Relating Structure to Function in the Hepatitis Delta Virus Antigen

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Hepatitis delta virus expresses two forms of a single protein, the small (δ Ag-S) and large (δ Ag-L) antigens, which are identical except for an additional 19 residues present at the C terminus of δ Ag-L. While δ Ag-S is required to promote genome replication, δ Ag-L potentially inhibits this process and also facilitates packaging of the viral genome by envelope proteins of the helper virus (hepatitis B virus). Regions within the antigens responsible for nuclear localization, RNA binding, and dimerization have been identified, yet it is not clear how these particular activities contribute to the ultimate replication and packaging phenotypes. Here we report the following findings. (i) Although the removal of the nuclear localization signal from either antigen resulted in significant cytoplasmic accumulation, both proteins still had access to the nucleus. As a consequence, no functional defect was observed with either mutant. (ii) The RNA-binding domain, although necessary for δ Ag-S function, could be deleted from δ Ag-L without compromising its ability to either inhibit replication or promote packaging. (iii) In contrast, the coiled-coil dimerization domain was required for both the activation of replication by δ Ag-S and the inhibition of replication by δ Ag-L. This region, with an additional 20 amino acids C-terminal to it, was necessary and sufficient to potentially inhibit replication by interacting with the small antigen. (iv) The packaging property of δ Ag-L required a C-terminal Pro/Gly-rich region which is hypothesized to interact with the hepatitis B virus envelope proteins during the assembly process.

Hepatitis delta virus (HDV) is a novel human subviral pathogen which is able to infect only persons who were previously or are simultaneously infected with hepatitis B virus (HBV) (27). The HDV genome is a single-stranded circular RNA of approximately 1.7 kb which is able to fold into an unbranched rod structure, in which 70% of its nucleotides are involved in intramolecular pairing (31). HDV is fully capable of genome replication in the absence of all helper virus functions; however, the hepadnaviral envelope proteins (s antigens) are necessary to promote the assembly and release of infectious virions (17, 26, 27). Replication occurs in the nucleus, where a host-encoded, RNA-directed polymerase is thought to synthesize the complement of the genome, termed the antigenome, which then serves as the template for genome synthesis (30).

HDV encodes a single protein, the 195-amino-acid (a.a.) delta antigen (δ Ag-S), which functions in the nucleus to promote genome replication (17). Currently, the mechanism underlying this *trans* activation is not understood, nor is it known at what stage in the replication cycle the antigen acts. From both in vitro and in vivo studies, however, it is clear that δ Ag-S binds to the HDV rod-structured RNA to form a ribonucleoprotein (RNP) which is found both within the nucleus and in virions (5, 8, 29). Although only δ Ag-S is synthesized early in infection, a second antigen variant, δ Ag-L, is observed later in infection (1, 31, 32). This protein is identical to δ Ag-S except that it has an additional 19 carboxy-terminal residues which result from the mutation of the δ Ag-S amber codon as a consequence of RNA editing (3, 22, 35). This editing event is essential for viral propagation because, unlike δ Ag-S, δ Ag-L is able to function with the hepadnaviral envelope proteins to direct the packaging of the HDV RNP structure into virions (4, 28). δ Ag-L differs phenotypically from δ Ag-S in a second way; not only is it

unable to support genome replication, but it acts as a *trans*-dominant inhibitor of this process. When δ Ag-L is expressed to only 1/10 the level of δ Ag-S, the number of genomes observed is reduced almost 10-fold (7). This degree of potency can be explained if a single δ Ag-L molecule interacts with a multimeric complex, such as the HDV RNP, somehow rendering it incompetent for replication yet competent for packaging.

How does the addition of 19 a.a. to the C terminus of δ Ag-S result in such drastic phenotypic changes? Part of the answer has recently been discovered by Glenn et al. (13), who found that the sole cysteine residue of δ Ag-L (position 211; not present in δ Ag-S) is isoprenylated. Furthermore, when this residue is mutated to serine to prevent the modification, packaging is abolished. The role of the carboxy-terminal 19 a.a. in inducing *trans*-dominant inhibition is less clear, however. For instance, an 11-a.a. C-terminal truncation mutant that lacks the isoprenylation site and should not therefore be packaged is still able to inhibit replication, indicating that the two phenotypes of δ Ag-L may be separable (14). This possibility has been confirmed by Chen et al., who described a mutant which is competent for packaging but unable to inhibit replication (10).

There is a growing body of genetic evidence concerning the locations of a number of functional domains within the delta antigen primary sequence (Fig. 1). A locale encompassing residues 89 to 163 has been shown, in vitro, to direct RNA binding (21). Within this region are two Arg-rich motifs, sequences that promote RNA binding in a class of proteins which includes bacteriophage antiterminators as well as the human immunodeficiency virus Tat and Rev *trans* activators (19). Although RNA binding is thought to be an essential activity required for the function of δ Ag-S, this has not yet been established experimentally, and the importance of this region to the phenotypes of δ Ag-L is unknown.

An adjacent region, a.a. 67 to 88, constitutes the nuclear localization signal (NLS) (33). Residues 69 to 75 can be

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FIG. 1. Diagram of the structural domains of the delta antigen and mutants constructed for this study. Deleted portions are blacked out or omitted in each diagram. ARM, arginine-rich motif.

considered the core NLS, as they display some nuclear targeting activity in the absence of additional sequence, while a.a. 85 to 88 are reported to enhance this function (33). Although even this core sequence is not conserved in all HDV δ Ag isolates, we note here that it includes an NLS consensus, K(K,R)X(K,R), and all isolates conform to this consensus (9). Although both δ Ag-S and δ Ag-L are thought to function in the nucleus, there has been no effort, prior to this work, to investigate the significance of this region in relation to the function of the delta antigens.

Indirect evidence indicates that the residues amino-terminal to the NLS (1 to 57) facilitate dimerization (33). Within this region, an $LX_6LX_6LX_6L$ motif was noted, prompting the authors to suggest that the antigen uses a particular type of coiled-coil structure known as the leucine zipper to dimerize. More recently, both sequence information from additional HDV isolates and mutational analysis suggest that the delta antigen has no strict requirement for leucine in the d position of the helix, as is typical for leucine zippers (6). Nevertheless, this region very likely does function as a coiled coil. A computer algorithm predicts with greater than 99% confidence that, at a minimum, residues 21 to 48 exist in this structure and that the coiled coil probably starts earlier, at position 13 (23). The role of dimerization in the replication phenotypes of δ Ag-S and δ Ag-L is unknown, although at least part of the coiled-coil region of δ Ag-L has been shown to be dispensable for packaging (10).

To date, no function has been ascribed to a potential structural domain which spans a.a. 145 to 214. Unlike the remainder of the protein, the region is not hydrophilic, is predicted to lack any helical structure, and is rich in proline and glycine. We set out to both elucidate the functional role of this region and determine how the other domains contribute to the ultimate phenotypes of the small and large antigens. Discrete regions within the context of each antigen were individually deleted, and the resulting mutants were tested for their ability to dimerize *in vivo*, localize to the

nucleus, *trans* activate or inhibit replication, and become packaged by the HBV small s antigen (sAg).

MATERIALS AND METHODS

Recombinant plasmids. The wild-type small and large delta antigen expression vectors, pDL444 and pDL445, respectively, were constructed by inserting the antigen open reading frame (ORF)-containing *ScaI*-*Bam*HI fragments from pSVL(Ag-S) and pSVL(Ag-L) into the *Sma*I and *Bam*HI sites of a plasmid composed of the *Nde*I-simian virus 40 (SV40) late promoter-*Sma*I region of pSVL and the *Nde*I-vector backbone-*Sma*I region of pGem4Z (7). The δ Ag(1-88)-producing plasmid pDL439 was generated by insertion of the delta ORF-containing *ScaI*-*Stu*I fragment into the *Sma*I site of pSVL. This construct was then restricted with *Bam*HI and blunted with Klenow fragment, and the synthetic *Nhe*I linker 5'-TAGCTAGCTAGCTA-3' was inserted into the site so as to introduce stop codons in all three reading frames. In addition to the first 88 a.a. of the delta antigen, δ Ag(1-88) contains six amino acids at its carboxy terminus (GELGSS) which are derived from the polylinker region. This plasmid was then used to generate the δ Ag(1-81)-expressing plasmid pDL476. The antigen ORF-containing *Sac*II-*Acc*I fragment was blunted at the *Acc*I site with Klenow fragment and inserted into the *Sac*II-blunted *Nhe*I backbone of pDL439. δ Ag(1-81) is extended by an additional 2 a.a. (A and S) at its C terminus. In order to delete 13 a.a. from the coiled-coil region, a pGem4Z derivative that contains the HDV *Bgl*II-*Eco*RI fragment inserted between the *Bam*HI and *Eco*RI sites was restricted with *Xho*I and religated to create a 39-nucleotide deletion. The deletion-containing *Sac*II-*Eco*RI fragment from this vector was then inserted into the same sites within pDL444 and pDL445 to generate the δ Ag-S(Δ 19-31)- and δ Ag-L(Δ 19-31)-producing plasmids pDL448 and pDL449, respectively.

The δ Ag-L(Δ 101-202)-expressing plasmid pDL497 was constructed by excising and purifying the antigen ORF-containing *Eco*RI-*Bst*XI fragment from pDL445, cleaving this with *Sau*3A, and then religating the products with the pDL445 *Eco*RI-*Bst*XI vector backbone. The same approach was used to construct pDL499, the δ Ag-L(Δ 89-145)-producing vector, as well as the δ Ag-L(Δ 69-146)-expressing plasmid pDL503 except that the purified *Eco*RI-*Bst*XI fragment was cleaved with *Stu*I and *Nae*I for pDL499 and with *Hae*III and *Nla*IV for pDL503. pDL499 was used to construct the δ Ag-S(Δ 89-145) producer pDL501 by inserting its antigen ORF-containing *Eco*RI-*Sma*I fragment into the same sites within pDL444. pDL503 was used in the same manner to generate the δ Ag-S(Δ 69-146) expressor pDL505. In the process of fusing the *Nla*IV site to *Hae*III in pDL503, a new *Apa*I site was created 18 nucleotides upstream and in frame with the *Apa*I site that naturally resides within the antigen ORF. Thus, the δ Ag-S(Δ 69-74)- and δ Ag-L(Δ 69-74)-producing plasmids pDL507 and pDL508 were generated by introducing the *Apa*I-*Bst*XI fragments from pDL444 and pDL445, respectively, into the same sites within pDL503. pDL500, which produces δ Ag-S(Δ 146-163), was generated by purifying the ORF-containing *Eco*RI-*Nae*I fragment of pDL444 and ligating it into the pDL444 *Eco*RI and *Sma*I sites.

The plasmid which initiates HDV replication, pDL481, comprises the *Nde*I-SV40 late promoter-*Sac*I fragment of pSVL and the *Sac*I-vector backbone-*Nde*I fragment of pGem4Z. Within the pGem polylinker, the 1.1-genome-length *Sall*-*Sall*-*Xba*I fragment of pSVL(D2M) has been inserted in the antigenomic orientation with respect to the

SV40 promoter (17). The HBV serotype AYW sAg expression clone pMSVgptSM was kindly provided by C. Mangold and contains the *Xho*I-sAg ORF-*Bgl*II fragment cloned into the *Bam*HI site of pNI2 (16).

DNA transfections and sample harvest. For all experiments, the human hepatoma cell line Huh7 was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and transfected at approximately 80% confluence with calcium phosphate-precipitated DNA (15). For packaging, immunofluorescence, and cross-linking experiments, samples were harvested at 3 days posttransfection and at 6 days for *trans* activation and *trans* inhibition studies, respectively. For protein analysis, samples were lysed and resuspended with Laemmli sample buffer (18), and RNA was isolated by a modification of the acid phenol-guanidinium-thiocyanate procedure (12). Secreted HBV sAg particles were recovered from the medium as described previously (28). Briefly, 10 ml of medium was clarified by low-speed centrifugation, and the particles were pelleted by high-speed centrifugation (32,000 rpm, 16 h, 4°C) through 2 ml of 20% isotonic sucrose. *In vivo* cross-linking was done by a modification of the method of Zapp et al. (34). The medium was supplemented with 0.3% methanol, 10 mM NaCl, 0.1 mM EDTA, and 0.01% glutaraldehyde and added to the cells for 20 min at 37°C, followed by a 3-h incubation at 4°C. Cross-linked cells were washed and then directly lysed and resuspended in sample buffer.

Immunoblot analysis. Protein samples were subjected to electrophoresis on 14% polyacrylamide gels by the method of Laemmli (18). They were then transferred to nitrocellulose electrophoretically, and delta antigen was detected by using rabbit polyclonal antiserum raised against bacterially expressed antigen and by incubation with ¹²⁵I-labeled protein A (Du Pont).

RNA analysis. Total cell RNA was separated electrophoretically on 1.5% agarose gels in the presence of ethidium bromide so that both the quality and quantity of RNA in each lane could be monitored. RNA was then electrophoretically transferred to a nylon membrane, and successful transfer was confirmed by visualization with UV light. RNA was UV cross-linked to the membrane and then hybridized with a radiolabeled RNA probe specific for the genomic strand as described previously (11).

Immunofluorescence assays. Cells were transfected and then fixed on coverslips by treatment with -20°C methanol for 3 min and then with -20°C acetone for 30 s. Fixed cells were incubated for 2 h at room temperature with a rabbit polyclonal antibody raised against recombinant delta antigen expressed in *Escherichia coli*, washed, and then incubated for 2 h at room temperature with rhodamine-conjugated protein A (Boehringer Mannheim).

RESULTS

Rationale for design of delta antigen mutants and their associated activities. Our goal was to determine the contributions that the coiled-coil, NLS, RNA-binding, and Pro/Gly-rich domains make in the function of the delta antigens. This question was addressed by individually deleting each region within both δ Ag-S and δ Ag-L and then testing the deletion's effect on the ability of the protein to localize to the nucleus, oligomerize, support or inhibit replication, and associate with HBV sAg in secreted particles. Figure 1 depicts the series of mutants constructed for this study. The mutants were designed to delete the following sequences: coiled-coil domain, δ Ag(Δ 19-31); nuclear localization signal, δ Ag(Δ 69-

74); RNA-binding domain, δ Ag(Δ 89-145); RNA-binding domain plus NLS, δ Ag(Δ 69-146); Pro/Gly-rich region, δ Ag(Δ 146-163); RNA-binding domain plus Pro/Gly-rich region, δ Ag(Δ 101-202); RNA-binding domain plus Pro/Gly-rich region plus isoprenylation site, δ Ag(1-88) and δ Ag(1-81). In most cases, a given deletion was created in both δ Ag-L and δ Ag-S; however, only the δ Ag-L mutants are diagrammed. The deletion mutation spanning residues 145 to 163 was introduced into both δ Ag-S and δ Ag-L, but its effects on the stability of the two proteins were very different. δ Ag-S(Δ 145-163) was readily detectable, whereas δ Ag-L(Δ 145-163) was either undetectable or barely detectable, depending on the time posttransfection that it was analyzed. For this reason, no conclusions about the phenotypes of this mutant could be drawn.

All proteins were expressed from SV40 late promoter-derived vectors via transient transfection of the human hepatoma cell line Huh7. The mutants were then assayed for relevant phenotypes.

The ability of a protein to localize to the nucleus was assessed by immunofluorescence. Glutaraldehyde cross-linking of intact living cells was used to determine whether a mutant could multimerize *in vivo*. The ability of each mutant to support genome replication was assayed by cotransfecting a plasmid that transcribes an antigenomic HDV RNA which contains a frameshift mutation in the antigen ORF and requires functional δ Ag-S in *trans* in order to replicate. Total cellular RNA was then subjected to Northern (RNA blot) analysis with a probe specific for the product of replication, i.e., HDV genomic RNA. *trans*-Dominant inhibition of replication was determined in the same manner except that in addition to the HDV RNA and mutant protein-producing constructs, cells were transfected with an equivalent amount of a vector which expresses wild-type δ Ag-S. A protein's ability to be packaged by the hepadnaviral envelope proteins was determined by cotransfecting a plasmid that produces the HBV sAg. Secreted sAg particles were then isolated and subjected to Western immunoblot analysis with delta antigen-specific antiserum. A summary of the phenotypes of the various mutants is given in Table 1.

Core nuclear localization signal is dispensable for the function of δ Ag-S and δ Ag-L. Both forms of the delta antigen are reported to show three distinct nuclear immunofluorescence staining patterns (33). With most cells, a punctate nucleolar pattern of localization is observed, in which both the nucleoli and additional subnuclear structures show preferential staining. However, in some cells, both the nucleoplasm and nucleoli are stained, whereas in others, antigen is localized to the nucleoplasm and excluded from the nucleoli. We have also observed these three patterns, and the first and third types can be seen in adjacent cells in Fig. 2A. Currently, we have no explanation for these differences in subnuclear localization, nor do we understand their relevance, if any, to the HDV life cycle. Consistent with earlier findings, we were unable to detect differences in the localization of δ Ag-S and δ Ag-L either in the context of the wild-type proteins or with the deletion mutants mentioned below.

It was previously shown that residues 1 to 88 of the delta antigen were sufficient to promote nuclear localization when fused to a cytoplasmic protein (33). Not surprisingly, therefore, our δ Ag(1-88) mutant localized to the nucleus with the same staining pattern as the wild-type protein. However, amino acids 85 to 88 are reported to enhance the function of the core NLS, yet when these residues were deleted [δ Ag(1-81)], we again observed wild-type localization (Fig. 2B) (33).

As would be expected, when the core NLS was removed

TABLE 1. Antigen mutants and their characteristics

Mutant	Packaged by HBV sAg	Copackaged δ Ag-S	Copackaged by sAg + δ Ag-L	Cross-linked oligomers	Localization ^a	<i>trans</i> activator	<i>trans</i> inhibitor
δ Ag-S	—		+	+	N	+	—
δ Ag-L	+	+		+	N	—	+
δ Ag-S(Δ 19–31)	—		—	—	N	—	—
δ Ag-L(Δ 19–31)	+	—		—	N	—	—
δ Ag-S(Δ 69–74)	—		+	n.d. ^b	W	+	—
δ Ag-L(Δ 69–74)	+	+		n.d.	W	—	+
δ Ag-S(Δ 89–145)	—		+	n.d.	N	—	—
δ Ag-L(Δ 89–145)	+	+		n.d.	N	—	+
δ Ag-S(Δ 69–146)	—		+	n.d.	W	—	—
δ Ag-L(Δ 69–146)	+	+		n.d.	W	—	+
δ Ag-L(Δ 101–202)	—		n.d.	n.d.	n.d.	—	+
δ Ag(1–88)	—		n.d.	+	N	—	+
δ Ag(1–81)	—		n.d.	+	N	—	+
δ Ag-S(Δ 146–163)	—		+	+	n.d.	—	—

^a N, nuclear; W, whole cell.^b n.d., not done.

in δ Ag-S(Δ 69–74) and δ Ag-L(Δ 69–74), a significant amount of protein was seen in the cytoplasm (Fig. 2C and D). These mutants were not excluded from the nucleus, however, and in fact, some cells showed preferential staining of the nucleoli (Fig. 2C and D). The presence of a portion of the NLS (a.a. 75 to 88) does not account for this residual nuclear staining, as δ Ag(Δ 69–146) localized in the same manner (data not shown).

The remaining nuclear access of the NLS deletion mutants is not a trivial issue, given their surprising phenotypes. In the *trans* activation assay, an HDV cDNA clone was unable to initiate replication, as assessed by the detection of genomic RNA in a Northern blot, unless functional δ Ag-S was supplied in *trans* (Fig. 3B, lanes a and b). The presence or absence of δ Ag-S was confirmed by Western blot analysis (Fig. 3A, lanes a and b). As shown in Fig. 3B, lane d, δ Ag-S(Δ 69–74) was fully capable of supporting genome replication with wild-type efficiency. In the *trans* inhibition assay, the cotransfection of a δ Ag-L-expressing plasmid with a plasmid expressing δ Ag-S and HDV RNA abolished replication (compare lanes b and l of Fig. 3B). δ Ag-L(Δ 69–74) displayed wild-type activity in its ability to inhibit replication (Fig. 3B, lane 3). Furthermore, in a packaging assay, δ Ag-L can be secreted into the tissue culture medium if HBV sAg is also expressed, and δ Ag-L(Δ 69–74) was similarly found to be efficiently packaged into sAg particles (Fig. 4, compare lanes b and B with lanes d and D).

Coiled-coil region needed for both *trans* activation of replication by δ Ag-S and *trans* inhibition by δ Ag-L. A program designed to recognize coiled-coil domains in protein primary sequences predicts that the deletion of a.a. 19 to 31 in the delta antigen will abolish the protein's capacity to assume this structure (23). This deletion was introduced into both δ Ag-S and δ Ag-L, and its effect on dimerization was evaluated by two independent methods. When cells which were transiently expressing wild-type δ Ag-S were subjected to in vivo cross-linking, a significant amount of a product that is consistent in size with a dimer and whose production is dependent on the addition of glutaraldehyde was observed (Fig. 5, lane 4). The assignment of this novel species as an δ Ag-S dimer was further substantiated by results obtained with the coiled-coil-containing truncation construct δ Ag(1–88). Glutaraldehyde-treated cells which expressed this protein did not produce a product of the same size as the δ Ag-S-expressing cells but rather two smaller products

whose sizes are what would be expected for δ Ag(1–88) dimers and trimers (Fig. 5, lane 2). Furthermore, in additional experiments with gels containing a lower percentage of acrylamide, dimers and trimers of both wild-type δ Ag-S and δ Ag-L could be resolved (data not shown). These results clearly established that both wild-type proteins as well as δ Ag(1–88) oligomerized in vivo. However, when cells producing δ Ag-L(Δ 19–31) were treated in the same manner, only monomeric protein was visualized (Table 1).

The inability of δ Ag-L(Δ 19–31) to oligomerize was also confirmed by packaging experiments. Previously, it was noted that although δ Ag-S is not packaged into hepadnaviral particles per se, it is copackaged if δ Ag-L is also present in the cell (28). This observation was interpreted as indicating a direct protein-protein interaction between δ Ag-S and δ Ag-L. Consistent with this notion, when the coiled-coil domain was disrupted in δ Ag-S(Δ 19–31), the protein was not copackaged by δ Ag-L (Table 1). Likewise, although δ Ag-L(Δ 19–31) did package itself, it was unable to copackage δ Ag-S (Table 1). In addition to providing further evidence that deletion of a.a. 19 to 31 inactivates the coiled coil, this result confirms a previous finding, that the packaging (although not the copackaging) phenotype of δ Ag-L is independent of dimerization (10). Disruption of the coiled-coil domain completely inactivated both the *trans* inhibition and *trans* activation phenotypes, as δ Ag-L(Δ 19–31) did not interfere with replication and δ Ag-S(Δ 19–31) was unable to promote it (Fig. 3B, lanes 2 and c).

Inhibition of replication by δ Ag-L does not require an RNA-binding domain but functions via interaction with δ Ag-S. To explain the potency of δ Ag-L in inhibiting replication, we proposed that the protein interacts with and “poisons” the replicating delta virus RNP structure (7). δ Ag-L might use its RNA-binding domain to gain access to this RNP. However, a number of our mutants indicated that the region which binds RNA is not needed for inhibition of replication. δ Ag(1–88), δ Ag(1–81), δ Ag-S(Δ 89–145), δ Ag-L(Δ 89–145), δ Ag-S(Δ 69–146), δ Ag-L(Δ 69–146), and δ Ag-L(Δ 101–202) all potentially inhibited replication, although none contain this domain (Table 1 and Fig. 3B, lanes 4 to 7).

How then do these proteins interact with their target? Since all of the mutants contain the coiled-coil domain, a region which we have just shown is required for inhibition, it seems likely that they could form heterodimers with δ Ag-S, which in turn would bind HDV RNA. This possibility was

FIG. 2. Immunofluorescence analysis of the localization of wild-type and mutant delta antigens. Huh7 cells were fixed 3 days posttransfection and incubated with a rabbit polyclonal antibody raised against recombinant delta antigen expressed in *E. coli*. (A) δ Ag-S; (B) δ Ag(1-81); (C) δ Ag-S(Δ 69-74); (D) δ Ag-L(Δ 69-74).

verified by *in vivo* cross-linking. When cells expressing both δ Ag-S and δ Ag(1-88) were cross-linked, a unique heterodimeric species was observed which was not seen in cells that expressed either protein individually (Fig. 5, compare lanes 2, 4, and 6). Although this heterodimeric species (289 a.a.) is similar in predicted size to and nearly comigrated with the δ Ag(1-88) trimer (282 a.a.), it could be discerned by its relative abundance. While the δ Ag(1-88) trimer was far less abundant than the dimer, the δ Ag(1-88)/ δ Ag-S heterodimer was in excess of either homodimeric species (Fig. 5, compare lanes 2 and 6). This result is in excellent agreement with what would be predicted theoretically. If δ Ag-S and δ Ag(1-88) are present in equimolar amounts and the energetics of homodimer and heterodimer formation are equivalent, then a 1:2:1 ratio of δ Ag-S dimer to δ Ag-S/ δ Ag(1-88) heterodimer to δ Ag(1-88) dimer would be expected.

Packaging of δ Ag-L into HBV sAg particles requires a C-terminal Pro/Gly-rich domain. Of the aforementioned mutants which potentially inhibited replication, several were unable to be packaged by HBV sAg. For instance, δ Ag(1-88)

was not found in sAg particles; however, when the Pro/Gly-rich region spanning residues 146 to 214 was appended to it [δ Ag-L(Δ 89-145)], packaging was restored (Table 1 and Fig. 4, lane F). Similarly, a mutant with an even larger deletion, δ Ag-L(Δ 69-146), was both packaged and competent in its ability to copackage δ Ag-S (Fig. 4, lane G). Since the δ Ag-S version of the a.a. 89 to 146 deletion both lacked the isoprenylation site and was not packaged, the restoration of packaging seen with the addition of a.a. 146 to 214 to δ Ag(1-88) might solely reflect the requirement for this site. Another mutant, δ Ag-L(Δ 101-202), however, indicated that the Pro/Gly-rich region was also needed, as this mutant contained the isoprenylation site but was still not packaged (Fig. 4, lane E).

DISCUSSION

We have systematically deleted domains within the delta antigens in order to determine their contributions to the various functions of these proteins. This approach has the general limitation that it is possible, in deleting a given

FIG. 3. *trans* activation and *trans* inhibition of replication. (A) Western blot analysis of delta antigen and mutant proteins expressed in Huh7 cells at 6 days posttransfection and probed with rabbit polyclonal anti-delta antigen antiserum. The predicted sizes of all proteins were in good agreement with actual sizes, as determined by comparison with nonradiolabeled, prestained protein standards. (B) Northern blot analysis of genomic HDV RNA synthesized in the presence of wild-type and mutant antigens in Huh7 cells at 6 days posttransfection. RNA was probed with uniformly labeled, *in vitro*-generated antigenomic RNA transcribed by using T7 RNA polymerase from a pGEM4Z derivative containing trimeric HDV cDNA. Lanes a to d, samples tested for *trans* activation; lanes 1 to 8, samples tested for *trans* inhibition. All samples were isolated from cells which were transfected with equal amounts of a vector which produces antigen ORF frameshift-containing antigenomic RNA (pDL481); the samples in lanes 1 to 8 were isolated from cells which were additionally transfected with a δ Ag-S expression plasmid. Lanes: a, no-protein control; b, δ Ag-S; c, δ Ag-S(Δ 19–31); d, δ Ag-S(Δ 69–74); 1, δ Ag-L; 2, δ Ag-L(Δ 19–31); 3, δ Ag-L(Δ 69–74); 4, δ Ag-L(Δ 101–202); 5, δ Ag-S(Δ 89–145); 6, δ Ag-L(1–81); 7, δ Ag-L(Δ 69–145); 8, δ Ag-S(Δ 146–163). As assessed by the intensity of ethidium bromide-stained RNA on the membrane after transfer, less RNA was loaded in lane b than in the other lanes, while lane 2 was somewhat overloaded.

domain, to alter the structure of the protein so as to affect other regions. The loss of a function associated with the deletion of a domain could indicate that domain's direct role in the process or may only reflect the inactivation of a second critical domain. For this reason, deletion mutants which maintain function are far more informative, as it can be concluded, without ambiguity, that the structure responsible for that activity has remained intact. As a multifunctional protein, the delta antigen has proved to be particularly amenable to this approach, since it has been possible to abolish one function while preserving others. All of the major conclusions that arise from this work were obtained by using mutants which retain the function of interest. These conclusions can be summarized as follows. (i) The core NLS is dispensable for the replication and packaging activities of δ Ag-S and δ Ag-L. (ii) Neither the packaging nor replication inhibition activities of δ Ag-L require the RNA-binding domain. (iii) Replication inhibition by δ Ag-L occurs via a protein-protein interaction with δ Ag-S. (iv) δ Ag-L uses a

FIG. 4. Packaging and copackaging assay; Western blot analysis of proteins expressed within cells and secreted from cells in the presence of HBV sAg. Lanes a to g show 1/100 of the cell protein from a 100-mm petri dish, and lanes A to G show 1/10 of the secreted material isolated from tissue culture medium from the same samples. Lanes: a, δ Ag-L(Δ 19–31); b, δ Ag-L plus δ Ag-S(Δ 69–74); c, δ Ag-L(Δ 69–74) plus δ Ag-S; d, δ Ag-L(Δ 69–74) plus δ Ag-S(Δ 69–74); e, δ Ag-L(Δ 101–202); f, δ Ag-L(Δ 89–145) plus δ Ag-S; g, δ Ag-L(Δ 69–146) plus δ Ag-S. The predicted sizes of each protein were in good agreement with actual sizes determined by comparison with nonradiolabeled, prestained protein standards.

C-terminal Pro/Gly-rich region in its association with the hepadnaviral envelope proteins.

We deleted six of the seven amino acids which were previously identified as the core of the nuclear localization signal (33), and as expected, these mutants displayed altered compartmentalization and were present in both the cytoplasm and the nucleus. Additional mutants demonstrate that the remaining amino acids of the NLS (a.a. 76 to 88) are not responsible for the residual access to the nucleus displayed by the a.a. 69 to 74 deletions. Surprisingly, and presumably as a consequence of this ability to reach the nucleus, δ Ag-S(Δ 69–74) is fully competent in supporting replication, whereas δ Ag-L(Δ 69–74) inhibits replication with wild-type efficiency and is packaged by HBV sAg. The small and large versions of δ Ag(Δ 69–74) may be entering the nucleus by passive diffusion, as the nuclear pore is thought to be permeable to molecules smaller than 50 kDa (25). Alternatively, since both proteins were present in the nucleoli of some cells, it remains possible that there is a secondary NLS within the first 68 a.a. Consistent with this possibility, Chang et al. recently reported that sequences within the first 50 a.a. of the delta antigen can, under some circumstances, direct a fusion protein to the nucleus (6).

So far, we have observed no defect in function after deletion of the core NLS. However, since several aspects of the viral life cycle have not been assayed in this study, it would be premature to conclude that these residues are truly dispensable. During the onset of infection, the viral RNP, having just entered the cell and shed its envelope, is expected to be far too large to freely diffuse through the nuclear pore. It seems possible, therefore, that the core NLS may be required to facilitate this process.

A number of mutants have enabled us to delineate the domain which governs the ability of δ Ag-L to be packaged by the hepadnaviral envelope proteins. Since both δ Ag-L(Δ 19–31) and δ Ag-L(Δ 69–146) are efficiently packaged, we can conclude that the residues which have been deleted are

FIG. 5. Western blot analysis of in vivo-cross-linked δ Ag-S and δ Ag(1-88). Samples in even-numbered lanes were isolated from cells for which 0.01% glutaraldehyde was added to the tissue culture medium, and samples in odd-numbered lanes were not cross-linked. Lanes: 1 and 2, δ Ag(1-88); 3 and 4, δ Ag-S; 5 and 6, δ Ag(1-88) plus δ Ag-S. Predicted sizes: δ Ag(1-88) monomer, 94 a.a.; δ Ag(1-88) dimer, 188 a.a.; δ Ag(1-88) trimer, 282 a.a.; δ Ag-S monomer, 195 a.a.; δ Ag-S/ δ Ag(1-88) heterodimer, 289 a.a.; δ Ag-S dimer, 390 a.a. The predicted sizes were in good agreement with actual sizes as determined by comparison with nonradiolabeled, prestained protein standards.

not involved in this process. Recently, Chen et al. (10) similarly showed that δ Ag-L(Δ 33-73) is proficient in this process. From these results, we can conclude that a.a. 19 to 146 are dispensable for packaging. Of the remaining residues, we have shown a direct role for a.a. 146 to 214. When this domain is appended to a packaging-deficient mutant, the wild-type packaging phenotype is restored.

Both the amino acid content and the predicted secondary structure of this domain are very different from the remainder of the antigen. More than 39% of the amino acids in this region are either proline or glycine, yet these residues constitute less than 12% of the amino-terminal portion. While residues 1 to 145 are extremely hydrophilic and predicted to include several α -helices, a.a. 146 to 214 are relatively nonpolar and are thought, because of the high content of proline and glycine, to be unable to assume this structure. These properties are consistent with a domain which is sequestered from the solvent, possibly by interaction with another protein. Although there is, as yet, no evidence that demonstrates the existence of an δ Ag-L/ δ Ag protein interface, it is difficult to imagine how the large antigen could be so efficiently and selectively packaged without such an interaction taking place. We propose that the Pro/Gly-rich region interacts directly with the HBV sAg to facilitate packaging.

It should also be noted that all of the packaging-competent mutants presented contain the 19 C-terminal residues unique to δ Ag-L, underscoring the importance of this region. The relevance of these 19 a.a. to replication inhibition is less obvious, however. Previously, we had observed that when 84 heterologous amino acids were appended to the C terminus of δ Ag-S, or when an *E. coli* protein (MalE) was fused to its amino terminus, the inhibitory phenotype was elicited (20). Neither protein contained any of the 19 a.a. unique to

δ Ag-L, and thus, the exact identities of these residues are irrelevant in relation to inhibition. We surmised that the C-terminal 19 a.a. as well as the other amino- and carboxy-terminal alterations act sterically to perturb the protein structure and thereby elicit the phenotypic change. The mutants presented here give further support to this hypothesis, as even δ Ag(1-81) and δ Ag-S(Δ 69-146), both of which lack the C-terminal 19 a.a. and have substantial deletions of other sequences, still inhibit replication with wild-type efficiency. Together, the results with the two mutants indicate that the antigen's first 68 residues are necessary and sufficient to confer this phenotype.

Within residues 1 to 68, there is a domain which is predicted to assume a coiled-coil structure that can potentiate multimerization. All of the inhibitory mutants from this study contain intact coiled-coil domains, although several lack the RNA-binding domain. Mutants in which a portion of the coiled-coil domain has been deleted are unable to either dimerize or inhibit replication. In addition, one inhibitory mutant, δ Ag(1-88), was shown to interact directly with δ Ag-S in vivo. The potency and stoichiometry of the inhibition exhibited by δ Ag-L can be explained if its target is a complex containing multiple δ Ag-S molecules. From a theoretical analysis of inhibition data, it was calculated that this complex should include 23 δ Ag-S monomers (7). Recently, Ryu et al. found that, based on equilibrium density centrifugation analysis, the HDV nuclear RNP contains approximately 25 δ Ag-S molecules, consistent with the notion that this structure is the target for δ Ag-L inhibition (29). The results presented here demonstrate that δ Ag-L (and the other *trans*-dominant inhibitor mutants) do not require the RNA-binding domain to access the RNP but can act via a coiled-coil interaction with δ Ag-S.

Ag-L could act through either a passive or active mechanism to poison the HDV RNP complex. In the passive model, any and all proteins which are themselves unable to support replication but can still interact with δ Ag-S would have access to the RNP and, by their mere presence, inhibit replication. This possibility is negated by the results obtained with δ Ag-S(Δ 146-163), which does not support replication and does not inhibit replication but does have a functional coiled coil (Table 1). The active model asserts that the inhibitor should not only interact with δ Ag-S but also must contain an activated function which interferes with the replication process.

What might this inhibitory function be? In addressing this question, we should make three points. First, there is no evidence to suggest that δ Ag-L directly prevents either the initiation, elongation, or maturation steps of HDV replication. All that is known is that, in the presence of δ Ag-L, fewer replicative products are observed. Thus, δ Ag-L could work through an indirect mechanism. Second, there is currently a gap in our understanding of how the HDV RNP, which is observed in the nucleus, can encounter and become packaged by the exclusively cytoplasmic hepadnaviral envelope proteins. Finally, there are a number of shuttle proteins, such as No38, nucleolin, and Nopp140, which show exclusively punctate nucleolar localization by immunofluorescence (2, 24). Despite this staining pattern, these proteins are not restricted to the nucleus. More sensitive techniques have been used to demonstrate that the proteins can "piggyback" larger molecules associated with them in and out of the nucleus (2, 24). All three of these proteins are phosphorylated and contain potentially complex nuclear localization signals with multiple NLS consensus sites. Similarly, δ Ag-L

is a nucleolar phosphoprotein which has a bipartite NLS (5, 33).

With these points in mind, we speculate that δ Ag-L can escort the HDV RNP from the nucleus to the cytoplasm. A nuclear export signal might reside within the first 68 a.a. of the antigen, which would be silenced in δ Ag-S but conformationally activated upon addition of the C-terminal 19 a.a. in δ Ag-L. δ Ag-L, through a coiled-coil interaction with the small antigen, would bind to the HDV RNP and move it to the cytoplasm, where the large antigen's isoprenylated Pro/Gly-rich region could interact with HBV sAg to induce packaging. This model also accounts for the *trans*-dominant inhibition simultaneously caused by δ Ag-L. As HDV RNP structures are removed from the nucleus, the nuclear pool of these species would be depleted, and as a consequence, replication would be reduced. Thus, a single-molecule mechanism, nuclear export, can account for the superficially different phenotypes of packaging and replication inhibition. A direct investigation of the ability of δ Ag-L to exit the nucleus will be required to test the validity of this model.

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