Mutations Associated with Occult Hepatitis B Virus Infection Result in Decreased Surface Antigen Expression *In Vitro*

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Abstract

Occult hepatitis B virus infection is characterized by the absence of detectable hepatitis B surface antigen (HBsAg) in the serum, despite detectable HBV DNA. Investigations of the mechanisms underlying the development of occult HBV infection are lacking in the current literature, although viral mutations in the surface region, resulting in decreased HBsAg expression or secretion, represent one potential mechanism. Wild-type HBsAg expression vectors were constructed from genotype-matched chronic HBV sequences. Site-directed mutagenesis was then utilized to introduce three genotype A mutations – M103I, K122R, and G145A – associated with occult HBV infection *in vivo*, alone and in combination, into the wild-type HBsAg vectors. Transfection of Huh7 and HepG2 cell lines was performed, and cell culture supernatants and cell lysates were collected over 7 days to assess the effects of these mutations on extracellular and intracellular HBsAg levels. The G145A mutation resulted in significantly decreased extracellular and intracellular HBsAg expression *in vitro*. The most pronounced reduction in HBsAg expression was observed when all 3 mutations were present. The mutations evaluated *in vitro* in the current study resulted in decreased HBsAg expression and potentially increased hepatic retention and/or decreased hepatic secretion of synthesized HBsAg, which could explain the lack of HBsAg detection that is characteristic of occult HBV infection *in vivo*.

Keywords

G145A; HBV/HIV co-infection; HBsAg mutants; hepatitis B surface antigen (HBsAg); occult hepatitis B virus

Introduction

Greater than 2 billion people have been exposed to hepatitis B virus (HBV) worldwide, and more than 350 million individuals are currently living with chronic HBV infection (CHBV) (1). While C-HBV infection is diagnosed by detection of serum hepatitis B surface antigen (HBsAg)(2), occult HBV infection (O-HBV) is characterized by a lack of detectable HBsAg in the serum despite low-level HBV replication(3). O-HBV infection was once defined by antibodies against HBV core (anti-HBc) alone(4), although cases have now been reported in

which HBV DNA is the only detectable marker of O-HBV infection(5). The clinical relevance of O-HBV has not been thoroughly explored and remains controversial(6); however, several studies have suggested a potential association of O-HBV infection with increased incidence of hepatocellular carcinoma (HCC) and severity of liver fibrosis(7-11). In addition, O-HBV is transmissible via blood transfusion in humans and primates, as well as solid organ transplantation(12-15), from which the recipient may develop either C-HBV or O-HBV.

Globally, studies have reported rates of O-HBV infection from 0% – 89.5%, depending on the population, laboratory methods, and inclusion/exclusion criteria(16,17). In the US, O-HBV prevalence ranges from 0% – 45% with higher rates in HIV and/or HCV co-infected patients(18,19). Few studies have included virologic comparisons between C-HBV and O-HBV patients from the same cohort, and functional analysis of mutations associated with O-HBV infection is rarely performed. Potential mechanisms resulting in O-HBV infection include 1) complexes of HBsAg with antibodies against surface (anti-HBs) that circulate in the serum but are not readily detected by standard HBsAg ELISAs, 2) interference of HBV replication by other viruses, 3) altered host immunologic responses, and/or 4) mutations within the HBV genome that change HBsAg expression(20). We previously identified mutations associated with O-HBV infection by comparing viral sequences from O-HBV infections and genotype-matched C-HBV infections from the same cohort(21). Three O-HBV mutations – M103I, K122R, and G145A – from a genotype A infection were significant by signature pattern analysis. M103I had been previously identified in 2 O-HBV infections, although these patients harbored genotype C or D(22). K122R – which indicates a sub-serotype change from d to y – has also been identified in one genotype A O-HBV infection(22). Finally, G145A was reported in one O-HBV infection of unknown genotype(23). Since these small HBsAg mutations have not been well characterized, the current study evaluated their effects on HBsAg synthesis and secretion *in vitro*.

Materials and Methods

Mutations Associated with O-HBV Infection

O-HBV-infected patients were identified by evaluating patient sera for markers of HBV infection, including real-time PCR for HBV DNA and ELISAs for serologic markers (HBsAg, anti-HBs, and anti-HBc)(5). Mutations associated with O-HBV were identified in the HBV Pre-Surface, Surface, and Polymerase regions as described previously(21). Three mutations within the small HBsAg – M103I, K122R, and G145A – from an individual with a genotype A infection, were evaluated in the current study, since genotype A represents the most common HBV genotype in the US(24). The most common amino acids at these positions among the representative GenBank reference sequences are listed in Table 1.

HBsAg Expression Vector Construction

The O-HBV-infected patient was matched to 2 C-HBV-infected, HIV-positive individuals from the same cohort, based on HBV genotype, age <5 years apart, gender, ethnicity, and HCV serostatus. HBV viral load for the O-HBV patient was 7.61×10^5 IU/mL, while the C-HBV patients were 1.48×10^7 and 9.74×10^2 IU/mL. First round PCR amplification was performed as previously described(25) with the Picomaxx High Fidelity PCR System (Agilent, Santa Clara, CA). Second round primers – sHBs-EcoRI-F (5' – CGA ATT CGG GAC CCT GTG ACG AAC) and sHBs-KpnI-R (5' – GGG GTA CCC ATC TCT TTG TTT TGT TAG) – designed to amplify the small S region (721bp, nt138-859, according to X97848), contained EcoRI and KpnI sites to facilitate cloning into pCMV-HA (Clontech, Mountain View, CA), producing 5'hemagglutinin (HA)-tagged small HBsAg proteins. Site-directed mutagenesis introduced O-HBV-associated mutations – separately or in

combination – into each C-HBV expression vector (Table 2) using the QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent).

Transfection and HBsAg Quantitation

Huh7 and HepG2 cells were maintained in DMEM (10% FBS, 1% penicillin/streptomycin) and RPMI-1640 (4% FBS, 4mM L-glutamine), respectively. Huh7 cells were seeded at 5×10^4 cells/well in 24-well plates, while HepG2 cells were seeded at 1×10^5 cells/well. 24 hours later, cells were transfected in duplicate with 2ug DNA/well with 3uL FuGENE6 (Roche, Indianapolis, IN). Media were changed after 24 hours, and collected every 48 hours thereafter. Supernatants collected on days 3 and 5 were pooled for the day 5 sample, while supernatants collected on days 3, 5, and 7 were pooled for the day 7 sample, and clarified by centrifugation at 10,000rpm for 5 minutes. Cells were washed three times with PBS and lysed with 200uL lysis buffer – Tris-HCl (50mmol/L, pH 7.8), NaCl (150mmol/L), EDTA (1mmol/L), 0.5% Nonidet P-40 (NP-40), and $1/100^{th}$ volume of protease inhibitors (Complete Mini Protease Inhibitor Cocktail, Roche). Plates were frozen, thawed, and lysates were clarified by centrifugation at 13,000g for 3 minutes. Cell viability post-transfection was determined by trypan blue staining on day 7. Cells were transfected with 2ug wild-type vector 1, G145A, M103I+K122R+G145A, or pCMV-HA. After 7 days, cells were washed, trypsinized, and resuspended in fresh media before staining with trypan blue and counting. Mean number of cells were determined for each construct and log-transformed for comparison (Supplemental Figure 1A). Additionally, cells were seeded to determine transfection efficiency. 50ng of plasmid p0Z-17, encoding for β–galactosidase, was co-transfected along with 2ug vector, while pCMV-HA alone served as a negative control. On day 7 post-transfection, cells were washed twice with PBS, and fixed for 5 minutes with 2% formaldehyde. Cells were incubated with 5mM phosphate ferricyanide, 5mM phosphate ferrocyanide, 2mM $MgCl_2$, and 1mg/mL X-gal in PBS, for 2 hours at 37°C. Five 25X fields of view were counted per well to determine the number of blue cells/well. The average was calculated and compared between constructs (Supplemental Figure 1B).

HBsAg was quantitated by ELISA (Biochain, Hayward, CA) with recombinant HBsAg (adr subtype) standards from 500ng/mL-0.25ng/mL with a lower limit of detection of 0.5ng/mL. The OD_{450} was measured and compared to a standard curve from 0.25ng/mL-10ng/mL. Samples with OD_{450} >10ng/mL were diluted and re-measured. Total nanograms of HBsAg were corrected for the proper dilution and lysate$_{HBsAg}$:supernatant$_{HBsAg}$ ratios were calculated for each construct.

Statistical Analysis

For each cell type, compartment, wild-type group, and time point, data were analyzed by a one-factor, repeated-measures ANOVA. Type of mutant (with wild-type as the reference level) was the repeated factor, and comparisons of each mutant condition to wild-type were performed using Dunnett's procedure for comparing multiple treatments to a common control condition. The maximum family-wise type I error rate for Dunnett's procedure was set at $\alpha=0.05$.

Results

Wild-type HBsAg Expression

Supernatant and lysate HBsAg levels from transfected Huh7 and HepG2 cells were assessed for wild-type (i.e., C-HBV) expression vectors on days 3, 5, and 7 post-transfection. Overall, HBsAg expression was higher in Huh7 cells (Figures 1A-B compared to 1C-D). No significant differences were observed between wells with regards to viable cell number (Supplemental Figure 1A) or transfection efficiency (Supplemental Figure 1B). Wild-type

supernatant HBsAg levels in Huh7 cells increased between days 3 and 5 post-transfection and decreased by day 7 (Figure 1A). Lysate HBsAg levels in Huh7 cells showed a similar trend (Figure 1B). These trends were also observed in HepG2 cells (Figures 1C-D) and did not vary when a second wild-type expression vector was utilized (Supplemental Figure 2). However, overall HBsAg levels were higher in the supernatant, while lysate HBsAg levels were lower, indicating increased HBsAg secretion with the second C-HBV backbone, presumably due to the 2 amino acid mutations (G164E and S212F) that differentiate the C-HBV sequences.

Effects of Single Point Mutations on HBsAg Expression

HBsAg expression was also assessed for the M103I, K122R, or G145A mutations. Supernatant levels of HBsAg were similar between wild-type, M103I, and K122R in both Huh7 and HepG2 cells (Figures 1A,C). In lysates, HBsAg levels were slightly lower for expression vectors containing the M103I and K122R mutations compared to wild-type (Figures 1B,D). In contrast, the G145A mutation resulted in significantly lower HBsAg levels in both cell supernatants and lysates. These trends were also observed for the second wild-type expression vector (Supplemental Figure 2).

Effects of Multiple Mutations on HBsAg Expression

When examining the combined effects of these mutations, M103I+K122R resulted in decreased HBsAg levels in both supernatants (Figures 2A,C) and lysates (Figures 2B,D), but remained well above the ELISA detection limit. In contrast, combinations of mutations containing G145A resulted in significantly lower HBsAg levels – some below detectable limits. The most pronounced reductions in HBsAg synthesis was observed with M103I +K122R+G145A (Figure 2, Supplemental Figure 3).

Effects of Mutations on HBsAg Secretion

Ratios of lysate (intracellular) to supernatant (extracellular) HBsAg were calculated to compare HBsAg secretion versus retention. At day 3 post-transfection, the wild-type vector resulted in higher intracellular levels of HBsAg in Huh7 cells (ratios >1 in **bold**, Table 3). Over time, HBsAg secretion increased, resulting in ratios <1. The G145A, M103I+G145A, K122R+G145A, and M103I+K122R+G145A vectors continued to have higher intracellular HBsAg levels at all time points in Huh7 cells, when above the detection limit. Similar results were observed in HepG2 cells, in which the only constructs with ratios >1 were those containing the G145A mutation alone or in combination. However, these findings were not observed with the second wild-type vectors (Supplemental Table 1), since lysate HBsAg levels were lower overall with these constructs.

Discussion

To date, most O-HBV studies have focused on prevalence(16,19) and/or identification of mutations associated with O-HBV(22,23). In contrast, functional analysis of specific HBV mutations on HBsAg synthesis has rarely been reported(26-28). Thus, to determine whether mutations associated with O-HBV infection may play a role in the lack of detectable HBsAg in these individuals, additional investigation is required. The current *in vitro* system specifically evaluates HBsAg protein expression, since the CMV-IE promoter drives transcription with little variation in mRNA expression. Thus, we examined the effects of three previously identified O-HBV mutations in the S region – M103I, K122R, and G145A – on HBsAg expression(21).

Of the three mutations characterized, G145A contributes the most to decreased HBsAg synthesis *in vitro*. Additional mutations at position 145 of HBsAg have been previously

characterized, although not always in O-HBV infections(29-31). The G145R mutation has been described as an immune escape mutant(31), as well as a potential contributor to lamivudine resistance(29); therefore, G145A may also represent a potential vaccine escape mutant(32). The G145R mutation has consistently resulted in decreased HBsAg levels and increased replication in lamivudine resistant mutants, especially among those with additional polymerase mutations, such as L180M and M204V(30). In this study, extracellular levels of HBsAg were slightly above detectable levels at times with the G145A mutation, indicating that the HBsAg secreted from infected hepatocytes would likely be below the limit of detection for commercial assays once diluted in the blood. Although the lack of detectable HBsAg could represent a diagnostic error(33-37), HBsAg synthesis was consistently detectable for G145A with the commercial ELISA kit utilized even at low levels. Western Blot to detect HBsAg was also attempted but was found to be much less sensitive than ELISA (data not shown). In addition, one study compared several commercial ELISA kits to assess detection of amino acid changes at position 145, including G145A(38). Although G145A had not been identified *in vivo*, it was detectable with all kits tested; thus, a false-negative HBsAg result *in vivo* seems unlikely.

The M103I or K122R mutations alone did not significantly affect HBsAg expression, although it is interesting to note that when these two mutations were combined, HBsAg expression was decreased. Although the K122R mutation results in a serotype change from 'd' to 'y', both K122R and M103I may have minor effects with respect to protein folding, antigenicity, detection by anti-HBs, and protein expression, due to their location within the antigenic determinant region of HBsAg(20,22,28,34,37,39).

For one wild-type construct, the small amount of HBsAg produced with the G145A mutation, alone or in combination, was retained within hepatocytes, as indicated by higher intracellular HBsAg ratios in Table 3. While this could represent an artifact of low overall HBsAg levels and was only observed for one of the two wild-type constructs, it is intriguing given that certain HBsAg mutations may result in a drastic increase in retention of viral proteins due to misfolding(40-42). However, the low levels of intracellular HBsAg observed indicate that if HBsAg misfolding is occurring, then proper proteasomal degradation is likely still taking place. Nonetheless, future investigations should evaluate the folding and sub-cellular localization of mutant forms of HBsAg.

The current investigation focused on the impact of HBsAg mutations on protein expression without influence from potential mutations in other HBV regions. While full-length HBV PCR was attempted, the concentration of PCR product was too low to permit subsequent generation of replication-competent vectors. A small number of prior studies have utilized full-length, replication-competent O-HBV genomes. For example, one polymerase mutation (terminal protein mutation T165P)(43) or several amino acid mutations throughout the genome(44,45) have resulted in defective replication or overall decreases in HBsAg and HBV DNA levels. Other studies identified a single nucleotide(26) or amino acid mutation(34) that lead to decreased PS2/S mRNA levels, resulting in decreased HBsAg. Additional investigations studied heavily mutated viral sequences within the PS1 and PS2/S promoters(46) or S region(27) that lead to altered HBsAg secretion. However, to date, only three studies have focused specifically on individual mutations within the S region and their effects on HBsAg synthesis. One study evaluated the Y100C mutation. Surprisingly, HBsAg levels were higher than wild-type; thus, why HBsAg was not detectable in the serum remains unclear(47). In the second study, HBsAg expression was decreased by 40% with the P142A mutation and by 60% with the D144V mutation; however, when both mutations were included, HBsAg expression was decreased by only 20%(28). Finally, in the third study, the I110M, G119E, and R169P mutations were each capable of impairing virion secretion(48). These studies, along with the current investigation, demonstrate that multiple mutations

within HBsAg have the potential to impact HBsAg expression and secretion, although the specific mutations may differ among O-HBV-infected patients.

Several aspects of the current investigation are unique compared to previously published studies. First, the specific mutations assessed in this study – M103I, K122R, and G145A – had not been included in previous functional analyses of HBsAg expression during O-HBV infection and, therefore, extend the small body of previously available research that utilized *in vitro* models to evaluate specific instances of undetectable HBsAg *in vivo*. The current study also examined changes in HBsAg expression over 7 days, since HBsAg synthesis and secretion are dynamic processes that may fluctuate over time. Additionally, both Huh7 and HepG2 cell lines were utilized to assess potential differences between these cell lines *in vitro*, since hepatocyte cell lines may not recapitulate infection in humans. Finally, two chronic/wild-type HBV sequences were used as backbones for the constructed HBsAg expression vectors to assess the consistency of the findings.

In summary, the S gene mutations M103I, K122R, and G145A were evaluated for their impact on HBsAg synthesis given that a lack of detectable HBsAg in the serum is a hallmark of O-HBV infection. The G145A mutation, alone and in combination, resulted in significantly decreased extracellular and intracellular HBsAg expression. Interestingly, the G145A mutation, also results in a W153C mutation in the reverse transcriptase region of the HBV polymerase. Previous mutations at this position, although not W153C, have been shown to decrease HBV replication(30). Additional cohorts will be utilized in the future to generate full-length, replication-competent viruses to further evaluate the simultaneous effects of these and other mutations on HBsAg synthesis and viral replication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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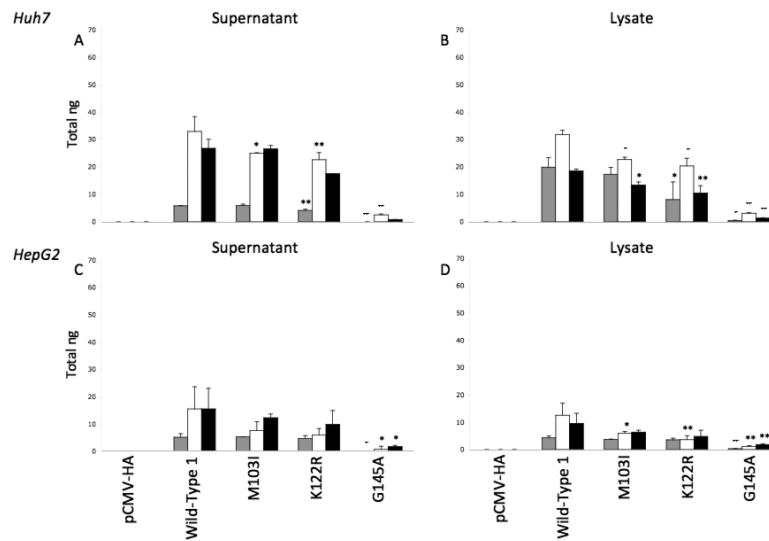
Figure 1.
Total ng of HBsAg produced by transfection of pCMV-HA alone, wild-type vector 1 alone or with mutations M103I, K122R, or G145A, were calculated for supernatants (**A,C**) and cell lysates (**B,D**) from Huh7 (**A,B**) and HepG2 (**C,D**) cells in duplicate and compared at days 3 (grey bars), 5 (white bars), and 7 (black bars) post-transfection. * p<0.05; ** p<0.01; [+] p<0.001; [++] p<0.0001.
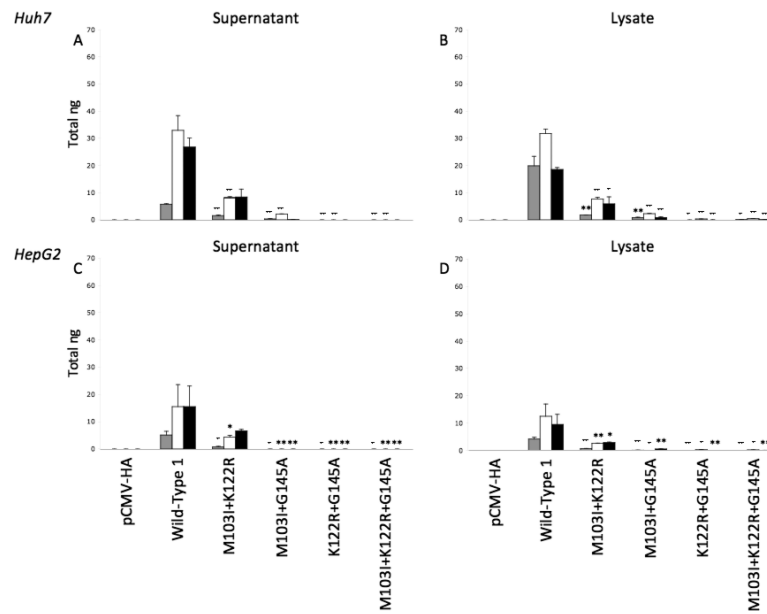
Figure 2.
Total ng of HBsAg produced by transfection of pCMV-HA alone, wild-type vector 1 alone or with mutation combinations M103I+K122R, M103I+G145A, K122R+G145A, or M103I +K122R+G145A, were calculated for supernatants (**A,C**) and cell lysates (**B,D**) from Huh7 (**A,B**) and HepG2 (**C,D**) cells in duplicate and compared for days 3 (grey bars), 5 (white bars), and 7 (black bars) post-transfection. * $p<0.05$; ** $p<0.01$; [+] $p<0.001$; [++] $p<0.0001$.

Table 1

Most Common Amino Acids at Positions of Interest by Genotype

Five GenBank references for each HBV genotype (A–H), used previously to identify mutations associated with O-HBV infection(21), were screened to identify the most common amino acid present at positions 103, 122, and 145 of the small HBsAg compared to the mutations associated with OHBV being examined.

Position	Mutation	A	B	C	D	E	F	G	H
103	I	M*	M	M	M	M*	M	M	M
122	R	K*	K	K*	R	R	K	K	K
145	A	G	G	G	G	G	G	G	G

*
indicates that the amino acid position was variable for the given genotype.

Table 2

Genotype A HBsAg Expression Vectors

Wild-type partial S ORF sequences were derived from 2 genotype A C-HBV infections and ligated into the pCMV-HA vector. The wild-type vectors served as positive controls and were the backbones into which mutations associated with O-HBV infection were introduced by site-directed mutagenesis. A total of 17 vectors were utilized in these experiments as listed above.

Genotype A HBsAg Expression Vectors	
Wild-Type 1	Wild-Type 2
Wild-Type 1 + M103I mutation	Wild-Type 2 + M103I mutation
Wild-Type 1 + K122R mutation	Wild-Type 2 + K122R mutation
Wild-Type 1 + G145A mutation	Wild-Type 2 + G145A mutation
Wild-Type 1 + M103I + K122R mutations	Wild-Type 2 + M103I + K122R mutations
Wild-Type 1 + M103I + G145A mutations	Wild-Type 2 + M103I + G145A mutations
Wild-Type 1 + K122R + G145A mutations	Wild-Type 2 + K122R + G145A mutations
Wild-Type 1 + M103I + K122R + G145A mutations	Wild-Type 2 + M103I + K122R + G145A mutations
pCMV-HA alone (negative control)	

Table 3

HBsAg$_{lysate}$:HBsAg$_{supernatant}$ Ratios

Ratios comparing lysate HBsAg to supernatant HBsAg (HBsAg$_{lysate}$:HBsAg$_{supernatant}$) were calculated at days 3, 5, and 7 post-transfection for all constructs with the wild-type 1 backbone in both Huh7 and HepG2 cells. Bold values indicate constructs with higher intracellular/lysate levels of HBsAg (values >1). UD – indicates undetectable levels of HBsAg (below the lower limit of detection for the ELISA, <0.5ng/mL) in the lysate and/or supernatant.

Construct	Day 3		Day 5		Day 7	
	Huh7	*HepG2*	*Huh7*	*HepG2*	*Huh7*	*HepG2*
Wild Type 1	**3.44**	0.83	0.98	0.85	0.70	0.63
M103I	**2.95**	0.68	0.90	0.83	0.51	0.52
K122R	**1.88**	0.76	0.90	0.60	0.60	0.50
G145A	**UD/0.43**	**UD/0.29**	**1.27**	**UD/1.09**	**1.61**	**1.11**
M103I+K122R	**1.18**	**1.12**	0.96	0.62	0.69	0.44
M103I+G145A	**2.64**	**UD/0.17**	**1.09**	UD/UD	**UD/0.85**	**UD/0.62**
K122R+G145A	UD/UD	UD/UD	**UD/0.31**	**UD/0.35**	UD/UD	UD/UD
M103I+K122R+G145A	**UD/0.08**	UD/UD	**UD/0.44**	**UD/0.26**	**UD/0.08**	UD/UD
pCMV-HA	UD/UD	UD/UD	UD/UD	UD/UD	UD/UD	UD/UD