similar observations with two independent nuclear localization signals have been made in

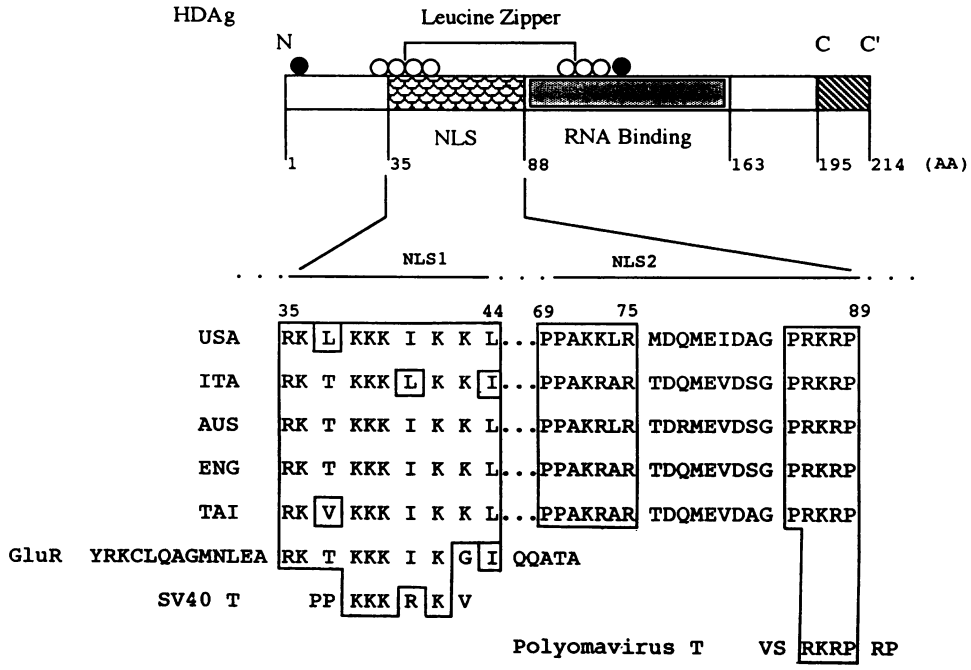

FIG. 7. Functional domains of HDAg. The RNA binding domain and the putative leucine zipper motifs of HDAg have been mapped previously (21). Open circles indicate the leucine residues of the motifs. The nuclear localization signals (NLS) of the glucocorticoid receptor (GluR), SV40 T antigen, and polyomavirus T antigen are shown, and their homologies to those of HDAg (NLS1 and NLS2) are indicated by boxes. Closed circles indicate the putative casein kinase II sites (S/T-X-X-D/E). C and C' represent the C termini of small and large HDAgs, respectively. USA, ITA, AUS, ENG, and TAI represent HDV isolates from the United States, Italy, Nauru Island, England, and Taiwan, respectively (6, 7, 23, 36, 43). AA, amino acids.

several cases, such as rat glucocorticoid receptor (27), influenza virus NS1 protein (12), *Xenopus laevis* N1 protein (16), polyomavirus T antigen (29), adenovirus DNA binding protein (25), and yeast ribosomal protein L29 (40).

Phosphorylation of the SV40 T antigen was linked to a dramatic effect on the kinetics of its nuclear transport (31). The phosphorylation occurred at the serine residues of a casein kinase II site (SSDDE-115) that has the consensus sequence S/T-X-X-D/E (30). Interestingly, HDAg is a phosphoprotein and is phosphorylated at serine residues (4). A computer search revealed that there are two possible casein kinase II sites (SRSE-5 and SREE-126) in HDAg (Fig. 7). Although we expect that phosphorylation at the possible casein kinase II sites may exhibit a great deal of selectivity and contribute to efficiency in the nuclear transport of HDAg, as suggested for SV40 T antigen (30), it is difficult to measure accurately the rate of nuclear transport by the transfection system used in this study. Other systems, such as microinjection of mutant proteins, are essential to defining the possible role of these casein kinase II sites in controlling the rate of nuclear transport of HDAg.

HDAg contains two putative leucine zipper motifs (leucine residues indicated by boldface type), 30-LERDLRKLKK KIKKL-44 and 108-LENKRKQLSSGGKSL-122. Although the putative N-terminal leucine zipper motif is not conserved among five HDV isolates (6, 7, 23, 36, 43), the motif in the middle portion and the leucine residue at position 44 are conserved. Evidence suggested that both of the putative leucine zipper motifs are important for the multiplication of HDV. The putative N-terminal leucine zipper motif of the large HDAg may be involved in the repression of HDV replication (8). By using site-directed mutagenesis, we recently found that Leu-115 in the putative middle leucine zipper motif of the small HDAg is essential for RNA binding activity and HDV replication (unpublished data). A change from Leu-115 to valine eliminated the binding activity of the small HDAg and its ability to support HDV replication. This change is thought to disrupt the putative leucine zipper motif because the valine residue cannot serve as an alternative to the leucine residue in leucine zipper motifs (20). However, the presence of helix-breaking residues, such as serines and glycines, between Leu-115 and Leu-122 may affect the formation of the leucine zipper motif in the middle domain (42).

We have found that HDAg forms dimers or oligomers in vitro (unpublished data). In this report, cotransfection experiments demonstrated that in the absence of either or both of the putative leucine zipper motifs, a full-length HDAg could drive the originally cytoplasm-localized N65Z fusion protein into the nuclei of transfected cells (Fig. 6). For fusion protein N35Z, this cotransport phenomenon was not observed, even when wild-type full-length HDAg was present (Fig. 5c), suggesting that the coiled-coil structure rather than the putative leucine zipper motif in the region between amino acid residues 35 and 65 of HDAg is essential for forming complexes in vivo. The coiled-coil structure between amino acid residues 29 and 46 of HDAg has a heptad repeat with hydrophobic residues present at the second and sixth positions of each seven-residue group and is conserved among five HDV isolates. A mutation that changes both Leu-37 and Leu-44 to valine disrupts the putative leucine zipper motif but not the coiled-coil structure. The possible coiled-coil structure required for the oligomerization of HDAg needs to be studied further.

The oligomerization of HDAg may be important for HDV replication. It was shown that the small HDAg is required for the replication of HDV in the nucleus (18). We have also shown that the middle one-third of HDAg can interact with HDV RNA (21). Therefore, it is possible that HDAg facilitates the entry of HDV RNA into the nucleus for replication upon viral infection. However, the putative leucine zipper motifs are not essential for the nuclear transport of HDAg, as supported by the cotransfection experiments and the nuclear localization of N88Z(M), from which both putative leucine zipper motifs have been deleted (Fig. 4c). The feature of nuclear localization for HDAg raises another interesting question regarding the assembly of HDV. HDAg is not a glycoprotein (4). It may be synthesized on free ribosomes in the cytoplasm and enter the nucleus thereafter (4). However, like the envelope of HDV, hepatitis B surface antigen is synthesized at the endoplasmic reticulum (10) and transported out of the Golgi apparatus after posttranslational modification. Questions of where and how the nuclear HDAg and the cytoplasmic hepatitis B surface antigen meet and when the HDV genomic RNA joins the antigens to form infectious HDV particles remain to be answered.

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