The Nucleolar Phosphoprotein B23 Interacts with Hepatitis Delta Antigens and Modulates the Hepatitis Delta Virus RNA Replication*

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Hepatitis delta virus (HDV) encodes two isoforms of delta antigens (HDAgs). The small form of HDAg is required for HDV RNA replication, while the large form of HDAg inhibits the viral replication and is required for virion assembly. In this study, we found that the expression of B23, a nucleolar phosphoprotein involved in disparate functions including nuclear transport, cellular proliferation, and ribosome biogenesis, is up-regulated by these two HDAgs. Using in vivo and in vitro experimental approaches, we have demonstrated that both isoforms of HDAg can interact with B23 and their interaction domains were identified as the NH2-terminal fragment of each molecule encompassing the nuclear localization signal but not the coiled-coil region of HDAg. Sucrose gradient centrifugation analysis indicated that the majority of small HDAg, but a lesser amount of the large HDAg, co-sedimented with B23 and nucleolin in the large nuclear complex. Transient transfection experiments also indicated that introducing exogenous full-length B23, but not a mutated B23 defective in HDAG binding, enhanced HDV RNA replication. All together, our results reveal that HDAg has two distinct effects on nucleolar B23, up-regulation of its gene expression and the complex formation, which in turn regulates HDV RNA replication. Therefore, this work demonstrates the important role of nucleolar protein in regulating the HDV RNA replication through the complex formation with the key positive regulator being small HDAg.

Hepatitis delta virus (HDV) is a negative-strand RNA virus and is also a subviral satellite of hepatitis B virus (HBV)1 (1).

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‡ The abbreviations used are: HDV, hepatitis delta virus; GST, glutathione S-transferase; HDAg, hepatitis delta antigen; HBV, hepatitis B virus; IP, immunoprecipitation; NLS, nuclear localization signal; NBS, nucleolin-binding site; kb, kilobase(s); PAGE, polyacrylamide gel electrophoresis; MT, metallothionein; HIV, human immunodeficiency virus.

Patients co-infected with HDV and HBV are at high risk for developing fulminant hepatitis, liver cirrhosis, and hepatocellular carcinoma (2). This virus has a single-stranded circular RNA genome of 1.7 kb which is folded into an unbranched rod-like structure (for review, see Ref. 3 and the references therein). Apart from its RNA genome, the HDV particle also contains hepatitis delta antigen (HDAg), the only known protein encoded by HDV, and an envelope provided by the surface antigen (HBsAg) of HBV. However, HDV RNA replication is independent of its HBV helper and is dependent on the presence of HDAg (4).

The HDAg exists as two protein species of 27 (214 amino acid residues) and 24 (195 amino acid residues) kDa, known as large and small HDAg, respectively (5). These two isoforms are translated from the same initiation codon of a single open reading frame, but the large HDAg contains an additional 19 amino acids at its carboxyl terminus, which is the result of RNA editing of the termination codon of small HDAg during the viral replication (6). The functional domains of HDAg have been well characterized (for review, see Refs. 3 and references therein). These include an NH2-terminal coiled-coil domain (amino acid residues 31–52) for HDAG oligomerization (7–10), two independent nuclear localization signals (NLSs) (amino acid residues 35–44 and 68–88) for nuclear transport of HDAG and HDV RNA (11–13), and a central HDV RNA-binding domain (amino acid residues 93–146) (14–16). In addition, the COOH-terminal portion of large HDAg contains a prenylation site, which is important for viral particle assembly with HBsAg (17, 18). Although containing similar sequences and functional domains, these two isoforms of HDAgs have different roles during HDV life cycle. Small HDAg functions to facilitate HDV RNA replication in a manner that is not yet understood (4), while large HDAg acts as a potent trans-dominant inhibitor for HDV replication (19, 20) and interacts with HBsAg during HDV viral particle assembly (18, 21, 22). Both HDAgs are phosphoprotein (23–26) and phosphorylation regulates HDV RNA replication but not viral assembly (25, 27). More recent studies suggested that HDAg has a RNA chaperone activity (28) and can interact with nucleolin for targeting to nucleolus (29). The trans-suppression ability of both HDAgs on RNA polymerase II-dependent transcription (30) and the observed trans-activation ability of the large HDAg on certain promoters (31), imply that HDAg has the ability to modulate host cellular gene expression.

The exact molecular mechanism for HDV RNA replication is still obscure. A double rolling-circle mechanism for HDV replication has been proposed whereby the host-encoded polymerase undergoes RNA-dependent RNA synthesis to produce a circular unit-length complement of the genomic RNA, termed the antigenome (for review, see Ref. 3 and references therein). The antigenomic RNA, which is also the coding strand for...
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HDAG, serves as a template for the synthesis of genomic RNA via the same rolling-circle mechanism. Since HDAG does not possess any RNA polymerase activity, the host-encoded RNA polymerase is required for HDV replication in a RNA-directed RNA synthesis manner. However, the identity of the responsible polymerase and the nature of the essential role of the small HDAG in the HDV replication remain unclear. Although host RNA polymerase II is suspected to be involved (32–34), a more recent study by Modahl et al. (35) suggested that this might not be the case. Notably, the finding that the HDAG-associated cellular factors, delta-interacting protein A and nucleolin, can modulate HDV RNA replication (29, 36), suggests that cellular factors other than RNA polymerase are involved in HDV replication.

In this study, we found that the mRNA level of the human nucleolar protein B23 gene is up-regulated in both HDV-replicating and HDAG-producing cell lines. B23 is a major nucleolar phosphoprotein that performs a plethora of activities. This protein-like nucleolin is present in abundance in both tumor cells and proliferating cells and constitutes a common signal for cell proliferation (37–39). It localizes in granular regions of nucleoli (40, 41), is associated with preribosomal particles (42, 43), and forms hexamers that may be important for the assembly of ribosomes (44, 45). B23 has the ability to shuttle between the nucleus and the cytoplasm (46), and binds to nuclear localization signal containing peptides (47), and thus serves as a shuttle protein in the nuclear import. For example, it forms a specific complex with the nucleolar protein, p120 (48), nucleolin (49), and several viral proteins such as Rex of human T-cell leukemia virus (50), the Rev (51) and Tat (52) proteins of human immunodeficiency virus (HIV). Its function is to facilitate their nuclear import. Interestingly, in this study we also found that the B23 protein can directly interact with the two isoforms of HDAG and the interaction domains of B23-HDAG reside within the fragment containing the nuclear localization signal of HDAG, suggesting that B23 may serve as a shuttle protein for transport of HDAG into the nucleus. Additionally, our results also indicated that the small HDAG is found in a complex of B23 and nucleolin with a size larger than 700 kDa, and the interaction of small HDAG with B23 regulates HDV RNA replication. Therefore, the association of the major nucleolar protein B23 and nucleolin with the small HDAG may represent an important mechanism for HDV RNA replication.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmid pSVL-d2g contains a head-to-tail tandem dimer of the full-length HDV cDNA (as 1.7-kb XbaI fragment) inserted at the XbaI site of the vector plasmid pSVL (Amer sham Pharmacia Biotech) (25). On transfection into mammalian cells, this plasmid can direct replication of the viral genome and expression of HDAG. Plasmids pGEM5L and pGEM5S contain a Scal-EcoRI fragment (1.1 kb) of HDV cDNA driven by the SP6 or the T7 phage promoter of pGEM-3Zf+ (53), which can allow the production of strand-specific probes by in vitro transcription and large or small HDAG by in vitro translation. Plasmids pGEX-3X-L and pGEX-3X-S, kindly provided by S. J. Lo (Yang-Ming University, Taiwan), contains the Scal-EcoRI fragment of the large or small HDAG open reading frame in-frame inserted into the plasmid pGEX-3X, which can direct the expression of either a GST-fused large HDAG (GST-DAG) or small HDAG (GST-dAG). Plasmid pCR3-FLAG-B23 is an expression construct of B23 in mammalian cells with an additional FLAG-tag at the NH2 terminus. This construct was generated by polymerase chain reaction using a NH2-terminal primer 5′-ACATGACACTAGCTACACACGAGGATGACGAACTTACGGGACGAGACGCCCATCGCTGACTGTTTCTTAATGGCTTGAGTTGAGCAG-3′ and a COOH-terminal polymerase chain reaction primer 5′-CCTCTCTTATTAGATTATATTTGAGCATTGATCTGACACCTAC-3′ was used. The COOH-terminal truncated variants of the in vitro translated B23 were produced by the TNT system (Promega) from the plasmid of pCR3-FLAG-B23 digested with the restriction enzymes EcoRI, BstNI, HhaI, or BglII, allowing generation of the NH2-terminal fragments of the 1.1-kb Scal-EcoRI fragment of the large or small HDAG cDNA sequence under human metallothionine (MT) promoter control. Plasmid pSVL-d contains a 1.1-kb Scal-EcoRI fragment of the small HDAG cDNA sequence under SV40 late promoter control. Plasmid pdAG-d17–56, a derivative of pGEM-3Zf+ (53), can direct the expression of in vitro translated small HDAG variant dAG-d17–56 with an in-frame deletion of residues 17–56 when prepared by the TNT system (Promega).

Cell Lines, Transfection, and Subcellular Fractionation—Human hepatocellular carcinoma cell lines HuH-7 and HepG2 were cultured as described in Yeh et al. (25). SVLDS-N1 cells (designated as N1) were HepG2 stable clones integrated with trimeric HDV cDNA and constitutively expressing both isoforms of the HDAGs and both HDV genomic and antigenomic RNA (54, 55). L10 and L10-N1 are the stable cell lines expressing the large HDAG in HepG2 cells, while S2 and S7 are the stable HepG2 cell lines expressing the small HDAG. These cell lines were established by transfected HepG2 cells (2–106 cells) with plasmid DNA of pMT-DAG or pMT-dAG (5 μg) together with pSV2-neo (0.5 μg) by electroporation. After 4 weeks selection with 0.8 mg/ml G418 (Sigma), cell colonies were picked up, cultured for another 2 weeks, and analyzed for the expression of HDAG by Western blotting. Detection of HDAG was performed with human anti-HDAG antisera as the primary antibody and horseradish peroxidase-conjugated goat anti-human antisera as the secondary antibody using the enhanced chemiluminescence detection method (ECL, Pierce). G418-resistant, HDAG-negative cell colonies (C4 and C5) were also picked up, expanded in G418-supplemented medium, and were used as control cell lines. For the transient transfection experiment, plasmid DNAs were transfected into HuH-7 cells by EffecteneTM transfection reagent (Qiagen). For preparation of total cell lysates and nuclear extracts, cells were harvested, lysed, and treated as described by You et al. (56, 57).

RNA Preparation, Northern Blotting, and mRNA Differential Display Method—Total RNAs from each cell line were extracted by using a TRIzol reagent (Invitrogen, San Diego) according to the instructions of the supplier. For Northern blot analysis, 20 μg of total cellular RNA samples was subjected to electrophoresis on a 6% formaldehyde, 1% agarose gel and then transferred into a nylon filter. The filter were pre-hybridized and then hybridized according to the standard method (58). For detection of genomic or antigenomic HDV RNA, the strand-specific HDV RNA probe was prepared by in vitro transcription of plasmid DNA using the SP6/T7 transcription kit (25). Differential display of mRNA was performed according to the instructions of the supplier using the RNAimage kit (GeneHunter Co.).

Immunofluorescence—Immunofluorescence was performed by a method modified from the published procedures (57, 59). The localization of HDAG and FLAG-tagged B23 protein in transfected cells was examined by fluorescence microscopy or confocal laser scanning microscopy (Leica TCS-NT). For immunofluorescence staining, the cells were fixed with acetone/methanol (1:1) (−20 °C) and probed with human anti-HDAG antisera or mouse monoclonal anti-FLAG M2 antibody (Kodak), followed by fluorescein isothiocyanate-conjugated rabbit anti-human IgG or rhodamine-conjugated rabbit anti-mouse IgG.

In Vitro Binding Analysis of HDAG and B23—The GST-HDAG fusion proteins expressed from pGEX-3X-L and pGEX-3X-S vectors were purified as described elsewhere (60, 61). The His-tagged HDAG fusion proteins expressed from various expression vectors (dAG-d17–195, NADag-d1–88, NMDag-d1–143, MdAg-d89–143, and Cdag-d89–195) (28) were affinity purified using a His-bound resin column according to the instructions of the supplier. For binding with the endogenous HDAG, HuH-7 nucleolar extracts (50 μg) were mixed with 2 μl of glutathione-Sepharose 4B beads prebound with GST or GST-HDAG (4 μg) or His resins prebound with His-tagged HDAG variants (4 μg) at 4 °C overnight under gentle agitation. The beads were washed four times with phosphate-buffered saline containing 0.3% Nonidet P-40. Proteins bound on beads were eluted using the sample buffer and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5% polyacrylamide gel), and processed for Western blotting.
HDV-producing N1 cells (N1), or HDAg-producing L7, L10, S2, and S7 cells (L7, L10, S2, and S7) was extracted, and 20 μg of these RNAs were analyzed by Northern blot using 32P-labeled B23 cDNA as a probe (see “Experimental Procedures”). The ethidium bromide-stained RNAs shown were used as a loading control. The results were normalized for the level of B23 expressed in HepG2 cells. Panel B, nuclear extracts (20 μg in lanes 1–6; 10 μg in lanes 7–9) of HepG2 (G2) and its derived cell lines (see “Experimental Procedures”) were analyzed by immunoblot using goat anti-B23 (Santa Cruz), mouse monoclonal anti-nucleolin antibody (97) (kindly provided by N.-H. Yeh), and human anti-HDAg antiserum for detection. The results were normalized to the expressed level of B23 in HepG2 cells. DAg, large HDAg; dAg, small HDAg.

**Fig. 1. Enhancement of cellular B23 expression in HDV and HDAg producing cell lines.** Panel A, total cellular RNA of HepG2 (G2), and HDV-producing N1 cells (N1), or HDAg-producing L7, L10, S2, and S7 cells (L7, L10, S2, and S7) was extracted, and 20 μg of these RNAs were analyzed by Northern blot using 32P-labeled B23 cDNA as a probe (see “Experimental Procedures”). The ethidium bromide-stained RNAs shown were used as a loading control. The results were normalized for the level of B23 expressed in HepG2 cells. Panel B, nuclear extracts (20 μg in lanes 1–6; 10 μg in lanes 7–9) of HepG2 (G2) and its derived cell lines (see “Experimental Procedures”) were analyzed by immunoblot using goat anti-B23 (Santa Cruz), mouse monoclonal anti-nucleolin antibody (97) (kindly provided by N.-H. Yeh), and human anti-HDAg antiserum for detection. The results were normalized to the expressed level of B23 in HepG2 cells. DAg, large HDAg; dAg, small HDAg.

**RESULTS**

The Expression of Nucleolar Phosphoprotein B23 Is Up-regulated in Both HDV- and HDAg-producing Cells—To search for the cellular target genes affected by HDV, we performed an mRNA differential display assay (see “Experimental Procedures”). The total RNAs from both HDV-producing N1 cells and its parental HepG2 cells were isolated and the differentially expressed RNAs were analyzed by the RNAimage kit (GeneHunter Co.). The identity of differentially expressed cDNA clones was determined by DNA sequencing and through sequence alignment, we found that one of the candidate genes was the human nucleolar phosphoprotein B23 (also known as nucleophosmin, NO38, or numatrin) (62–64). Northern blot analysis confirmed the up-regulation of the B23 transcript in the HDV-producing N1 cell line (Fig. 1A). To find out whether this enhancement of B23 expression resulted from HDAg, we also examined B23 expression in large and small HDAg-producing stable cell lines (L7 and L10 cell lines for large HDAG, S2, and S7 cell lines for small HDAG), parental HepG2 cells, and control cell lines which failed to show HDAg expression (C4 and C5) (see “Experimental Procedures”). Interestingly, although the expression level of the large HDAg is not comparable with that of the small HDAg in their respective producing cells as shown by Western blot analysis (Fig. 1B), the enhancement of B23 mRNA expression occurred to a similar extent as that found in the N1 cell line (about 3–4-fold increase) (Fig. 1A). As noted, there is about a 2-fold increase in the protein level of B23 in these HDV- or HDAg-producing cells with nuclear nucleolin as a loading control (Fig. 1B), further supporting the notion that B23 expression is up-regulated in both the HDV- and the HDAg-producing cell lines. However, the lack of apparent correlation of the expression level of two isoforms of HDAG and the extent of B23 expression is intriguing. Clearly, the observed up-regulation of B23 expression in these HDAG-positive cells is HDAG-specific but not due to clonal selection against G418, since both G418-resistant, HDAG-negative cell lines C4 and C5 did not have any effect (Fig. 1B, compare lanes 8 and 9 with lanes 2–6). Presumably, this may reflect the differential trans-activation ability of two isoforms of HDAG, a property noted previously (31).

**HDAg Co-localizes with the B23 Protein in the Nucleoli—** Previous studies have demonstrated the nuclear or nucleolar localization of HDAG (12, 23, 65, 66). Since B23 is also abundant in nucleolus, we investigated the possibility of co-localization of these two proteins. Consistent with previous findings, both HDAG and B23 were nucleolar proteins when expressed in HeLa cells by transiently transfected with the HDV replication plasmid pSVL-d2g or the plasmid FLAG-tagged B23 and detected by the indirect immunofluorescence staining using the anti-HDAg or the anti-FLAG antibody (Fig. 2A, panels a-f). Furthermore, when the expression plasmids of HDAG (pMT-
DAg or pMT-dAg) and FLAG-tagged B23 were co-transfected into HeLa cells, confocal microscopy analysis using the indirect immunofluorescence staining indicated that both forms of HD-Ags co-localized with the FLAG-B23 in nucleoli (Fig. 2B, panels j-o). Similar results were obtained in cells transiently co-transfected with the HDV-replication plasmid pSVL-d2g (panels g-i), pMT-DAg (panels j-l), pMT-dAg (panels m-o) as indicated. The distributions of B23 and HD-Ags were assessed by indirect immunofluorescence staining (see "Experimental Procedures"). For double immunofluorescence staining, cells were stained with human anti-HDAg antiserum and mouse anti-FLAG M2 monoclonal antibody, followed by fluorescein isothiocyanate-conjugated goat anti-human IgG or rhodamine-conjugated goat anti-mouse IgG. Panels c and f are the phase-contrast images of panels a, b, d, and e. Cell nuclei were also visualized by Hoechst 33258 staining. Preparations in panels a-f were examined by fluorescence microscopy, while in panels g-o the immunofluorescence patterns were recorded by confocal laser scanning microscopy. The left panels g, j, and m show the merged image of the co-localization of HD-Ag with FLAG-B23 in the nucleoli. Bars, 10 μm.

Fig. 2. Both FLAG-B23 and HD-Ag co-localize inside a cell. HeLa cells were transfected with pSV-d2g (panels a-c) or FLAG-tagged B23 construct pCR3-FLAG-B23 (panels d-f) or co-transfected pCR3-FLAG-B23 together with various forms of HDV construct including pSVL-d2g (panels g-i), pMT-DAg (panels j-l), pMT-dAg (panels m-o) as indicated. The distributions of B23 and HD-Ags were assessed by indirect immunofluorescence staining (see "Experimental Procedures"). For double immunofluorescence staining, cells were stained with human anti-HDAg antiserum and mouse anti-FLAG M2 monoclonal antibody, followed by fluorescein isothiocyanate-conjugated goat anti-human IgG or rhodamine-conjugated goat anti-mouse IgG. Panels c and f are the phase-contrast images of panels a, b, d, and e. Cell nuclei were also visualized by Hoechst 33258 staining. Preparations in panels a-f were examined by fluorescence microscopy, while in panels g-o the immunofluorescence patterns were recorded by confocal laser scanning microscopy. The left panels g, j, and m show the merged image of the co-localization of HD-Ag with FLAG-B23 in the nucleoli. Bars, 10 μm.

Interaction of HD-Ags and B23—Co-localization of B23 with HD-Ag suggests that HD-Ag may interact with B23. To examine this possibility, a protein binding assay of GST-HDAg fusion protein to in vitro translated 35S-labeled B23 was performed. As shown in Fig. 3, A and B, the in vitro translated B23 bound to both forms of GST-HDAg fusion protein (GST-DAg and GST-dAg) but not GST. We further tested the interaction of B23 and HD-Ag by co-immunoprecipitation of in vitro translated 35S-labeled B23 and HD-Ags (Fig. 4A). As shown in Fig. 4B, B23 together with both large and small HD-Ag could be co-immunoprecipitated by the anti-HDAg antisera (lanes 5–7). This co-precipitation was specific, since no B23 was precipitated in the absence of HD-Ag (Fig. 4B, lane 2). The interaction of HD-Ag with cellular B23 was also investigated using a GST-HDAg
pull-down analysis. As shown in Fig. 3C, when HeLa cell nuclear extracts were incubated with partially purified GST-HDAg fusion proteins, and the bound proteins were detected by anti-B23 antibody, results indicated that cellular B23 protein was pulldown by both isoforms of GST-HDAg fusion protein. An in vivo co-immunoprecipitation experiment using anti-HDAg antibody suggested that B23 in HDV-producing N1 cells as detected by immunoblot was co-precipitated by the small HDAg (Fig. 4, C and D, lanes 5 and 6). This co-immunoprecipitation experiment was specific, since no such co-precipitation was observed when using the anti-FLAG antibody for immunoprecipitation (Fig. 4, C and D, lanes 3 and 4). Taken together, based on the four different approaches, our results indicated that both isoforms of HDAg could interact with B23 both in vitro and in vivo.

Mapping the Interaction Domains of B23 and HDAg—
The interaction domain of B23 within HDAg was mapped using the His-tagged HDAg fragments harboring the full-length (dAg, 1–195 residues), the NH2 terminus (NdAg, 1–88 residues), the middle (MdAg, 89–143 residues), the NH2 terminus plus the middle (NmDag, 1–143 residues), and the COOH terminus (CdAg, 89–195 residues) of small HDAg (Fig. 5A). As shown in Fig. 5C, apart from the full-length protein, only the HDAg variants containing the NH2-terminal fragment, NdAg and NmDag, but not those harboring the COOH-terminal (CdAg) or middle fragment (MdAg), could bind to the cellular B23, suggesting that the interaction domain of B23 is within amino acid residues 1–88 of HDAg. Since this NH2-terminal fragment harbors a coiled-coil domain (residues 31–52) responsible for HDAg oligomerization (8–10) and two independent NLSs (residues 35–44 for NLS1 and residues 68–88 for NLS2) for targeting HDAg to the nucleus (11, 12), it was of interest to further define the interaction region within HDAg. As shown in Fig. 5E, like the wild-type full-length protein, the in vitro
translated small HDAg variant dAg(17–56) which lacks the regions of coiled-coil and NLS1 but retains the intact NLS2, could be co-immunoprecipitated with the in vitro translated FLAG-B23 (lanes 4 and 5). This suggests that the interaction of B23 is not mediated through the coiled-coil domain and NLS1 but rather through the NLS2 region.

The interaction domains of both HDAgs within the B23 protein was also determined by the in vitro binding analysis using the GST-HDAg fusion proteins (GST-DAg or GST-dAg) and a series mutants of in vitro translated GST-DAg (lanes 4 and 5). This suggests that the interaction of B23 is not mediated through the coiled-coil domain and NLS1 but rather through the NLS2 region.

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HDAG Forms a Large Complex with B23 in Vivo—In the nucleolus, the protein B23 is expected to be largely in the hexameric form with a molecular mass of 230–255 kDa (44). Since B23 can associate with a wide spectrum of cellular factors, it was necessary to investigate the status of in vivo complex formation between these two nuclear proteins. To this end, the sedimentation behavior of B23 and HDAg in the nuclear fractions of both types of HDAg-producing cells was examined using the sucrose gradient centrifugation. As shown in Fig. 7A, the nuclear B23 in all the cell lines examined, including L10, S7 cells, and the parental HepG2 (G2) cells, sedimented in two predominant peaks at fractions 5–8 (peak I) and fractions 15–17 (peak II). Based on the sedimentation profiles of two standard proteins, catalase (fraction 7, 232 kDa) and thyroglobulin (fraction 10, 699 kDa), it seems that the nuclear B23 is either present as a hexameric form (peak I, molecular mass 230 kDa) or associated with other cellular components (peak II). Intriguingly, the small HDAg in HDAg-producing S7 cells co-sedimented with the peak II complex of B23, while in the large HDAg-producing L10 cells, HDAg was distributed more broadly from fraction 12 to 17 and peaked at fractions 12–14. Thus, large HDAg was only partially superimposed with the B23 profiles at peak II. This reflects a partial separation of the
large HDAg from a B23-associated complex. Notably, the small HDAg in S7 cells also appeared as a minor species at fraction 13, unassociated with B23. These results strongly suggest that unlike the case of large HDAg, the cellular small HDAg predominantly co-sedimented with a high molecular weight form of B23 in vivo. Additionally, when considering that both HDAg and B23 can associate with another nucleolar protein nucleolin (29, 49), it was important to examine the status of nucleolin in the sucrose gradient centrifugation. Our results indicated that apart from its monomeric form (~100 kDa) which appeared at fractions 4–6, about half the population of nucleolin co-sedimented with B23 and HDAg at fractions 15–17 (Fig. 7A). Co-immunoprecipitation indicated that the co-sedimentation of these three nucleolar proteins (HDAg, B23, and nucleolin) at fractions 15–17 of sucrose gradients of L10 or S7 cells is not coincidental because both B23 and nucleolin in these two HDAg-expressing cells, but not in the control cells, were co-immunoprecipitated by anti-HDAg antibody (Fig. 7B, lanes 2–4 and 6–8). This supports the notion that both forms of HDAg form a large complex with B23 and nucleolin in vivo.

B23 Enhances the HDAg Replication—The above results show that HDAg elicits two effects on B23, the up-regulation of its expression and the formation of a complex. Thus, it is rather important to know whether these two features of HDAg on B23 have any role in regulation of HDV replication. To explore this possibility, the effect of B23 on HDV replication was examined by introducing increasing amounts of B23 expression construct together with the HDV-replication plasmid pSVL-d2g into HuH-7 cells and the level of HDV replication was then analyzed by examining HDAg expression and HDV genomic/antigenomic RNA production. As shown in Fig. 8A, introducing 0.2 or 1.0 μg of the FLAG-B23 construct enhanced the production of the small HDAg as well as increasing the HDV genomic/antigenomic RNA (about 2–4-fold) in a dose-dependent manner. Notably, this is not the case for cells co-transfected with the B23 mutant construct FLAG-B23-(1–127) which is defective in binding with HDAg (Fig. 8D). This enhancement of HDV replication by B23 is also not due to the effect of B23 on the promoter activity of the SV-40 late promoter used to drive the HDV replication plasmid pSVL-d2g, since no such enhancement of HDAg production was observed for the HDAg gene under the SV40 late promoter control in the construct pSVL-d (Fig. 8C). Therefore, our results clearly indicate that B23 modulates HDV replication through the formation of a complex with HDAg.

DISCUSSION

This study has shown that the steady state level of B23 mRNA and protein is increased in both HDV-replicating and HDAg-expressing cells (Fig. 1). Overexpression of B23 mRNA is usually found in proliferative or tumor cells (37–39) and thus our results strongly support the notion that HDV infection may reflect a similar status of proliferation. However, the mechanism by which HDAg up-regulates the B23 gene expression is still unclear. According to previous studies, the large HDAg but not its small isoform has a trans-activation ability on certain promoters (31) or conversely, both HDAg have a trans-suppression ability on RNA polymerase II-dependent transcription (30). Therefore, it would appear that the mechanism involved in the up-regulation of B23 expression by these two isoforms of HDAg is distinct from those mechanisms (Fig. 1). When considering that the transcriptional regulatory region of human B23 gene contains a transcription factor YY1-binding site (67) and protein B23 can associate with this transcriptional factor and modulate its transcriptional activity (68), it is likely that the up-regulation of B23 gene expression by HDAg is mediated through an effect on the transcriptional activity of YY1.

Apart from the up-regulation of B23 expression, the current study based on four different lines of evidence reflecting both in vivo and in vitro situations, has demonstrated that both isoforms of HDAg can associate with the major nucleolar protein B23. The confocal microscopy analysis showed the co-localization of B23 and HDAg in nucleoli (Fig. 2). The immunoprecipitation experiment in HDV-replicating N1 cells transiently transfected with the expression plasmid of FLAG-B23 provided further support for the in vitro binding of B23 and HDAg (Fig. 4, C and D). Additionally, by using both co-immunoprecipitation (Fig. 4, A and B) and the GST-HDAg fusion protein pull-down assay (Fig. 3), both in vitro translated and cellular B23 have been shown to interact with HDAg. The in vitro binding
The immunoprecipitates were analyzed by SDS-PAGE followed by immunoblot using goat anti-B23, human anti-HDAg antisera, or mouse monoclonal anti-nucleolin antibody and the ECL method used for detection. Protein standards (catalase, 232 kDa; thyroglobulin, 669 kDa) were run in a parallel experiment and their positions are indicated. Panel A, the nuclear extracts of HepG2 (G2), and its HDAg-producing cells (L10 or S7) (500 μg) were prepared and analyzed by sucrose gradient centrifugation (see "Experimental Procedures"). Aliquots of each fraction were analyzed by immunoblot using goat anti-HDAg antibody. Protein extracts of HepG2 (G2), and its HDAg-producing cells (L10 or S7) (500 μg) were prepared and analyzed by sucrose gradient centrifugation (see "Experimental Procedures"). Aliquots of each fraction were analyzed by immunoblot using goat anti-HDAg antibody.

![Diagram of in vivo complex formation between HDAg and B23 by sucrose gradient centrifugation. Panel A, the nuclear extracts of HepG2 (G2), and its HDAg-producing cells (L10 or S7) (500 μg) were prepared and analyzed by sucrose gradient centrifugation (see "Experimental Procedures"). Aliquots of each fraction were analyzed by immunoblot using goat anti-HDAg antibody. Protein extracts of HepG2 (G2), and its HDAg-producing cells (L10 or S7) (500 μg) were prepared and analyzed by sucrose gradient centrifugation (see "Experimental Procedures"). Aliquots of each fraction were analyzed by immunoblot using goat anti-HDAg antibody.](image)

**Fig. 7.** Analysis of in vivo complex formation between HDAg and B23 by sucrose gradient centrifugation. Panel A, the nuclear extracts of HepG2 (G2), and its HDAg-producing cells (L10 or S7) (500 μg) were prepared and analyzed by sucrose gradient centrifugation (see "Experimental Procedures"). Aliquots of each fraction were analyzed by immunoblot using goat anti-HDAg antibody. Protein extracts of HepG2 (G2), and its HDAg-producing cells (L10 or S7) (500 μg) were prepared and analyzed by sucrose gradient centrifugation (see "Experimental Procedures"). Aliquots of each fraction were analyzed by immunoblot using goat anti-HDAg antibody. Protein extracts of HepG2 (G2), and its HDAg-producing cells (L10 or S7) (500 μg) were prepared and analyzed by sucrose gradient centrifugation (see "Experimental Procedures"). Aliquots of each fraction were analyzed by immunoblot using goat anti-HDAg antibody. Protein extracts of HepG2 (G2), and its HDAg-producing cells (L10 or S7) (500 μg) were prepared and analyzed by sucrose gradient centrifugation (see "Experimental Procedures"). Aliquots of each fraction were analyzed by immunoblot using goat anti-HDAg antibody.
pressed HDAg or B23 molecule has oligomerization property and readily associates with host proteins to form a high molecular weight complex (44, 45, 75, 77, 78), likely the HDAg or B23 in this B23-nucleolin-HDAg containing complex is in the oligomer form.

An intriguing finding emerged from this study was the preferential association of the small HDAg with B23 in vitro (Fig. 7A). In vitro, recombinant large and small HDAGs could bind to B23 (Figs. 3–6), while in vivo about half of the large HDAg-containing complexes were not associated with B23 (Fig. 7A). This difference in the sedimentation profiles of large and small forms of HDAG-containing complexes present in the nuclear extracts may reflect that they associate with different cellular partners depending on their relevant biological functions. In view of the essential role of small HDAg in HDV RNA replication, the strong co-localization of the small HDAg with the two nucleolar proteins, B23 and nucleolin, suggests a potential functional role for this complex in replication. Although the precise components making up this B23-nucleolin-small HDAg-containing complex and its biochemical activity are yet to be determined, it is tempting to speculate that this complex is the active one involved in HDV RNA replication. Consistent with this, we found that exogenous B23 appears to have a stimulatory effect on HDV RNA replication (Fig. 8A) and the deletion of the HDAg-binding site impaired this effect (Fig. 8B). Similarly, the observation that introducing the exogenous nucleolin enhances HDV RNA replication (29) agrees with this hypothesis. If this is the case, an important question arises as to where these two nucleolar proteins in HDV replication. In the view of the known functions of B23 and nucleolin (for review, see Ref. 79), several hypotheses can be put forward. First, that B23 or nucleolin binding to HDAg may be the mechanism by which HDAg enters the nucleus/nucleoli. In this regard, there is evidence that the HDV RNA replication occurs in the nucleus (13, 29, 65, 80–84). In this scenario, B23 and its binding molecule nucleolin may well play a role in HDV RNA replication by transporting HDAg from cytoplasm to nuclei/nucleoli. Second, it can be suggested that since both B23 and nucleolin are involved in several aspects of nucleolar structure and transcriptional regulation (46, 68, 79, 85–88), one possible role for B23 or nucleolin binding is to target HDAg to sites of transcription. This hypothesis is consistent with previous work indicating that there are interactions between B23 and transcriptional proteins (68, 89) and also agrees with the fact that nucleolin can serve as a transcriptional factor (86, 88). Therefore, B23 or nucleolin may tether HDAg to the transcription complex, which in turn confers a regulatory role for HDAg on HDV RNA replication. More significantly, nucleolin or B23 may act as an assembly factor to recruit, by protein-protein interaction, other factors including HDAg into the HDV replication process. Indeed, the possibility that the HDV antigenomic RNA synthesis may be mediated by the nucleolar RNA polymerase I, as suggested by a recent study of Modahl et al. (35), fits the roles one may expect from B23 or nucleolin, since both these two nucleolar proteins are known to be involved in ribosome biogenesis as directed by nucleolar RNA polymerase I (40, 43, 79, 90–92). Alternatively, when considering that both nucleolar proteins have RNA binding, reannealing, and the RNA duplex destabilizing activity (72, 73, 79, 93–95), they may provide a RNA chaperone activity for HDV RNA replication. Furthermore, the molecular chaperone activity of B23 (75) may facilitate the proper assembly of the HDV replication machinery or aid the transport of HDV ribonucleoprotein particles. Finally, our previous findings that HDV RNA replication but not viral particle assembly is regulated by casein kinase II (25, 27), and the observations that both B23 and nucleolin are nuclear matrix-associated (62, 86) and casein kinase II-regulated (47, 69, 95), may not be coincidental. It implies that these two nucleolar proteins together with HDAg, casein kinase II, and other cellular factors (e.g. RNA polymerase or transcription factor) may interact within the nuclear matrix and participate in the HDV RNA replication cooperatively in a similar manner to the SWAP complex, which contains B23, nucleolin, poly(ADP-ribose) polymerase, and SWAP-70 and is involved in B-cell DNA recombination (96).

In summary, we have found that HDAg can up-regulate the nucleolar protein B23 expression, and both in turn form a complex which facilitates HDV RNA replication. This study together with previous work on nucleolin-HDAg interaction reinforces the important role of nucleolar proteins in HDV
replication. However, further insights into the replication machinery of HDV are required to define the role of nucleolar components in HDV RNA replication.

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