Redistribution of the Delta Antigens in Cells Replicating the Genome of Hepatitis Delta Virus

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When the small form of the delta antigen (δ Ag-S) was expressed from a cDNA expression plasmid and subsequently detected by immunofluorescence, it was found localized to the nucleoli. However, if the cDNA was cotransfected with a cDNA expressing a mutated hepatitis delta virus (HDV) genome that could only replicate by using the δ Ag-S provided by the first plasmid, then most of the δ Ag-S was redistributed to the nucleoplasm, largely to specific discrete nucleoplasmic sites or speckles; this pattern was stable for at least 50 days after transfection. These speckles coincided with those detected with an antibody to SC35, an essential non-small nuclear ribonucleoprotein splicing factor. Others have shown that SC35 speckles correspond to active sites of DNA-directed transcription by RNA polymerase II and also of RNA processing. We also found, in contrast to the cotransfections with the mutant HDV and the δ Ag-S provided in *trans*, that cells transfected with wild-type HDV showed a variable pattern of staining. The SC35-like speckle pattern of accumulation of delta antigen (δ Ag) was maintained for only 6 days, after which the pattern began to change. By 18 days posttransfection, a variety of different δ Ag staining patterns were observed. This pattern of change occurs at a time when the large form of the delta antigen (δ Ag-L) appears and HDV RNA synthesis begins to shut down. Our studies therefore support the interpretation that HDV RNA and δ Ag-S accumulate at SC35 speckle sites in the nucleoplasm. We speculate that these may be the sites at which HDV RNA is transcribed by RNA polymerase II and/or sites of HDV RNA processing. Furthermore, when δ Ag-L, as well as other mutant δ Ag accumulate, the speckle association is disrupted, thereby stopping HDV RNA replication.

Hepatitis delta virus (HDV) is a subviral satellite of hepatitis B virus (38). Replication of the small circular RNA genome of HDV can be achieved in the absence of the helper virus (22). HDV genome replication takes place in the nuclei of infected cells (43), probably by the host RNA polymerase II (14, 31). This replication is absolutely dependent on the single protein encoded by HDV, the small delta antigen (δ Ag-S) (22). The 195-amino-acid (aa) δ Ag-S has the ability to specifically bind HDV RNA (27) and localizes to the nucleus (8). During HDV replication, posttranscriptional RNA editing leads to a change in the termination codon for δ Ag-S (28); this allows the synthesis of a delta antigen (δ Ag) with an additional 19 aa. This 214-aa large form of the delta antigen (δ Ag-L) is functionally different from δ Ag-S: it does not support genome replication, it is a potent dominant-negative inhibitor of replication (9), and it plays an essential role in the assembly and secretion of new HDV particles (7).

Many previous studies have used immunofluorescence and in situ hybridization to study the intracellular distribution of HDV proteins and RNA in infected cells and tissues (10, 16, 23-25, 30, 32, 43, 47). There is agreement that replication of the genomic RNA occurs in the nucleus and that the majority of δ Ag is in the nucleus. It also seems to be clear that in the absence of genome replication, δ Ag localizes to the nucleoli (24, 30, 32). However, when genome replication occurs, a variety of patterns of intranuclear distribution have been reported. In some studies the distribution is still nucleolar, while in others it is nucleoplasmic, or both, and in others, the results are varied for different cells within a single experiment (30, 34). The basis for the variation has not been determined.

In the current report, we have investigated how the intracellular staining pattern of δ Ag-S was influenced by viral RNA synthesis and the presence of δ Ag-L. As expected, δ Ag-S was localized to nucleoli when expressed in the absence of viral RNA synthesis or δ Ag-L. Viral RNA synthesis changed this pattern, causing δ Ag-S to localize to the nucleoplasm and specific nucleoplasmic speckles. Subsequent investigations considered how this localization was changed by the appearance of δ Ag-L and mutated forms of δ Ag.

MATERIALS AND METHODS

Plasmids. Plasmid pSVL(D3) was constructed by insertion of a trimer of unit-length cloned HDV cDNA (wild type) into the expression vector pSVL (Pharmacia) as described previously (22). pSVL(D2m) and pSVL(D1.2m), are mutants with a 2-nucleotide deletion adjacent to the unique *EcoRI* site, creating a frameshift near the N terminus of the δ Ag open reading frame; the corresponding mutant genomes can only replicate when δ Ag-S is provided in *trans* (22). The pSVL constructs for δ Ag-S and δ Ag-L, pDL444 and pDL445, respectively, were as described previously (25). A deletion mutant of δ Ag-S (pDL507) and several in δ Ag-L (pDL449, pDL503, and pDL508) were also as described previously (25). A final construct, pMB006, represents the insertion into pSVL of the complete δ Ag-L with the addition at the N terminus of six histidines and the factor Xa site from a pGEMEX2 vector (Qiagen).

Antibodies. Antibody binding to both δ Ag-S and δ Ag-L was raised in a rabbit by inoculation of δ Ag-S prepared in *Escherichia coli*. Human antiserum to δ Ag that was partially purified and then labeled with fluorescein was provided by J. Gerin. Rabbit antibody rab-LP3, provided by S. Lemon and J. Cullen, was raised against the 15-aa peptide DILFPADPPFSPQSC, representing the carboxy terminus of δ Ag-L (residues 197 to 211), and recognizes δ Ag-L but not δ Ag-S (45). Antibody raised in a rabbit and specific for a 24-aa synthetic peptide, CDLHRYSSQPIRPFLPRVVDPSSE, was provided by Y. Khudyakov. This peptide, designated peptide K, represents the carboxy terminus of ORF-K on the HDV genome (3, 21). Mouse monoclonal antibody to transcription factor SC35 was provided by X.-D. Fu (15).

Transfection. Huh7 human hepatoma cells (33) were seeded on glass coverslips placed in plastic 35-mm-diameter tissue culture wells (Costar). Lipofectamine (Bethesda Research Laboratories) was used to transfect subconfluent cells in the presence of Opti-MEM (Bethesda Research Laboratories), as recommended by the manufacturer (18), with the modifications described previously (48).

Immunofluorescence. Monolayer cultures on glass coverslips were fixed with

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methanol, permeabilized with acetone, and stained, as described previously (2, 35). Alternatively, cells were fixed with 1.75% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min on ice, washed, and permeabilized with 0.5% Triton X-100–2 mM MgCl₂ in PBS for 20 min at room temperature. Double staining (Fig. 2 and 3) of cells was achieved by using rabbit anti- δ Ag antibody as primary antibody, followed by the addition of 2 mg of 4',6-diamidino-2-phenylindole (DAPI [Sigma]) to stain the cellular DNA and then secondary antibody (goat anti-rabbit immunoglobulin G polyclonal antibody conjugated with rhodamine [Sigma]). Triple staining (Fig. 1) of cells was achieved by the addition of human polyclonal anti- δ Ag antibody, conjugated with fluorescein, to this mixture. To study colocalization of δ Ag or domain K with splicing factor SC35, mouse anti-SC35 antibody was used in combination with rabbit antibodies specific for either total δ Ag, δ Ag-L, or domain K. The mixture of secondary antibodies contained goat anti-rabbit immunoglobulin G polyclonal antibody conjugated to rhodamine (Sigma), goat anti-mouse immunoglobulin G + M polyclonal antibody conjugated to fluorescein (Sigma), and DAPI. After being mounted, the samples were viewed with a Zeiss Axiophot microscope with a $\times 40$ objective and specific filter blocks. Images were acquired by using a charge-coupled device camera (Photometrics model CH 250) and processed using Adobe Photoshop and Canvas software.

Some slides were also examined using a Bio-Rad MRC600 confocal microscope with a $\times 60$ oil immersion Plan Apo objective. The 488- and 568-nm lines of the krypton/argon laser were used to excite fluorescein isothiocyanate and rhodamine, respectively. Optical sections of 0.7 μ m were used. The dual-channel images were captured on a Gateway 2000, model 4DX2-66E, computer equipped with Bio-Rad Comos software. The final images were rendered on an SGI Indy R5000 computer using Voxelfview 2.5 software and finally processed using Adobe Photoshop and Canvas software.

RESULTS AND DISCUSSION

δ Ag-S alone. The expression in cells of δ Ag-S leads to accumulation in the nucleoli (Fig. 1A) (22, 32). This accumulation may be related to the RNA-binding activity of δ Ag combined with the fact that most of the nascent RNA in a cell is rRNA being synthesized in nucleoli. The biological significance, if any, of nucleolar accumulation, both for the cell and for HDV replication, is unclear; with one exception (34) nucleolar staining has not been detected in studies of infected liver tissues (10, 20, 37, 38). The nucleolar accumulation may also be to some extent an artifact produced by inappropriately high levels of expression of δ Ag. For example, overexpression of the retroviral Tat and Rev proteins (29, 42) and the cellular nuclear matrix protein NMP-1 (which corresponds to the RNA polymerase II transcription factor YY1) (17) leads to their accumulation in nucleoli. It is worth noting that δ Ag, Rev, and Tat, all have similar arginine-rich RNA-binding domains (26).

δ Ag-S in the presence of a protein-deficient replicating genome. We examined whether this nucleolar localization of δ Ag-S would be changed by the presence of a replicating HDV genome. Cells were transfected with the δ Ag-S construct along with pSVL(D2m), a mutant form of the HDV genome. This mutant, because of a particular 2-nucleotide deletion, cannot make any viral protein; nevertheless, in the presence of δ Ag-S provided by a second plasmid, it is able to replicate (22). In cotransfected cells, δ Ag-S was no longer detected in the nucleoli by 6 days posttransfection (Fig. 1B). The majority of δ Ag-S was found in the nucleoplasm, primarily at discrete sites. This new distribution was surprisingly stable. The intensity of this staining ultimately decreased with time, and after 60 days we were unable to detect positive cells (4). Nevertheless, even after 50 days, the speckled pattern of nucleoplasmic staining was qualitatively the same (4). In another experiment, the mutant HDV genome was introduced into cells not as cDNA but as RNA transcribed *in vitro*, and identical results were obtained (4).

For each cotransfection experiment described here, replication of the mutant HDV genome was confirmed by Northern (RNA) analyses using probes specific for genomic and antigenomic strands of HDV RNA, as described elsewhere (14). The

major species of genomic and antigenomic RNA detected were of unit length and circular (4).

By means of double-immunofluorescent labeling we studied not only the distribution of δ Ag-S in the nucleoplasm but also the non-small nuclear ribonucleoprotein (non-snRNP) spliceosomal factor SC35 (Fig. 1F). We observed a very similar nucleoplasmic speckle pattern. By image superimposition, we found the colocalization was precise (Fig. 1J). This finding was in dramatic contrast to what we found in cells transfected with only δ Ag-S, for which there was no detectable alignment between δ Ag-S and SC35 (Fig. 1I).

A possible problem with the alignment between the δ Ag and SC35 signals seen in Fig. 1J is that standard fluorescence microscopy cannot distinguish between direct colocalization and a close association. To address this we examined the same sample, using laser confocal microscopy, at a higher magnification. An optical section of 0.7 μ m is shown in Fig. 1Q, and it is now clearer that the two signals actually colocalize.

SC35 was first studied by Fu and Maniatis, who made monoclonal antibodies to purified mammalian spliceosomes (15). They obtained an antibody to this 35-kDa protein which proved to be an essential non-snRNP spliceosomal factor (46); this antibody stains the nucleoplasm and especially 20 to 50 nucleoplasmic speckles. Many studies have attempted to address both the composition of these speckles and also their possible function(s); it would seem from both electron and light microscopy studies, that these speckles contain additional non-snRNP splicing factors, snRNPs, and are also associated with the nuclear matrix (reviewed in reference 41). By *in situ* hybridization it would seem that the SC35 speckles are sites of active transcription from DNA by RNA polymerase II (19, 49) as well as sites of RNA processing by splicing and polyadenylation (50).

If SC35 speckles are indeed sites of active DNA-directed transcription by polymerase II and if HDV genome replication is achieved by redirecting polymerase II (14, 31), then it is tempting to interpret our observed replication-dependent accumulation of δ Ag-S in SC35 speckles according to the following model. HDV genome replication is initiated by the migration of HDV RNA, as a ribonucleoprotein complex with δ Ag-S, to these nucleoplasmic domains where transcription occurs via RNA polymerase II, after which the products are processed by cleavage and ligation (to produce unit-length circular RNAs) and ultimately accumulate at this same location. Presumably, these processes of HDV RNA transcription, processing, and accumulation will depend on the concurrent recruitment of δ Ag-S newly translated in the cytoplasm.

A qualification of this model is that although both δ Ag-S and SC35 were prominent in nucleoplasmic speckles, there was also for both some nucleoplasmic staining that was diffuse. The next step, to test this model further, will be to couple immunofluorescence microscopy with high-resolution *in situ* hybridization.

It is important to point out that neither δ Ag-S expression alone nor HDV genome replication leads to any detectable rearrangement of the SC35 speckles (Fig. 1E through H) (4). This finding is in contrast to what happens during infection with certain animal viruses, such as herpesviruses and adenoviruses, in which genome replication and expression lead to a disturbance of the SC35 speckles (5, 6, 36, 39).

δ Ag-S provided by a replication-competent genome. In the above experiments, mutant HDV genome replication was supported by a plasmid encoding δ Ag-S. When we changed our experimental system to one in which δ Ag-S was not provided by a separate plasmid but by a wild-type HDV genome, we obtained a similar intracellular staining pattern with the δ Ag

FIG. 1. Immunofluorescence of cells transfected with HDV cDNAs and examined by standard (A through P) or confocal microscopy (Q through T). (A through L) Four fields of cells stained three ways: for δ Ag (A to D), SC35 (E to H), and superpositions for δ Ag, SC35, and DAPI (I to L). Transfections were as follows: δ Ag-S, day 6 (A, E, and I); δ Ag-S plus pSVL(D2m), day 6 (B, F, and J); pSVL(D3), day 6 (C, G, and K); pSVL(D3), day 18 (D, H, and L). (M through P) Separate fields of cells, all at day 9 after transfection with pSVL(D3). DAPI and staining for the following different combinations of proteins: SC35 plus total δ Ag (M); SC35 plus δ Ag-L (N); SC35 plus domain K (O); total δ Ag plus δ Ag-L (P). (Q through T) Four of the above-described transfections and stainings (except that DAPI was omitted) by confocal microscopy at higher magnification: Q=J, R=K, S=N, and T=L. Throughout the figure DAPI is shown as blue, SC35 as green, δ Ag as red (with one exception; in panel T, total δ Ag are green). The bars in panels P and T indicate the scales for panels A through P and Q through T, respectively.

FIG. 2. Immunofluorescence of cells at 18 days after transfection with pSVL(D3). At this time, certain groups of cells showed quite distinct patterns of staining for δ Ag (B, D, F, and H). These same groups were also stained by DAPI (A, C, E, and G).

associated with SC35 speckles for at least 6 days after transfection, a time before the peak of HDV genome accumulation (Fig. 1C). Again, the SC35 distribution was not altered (Fig. 1G) and the colocalization of δ Ag and SC35 was still precise (Fig. 1K). This colocalization was again made clearer by confocal microscopy (Fig. 1R). However, after 18 days, though the SC35 pattern remained unchanged (Fig. 1H), the δ Ag staining had altered (Fig. 1D). Even though δ Ag was still largely nucleoplasmic and in a speckled pattern, the pattern was no longer the same as that of SC35, i.e., the two patterns were no longer superimposed (Fig. 1L). This lack of alignment was more obvious when the sample was viewed by confocal microscopy (Fig. 1T).

From a study of cells at intermediate times after transfection with wild-type HDV, we were able to detect early events in the time-dependent progression from day 6, when colocalization with the SC35 speckles was precise, to day 18, when there was no colocalization at all. After 9 days, when the peak of accumulation of HDV genomes had been reached (48), most of the δ Ag-positive speckles failed to colocalize with SC35 speckles (Fig. 1M). In some cases, it was as if the number of δ Ag speckles was reduced and their location was not coincident but rather adjacent to a subset of SC35 speckles.

From previous studies of HDV genome replication, it is known that posttranscriptional RNA editing acts on HDV to allow the synthesis of δ Ag-L, which is a potent dominant-negative inhibitor of genome replication (9, 28). Using an antibody specific for the unique C-terminal extension of the large form (44), we found that speckles positive for total δ Ag were also positive for δ Ag-L (Fig. 1N). Here it can again be seen that most of the new speckles were not coincident but rather appear to be adjacent to the SC35 speckles. This association was confirmed and made much clearer when the sample was examined by confocal microscopy (Fig. 1S). Interestingly, in some studies of gene transcription by in situ hybridization, the RNA and/or the DNA template has often been reported to be eccentrically located relative to the SC35 signal (49).

We next used double immunostaining to determine if speck-

les detected with antibody to total δ Ag corresponded to those detected with antibody specific to δ Ag-L (Fig. 1P). The correspondence was precise. Since one of the antibodies detected both forms of δ Ag, further experiments were carried out using consecutive staining. Specifically, cells were first stained for δ Ag-L and photographically documented. Then, the same coverslips were stained for total δ Ag and the same fields were again documented. The observation was that few if any additional positive cells appeared with the second staining (4). Thus, at day 9, virtually all the cells that contained δ Ag-S also contained δ Ag-L in the same nuclear speckles.

Figure 1D shows one example of a δ Ag staining pattern at day 18 that is quite different from the SC35 pattern. Actually, after 18 days, we observed a series of different altered staining patterns for δ Ag. Four more examples are shown in Fig. 2, using a $2\times$ lower magnification. Each field of cells is shown both as stained with DAPI (Fig. 2A, C, E, and G) and with antibodies to δ Ag (Fig. 2B, D, F, and H). Groups of δ Ag-positive cells can be seen (Fig. 2B, D, F, and H). Our interpretation is that each cell group is probably the progeny of a single transfected cell. The staining pattern was uniform within each group. All groups had nucleoplasmic staining with (non-SC35) speckles; however, there were additional and variable characteristics between groups. For example, one group had just nucleoplasmic staining (Fig. 2B). One group had more staining in the cytoplasm than in the nucleus (Fig. 2D). Other groups also had staining over the Golgi apparatus (Fig. 2D, F, and H). This apparent association of δ Ag with the Golgi apparatus was confirmed by double labeling with anti- δ Ag antibody and one of two Golgi markers: rabbit polyclonal antibody to β -COP (1, 40) or lectin from *Lens culinaris* coupled to fluorescein (both provided by V. Hsu) (4). Furthermore, the δ Ag was probably outside rather than inside the Golgi system because prior treatment of cells with brefeldin A caused redistribution of the β -COP signal (11, 12) but not that of δ Ag (4).

Our interpretation of the varied patterns of δ Ag staining is that each group of cells contains its own spectrum of wild-type and mutated HDV genomes, with corresponding mutated pro-

FIG. 3. Immunofluorescence of cells at 6 days after transfection with cDNAs that express various forms of δ Ag. (A and B) δ Ag-L (pDL445); (C and D) δ Ag-L with a deletion of aa 19 to 31 (pDL449); (E and F) δ Ag-L with a deletion of aa 69 to 146 (pDL503); (G and H) δ Ag-L with an N-terminal addition (pMB006); (I and J) δ Ag-S with a deletion of aa 69 to 74 (pDL507); (K and L) δ Ag-L with the same deletion (pDL508). Each group of cells was stained for both δ Ag (B, D, F, H, J, and L) and DAPI (A, C, E, G, I, and K).

teins, and that each group represents a unique quasispecies population (13).

δ Ag mutants. The interpretation of studies with wild-type HDV, that during genome replication altered forms of δ Ag arose and displayed characteristic intracellular distributions (Fig. 2), was tested indirectly. We transfected cells with a series of six variants of δ Ag, expressed from cDNA constructs, in the absence of genome replication. Each field of cells is shown both as stained with DAPI (Fig. 3A, C, E, G, I, and K) and with antibodies to the δ Ag (Fig. 3B, D, F, H, J, and L). As in Fig. 2, we observed groups of transfected cells with a shared pattern of δ Ag staining. The δ Ag-L was localized to the nucleoli (Fig. 3B), just like δ Ag-S (Fig. 1A). The other five constructs expressed mutant forms of either δ Ag-S or δ Ag-L and gave quite different staining patterns.

With a construct in which part of the coiled-coil domain was deleted from δ Ag-L, the distribution was quite different from that of either the natural δ Ag-S or δ Ag-L; the signal was excluded from the nucleoli and generally diffusely nucleoplasmic (Fig. 3D). For another construct, both the nuclear localization signal and the adjacent RNA-binding domain from δ Ag-L were deleted; now the staining was nucleoplasmic but with discrete speckles (Fig. 3F), with a partial correspondence

with cellular SC35 speckles (4). The deletion of these particular domains may not be the basis for this particular altered distribution since with another mutant, in which 13 aa, including six histidines, were added to the N terminus of δ Ag-L, we obtained virtually the same nuclear distribution (Fig. 3H).

In contrast to the above findings, two other mutants gave accumulation in the cytoplasm. For δ Ag-S with a deleted nuclear localization signal, the staining was mainly cytoplasmic (Fig. 3J). δ Ag-L bearing the same deletion also gave cytoplasmic staining but with a prominent signal over the Golgi apparatus (Fig. 3L).

Consistent with our original hypothesis, the intracellular distribution of some of these mutant proteins did mimic what was seen during genome replication, for example, accumulation in the cytoplasm and over the Golgi apparatus. However, during genome replication there probably is not one but multiple forms of δ Ag being expressed in a single cell. For instance, we already know that both δ Ag-S and δ Ag-L are being expressed in the same cell (Fig. 1P). Maybe we detected variants of both δ Ag-S and δ Ag-L.

Mutant forms of δ Ag probably arise not only during genome replication in cultured cells but also in the livers of infected animals. Recently we described one such novel form; it in-

volved the expression of an alternative reading frame, ORF-K, which partially overlaps the C-terminal part of the δ Ag open reading frame (3). We found that in cells which expressed this ORF-K there was colocalization with δ Ag-L (4); these two proteins were not colocalized with SC35 speckles but were adjacent to a subset of such speckles (Fig. 1N, O, and S).

Conclusions. Our studies support the interpretation that SC35 speckle sites in the nucleoplasm are sites of HDV RNA accumulation and possibly also sites of synthesis and/or processing. Furthermore, this linkage is clearly and stably maintained if we use genomes that depend on δ Ag-S provided by a separate cDNA construct. However, in a more biologically relevant situation, in which δ Ag-S is produced via the replicating HDV genome, the δ Ag staining pattern is only like this at early stages up to 6 days. By day 9, after genome accumulation has reached its peak value (48), we detected δ Ag patterns with a reduced level of relationship to SC35 speckles. However by day 18, the patterns were completely different from SC35 and moreover, we observed not one but several novel staining patterns.

At the intermediate time, we detected the appearance of δ Ag-L, which is an inhibitor of genome replication (9). Although this protein was in the same altered speckled pattern as δ Ag-S (Fig. 1P), we do not yet know whether it was the appearance of δ Ag-L per se that caused the alteration. We do know that synthesis of δ Ag-L is not the only way to inhibit genome replication; virtually all mutants of δ Ag-S also act as dominant-negative inhibitors (25). Furthermore, if the SC35 speckles are in fact sites of synthesis, processing, and accumulation of HDV RNA, then a further speculation would be that this redistribution away from the SC35 pattern, represents the inhibition of genome replication.

We have interpreted the additional changes in the patterns of δ Ag staining, which are detected at later times after transfection, as a consequence of the appearance of variant HDV genomes and corresponding variant forms of δ Ag. These patterns persist up to 18 days after transfection, during at least several rounds of cell division. According to our interpretation, this putative level of residual genome replication must be relatively low since we can no longer detect in these cells any colocalization of δ Ag with SC35 speckles (Fig. 1P).

All the distributions and redistributions of δ Ag that we have detected took place in the absence of the hepadnavirus surface antigens, and therefore, our studies do not address the later events of virus particle assembly and release.

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