Zhang *et al.* showed that HBcAg downregulated TLR4 expression on the CD14 positive monocytes in HBV-infected patients in comparison to healthy controls (67). Another study identified that HBsAg stimulated monocytes to release IL-10, which resulted in activation of JAK/STAT3 pathway (51) and consequently suppression of IRAK1, IRAK2, TRAF6, and MAPK pathways (Fig. 1). Second, the polymorphisms within the gene of TLR4 may be another reason for downregulation of this molecule in long-term HBV infection. Accordingly, Cussigh *et al.* reported that the polymorphism within +299 region of TLR4 was significantly associated with chronic HBV infection (20). A study on the Taiwanese population revealed that TLR4 rs4986790 (p.Asp299Gly) polymorphism was significantly associated with HBsAg seroclearance/seroconversion in chronic HBV-infected patients (61). Zhou *et al.* showed that the polymorphism within 3′-untranslated position of the TLR4 gene was significantly associated with protection from HBV recurrence after liver transplantation in the Chinese population (68). Epigenetic factors are the third plausible candidates for regulation of TLR4 expression in prolonged HBV-infected patients. In parallel with the hypothesis, several investigations demonstrated that miRNAs, as important epigenetic factors, play key roles in TLR4 expression, as well as its molecular signaling (19,38,46). Interestingly, our unpublished data showed that expression of miRNA-1, 21, 125, and 155 were significantly increased in Iranian HBV chronically infected patients. Moreover, previous studies revealed that immune tolerance to HBV antigens is plausible in long-term hepatitis B. Hence, it seems that alteration in TLR4 expression and its intracellular signaling molecules is a probable mechanism to induce immune tolerance to HBV antigens. Interestingly, one study revealed that lipopolysaccharide/TLR4 interactions could lead to activation of T regulatory lymphocytes, which play significant roles in induction of immune tolerance to hepatitis B antigens (67). It appears that additional studies using TLR4 agonists as adjuvants can improve our knowledge about the roles of TLR4 and its related signaling pathways in modulation of immune responses against HBV.

TLR4 and Hepatitis B Liver Complications

In contrast to the roles played by TLR4 in induction of appropriate immune responses against HBV, it appears that there is a different scenario regarding the roles of TLR4 in the pathogenesis of hepatitis B–related complications such as cirrhosis and HCC. For example, Cheng *et al.* revealed that endotoxin is upregulated during active phases of hepatitis B (17). They also showed that mRNA levels of TLR4 are significantly increased during endotoxin stimulation (17), which leads to production of pro-inflammatory cytokines, including TNF-α, IL-1, and IL-6. The phenomenon results in the aggravation of the hepatitis B. Soares *et al.* reported that the expression of TLR4 on hepatocytes was increased in hepatitis B and its related complications, including cirrhosis and HCC (53). It has also been identified that hepatic stellate cells (HSCs), the important cells involved in induction of liver cirrhosis, express TLR4 and respond to TLR4 ligands vigorously (3). Wang *et al.* showed that transfection of HK-2 cells, an immortalized proximal tubule epithelial cell line, with HBx gene results in upregulation of TLR4 *in vitro* (58). Lian *et al.* revealed that the expression of TLR4 was significantly upregulated in patients with liver cirrhosis when compared with noncirrhotic chronic HBV-infected patients (36). The main responsible mechanisms that lead to cirrhosis and HCC via TLR4 pathway have yet to be completely clarified, but the authors of the current review article suggest that several directly and indirectly plausible mechanisms could be responsible for induction of cirrhosis and HCC as follows. First, as a probable direct mechanism, it has been evidenced that MYD88-dependent intracellular signaling results in activation of several pro-inflammatory transcription factors, including NF-κB and AP-1, which are the main reasons for induction of HCC (37,40). Based on the fact that TLR4/ligands interactions lead to immune cells activation via MYD88-dependent pathway, it may be hypothesized that TLR4 may promote tumoregenesis pathways in the HBV-infected hepatocytes. Second, as plausible indirect mechanisms, studies have revealed that angiotensin-II is an important molecule that participates in induction of liver fibrosis (50). TLR4 has a pivotal cross-talk with angiotensin-II (52). Hence, it may induce cirrhosis via upregulation of angiotensin-II. It has also been identified that inflammation plays pivotal roles in the induction of cirrhosis and HCC (41,54). TLR4 is a main receptor for PAMPs and DAMPs, which stimulate immune cells to produce inflammation in the infected tissues. Thus, it appears that the induction of inflammation via TLR4/ligands interactions may be considered as another mechanism to induce cirrhosis and HCC. Furthermore, researchers have demonstrated that high-mobility group box 1 (HMGB1) stimulates proliferation, migration, and profibrotic effects of HSCs (56). Wang *et al.* reported that TLR4/ligands interaction leads to upregulation of HMGB1 (56). Transforming growth factor-β1 (TGF-β1) also plays important roles in the pathogenesis of fibrosis (18). It has been documented that TLR4 promotes the fibrotic effects of TGF-β1 on the fibroblasts (11). It has been documented that T regulator lymphocytes plays significant roles in induction of fibrosis by production of TGF-β1 (18). Lian *et al.* reported that TLR4 expression has a positive correlation with the frequency of T regulatory lymphocytes in HBV-infected patients with liver cirrhosis (36). Therefore, it appears that TLR4 may participate in the pathogenesis of liver complications, and future therapeutic strategy should be directed to the utilization of TLR4 antagonists for the treatment and prevention of hepatitis B–related liver cirrhosis and HCC.

Concluding Remarks

According to the information presented by the aforementioned investigations, it may be concluded that TLR4 plays important roles in the stimulation of appropriate immune responses against HBV. Thus, the virus targets this receptor and also its intracellular signaling molecules to suppress immune responses in long-term hepatitis B infection. Additionally, based on the presented studies, it seems that TLR4 and its intracellular molecular signaling potentially participate in the pathogenesis of hepatitis B–related complications, including liver cirrhosis and HCC. Accordingly, it appears that future therapies should focus on treatments of noncirrhotic/HCC and cirrhotic/HCC hepatitis B–infected patients with agonists and antagonists of TLR4, respectively, to promote immune responses for eradication

of HBV in noncirrhotic/HCC and regulate inflammation and pro-inflammatory transcription factors in cirrhotic/HCC hepatitis B–infected patients.

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Author Disclosure Statement

AU4 ▶ No competing financial interests exist.

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Resistance to cyclosporin A derives from mutations in hepatitis C virus nonstructural proteins

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ABSTRACT

Cyclosporine A (CsA) is an immunosuppressive drug that targets cyclophilins, cellular cofactors that regulate the immune system. Replication of hepatitis C virus (HCV) is suppressed by CsA, but the molecular basis of this suppression is still not fully understood. To investigate this suppression, we cultured HCV replicon cells (Con1, HCV genotype 1b, FLR-N cell) in the presence of CsA and obtained nine CsA-resistant FLR-N cell lines. We determined full-length HCV sequences for all nine clones, and chose two (clones #6 and #7) of the nine clones that have high replication activity in the presence of CsA for further analysis. Both clones showed two consensus mutations, one in NS3 (T1280V) and the other in NS5A (D2292E). Characterization of various mutants indicated that the D2292E mutation conferred resistance to high concentrations of CsA (up to 2 μM). In addition, the missense mutation T1280V contributed to the recovery of colony formation activity. The effects of these mutations are also evident in two established HCV replicon cell lines—HCV-RMT ([1], genotype 1a) and JFH1 (genotype 2a). Moreover, three other missense mutations in NS5A—D2303H, S2362G, and E2414K—enhanced the resistance to CsA conferred by D2292E; these double or all quadruple mutants could resist approximately 8- to 25-fold higher concentrations of CsA than could wild-type Con1. These four mutations, either as single or combinations, also made Con1 strain resistant to two other cyclophilin inhibitors, N-methyl-4-isoleucine-cyclosporin (NIM811) or Debio-025. Interestingly, the changes in IC_{50} values that resulted from each of these mutations were the lowest in the Debio-025-treated cells, indicating its highest resistant activity against the adaptive mutation.

1. Introduction

The genome of the hepatitis C virus (HCV) is a single-stranded RNA with positive polarity and is classified in the *Flaviviridae* family. HCV frequently establishes chronic infections that lead to liver cirrhosis and hepatocellular carcinoma (HCC) [2]. An estimated 130–200 million people worldwide are now infected with HCV [3]. HCVs have been classified into six major genotypic groups (genotypes 1–6); genotype 1 is the most prevalent over most of the world. Treatments with alpha interferon (IFNα), together with the nucleoside analog ribavirin (RBV), greatly increased the percentage of HCV chronically infected patients able to reach a sustained anti-viral response (SVR). Covalent attachment of polyethylene glycol (PEGylated) IFN-α-plus-RBV therapy has a success rate of ~80% in patients with genotype 2 or 3 infections, but only ~50% in patients with genotype 1 infections [4,5]. The recently approved protease inhibitors boceprevir and telaprevir each improved the efficacy of IFN-α-plus-RBV therapy [6]. These direct-acting agents (boceprevir, simeprevir, sofosbuvir, faldaprevir and telaprevir, etc.) each have the advantage of being highly specific, but each may select for specific resistant mutations, limiting their long-time efficacy. Therefore, antiviral inhibitors targeting host factors crucial for viral replication should be developed to overcome these problems.

Abbreviations: HCV, hepatitis C virus; CsA, cyclosporine A; HCC, hepatocellular carcinoma; IFNα, alpha interferon; Cyp, cyclophilins; SVR, sustained anti-viral response.

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Reportedly, several HCV proteins interact with cyclophilins (Cyp) and modulate HCV replication [7–9]. To date, three Cyp inhibitors—Debio-025, NIM811, and SCY-635—have been deemed safe and effective for patients with HCV in phase I and II studies [10–12]. Development of Debio-025 has advanced the farthest through phase II studies, and Debio-025 has approved and showed a great deal of promise for decreasing HCV viremia in infected patients. However, emergence of drug-resistant HCV mutants could limit the therapeutic potential of CsA and Cyp inhibitors.

The HCV genome is a positive-sense, single-stranded RNA (about 9.6 kb) that encodes at least 10 viral proteins; these are categorized as structural core proteins (E1, E2) or nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [13,14]. The nonstructural proteins are involved in HCV RNA replication [14]. NS5A protein comprises three domains linked by two low-complexity sequences (LCS) that are either serine or proline rich; domain I is a highly structured zinc binding domain whose three-dimensional structure shows two dimeric conformations [15,16]. Domains II and III have been shown to be unstructured in their native states, but nuclear magnetic resonance and circular dichroism have shown that elements of secondary structure run throughout each of these domains [17–19]. NS5A is anchored to membranes by an N-terminal amphipathic helix and is an essential component of the viral genome replication complex; it also interacts with other non-structural proteins [20] or cellular factors. NS5A domain II is a substrate for the peptidyl-prolyl cis/trans isomerase activity of Cys A and B [21], and NS5A domain III is reportedly a substrate of CypA [22].

In this study, we used CsA to select for and isolate drug-resistant HCV mutants; we then performed virus genome sequencing to investigate the molecular mechanisms of this drug resistance.

2. Materials and methods

2.1. Cells, electroporation and ethics statement

HuH-7 cells were cultured in DMEM-GlutaMax-I (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Invitrogen). Replicon cells were maintained in the same medium supplemented with 300 μg/ml G418 (Invitrogen). Cells were passaged three times a week, and at each passage, each culture was split into four subcultures. Electroporation of replicon RNA and G418 selection were performed as previously described [23]. All experimental protocol was approved by the regional research institute.

2.2. Establishment of cyclosporin A resistant replicon clones

FLR3-1 cells derived from Con1 (AJ238799)-based, luciferase-harboring HCV sub-genomic replicon cell were treated with both 2 μM of cyclosporin A and 0.5 mg/ml of G418 for 24 days. Surviving cells were further treated with 3 μM CsA for 2 days, 4 μM for 4 another days, and finally 6 μM for the last 10 days. Using limiting dilution cloning, we established nine clonal cell lines. Using real-time RT-PCR (ABI 7700 system, Applied Biosystems, Foster City, CA, USA) as described previously [24], we systematically measured HCV RNA copy number in each of these nine clonal lines.

2.3. Determination of consensus sequence of resistant clones

LongRange Reverse transcriptase (QIAGEN, Valencia, CA, USA) and an oligonucleotide primer (antisense sequence 9549–9569 of HCV-Con1) were used to reverse transcribe purified RNA (1 μg). The resulting cDNA, Phusion DNA polymerase (Finnzymes, Vantaa, Finland), and primers recognizing each non-coding region were used for PCR amplification of the entire non structural protein coding region of the sub-genomic replicon. The TA cloning kit (Invitrogen) was used to introduce each fragment into a separate plasmid; we picked up eight clones from each resistant cell line and their nucleotide sequences were determined.

2.4. Construction and RNA transcription

The pFK I389neo/NS3-3′/5.1 and pFK I389luc/NS3-3′/5.1 plasmids (ReBlikon, Baden-Württemberg, Germany) were used to generate HCV constructs with regions of the sub-genomic replicon with mutations (Fig. 2A). The QuikChangeII kit (Stratagene, La Jolla, CA, USA) was used to introduce specific mutations into the HCV sequences. To generate RNA, plasmids were digested with *XbaI* and used as a template for RNA transcription; RiboMax (Promega, Madison, WI, USA) was used for each transcription reaction.

2.5. Drug treatment

For the drug resistance assays, established CsA-resistant replicon clones were seeded onto 24-well tissue culture plates (10,000 cells/well) and cultivated overnight. Then cells were treated with various concentrations of CsA (0–8 μM) for 4 days. Surviving cells were stained with crystal violet.

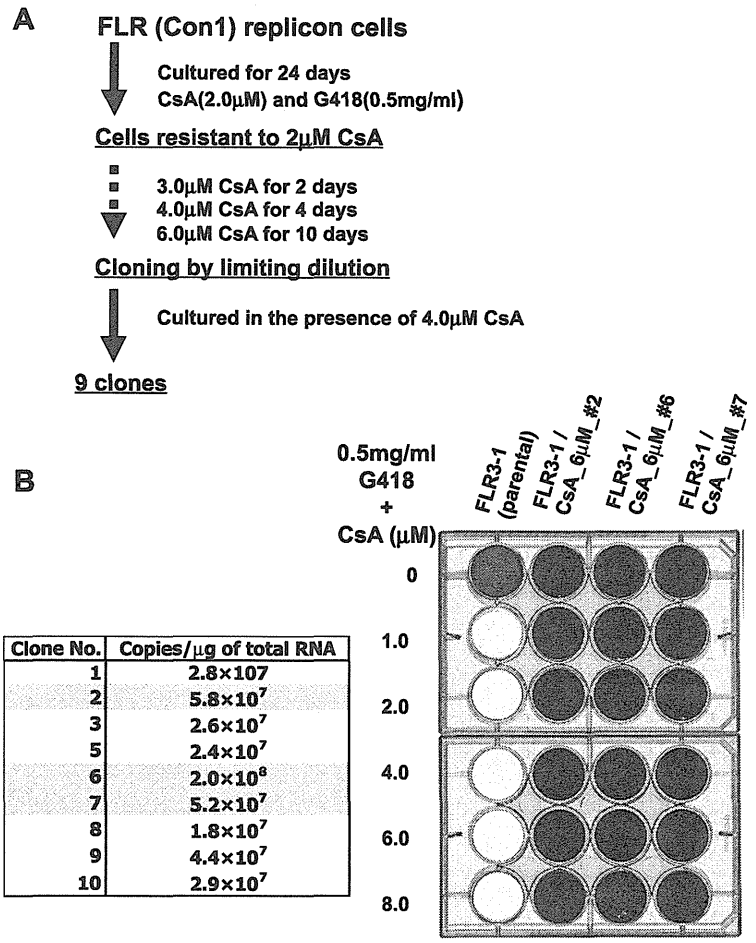
For HCV replication inhibition assays, replicon cells were seeded in 96-well tissue culture plates (5000 cells/well) and cultivated overnight. Serial dilutions of CsA (Fluka Chemie, Buchs, Switzerland) or NIM811 (Novartis) and Debio025 (Debiopharma) were then added to sets of wells. After incubation for 72 h, ABI prizm 6100 (Applied Biosystems) was used to extract total RNA from cells, and HCV-RNA was measured as described above. Each assay was carried out in triplicate.

For another HCV replication inhibition assay, mutant replicon RNA derived from pFK I389luc/NS3-3′/5.1 plasmid were introduced into HuH7 cells via electroporation, and the transformed/transfected cells were seeded to 96-well tissue culture plates. Drugs were added 24 h after electroporation. Luciferase activities were evaluated 4 h or 72 h after electroporation, which corresponded to 20 h before drug treatment or 48 h after drug treatment, respectively; the Blight-Glo kit (Invitrogen) and Envision (Perkin Elmer, Waltham, MA, USA) were used to take all measurements, and values at 72 h were normalized relative to the values from 4 h.

3. Results

3.1. Establishment of CsA-resistant clones

To establish CsA-resistant clones, we treated HCV FLR-N replicon cells with CsA (Fig. 1A) and obtained nine resistant clonal cell lines. We measured the amount of HCV RNA in each resistant clonal line and chose for further study the three lines that consistently had the largest amount of HCV RNA (Fig. 1B). We then determined the entire HCV sequence from 16 subclones; we isolated two groups of eight subclones (one group each from clones #6 and #7), because we could not establish clone #2; each subclone was isolated by treating a CsA-resistant clone (#6 or #7) with 6 μM CsA (Table 1). Although there were several mutations in the NS3–NS5B protein-coding regions, common mutations were isoleucine (I) to valine (V) at amino acid 1280 (T1280V) and aspartic acid (D) to glutamic acid (E) at amino acid 2292 (D2292E). At 1280, original Con1 has threonine (T) and was mutated into (I) in Con1 replicon cells.



Clone No.	Copies/μg of total RNA
1	2.8×10^{7}
2	5.8×10^{7}
3	2.6×10^{7}
5	2.4×10^{7}
6	2.0×10^{8}
7	5.2×10^{7}
8	1.8×10^{7}
9	4.4×10^{7}
10	2.9×10^{7}

Fig. 1. Basic characteristics of the nine cyclosporin A-resistant clones. (A) Flow chart outlining the selection of cyclosporin A-resistant HCV replicon clones. (B) Real-time PCR was used to determine the copy number of each Cys A-resistant clone. The three clones with the highest HCV genome copy number are highlighted in green (Left). Colony formation assay of mutant #2, 6 and 7 (Right). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Identification of mutations responsible for CsA resistance

To define the mutations responsible for CsA resistance, we constructed various chimeric clones that each contained specific mutation that arose from CsA selection (Fig. 2A). We could thereby evaluate each mutation with regard to its effect on CsA resistance. We found that mutations in two proteins—NS5A and NS4A—significantly enhanced the resistance against CsA treatment (Fig. 2B). We also cultured replicon cells with these mutants in the presence of CsA (up to 2 μM); we found that cells with a D2292E mutation could survive, but cells with wild-type NS5A or T1280V mutation could not (Fig. 3A).

The effect of T1280V mutation on colony formation was further evaluated (Fig. 3B). Introduction of the T1280V mutation in *cis* to the D2292E mutation rescued the colony-formation defect of the D2292E mutant replicon cells; specifically, the T1280V–D2292E double-mutant replicon cells had the same colony-forming ability as the parental replicon cells.

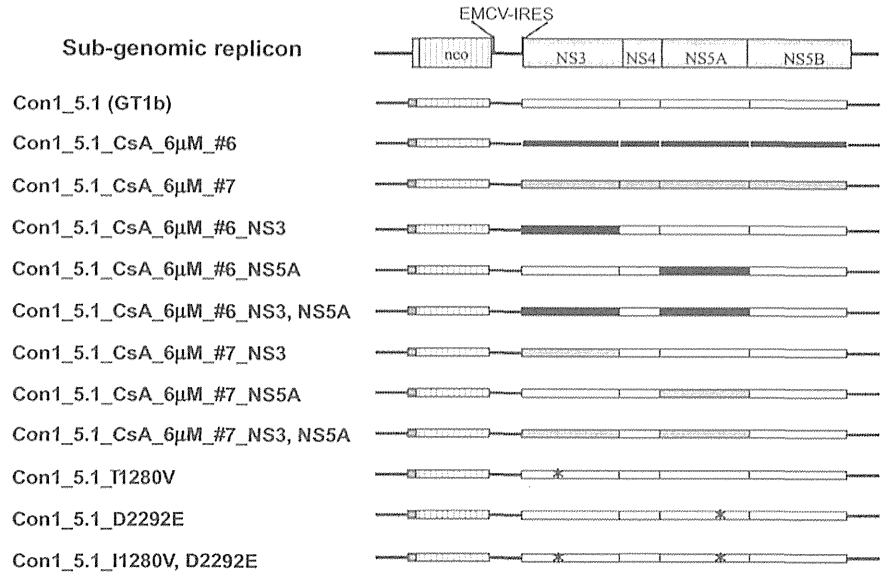
3.3. Evaluation of mutations for CsA resistance in other HCV genotypes

We evaluated whether the mutations that conferred CsA resistance to the HCV Con1 strain (genotype 1b) also conferred CsA resistance to the RMT (genotype 1a; AB520610) and JFH1 (genotype 2a; AB047639) strains (Fig. 4A, B and Table 2) [1]. D2292E conferred CsA resistance to the HCV strains RMT and JFH1, but T1280V did not (Table 2), as observed with HCV Con1 strain (Fig. 2E). The amino acid sequences surrounding mutations other than D2292E showed some differences among three genotypes (1a, 1b, and 2a) (Fig. 4B). D2292E mutants of these three genotypes showed resistance to CsA (Fig. 2E, Table 2) but the fold increase of resistance in genotype 1a and 2a was lower than that of genotype 1b (Tables 2 and 3). Therefore, there might be some residue(s) other than D2292E to influence the resistance to CsA.

3.4. Efficacy of mutations in NS5A for conferring CsA resistance

Although D2292E clearly conferred CsA resistance to HCV, other mutations in NS5A may also have had an effect because constructs with all four of the original NS5A mutations found in clone #6 mutations were more resistant to CsA than were constructs with only the D2292E mutation (Fig. 2B and E). We constructed HCV-luciferase replicons, each with one or more of four mutations (D2292E, D2303H, S2362G, and E2414K). HuH-7 cells were transiently transfected with RNA of each construct; we then treated the transfected cells with CsA (Table 3). Of the four single mutants, all but S2362G conferred some CsA resistance to HCV-luciferase replicons; notably, combinations of mutations had additive effects

A



B

Cyclosporin A	Replacement or Mutation			IC_{50} (μM)	Fold change
	NS3	NS4A	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.18	1.0
	Cs6#6	-	-	0.21	1.2
	-	-	Cs6#6	2.10	11.7
	Cs6#6	-	Cs6#6	2.20	12.2
	Cs6#6	V1681A	Cs6#6	5.40	30.0

C

NIM811	replacement or mutations			IC_{50} (μM)	Fold change
	NS3	NS4A	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.09	1.0
	Cs6#6	-	-	0.10	1.1
	-	-	Cs6#6	0.49	5.4
	Cs6#6	-	Cs6#6	0.50	5.6
	Cs6#6	V1681A	Cs6#6	1.80	20.0

D

Debio025	Replacement or mutation			IC_{50} (μM)	Fold change
	NS3	NS4A	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.05	1.0
	Cs6#6	-	-	0.04	0.8
	-	-	Cs6#6	0.43	7.8
	Cs6#6	-	Cs6#6	0.40	8.0
	Cs6#6	V1681A	Cs6#6	0.75	13.7

E

Cyclosporin A	Replacement or mutation			IC_{50} (μM)	Fold change
	NS3	NS4B	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.54	1.0
	Cs6#7	-	-	0.59	1.1
	-	-	Cs6#7	2.36	4.4
	Cs6#7	-	Cs6#7	2.51	4.6
	Cs6#7	I1797V	Cs6#7	2.44	4.5
	T1280V	-	-	0.57	1.1
	-	-	D2292E	1.71	3.1
	T1280V	-	D2292E	1.76	3.3

Fig. 2. (A) Schematic representations of 12 Con1 replicon-derived constructs. (B–D) Evaluation of Cs6#6 constructs with regard to resistance to CsA or to each of two CsA derivatives (NIM811 and Debio025). Real-time PCR was used to measure HCV sub-genome copy number in cells, and IC_{50}s were then determined from the copy number values. For each construct, the fold change represents the ratio of IC_{50} values from the construct and the parental Con1 replicon (IC50Construct:IC50Parental). (E) Resistance to CsA of three Cs6#7 derivative constructs that represent the T1280V and D2292E mutations as each single mutation or as a double mutation.

and conferred greater CsA resistance than any single mutation. The HCV replicon with all four mutations showed the strongest CsA resistance.

3.5. Evaluation of CsA-resistant mutants for resistance to cyclophilin inhibitors

We further evaluated each of the NS5A mutants for their ability to confer resistance to each of two other cyclophilin inhibitors, N-methyl-4-isoleucine-cyclosporin (NIM811, Table 4) and Debio-025 (Table 5). Of the four single mutants, D2292E conferred the highest resistance, and the combination of all four mutations conferred the overall highest resistance to NIM811 and to Debio-025. When we compared CsA, NIM811, and Debio-025, the mutation-mediated increases in IC_{50} values were lowest with the Debio-025 treatment (Tables 3–5).

4. Discussion

Here, we investigated two of nine HCV sub-genomic replicon cell clones (CsA-resistant HCV mutants) isolated following long-term dual treatment with CsA and G418. Comparing the HCV sequences of these two clones (#6 and #7), only two of many mutant sites were shared between the mutant HCV sequences. Specifically, both clones #6 and #7 had a D2292E missense mutation in NS5A and a T1280V missense mutation in NS3. D2292E is

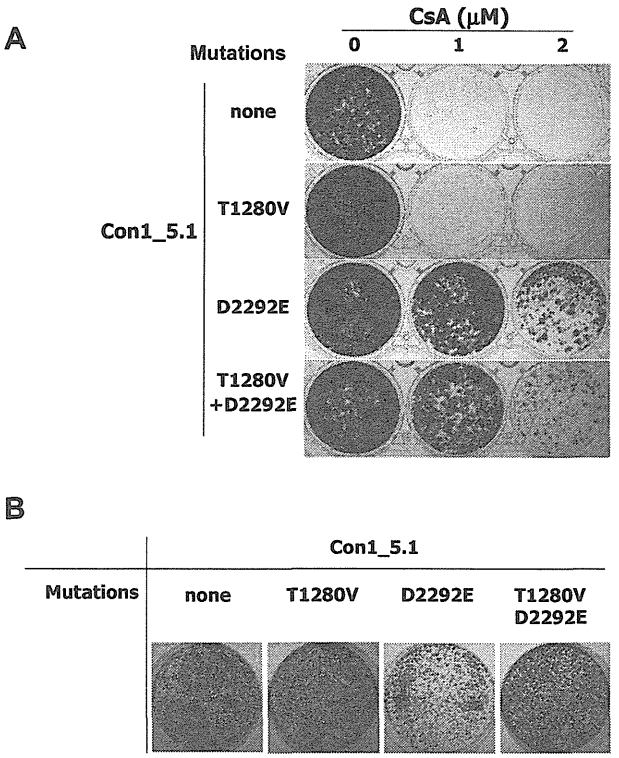


Fig. 3. (A) Resistance to CsA of T1280V and D2292E mutants. While under G418 selection, established replicon cells were treated with CsA at the indicated doses. (B) Standard methods described in Section 2 were used to determine the colony-forming abilities of T1280V and D2292E mutants.

known to confer CsA resistance to some HCV genotypes [25–28], and as a single mutation, it conferred CsA resistance to three separate HCV strains in our hands. In contrast, T1280V in NS3 was not previously identified as a CsA-resistance mutant, and in our hands, it had no impact on CsA resistance as a single mutation (Figs. 2E and 3A).

D2292E was the most significant resistance mutation in this study (Fig. 4C). This mutation is also significant in the regulation of HCV genome replication [29], and close to the CypA binding region [30] (Supplementary Fig. 1). With several genotypes (1a, 1b, 2a, 3, 4, and 6), D2292E is frequently observed after Debio-025 selection [28,31]. Other different mutations in NS5A and NS5B were identified in other studies of CsA resistance [7]; therefore, various mutations could influence HCV resistance to CsA.

In addition to the D2292E mutation, the T1280V mutation in NS3 was present in both clones #6 and #7. Despite its presence in both clones, it did not confer CsA resistance as a single mutant, nor did it enhance the effects of the NS5A CsA-resistant mutants (Fig. 2E). Instead, it partially rescued the colony-forming defect caused by D2292E (Fig. 3B). We used three assays—colony formation assay without CsA treatment (Fig. 3B), cell survival assay of established replicon cells with CsA and G418 dual-treatment (Fig. 3A), and HCV replication inhibition assay without G418 treatment (Fig. 2E and Table 2)—to evaluate the HCV replication competence of each of these two mutations (D2292E, T1280V). It is difficult to fully explain all of the results, and comparison of the two CsA-resistant clones (clone #6 and #7) leaves some questions unanswered. These clones were similar to each other when considering survival during CsA and G418 dual-treatment (Fig. 1B), but they show differences in their resistance in HCV sub-genome replication assay (Fig. 2B and E). Apparently, each mutation in clone #7, except for D2292E, had no effect on the results of the HCV sub-genome replication inhibition assay with CsA. These findings might suggest that these mutations were important to G418 resistance, but not to the resistance of HCV to CsA treatment. In contrast, each of three other mutations in NS5A (D2303H, S2362G, and E2414K) that were found in clone #6 were required for the maximum level of drug resistance conferred by a mutant NS5A in this study. To our knowledge, D2303H is a novel CsA-resistant mutation, and as a single mutation, it conferred CsA resistance comparable to D2292E. D2303H, like D2292E, was located in carboxy-terminal of domain II of NS5A, which is reportedly a CypA binding site [9]. S2362G and E2414K were mutations in domain III of NS5A, and these mutations may have influenced the peptidyl-prolyl isomerase enzymatic catalytic activity of CypA [22]. The V1681A mutation in NS4A identified in clone #6 greatly enhanced the CsA resistance of a HCV construct that had NS3 and NS5A replaced with Cs6#6 sequences (Fig. 2B–D). Though

Table 1
The list of each mutated amino acid sequences in 16 clones throughout whole non-structural region.

		NS3						4A	4B	NS5A										5B
A.A. No.		1062	1275	1280	1560	1609	1612	1681	1797	2109	2179	2197	2231	2269	2292	2303	2320	2362	2387	2414	2992
p5.1		V	D	T	S	K	I	V	I	D	S	P	L	S	D	D	K	S	S	E	M
Cs6#6	1	V	D	V	G	K	T	A	I	D	S	P	L	S	D	D	K	G	S	K	M
	2	V	D	V	S	E	I	V	I	N	S	P	L	S	D	D	K	S	S	E	M
	3	V	D	V	G	K	T	A	I	D	S	P	L	S	E	H	K	G	S	K	M
	4	V	D	V	G	K	T	A	I	D	S	P	L	S	E	H	K	G	S	K	M
	5	V	D	V	G	K	T	A	I	D	S	P	L	S	E	H	K	G	S	K	M
	6	V	D	V	G	K	T	A	I	E	S	P	L	S	E	H	K	G	S	K	M
	7	V	D	V	G	K	T	A	I	E	S	P	L	S	E	H	K	G	S	K	M
	8	V	D	V	G	K	T	A	I	D	S	P	L	S	E	H	K	G	S	K	M
Cs6#7	1	I	G	V	S	E	I	V	I	N	S	P	P	P	E	D	K	S	P	G	T
	2	V	D	V	S	E	I	V	I	N	S	P	P	P	E	D	K	S	P	G	T
	3	I	G	V	S	K	I	V	V	N	P	L	L	S	E	D	T	S	S	E	M
	4	I	G	V	S	K	I	V	V	D	P	L	L	S	E	D	M	S	S	E	M
	5	I	G	V	S	K	I	V	V	D	P	L	L	S	E	D	T	S	S	E	M
	6	I	G	V	S	K	I	V	V	D	P	L	L	S	E	D	T	S	S	E	T
	7	I	G	V	S	K	I	V	V	D	P	L	L	P	E	D	K	S	P	G	T
	8	V	D	V	S	E	I	V	I	N	S	P	P	P	E	D	K	S	P	G	T

The two gray-highlighted lines were selected as the representative sequences of CsA_6μM_#6 and #7 and used to generate the derivative constructs.

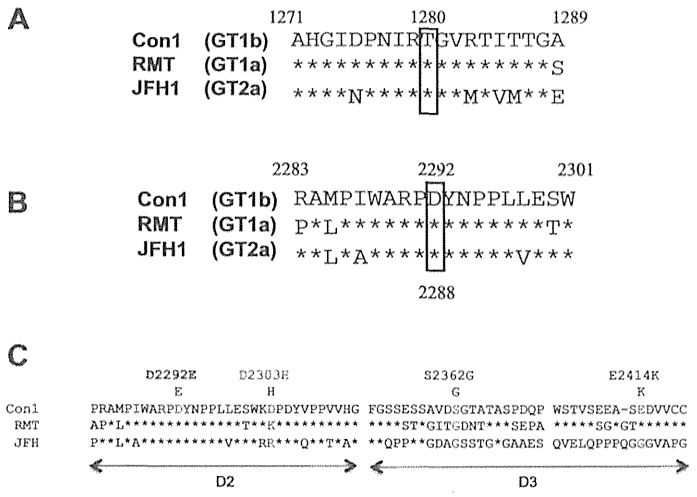


Fig. 4. Amino acid sequences of HCV-RMT-tri (GT1a) and HCV-JFH1 (GT2a) around (A) T1280V and (B) D2292E. (C) Location of the CsA resistant mutations in NS5A. Amino acid sequences around the positions of four CsA resistant mutations.

Table 2
Evaluation of resistance to CsA of mutants that have single mutations or combinations of multiple mutations.

	Mutations		IC_{50} (μm)	Fold change
	NS3	NS5A		
RMT-tri (RMT, GT1a)	–	–	0.79	1.0
	–	D2292E	2.1	2.7
	T1280I	–	0.96	1.2
	T1280I	D2292E	2.46	3.1
	T1280V	–	0.91	1.2
	T1280V	D2292E	2.54	3.2
JFH1 (JFH1, GT2a)	–	–	0.49	1.0
	–	D2292E	1.3	2.7
	T1280I	–	0.51	1.0
	T1280I	D2292E	1.38	2.8
	T1280V	–	0.69	1.4
	T1280V	D2292E	1.2	2.4

Threonine at site 1280 (RMT-tri or JFH1) were mutated to isoleucine (adaptive mutation of Con1 replicon) or valine (major mutation of CsA resistant clones). Aspartic acid at 2292 was mutated to glutamic acid.

Table 3
Evaluation of amino acid mutations in NS5A that conferred CysA resistance.

	Mutations in NS5A				IC_{50} (μm)	Fold change
	D2292E	D2303H	S2362G	E2414K		
Con1_5.1 (GT1b)					0.11	1.0
	○				0.88	7.9
		○			0.52	4.7
			○		0.12	1.0
				○	0.30	2.7
		○	○	○	1.0	9.4
	○	○			1.8	16.6
	○		○		0.95	8.5
	○			○	1.5	13.1
	○	○	○	○	2.80	25.7

we have not assessed V1681A as single mutant, analyzing its mechanism of CsA resistance and its cooperation with other mutations in NS3 and NS5A must be worthwhile because V1681A greatly enhanced the CsA resistance of some constructs.

In all, we evaluated three cyclophilin inhibitors—CsA, NIM811, and Debio-025. Among them, Debio-025 showed the strongest inhibition (IC_{50} values to any mutants) and was tolerated by CsA-resistant mutations (IC_{50} index change values, Fig. 2 and

Table 4
Evaluation of amino acid mutations in NS5A that conferred NIM811 resistance.

	Mutations in NS5A				IC_{50} (μm)	Fold change
	D2292E	D2303H	S2362G	E2414K		
Con1_5.1 (GT1b)					0.054	1.0
	○				0.324	6.0
		○			0.184	3.4
			○		0.056	1.0
				○	0.125	2.3
		○	○	○	0.455	8.4
	○	○			0.635	11.8
	○		○		0.403	7.5
	○			○	0.599	11.1
	○	○	○	○	0.923	17.1

Table 5
Evaluation of amino acid mutations in NS5A that conferred Debio-025 resistance.

	Mutations in NS5A				IC_{50} (μm)	Fold change
	D2292E	D2303H	S2362G	E2414K		
Con1_5.1 (GT1b)					0.024	1.0
	○				0.095	4.0
		○			0.074	3.1
			○		0.028	1.2
				○	0.024	1.8
		○	○	○	0.139	5.8
	○	○			0.198	8.3
	○		○		0.139	5.8
	○			○	0.185	7.8
	○	○	○	○	0.263	11.0

Table 3–5). It was interesting that the resistant mutants differed so greatly in their tolerance of these three inhibitors because all three inhibitors have the same mode of action. Garcia-Rivera et al. concluded that CsA resistance of HCV mutants were solely derived from dependence of the NS5A proteins on cyclophilins [28]. Our results might indicate that other factors are important to CsA resistance, in addition to residual cyclophilin activity.

Drugs that are intended to treat chronic HCV infection and that target important nonstructural HCV proteins—the serine protease NS3/4A, the large phosphoprotein NS5A, or the RNA-dependent RNA polymerase NS5B—have reached the clinical trial stage of drug development [32–34]. Two oral HCV protease inhibitors were approved by the FDA, and some of the drugs could achieve a sus-

tained virologic response (SVR) [35]. However, to develop treatments that eradicate individual chronic HCV infections, additional studies on the emergence of drug-resistant HCV mutants and on the molecular interactions at HCV replication complexes are necessary.

Our new findings provided insights into the way by which HCV acquires resistance to cyclophilin inhibitors, and these insights will facilitate the development of this type of anti-HCV drug for clinical use.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.053.

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Recent Insights into Hepatitis B Virus–Host Interactions

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Hepatitis B virus (HBV) poses a threat to global public health mainly because of complications of HBV-related chronic liver disease. HBV exhibits a narrow host range, replicating primarily in hepatocytes by a still poorly understood mechanism. For the generation of progeny virions, HBV depends on interactions with specific host factors through its life cycle. Revealing and characterizing these interactions are keys to identifying novel antiviral targets, and to developing specific treatment strategies for HBV patients. In this review, recent insights into the HBV–host interactions, especially on virus entry, intracellular trafficking, genome transcription and replication, budding and release, and even cellular restriction factors were reviewed. ***J. Med. Virol. 86:925–932, 2014.*** © 2014 Wiley Periodicals, Inc.

KEY WORDS: HBV; host factors; receptors; cccDNA; replication; new target

INTRODUCTION

Hepatitis B virus (HBV) is the prototype of the family *Hepadnaviridae*, which is characterized by enveloped virions, incomplete double-stranded circular DNA genomes, a retrovirus-like replication strategy which depends on reverse transcription, and hepatotropic infection with a high species-specificity [Summers and Mason, 1982]. HBV virions are composed of a viral envelope with a diameter of about 42 nm that surrounds viral nucleocapsid [Dane et al., 1970]. The nucleocapsid harbors the DNA genome that is covalently linked to the viral polymerase (Fig. 1A) [Seeger and Mason, 2000]. The HBV genome (3.2 kb) contains four overlapping open reading frames (ORFs) (Fig. 1B) [Liang, 2009]. The surface (S) ORF encodes the envelope protein, which actually consists of three separate surface proteins: large (L), middle (M), and small (S) proteins. The polymerase (P) ORF encodes a multifunctional protein that is involved in encapsidation, initiation of minus strand DNA synthesis, reverse transcriptation, and degradation of pregenomic RNA (pgRNA). The core (C) ORF encodes both hepatitis B core antigen (HBcAg), which is a structural nucleocapsid core protein, and hepatitis B e antigen (HBeAg), which is a soluble nucleocapsid protein (Fig. 1C). The X ORF encodes hepatitis B x protein (HBx), which plays roles in signal transduction, transcriptional activation, DNA repair, and inhibition of protein degradation [Neuveut et al., 2010].

Abbreviations: APOBEC3, apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3; cccDNA, covalently closed circular DNA; DHBV, duck hepatitis B virus; FTL, ferritin light chain; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBx, hepatitis B x protein; HCC, hepatocellular carcinoma; miRNAs, microRNAs; MVB, multivesicular body MVB; NTCP, sodium taurocholate cotransporting polypeptide; pgRNA, pregenomic RNA; rcDNA, relaxed circular DNA; SCCA1, squamous cell carcinoma antigen 1

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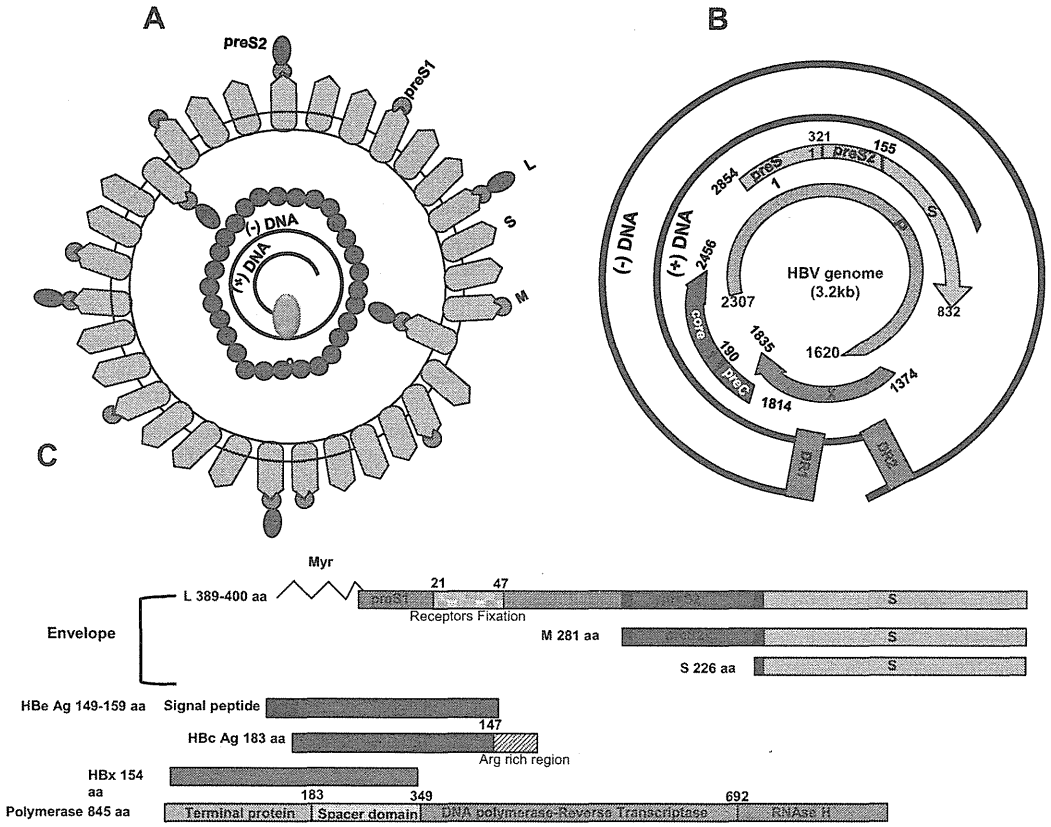

Fig. 1. Schematic structure of the HBV particle and viral proteins with domain structures. **A**: Schematic structure of HBV virion. **B**: HBV genome organization. The genome of HBV is a double-stranded DNA (3.2 kb), which contains four overlapping open reading frames (ORFs) coding for viral envelope (pre-S1/pre-S2/S), core proteins (preC/C), viral polymerase, and HBx protein (X) are shown. Two 11-bp repeats, DR1 and DR2, located at the 5′ ends of the minus and plus strands, play a critical role in viral DNA replication. **C**: Schematic representation of HBV proteins. The HBV envelope proteins consisting of L, M, and S proteins locate on the viral membrane. The L proein with 389–400 aa plays a pivotal role in receptor attachment and conatins preS1 (green), preS2 (red), and S domains (brown): the myristic acid attaches to glycine at aa position 2 of the N-terminus of preS1. The M protein with 281 aa conatins preS2 and S. The S protein encods for only the S domain (226 aa) that is crucial for virus assembly and infectivity. HBx gene is the smallest of the four partially ORF of HBV. It comprises 452 nucleotides that encode a 154-amino acid regulatory protein wich is known as a transcription factor. The precore mRNA is translated into precore protein (HBeAg), a secretory protein with 149–159 aa. The pgRNA serves as mRNAs for both viral polymerase and the core protein (183 aa) that contains an arginine rich domain (ARD) containing four stretches of clustering arginines at the C-terminus. The pgRNA subsequently functions as a template for progeny viral DNA genomes synthesized by reverse transcription. HBV polymerase protein (P, pink) with 845 aa comprises four domains: a terminal protein domain for priming of HBV replication, a spacer domain with unknown function, a reverse transcriptase (RNA-dependent DNA polymerase) domain for viral RNA transcription and replication, and an RNase H domain for degradation of pgRNA.

The clinical course of HBV infection is variable and includes acute self-limited infection, fulminant hepatic failure, an inactive carrier state, and chronic hepatitis with progression to cirrhosis and hepatocellular carcinoma (HCC) [McMahon, 2005]. Although an effective HBV vaccines is available, HBV infection is a major public health problem, and an important cause of infectious disease mortality worldwide [WHO, 2013]. Indeed, there are more than 350 million chronic carriers of HBV worldwide [Lee, 1997; Hoofnagle et al., 2007] and about 600,000 people die every year due to the complications of HBV-related chronic liver disease [WHO, 2013]. Serum hepatitis B surface antigen (HBsAg) is used as a diagnostic marker of HBV infection. Also, antibodies against HBsAg signify recovery or immunization.

Eradication of HBV infection is rendered difficult by occasional integration of covalently closed circular DNA (cccDNA) into cellular chromosomes and/or due to the unusual persistence of the episomal cccDNA in infected cell nuclei [Dejean et al., 1986]. Interferon alpha and nucleos(t)ide analogues are currently approved as antiviral agents to reduce the severity of HBV-related diseases, although these antiviral agents have limited efficacy and do not result in sustained virological response in most cases [Zoulim and Locarnini, 2009, 2012]. In addition, nucleos(t)ide analogues can result in the selection of single or even

broad antiviral-resistance mutants [Zoulim and Locarnini, 2009]. Therefore, the development of novel therapeutic strategies that interfere with other steps of the viral replication cycle and improve treatment outcomes are needed. To this end, an understanding of HBV biology and pathogenesis is important. In this review, available literatures concerning molecular mechanisms underlying HBV life cycle, especially the interactions between viral and host factors are considered.

HBV ENTRY AND INTRACELLULAR TRAFFICKING

The first step in terms of the HBV life cycle is the recognition of cellular receptor by viral envelop protein; co-receptors may also contribute to binding to the cell surface and/or to host specificity and tissue tropism (Fig. 2). The L protein plays a pivotal role in the attachment to receptors, and exhibits mixed topologies one cytoplasmically oriented (an inward topology) with as essential role in nucleocapsid envelopment, and another outward topology, displaying preS1 on the exterior of the virion envelope to mediate infection via a cell-surface receptor (or receptors) [Ostapchuk et al., 1994; Prange and Streeck, 1995; Awe et al., 2008]. In terms of viral factors required for the HBV receptor recognition, it has been demonstrated that the myristoylated preS1 domain of HBV L protein plays a key role in viral infectivity by mediating attachment to specific receptor molecule(s) [Gripon et al., 1995; Schulze et al., 2007]. Recent studies by using chemically synthesized lipopeptide fragments of the HBV L-protein showed that HBV hepatotropism is mediated through specific binding of the myristoylated N-terminal preS1-subdomain of the HBV L-protein to a hepatocyte specific receptor. Moreover, the restricted infectivity of HBV to human primates is not generally determined by the absence of this binding receptor in non-susceptible hosts, but is probably related to the lack of cofactor involved in membrane fusion [Meier et al., 2013; Schieck et al., 2013]. In addition, other studies reported that a short peptide fragment encompassing amino acids (aa) 21–47 of the preS1 domain in genotypes A, B, and C (corresponding to aa 10–36 in genotypes D, E, and G) was sufficient for HBV to bind HepG2 cells (human hepatocellular liver carcinoma cell line) [Neurath et al., 1986]. This finding is consistent with the observation that aa 3–77 of the preS1 domain are crucial for viral infectivity [Le Seyec et al., 1998; Schulze et al., 2010; Zoulim and Locarnini, 2012].

Identification of cellular receptors for HBV has received substantial attention over the years. Reliable in vitro HBV infection systems, however, have not been available for a long time. Initially, cultures of primary human hepatocytes, obtained by immediate perfusion of surgically resected liver sections, had been used to study HBV infectivity [Gripon et al., 1988]. Major problems with the use of primary human hepatocytes are their limited availability, and the heterogeneity in the quality of liver cell preparations. Against such a background, the first insight into a host factor involved in HBV entry was obtained by using duck hepatitis B virus (DHBV), which also belongs to the family *Hepadnaviridae*, as a model virus: carboxypeptidase D (gp180) was identified as a host factor that binds DHBV particles with high affinity on duck hepatocytes [Kuroki et al., 1995; Tong et al., 1995; Urban et al., 1998], although it remains uncertain whether gp180 function as a receptor for DHBV infection. Primary hepatocyte cultures from *Tupaia belangeri* were also demonstrated to be infected with HBV as efficiently as primary human hepatocytes cultures of good quality [Walter et al., 1996]. Using the primary tupaia hepatocyrtes-based in vitro system, several candidates of hepatocyte membrane receptors and co-receptors for HBV have been identified [Schulze et al., 2007; Leistner et al., 2008; Yan et al., 2012].

The recent development of a proliferating HepaRG cell line, which is a human bipotent progenitor cell line capable differentiating into two different cell phenotypes (i.e., biliary-like and hepatocyte-like cells) has been established from a liver tumor associated with chronic hepatitis C [Gripon et al., 2002]. The latter has presented new possibilities to explore HBV infection in a more specific and accurate manner. This experimental tool of human-origin can be employed for investigations addressing HBV entry (attachment, receptor interaction and viral uptake). By using this newly established in vitro system, the relevance of the initial attachment to the carbohydrate side chains of hepatocyte-associated heparan sulphate proteoglycans (HSPGs) as attachment receptors for HBV infection was reported [Schulze et al., 2007; Leistner et al., 2008]. However, because of the ubiquitous expression of heparan sulfate proteoglycans, this finding does not explain the hepatotropism of HBV. Rather, it may represent a first, non-specific step of a multistep entry process.

In 2012, very interesting data regarding the cellular receptor for HBV was presented [Yan et al., 2012]. In this study, they used a photoreactive ligand peptide derived from aa 2–47 of the preS1 domain of HBV L protein as "'bait" to identify interacting proteins expressed in primary tupaia hepatocyrtes to screen for putative HBV receptor molecules. The cross-linked peptide-protein complexes were purified and subjected to mass spectrometry analysis to identify cellular proteins. Comparing the mass spectrometry results of the captured proteins with a tupaia protein datasets obtained by transcriptome, they identified sodium taurocholate cotransporting polypeptide (NTCP, also known as SLC10A1) as a hepatocyte surface molecule binding the preS1 domain. NTCP is a member of the solute carrier family 10 (SLC10), the major bile acid uptake system in human hepatocytes, and localized to the

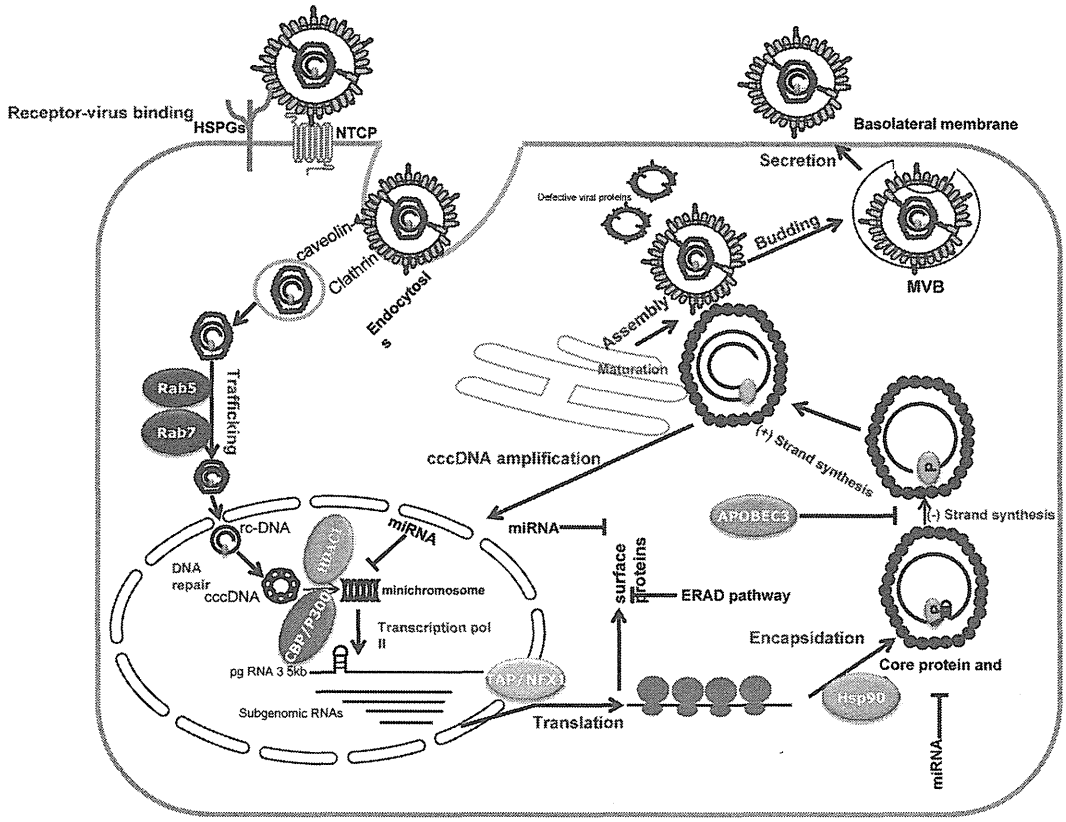

Fig. 2. Model of hepatitis B virus life cycle in polarized hepatocytes with main host factors. HBV binding receptor on hepatocytes is internalized by endocytosis and uncoated in endosome. rcDNA genome in nucleocapsid is delivered into the nucleus. The rcDNA genome, which is incomplete double-stranded circular DNA, is converted to cccDNA, which is covalently closed circular DNA by DNA repair. The cccDNA serves as a template for the viral RNA transcription mediated by the host RNA polymerase II. Viral RNAs, including the pgRNAs and subgenomic RNAs, are exported to the cytoplasm where viral RNA transcription and replication occur. The subgenomic RNAs are translated to viral proteins, the pgRNA is packaged together with polymerase protein (P) into immature nucleocapsids consisting of core proteins and reverse transcribed to the rcDNA. Mature nucleocapsids containing rcDNA are then either recycled to the nucleus to amplify the cccDNA or enveloped by the viral envelope proteins and secreted extracellularly as progeny virions. HSPGs, Heparan sulphate proteoglycans; NTCP, sodium taurocholate cotransporting polypeptide; rcDNA, relaxed circular DNA; cccDNA, covalently closed circular DNA; pgRNA, pregenomic RNA; TAP, tip-associated protein; NFX1, nuclear export factor-1, NFX ; P, HBV polymerase; Hsp 90, heat-shock protein 90; ERAD, ER-associated degradation; APOBEC3, apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3; HDAC1, histone deacetylase 1; MVB, multivesicular body.

basolateral hepatocyte membrane [Kullak-Ublick et al., 2000]. Silencing the NTCP expression by small interfering RNAs in primary tupaia hepatocyrtes, HepaRG or primary human hepatocytes led to the reduced HBV infection. Phylogenetic analysis and mutagenesis studies showed that aa 157–165 in NTCP serve as determinants for species-specificity. Using ectopic expression of tupaia and human NTCPs in non-permissive HepG2 or HuH-7 hepatoma cells rendered these cells susceptible to HBV infection at low levels, suggesting that NTCP may not be the sole host factor supporting HBV entry. More recent data suggested that both ferritin light chain (FTL), and squamous cell carcinoma antigen 1 (SCCA1) may serve as co-receptors in HBV cellular attachment and viral entry into hepatocytes [Hao et al., 2012].

For HBV entry into cells, receptor binding is followed by endocytosis mediated by host factors. A recent in vitro study showed that a caveolin-1-mediated endocytosis is required to initiate HBV entry in HepaRG cells [Macovei et al., 2010]. In addition, disrupting the epithelial barrier of HepaRG cells was demonstrated to increase HBV infection, suggesting that the entry of HBV into hepatocytes occurs in a polarized manner, and the resulting transformation in membrane polarity renders HepaRG cells susceptible to infection by allowing access to the basolateral domain [Schulze et al., 2012].

Early reports indicated that HBV replication was cell cycle-dependent and was inversely correlated with cellular DNA synthesis: these findings may explain the elimination of virus during cell regeneration that has been observed during severe acute hepatitis B or HBV reactivation [Ozer et al., 1996]. Following endocytosis, HBV must travel through the complex network of the endocytic pathway to reach

the cell nucleus where HBV genome transcription and replication occur (Fig. 2). The intracellular trafficking events which are critical for the initiation of a productive infection by providing the appropriate environment for virus uncoating and nucleocapsid release, have remained completely obscure for HBV. Indeed, recent study showed that HBV infection strongly depends on the expression of host factors Rab5 and Rab7 [Macovei et al., 2013], both of which are GTPases involved in the endosome biogenesis [Somsel Rodman and Wandinger-Ness, 2000]. In this study, authors investigated the effect of the host factors on trafficking of HBV particles internalized in HepaRG cells by using a stable and inducible short hairpin RNA expression system. The results showed that silencing of either Rab5 or Rab7 expression results in significant inhibition of the early stages of HBV infection, indicating that HBV transport from early to mature endosomes is required for HBV life cycle.

An understanding of the HBV entry mechanism may represent a rather new and attractive therapeutic concept to combat HBV infection both in the acute and chronic phases. In fact, several studies have shown that a lipopeptide derived from the preS1 domain is a promising drug additive that can be used to improve patient treatment outcomes [Gripon et al., 2005; Petersen et al., 2008; Volz et al., 2013].

HBV GENOME TRANSCRIPTION AND REPLICATION

After fusion of viral and cellular membranes in endosomes, the capsid delivers its relaxed circular DNA (rcDNA), which is held in circular conformation by a short cohesive overlap between the 5′ ends of the two DNA strands, into the nucleus through nuclear pore complex (NPC). Passage through the NPC is mediated by the interaction between a nuclear localization signal on the C terminal of the viral capsid proteins and nuclear import receptors importin α and β [Kann et al., 1999]. Upon translocation to the nucleus, the rcDNA is converted to a cccDNA by a mechanism largely unknown and most probably involves cellular repair enzymes [Wei et al., 2010]. The minus strand DNA of the cccDNA serves as a template for transcription of both pgRNA and subgenomic RNA, of which the former in turn serves as the template for the reverse transcriptional synthesis of viral DNA, and the latter as the message for viral proteins by the host cell RNA polymerase II [Seeger and Mason, 2000]. The cccDNA also functions as an HBV reservoir responsible for persistent replication, and thus is considered to be a reliable marker for HBV infection [Levrero et al., 2009]. Viral HBx has been shown to be essential for the initiation and maintenance of transcription from HBV cccDNA: HBx stimulates the acetylation of histones associated with cccDNA, and is required for transcription in the context of HBV infections [Lucifora et al., 2011].

In a DHBV model, several cellular transcription factors, such as C/EBP, HNF1, and HNF3, have been demonstrated to bind the DHBV cccDNA enhancer region in duck liver extracts [Liu et al., 1994]. HBx has been proposed to promote HBV gene expression by recruiting the histone acetylases CBP/p300 and PCAF/GCN5 to the cccDNA [Belloni et al., 2009]. In addition, a recent study showed that HBx promotes gene expression from the natural HBV cccDNA, but not from a chromosomally integrated HBV [van Breugel et al., 2012]. It has been reported that HBV replication is regulated by the acetylation status of histones H3 and H4 bound to cccDNA minichromosome [Pollicino et al., 2006]. Furthermore, it appears that cellular acetyltransferases, p300 and CBP, and a cellular deacetylase, HDAC1, are recruited to bind the HBV cccDNA in vitro and in vivo [Belloni et al., 2009]. Recently, several studies have reported that a number of liver-enriched transcription factors and nuclear receptors, including STAT3 and HNF1/4, binds HBV promoter/enhancer elements [Wang et al., 2009], and to be critical in the regulation of HBV transcription [Quasdorff and Protzer, 2010]. Interestingly, most of these transcription factors/nuclear receptors are potential linkers between major cellular events in the hepatocyte (i.e., hepatic gluconeogenesis and lipid metabolism, etc.) and HBV life cycle [Bar-Yishay et al., 2011].

HBV pgRNA in the nucleus was demonstrated to be exported to the cytoplasm by associating with a cellular Tip-associated protein/nuclear export factor-1 (TAP/NFX1) and HBcAg [Li et al., 2010]. In the cytoplasm, the pgRNA is translated to HBV core protein and polymerase [Chang et al., 1990]. Binding of the HBV polymerase to RNA stem-loop structure epsilon of pgRNA initiates packaging of the single viral RNA molecule into immature nucleocapsids [Summers and Mason, 1982]. A recent study using DHBV showed that host chaperones Hsc70, Hsp40, Hsp90 plus ATP regulate reverse transcriptional activity of the viral polymerase [Stahl et al., 2007]. Reverse transcription of pgRNA into minus strand DNA is followed by degradation of the pgRNA by the RNAse H domain in the HBV polymerase. The degradation is complete except for its 5' terminal, 15–18 nucleotides which serve as a primer for plus-strand DNA synthesis resulting in rcDNA formation [Beck and Nassal, 2007; Nassal, 2008]. Mature capsids containing rcDNA can be either recycled for intracellular cccDNA amplification [Tuttleman et al., 1986; Locarnini and Mason, 2006], or assembled with viral surface proteins in the endoplasmic reticulum to form progeny viral particles that will be released from the cell [Ganem, 1991; Locarnini and Zoulim, 2010; Dandri and Locarnini, 2012].

HBV BUDDING AND RELEASE

HBV virions are assembled in the endoplasmic reticulum (ER)-Golgi compartment [Patient et al., 2007]

(Fig. 2). A recent report showed that HBV activates the ER-associated degradation (ERAD) pathway, which in turn, reduces the levels of HBV envelope proteins, possibly as a mechanism to control the level of viral particles in infected cells, and facilitate the establishment of chronic infections [Lazar et al., 2012]. The budding and release of HBV virions from hepatocytes have been suggested to involve the machinery of multivesicular body (MVB), including the interaction of host factors γ2-adaptin, Nedd4 ubiquitin ligase, Vps4, VPS4B, and AIP1 [Hartmann-Stuhler and Prange, 2001; Rost et al., 2006; Watanabe et al., 2007]. The molecular mechanisms underlying HBV budding and release, remain largely unknown. However, knowledge in this area has the potential to lead to a new class of therapeutic agents.

HBV RESTRICTION FACTORS

Recently, some variants of human apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3 (APOBEC3), that is, APOBEC3B, APOBEC3C, APOBEC3F, APOBEC3G, and APOBEC3H, have been shown to affect HBV by two ways: introducing G-to-A hypermutations into the nascent minus strand DNA of HBV by their deaminase activities [Turelli et al., 2008; Henry et al., 2009; Noguchi et al., 2009; Ezzikouri et al., 2013] and by inhibiting HBV reverse transcription independent of the deaminase activities [Rosler et al., 2004; Turelli et al., 2004]. Previous studies have shown that the prevalence of hypermutated HBV genomes (G > A transitions) varies between 2% and 35% [Noguchi et al., 2009; Vartanian et al., 2010; Ezzikouri et al., 2013]. The role of these restriction factors listed above in the regulation of HBV replication needs further investigation to fully elucidate their therapeutic potential.

MicroRNAs (miRNAs) are important small noncoding RNAs that regulate post-transcriptional gene expression in diverse biological processes such as development, immune response, and tumorigenesis [Lindsay, 2008; Pedersen and David, 2008; Nana-Sinkam and Croce, 2013]. Several studies have shown that HBV replication is also regulated by several miRNAs (miR-1, miR-141, miR-449a, miR-210, miR-152, miR-148a, etc.) that lead to modification of host gene expression [Liu et al., 2011; Zhang et al., 2011; Hu et al., 2012]. In fact, miR-141 suppressed HBV replication by reducing HBV promoter activities through the down-regulation of peroxisome proliferator activated receptor alpha [Hu et al., 2012] and miR-125a-5p interferes with the HBV translation and down-regulation of the expression of the surface antigen, thus reducing the amount of secreted HBsAg [Potenza et al., 2011]. In addition, other group showed that miR-1 was able to enhance the HBV core promoter transcription activity by up-regulating of farnesoid X receptor alpha expression [Zhang et al., 2011].

More recently in chronic hepatitis B patients, the miR-122 was found to be specifically suppressed and led to enhanced HBV replication [Wang et al., 2012]. The loss of miR-122 expression by viral mRNAs and/or chronic inflammation leads to upregulation of its target binding factor, which initiates pituitary tumor transforming gene (PTTG1) nuclear translocation, promoting PTTG1 transcriptional activity and thus enhancing cell growth and invasion [Li et al., 2013]. These data provide a potential new strategy for the development of novel therapies to prevent the development of HCC under HBV infection. However, the mechanism underlying the miR-122-mediated regulation of viral mRNAs is unknown. The relationship between miRNAs and HBV infection offers a promising miRNA-based HBV therapy in the future.

CONCLUSIONS AND PERSPECTIVES

To gain insights into HBV infection, advances in molecular virology are indispensable. The studies reviewed above have significantly enhanced the understanding of some cell biological aspects of HBV–host interactions. Despite significant experimental hurdles, numerous *bona fide* HBV host interactions have been defined with the most recent data about the discovery of NTCP that allows new insight in the improvement of cell culture systems, such, HepaRG-NTCP, and HepG2-NTCP cells. These in vitro systems will be also accelerated the acquisition of data revealing the interplay between HBV and host factors and design new therapy. The most challenging goal, will be to understand the assembly, budding, and release of HBV particle and to develop a small animal model system for HBV studies, which will have strong implications for drug development and the decipher of hepatitis B pathogenesis.

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RNA Polymerase III Regulates Cytosolic RNA:DNA Hybrids and Intracellular MicroRNA Expression *

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*Running Title: *POL III links cytosolic RNA:DNA hybrids to miRNAs*

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Background: RNA:DNA hybrids exist in the nucleus and mitochondria, but not in the cytosol except viral infection.
Results: RNA:DNA hybrids exist in the cytosol of various human cells is mediated by RNA polymerase III, where RNA polymerase III regulates the microRNA machinery.
Conclusion: Cytosolic RNA:DNA hybrids is regulated by RNA polymerase III.
Significance: Previous unknown cytosolic RNA:DNA hybrids may have physiological relevance to miRNA machinery and RNA transport.

ABSTRACT

RNA:DNA hybrids form in the nuclei and mitochondria of cells as transcription-induced R-loops or G-quadruplexes, but only exist in the cytosol of virus-infected cells. Little is known about the existence of RNA:DNA hybrids in the cytosol of virus-free cells, in particular cancer or transformed cells. Here, we show that cytosolic RNA:DNA hybrids are present in various human cell lines, including transformed cells. Inhibition of RNA polymerase (III), but not DNA polymerase abrogated cytosolic RNA:DNA hybrids. Cytosolic RNA:DNA hybrids bind to several components of the miRNA machinery-related proteins including AGO2 and DDX17. Furthermore, we identified miRNAs that were specifically regulated by RNA polymerase III, providing a potential link between RNA:DNA hybrids and the miRNA machinery. One of the target genes, exportin-1, was shown to regulate cytosolic RNA:DNA hybrids. Taken together, we reveal previously unknown mechanism by which the RNA polymerase III regulates the presence of cytosolic RNA:DNA hybrids and miRNA biogenesis in various human cells.

RNA:DNA hybrids can occur during transcription and replication of DNA (1). The DNA primase generates short RNA:DNA fragments during replication of the lagging strand (2,3). Short hybrids also form during the transcription of DNA by RNA polymerases. In contrast, long RNA:DNA hybrids are events that can occur during stalling of the RNA polymerase or during replication of mitochondria DNA (4). Stalling of the RNA polymerases can lead to the formation of R-loops, which consist of long RNA:DNA hybrids and the displaced non-template DNA strand. Long RNA:DNA hybrids also occur in G-quadruplexes, which promote class switch recombination in B cells (5). Recent evidence suggest that R-loops and G-quadruplexes may occur more frequently than previously assumed and interfere with gene expression and threaten genome stability (6-8).

While many studies have focused on the generation of nuclear RNA:DNA hybrids, it is unclear how nuclear RNA:DNA hybrids are resolved and their role in diseases related to genomic instability such as cancer.

We recently found the presence of ssDNA and double-stranded (ds) DNA in the cytosol of B-cell lymphoma cells (9). Inhibition of ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) kinases, which initiate the DNA damage response (DDR) lead to the disappearance of cytosolic DNA. Conversely, the levels of cytosolic ssDNA and dsDNA increased in response to DNA damage suggesting that constitutive nuclear DNA damage and the ensuing DDR induces the presence of cytosolic ssDNA and dsDNA in B-cell lymphoma cells. Cytosolic DNA in B-cell lymphoma cells induced STING-dependent DNA sensor pathways leading to the expression of ligands for the activating immune receptor NKG2D (9). Delocalized DNA is important for innate immune recognition of pathogens and recent reports suggest that TLR9 and the NLRP3 inflammasome sense pathogen-derived RNA:DNA hybrids in dendritic cells (10-13). However it is not known if RNA:DNA hybrids exist in the cytosol of non-infected cells.

RNA polymerase III (POL III) is the largest RNA polymerase consisting of 17 subunits including a DNA binding site (14-16). It catalyzes the transcription of genes required for transcription and RNA processing such as tRNAs, ribosomal 5S rRNA, and U6 snRNAs. It also transcribes short interspersed elements (SINEs) and repeated elements in the human genome (14). *POL III* expression is regulated by oncogene products, tumor suppressors such as p53, and POL III-associated transcription factors (17-19). Consistent with these observations, POL III activity is increased in many cancers including melanomas, myelomas and carcinomas (20). Although POL III is mostly present in the nucleus (20,21), cytosolic POL III was proposed to play a role in the sensing of AT-rich DNA via the retinoic acid inducible gene I (RIG-1) pathway (22-24). Despite the regulation of *POL III* by genes associated with tumorigenesis, little is known about the role of POL III in cellular function of transformed cells.

Here, we identified the presence of cytosolic RNA:DNA hybrids in immortalized and transformed human tumor cells. Chemical inhibition of POL III abrogated the presence of cytosolic RNA:DNA hybrids in cells. Cytosolic RNA:DNA hybrids were bound by miRNA-machinery-associated proteins such as DDX17 and AGO2. We also identified POL III-regulated intracellular miRNAs in lung cancer A549 cells. In summary, we demonstrate that the constitutive presence of cytosolic RNA:DNA hybrids in a variety of cell lines, and this accumulation depends on RNA POL III in at least A549 lung carcinoma.

EXPERIMENTAL PROCEDURES

Cells – The human lung adenocarcinoma (A549), colorectal adenocarcinoma (LoVo and HT29), colorectal carcinoma (HCT116), acute monocytic leukemia (THP-1), human cervix carcinoma cell line (HeLa), and normal lung tissue derived (MRC-5) cell lines were purchased from ATCC (USA). Cells were grown in Dulbecco's modified Eagle's medium (Nacalai Tesque, Japan), supplemented with 10% fetal bovine serum (Cell Culture Bioscience, Japan), 1% penicillin/streptomycin (Nacalai Tesque), and 2% HEPES (Life Technologies, Japan). Cells were maintained with 5 μg/ml of Plasmocin (Invivogen, USA) to prevent mycoplasma infection.

Reagents and cell treatments – Cytarabine (Ara-C) was purchased from Wako Chemicals (Japan). Aphidicolin (APH), RNA Pol III inhibitor, ML-60218 and Leptomycin B was purchased from Calbiochem (Germany). ATM inhibitor, KU60019 (Tocris Bioscience, United Kingdom) and ATR inhibitor, VE821 (Axon Med Chem, Netherlands) were used at 10 μM. PicoGreen dsDNA reagent (Life Technologies, USA) was used at a 1:100 dilution. Mitotracker (Life Technologies) was dissolved in DMSO and used at 500 nM. Fixed cells were treated with 0.5 U/ml RNase H (NEB, USA) for 3 hrs at 37°C.

Immunofluorescence studies – Cells were fixed with 4% paraformaldehyde for 10 min, and permeabilized in 0.2% Triton X-100 for 15 min. Non-specific sites were blocked with 2% goat serum and 1% BSA in 0.2% Triton X-100 for 1 h. Transfected cells were stained with anti-COX IV antibody (ab16056, Abcam, United Kingdom), anti-POLR3G (LS-C163858, LS Bio, USA), or anti-DDX17 (19910-1-AP, Proteintech, USA). The RNA:DNA hybrid-specific S9.6 antibody was a kind gift of Dr. D. Koshland, University of