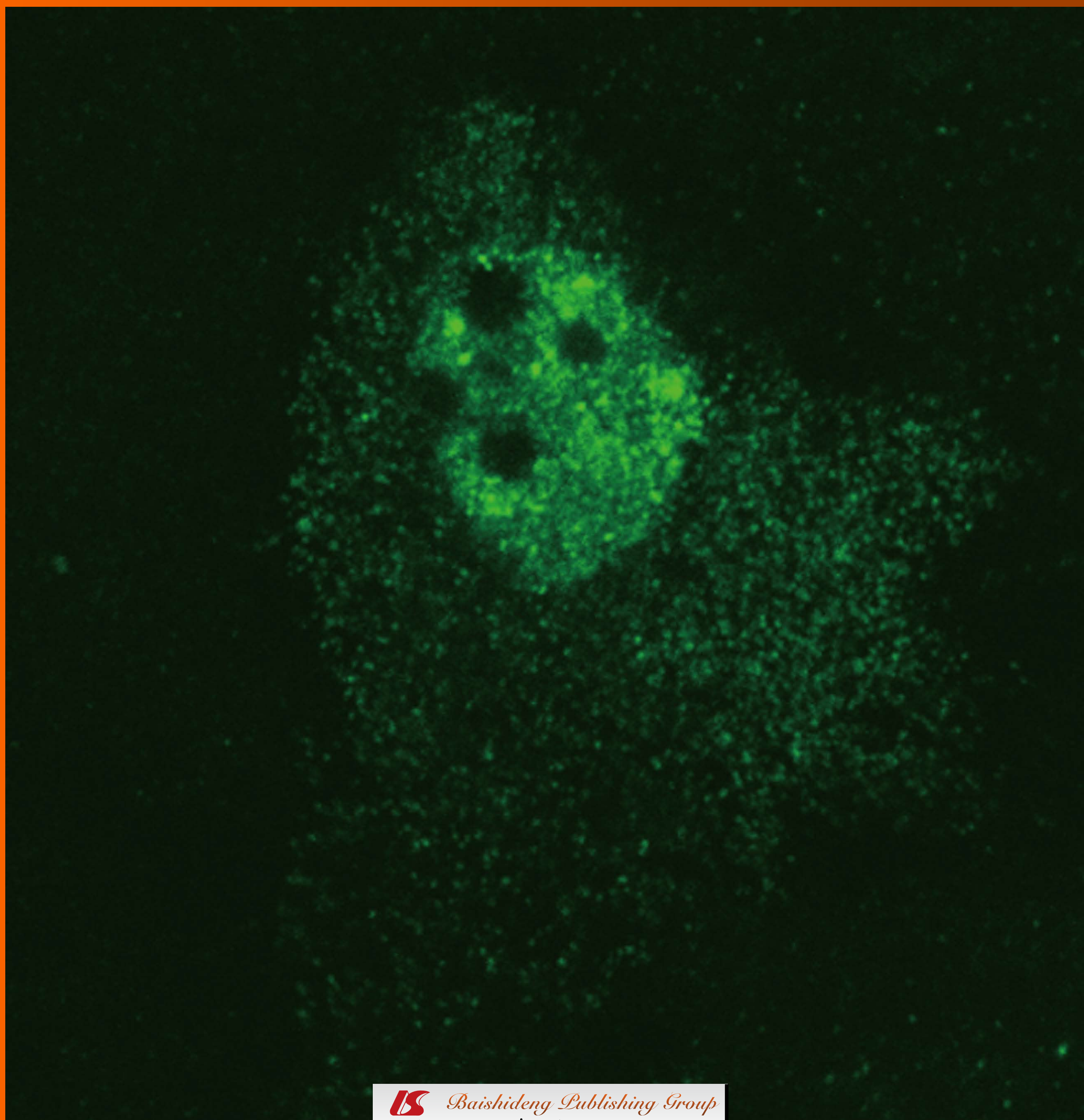
ISSN 2220-3249 (online)

World Journal of *Virology*

World J Virol 2013 August 12; 2(3): 110-138



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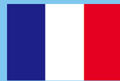
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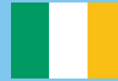
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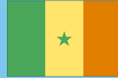

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http://www.wjgnet.com/2220-3249/full/v2/i3/123.htm
http://dx.doi.org/10.5501/wjv.v2.i3.123

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NAME OF JOURNAL
World Journal of Virology

ISSN
ISSN 2220-3249 (online)

LAUNCH DATE
February 12, 2012

FREQUENCY
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PUBLICATION DATE
August 12, 2013

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doi:10.5501/wjv.v2.i3.110

World J Virol 2013 August 12; 2(3): 110-122
ISSN 2220-3249 (online)
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REVIEW

Nuclear domain 10 of the viral aspect

Yisel A Rivera-Molina, Francisco Puerta Martínez, Qiyi Tang

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Supported by A Pilot Grant from the National Center for Research Resources (G12 RR003050); and the National Institute on Minority Health and Health Disparities (8G12MD007579-27), both of the National Institutes of Health (To Tang Q), an American Cancer Society Grant (117448-RSG-09-289-01-MPC) (To Tang Q), and NIH/NCRR U54RR022762 (To Tang Q)
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Received: January 27, 2013 Revised: May 31, 2013
Accepted: May 31, 2013
Published online: August 12, 2013

Abstract

Nuclear domain 10 (ND10) are spherical bodies distributed throughout the nucleoplasm and measuring around 0.2-1.0 μm. First observed under an electron microscope, they were originally described as dense bodies found in the nucleus. They are known by a number of other names, including Promyelocytic Leukemia bodies (PML bodies), Kremer bodies, and PML oncogenic domains. ND10 are frequently associated with Cajal bodies and cleavage bodies. It has been suggested that they play a role in regulating gene transcription. ND10 were originally characterized using human autoantisera, which recognizes Speckled Protein of 100 kDa, from patients with primary biliary cirrhosis. At the immunohistochemical level, ND10 appear as nuclear punctate structures, with 10 indicating the approximate number of dots per nucleus observed. ND10 do not colocalize with kinetochores, centromeres, sites of mRNA processing, or chromosomes. Resistance of ND10 antigens to nuclease digestion and salt extraction suggest that ND10 are associated with the nuclear matrix. They are often identified by immunofluorescent assay using specific antibodies against PML, Death domain-associated protein, nuclear dot protein (NDP55), and so on. The role of ND10 has long been the subject of investigation, with the specific connection of ND10 and viral infection having been a particular focus for almost 20 years. This review summarizes the relationship of ND10 and viral infection. Some future study directions are also discussed.

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Key words: Nuclear domain 10; Promyelocytic Leukemia; Speckled protein of 100 kDa; Death domain-associated protein; Virus; Viral replication

Core tip: We, for the first time, discussed the function of nuclear domain 10 (ND10) as a nuclear structure. Although the ND10 components, especially Promyelocytic Leukemia bodies, Speckled Protein of 100 kDa and death domain-associated protein, have been widely investigated for their roles in viral gene expression and viral replication, individual virus interacts with ND10 differentially as we summarized up in this review. This review is expected to guide readers especially virologists and cell biologists to understanding the interaction of ND10 with viruses.

Rivera-Molina YA, Martínez FP, Tang Q. Nuclear domain 10 of the viral aspect. *World J Virol* 2013; 2(3): 110-122 Available from: URL: http://www.wjgnet.com/2220-3249/full/v2/i3/110.htm DOI: http://dx.doi.org/10.5501/wjv.v2.i3.110

ND10: GENERAL INFORMATION

Mammalian cells contain differentially functional compartments called organelles, which are separated from the cytoplasm by a lipid bilayer membrane. The nucleus is an extremely dynamic organelle and highly organized compartment with multiple functions (reviewed in Dundr

et al[1], Dundr *et al*[2], and Zhao *et al*[3]) the nucleoplasm consists of soluble and insoluble materials that keep the genomic structure intact and host the complicated process of gene transcription. Some insoluble and soluble materials congregate together to form shaped structures such as nuclear domain 10 (ND10 can also refer to nuclear dot 10)[4]. When analyzed by indirect immunofluorescence microscopy, many nuclear proteins are seen to localize in distinct structures with punctate staining patterns[5,6]. Nuclear structures, such as speckles, paraspeckles, nucleoli, Cajal bodies, polycomb bodies, and ND10, are formed primarily by protein-protein, protein-RNA, or protein-DNA interactions[1]. Each nuclear body has a matrix protein that is essential for the formation of the specific nuclear body. ND10 are subnuclear structures that gather many different SUMOylated nuclear proteins (such as Daxx and SP100). The formation of ND10 depends on Promyelocytic Leukemia (PML) protein. Past observations confirm that PML knockout cells lack ND10 and that transfecting exogenous PML into PML knockout cells results in the restoration of ND10[7,8]. Most DNA viruses replicate DNA and transcribe genes in the nucleus after their genomic DNA enters the nucleus by facilitated transport through the nuclear pore complex[9]. Once inside the nucleus, viral genomes distribute randomly, but it appears that only those at ND10 replicate and transcribe predominantly[10-13], suggesting specifically that the environment at ND10 is particularly advantageous for the virus. However, the ND10 proteins [such as PML, Speckled Protein of 100 kDa (SP100), and Daxx] are interferon-upregulated and have repressive effects on viral replication[14-25]. Moreover, most DNA viruses encode an immediate-early protein that induces the dispersion of ND10[10,26-29], and in the absence of these viral proteins, replication is severely retarded[13,29,30]. These findings have led to the hypothesis that ND10 are also part of nuclear defense mechanism[4]. At this point, the effects of ND10 on viral replication remain to be settled.

A HISTORIC OVERVIEW OF ND10

There are five hallmark events in the history of studying ND10. First, a French paper in 1960 described an unknown nuclear structure in rabbit cells with the Papillomavirus as an electron-dense body[31]. It was the earliest description of the nuclear structure, but it left everything unexplained, other than providing that observation. Second, there was not any other information that could lead to a deeper investigation of these nuclear structures until they were first identified (by immunofluorescence analysis, using specific antibodies that were later revealed to be against SP100 and NDP55) as ND10 in 1991 by Ascoli *et al*[32]. SP100 was later proved to be essential for the formation of ND10[33]; NDP55 has not been characterized so far. Ascoli *et al*[32] investigated the structure in different types of cells. A combination of immunofluorescence analysis and electronic microscopy confirmed that ND10 are the structures that were previously observed in 1960. Third, in the process of investigating the function of ND10, ND10 were found to be related to herpes simplex virus type 1 (HSV-1) infection[26,34] in 1993 and 1994; these studies (by Maul GG, the Wistar Institute, the United States of America, and Everett RD, the MRC Virology Unit, Glasgow, United Kingdom) awakened the interest of virologists with regard to the interaction of ND10 and many different viruses. The interactions of ND10 and a variety of viruses will be discussed in this review. Fourth, it was determined that PML knockout mice lack ND10, which provided direct evidence that supported the hypothesis that the protein PML is essential to the formation of ND10. It was confirmed, as well, by the experimental results that demonstrate that the transfection of PML into PML-/- cells restores ND10[26,35]. PML-/- mice live normally, which further obscured the function of PML, though later studies were able to determine that PML-/- have a greater tendency to develop cancer than do their PML+/+ counterparts[36]. Fifth, ND10 components were identified. Even though more than 60 nuclear proteins have been shown to be more-or-less related to ND10[37,38], three components are thought to be the primary ND10 proteins (called the prototype proteins of ND10): PML, Daxx, and SP100. Other important events relating to the study of ND10 will be discussed in the following sections.

MOLECULAR ASPECT OF ND10

Promyelocytic leukemia

The molecular mechanism of the biogenesis of ND10 was a complete mystery until PML was identified as forming the matrix of ND10. PML is a tumor-suppressor protein that in both humans and mice is encoded by the PML gene. This gene was found to be involved in translocation with the retinoic acid receptor alpha (*RARalpha*) gene, causing acute promyelocytic leukemia (APL) (see the review by de Thé *et al*[39]). The protein encoded by this gene was therefore named after PML. PML is also called tripartite motif (TRIM) 19 because it is a member of the TRIM family[40]. The TRIM motif includes three zinc-binding domains, a RING, two B-boxes, and a coiled-coil region. Phosphorylation is required for the high SUMOylation of PML; SUMOylated PML localizes to ND10, where it functions as a transcription factor and tumor suppressor[41]. Its expression is cell-cycle related; therefore ND10 morphology and number in the nucleus are dependent on the cell cycle[42]. It regulates the p53 response to oncogenic signals, which might explain how the translocation of PML with RARalpha causes APL. Right after its identification, ND10 were shown to be important in cell differentiation and cell growth; this was first indicated in studies of promyelocytes from patients suffering from APL[43-45]. In the promyelocytes from these patients, ND10 cannot be detected. When cells are treated with retinoic acid (RA) or with arsenic trioxide, ND10 are restored and the APL phenotype is reversed and the patients can be cured with these agents (reviewed by Melnick and Licht[46]). PML has about 11 isoforms that are caused by the extensive

alternative splicing of this gene. PML isoforms vary in the protein's central and C-terminal regions; all variants encode the same N-terminus[47]. Some isoforms of PML are cytoplasmic, but most of the isoforms are nuclear proteins important for ND10 formation.

Speckled protein of 100 kDa

SP100 was first identified by immunofluorescence using autoantibodies from patients with primary biliary cirrhosis, and its cDNA was then isolated and cloned; it was found to encode a human nuclear antigen distributing in the nucleus as speckles[48]. SP100 is a single-copy gene sited in human chromosome 2q37 and, like PML, it is IFN upregulated. The *SP100* gene needs to be spliced and gives rise to a number of speckled protein of 100 kDa (SP100) isoforms: SP100A, -B, -C, and -HMG[48-52]. The four SP100 isoforms share a homologous 476 N-terminal amino acid, but differ in their C-terminal part. The most abundant isoform is SP100A, which has 480 amino acids and migrates to 100 kDa on SDS-PAGE[51]. SP100A most likely does not bind to DNA alone because it lacks all other domains of SP100B, -C, and -HMG. It may be recruited to DNA *via* association with DNA-binding proteins such as hHMG2/DSP1[53], the B-cell-specific transactivator Bright[54], or ETS-1[55]. SP100B contains a SAND domain (SAND stands for SP100, AIRE, NucP41/75, and DEAF1), SP100HMG contains a SAND domain and an HMG box, and newly described SP100C contains SAND, PHD, and Bromo domains[52,56]. SP100 is one of the prototypical proteins of ND10, and it colocalizes with Daxx and PML in ND10. SP100B, -C, and -HMG isoforms contain SAND, PHD, Bromo, and HMG domains and are highly SUMOylated. All the domains are suggestive of a role in chromatin-mediated gene regulation. The three minor isoforms contain a SAND domain that binds to DNA and is required if SP100 is to have transcriptional regulating activity.

Death domain-associated protein

Upon its discovery, death domain-associated protein (Daxx beta) was found to be a protein of the classical death receptor[57]. It was found to bind specifically to the Fas death domain *via* its C-terminal portion. Overexpression of Daxx enhances Fas-mediated apoptosis through activating the Jun N-terminal kinase (JNK) pathway. It was later found that Daxx interacted with CENP-C, one of the few known intrinsic proteins of the human centromere[58]. CENP-C is thought to play structural as well as regulatory roles crucial to proper chromosome segregation and mitotic progression. The interaction between CENP-C and Daxx was then confirmed by an immunofluorescence assay that found the colocalization of these two proteins at discrete spots in the nuclei of some interphase cells[58]. The other Daxx-binding proteins include the transcription factor Pax3[59] and DNA methyltransferase I [60]. They both are related to centromeres such as CENP-C and are not related to ND10. Therefore, Daxx is a protein of centromere. However, Ishov *et al*[7] found that PML recruited Daxx to ND10. Interestingly, in PML-/- cells, Daxx totally stays in the centromere. Therefore, Daxx might travel from centromere to ND10 or from ND10 to centromere. Ishov *et al*[42] also found that Daxx and the SWI/SNF protein ATRX are both associated with two intranuclear domains: ND10 and heterochromatin. The accumulation of ATRX at ND10 was mediated by its interaction with the N-terminus of Daxx. Although ATRX was present in heterochromatin during the entire cell cycle, Daxx was actively recruited to this domain at the end of the S-phase. Daxx functions as an adapter for ATRX accumulation at ND10[42]. Daxx can be highly SUMOylated, and SUMOylation was found to be crucial for targeting Daxx to PODs and for the trans-repression of several SUMOylated transcription factors, including the glucocorticoid receptors (GR)[61]. Recently, two variants of Daxx were identified. The two novel variants of Daxx were termed Daxx- and Daxx-γ, and these variants are generated by alternative splicing. They have a truncated regulatory C terminus, and Daxx- and Daxx-γ show markedly decreased affinities to PML and have a different nuclear distribution[62].

In summary, all three of the prototypical proteins (PML, SP100, and Daxx) of ND10 share some similar characteristics: (1) They colocalize with ND10. Their colocalization in ND10 depends on PML[7,8], and SP100 can also affect ND10 formation[33]. These proteins are prototypical components of ND10; (2) They can be up regulated by interferon, which provided the first evidence to support the hypothesis that ND10 are defensive against viral infection[4]; (3) The prototypical proteins of ND10 are all highly SUMOylated, SUMOylation is important for the formation of ND10, Daxx function, and the interaction of the three proteins; (4) They are all cancer gene repressors. Although PML-/- mice can still live normally, they are shown to have a higher chance of developing cancer[63]; (5) All three genes produce different isoforms *via* alternative splicing; and (6) They are all viral replication inhibitors, which will be discussed in the review below.

ND10 function

ND10 came to the forefront because it was found that t(15; 17) translocation causes the fusion of PML and RARA (generating PML/RARA) and the dysfunction of both PML and RARA (consequently resulting in APL). The oncogenic PML/RARA protein disrupts ND10 in a reversible manner upon being treated with retinoic acid and/or arsenic, either of which treatment can cure the patients with APL[64-68]. ND10 number and size are regulated in several cellular responses: viral infection[69], DNA-damage, transformation[70-72], and oxidative stress[73,74]. The transcriptions of PML, SP100, and Daxx are dramatically enhanced by interferons. However, PML-/- mice develop normally and live well without the formation of ND10, demonstrating that ND10 are not required for most basic biological functions. Nevertheless, recent data have implicated PML in the control of cellular senescence and stem

cell self-renewal, extending the fields of the investigation of PML function[75,76].

ND10 studies have been so intense in recent years that novel information about these structures is being uncovered continuously; however, the function of PML bodies is still not fully understood. Three models have been proposed: the Depot or Sequestration model; the Hotspot model, and a site of specific nuclear activities. These models are described in the following paragraphs.

Depot or sequestration model

The nuclear domains are proposed to be aggregations of excess nucleoplasmic protein[77]. This model suggests that the ND10 components in the nucleoplasm have a dynamic nature, that is, they move from ND10 to the functional sites where they are needed. In other words, the aggregated proteins in ND10 are sequestered. This sequestration is evidenced by the fact that the PML partners in ND10 vary significantly between individual partners and levels of PML expression, as well as SUMOylation. A well-studied sequestered component in ND10 is Daxx, a potent repressor that forms partitions between SUMOylated proteins, including PML and many transcription factors. Sequestration of Daxx by ND10-associated, SUMOylated PML releases transcriptional repression by DNA-bound SUMOylated transcription factors[61,78-80].

Hotspot model

This model proposes that ND10 are the sites of the post-translational modification and the degradation of proteins. It is supported by the facts that SUMO-1 molecules aggregate in ND10 and ND10 might be the hot sites for SUMOylation, that the acetylation and phosphorylation of p53 at PML bodies enhance the activity of p53[16,81,82], and that the 19S and 20S proteasome subunits localize at some PML bodies[83].

Third model

Proposes ND10 to be sites of specific nuclear activities, such as transcriptional regulation and DNA replication. This model is supported by the detection of nascent RNA around ND10[84], the association of ND10 with regions of high transcriptional activity[85], and the non-random nature of PML body assembly (based on the conservation of their size and position) following dissociation and re-formation as a result of cellular stress[86].

INTERACTIONS OF ND10 AND VIRUSES

Herpesviruses

Human herpesviruses are divided into three subfamilies: alpha, beta, and gamma. The alpha subfamily includes Herpes Simplex Virus 1 and 2 (HSV-1 and HSV-2) and the Varicella Zoster Virus (VZV). The beta subfamily has cytomegalovirus (CMV) and human herpesvirus 6 and 7 (HHV-6 and HHV-7). Kaposi's sarcoma-associated herpes virus (KSHV) and the Epstein-Bar virus (EBV) are in the gamma subfamily. These viruses are characterized by their practice of setting up latency in the host after primary infection. After entering to the nucleus through nuclear pores, these DNA viruses replicate their DNA and transcribe their genes inside the nucleus, preferably at ND10[4]. Therefore, the interaction of ND10 and herpesviruses occurs at the very early stage of infection.

Herpes simplex

The first virus found to be connected to ND10 was herpes simplex (HSV)-1. In 1993, Maul *et al*[28] were the first to discover that Vmw110 (ICP0-infected cell protein 0) localizes to ND10. Interestingly, they also showed that the C-terminal portion of ICP0, when linked to a heterologous protein, disrupts the normal distribution of PML. These observations presented the first link between processes involved in the control of cell growth and viral infection and latency. Later, Maul and Everett[11,26,34,87] systemically collaborated on the investigation of the interaction of ND10 and HSV-1, which collaboration typically combined the views from a cell biologist (Maul) and a virologist (Everett) on the direction to revelation of the phenomenal interaction of viral molecules and ND10. This, according to the authors' opinion, could be the most important contribution to the ND10 field.

It is now known that ICP0 disrupts ND10 through mediating the loss of the SUMO-1-modified forms of PML and the subsequent proteasome-mediated degradation of the PML protein[14-15,88-90]. The results were consistent with the finding that PML residue lysine 160 is the SUMOylation site and the mutation of this residue makes PML resistant to degradation by ICP0[91]. ND10 function might not be so critical for HSV-1 lytic infection because ICP0-deleted HSV-1 can replicate well, especially at a high multiplicity of infection (MOI).

It was visualized that both parental and replicated HSV-1 amplicon genomes were in association with ND10 in live cells[92]. It is likely that the genomes situated at ND10 preferentially form viral replication compartments. Tang *et al*[12] further figured out that there exist minimal viral DNA sequences and viral proteins that are essential and sufficient for the replication of DNA and the transcription of RNA at ND10 by the virus. For HSV-1 we found that a specific viral DNA sequence, OriS, and the viral immediate-early proteins ICP4 and ICP27 are sufficient for a reporter gene placed in cis at the OriS sequence to transcribe RNA at ND10[12]. HSV-1 DNA replication results in formation of compartments in the nucleus; it has been shown that some, but not all, PML isoforms are recruited to the replication compartments[93]. Viral DNA replication compartments also contain many other viral and cellular proteins that have different functions, many of which are required for DNA replication, DNA repair, and DNA stabilization[94]. However, the function of ND10 proteins in the DNA replication compartments is not fully understood.

HSV-1 with deleted ICP0 has an obvious defect in viral gene expression and plaque formation in limited-pas-

sage human fibroblasts (though not in mouse fibroblast cells)[95,96]. This suggests both that ND10 have defensive effect on HSV-1 infection and that ICP0 can abolish the defensive effect of ND10 in human fibroblasts. ICP0 is a RING finger E3 ubiquitin ligase that induces the degradation of PML. Depletion of PML from human fibroblasts increases ICP0-null mutant *HSV-1* gene expression but not to wild-type levels[96]. Another major ND10 protein, SP100, has a similar effect on ICP0-deleted HSV-1 gene expression[96]. It has been shown that all four SP100 isoforms stabilize ND10 and protect PML from ICP0-based hydrolysis[18]. Depletion of either all PML isoforms or all SP100 isoforms reduces the other constituent ND10 protein, suggesting that different ND10 proteins use different mechanisms to inhibit virus infection at the immediate-early stage of HSV-1 infection[18]. Simultaneous depletion of both PML and SP100 proteins complements the mutant virus to a greater degree, implying that PML and SP100 could have additive or synergistic effects on viral replication[96].

HSV-1 ICP0 might be important for the activation of lytic infection and the countering of the cell-mediated repression of viral gene expression by HSV-1. This repression is defended by preexisting cellular proteins, and those proteins function as intrinsic antiviral resistance or intrinsic defense. PML and SP100, as we discussed above, are two of the core components of ND10 and contribute to intrinsic resistance. But how about other ND10 proteins, such as, ATRX and Daxx? ATRX and Daxx are known to comprise components of a repressive chromatin-remodeling complex. It has been shown that the infection of ICP0-deleted HSV-1 (not wild-type HSV-1) can replicate at a greater level in both ATRX- and hDaxx-depleted cells than it can in normal cells[97], suggesting that ATRX and hDaxx act as a complex to play intrinsic antiviral resistance to HSV-1 infection, which is counteracted by ICP0.

Cytomegalovirus

Cytomegalovirus (CMV) infection differs from that of HSV-1 in host range and replication. HCMV can infect only human cells productively and causes diseases in humans only, and it replicates slowly in cell culture. HCMV is similar to HSV-1 in many ways: (1) setting up latency after primary infection in host; (2) sequential viral gene expression; and (3) viral DNA replication at ND10, preferentially. Following the studies of ND10 and HSV-1 interaction, many ND10 components have been demonstrated to have a repressive effect on CMV gene expression and viral replication (reviewed by Saffert and Kalejta[98]). The first ND10 protein investigated for its role in *HCMV* gene expression and viral replication was Daxx. In that study, Daxx was found to interact functionally with HCMV tegument protein pp71[16]. The Stamminger group[99] also investigated PML to see whether PML could have any effects on viral gene expression or on viral replication. After comparing HCMV replication in PML-kd or hDaxx-kd cells with that in normal cells, they revealed that immediate-early (IE) gene expression increased to a similar extent, regardless of whether PML or Daxx was depleted[98]. Their experimental results suggest that PML and Daxx might function using different mechanisms to suppress HCMV replication; double-knockdown cells depleted of both PML and hDaxx support the additive enhancement of HCMV infection in the replication efficacy of HCMV compared to that of single-knockdown cells[99]. Finally, they also found that the infection of SP100 knockdown (kd) cells with HCMV resulted in a significantly increased plaque-forming ability[99,100].

Like HSV-1, HCMV infection can also disrupt ND10, but the mechanisms of dispersing ND10 might be different. HSV-1 ICP0 induces the loss of the SUMO-1-modified forms of PML and the proteasome-mediated degradation of the PML protein[14,15,88-90]. However, in CMV-infected cells, PML is not degraded[13,101]. For cytomegaloviruses (including MCMV and HCMV), IE1 has been identified to disperse ND10 by an as yet unknown mechanism, but it is not able to degrade PML[27,101-104]. HCMV IE1's induction of PML deSUMOylation, reported by Lee *et al*[101], needs to be investigated for MCMV IE1.

Species-specificity is one of the major characteristics of cytomegaloviruses (CMVs) and is the primary reason for the lack of a mouse model for the direct infection of human CMV (HCMV). It has been determined that CMV cross-species infections are blocked at the post-entry level by intrinsic cellular defense mechanisms[105,106], but few details are known. We discovered that ND10 of human cells is not disrupted by murine CMV (MCMV) and that the ND10 of mouse cells is not disrupted by HCMV[107], although the ND10-disrupting protein, immediate-early protein 1 (IE1), also colocalize with ND10 in cross-species infections[107]. In addition, we found that the UL131-repaired HCMV strain AD169 (vDW215-BADrUL131) can infect mouse cells to produce immediate-early (IE) and early (E) proteins but that neither DNA replication nor viral particles are detectable in mouse cells. Unrepaired AD169 can express only IE1 in mouse cells. In both HCMV-infected mouse cells and MCMV-infected human cells, the knocking-down of ND10 components (PML, Daxx, and SP100) resulted in significantly increased viral-protein production. Our observations provide evidence to support our hypothesis that ND10 and ND10 components might be important defensive factors against CMV cross-species infection.

EPSTEIN-BARR VIRUS AND KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS

The relationship of Epstein-Barr Virus (EBV) or Kaposi's sarcoma-associated herpesvirus (KSHV) with ND10 has been less investigated than has that of HSV-1 or CMV (with ND10). The first study of the interaction of EBV and ND10 also came from the Maul group. Bell *et al*[108] studied the effect of the EBV on ND10, and its (EBV's) spatial distribution in the nucleus of cells during latency and lytic reactivation. In EBV, latently-infected Burkitt's lymphoma,

lymphoblastoid, and D98/HR1 cells, ND10 were intact. Fluorescent *in situ* hybridization (FISH) revealed no association between viral episomes and ND10 during latency, implying that the maintenance replication of EBV, which depends on host cell proliferation, occurs independently of ND10. Upon lytic activation, ND10 become dispersed in cells expressing lytic proteins. Thus, latency does not require or induce the interaction of EBV and ND10 for transcription or replication, whereas lytic replication triggers the dispersion of ND10 proteins and occurs in close association with PML aggregates. The required movement of chromosome-attached latent EBV episomes to ND10 after reactivation from latency might include the physical release of chromosome-bound episomes. Only episomes that come in contact with ND10 after such a release might be able to begin the process of lytic replication[108]. The dispersion of ND10 by EBV in lytic infection might be through molecular and functional interactions between the EBV BZLF1 protein and the PML[109].

There are many fewer functional studies of ND10 proteins in EBV infection or reactivation than there are of those proteins in HSV-1 or CMV. So far, SP100 appears to be an effective ND10 protein that is related to *EBV* gene expression and viral reactivation. The EBV EBNA-LP protein is a potent gene-specific coactivator of the viral transcriptional activator, EBNA2. Ling *et al*[17] found that EBNA-LP interacts with ND10 protein SP100 and displaces SP100 and heterochromatin protein 1alpha (HP1alpha) from ND10. Their experimental results suggest that SP100 is a major mediator of EBNA-LP coactivation[17]. Recently, Tsai *et al*[110] showed that the EBV major tegument protein BNRF1 interacts with host-cell ND10 proteins and promotes viral early gene activation. Specifically, they demonstrated that BNRF1 interacts with the Daxx at ND10 and interferes with the formation of the Daxx-ATRX chromatin remodeling complex. Furthermore, the knockdown of Daxx and ATRX induces the reactivation of EBV from latency in infected lymphoblastoid cell lines, suggesting that Daxx and ATRX play a role in the regulation of viral gene expression and viral replication.

KSHV interacts with ND10 at the very early stage after reactivation. Although EBV and KSHV are so similar in many aspects that they are classified into the gama-herpesviral subfamily, they are different in many other characteristics. For example, KSHV might not be able to disrupt ND10, even though that particular claim is arguable. Wu *et al*[111] first studied the interaction of ND10 and KSHV and found that the KSHV protein, K8, interacted with PML; nevertheless, they clearly demonstrated that KSHV infection (latent or lytic) cannot disrupt ND10[111,112]. Our unpublished data also support their conclusion that ND10 are not dispersed by KSHV infection. However, this has been recently challenged by other studies[111,112]. In one such study, Marcos-Villar *et al*[113] stated that the KSHV protein LANA2 increased the levels of SUMOylated PML and induced the disruption of ND10 by a proteasome-mediated mechanism. They also reported that ND10 disruption needs both the integrity of a SUMO interaction motif (SIM) in LANA2 and the lysine 160 from PML. Moreover, they showed that the depletion of LANA2 in PEL cells led to an increase in the PML levels[111,112]. Arguably, KSHV's dispersion of ND10 was not clearly shown in the published pictures. Interestingly, the authors didn't cite the paper by Wu *et al*[111] that is intimately related to the subject.

As for the molecular and functional interaction of KSHV and ND10 proteins, only a few publications have been presented. First, Murakami *et al*[114] reported that Daxx is a LANA-binding protein and that interaction made LANA inhibit the repressive effect of Daxx on VEGF expression. Their results suggest that LANA contributes to the high expression of the vascular endothelial growth factor (VEGF) receptors in KS lesions by interfering with the interaction of Daxx and Ets-1[114]. Other studies showed the existence of an interaction between PML and KSHV proteins (including K8 and LANA2)[111,115]. The biological significance of this interaction is still unclear.

OTHER DNA VIRUSES

Adenovirus

Adenovirus (Adv) is another virus that interacts with ND10. It was found that Adv infection changed the morphology of ND10 from being spherical punctate structures to being fibrous ones. This morphological change is caused by the molecular interaction of the Adv protein, E4 ORF3, and PML[116]. The other Adv protein found to interact with PML was E1A, which is an oncoprotein[116]. This study suggests that PML in ND10 might be involved in the cancerous consequence of Adv infection. More recently, a study by Hoppe *et al*[117] showed the PML isoform interacting directly and specifically with Adv E4 Orf3 *in vitro* and *in vivo*. Moreover, Hoppe *et al*[117] reconstructed ND10 in PML-null cells by inducing the transient transfection of different PML isoforms. They observed that only those ND10 formed from PML isoform Ⅱ were morphologically changed by E4 Orf3. Their data suggest that the interaction of E4 Orf3 and PML isoform Ⅱ is required for ND10 rearrangement[117].

The E4 ORF3 protein is required for Adv DNA replication when the cells are in the interferon (IFN)-induced antiviral state. ND10 prone proteins are all IFN-upregulated. This may reflect the fact that PML, Daxx, and SP100 are encoded by an interferon-stimulated gene. If so, can the interaction of E4 ORF3 and ND10 have any effect on Adv replication or viral gene expression? Ullman *et al*[118] demonstrate that the interaction of E4 ORF3 and ND10 antagonizes an innate antiviral response mediated by both PML and Daxx. Depleting any one of these proteins makes it possible to restore the replicative capacity of the virus using the E4 ORF3 protein deleted in the IFN-induced antiviral state. The interaction of Adv and ND10 has been also investigated with respect to SP100. Obviously, SP100 SUMOylation

is also affected by E4 ORF3, which in part contributes to the morphological change of ND10[89]. We think that it is critical to investigate whether E4 ORF3's interaction with ND10 plays a role in the oncogenesis of Adv. The interaction of E1A (an oncoprotein of Adv) and ND10 might be more important in the field of ND10 and viruses.

Human papillomavirus

Using indirect immunofluorescence in combination with fluorescence in situ hybridization, Swindle *et al*[119] found that human papillomavirus (HPV) DNA replication is targeted to host nuclear domains that are active during the late S phase, when such domains are limited in number. It was also observed that E1 and E2 partially or completely colocalize with ND10. The observation suggests that HPV DNA amplification might be partially coupled to virion assembly[119]. Interestingly, Florin *et al*[120] showed that the minor capsid protein L2 of HPV interacted with ND10-associated proteins. They observed that (1) the PML was unaffected by L2; (2) SP100 was released from ND10 upon L2 expression; and (3) In contrast to SP100, Daxx was recruited to ND10 by L2 expression. These studies suggested that ND10 might be involved in HPV capsidation.

Simian virus 40

Simian virus 40 (SV40) is a small DNA virus. Like other DNA viruses, SV40 starts transcription and replication adjacent to ND10. In an early study, we identified a specific viral DNA sequence and its binding protein that determine the location of these synthetic activities at such restricted nuclear sites[121]. A beta-galactosidase gene was introduced into an expression vector that contains partial and overlapping SV40 sequences. Transcripts derived from control plasmids were found throughout the nucleus and at highly concentrated sites but not at ND10. SV40 genomic segments supported ND10-associated transcription only when the origin and the coding sequence for the large T antigen were present. When the large T-antigen coding sequence was eliminated but the T antigen was constitutively expressed in COS-7 cells, the viral origin was sufficient to localize transcription and replication to ND10. Large T antigen expressed from plasmids without the viral core origin did not bind or localize to ND10. Blocking of DNA replication prevented the accumulation of transcripts at ND10, indicating that only sites with replicating templates accumulated transcripts. Transcription at ND10 did not enhance total protein synthesis of plasmid transcripts. These findings suggest that viral transcription at ND10 may only be a consequence of viral genomes directed to ND10 for replication. Although plasmid transcription can take place anywhere in the nucleus, T-antigen-directed replication is apparently restricted to ND10[121].

RNA VIRUSES

The first RNA virus studied for its interaction with ND10 was the lymphocytic choriomeningitis virus (LCMV), a single strand RNA virus, but interestingly, the interaction occurs in the cytoplasm. In cells infected with LCMV, the viral zinc-finger (Z) protein forms large bodies primarily in the cytoplasm. Z protein can redistribute PML from the nucleus to the cytoplasm, and PML and Z protein colocalize in the cytoplasm[35,122]. The similar function of Z protein was also found in other viruses of Arenaviridae[35,122]. The interaction of PML and Z proteins may influence certain unique characteristics of arenavirus infection.

Another RNA virus is hepatitis delta virus (HDV). HDV is a single-stranded RNA virus and has only one coding region producing the hepatitis delta antigen (HDAg). HDAg is expressed in two isoforms, small (S-HDAg) and large (L-HDAg). S-HDAg is required for the replication of HDV, while L-HDAg inhibits viral replication and is required for the envelopment of the HDV genomic RNA by hepatitis B virus proteins[123]. Bell *et al*[124] found that over half of the L-HDAg domains were localized beside ND10. At later times, ND10-associated proteins such as PML were found in larger HDAg complexes, in which PML was found chiefly in the rims of the spheres. Other ND10 components (SP100, Daxx, and NDP55) were found in the centers of the spheres. HDV genomic RNA was distributed more uniformly throughout the nucleus, but nascent viral RNA colocalizes with L-HDAg and the transcriptional repressor PML. These results suggest that this RNA virus, like DNA viruses, can alter the distribution of ND10-associated proteins and preferably transcribe mRNA at ND10. It is not clear whether the ND10-associated proteins (PML) play a role in the regulation of HDV RNA synthesis.

As for human immunodeficiency virus (HIV), the results have been controversial. Bell *et al*[125] reported that no significant relationship was observed between ND10 or any of the following: HIV-1 DNA, transcription foci, and integrated DNA. Their results showed that HIV-1 did not modify ND10 at early or late times of infection[125]. However, Turelli *et al*[126] reported that incoming retroviral preintegration complexes trigger the exporting-mediated cytoplasmic export of PML. They further described how the HIV genome associates with PML before nuclear migration. Further experiments are needed to reveal the detailed interaction of HIV and ND10.

VIRAL DISPERSION OF ND10

During viral infection, viruses and ND10 interact differently. The modification of ND10 structure can include (1) an increase in the size and number of ND10 per nucleus by double strand RNA viruses because their infection can induce IFN; (2) a change to the shape of ND10; (3) a decrease in the size or the number (of ND10) per nucleus; and (4) a total dispersal of ND10. Only CMV, EBV, and HSV have been clearly shown to disperse ND10. Here we take the MCMV infection as an example to show the real dispersing of ND10 (Figure 1). As we stated above,

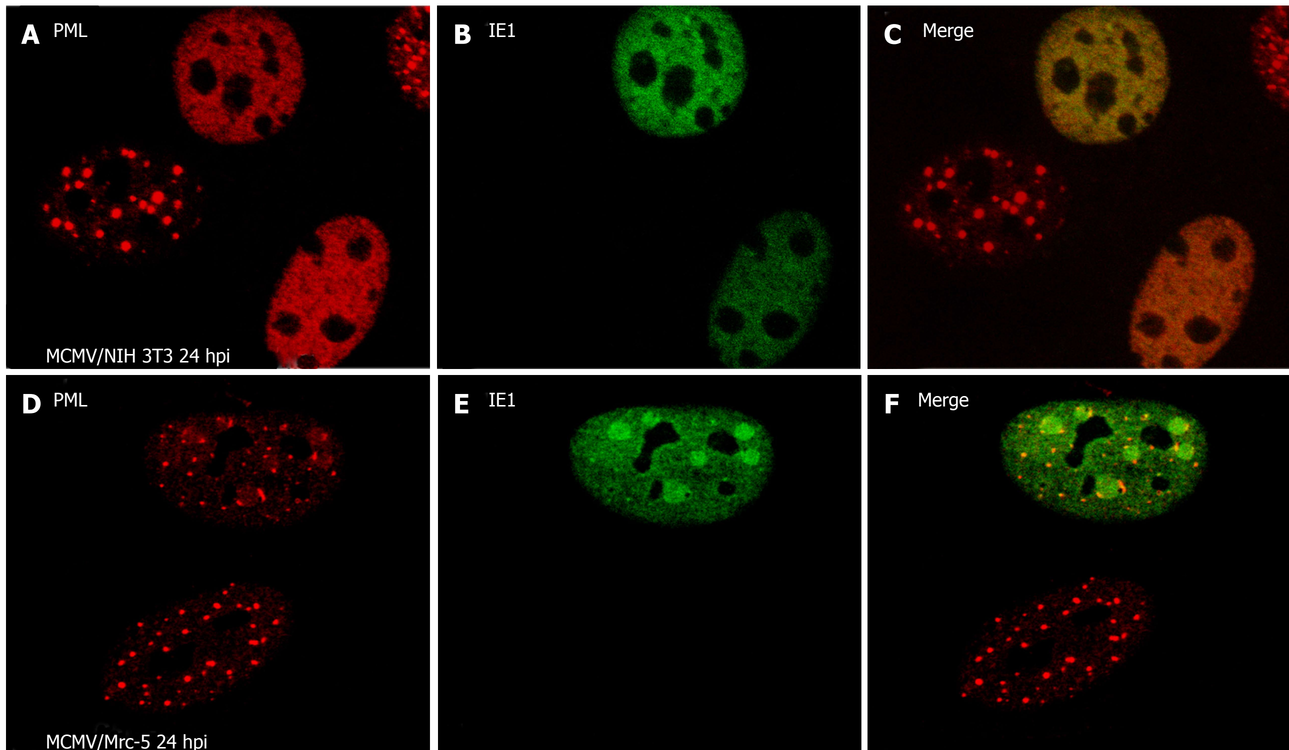

Figure 1 Immunofluorescent assay to show cytomegalovirus infection and nuclear domain 10. A: After murine cytomegalovirus (MCMV) infection in NIH3T3 cells for 24 h, cells were stained with anti-Promyelocytic Leukemia bodies (PML) antibody (rabbit) to show nuclear domain 10 (ND10) (in red); B: Anti-IE1 antibody (mouse) was used to show IE1 (in green); C: The merged picture is shown in; D: After MCMV infection in Mrc-5 cells for 24 h, cells were stained with anti-PML antibody (rabbit) to show ND10 (in red); E: Anti-IE1 antibody (mouse) was used to show IE1 (in green); F: The merged picture is shown.

CMV infection is species-specific. Interestingly, we discovered that murine CMV (MCMV) infection in human cells cannot disperse ND10[107], suggesting the involvement of ND10 in species-specificity. We performed comparative IFA to analyze the ability of IE1 to disperse ND10 in cross-species-infected cells as opposed to in natively infected (mouse) cells. We infected wt-MCMV into both Mrc-5 cells and NIH3T3 cells for 24 h. Cells were fixed and permeabilized and stained with anti-PML to show ND10 (red, Figure 1A, D) and with anti-IE1 to show the distribution of IE1 (green, Figure 1B, E). As can be seen in the MCMV-infected mouse cells, IE1 was diffusely distributed in the nucleus at 24 hpi. Interestingly, the IE1 of MCMV formed domains (Figure 1E) in human cells and lost the ability to disperse ND10, their distribution being different from that found in MCMV-infected mouse cells (Figure 1A-C). There is no standard for judging the level of viral effect on ND10 structure because ND10 number or size can vary in different cell cycles. Therefore, one has to be careful to make conclusions of dispersing or disrupting ND10 by any viral infection or transient transfection.

FUTURE DIRECTIONS FOR INVESTIGATIONS INTO THE VIRAL ASPECT OF ND10

A great deal of progress regarding the interaction of ND10 and viruses has been made in the past decades. A lot of questions are still left behind us, which makes the future direction of studies in the ND10-viruses field: (1) ND10 structure and ND10 protein are clearly related to cancer development (at least to some types of cancers). Therefore, the interaction of tumor viruses and ND10 should be the future focus of research in this field; (2) ND10 aggregate a lot of nuclear proteins that have different functions; we already know that SUMOylation is important for the formation of ND10. Are there any other nuclear functions needed for ND10 formation? Why do so many nuclear proteins meet in this place? (3) ND10 have been shown to be positioned beside SC35; SC35 is also related to transcribed RNA. What is the functional connection between ND10 and RNA? and (4) HIV DNA locates at SC35, not at ND10. HIV DNA is replicated and not integrated DNA (leftover). Given the fact that ND10 are located next to SC35, is it possible that they have any role with regard to HIV DNA?

ACKNOWLEDGEMENTS

This review is dedicated to the memory of Gerd Maul who was named the Father of ND10 in the international herpesvirus workshop. Dr. Maul was a talented, energetic, and creative cell biologist from the Wistar Institute (Philadelphia, PA) who passed away in August 2010, after devoting over 20 years of his life to ND10.

We apologize to those friends and colleagues whose pri-

mary work could not be cited because of space restrictions.

We thank Bob Ritchie of the Ponce School of Medicine and Health Sciences/RCMI Publications Office for his help with manuscript preparation.

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P- Reviewers Berardinis PD, Devaux CA, Laassri M, Kawasaki H
S- Editor Wen LL **L- Editor** A **E- Editor** Lu YJ

World Journal of Virology

Online Submissions: http://www.wjgnet.com/esps/
wjv@wjgnet.com
doi:10.5501/wjv.v2.i3.123

World J Virol 2013 August 12; 2(3): 123-135
ISSN 2220-3249 (online)
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ORIGINAL ARTICLE

Searching for nuclear export elements in hepatitis D virus RNA

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Supported by A grant from Fundação para a Ciência e Tecnologia, Portugal to Freitas N
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Received: May 7, 2013 Revised: July 26, 2013
Accepted: August 8, 2013
Published online: August 12, 2013

Abstract

AIM: To search for the presence of cis elements in hepatitis D virus (HDV) genomic and antigenomic RNA capable of promoting nuclear export.

METHODS: We made use of a well characterized chloramphenicol acetyl-transferase reporter system based on plasmid pDM138. Twenty cDNA fragments corresponding to different HDV genomic and antigenomic RNA sequences were inserted in plasmid pDM138, and used in transfection experiments in Huh7 cells. The relative amounts of HDV RNA in nuclear and cytoplasmic fractions were then determined by real-time polymerase chain reaction and Northern blotting. The secondary structure of the RNA sequences that displayed nuclear export ability was further predicted using a web interface. Finally, the sensitivity to leptomycin B was assessed in order to investigate possible cellular pathways involved in HDV RNA nuclear export.

RESULTS: Analysis of genomic RNA sequences did not allow identifying an unequivocal nuclear export element. However, two regions were found to promote the export of reporter mRNAs with efficiency higher than the negative controls albeit lower than the positive control. These regions correspond to nucleotides 266-489 and 584-920, respectively. In addition, when analyzing antigenomic RNA sequences a nuclear export element was found in positions 214-417. Export mediated by the nuclear export element of HDV antigenomic RNA is sensitive to leptomycin B suggesting a possible role of CRM1 in this transport pathway.

CONCLUSION: A cis-acting nuclear export element is present in nucleotides 214-417 of HDV antigenomic RNA.

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Key words: Hepatitis D virus; Genomic RNA; Antigenomic RNA; Nuclear export; Nuclear export element

Core tip: Hepatitis D virus (HDV) replicates in the nucleus and export of HDV RNPs to the cytoplasm is thought to be mediated by cis-elements present in virus RNA. We used a chloramphenicol acetyl-transferase reporter system in an attempt to identify the RNA sequences that mediate export to the cytoplasm. Several cDNA constructs coding for different HDV RNA (genomic and antigenomic) sequences were tested. Our results show that a cis-acting nuclear export element is present in positions 214-417 of antigenomic RNA. Two regions in genomic RNA were found to promote nuclear export with efficiency higher than the negative control although lower that the positive control.

Freitas N, Cunha C. Searching for nuclear export elements in hepatitis D virus RNA. *World J Virol* 2013; 2(3): 123-135 Available from: URL: http://www.wjgnet.com/2220-3249/full/v2/i3/123.htm DOI: http://dx.doi.org/10.5501/wjv.v2.i3.123

INTRODUCTION

Hepatitis D virus (HDV) is the only member of the Deltavirus genus and is considered to be a satellite virus of the hepatitis B virus (HBV)[1]. When compared with HBV alone, infection of human hepatocytes with both viruses increases liver damage and the risk of cirrhosis and fulminant disease[2,3]. The two viruses are associated due to the fact that the outer envelope of HDV consists of HBV surface antigens (HBsAgs) which are necessary for virus packaging and propagation of infection[4].

The HDV genome consists of a circular, closed ss-RNA molecule of approximately 1.7 kb and negative polarity. It is estimated that about 70% of this RNA molecule is internally base-paired resulting in the formation of a rod-like structure similar to plant viroids[5]. There is still a considerable lack of information, and even some controversy, concerning the mechanisms and host factors involved in HDV RNA replication. It seems to be generally accepted that replication occurs through a double rolling-circle mechanism involving the participation of at least host RNA polymerase Ⅱ, and resulting in the synthesis of multimeric antigenomic molecules[6,7]. Subsequently, these multimeric antigenomic molecules are self-cleaved and ligated at precise monomeric intervals by the HDV RNA ribozyme activity[8]. The monomeric antigenomes serve as templates for a second round of replication, by a similar mechanism, thus resulting in the synthesis of monomeric genomic RNA molecules. The HDV genome contains a single ORF that codes for a 24 kDa protein, the so-called small delta antigen (S-HDAg)[9]. As a consequence of an editing mechanism that converts an amber stop codon UAG into a tryptophan codon UGG in the antigenome, the ORF is extended by 19 additional aminoacids[10]. As a result, a 27 kDa protein, the large delta antigen (L-HDAg) is produced. These two proteins are thought to play different roles in the HDV replication cycle. S-HDAg is necessary for accumulation of virus RNA[11] and positively regulates ribozyme activity[12], and L-HDAg inhibits replication and interacts with HBsAgs to promote virus packaging[13,14]. HDV packaging occurs in the cytoplasm where the newly synthesized RNPs meet the HBsAgs to assemble mature virions. It has been previously shown that HDV RNPs shuttle continuously between the nucleus and the cytoplasm[15]. While nuclear import of virus RNPs is mediated by a nuclear localization signal in HDAgs[16], the export to the cytoplasm is believed to be mediated by a cis element present in the RNA molecule. This is supported by the fact that export of both genomic and antigenomic HDV RNAs was found to be independent of the presence of HDAgs[15]. Furthermore, Macnaughton and Lai reported that both genomic and antigenomic RNAs (gRNA and agRNA, respectively) are exported with similar efficiency at early times during replication[17]. Although cells expressing L-HDAg, HBsAgs, and agRNA were found to secret virus-like particles containing HDV agRNA[18], it is widely accepted that only gRNA molecules are packaged into newly synthesized virions. This observation led to the hypothesis that packaging is restricted to gRNA molecules due to the nuclear retention, and eventual further degradation, of HDV antigenomes. However, to our knowledge, no experimental evidences were obtained supporting this idea.

Simple retroviruses such as simian type D retroviruses have evolved mechanisms of RNA export based on the direct interaction of a cis-acting transport element [constitutive transport element (CTE)] with cellular transport receptors. The TAP protein, the human homologue of yeast Mex67p, is one of best studied host factors shown to interact with the CTE to promote nuclear export of unspliced simian retrovirus type D mRNAs[19]. TAP was also identified as one of the proteins responsible for export of cellular mRNAs[20]. On the other hand, complex retroviruses were shown to use a different pathway for export of intron-containing mRNAs. This pathway involves the participation of the cellular protein CRM1[21]. In the case of human immunodeficiency virus-1 (HIV-1), the association of CRM1 with intron-containing mRNAs is mediated by the virus protein Rev which recognizes a specific sequence named rev-responsive element (RRE)[22]. Additionally, the HBV posttranscriptional regulatory element (PRE), which was reported to play a crucial role in export of virus mRNAs to the cytoplasm, seems to use a distinct, not yet identified nuclear export pathway[23].

In an attempt to clarify whether HDV gRNA and agRNA contain cis elements capable of promoting the export to the cytoplasm, we made use of a chloranphenicol acetyl-transferase (CAT) reporter system, in transfection experiments, to identify and characterize putative nuclear export elements.

MATERIALS AND METHODS

Cell culture and transfection

HuH-7 cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10%FBS (Invitrogen). Cells were grown as monolayers at 37 ℃, in a humidified atmosphere containing 5%CO_2. Transfection assays were performed using the Fugene6 Transfection Reagent (Roche) and 1 μg plasmid DNA per 35-mm well, according to the manufacturer's instructions. To control for transfection effiency in CAT assays, 20 ng of plasmid pSV-β-galactosidase (Promega) were cotransfected with 100 ng of reporter CAT constructs, and 1.88 μg pUC19. Cells were analysed 24 h post-transfection. In some experiments, 10 nmol/L leptomycin B was added to the medium 18 h after transfection and cells were subsequently incubated for 6 h before analysis as earlier described[24].

Plasmid constructs

Plasmid pDM138[25,26] was a kind gift of Tristram Parslow (Emory University School of Medicine, Atlanta, United States). Plasmids pDM138-PRE(+) and pDM138-PRE(-) were generously provided by Benedict Yen (University of California, San Francisco, United States). Plasmids pDM138-PRE(+) and pDM138-PRE(-) contain a DNA fragment of approximately 570 bp that codes for the

Table 1 Primers used to amplify hepatitis delta virus cDNA fragments

HDV cDNA fragments	*Forward primer* 5'→3'	*Reverse primer* 5'→3'	Fragment length (bp)	Genome location (Sense)	Antigenome location (Antisense)
A1	CGCATCGATACTCCCTGCAGATTGGGGA	CGCATCGATATTCACCGACAAGGAGAGGC	243	360-602	1078-1320
A2	CGCATCGATGCCTCTCCTTGTCGGTGAAT	CGCATCGATGAGACCTCCGGAAGACAAAGA	204	176-379	1301-1504
A3	CGCATCGATTTCCGGAGGTCTCTCTCGAGT	CGCATCGATTCTCCTCGCTCGGAACTTG	214	1654-679/188	1492-1679/26
A4	CGCATCGATTTCCTCGGTCAACCTCCTGA	CGCATCGATATAAGGATGGAGAGGGGGCT	224	266-489	1191-1414
A5	CGCATCGATCCCCTCTCCATCCTTATCCT	CGCATCGATAGGGAGAGAAGAGATCCTCGA	172	114-285	1395-1566
A6	CGCATCGATCGAATGGGACCCACAAATCT	CGCATCGATTCCCCAATCTGCAGGGAGT	337	584-920	760-1096
A7	CGCATCGATCCCAATCCCAGATCTGGAGA	CGCATCGATTTTGCTTTCCTCCTCGCTTC	204	1263-1466	214-417
A8	CGCATCGATAAAGAAAGCAACGGGGCTAG	CGCATCGATGGGAGTCGGAATCGAGCAT	199	1067-1265	415-613
A9	CGCATCGATATGCTCGATTCCGACTCCC	CGCATCGATCCTAGAGAGATTTGTGGGTCCC	191	895-1085	595-785
A10	CGCATCGATCAAGTTCCGAGCGAGGAGAC	CGCATCGATTCTCCAGATCTGGGATTGGG	227	1446-1672	8-234

Nucleotide sequence of the primers used in PCR reactions to amplify hepatitis delta virus (HDV) cDNA fragments. The *Cla* I restriction site is underlined. The size of the amplicon and the corresponding location in the genome, when cloned in sense and antisense orientations, are indicated.

Table 2 Primers used to amplify truncated forms of the A7AS agRNA sequence

HDV cDNA fragments	*Forward primer* 5'→3'	*Reverse primer* 5'→3'	Fragment length (bp)
269-417	CGCATCGATGGGAGGAATCCACTCGGAGA	CGCATCGATTTTGCTTTCCTCCTCGCTTC	149
214-379	CGCATCGATCCCAATCCCAGATCTGGAGA	CGCATCGATGCATCTCCTCCTATCGCTATGG	166
214-403	CGCATCGATCCCAATCCCAGATCTGGAGA	CGCATCGATGCTTCGGTCTCCCCCTACTC	190
244-417	CGCATCGATCCCGAAGGGTTGAGTAGCAC	CGCATCGATTTTGCTTTCCTCCTCGCTTC	174
244-403	CGCATCGATCCCGAAGGGTTGAGTAGCAC	CGCATCGATGCTTCGGTCTCCCCCTACTC	160
314-417	CGCATCGATACCCCTTCAGCGAACAAGAG	CGCATCGATTTTGCTTTCCTCCTCGCTTC	104
214-322	CGCATCGATCCCAATCCCAGATCTGGAGA	CGCATCGATTGAAGGGGTCCTCGGAGGT	109
269-379	CGCATCGATGGGAGGAATCCACTCGGAGA	CGCATCGATGCATCTCCTCCTATCGCTATGG	111

Nucleotide sequence of the primers used to amplify eight truncated forms of the A7AS sequence localized in position 214-417 in the hepatitis delta virus (HDV) antigenomic cDNA. The underlined sequence corresponds to a *Cla* I restriction site.

HBV post-transcriptional regulatory element (PRE). This fragment was inserted in the unique *Cla* I site of pDM138 in both sense and antisense orientations, originating plasmids pDM138-PRE(+) and pDM138-PRE(-), respectively[27].

Twenty vectors containing cDNA inserts corresponding to 10 different regions of the HDV agRNA cloned, in both orientations, in the unique *Cla* I site of pDM138 were generated by polymerase chain reaction (PCR), using the primers listed in Table 1, and plasmid pDL481[18] as template. This plasmid was designed to code for full-length HDV antigenomic RNA and was a kind gift of John Taylor (Fox Chase Cancer Center, Philadelphia, United States). The primers were designed in order to include a *Cla* I site in the 5' end. The obtained PCR fragments were purified using the GFX PCR and Gel Band kit (GE Healthcare) and ligated with *Cla* I digested pDM138 using the Rapid DNA Ligation kit (Roche) according with the instructions of the manufacturer. The correct insertion in sense or antisense orientations of the fragments was first monitored by restriction endonuclease analysis with *Ban* II, *EcoR* I, *Ban* II and *Nhe* I, *Pst* I, *Xho* I, *Bgl* II, and *Bam*HI (Fermentas) followed by DNA sequencing.

Additionally, we constructed eight pDM138 derived vectors containing different portions of the cDNA complementary to the HDV agRNA sequence comprised between nt 214 and 417. The strategy was similar to the one described above and the primers used in PCR reactions are listed in Table 2.

Plasmid pDL481ΔNEE, containing full-length HDV agRNA from which the sequence corresponding to the putative nuclear export element (NEE) was removed (nt 2473-2696), was constructed as follows: first we digested plasmid pDL481 with *Apa* I (Invitrogen) which cuts at positions 2696 and 3208. The two resulting 5762 bp and 512 bp fragments were separated by electrophoresis, and the 5762 bp fragment was recovered and purified using the GFX PCR DNA kit (GE Healthcare). Subsequently, this fragment was further digested with *Nhe* I (GE Healthcare). Two fragments were obtained with 5539 bp and 223 bp, respectively. The 5539 bp fragment was purified as above. The next step consisted of the amplification of the 2696-3208 nt region of plasmid pDL481. To do this, we used the following primers: Fwd 5'GGGCCCGCTTAGCGCCCCTTTTTCTTCCACCTT 3' in which a *Apa* I and a *Nhe* I restriction sites were included in the 5' end, and Rev 5' GGGCCCACCGGTGCCCCCTCTCCATCCTTAT 3' in which a *Apa* I (underlined) and a *Age* I (grey box) restriction sites were also added at the 5' end. The amplified 512 bp fragment was purified as above, and the two 512 bp and 5539 bp

fragments were then ligated using the Rapid DNA Ligation kit (Roche) according to the specifications of the manufacturer. The correct construction of the recombinant vector was tested by digestion with *Xho* I followed by DNA sequencing.

Plasmid pDL481Δδ was constructed by removing a 189 bp sequence, comprised between nucleotides 3208 and 3397 in pDL481. This sequence is complementary to the putative NEE in the HDV antigenome. To do this we first digested plasmid pDL481 with *Bpi* I (Fermentas) which cuts at positions 3098 and 3397 generating two 5975 bp and 299 bp fragments, respectively. Next, the two fragments were purified and incubated with 5 U Klenow enzyme (Fermentas), 0.05 mmol/L dNTPs, and Klenow buffer (Fermentas), for 10 min at 37 ℃ to generate blunt ends. The resulting blunt-ended fragments were digested with *Apa* I (Fermentas) and 4 fragments were obtained with 110, 189, 402 and 5573 bp, respectively. The 5573 bp fragment was purified as above and used in ligation reactions with the 512 bp fragment of pDL481 (nt 2696-3208) which was amplified by PCR as described before. Prior to ligation, compatible ends were generated in the 512 bp amplified DNA fragment. To do this, we first digested this fragment with *Age* I . Following incubation with Klenow and dNTPs to generate blunt ends, as above described, this fragment was next digested with *Apa* I . After digestion, the DNA was purified using the GFX PCR DNA kit (GE Healthcare), and subsequently used in ligation reactions with the 5573 bp fragment. Ligations were performed using the Rapid DNA Ligation kit (Roche) following the instructions of the manufacturer. The correct construction of the recombinant vector was monitored by restriction endonuclease analysis with *Xho* I followed by DNA sequencing.

Plasmid pDL481ΔNEEδ, from which the putative NEE and the corresponding complementary sequence in the HDV antigenome were deleted, was generated by removing a 223 bp sequence between positions 2473 and 2696 and a 189 bp sequence comprised between nucleotides 3208 and 3397 in plasmid pDL481. The first approach consisted of digesting plasmid pDL481 with *Bpi* I followed by generation of blunt ends with Klenow enzyme, as above described for plasmid pDL481Δδ. Next, we digested the two resulting fragments with *Nhe* I . Three fragments of 299, 625 and 5350 bp, respectively, were obtained and separated by agarose gel electrophoresis. The 5350 bp fragment was purified from the gel using the GFX PCR DNA kit (GE Healthcare) and used in subsequent ligation reactions. Before ligation with the 512 bp fragment of pDL481 (nt 2696-3208) obtained by PCR, compatible ends were generated. This was performed by digesting the 512 bp fragment with *Age* I . After filling the resulting cohesive ends with dNTPs, as above described, the obtained blunt fragment was further digested with *Nhe* I . After purification, this DNA fragment was finally ligated with the previously obtained 5350 bp DNA fragment, as described. Finally, we tested the correct construction of the recombinant plasmid by digestion with *Xho* I followed by DNA sequencing.

In situ hybridization

In situ hybridization was performed on pDL481 and pDL542 transfected HuH-7 cells essentially as described[15]. After transfection, cells were incubated at 37 ℃ for 24 h. All fixation, permeabilization, and denaturation steps were exactly as described[15]. Plasmid pSVL(D3) was labeled by nick-translation with digoxigenin-11-dUTP and used as a probe. This plasmid contains a trimer of full-length HDV cDNA cloned in pSVL (GE Healthcare). Hybridization was performed overnight at 37 ℃ and the probe was detected using a monoclonal anti-digoxigenin antibody conjugated with FITC (Roche) and a secondary anti-FITC antibody conjugated with Alexa-488 (Jackson ImmunoResearch Laboratories). Samples were analyzed under a Zeiss META LSM 510 microscope calibrated with multicolor fluorescent beads (Molecular probes). Green fluorescence was detected using a 488 nm Argon laser.

Northern blotting

For Northern blotting, cytoplasmic mRNA was extracted from HuH-7 cells using the Oligotex Direct mRNA Mini kit (Qiagen). For each obtained sample, 10 μg mRNA was separated by formaldehyde agarose gel electrophoresis and transferred to Nylon membranes (Hybond-N, GE Healthcare) using standard protocols[28]. Hybridization was performed using a digoxigenin-11-dUTP (dig-11-dUTP) labeled DNA probe. Plasmid pDM138 was used as template to amplify and label, by asymmetric PCR, a 481 bp region in the ORF of the CAT protein (nucleotide position 109-590). The primers used in PCR reactions were: Fwd 5' GTTCAGCTGGATATTACGGCC 3' and Rev 5' TCACAGACGGCATGATGAAC 3'. Typically, reaction mixtures contained 2 mmol/L $MgCl_2$, 0.2 mmol/L dATP, dCTP and dGTP, 0.13 mmol/L dTTP, 0.07 mmol/L dig-11-dUTP (Roche), 0.1 μmol/L forward primer, 1 μmol/L reverse primer, 10 ng template DNA, 2.5 U Taq DNA polymerase (Fermentas), in PCR buffer for a final volume of 50 μL. After amplification and labeling, probes were purified using the GFX PCR DNA kit (GE Healthcare), and used for hybridization.

Hybridization was performed according to standard protocols[28] and the hybridized probe was detected with a monoclonal anti-digoxigenin antibody conjugated with peroxidase (Roche). Membrane development was achieved with the Lumi-light^{PLUS} Western Blotting Kit, Mouse/Rabbit (Roche) under the conditions indicated by the manufacturer.

Real-time PCR

Nuclear and cytoplasmic HuH-7 cell fractions were obtained according to a previously described method[29], and used for isolation of RNA with the NucleoSpin® RNA/protein kit (Macherey-Nagel) following the manufacturer's specifications. The RNA samples were then treated with DNase I using the DNA-free™ kit (Ambion), also ac-

cording to the instructions of the manufacturer, and used as templates for synthesis of cDNA. cDNA synthesis reactions typically contained approximately 5 μg total RNA, 0.2 μg random primers, 2 mmol/L dNTPs, 200 U Revert Aid™ M-MuLV Reverse Transcriptase (Fermentas), and 20 U RNase inhibitor (Fermentas) in a final volume of 20 μL. Reactions were performed at 42 ℃, for 1 h, and the obtained cDNA was finally purified using the GFX PCR DNA and Gel Band purification kit (GE Healthcare).

Real-time PCR experiments were performed essentially as described[30]. The qPCR Core kit for SYBR® Green I (Eurogentec) was used following the specifications of the manufacturer. Reaction mixtures typically contained 3.5 mmol/L $MgCl_2$, 200 μmol/L each dNTP, 300 nmol/L each primer, 0.025 U/μL HotGoldStar enzyme, and reaction buffer in a final volume of 20 μL. Reactions were performed in 96-well plates with optical caps in a GeneAmp® 5700 Sequence Detector System (all from Applied Biosystems). The PCR program used for amplification was: 10 min at 95 ℃, 40 cycles with 15 s at 95 ℃ and 1 min at 60 ℃. Each sample was assayed in triplicate and analysed with the GeneAmp® 5700 SDS v1.1 software and Microsoft Excel.

The relative quantification of RNA was performed according to the $2^{-\Delta\Delta Ct}$ method earlier described[31]. The β-2-microglobulin gene (*β2MG*; GenBank accession number NM_004048) was used as reference gene to which all the samples were compared with. The program Primer Express™ (Applied Biosystems) and the bioinformatics tool Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html) were used to design primers for the reference gene and target HDV cDNA sequence (GenBank accession number M21012). Melting temperature, GC content, length, and secondary structure were taken in consideration for primer design. The cDNA sequences were obtained from GenBank database from NCBI. The primers used in these experiments were, respectively: HDV Fwd 5’ CAGAGATTCTCCGGCGTTGT 3’, Rev 5’ CGGTAAAGAGCATTGGAACG 3’; β2MG Fwd 5’ GGCTATCCAGCGTACTCCAA 3’, Rev 5’ TCACACGGCAGGCATACTC 3’.

Western blotting

For western blot, protein extracts were prepared using with the NucleoSpin® RNA/protein kit (Macherey-Nagel) according to the manufacturer’s instructions and dissolved in sample buffer. Proteins were separated by electrophoresis on 12%SDS-polyacrylamide gels, and subsequently electroblotted onto nitrocellulose membranes (Schleicher and Schuell) as previously described[32]. Membranes were blocked with 5% low fat milk powder in PBS, and incubated for 1 h with 1 μg/mL of a primary mouse monoclonal antibody anti-GAPDH (Ambion). After washing with 2% low fat milk powder in PBS, membranes were further incubated with a secondary anti-mouse IgG antibody conjugated with horseradish peroxidase (BioRad). After washing, membranes were rinsed with PBS and subsequently developed using the ECL™ Western blotting analysis system (GE Healthcare).

CAT assay

Determination of CAT expression was performed using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Roche). Briefly, 24 h post-transfection HuH-7 cells were washed with ice-cold PBS and incubated with 500 μL lysis buffer for 30 min, at room temperature. After centrifugation, the supernatants were collected, and 200 μL were added to individual wells of the ELISA plate. CAT detection was performed with a polyclonal anti-CAT antibody conjugated with digoxigenin followed by incubation with a monoclonal anti-digoxigenin antibody conjugated with peroxidase (Roche), as indicated by the manufacturer. The concentration of unknown samples was determined from a standard curve constructed from 1:2 serial dilutions of the standards.

To normalize for transfection efficiencies HuH-7 cells were cotransfected with plasmid pSV-β-galactosidase (Promega) and β-galactosidase (β-Gal) expression was monitored using a commercial ELISA kit (Roche). Briefly, 200 μL of the same cell lysis supernatants obtained as above described were loaded onto individual wells of the ELISA plate. The ELISA assay was performed according to the specifications of the manufacturer and β-Gal concentrations were determined from a standard curve obtained from 1:2 serial dilutions of the standards. All assays were performed in triplicate.

RESULTS

HDV gRNA and agRNA are exported to the cytoplasm

It was earlier reported that both HDV gRNA and agRNA are exported to the cytoplasm in HuH-7 cells[15,17]. This export is independent of the presence of HDAgs, and thus it is likely to rely on the direct interaction of the virus RNA with host factors[15]. Furthermore, Northern blot analysis of HuH-7 transfected cells seemed to indicate that the relative amounts of gRNA and agRNA in the nucleus and cytoplasm remained nearly equimolar up to 28 h after transfection[17].

In order to confirm that HDV agRNA is efficiently exported to the cytoplasm of HuH-7 cells we made use of plasmid pDL481[18], which codes exclusively for HDV agRNA, in transfection experiments. Plasmid pDL542[18], which codes exclusively for gRNA, was used in parallel experiments. Preliminary *in situ* hybridization analysis confirmed that both HDV gRNA and agRNA can be detected in the nuclear and cytoplasmic compartments of HuH-7 cells (Figure 1) 24 h post-transfection.

Since this approach did not allow us to determine if gRNA and agRNA are exported to the cytoplasm with similar efficiency with decided to quantify, by Real-time PCR, the amounts of both molecules in the nuclear and cytoplasmic compartments. To do this, HuH-7 cells were transfected with plasmids pDL481 and pDL542 respectively. After 24 h incubation, RNA samples were obtained

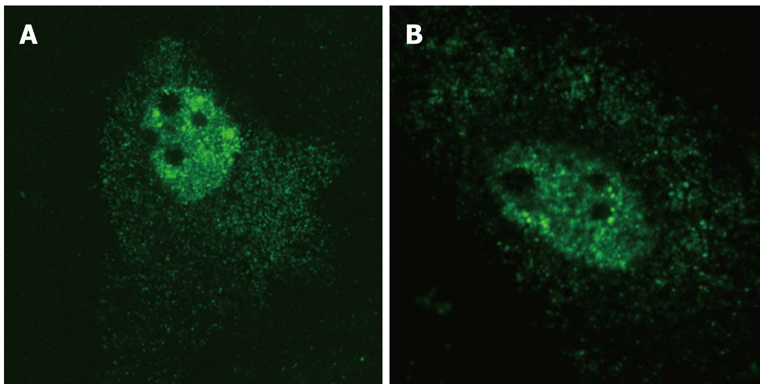

Figure 1 Intracellular localization of hepatitis delta virus gRNA (A) and agRNA (B). HuH-7 cells were trasnfected with plasmids pDL542 and pDL481, respectively, and virus RNA was detected by *in situ* hybridization with a dig-11-dUTP labeled probe. Both gRNA and agRNA can be observed in the nucleus and cytoplasm (green).

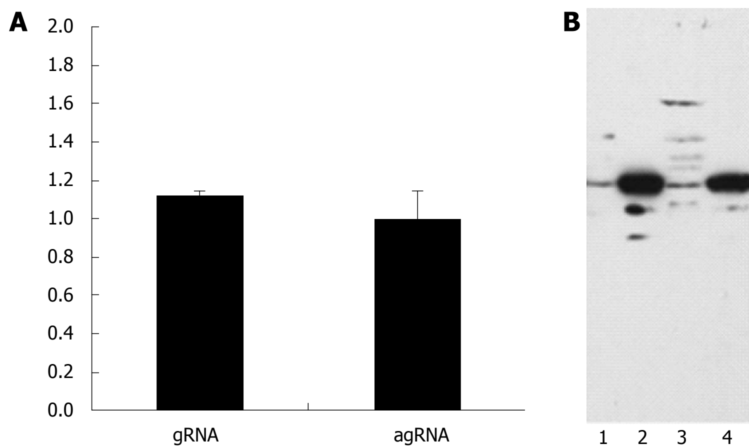

Figure 2 Nucleo-cytoplasmic distribution of hepatitis delta virus gRNA and agRNA. HuH-7 cells were trasnfected with plasmids pDL542 and pDL481, respectively. A: The relative quantification of HDV RNA was performed by real time-polymerase chain reaction using the $2^{-\Delta\Delta Ct}$ method. Results are presented as the cytoplasmic /nuclear ratio (C/N) and correspond to the mean of three independent experiments. Bars indicate the standard deviation; B: Western blotting analysis of nuclear and cytoplasmic HuH-7 cell protein fractions. Equivalent amounts of nuclear (lanes 1 and 3) and cytoplasmic (lanes 2 and 4) protein fractions used for quantification of gRNA (lanes 1 and 2) and agRNA (lanes 3 and 4) were separated in 12% SDS-PAGE gels. The possible contamination of nuclear fractions was monitored by using an anti-GAPDH antibody.

from nuclear and cytoplasmic fractions for subsequent use in Real-time qPCR experiments. The possible cross contamination of nuclear and cytoplasmic fractions was monitored by Western blotting using an anti-GADPH antibody (Figure 2B). The reference gene in qPCR experiments was β-2-microglobulin (Genbank accession number P61769).The obtained results are displayed, as the cytoplasm to nuclear ratio of gRNA and agRNA, respectively, in Figure 2A. The HDV agRNA was found to be distributed in equimolar amounts between the nucleus and cytoplasm of HuH-7 cells 24 h after transfection. At the same time point the HDV gRNA was found in slightly higher amounts in the cytoplasm. These results are consistent with previously reported data and clearly indicate that the virus agRNA is efficiently exported to the cytoplasm.

Identifying nuclear export elements in HDV genomic and antigenomic RNA

After establishing that HDV gRNA and agRNA are exported to the cytoplasm, in the absence of HDAgs, with similar efficiency, until at least 24 h after transfection, we decided to investigate the eventual presence of a cis-acting nuclear export element in both RNA molecules. To do this, we made use of plasmid pDM138[25,26]. This plasmid codes for the second half of the HIV-1/SF2 genome under the control of the SV40 promoter. The DNA sequence coding for the CAT gene was inserted in the HIV-1 envelope gene intron, and the RRE was substituted by a linker containing a unique *Cla* I restriction site. Nuclear export of mRNAs derived from pDM138, and subsequent expression of the reporter CAT protein, is thus dependent on the insertion of a functional transport element in the *Cla* I restriction site.

Initially, we amplified by PCR 20 cDNA fragments covering the entire HDV genome (10 fragments) and antigenome (also 10 fragments). The size of the obtained fragments ranged from 167 to 337 nt and the respective location in the genome is displayed in Table 1. Each fragment was subsequently cloned in the unique *Cla* I site of plasmid pDM138 in both sense and antisense orientations. We thus obtained 20 different constructs which, after being sequenced to confirm the correct insertion and orientation, were used to transfect HuH-7 cells. As positive and negative controls in these experiments we used plasmids pDM138-PRE(+) and pDM138-PRE(-), respectively. These plasmids contain the HBV post-transcriptional regulatory element, cloned in sense and antisense orientations, respectively[27]. In all experiments, plasmid pSV-β-Gal (Promega) was used to cotransfect HuH-7 cells in order to normalize for transfection efficiencies. Twenty four hours after transfection, total protein extracts were prepared and the production of CAT and β-Gal was determined by ELISA. Figure 3 displays the obtained results.

When analyzing gRNA all the tested sequences were found to be unable to promote the export of CAT mRNAs with efficiency as high as that determined for the

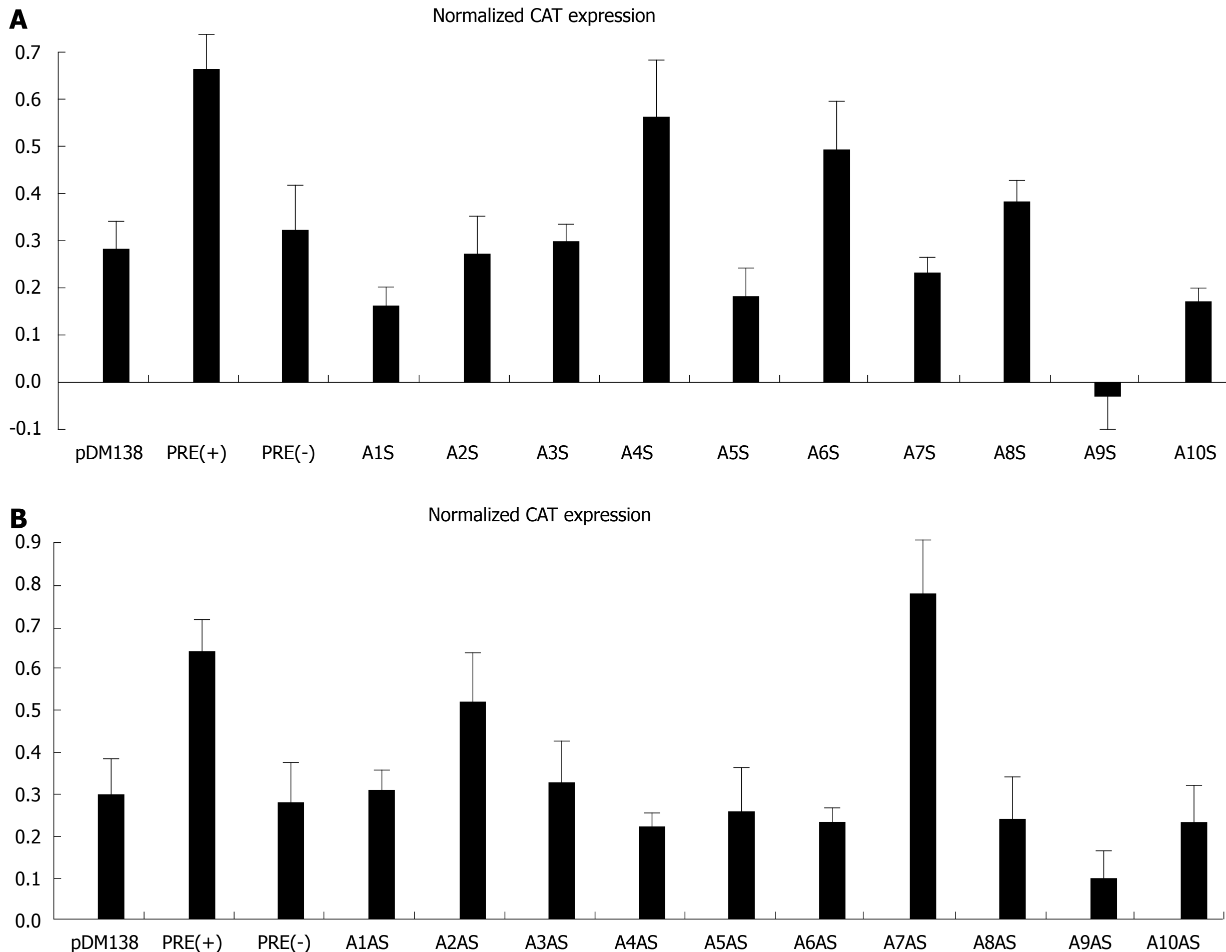

Figure 3 Analysis of chloranphenicol acetyl-transferase expression in HuH-7 cells transfected with plasmids pDM138, pDM138 (PRE+), pDM138 (PRE-), pDM138 A1S-pDM138 A10S (A), and pDM138 A1AS-pDM138 A10AS (B). In order to normalize for transfection efficiency, cells were co-transfected with plasmid pSV-β-Gal (Promega). Chloranphenicol acetyl-transferase (CAT) and β-Gal expression levels were determined by ELISA. Normalization of CAT expression levels was calculated by dividing the values obtained for the CAT protein by the values obtained for the β-Gal protein. The results correspond to the mean of three independent experiments. Bars represent the standard deviation.

positive pDM138-PRE(+) control. Nevertheless, two of the analyzed gRNA sequences, corresponding to nucleotides 266-489 and 584-920 (A4S, and A6S, respectively), showed an export-promoting ability slightly lower than the positive control albeit clearly higher than the negative control pDM138-PRE(-).

The analysis of agRNA coding sequences, however, showed that in HuH-7 cells transfected with plasmid pDM138-A7AS, which contains the HDV agRNA sequence corresponding to nucleotides 214-417, the expression levels of the reporter CAT protein are higher than those detected for the pDM138-PRE(+) positive control.

With the exception of A7AS and A2AS sequences, all the remaining tested constructs were found to be unable to promote the export of heterologous intron-containing mRNAs since the detected CAT expression levels were comparable or even lower than those observed in negative pDM138-PRE(-) transfected HuH-7 cell controls. The CAT expression values obtained for the A2AS sequence, however, were found to be intermediate between those obtained for the positive pDM138-PRE(+) and negative pDM138-PRE(-) controls.

According to what has been previously reported for the HBV PRE[27], the export promoting activity of the identified A7AS sequence in the HDV antigenome is dependent on its orientation relative to the ORF of the reporter gene. In fact, when cloned in opposite orientation (A7S), the A7AS sequence (nt 214-417) was not functional, since the observed CAT expression levels were similar to those found for the pDM138-PRE(-) negative control (data not shown).

Since the quantification of CAT expression levels by ELISA represents an indirect approach for the determination of intron-containing mRNAs accumulation in the cytoplasm, we decided to investigate the presence of CAT mRNAs in cytoplasmic fractions, after transfection, by Northern blot. To do this, HuH-7 cells were transfected with plasmid pDM138-A7AS or plasmids pDM138-PRE(+) pDM138-A9AS as positive and negative controls, respectively, and after 24 h incubation total and cytoplasmic fractions were prepared and used for further mRNA extraction. After electrophoresis, the mRNA samples were transferred to nylon membranes, and a single-stranded dig-11-dUTP labeled DNA probe,

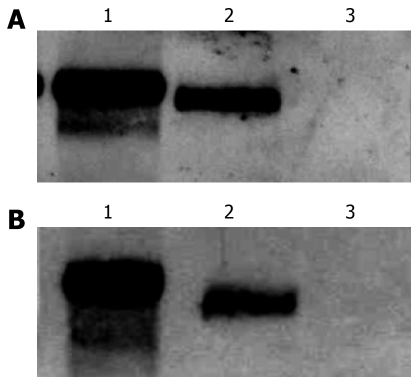

Figure 4 Northern blotting analysis of reporter chloranphenicol acetyltransferase mRNA in total (A) and cytoplasmic (B) fractions of HuH-7 cells transfected with plasmids pDM138 (PRE+), pDM138 A7AS, and pDM138 A9AS (lanes 1, 2 and 3 respectively). Hybridization was performed using a dig-11-dUTP labeled probe. A peroxidase conjugated anti-digoxigenin antibody was used to detect the hybridized probe.

which specifically hybridizes with CAT mRNA, was used in Northern blot assays. As expected, it was possible to detect the presence of CAT mRNA in cytoplasmic fractions of pDM138-PRE(+) and pDM138-A7AS transfected HuH-7 cells but not in pDM138-A9AS transfected cells (Figure 4).

The absence in Northern blot experiments of detected CAT mRNA in total and cytoplasmic fractions of pDM138-A9AS transfected cells is compatible with the data obtained by ELISA. The A9AS sequence includes the agRNA autocatalytic ribozyme domain. It is, thus, possible that CAT mRNAs that include the A9AS sequence are degraded in the nucleus before export to the cytoplasm. In conclusion, these results indicate that the HDV agRNA sequence located between nucleotides 214-417 can efficiently promote the export of heterologous intron-less RNAs. Moreover, the increase in CAT expression levels observed in pDM138-A7AS transfected cells is a consequence of the export and accumulation of the respective reporter mRNAs in the cytoplasm.

Analysis of the structure of the nuclear export element in agRNA

Having established that the HDV agRNA sequence corresponding to nucleotides 214-417 (A7AS) is able to promote the nuclear export of heterologous intron-containing RNAs, we next decided to analyze it in more detail. First, we generated by PCR eight truncated forms of the A7AS motif and cloned them in the unique *Cla* I site of plasmid pDM138. These truncated forms correspond to the A7AS sequence from which several nucleotides were removed from the 5’ and 3’ ends. The constructs were subsequently used to transfect HuH-7 cells, and after 24 h CAT expression was determined by ELISA. The obtained CAT expression values were normalized for transfection efficiency by cotransfection with plasmid pSV-β-Gal followed by determination of β-Gal expression. The obtained results allowed us to conclude that the agRNA sequences comprised between nucleotides 214-322, 214-379, 214-403, 244-403, and 244-417 induce an increase in CAT expression comparable to the observed for the HBV PRE(+) positive control (Figure 5) suggesting that these sequences are sufficient to promote export of heterologous RNAs.

Additionally, the first 30 nucleotides localized 3’ in the A7AS sequence seem not to be crucial to promote nuclear export since its deletion did not significantly affect the detected amounts of CAT expression. All the remaining analyzed sequences, 269-417, 314-417, and 269-379 were found to be considerably less efficient in promoting nuclear export.

Analysis of the NEE function in the context of the full-length agRNA

The identified NEE is localized in the central region of the rod-like full-length agRNA molecule. It could be possible that the complementary RNA sequence that pairs with the NEE in the antigenome is possibly also involved in the nuclear export of the agRNA. To test this hypothesis we constructed three deletion mutants of plasmid pDL481, which codes for the complete HDV agRNA molecule: pDL481ΔNEE in which the NEE was deleted (nt 214-417), pDL481Δδ in which the complementary to the NEE sequence was deleted (nt 1179-1385), and pDL481ΔNEEδ which does not contain the NEE and the corresponding complementary sequence (nt 214-417 and 1179-1385, respectively).

Plasmids pDL542, pDL481, and the obtained deletion constructs were used to transfect HuH-7 cells. Twenty four hours post-transfection, nuclear and cytoplasmic fractions were prepared and the RNAs derived from pDL542, pDL481, pDL481ΔNEE, pDL481Δδ, and pDL481ΔNEEδ, respectively, were quantified by qRT-PCR. Possible cross contaminations of nuclear and cytoplasmic fractions were monitored by western blot using an anti-GAPDH antibody as described before. The obtained results confirmed that both gRNA and agRNA are efficiently exported to the cytoplasm 24 h post-transfection. Additionally, the RNA derived from plasmid pDL481Δδ, in which the sequence coding for the region complementary to the NEE was deleted, showed to be efficiently exported (Figure 6). In contrast, the RNAs coded by plasmids pDL481ΔNEEδ and pDL481ΔNEE were mostly retained in the nucleus. Only about 40% of the total amount of these RNAs was found in the cytoplasm of transfected cells when compared to wt pDL481. These results indicate that the complementary to the NEE sequence in the HDV antigenome (nt 1179-1385) is not involved in nuclear export since its deletion did not reduce the amounts of agRNA detected in the cytoplasm.

HDV agRNA export is sensitive to leptomycin B

Export of cellular RNAs is accomplished using different pathways which involve the participation of distinct transport receptors. Typical examples include the export of mRNAs, which is mediated by members of NXF family of proteins, namely the TAP protein, and the

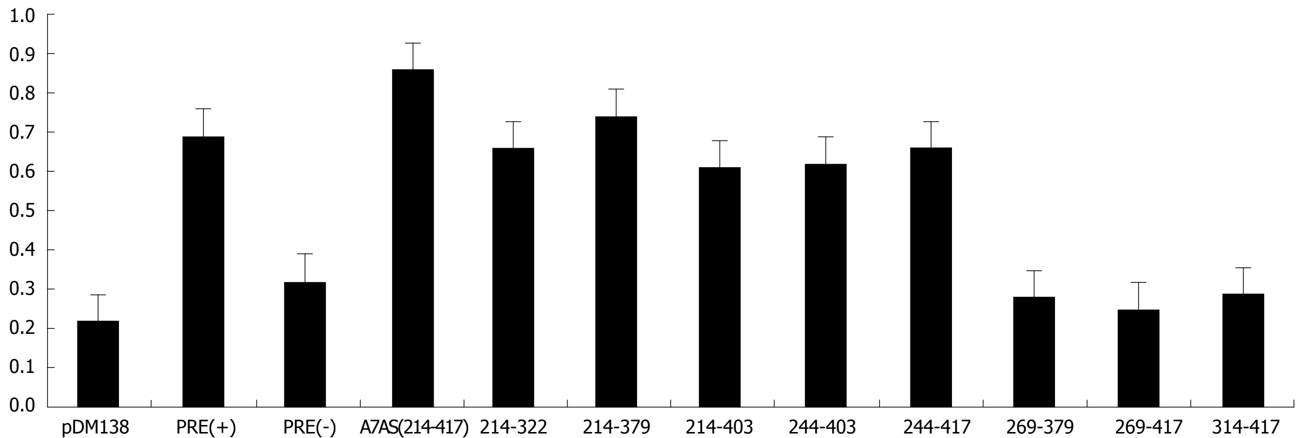

Figure 5 Analysis of chloranphenicol acetyl-transferase expression in HuH-7 cells transfected with plasmids pDM138, pDM138 (PRE+), pDM138 (PRE-), pDM138 A7AS (214-417), pDM138-314-417, pDM138-214-322, pDM138-269-379, pDM138-244-379, pDM138-269-417, pDM138-214-403, pDM138-244-403, and pDM138-244-417. Chloranphenicol acetyl-transferase (CAT) and β-Gal expression levels were determined by enzyme-linked immunosorbent assay. The CAT expression values were normalized for transfection efficiency by transfecting HuH-7 cells with plasmid pSV-β-Gal (Promega) followed by determination of β-Gal expression. CAT expression values were divided by the corresponding β-Gal expression values, and the displayed results correspond to the mean of three independent experiments.

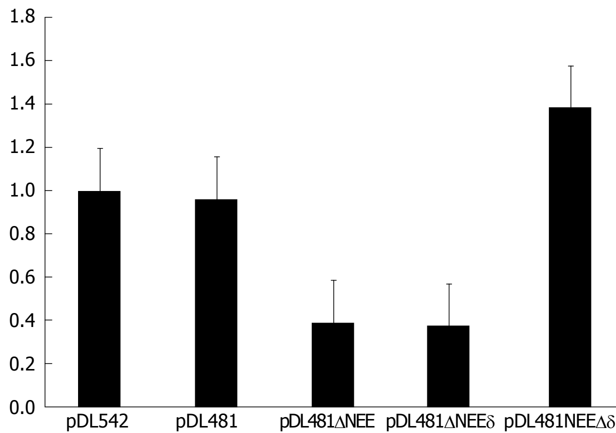

Figure 6 Nucleo-cytoplasmic distribution of hepatitis delta virus RNA in HuH-7 cells transfected with plasmids pDL481, pDl542, pDL481ΔNEE, pDL481ΔNEEδ, and pDL481Δδ. The RNA in nuclear and cytoplasmic cell fractions was determined by real time-polymerase chain reaction using the $2^{-\Delta\Delta Ct}$ method. Results are presented as the cytoplasmic /nuclear ratio (C/N) and correspond to the mean of three independent experiments. Bars indicate the standard deviation.

export of UsnRNAs which is mediated by the exportin CRM1[20]. This specific export pathway can be inhibited in the presence of leptomycin B (LMB)[33]. In order to investigate whether HDV agRNA is exported to the cytoplasm using the pathway mediated by CRM1, we decided to analyze the effect of LMB on CAT protein expression in pDM138-A7AS transfected HuH-7 cells. To do this, 10 nmol/L LMB was added to the culture medium 18 h after transfection, and cells were further incubated for 6 h. Total protein extracts were then prepared and used to determine the concentration of CAT by ELISA. As negative control pDM138-PRE(+) transfected HuH-7 cells were used since it was previously reported that export of HBV PRE(+) is not sensitive to LMB[23,34]. Figure 7 displays the obtained results. As expected, in the absence of LMB both the HDV agRNA A7AS and HBV PRE(+) sequences promote the export of CAT mRNA thus confirming our previous data. In the presence of LMB the export capacity of the HBV PRE(+) sequence is not affected, and we could detect a slight increase in CAT expression. This observation is in accordance with the data obtained by others when measuring CAT enzyme activity in similar experiments[34]. In contrast, the export promoting activity of the HDV agRNA A7AS sequence was found to be affected in the presence of LMB. In fact, we observed a 60% reduction in CAT production when HuH-7 cells were transfected with pDM138-A7AS in the presence of 10 nmol/L LMB. These results seem to indicate that the nuclear export mediated by the A7As agRNA sequence is dependent, at least partially, on CRM1 activity and suggest the involvement of this cellular protein in HDV agRNA export.

DISCUSSION

Although HDV RNA replication occurs in the nucleus of liver cells, virus packaging takes place in the cytoplasm where HDV RNPs meet HBsAgs to assemble newly synthesized virions. Noteworthy, only gRNA molecules were found, until now, to be packaged into mature hepatitis delta virions. This restriction could be due to a possible impairment of export of agRNA molecules to the cytoplasm. However, agRNA was found in HDV virus-like particles secreted by cells expressing agRNA, L-HDAg, and HBsAgs[18].

A previous work showed that HDV RNPs are exported to the cytoplasm independent of the presence of HBsAgs[15]. Additionally, it was demonstrated that export of HDV RNPs is not mediated by a putative nuclear export signal present in delta antigens but is rather promoted by cis elements in virus RNA. In fact, both HDV gRNA and agRNA are exported to the cytoplasm in the absence of

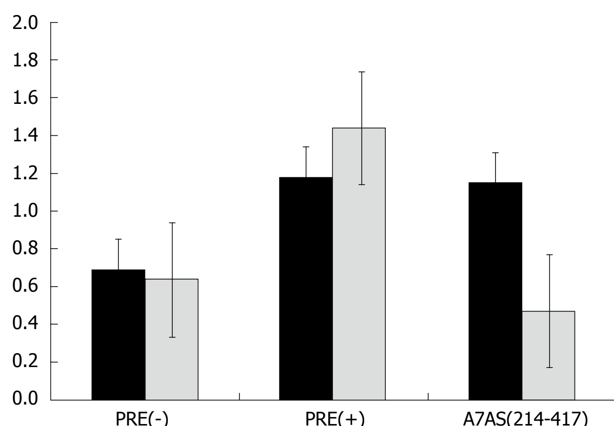

Figure 7 Analysis of chloranphenicol acetyl-transferase expression in HuH-7 cells transfected with plasmids pDM138 (PRE-), pDM138 (PRE+), and pDM138 A7AS, in the absence (black columns) and presence of 10 nmol/L leptomycin B (grey columns). In order to normalize for transfection efficiency, cells were co-transfected with plasmid pSV-β-Gal (Promega). Chloranphenicol acetyl-transferase (CAT) and β-Gal expression levels were determined by enzyme-linked immunosorbent assay. Normalization of CAT expression levels was calculated by dividing the values obtained for the CAT protein by the values obtained for the β-Gal protein. The results correspond to the mean of three independent experiments. Bars represent the standard deviation.

HDAgs, an observation also reported by Macnaughton *et al*[17].

Our first approach was designed to confirm that both HDV genomic and antigenomic RNA molecules are exported to the cytoplasm of HuH-7 cells. Using plasmids pDL542 and pDL481 which express exclusively HDV gRNA and agRNA, respectively, we were able to detect both molecules, by *in situ* hybridization, in the nuclear and cytoplasmic compartments of HuH-7 transfected cells. Furthermore, nuclear export of HDV gRNA and agRNA seems to occur with the same efficiency since similar amounts of genomic and antigenomic RNA molecules could be detected, by real time-PCR, in the nuclear and cytoplasmic compartments of HuH-7 cells transiently transfected with plasmids pDL542 and pDL481. The finding that both genomic and antigenomic HDV RNA molecules are exported with similar efficiency suggests a possible biological function associated with the presence of HDV agRNA in the cytoplasm. However, further research is mandatory to clarify the biological significance of these findings.

The above described observations are suggestive of a possible presence of cis-acting nuclear export elements in HDV RNA, both genomic and antigenomic. In an attempt to identify putative cis elements in the HDV gRNA and agRNA capable of mediating the export to the cytoplasm, we used a well characterized CAT reporter system previously used by others to investigate the role of the HBV PRE in export of intronless mRNAs[27]. This system is based on plasmid pDM138 which was generated in order to contain the second half of HIV-1 cDNA under the control of the SV40 promoter[25,26]. This vector was further engineered in order to remove the initiation codons for the Rev and Env proteins, to include the cDNA sequence coding for the CAT protein, and to substitute the HIV-1 RRE sequence by a linker containing a *Cla* I restriction site. After transcription, an mRNA containing the ORF for the CAT protein inserted in the intron of the HIV-1 Env proteins is produced. As positive and negative controls we used plasmids pDM138-PRE(+) and pDM138-PRE(-), respectively. These plasmids contain the HBV post-transcriptional regulatory element (PRE) inserted in the unique *Cla* I site of pDM138, in sense and antisense orientations, respectively[27]. We cloned several cDNA fragments, covering the entire HDV gRNA and agRNA, in plasmid pDM138. After transfection of HuH-7 cells, CAT production was determined by ELISA. This approach did not allow identifying unequivocally a nuclear export element in gRNA. Although two gRNA fragments corresponding to nucleotides 266-489 and 584-920, (A4S and A6S, respectively) were found to promote export of CAT mRNAs, the efficiency was in all experiments lower than the determined for the positive control pDM138-PRE(+). This could be possibly due to the lack of crucial nucleotides in the ends of the analyzed sequences. Clearly, additional experiments are mandatory to clarify this point.

However, we were able to identify a region (A7AS) in the HDV agRNA, located between positions 214-417, which promoted the export of CAT mRNA with slightly higher efficiency than that observed for the pDM138-PRE(+) positive control. This result was further confirmed by Northern blot analysis of total and cytoplasmic mRNAs prepared from pDM138-PRE(+) and pDM138A7AS transfected HuH-7 cells. Moreover, when inserted in the opposite orientation in pDM138, the fragment A7AS was not able to mediate export of CAT mRNAs (data not shown). All the other fragments tested were not capable of promoting export of CAT mRNA at levels comparable to those observed for the pDM138-PRE(+) positive control. In particular, the fragment A9AS which includes the HDV RNA ribozyme sequence displayed a CAT mRNA export capacity significantly lower than the calculated for the negative control. This may be a consequence of instability of the produced mRNA molecules due to the self-cleavage activity of the HDV ribozyme. Besides the A7AS sequence, the only exception to the low export promoting capacity of the analyzed fragments, concerns a cDNA fragment corresponding to positions 1301-1514 in the antigenome (A2AS). This agRNA fragment was able to promote CAT mRNA export with efficiency higher than the negative control but still lower than the determined for the positive control. This fragment, A2AS, includes part of the HDAg ORF. This observation may allow speculating about a possible presence of a cis element in the HDV mRNA involved in export to the cytoplasm. Huang and Carmichael have previously shown that export on intronless histone H2a mRNAs is mediated by a signal present in the coding region[35]. However, further experiments are needed to clarify the possible involvement of a similar signal in export of HDAg mRNA.

In an attempt to analyze in more detail the A7AS sequence in the HDV agRNA we generated, by PCR, eight truncated forms of this motif which were subsequently cloned in pDM138 and used in transfection experiments to determine CAT expression by ELISA. These truncated forms corresponded to nucleotides 314-417, 269-417, 244-417, 244-403, 214-403, 269-379, 214-379, and 214-322. The obtained results showed that deletion of the first 30 nucleotides in the 5' end of the A7AS sequence did not significantly affect the export promoting capacity. However, deletion of the first 55 nucleotides in the 5' end results in loss of the export capacity of the A7AS sequence. In contrast, deletions in the 3' end of the A7AS motif, did not significantly affect the ability to promote nuclear export. In fact, removal of as much as the first 95 nucleotides in the 3' end still resulted in the production of the CAT protein at intermediate levels between the negative and positive controls. Noteworthy, all the analyzed A7AS truncated forms were less efficient in promoting RNA export when compared to the wild-type sequence.

However, analysis of the secondary structure of the entire agRNA molecule did not allow predicting a similar branched structure in the region where the A7AS motif is located. In order to clarify a possible role of a, at least partially, complementary to the A7AS motif sequence in the antigenome, in nuclear export, we constructed deletion mutants of plasmid pDL481. These constructs were designed as follows: pDL481ΔNEE lacks the nuclear export element (A7AS sequence), pDL481Δδ lacks the complementary to the NEE sequence in the antigenome, and pDL481ΔNEEδ lacks both the NEE and the respective complementary sequence. After transfection of HuH-7 cells, the relative amounts of agRNA in nuclear and cytoplasmic fractions were determined by qRT-PCR. The obtained results showed that deletion of the NEE (pDL481ΔNEE) reduces export of agRNA by 60% when compared with wt pDL481. Additionally, deletion of both the NEE and the respective complementary sequence (pDL481ΔNEEδ) results in a similar reduction (62%) of detected cytoplasmic RNA. Finally, deletion of only the complementary to the NEE sequence did not impair the capacity of the agRNA to be exported to the cytoplasm. Taken together, these results indicate that the identified NEE is important to promote nuclear export of the HDV antigenome, and that export efficiency is not diminished by deletion of the respective putative complementary sequence.

Nuclear export of host and virus RNAs may be promoted by several cellular factors that participate in distinct pathways. One of these pathways is mediated by the exportin CRM1 which belongs to the kariopherin-β family of proteins. CRM1 mediates the export of the majority of proteins containing a nuclear export signal (NES) and of two classes of cellular non-coding RNAS, rRNAs and UsnRNAs[21]. The CRM1 export pathway may be specifically inhibited in the presence of LMB which binds to a cysteine residue in the central region of the protein[33]. In order to investigate a possible involvement of CRM 1 in export of HDV agRNA we used LMB to inhibit this pathway in pDM138-A7AS transfected HuH-7 cells. As positive and negative controls we used plasmids pDM138-PRE(+) and pDM138-PRE(-), respectively, since export of the HBV PRE was previously reported to be insensitive to LMB. The obtained results showed that RNA export mediated by the NEE of HDV agRNA is partially inhibited in the presence of LMB, displaying a 50% reduction of CAT expression when compared with the parental pDM138 vector. This suggests an involvement of the CRM1 protein in export of HDV agRNA. However, since CRM1 binds to RNA molecules indirectly through interaction with other NES-containing proteins this implies the participation of other not yet identified host factors in HDV agRNA export. Not surprisingly, using coimmunoprecipitation assays we couldn't detect complexes between HDV RNPs and CRM1 (our unpublished data).

In this work we attempted to identify nuclear export elements present HDV gRNA and agRNA. Although two regions in gRNA were found to be able to promote export of heterologous RNAs, an unequivocal NEE could not be identified in gRNA. However, it was possible to identify a NEE in HDV agRNA located in positions 214-417. This cis element is not only capable of promoting the nuclear export of heterologous intronless RNAs but is also involved in export of HDV antigenomes. Analysis of the export capacity of several truncated forms of the NEE showed that the two mini-helices seem to play a crucial role in mediating RNA export. Cytoplasmic export of HDV agRNA was found to be sensitive to leptomycin B suggesting a possible involvement of a CRM1 mediated pathway.

ACKNOWLEDGMENTS

We thank Dr. Tristram Parslow (Emory University School of Medicine, Atlanta, United States) for kindly providing plasmid pDM138, Dr. Benedict Yen (University of California, San Francisco, United States) for the generous gift of plasmids pDM138-PRE(-) and pDM138-PRE(+), and Dr. John Taylor (Fox Chase Cancer Center, Philadelphia, United States) for plasmid pDL481.

COMMENTS

Background

Hepatitis delta virus (HDV) is a satellite of hepatitis B virus (HBV). The two viruses share the same envelope which consists of HBV surface antigens (HBsAg). HBsAgs are coded exclusively by the HBV genome and are localized in the cytoplasm of infected cells. In contrast HDV replicates in the nucleus and assembly of mature virions takes place in the cytoplasm. Accordingly, HDV RNPs need to be exported to the cytoplasm and this process is thought to be mediated by cis-elements present in the virus RNA.

Research frontiers

Export of RNA molecules to the cytoplasm depends on the interaction of cellular transport receptors with specific nucleotide sequences. These sequences were previously studied in simple and complex retroviruses as well as in HBV. The cellular proteins and pathways involved in virus RNA export were also identi-

fied. Here, the research hotspot is the identification of nucleotide sequences in HDV RNA capable of promoting export of heterologous RNAs and thus being able to function as nuclear export elements.

Innovations and breakthroughs

This is the first report describing a comprehensive analysis of HDV genomic and antigenomic RNA sequences aiming to identify cis-elements capable of promoting export to the cytoplasm. Two possible regions corresponding to nucleotides 266-489 and 584-920, respectively, were identified in HDV genomic RNA. In addition, analysis of HDV antigenomic RNA sequences allowed finding a nuclear export element in positions 214-417. Furthermore, export mediated by the nuclear export element of HDV antigenomic RNA was found to be sensitive to leptomycin B suggesting a possible role of the cellular protein CRM1 in this transport pathway.

Applications

The results herein described may contribute to a better understanding of an essential step in the HDV replication cycle-the export of virus RNPs to the cytoplasm where they interact with HBsAgs in order to assemble new virions.

Terminology

Nuclear export element is an RNA sequence capable to interact with cellular proteins that promote export from the nucleus to the cytoplasm.

Peer review

The paper is of good quality as research article, its english is perfect and its scientific content is original.

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P- Reviewer Kamal SA **S- Editor** Zhai HH
L- Editor A **E- Editor** Lu YJ

World Journal of Virology

Online Submissions: http://www.wjgnet.com/esps/
wjv@wjgnet.com
doi:10.5501/wjv.v2.i3.136

World J Virol 2013 August 12; 2(3): 136-138
ISSN 2220-3249 (online)
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LETTERS TO THE EDITOR

Identification of novel silent HIV propagation routes in Pakistan

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Received: April 9, 2013 Revised: June 19, 2013
Accepted: July 23, 2013
Published online: August 12, 2013

Abstract

Human immunodeficiency virus (HIV) is rapidly increasing in both high risk groups and the general population. In this study, silent routes of propagation in teenaged Pakistanis are discussed. In order to promote sexual activity in youths, regular clients write contact details of sex workers on the doors of public washrooms. HIV prevalence is much higher among Hijra sex workers. Hijra sex workers have apparently stepped into the profession of begging at public places, where they earn money by both begging and distributing visiting cards offering unsafe sex. In many educational institutes, sex education is lacking or absent; if delivered *via* teachers, government agencies and nongovernmental organizations this could prevent a future epidemic of sexually transmitted infections in Pakistan.

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Key words: Human immunodeficiency virus; Pakistan; Sex workers; Silent route; Youth

Core tip: Human immunodeficiency virus (HIV) is an emerging threat for rising generations in Pakistan. The prevalence of HIV is gradually increasing in different high risk populations due to the rapid increase of sexual activity among Pakistani youth. Increased sexual desperation among Pakistani youngsters has outranked the rest of the world. The rate of HIV spread in Pakistan is much greater than in any of the western countries, therefore adequate preventive measurements should be implemented as soon as possible, otherwise it will be too late.

Saeed U, Waheed Y, Manzoor S, Ashraf M. Identification of novel silent HIV propagation routes in Pakistan. *World J Virol* 2013; 2(3): 136-138 Available from: URL: http://www.wjgnet.com/2220-3249/full/v2/i3/136.htm DOI: http://dx.doi.org/10.5501/wjv.v2.i3.136

TO THE EDITOR

Human immunodeficiency virus (HIV) is an emerging threat for rising generations in Pakistan. The prevalence of HIV is gradually increasing in different high risk populations due to rapid increase of sexual activity among Pakistani youth. Maximum viral spread and stable maintenance in the environment depends upon effectively penetrating protecting barriers[1]. Prevalence of sexually transmitted diseases was limited to certain areas but unfortunately, now, its prevalence has rapidly increased in different cities. Pakistan is an Islamic republic, where religion, law, culture and society strictly forbid sex outside marriage. Despite the recognition of sex outside marriage as a crime, commercial sex is being offered secretly in various brothels, kothikhana, client homes, rental houses near truck stops and market places, massage parlors and hotels. Strict punishments against sex outside marriage, if implemented by any nation, could prevent individuals from extra marital sexual activity.

We have identified silent routes stimulating sexual

activity among teenaged Pakistanis. Most of the regular clients of sex workers write sexually attractive sentences about female sex workers on the doors of public toilets (in public places such as markets, parks, picnic places, hotels and bus stands) in order to promote sex in the younger generation. Contact details of female sex workers are randomly distributed in public washrooms. Sexually desperate youngsters, introduced to the opportunity, indulge in risky sexual activities which expose them to sexually transmitted infections like HIV and acquired immunodeficiency syndrome. At the moment, fortunately, the prevalence of HIV in the general population is very low. However, if unsafe sexual practices stimulated by this rapid advertisement route, were to be carried out by youngsters then this would probably generate an HIV epidemic in society which could create an extra burden on the economy. Another highly dangerous route of sexual advertisement is through Hijra sex workers. In Pakistan, the prevalence of HIV is highest among injecting drug users (IDUs) and Hijra sex workers. The greatest HIV prevalence (37.8%) was found in IDUs from different cities of Pakistan[2,3]. An extremely high HIV prevalence (27.6%) was observed in Hijra sex workers of Larkana Sindh, Pakistan[4]. Similarly another study conducted in Rawalpindi (the city adjoining Pakistan's capital) reported a high prevalence of HIV (21.6%) among transgender men[5]. The rate of HIV spread in Pakistan is much higher than in any of the western countries, therefore adequate preventive measurements should be introduced as soon as it is possible.

Hijra is a distinct type of gender role which includes Khusras (true hermaphrodites), Zananas (crossdressers) and Narnbans (eunuchs)[6]. We have identified that the majority of Hijra sex workers have started promoting sexual activity in the younger generation by distributing various visiting cards among shop keepers and youth as sexual advertisements. Mobile numbers and visiting addresses are written on those cards. Sexually active people usually make a phone call and arrange a time and place for unsafe sexual activities. Hijra sex workers have also stepped into begging at various public sectors including bus stands, markets and traffic signals, where they not only earn money by begging but also promote sex work by contacting youths. Hijras usually flatter the physical beauty and power of their clients thus promoting sexual relationships. Most of the youngsters who indulge in risky sexual relationships are illiterate and have a poor understanding of sexually transmitted diseases and their consequences. Most of them lack awareness and knowledge, and are therefore unable to comprehend the seriousness of this issue.

In educational institutes such as schools and colleges, sex education is mostly avoided. Teachers are often reluctant to educate students with knowledge about awareness and prevention of sexually transmitted disease. If appropriate seminars were arranged for teenaged students this would be a positive step towards awareness and prevention of HIV and other sexually transmitted diseases. In order to reduce any future epidemic of sexually transmitted infections, harm reduction strategies must be implemented rather than intimidation by law enforcement agencies. The mysterious sex industry is based upon the principle of demand and supply. Sexually desperate unemployed youths, tourists, business men, public officials, migrant workers, truck drivers and traders with conspicuous wealth create a strong demand for sexual services. On the other hand, various social and economic factors like acute poverty, unemployment, limited knowledge and skills and the opportunity of a high income through sex, usually compel poor individuals towards offering sex. In such circumstances, suppressing measures *via* law enforcement agencies could further aggravate the spread of hidden risky behavior in society. It has been observed that Hijra sex workers have a strong wish to earn money by positive means but unfortunately our society does not accept them in either the private or public sector. The majority of Hijra sex workers are unable to earn money by positive means due to problems of sexual harassment from sexually active people. By arranging positive support for sex workers, *via* assistance from government sectors and nongovernmental organizations (NGOs) to earn an honorable livelihood, their risky sexual activities and behaviors can be reduced significantly.

Policy makers should not only provide wider opportunities for the dissemination of awareness and knowledge about risk factors associated with viral transmission among populations at risk; but also focus on identification of epidemiological patterns associated with sexually transmitted infections among various high risk populations in Pakistan. At the moment, an inadequate surveillance system for sexually transmitted infections exists with inadequate identification of subpopulations where deadly pathogens are secretly propagating. There is very little knowledge in the general and high risk populations about access to anti-retroviral therapy clinics. Expanded scientific research is also an important factor for successful surveillance of sexually transmitted infections. Among target populations, simple diagnostic tests against HIV could provide information regarding prevalence and trend of HIV infection. If the serum sub-samples are screened against other sexually transmitted infections (like hepatitis C virus and herpes simplex virus 2) after recording valuable basic demographic and behavioral data, this would help to identify behavior risk trends over time and aid surveillance. Another possible way towards harm reduction in Pakistan is by supporting community organizations and NGOs. The mysteries of complex HIV (and other sexually transmitted infection) dynamics could also be investigated using molecular epidemiology studies and mathematical modeling.

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World J Virol 2013 August 12; 2(3): I-V
ISSN 2220-3249 (online)
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Name of journal

World Journal of Virology

ISSN

ISSN 2220-3249 (online)

Launch date
February 12, 2012

Frequency
Quarterly

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World Journal of Virology
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No. 62 Dongsihuan Zhonglu, Chaoyang District,
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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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15 Morse SS. Factors in the emergence of infectious diseases.

Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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