Expression of wild-type or G1862T mutant HBe antigen of subgenotype A1 of hepatitis B virus and the unfolded protein response in Huh7 cells

Nimisha Harshadrai Bhoola and Anna Kramvis*

Abstract

The G1862T mutation, which occurs most frequently in subgenotype A1 of the hepatitis B virus (HBV), results in a valine to phenylalanine substitution at the –3 position of the signal peptide cleavage site at the amino end of the precore/core (preC/C) precursor protein. The objective of this study was to functionally characterize the G1862T mutation relative to its wild-type counterpart in subgenotype A1. Huh7 cells were transfected with subgenotype A1 replication-competent plasmids, with and without G1862T. Secretion of HBsAg and HBeAg, preC/C/HBeAg expression in the secretory pathway, activation of the unfolded protein response (UPR) and subsequent activation of apoptosis were monitored. The introduction of G1862T did not affect HBsAg expression. Cells transfected with the G1862T subgenotype A1 plasmid showed decreased expression of intracellular HBcAg and of nuclear preC/C/HBeAg and extracellular HBeAg, when compared to cells transfected with its wild-type counterpart as a result of the accumulation of the mutant protein in the endoplasmic reticulum (ER) and ER–Golgi intermediate compartment (ERGIC). This accumulation of preC/C/HBeAg protein in the ER led to the earlier activation of the three UPR pathways, but not to an increase in apoptosis. Therefore, it is evident that the presence of G1862T in subgenotype A1 does not completely abolish HBeAg expression, but affects the rate of HBeAg maturation, its passage through the secretory pathway and activation of the UPR. Increase in ER stress can result in liver damage, which has been shown to be a contributing factor to hepatocarcinogenesis and may explain why G1862T is frequently found in subgenotype A1 from liver disease patients.

INTRODUCTION

The hepatitis B virus (HBV) is the prototype member of the family Hepadnaviridae [1, 2] that is prevalent in the black population in southern Africa [3]. In this region, genotype A is the dominant genotype, with subgenotype A1 prevailing [4, 5]. Characteristic molecular and clinical features of subgenotype A1 include lower HBV DNA levels, early seroconversion from HBeAg to anti-HBe and rapid disease progression, including high rates for developing hepatocellular carcinoma (HCC), even in the absence of cirrhosis [4, 6–13]. This high rate of HBeAg negativity in subgenotype A1 is attributable to the presence of mutations in the basic core promoter (BCP)/precore (preC) that affect HBeAg expression. These mutations can affect synthesis of HBeAg at the: (i) transcriptional [1762T1764A (when numbered from the EcoRI recognition site [14]) mutations [15]], (ii) translational (substitutions at 1809–1812, resulting in a TCAT [16]) and (iii) post-translational (G1862T transversion mutation) [17, 18] levels.

The precore/core (preC/C) open reading frame of HBV, containing two alternative initiation codons, encodes for the preC/C protein that is the precursor of HBeAg [19]. This preC/C protein is post-translationally modified by cleavage at a fixed site on the amino end in the endoplasmic reticulum (ER) and at a variable site on the carboxyl end in the Golgi apparatus, giving rise to the mature heterogeneous non-particulate soluble HBeAg protein that is secreted into the serum [19–22].

The G1862T mutation occurs in the bulge of the RNA encapsidation (e) signal, at a position that is 39 nucleotides upstream of the C gene initiator codon [23]. The viral polymerase/reverse transcriptase acts as a primer of RNA-directed
DNA synthesis by binding to the bulge of the $\varepsilon$ signal [23]. While the base pairing of the template at 1862 is not as essential as its binding at 1864 and 1865 [24], the presence of the mutation at 1862 may decrease the efficiency of reverse transcription, resulting in the decrease in a level of viral DNA [23, 25]. Studies have shown that the presence of the mutation decreased viral replication in conjunction with decreased HBeAg expression [8, 17, 18]. Furthermore, the G1862T mis-sense mutation occurs in the preC region coding for the amino terminal of the PreC region at codon 17. This mutation results in a phenotypic change from valine to phenylalanine at amino acid positions 19 and 20 [8, 26–28]. Phenylalanine, which is an aromatic and bulky amino acid, is a ‘forbidden’ amino acid at this position because of the $−3, −1$ rule [29–31], resulting in the abrogation of signal peptide cleavage, as seen by a decrease in signal peptide cleavage efficiency [8, 17, 30], which has also been shown for Escherichia coli alkaline phosphatase [32]. This leads to the G1862T preC/C precursor protein failing to meet the productive folding requirements of the ER and its retention in the ER for further folding by folding enzymes and molecular chaperones, and the prevention of synthesis and secretion of HBeAg [17, 33, 34]. Thereafter, the misfolded G1862T preC/C precursor protein that still failed to meet the productive folding needs of the ER is exported from the ER to the cytosol for degradation, as measured by increased expression of ubiquitin and proteasomes [17, 35]. In addition, the misfolded G1862T preC/C precursor protein results in the formation of ubiquitin-rich mini-aggregates along the microtubules in the cytosol [17, 36].

The ER quality control (QC) mechanism ensures that only correctly folded polypeptide chains traverse along the secretory pathway [37]. This ER QC mechanism can be compromised when unfolded or misfolded- and viral-proteins accumulate and aggregate in the lumen of the ER, resulting in ER stress [38–40]. In order to overcome this ER stress, all eukaryotic cells have evolved a specific response mechanism known as the unfolded protein response (UPR) or ER stress response [40–42]. In mammalian cells, initiation of the UPR occurs through activation of signalling cascades in a sequential order by three ER-localized transmembrane transducers, which include: (1) double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK), (2) activating transcription factor (ATF) 6, and (3) inositol-requiring enzyme (IRE1) [41, 42]. Activation of these signalling cascades alters the cell's transcriptional and translational programmes to cope with stressful conditions and resolve protein-folding defects, thus ensuring cell survival [40–42]. If the UPR fails, cells initiate lethal programmes such as autophagy or cell death by either apoptosis or necrosis [41, 43, 44].

The objective of the present study was to compare HBV protein expression, HBeAg expression in the secretory pathway, activation of the UPR and subsequent activation of apoptosis in Huh7 cells transfected with replication-competent plasmids belonging to either wild-type or G1862T subgenotype A1.

### Results

#### Intracellular and extracellular expression of HBV proteins

Immunoblotting was performed to determine the intracellular expression of HBV proteins in Huh7 cells that were transfected with either wild-type- or G1862T-A1 plasmids. No HBV protein bands were detected in cells transfected with replication-deficient HBV plasmids (Fig. 1a). There was no change in HBsAg expression when cells were transfected with either wild-type- or G1862T-A1 replication-competent plasmids (Fig. 1a). Cells transfected with wild-type A1 plasmid had higher expression of HBCAg and/or preC/C/HBeAg relative to cells transfected with the G1862T A1 plasmid (Fig. 1a).

ELISAs were performed to determine the extracellular expression of HBsAg and HBeAg in Huh7 cells that were transfected with either wild-type- or G1862T-A1 plasmids. No expression of HBsAg or HBeAg was observed in cells transfected with replication-deficient HBV plasmids (Fig. 1a). In the case of cells transfected with either wild-type- or G1862T-A1 plasmids, the expression of HBsAg was observed to occur earlier than that of HBeAg (Fig. 1b, c). From days 1 to 3, a statistically significant increase in the expression of HBsAg expression was observed for cells transfected with either wild-type- or G1862T-A1 plasmids (P<0.05, Fig. 1b). This increase in HBsAg expression was not maintained from day 3 to day 5, where a decrease in HBsAg expression was observed for cells transfected with either wild-type- or G1862T-A1 plasmids (Fig. 1b). HBeAg was not measured beyond day 5 and thus we could not determine whether this decrease was maintained or whether the difference was as a result of fluctuations of maximum levels of expression on days 3 and 5. From days 1 to 3 and days 3 to 5, there was a statistically significant increase in the expression of HBeAg observed in cells transfected with either wild-type- or G1862T-A1 plasmids (P<0.05, Fig. 1c). There was no statistically significant difference in the expression of HBsAg on days 1, 3 and 5 when cells were transfected with wild-type A1 plasmid as compared to G1862T A1 plasmid (Fig. 1b). On day 1, expression of HBeAg was statistically significantly lower for cells transfected with G1862T A1 plasmid compared to cells transfected with wild-type subgenotype A1 plasmid (P<0.05, Fig. 1c).

#### Subcellular localization of HBeAg in the early secretory pathway

No preC/C/HBeAg expression was detected when cells were mock-transfected (Fig. 2a, b: 1–8). In order to differentiate between the nucleus and the cytoplasm, cells were counterstained with DAPI (Fig. 2a, b: 1, 5, 9, 13, 17 and 21) and intracellular organelle markers were used to follow the expression of preC/C/HBeAg in the secretory pathway. Protein disulphide isomerase (PDI), which locates the ER, showed a fine reticular staining pattern that extended throughout the cytoplasm (Fig. 2a: 2, 6, 10, 14, 18 and 22), whereas the ER Golgi intermediate compartment (ERGIC)-53, which locates the ERGIC, showed a more punctate and dense staining pattern (Fig. 2a: 1, 5, 9, 13, 17 and 21) and presented throughout the cytoplasm (Fig. 2a: 7, 11, 15, 19 and 23).
The mouse monoclonal antibody against HBeAg binds to the preC region and thus does not differentiate between the preC/C and mature HBeAg. Both wild-type- and G1862T-A1 plasmids expressed HBeAg. Nuclear preC/C/HBeAg expression for cells transfected with either the wild-type- or G1862T-A1 plasmids showed a diffuse reticular and finely granular appearance on days 3 and 5 (Fig. 2a, b: 11, 15, 19 and 23). The relative frequency of the nuclear localization was higher for cells transfected with the wild-type A1 plasmid compared to cells transfected with the G1862T A1 plasmid. Cytoplasmic preC/C/HBeAg expression for cells transfected with wild-type A1 plasmid showed a diffuse and finely reticular expression (Fig. 2a, b: 11 and 15), whereas cells transfected with G1862T A1 plasmid showed a more punctate and reticular expression (Fig. 2a, b: 19 and 23), on days 3 and 5. The magnitude of expression of preC/C/HBeAg through the secretory pathway as visualized by the co-localization of preC/C/HBeAg with the ER and ERGIC remained the same when cells were transfected with the wild-type A1 plasmid (Fig. 2a, b: 12 and 16) on days 3 and 5, while it was relatively increased from days 3 to 5 when cells were transfected with the G1862T A1 plasmid (Fig. 2a, b: 20 and 24). These results suggest that the presence of the G1862T mutation leads to the temporal retardation of the preC/C/
HBeAg in the ER and ERGIC and decreased nuclear expression of the protein.

**Activation of the UPR**

We investigated if one or all three UPR pathways were activated following transfection of Huh7 cells with wild-type- or G1862T-A1 plasmids. Activation of the PERK pathway is the first and least stringent adaptive response pathway of the UPR that is initiated in cells in response to ER stress. Activation of the PERK pathway was determined by immunoblotting for the phosphorylation of eIF2α, which has a molecular weight of 38 kDa. Phosphorylation of eIF2α occurs subsequent to the dissociation of immunoglobulin heavy-chain binding protein (BiP) from PERK, resulting in its activation [40–42]. Activation of the PERK pathway was seen in all samples, with the activity of the PERK pathway being higher on day 3 compared to day 5 (Fig. 3). On day 5, cells transfected with wild-type A1 plasmid had a higher expression of phosphorylated eIF2α when compared to cells transfected with G1862T A1 plasmid (Fig. 3). This implies that the activation of the PERK pathway occurred earlier for cells transfected with G1862T A1 plasmid than cells transfected with wild-type subgenotype A1 plasmid.

Activation of the ATF6 pathway is the second and intermediate stringent adaptive response pathway of the UPR that is activated in cells in response to ER stress which is not overcome by the PERK pathway. Activation of the ATF6 pathway...
was determined by immunoblotting for the active form of ATF6α, which has a molecular weight of 50 kDa [40–42]. Activation of the ATF6 pathway was seen in all samples with activity being relatively higher on day 3 compared to day 5 (Fig. 4). On both day 3 and day 5, cells transfected with G1862T A1 plasmid had a relatively higher expression of phosphorylated ATF6 when compared to cells transfected with wild-type A1 plasmid (Fig. 4). This implies that the activation of the ATF6 pathway occurred earlier for cells transfected with the wild-type A1 plasmid than cells transfected with the G1862T A1 plasmid.

Activation of the IRE1/XBP1 pathway is the third and most stringent adaptive response pathway of the UPR that is activated in cells in response to ER stress which is not overcome by the PERK and ATF6 pathways. Activation of this pathway was determined by reverse-transcriptase PCR (RT-PCR) analysis, through the amplification of the spliced and unspliced form of XBP1s. The expression of XBP1s was normalized to the expression of GAPDH, which was used as a control gene.
forms of XBP1 mRNA, which represents the active and inactive forms of XBP1, respectively [40–42]. Activation of the IRE1/XBP1 pathway was seen in all samples, with activity being higher on day 5 compared to day 3 (Fig. 5). On day 3, there was no difference in the IRE1/XBP1 activity between cells transfected with either wild-type- or G1862T-A1 plasmids (Fig. 5), whereas on day 5, cells transfected with wild-type A1 plasmid showed higher IRE1/XBP1 activity when compared to cells transfected with G1862T A1 plasmid (Fig. 5). Unfortunately, because of the limitations of our study, we were unable to determine whether the activation of the IRE1/XBP1 pathway occurred earlier for cells transfected with wild-type- or G1862T-A1 plasmids.

**Activation of apoptosis and induction of total cell death**

Expression of cleaved soluble CK18 fragments serves as a marker of apoptosis, whereas expression of total CK18 serves as a marker of total cell death occurring as a result of apoptosis and necrosis [45–47]. On day 5, a statistically significantly higher extracellular expression of cleaved CK18 fragments was observed for cells transfected with wild-type A1 plasmid when compared to cells transfected with G1862T A1 plasmid (P<0.05, Fig. 6a). On both days 3 and 5, no statistically significant difference was observed in the expression of total CK18 when cells were transfected with either wild-type- or G1862T-A1 plasmids (Fig. 6b).

**DISCUSSION**

Using a replication-competent plasmid containing an endogenous HBV promoter and subgenotype A1 backbone, we functionally characterized G1862T, which occurs most frequently in subgenotype A1. This mutation is found in the preC region of the preC mRNA transcript and overlaps with the region that encodes the ε signal in the pgRNA and may therefore affect HBV replication [23, 25, 48] and/or decrease HBeAg expression [8, 26, 28]. As expected, HBsAg expression was not affected by cells transfected with wild-type A1 plasmid when compared to cells transfected with G1862T A1 plasmid (Fig. 7).

![Western Blot](image)

**Fig. 4.** Activation of the ATF6 pathway of the UPR by wild-type or G1862T-A1 HBV plasmid. Activation of the ATF6 pathway of the UPR was determined from Huh7 cells that were transfected with either wild-type- or G1862T-A1 plasmids and harvested at days 3 and 5 post-transfection. Tunicamycin, thapsigargin and ionomycin served as positive controls for the activation of the UPR. Activation of the ATF6 pathway was determined by immunoblotting. The immunoblot shows the expression of control proteins: α-tubulin and GFP (a), and experimental protein: cleaved ATF6α (b). α-Tubulin was detected by the binding of primary mouse monoclonal antibody against α-tubulin. GFP was detected by the binding of primary rabbit polyclonal antibody against GFP. Cleaved ATF6α was detected by the binding of primary rabbit polyclonal antibody against ATF6α. All primary antibodies were detected using either secondary goat anti-mouse IgG (H+L)-HRP antibody or goat anti-rabbit IgG (H+L)-HRP antibody. The expression of the protein of interest was determined relative to that of α-tubulin. To facilitate analysis, results from one blot are shown as two different blots.
and HBeAg, we showed a reduction in HbcAg when cells were transfected with the G1862T A1 plasmid when compared to cells transfected with the wild-type A1 plasmid. This is in agreement with previous studies, which showed that the presence of G1862T and/or G1888A independently caused a reduction in HbcAg expression [18, 49]. An additive decreased expression in HbcAg was observed when G1862T and G1888A occurred together [18]. Other studies have shown that changes upstream of the C gene initiation codon leads to changes in the secondary structure of the pgRNA and the formation of minicistrons, which may affect HbcAg expression [49–51]. G1862T is found 39 nucleotides upstream of the C gene initiator codon and further studies will be needed to elucidate the mechanism for reduced expression of HbcAg. In addition, primary antibodies that bind to HbcAg alone and not to HBeAg will be needed to confirm this reduction in HbcAg.

Fig. 5. Activation of the IRE1/XBP1 pathway of the UPR by wild-type- or G1862T-A1 HBV plasmid. Activation of the IRE1/XBP1 pathway was determined from Huh7 cells that were transfected with either wild-type- or G1862T-A1 plasmids and harvested at days 3 and 5 post-transfection. Tunicamycin, thapsigargin and ionomycin served as positive controls for the activation of the UPR. Activation of the IRE1/XBP1 pathway was determined by reverse-transcriptase PCR and restriction digestion. Comparison of the activation of the IRE1/XBP1 pathway was determined semi-quantitatively by using 500 ng total RNA for each sample. PstI results in the digestion of the unspliced form of XBP1. All lanes labelled ‘A’ show the amplification of total (spliced- and unspliced-form) XBP1 mRNA after PCR. All lanes labelled ‘B’ show the digestion of the total PCR product.
A1 correlated well with the expression of HBeAg seen in cells transfected with G1862T ER and ERGIC. The 22% reduction in the extracellular showed that G1862T HBeAg temporally accumulated in the early secretory pathway for wild-type- and G1862T A1 plasmid. Moreover, the expression of preC/C/HBeAg in the plasmid as compared to cells transfected with wild-type A1 reduced when cells were transfected with the G1862T A1.

Expression of preC/C/HBeAg on day 5 were markedly extracellular expression of HBeAg on day 1 and intracellular expression of preC/C/HBeAg on day 5 were markedly reduced when cells were transfected with the G1862T A1 plasmid as compared to cells transfected with wild-type A1 plasmid. Moreover, the expression of preC/C/HBeAg in the early secretory pathway for wild-type- and G1862T A1 showed that G1862T HBeAg temporally accumulated in the ER and ERGIC. The 22% reduction in the extracellular expression of HBeAg seen in cells transfected with G1862T A1 correlated well with the in silico prediction of a 24% decreased efficiency of the signal peptide in the ER, but was lower than the 54% reduction observed when cells were transfected with a HBeAg-expressing plasmid driven by an exogenous CMV promoter, to which G1862T had been introduced [17]. Cells transfected with G1862T A1 plasmid caused a temporal retardation in HBeAg expression, which confirmed a previous observation where the preC/C protein, p22, accumulated in the ER and did not transfer through the downstream secretory pathway when HBeAg-expressing clones driven by an exogenous CMV promoter were utilized [17]. Transfection with G1862T-HBeAg-expressing plasmids in the latter study, in addition to accumulating in the ER and ERGIC, also resulted in the accumulation of G1862T preC/C/HBeAg in the Golgi apparatus and in the formation of aggresomes, which were not seen in the present study [17]. These differences may be temporal because, as we demonstrated, the presence of an exogenous CMV promoter as opposed to an authentic HBV promoter leads to an earlier expression of HBeAg through the early secretory pathway [52]. Therefore, it is evident from this and previous studies that the secretion of HBeAg is not completely abolished but rather reduced by the presence of G1862T [17, 33]. Extending the follow-up of the transfection with the replication-competent plasmids used in our study for more than 5 days may result in its accumulation in the Golgi apparatus and in the development of aggresomes.

We found that in addition to being expressed in the early secretory pathway in the cytoplasm, preC/C/HBeAg was also expressed in the nucleus, as observed previously [53–55]. As suggested, this nuclear expression of preC/C/HBeAg may be the p22 form of the preC/C protein that is generated by signal
peptide cleavage in the ER [19, 56, 57]. Two different types of p22 exist: the secretory form and the cytosolic form [56, 58]. The cytosolic form of p22 is generated because of the inefficient translocation of the secretory form of p22 from the ER into the rest of the secretory pathway, which subsequently leads to its translocation into the cytoplasm through exploitation of the ERAD pathway; and is prevented from being degraded because of its low lysine content [56, 58, 59]. Thereafter, the cytosolic form of p22 can then traverse into the nucleus because of the presence of two nuclear localization signals in the arginine-rich carboxyl-terminal end, which are regulated by the presence of the extra 10 amino acids (preC region) on its amino end [56, 58, 60]. There was higher degree of localization of preC/C/HBeAg in the nucleus of cells transfected with wild-type A1 plasmid compared to G1862T A1 plasmid. Localization of p22 in the cytoplasm and nucleus could lead to the persistence of viral infection [59] since a higher prevalence of nuclear localization of preC/C/HBeAg was observed in liver biopsies of patients with chronic hepatitis than in those with acute hepatitis [53, 55]. Therefore, further studies that are able to differentiate between the cytosolic and secretory form of p22 will need to be performed to verify this hypothesis.

The activity of the PERK and IRE1/XBP1 pathways appeared to be decreased whereas the ATF6 pathway appeared to be increased for cells transfected with G1862T subgenotype A1 replication-competent plasmid when compared to the wild-type counterpart. Apoptosis was lower in cells transfected with G1862T A1 plasmid when compared to cells transfected with wild-type A1 plasmid. Moreover, the relative levels of apoptosis decreased over time in cells transfected with G1862T A1 plasmid but not in cells transfected with wild-type A1 plasmid. The temporal decrease in apoptotic activity seen in the presence of G1862T correlates well with a previously suggested hypothesis that postulates that adaptation of cells to chronic ER stress occurs through the regulation of the downstream effector molecules upon activation of the three pathways of the UPR [61]. This may occur by altering the capacity of the ER to process the proteins to an extent that would be sufficient to alleviate the perturbing effects of the stressor [61]. Further UPR activation does not occur, leading to a new homeostasis of the ER, where active UPR signalling maintains the improved processing capacity but does not result in the activation of apoptosis [61].

G1862T led to a decreased expression of HBeAg and preC/C/HBeAg. The decreased expression of extracellular HBeAg was probably the result of decreased cleavage efficiency by the signal peptide leading to the retardation of the preC/C/HBeAg protein in the ER and ERGIC and its decreased expression in the nucleus. This retardation and accumulation led to the earlier activation of all three UPR pathways, but not to an increase in apoptosis. In response to the accumulation of HBeAg in the ER, the UPR was activated, altering the capacity of the ER to overcome the cellular stress.

HBeAg and HBeAg share antigenic epitopes. Processed HBeAg peptides presented by human leukocyte antigen (HLA)s are a major target of HBV specific cytotoxic T cells. HBeAg modulates T cell immunity to HBeAg and reduces immune pathogenicity caused by HBeAg-specific T cells [62–64]. The decreased expression of HBeAg in patients infected with the G1862T mutant results in the immune response being directed to the hepatocytes, and together with the increased ER stress, it can result in liver damage, which has been shown to be a contributing factor to hepatocarcinogenesis. G1862T frequently develops in subgenotype A1 and this mutation can contribute to the higher hepatocarcinogenic potential of this subgenotype [65].

**METHODS**

**Construction of G1862T subgenotype A1 1.28 mer replication-competent plasmid**

The wild-type subgenotype A1 1.28 mer replication-competent plasmid was constructed and shown to replicate and express HBV proteins in vitro [66]. A G1862T subgenotype A1 1.28 mer replication-competent plasmid was generated using the strategy described previously [66]. This plasmid has the same backbone as wild-type subgenotype A1, which ensured that any differences observed in vitro were as a result of the presence of the mutation. Ethics approval to perform this study was obtained from the University of the Witwatersrand Research Ethics Committee (Medical), Johannesburg, South Africa.

**Cell culture and transfection**

Huh7 cells were transfected with either wild-type- or G1862T subgenotype A1 replication-competent plasmids as described previously [52, 66]. Twenty-four hours prior to transfection, cells were plated as follows, depending on the technique to be performed downstream: 1.2×10⁶ cells were plated into each 10 cm culture dish for Southern blotting; 6×10⁵ cells were plated into each of the 6 cm culture dishes containing either six coverslips for indirect immunofluorescence or no coverslips for immunoblotting. ELISA for measurement of HBsAg and HBeAg and reverse transcriptase PCR (RT-PCR); and 6×10⁴ cells were plated into 96-well tissue culture plates for ELISA for measurement of cleaved and uncleaved cytokeratin (CK) 18. Plated cells were incubated overnight at 37°C in a humidified incubator containing 5% CO₂. Transfection efficiency was measured 1 day post-transfection by determining the number of cells that had been successfully transfected with the eGFP plasmid using an Olympus IX71 fluorescence microscope system (Olympus Soft Imaging Solutions). All transfection reactions were carried out in triplicate.

For the determination of the activation of the UPR, stress-inducing compounds were included as controls and added to Huh7 cells 24 h prior to cell harvest: (1) 5 μg ml⁻¹ tunicamycin, a nucleoside antibiotic, which functions in the inhibition of N-linked glycosylation resulting in the accumulation of unfolded proteins in the ER [67, 68]; (2) 2 μg ml⁻¹ thapsigargin, which functions in Ca²⁺ depletion from the ER and consequently results in ER stress [67]; and (3)
5 µg ml⁻¹ ionomycin, which functions in Ca²⁺ depletion from the ER and consequently results in ER stress [68].

For the determination of the activation of cell death, the following controls were included and added to Huh7 cells 24 h prior to cell harvest: (1) 7 µg ml⁻¹ cycloheximide, a protein synthesis inhibitor that induces the activation of the JNK pathway and subsequent apoptosis [69]; and (2) 1 : 1000 H₂O₂, which functions in the dose-dependent activation of necrosis through the activation of the activator protein (AP) 1 [70].

**Immunoblotting**

Expression of HBV proteins (HBsAg and HBeAg/HBeAg) and activation of the PERK and ATF6 pathways was determined by immunoblotting analysis. Expression of the HBV proteins was determined on day 5 post-transfection, while activation of the PERK and ATF6 pathways was determined on days 3 and 5 post-transfection. The protocol for immunoblotting was as described previously [52, 66]. The nitrocellulose membrane was incubated overnight at 4°C with one of the following primary antibodies: 1 : 50 000 mouse monoclonal antibody against α-tubulin (clone B-5-1-2) (Sigma-Aldrich), 1 : 25 000 rabbit polyclonal antibody against GFP (ab6556), 1 : 2000 rabbit polyclonal antibody against ATF6α (ab37149) (both from Abcam), 1 : 4000 mouse monoclonal antibody against the preS1 region of HBsAg (MA18/7), 1 : 1000 mouse monoclonal antibody against the preS2 region of HBsAg (Q19/10) (both kindly donated by Dr Dieter Glebe, Justus-Liebig University, Giessen, Germany), 1 : 1000 mouse monoclonal antibody cross-reacting against HBCAg and HBeAg (ID8) (kindly donated by Dr Peter Revill, Victorian Infectious Diseases Reference Laboratory, Melbourne, Victoria, Australia) and 1 : 1000 rabbit polyclonal antibody against phospho-eukaryotic initiation factor (eIF) 2α [serine (Ser) 51 (9721)] (Cell Signalling Technology). The following day, the nitrocellulose membrane was incubated with either 1 : 3000 goat anti-mouse IgG (H+L)-horseradish peroxidase (HRP) conjugate or 1 : 3000 goat anti-rabbit IgG (H+L)-HRP conjugate, depending on the species of the primary antibody, and 1 : 10 000 Precision Protein StreptTactin–HRP conjugate, which detects for the Precision Plus Protein WesternC Standards (all from Bio-Rad Laboratories). Thereafter, the protein signal of interest was developed with SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology by Thermo Fischer Scientific). Chemiluminescent detection of the protein signal of interest was performed using the GelDoc MP system camera (Bio-Rad Laboratories) and Image Lab, version 3.0 software.

**Enzyme-linked immunosorbent assay (ELISA)**

The amount of extracellular HBsAg, HBeAg, cleaved CK18 fragments and soluble total (cleaved and uncleaved) CK18 was determined by ELISA. Expression of HBsAg and HBeAg was determined on days 1, 3 and 5 using the Monolisa HBsAg ULTRA and Monolisa HBeAg-Ab PLUS (both from Bio-Rad Laboratories), respectively. Extracellular cleaved CK18 fragments, which are generated through the cleavage of CK18 by active cysteiny1 aspartate-specific protease (caspase)-9, which acts as a marker of apoptosis in epithelial cells, including hepatocytes, were quantified using the M30-Apoptosense ELISA kit (Peviva AB) [45, 46]. Total cell death was determined extracellularly by the quantitative measurement of soluble total (cleaved and uncleaved) CK18, which is expressed at high levels in most cells and in serum when released from epithelial cells during cell death, and was detected using the M65 ELISA kit [46, 47].

**Indirect immunofluorescence**

On days 3 and 5, Huh7 cells grown on coverslips and transfected with either the wild-type- or G1862T- subgenotype A1 replication-competent plasmids were prepared for indirect immunofluorescence as previously described [52]. The primary antibodies used were mouse monoclonal antibody against HBeAg (7e9), which does not react with HBcAg [71] (kindly donated by Dr Peter Revill, Victorian Infectious Diseases Reference Laboratory, Melbourne, Victoria, Australia) in combination with either rabbit monoclonal antibody against PDI (C8H16) (Cell Signalling Technology) or rabbit polyclonal antibody against ERGIC-53/p58 (Sigma-Aldrich), which bind to their respective proteins, located in the ER and ERGIC, respectively. The secondary antibodies used were 1 : 1000 donkey anti-mouse (IgG) (H+L) antibody labelled with AlexaFluor-488 or -546, and 1 : 1000 goat anti-rabbit (IgG) (H+L) antibody labelled with AlexaFluor-546 or -488 (all from Invitrogen). Coverslips were mounted onto tissue slides using ProLong Gold antifade reagent with 4’,6-diamidino-2-phenyindole (DAPI) (Invitrogen). The cells were viewed and the images captured and analysed using an LSM 780 confocal laser scanning microscope and camera and ZEN 2010, release version 6.0 software (Carl Zeiss Microimaging).

**RT-PCR and restriction digestion analysis**

Activation of the IRE1/XBP1 pathway was determined on days 3 and 5 post-transfection by RT-PCR and restriction digestion analysis. The protocol for RT-PCR and restriction digestion analysis was as described previously [52]. Briefly, total RNA was extracted using the Trizol Plus RNA purification kit (Invitrogen) following the manufacturer’s protocol. An aliquot of 500 ng RNA was reverse transcribed to generate cDNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories). Subsequently, the cDNA was used as a template to amplify part of the spliced and unspliced XBPI mRNA of 174 and 198 bp, respectively. Confirmation of the unspliced form of the XBPI PCR product was achieved using digestion with PsI, which is only found in the unspliced form of XBPI mRNA, resulting in the generation of two fragments of 114 and 84 bp.

**Statistical analysis**

Quantification of HBV DNA and extracellular expression of HBsAg and HBeAg, cleaved and total CK18 was analysed statistically using STATA 12.0 data analysis and statistical software (StataCorp LP). The significance of the difference...
observed between samples was determined using the one-way ANOVA test and the Scheffe comparison method. Probability $P<0.05$ was regarded as statistically significant. Analysis was obtained from three independent transfection experiments performed in triplicate.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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