Hepatitis D virus RNA editing is inhibited by a GFP fusion protein containing a C-terminally deleted delta antigen

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During its life cycle, hepatitis D virus (HDV) produces two forms of delta antigen (HDAg), small delta antigen (SDAg) and large delta antigen (LDAg), which differ in their C-terminal 19 amino acids. Host enzymes termed ADARs (adenosine deaminases that act on double-stranded RNA) are required for LDAg production. These enzymes change the stop codon (UAG) of SDAg to a tryptophan codon (UGG). However, the temporal and spatial regulation of HDV RNA editing is largely unknown. In this study, we constructed three GFP fusion proteins containing different lengths of SDAg and characterized their cellular localization and effects on HDV replication. One of these fusion proteins, designated D(1–88)-GFP, inhibited LDAg but not SDAg production, suggesting that D(1–88)-GFP inhibits HDV RNA editing. Two experiments further supported this supposition: (i) RT-PCR analysis combined with NcoI restriction enzyme digestion revealed that HDV RNA editing was reduced by 42% in HeLa-D(1–88)-GFP when compared with HeLa cells; and (ii) the ratio of SDAg/LDAg production from the reporter RNAs was reduced in cells co-transfected with ADAR-expressing and reporter plasmids in the presence of D(1–88)-GFP. Double fluorescence microscopy found that D(1–88)-GFP was either associated with SC-35 or was adjacent to PML (premyelocytic leukaemia antigen) at nuclear speckles, but D(1–88)-GFP was not co-localized with ADAR, which was mainly located in the nucleolus. In situ hybridization showing co-localization of HDV RNA with D(1–88)-GFP at nuclear speckles suggested that HDV RNA editing might occur in the nuclear speckles and require other nuclear factor(s), in addition to ADAR.

INTRODUCTION

Hepatitis D virus (HDV) is a subviral pathogen that causes acute and chronic liver diseases during co- or superinfection with hepatitis B virus (HBV) (Bonino et al., 1986; Chen et al., 1997). HDV requires HBV surface antigens (HBsAgs) to form a mature virion that can re-enter hepatocytes for a second round of infection (Sureau et al., 1991; reviewed by Lai, 1995). In addition to HBsAg present on the surface, the HDV virion contains a 1·7 kb single-stranded circular RNA genome and two isoforms of the hepatitis delta antigen (HDAg), which form a ribonucleoprotein (RNP) complex located inside the virion (Lai, 1995).

Two forms of HDAg, small and large (SDAg and LDAg, respectively), are encoded by a single open reading frame (ORF) of antigenomic RNA (Lai, 1995). The SDAg contains 195 amino acids while the LDAg contains 19 additional amino acids at its C terminus (Taylor, 1990). The SDAg trans-activates HDV RNA replication while the LDAg suppresses this process and interacts with HBsAgs to assemble mature virions (Chao et al., 1990; Chen et al., 1993; Ryu et al., 1992; for a review see Lai, 1995, and references therein). The sequential appearance of SDAg and LDAg is achieved via an RNA editing event in which the amber stop codon (UAG) for the SDAg ORF is converted to a tryptophan codon (UGG) (Casey & Gerin, 1995; Polson et al., 1996, 1998). Thus, RNA editing is essential to the HDV life cycle since without it there would be no LDAg production and subsequently no mature HDV formation.

HDV RNA editing occurs on the rod-structured antigenomic RNA (Casey & Gerin, 1995) by host enzymes called ADARs (adenosine deaminases that act on double-stranded RNA) (Wong & Lazinski, 2002). Three members of the ADAR gene family have been identified and characterized in recent years (Bass, 2002; Gott & Emeson, 2000). ADAR1 and ADAR2 are capable of editing adenosines in double-stranded RNA of mammalian cells (Melcher et al., 1996; Seeburg et al., 1998), while ADAR3 was found to play a...
regulatory role in RNA editing (Chen et al., 2000). The ADAR1 homologues from *Xenopus laevis*, HeLa nuclear extract and *Drosophila melanogaster* embryo nuclear extract have been demonstrated to edit HDV RNA efficiently (Polson et al., 1996; Casey & Gerin, 1995). Recently, overexpression of human ADAR1 and ADAR2 was shown to increase the editing efficiency of a reporter mRNA in transfected HEK293 cells and to inhibit HDV RNA replication in HuH-7 cells (Sato et al., 2001; Jayan & Casey, 2002).

Although the host enzymes and the RNA substrate structure of HDV RNA editing have been well studied (Wong et al., 2001; Sato et al., 2001), the involvement of other host factors and the nuclear locations of the event remain largely unknown. Previously, we demonstrated that GFP fusion proteins are powerful tools in understanding the locations and the functions of the components involved (Shih & Lo, 2001). In this study, we generated three new GFP fusion proteins containing various lengths of HDAg and demonstrated that one of these fusion proteins, designated D(1–88)-GFP, inhibited HDV RNA editing. This fusion protein allowed us to investigate the locations of HDV RNA editing.

### METHODS

#### Plasmid construction and features.

The eukaryotic expression vector pEGFP-N2, which contains the GFP gene of jellyfish, was obtained from Clontech Laboratories. The coding region of full-length HDV SDAg spanning nt 1605–1010 of HDV cDNA was amplified by PCR using a primer containing a Kozak's sequence and cloned into pGEMT to yield pGEMT-SD. The full-length HDV SDAg fragment was then excised from pGEMT-SD and inserted into the BgIII and EcoRI sites of pEGFP-N2 to yield pSD-GFP. The pD(1–88)-GFP and pD(1–163)-GFP plasmids were constructed by inserting the HDV *XhoI–Stul* or *XhoI–SmaI* DNA fragment, respectively, from the previously established pGEM3(−)L (Hu et al., 1996) into pEGFP-N2. The plasmid pCMVDag2 was derived from pEGFP-N2 by replacing the GFP fragment with the dimer of 1.7 kb HDV cDNA. Four other HDV cDNA-containing plasmids, pSVL-d2g, pGFP-LD, pGFP-LDM and pGFP-LD(31–214), used in this study have been reported previously (Yeh et al., 1996; Shih & Lo, 2001). The features of these plasmids are summarized as follows: (i) pSVL-d2g containing the SV40 late promoter is similar to pCMVDag2, which contains the CMV promoter to produce a replication-competent HDV RNA and two HDAg; (ii) pGFP-LD produces GFP fused to wild-type LDAg; (iii) pGFP-LDM produces GFP fused to a non-isoprenylation mutant of LDAg; and (iv) pGFP-LD(31–214) produces GFP fused to an N-terminal deletion (aa 1–30) mutant of LDAg. The editing reporter plasmid pSS74 contains antigenomic HDV cDNA sequences from nt 1625 to 211, in which the polyadenylation signal and the ribosome are mutated (Sato et al., 2001). Two plasmids, pDL700 encoding ADAR1 fused to a haemagglutinin tag (ADR1–HA) and pMS40 encoding ADAR2–HA, have been described previously (Sato et al., 2001).

#### Cell culture and transfection of HuH-7 and HeLa cells.

Two human cell lines were used for plasmid transfection in this study, HuH-7, a well-differentiated human hepatoma cell line, and HeLa, an epithelioid carcinoma cell line. Both cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, penicillin (100 IU ml⁻¹), streptomycin (100 µg ml⁻¹), Fungizone (50 µg ml⁻¹) and 2 mM l-glutamine and grown at 37°C under 5% CO₂. Plasmids in a supercoiled form were obtained using the Qiagen Plasmid Maxi Kit and used for transfection. HeLa and HuH-7 cells were grown in 10 cm Petri dishes to 60% confluence and transfected with 20 µg of the indicated plasmids by the calcium phosphate/DNA precipitation method (Graham & van der Eb, 1973). To obtain cells that permanently expressed the plasmids, the transfected HeLa cells were scraped off, replated into 10 cm Petri dishes and selected with G418. Single colonies were generated by limiting dilution cloning as described previously (Shih & Lo, 2001). HeLa cells expressing various GFP fusion proteins were designated HeLa-D(1–88)-GFP, HeLa-D(1–163)-GFP, HeLa-SD-GFP and HeLa-GFP-LD(31–214).

#### Fluorescent microscopy.

Transiently or permanently transfected cells that expressed GFP-SD or other variants were cultured on 22 x 22 mm coverslips. Cells were fixed using 4% paraformaldehyde in PBS for 30 min at room temperature, probed with anti-HA (human influenza virus haemagglutinin), anti-SC-35 (a splicing factor) or anti-PML (premyelocytic leukaemia antigen) antibodies and visualized with a secondary goat anti-mouse rhodamine-conjugated antibody. Finally, cells were stained with Hoechst 33258, mounted on glass slides using mounting solution and visualized using a fluorescent microscope (Olympus B-Max 60) or a confocal microscope (Leitz).

#### Western blotting.

To identify the presence of various antigens, protein samples obtained from whole-cell extracts or sucrose-gradient fractions were subjected to Western blot analyses. For sucrose-gradient fractionation, nuclear extracts were obtained from cells expressing various GFP fusion proteins. Samples were layered on to a 10–50% (w/v) discontinuous sucrose gradient and subjected to centrifugation at 38 000 r.p.m. in an SW41 rotor for 16 h at 4°C. The gradients were fractionated into 18 tubes, each containing 0.6 ml. The density of each fraction was determined from the refractive index using a refractometer. Protein samples were separated by SDS-PAGE and electrotransferred on to PVDF membranes (Immobilon-P; Millipore) as described by Towbin et al. (1979). The membranes were incubated with 5% non-fat milk followed by human anti-HDAg sera (Yeh et al., 1996), anti-GFP or anti-HA primary antibodies. After incubation with horseradish peroxidase-conjugated anti-human or anti-mouse secondary antibody, the blot was developed by enhanced chemiluminescence (ECL) using a commercial kit (Amersham Japan) or developed using 4-chloro-1-naphthol as the substrate. The intensity of protein bands was quantified by ImageQuant TL software (Amersham Biosciences).

#### Analysis of RNA editing efficiency by RT-PCR.

Total RNA was prepared from wild-type or GFP-expressing HeLa cells that had been transfected with pCMVDag2 using a REzol C&T RNA extraction kit (PROtech Technologies). RNA samples were treated with DNase and either treated with or without RNase. Samples were then subjected to RT-PCR using PCR primers 877 (5' -GAGGTTGGAGATGC-3', genomic strand) and T37A (5' -CTCGGCCAGACCAAAGAA-3', antigenomic strand). The effectiveness of DNase treatment was confirmed by the absence of PCR products after PCR amplification in the absence of reverse transcription. The PCR products were analysed by Ncol restriction enzyme digestion, which generated two DNA fragments (509 and 134 bp). The RT-PCR products from the pMTLD-transfected cells served as the standard index because pMTLD contains the Ncol site and can express LDAg without RNA editing (Hu et al., 1996).

#### In situ hybridization.

HeLa cells transfected with pCMVDag2 were grown directly on coverslips to 60% confluence, washed once in PBS and fixed for 20 min at room temperature in 4% formaldehyde. After penetration with ice-cold acetone for 3 min, cells were dehydrated using increasing concentrations of ethanol and grown at 37°C.
hybridized overnight at 37 °C in hybridization buffer (10% dextran sulfate, 2 mM vanadyl-ribonucleoside complex, 50 μg salmon sperm DNA, 2× SSC, 50% formamide) with 20 ng HDV DNA probe labelled with digoxigenin or biotin. After hybridization, cells were washed twice at 45 °C for 30 min in 2× SSC, 50% formamide and once in 0.1× SSC, 0.1% Tween-20. Digoxigenin-labelled probes were then detected with mouse anti-digoxigenin primary antibody and goat anti-mouse secondary antibody conjugated to rhodamine. Biotin-labelled probes were detected directly with avidin conjugated to rhodamine. For the fluorescence detection, cells were visualized with the Olympus B-Max 60 fluorescent microscope or the Leitz confocal microscope and photographed.

RESULTS

Various forms of SDAg fused to GFP display different nuclear distribution patterns and form complexes of different densities

The functional domains of HDAs, such as coiled-coil, nuclear localization and RNA binding domains, have been well characterized (Chen et al., 1993; Chang et al., 1992; for a review see Lai, 1995), as summarized in Fig. 1(A). To test whether different functional domains of SDAg affect localization in transfected cells, we constructed three plasmids expressing GFP fusion proteins. The plasmids contained the full-length wild-type or C-terminally truncated mutant (deletions of aa 164–195 or 89–195) of SDAg fused to the N terminus of GFP and were designated pSD-GFP, pD(1–163)-GFP and pD(1–88)-GFP, respectively (Fig. 1B). These plasmids were compared with three previously created plasmids that express GFP fusion proteins containing various lengths or mutations of LDAg (Fig. 1B).

Fluorescent microscopy showed a unique GFP distribution pattern for each fusion protein in HeLa cells both transiently and permanently expressing the transfected plasmids (Fig. 2A). This unique pattern was also seen in the human hepatoma cell line, HuH-7 (data not shown). The SD-GFP protein was present in the nucleolus of HeLa

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**Fig. 1.** Diagrammatic representation of the plasmids used in this study. (A) Functional domains have been identified in the SDAg and LDAg, including the coiled-coil domain (aa 12–60), nuclear localization domain (NLS, aa 31–52 and 68–88) and RNA binding domain (aa 79–163). The numbers indicate the positions of amino acids. (B) SDAg and its derivatives were fused to the N terminus of GFP, while LDAg and its derivatives were fused to the C terminus of GFP. Amino acid positions are indicated. pSD-GFP, pD(1–163)-GFP and pD(1–88)-GFP were constructed in this study, while pGFP-LD, pGFP-LDM and pGFP-LD(31–214) have been described previously (Shih & Lo, 2001).
cells as well as in the nuclear speckles (Fig. 2A, right panel). In contrast, the D(1–163)-GFP appeared in the nucleolus only (Fig. 2A, middle panel), while D(1–88)-GFP was restricted to the nuclear speckles, ranging from 2 to 16 dots per cell (Fig. 2A, left panel).

To test whether the different distribution patterns of the GFP fusion proteins resulted from their association with different cellular factors (due to the different domains and/or different conformations), sucrose density fractionation was performed. Results showed that SD-GFP with an expected molecular mass of 51 kDa was widely distributed, being found in fractions 9–18 with densities from 1·44 to 1·80 g ml$^{-1}$ (Fig. 2B, bottom panel) and was concentrated in fractions 10–13. The distribution of D(1–163)-GFP, which has a predicted molecular mass of around 45 kDa, was mostly concentrated in fractions 14–18 (Fig. 2B, middle panel); in contrast, the D(1–88)-GFP protein with a molecular mass of 37 kDa was found only in fractions 11–13 (Fig. 2B, top panel). These results indicated that GFP fusion proteins localized to the nucleolus above a density of 1·62 g ml$^{-1}$. Furthermore, the lower density of D(1–88)-GFP might result from its association with different nuclear factors, restricting it to speckles.

**HDV RNA editing is inhibited by the D(1–88)-GFP fusion protein**

To examine possible correlation between the specific localization of GFP fusion proteins and their biological activities,
we transfected pSVL-d2g into HeLa-SD-GFP, HeLa-D(1–163)-GFP or HeLa-D(1–88)-GFP cells. Because pSVL-d2g expresses the replication-competent 1.7 kb HDV cDNA dimer from the SV40 late promoter, HDV replication could be assessed. Similar experiments were performed in cells expressing GFP-LD, GFP-LDM and GFP-LD(31–214). At 4 days post-transfection, cells were harvested and lysed for analysis of HDV RNA and HDAg. Northern blot results showed that HDV RNA replication was completely inhibited in HeLa-SD-GFP, HeLa-D(1–163)-GFP, HeLa-LD, HDV-GFP-LD and HeLa-GFP-LDM cells, but not in cells expressing D(1–88)-GFP or GFP-LD(31–214) (data not shown). To determine whether HDV replication was related to the presence of GFP fusion proteins, Western blots were performed, (Fig. 3A). The gel migration patterns of GFP-LD, SD-GFP and D(1–88)-GFP were as expected. The inhibition of HDV RNA replication by SD-GFP appeared to result from the inhibition of authentic HDAg expression as detected by Western blot (Fig. 3B, lane 3). This result is similar to the previous finding that cells expressing GFP-LD and GFP-LDM allow neither HDAg expression nor HDV RNA replication (Shih & Lo, 2001). As expected, both SDAg and LDAg were detected in parental HeLa cells and in HeLa-GFP-LD(31–214) cells transfected with the pSVL-d2g plasmid, in which HDV RNA replication occurred (Fig. 3B, lanes 1 and 4). Although HDV RNA replication also occurred in the HeLa-D(1–88)-GFP cells transfected with pSVL-d2g, only SDAg was detected (Fig. 3B, lane 2). With normal levels of SDAg but no LDAg expression in the HeLa-D(1–88)-GFP cells transfected with pSVL-d2g, we hypothesized that D(1–88)-GFP might have the ability to inhibit HDV RNA editing. To confirm the hypothesis that the lack of LDAg resulted from inhibition of RNA editing in the HeLa-D(1–88)-GFP cells, we conducted two highly sensitive experiments, RT-PCR followed by a restriction enzyme digestion (Casey & Gerin, 1995), and an RNA editing reporter assay (Sato et al., 2001). If HDV RNA editing occurs, the cDNA corresponding to the LDAg mRNA will generate a novel Ncol site. The ratio of the two small fragments (134 and 509 bp resulting from Ncol digestion) to the full-length fragment (643 bp)
reflects the efficiency of RNA editing. Using pMTLD as a standard index, 12.8% HDV RNA editing was detected in the parental HeLa cells but only 7.5% in the HeLa-D(1–88)-GFP cells (Fig. 4, lane 3 and lane 4). The demonstration that HDV RNA editing was reduced by 42% in the HeLa-D(1–88)-GFP cells compared with parental HeLa cells supported our hypothesis.

In the RNA editing reporter assay, the pSS74 plasmid produces a reporter mRNA encoding either SDAg or LDAg depending on the occurrence of RNA editing. pSS74 was transiently transfected or not into HeLa or HeLa-D(1–88)-GFP cells with or without co-transfection of plasmids expressing ADAR1-HA or ADAR2-HA. The amount of SDAg and LDAg produced under these conditions was analysed by Western blot. As shown in Fig. 5(A), the amount of LDAg found in HeLa cells increased approximately fourfold when both ADAR1-HA and ADAR2-HA were expressed (compare lanes 3 and 4 with lane 2). In the absence of exogenous ADAR (Fig. 5B, compare lanes 2 and 5 with lanes 3 and 4), a minor degree of HDV RNA editing produced a small amount of LDAg (Fig. 5A, lanes 2 and 5), suggesting that HeLa cells constantly express HDV RNA editing enzymes. However, this enzyme activity was totally inhibited by D(1–88)-GFP, since no LDAg was observed in the HeLa-D(1–88)-GFP cells (Fig. 5A, compare lanes 6 and 9 with lanes 2 and 5). Overexpression of ADAR1-HA or ADAR2-HA (Fig. 5B, lanes 7 and 8) could elevate the production of LDAg in D(1–88)-GFP cells. However, the amount of LDAg produced was about 30–54% in HeLa-D(1–88)-GFP cells compared with that in HeLa cells expressing ADAR1-HA or ADAR2-HA (Fig. 5A, compare lanes 7 and 8 with lanes 3 and 4). The results of both LDAg production (Fig. 5A) and RNA editing (Fig. 4) are consistent with the Western blot results shown in Fig. 3 and thus support the hypothesis that D(1–88)-GFP inhibits ADAR activity and HDV RNA editing.

The nuclear distribution pattern of D(1–88)-GFP is similar to HDV RNA, SC-35 and PML, but not ADAR-HAs

To understand whether D(1–88)-GFP inhibits the HDV RNA editing through a direct interaction with ADAR, we transiently transfected ADAR-HA expression plasmids into HeLa-D(1–88)-GFP cells. At 4 days post-transfection, cells were fixed and immunostained with the anti-HA antibody to detect ADAR1-HA or ADAR2-HA. The distribution of ADAR was observed and recorded with a fluorescent microscope. As shown in Fig. 6, ADAR1-HA and ADAR2-HA mainly accumulated in the nucleolus and only a minor population was distributed in speckles (Fig. 6B and F). Two different fluorescent pictures containing the same field of cells were merged and showed no co-localization (yellow) of D(1–88)-GFP with ADAR1-HA or ADAR2-HA (Fig. 6C and G). These results indicated that D(1–88)-GFP is unlikely to be associated with ADARs or was undetectable by the method of fluorescent microscopy.

To determine whether D(1–88)-GFP co-localized with known nuclear factors that co-localize with HDAg (Bichko & Taylor, 1996; Bell et al., 2000), the HeLa-D(1–88)-GFP
Fig. 6. Co-localization of D(1–88)-GFP and ADARs. D(1–88)-GFP cells were transfected with plasmids producing ADAR1-HA (right column) or ADAR2-HA (left column). The cellular location of ADAR was detected using anti-HA primary antibody and rhodamine-conjugated secondary antibody. D(1–88)-GFP (green) was visualized using the FITC channel and appeared in speckles (A, E). ADAR (red) was visualized using the rhodamine channel and appeared in the nucleolus (B, F). The same field of cells was merged to show no co-localization between D(1–88)-GFP and ADAR (C and G). The nucleus of the cells was stained with DNA dye Hoechst 33258 (D, H). Bar, 10 μm.
Fig. 7. Co-localization of D(1–88)-GFP with PML (premyelocytic leukaemia antigen) and SC-35 (a splicing factor). D(1–88)-GFP cells were stained with anti-PML primary antibody (left column) or anti-SC-35 primary antibody (right column) followed by rhodamine-conjugated secondary antibody. D(1–88)-GFP (green) was visualized using the FITC channel (A, D) while PML and SC-35 (red) were visualized using the rhodamine channel (B and E, respectively). Two pictures of the same cell were merged to show the co-localization of D(1–88)-GFP with PML (C) or D(1–88)-GFP with SC-35 (F). Arrows indicate the co-localization of nuclear proteins and D(1–88)-GFP (yellow). Bar, 10 μm.
cells were stained with anti-SC-35 or anti-PML. The merged pictures from FITC and rhodamine fluorescence channels showed that 90% of D(1–88)-GFP co-localized with SC-35 splicing factors (Fig. 7F) and 20% of D(1–88)-GFP co-localized with or was adjacent to PML speckles (Fig. 7C).

Since ADAR and D(1–88)-GFP did not co-localize, we wondered where the HDV antigenome, the RNA substrate of ADAR, was located. We transfected pCMVDag2 alone or with either ADAR1-HA or ADAR2-HA expression constructs into HeLa-D(1–88)-GFP cells. At 4 days post-transfection, cells were harvested and in situ RNA hybridization was performed with HDV-specific probes. Meanwhile, some cells were immunostained with anti-HDAg or anti-HA antibodies for the detection of HDAs or ADAR-HAs. Since D(1–88)-GFP is always present in speckles, the positive nucleolus signal (Fig. 8A) stained by anti-HDAg antibody was presumed to be due to the expression of SDAg, as found in Fig. 3B (lane 2). In the same cells, HDV RNA was detected by digoxigenin-labelled HDV probe and was found mostly in speckles (Fig. 8B). When the two pictures were merged, co-localization of HDAs and HDV RNA (yellow) was clearly seen in speckles but not in the nucleolus (Fig. 8C). Without the anti-HDAg antibody, HDV RNA and D(1–88)-GFP were consistently co-localized in the speckles (Fig. 8G). In contrast, no co-localization between HDV RNA and ADAR-HAs was observed (data not shown).

**DISCUSSION**

The effect of RNA editing on the coding potential of mRNA, tRNA and rRNA has been widely demonstrated in organisms from unicellular protozoa to humans (Gott & Emeson, 2000). RNA editing in virally encoded RNAs producing a variety of proteins has been found in Sendai virus, HIV and HDV (Hausmann *et al.*, 1999; Bourara *et al.*, 2000; Casey & Gerin, 1995). Among these viruses, RNA editing in HDV appears to be more strictly regulated in its life cycle since the presence of LDAg inhibits HDV replication, and genomes copied from edited antigenome are non-replicable. In recent years, host enzymes participating in and the mechanism regulating HDV RNA editing have been explored (Cheng *et al.*, 2003; Casey, 2002; Hsu *et al.*, 2002; Wong & Lazinski, 2002). However, the temporal and spatial control of HDV RNA editing remains largely unclear. In this study, we constructed a GFP fusion protein, D(1–88)-GFP, which inhibits LDAg production in an HDV RNA replication system (Fig. 3, lane 2). This protein has potential in revealing the mechanisms that regulate HDV RNA editing.

Previously, we showed that a GFP fusion of LDAg allowed study of LDAg movement inside the nucleus (Shih & Lo, 2001). In this study, we further demonstrated the usefulness of GFP fusion proteins in localization, as well as in inhibiting HDV RNA replication. Unlike the authentic SDAg, which can promote HDV replication, a full-length SDAg fused to GFP, SD-GFP, suppressed both SDAg and LDAg expression and resulted in no HDV replication (Fig. 3, lane 3). This was possible through an interaction between SDAg and SD-GFP to antagonize the transactivation ability of SDAg for replication, similar to a dominant-negative effect (Chao *et al.*, 1990; Dridi *et al.*, 2003). Another possibility is that SD-GFP occupied the co-factors required for SDAg function in the nucleolus. This supposition was supported by the finding that three GFP fusion proteins that retain the coiled-coil domain, D(1–163)-GFP, GFP-LD and GFP-LDM, localized to the nucleolus and inhibited HDV replication (Fig. 2A and Fig. 3; Fig. 1 and Fig. 5 in Shih & Lo, 2001). In contrast, GFP-LD(31–214), which lacks the coiled-coil domain, was dispersed in the nucleoplasm and could not suppress HDAg production or HDV replication (Fig. 3, lane 4). The observation that D(1–88)-GFP localizes in speckles suggests that D(1–88)-GFP is unlikely to form a complex with SDAg in the nucleolus, thus allowing HDV RNA replication to occur, although it retains the coiled-coil domain.

Nevertheless, the location and function of D(1–88)-GFP allow us to speculate about the site of HDV RNA editing. Based on the observation that ADAR is found in the nucleolus (Fig. 6), one could hypothesize that HDV RNA editing takes place in the nucleolus. If this is true, HDV RNA editing requires other nuclear factors in addition to ADAR and these factors may be trapped by D(1–88)-GFP in the speckles. Alternatively, HDV RNA editing could take place in D(1–88)-GFP-occupied speckles, but ADAR is not able to contact its substrate. The evidence that HDV RNA and D(1–88)-GFP co-localize in speckles (Fig. 8) supports this hypothesis. Although we favour the second hypothesis, one could still argue against the hypothesis that ADAR is located solely in the nucleolus. To correlate with the fact that the small amount of HDV RNA is edited, one might suggest that an undetectable amount of ADAR could shuttle between speckles and the nucleolus, allowing HDV RNA editing to occur. Using the photobleaching technique, a recent study has demonstrated that ADAR2 can shuttle between the nucleolus and nucleoplasm and, furthermore, has suggested that the nucleolus is the site of RNA editing (Sansam *et al.*, 2003). Although their demonstration supports our hypothesis that SC-35 is the site of HDV RNA editing, this hypothesis requires further study. Compared with the previous finding that HDV RNA editing takes place in the nucleus (Wong & Lazinski, 2002), we have narrowed the possible locations to either the nucleolus or speckles.

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Fig. 8. Co-localization of HDV RNA and HDAg. D(1–88)-GFP cells were transfected with pCMVDag2, which provides a replication-competent HDV RNA. The location of HDV RNA was then detected by in situ hybridization (B, F). Cells were either stained with (left column) or without (right column) anti-HDAg antibody. The presence of HDAg or D(1–88)-GFP (green) was detected using the FITC channel (A, E) and HDV RNA (red) was detected using the rhodamine channel (B, F). The same field of cells was merged to show co-localization between HDAg and HDV RNA (C) or D(1–88)-GFP and HDV RNA (G) (yellow). The nucleus of the cells was stained with DNA dye Hoechst 33258 (D, H). Bars, 10 μm (A–D) and 5 μm (E–H).
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