漿は脂肪細胞からの肝細胞への遊離脂肪酸の取り込みを増加させている。

3. HCV感染が宿主エネルギー代謝に与える影響

我々は、JFHウイルスをHuh-7細胞に感染させ、感染が全ての細胞に広がった９日目に細胞のライセートを質量分析法でメタボローム解析を行った。クエン酸回路、プリン・ピリミジン合成系など蛋白核酸合成等は低下し、ATP、GTP、phosphocreatine等のエネルギー供与体は減少し、一方、解糖系は著明に亢進していた。クエン酸回路や電子伝達系を有し、生体内でエネルギー供給するATPを産生する場所がミトコンドリアである。HCV感染した細胞を電子顕微鏡で観察したところ、ミトコンドリアのクリステ構造が破壊されていた。さらに、蛍光抗体法による観察では、HCV蛋白発現部位でミトコンドリア機能低下が見られた。このようなHCVによるミトコンドリア障害はエネルギー産生低下をもたらす可能性がある。さらに、我々はHCV複製による細胞におけるATP消費量の変化を調べた(5)。レプリコン細胞から複製複合体を含む画分を分離し、ATPを添加しその減少量を比較したところ、オリジナルの細胞に比べてATP消費量が亢進していた。ATPが存在するとビーナスとCFPが結合しFRET蛍光を発する、ATP濃度測定プローブATeam を用いて、生細胞内のATPの量と局在の解析を目指した。Ateamをレプリコン RNAのNS5Aの下流に挿入することにより、FRET強度からATPの量を評価するとともに、ドット状の蛍光から複製複合体の局在を識別した。レプリコン細胞では複製複合体で強いFRETシグナルを観察した。我々は、レプリコン細

胞から複製複合体を粗精製し(2)、そこに含まれるHCVのゲノム複製に関与する宿主因子をプロテオミクスの手法を利用して探索し、creatine kinase B(CKB)を見出した(6)。CKは、エネルギーを多く必要とする、あるいは急速に必要とする組織でのATPの供給、ATPレベルの維持に重要であるとされており、CKBはNS4Aとの結合を介してHCV複製複合体にリクルートされ、ATPを供給することでHCV複製活性の維持に重要であると考えられた。

4. おわりに

HCVは感染した細胞に脂質の蓄積という、HCV増殖に好都合な環境を作り出しているものと考えられる。また、感染細胞のメタボローム解析の結果、脂質代謝だけでなく、アミノ酸合成、RNA核酸合成、TCA回路、エネルギー産生系、糖新生・解糖系などに多彩な影響を与えている可能性が示された。このような解析はC型肝炎患者の病態の理解につながると期待できる。

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4) HCV 粒子形成に関与する脂肪滴周辺膜蛋白の同定と機能解析

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はじめに

エンベロープウイルスは小胞体，ゴルジ体，形質膜など細胞の生体膜を被って出芽するため，細胞の生体膜は粒子形成に重要な役割を果たしていると考えられる[1,2]．近年，C 型肝炎ウイルス（HCV）粒子形成に細胞内脂肪滴（LD）が重要であることが示された[3]．JFH-1[4] 感染細胞を電顕で観察すると，LD と粗面小胞体が結合している部分に黒い点状のものが認められた．われわれは，この LD 周辺の膜構造が HCV 粒子産生の場と考え，LD 近傍膜のプロテオーム解析を行い，ウイルス粒子産生に関与する宿主因子の同定と機能解析を行った．

1．LD 周辺膜に含まれる蛋白の同定

はじめに感染細胞のライセートを遠心法により分画した．ショ糖濃度は LD を分けやすい 0%から，膜を分けやすい 8.5%までを用い，感染性は軽い分画に存在していた．そこで次にこの軽い分画に含まれている蛋白について解析した．この感染性の高い分画からは，コア蛋白，E2，NS5A などの HCV 蛋白，それに小胞体（ER），ゴルジ体，脂質ラフトなどに含まれる蛋白が検出された．一方，リソソーム，細胞質などに含まれる蛋白は検出されなかった．LD 由来蛋白が強く残っていることから，LD が強く濃縮されていたことが分かった．この分画から LD を精製したところ，膜蛋白も HCV 蛋白も検出できなくなった．以上のことから，HCV 粒子産生の場は LD そのものではなく，その周辺膜であると考えられた．

そこで，LD 周辺膜に含まれる宿主蛋白質同定のため，比較プロテオーム解析を行ったところ，LD および LD 周辺膜を含む蛋白質として 75 の蛋白が同定された．また，そこから精製した LD からは約 30 の蛋白が検出できた．そこで，これら 30 の蛋白を除く 45 の蛋白を LD 周辺膜蛋白と仮定して解析を行った．その中には，さまざまな機能の蛋白が含まれていたが，そのうち Apolipoprotein E（ApoE）等は既に HCV の生活環に加わっていることが報告されている[5]．これらの蛋白について siRNA でスクリーニングを行い，候補蛋白が HCV 粒子感染性および複製に与える影響について調べた．その結果，24 dehydrocholesterol reductase（DHCR24），17 beta hydroxysteroid dehydrogenase（HSD）11，protein disulfide isomerase（PDI），ApoE の SiRNA は培養上清中および細胞内の HCV 粒子の感染性を低下させることが分かった．さらに，レプリコン細胞を siRNA で処理したところ，

PDI, DHCR24 の低下が複製に影響を与えることが分かった．ApoE はウイルス粒子感染に重要な役割を果たすことが知られており[5]，同様に HSD11 は複製には関与しないものの感染性粒子産生に重要な役割を果たすと想定されたので，以降は HSD11 に注目して研究を進めた．

2. HSD11 の HCV 感染性粒子産生における役割

まず標的配列の異なる HSD11 siRNA が HCV 粒子感染性に与える影響について検討した．3 種類の HSD11 siRNA を用いたところ，いずれも mRNA の低下が認められ，感染性を強く抑制することが分かった．次に trans-packaging system を用いて HSD11 siRNA が HCV 粒子感染性に与える影響を調べた．trans-packaging system ではレプリコン細胞に外から構造蛋白を加えることにより，再感染しない粒子を作製可能である[6]．その結果，再感染性のない粒子に対しても HSD11 siRNA は培養上清中の HCV 粒子感染性を抑制した．われわれは Huh7 細胞に HSD11 shRNA を恒常的に発現する HSD11 発現抑制細胞を樹立し，同細胞では HSD11 mRNA 発現が著明に抑制されていたことを確認している．そこでこの標的部分のアミノ酸変異を伴わないヌクレオチド変異を導入した shRNA resistant な HSD11 を作製し，HSD11 発現抑制細胞に発現させたところ感染性が回復した．以上より，HSD11 は HCV の感染性粒子形成に重要な役割を果たしていると考えられた．

3. HSD11 の細胞内局在と HCV 感染粒子形成性への影響

HSD11 はステロイドホルモンの代謝に関与する遺伝子として発見され，多くの臓器で発現していることが知られている[5]．しかしながら，肝細胞における生理的な役割については分かっていない．また，Peroxisome proliferator-activated receptor-α（PPARα）により，肝臓での発現が制御されていることが知られている．肝では ER と LD に存在するが，LD 形成誘導下では主に LD に移行する．さらにマウスの HSD11 遺伝子は N 末端領域に LD 局在シグナルを有することが分かっている．

そこでヒト HSD11 の野生型および LD 局在シグナル欠損 HSD11 の細胞内局在について調べた．一般に HSD11 は ER に存在しているが，オレイン酸を用いて LD 産生を亢進させると HSD11 発現は LD に移行した．また，LD 局在シグナル欠損 HSD11 を用いると LD 局在は消失した．次に HCV 感染細胞での HSD11 局在を検討したところ，野生型の HSD11 は LD と共局在していたが，LD 局在シグナル欠損型では共局在が見られなかった．また，野生型および LD 局在シグナル欠損型 HSD11 の HCV 感染性に与える影響について調べた．野生型の HSD11 を強制発現させると，培養上清中および細胞内の HCV 感染性は増加したが，LD 局在シグナル欠損型 HSD11 の強制発現は感染性を減少させた．

4. HSD の感染性 HCV 粒子形成のメカニズムの検討

次に HSD が HCV の生活環にどのように関わっているかを調べた．まず HSD11 と HCV 蛋白の免疫沈降を行ったところ，1b 型および 2a 型 HCV の両方の NS5A と HSD11 の相互作用が認められた．また，NS5A のどの領域が HSD11 と結合するかを調べたところ，NS5A の N 末端領域と HSD11 が結合していることが分かった．そこで NS5A・野生型 HSD11 結合体と LD の感染細胞内局在について

proximity ligation assay を用いて調べたところ，NS5A・野生型 HSD11 結合体は LD の周辺部分に存在していた．

次に HSD11 siRNA が HCV の比重および感染性に与える影響について調べた．HSD11 siRNA 処理により，HCV 粒子の比重は高い方に移行し，感染性は低下した．高い比重への移行は HCV 粒子の脂質量の低下あるいは超低比重リポ蛋白（VLDL）との結合低下などの可能性が考えられた．

5．HSD の肝細胞における生理的な役割の解析

次に HSD11 の肝細胞内における生理的な役割の解析を行った．Huh7 細胞では LD は発達していないが，LD 高産生状態の細胞に野生型 HSD11 を導入すると，LD が強く発達した．そこで HSD11 が LD 産生あるいは成熟化に関与している可能性について検討するため，LD 局在シグナル欠損 HSD11 を発現させたところ，発現細胞および発現部位において LD が観察できなかった．次に，LD 合成亢進 Huh7 細胞への野生型，LD 局在シグナル欠損 HSD11 の siRNA，shRNA 導入により細胞内の脂質発現の変化を調べた．その結果，野生型を発現させた場合はリン脂質，中性脂肪が増加したが，LD 局在シグナル欠損 HSD11 siRNA，shRNA の導入は各種脂質の減少をもたらした．また，

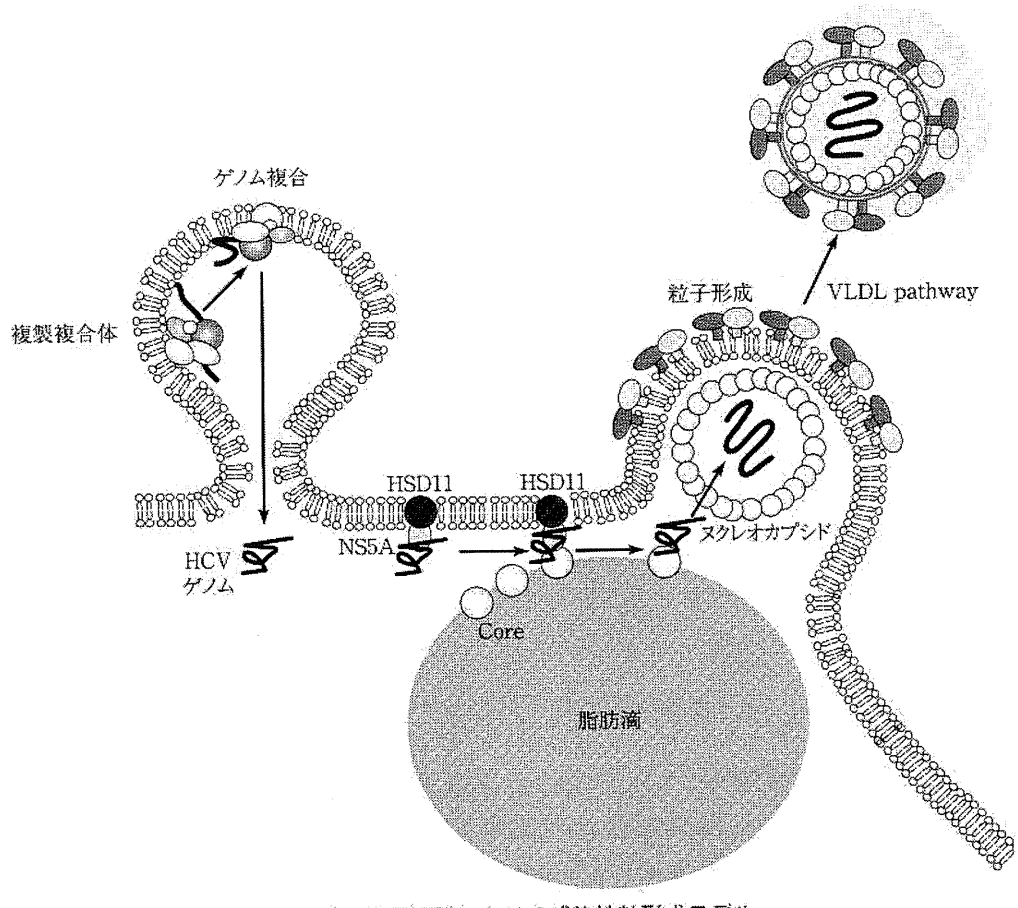

図　脂肪滴周辺膜における感染性粒形成モデル

LD 分画の中性脂肪も同様に HSD11 siRNA，局在シグナル欠損 HSD11 で減少し，野生型で増加した．さらに HSD11 siRNA が中性脂肪の合成酵素である DGAT 活性に与える影響を調べたところ，HSD11 siRNA 処理により Diacylglycerol acyltransferase (DGAT) 活性は低下した．以上の結果から，HSD11 は LD 合成亢進時に LD 産生，成熟化に関与していた可能性が示された．

まとめ

比較プロテオーム解析により，LD 周辺膜蛋白として約 45 の蛋白が同定され，その中で HSD11 が感染性粒子形成に重要な役割を果たしている可能性が示唆された．さらに HSD11 は肝細胞内で LD 産生あるいは成熟化に関与している可能性が示唆された．以上の結果から，NS5A 蛋白は HSD11 に結合することによって LD 周辺にリクルートされ，そこで粒子が形成されると推定できる（図）．HCV は感染・複製・粒子形成・放出などその生活環の多くのステップで宿主の生体膜の脂質を巧みに利用していることが分かってきた[8]．さらに，HCV は感染した宿主細胞に脂質の蓄積，LD 産生亢進という，HCV 増殖に好都合な環境を作り出しているものと考えられた．このような解析は C 型肝炎患者の病態の理解と治療につながると期待できる．

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【C型肝炎ウイルスの生活環】

Title □□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□

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Key words
C型肝炎ウイルス，生活環，
脂質，脂肪滴

要　約

本原稿執筆にあたり，前回2001年10月に執筆させていただいた本誌を紐解いたところ，「HCVの研究はHCV cDNAの発現産物の解析による人工的なものばかりであり，ウイルス本来の性状やウイルスの生活環はわかっておらず，HCV増殖が効率良く観察できる細胞培養系の確立が最重要研究課題である」と記述していた。2005年に，JFH-1株のゲノムRNAをHuh-7細胞に導入することにより，感染性ウイルス粒子を効率良く作製する技術が確立された。これにより，HCVの生活環のすべてを再現可能な実験系が確立したことになり，HCV研究は目覚ましい進歩を遂げている。その結果，C型肝炎の治療法もインターフェロン中心の治療から，NS5A阻害剤，ポリメラーゼ阻害剤の導入も間近であり，経口薬だけでHCVを撲滅するのも夢ではないと期待できる昨今である。本稿ではHCV増殖系により解明されたHCV生活環を中心に紹介する。

1.HCV培養細胞系確立の試み

1989年ホートンら米国カイロン社のグループにより感染チンパンジーの血漿からHCV遺伝子断片が発見された当時から，C型肝炎患者血清中のHCVを培養細胞に感染させウイルス増殖細胞系を作製する試みが行われてきた。リンパ球系細胞や肝細胞由来細胞株を用いて数多くの感染実験が試みられてきたが，観察できるウイルス量は少なく，ウイルス研究への応用は難しい状況であった。肝細胞を三次元培養すると細胞あたりのアルブミン産生，分泌能や肝特異的薬物代謝酵素の発現が亢進すること，極性細胞の特徴である細胞間ジャンクション構造が形成されることが報告されているので[1]，我々は単層培養系に比べより本来の肝組織に近い立体的な培養環境化の方が肝炎ウイルスの感染増殖に適しているのではないかと期待し，三次元化細胞培養システムであるラジアルフロー型バイオリアクター（RFB）および温度感受性ハイドルゲル（TGP）を利用してHCV培養細胞系の構築を試みた。RFB培養肝細胞株に患者血清を感染，またHCVゲノムcDNAをトランスフェクションし高密度培養を行うことでウイルスの複製増殖を確認した[2]。さらに，遺伝子型1bのダイシストロニックゲノム（全長レプリコン）を保持した細胞株をTGP培養することで，単層培養系では認められなかったウイルス粒子の産生を観察した[3]。しかしながら，依然としてそのHCV増殖レベルは必ずしも高いものではなく，よりウイルス産生効率に優れ，汎用性の高い培養系の開発が待望されていた。

2005年，Wakitaらは東京慈恵会医科大学付属第三病院の劇症肝炎症例の急性期血清からHCV株JFH1を分離した。このクローンを用いて全長のウイルスゲノムRNA合成し，Huh7細胞に導入することにより，ウイルス粒子が産生された[4]。このウイルス粒子は新たなHuh7細胞に感染性を示し，その感染はHCVのエンベロープに対する抗体やHCVの受容体CD81に対する抗体で阻止された。また，培養細胞で作製したウイルスは短期間ながらチンパンジーにも感染した。この系により，ウイルスの感染から分泌までの全ての過程が培養細胞内で解析可能になった。

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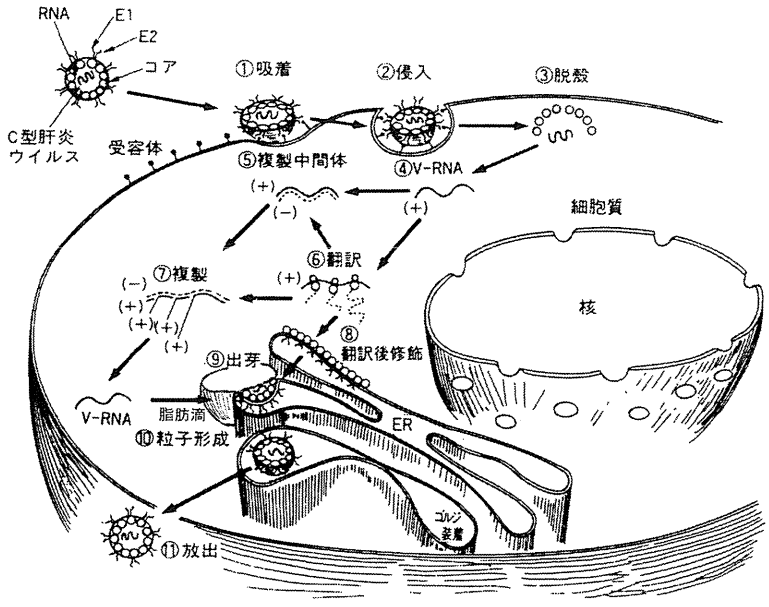

図1　HCVの生活環

2.HCVの生活環

推定されているHCVの生活環を図1に示す。HCVがレセプターを介して肝細胞に吸着，侵入し，粒子よりウイルスRNAが放出され（脱核），これがメッセンジャーRNAとして翻訳され，大きな前駆体蛋白が合成される。この前駆体蛋白は，細胞のシグナラーゼによってウイルス粒子を形成する構造蛋白であるコア蛋白と2つのエンベロープ蛋白E1, E2がプロセスされる。また，ウイルス自身がコードするプロテアーゼによって，プロテアーゼ，ヘリカーゼ，RNA依存性RNAポリメラーゼなどウイルスの複製に必須な非構造蛋白がプロセスされる。ウイルス由来酵素や宿主因子によってゲノムRNAからマイナス鎖RNAが転写され，複製複合体が形成される。これを基にしてプラス鎖RNAが合成され（複製），ウイルスRNAやmRNAとして働く。ウイルスRNAがコア蛋白と結合してヌクレオカプシドを形成し，さらにエンベロープ蛋白が邂逅してERでウイルス粒子が成熟し（出芽），トランスゴルジを通り細胞膜に達して細胞外へ放出されるものと考えられている。以下に各過程について解説する。

（1）吸着と侵入

宿主細胞の受容体とウイルス粒子表面の蛋白の特異性結合はウイルスの組織特異性や宿主域を決定する。HCVは細胞表面に存在するヘパリンやヘパラン硫酸などの硫酸多糖類に捕捉されて濃縮された後，エンベロープ蛋白質を介して親和性の高い蛋白質性受容体に結合し，エンドサイトーシスによってエンドソームに取り込まれる。HCVの感染受容体候補分子として，現在までにHeparansulphate Proteoglycan（HSPG），C型レクチン（DC-SIGN，L-SIGN），Low-Density Lipoprotein（LDL）受容体，CD81，ヒトスカベンジャー受容体クラスB-1型（SR-BI），Claudin-1などが知られている。

（2）翻訳

フラビウイルスやピコルナウイルスなどのウイルスRNAはキャップ構造を持たず，キャップ非依存的なIRESによる翻訳がおこなわれている。HCVでは，40Sリボゾームサブユニットが IRES（38-46番目）に直接結合するところから始まる。ここに，eukaryotic initiation factor（eIF）3などの翻訳開始因子が結合する。IRESに結合する他の宿主因子としては，La蛋白質，heterogeneous ribonucleoprotein L，poly-C binding protein，Pyrimidine Tract-Binding protein（PTB）が知られており，IRES活性の調節に関与している。HCVゲノムの翻訳産物であるポリプロテインは，細胞およびウイルス由来のプロテアーゼにより切断され，成熟した構造および非構造蛋白になる。各構造蛋白質間及びp7/NS2間の切断は宿主細胞小胞体のシグナルペプチダーゼによって行われる。各NS蛋白質間の切断のうち，NS2/NS3間の切断はNS2/NS3金属要求性プロテアーゼにより早期に起こる。一方，NS3からNS5Bの切断はNS3セリンプロテアーゼによる。

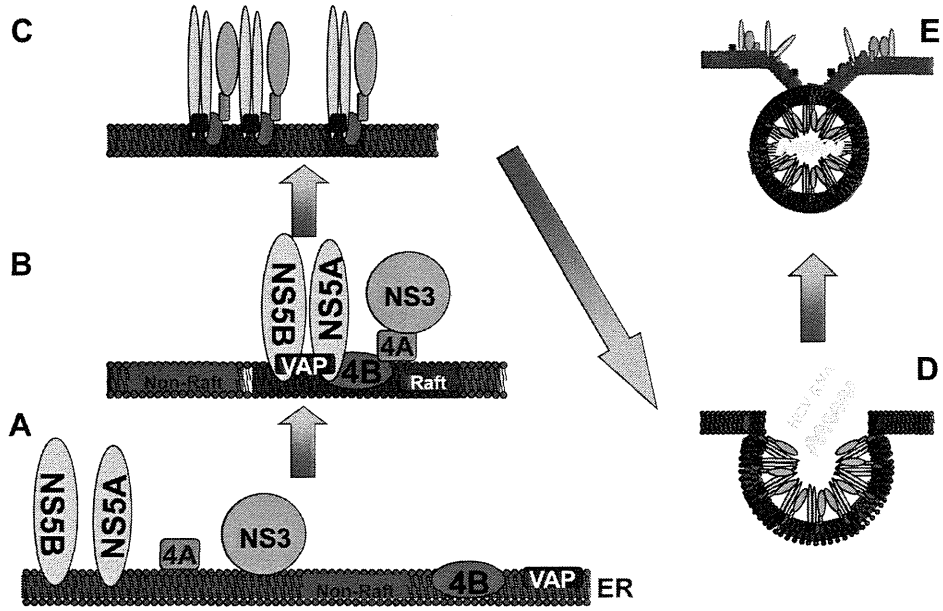

図2　脂質ラフト上でのHCV複製複合体形成モデル

（3）複製

1999年，ドイツのグループは，本来HCVゲノムの中でウイルス粒子を形成する構造タンパク質領域を薬剤耐性遺伝子に置き換え，その下流に，より強力にHCVゲノムの内部から翻訳させる働きを有するEncepharomyocarditis Virus（EMCV）のIRESを挿入したRNAレプリコンを作成した[5]。このRNAをトランスフェクトした細胞を薬剤存在下で培養することで，自律複製するために必要な適応変異を獲得したHCVゲノムと，更にこのHCV遺伝子が複製しうる細胞を選択することを可能にした。レプリコン細胞を電子顕微鏡で観察すると「membranous web」と呼ばれる小胞様構造物が認められることが報告されており[6]，HCVの複製複合体は感染細胞のmembranous webに存在しているものと考えられている。

次に，生化学的手法を用いて，複製活性を維持したままのHCV複製複合体を粗精製し解析したところ，HCV RNAとNS蛋白の大部分は界面活性剤不溶性膜画分（DRM）に残り，HCV RNA複製活性はDRMに検出された[7]。以上のことから，このDRMに複製活性を保持したHCV複製複合体が存在することが判明したことから，HCV複製複合体が脂質ラフト上で形成される可能性が示唆された（図2）。脂質ラフトはコレステロールとスフィンゴ脂質からなると考えられており，HMG-CoAレダクターゼ阻害剤，またはスフィンゴ脂質合成阻害剤が脂質ラフト形成を抑制することで，ウイルス複製を抑えるという報告もあり，脂質ラフトの存在する膜上で複製が起こるという仮説が支持された。HCV複製複合体は脂質ラフトを含む膜小胞構造内に存在し，内部に存在するHCV RNAやNS蛋白は外部からのRNA分解酵素やプロテアーゼに対して保護されているものと考えられた。

（4）粒子形成，分泌

HCV粒子の形成，分泌過程の解析もウイルス培養系の開発により可能となり，脂肪滴の役割が注目されている。Miyanariらはウイルス感染細胞内で脂肪滴をコア蛋白質が被い，さらにそのコア蛋白をNS5A蛋白が被っていることを発見した[8]。NS5A蛋白はER膜上で形成され，脂肪滴上に移行し，そこに存在するコア蛋白と結合していた。コアとNS5A蛋白が結合できない変異をウイルスゲノムに導入すると，感染性ウイルス粒子の形成がなくなったことから，感染性ウイルス粒子の形成には脂肪滴上でのコアとNS5A蛋白質の結合が重要であると考えられた。

脂肪滴周辺で形成されたHCV粒子は，リポ蛋白形成や分泌を抑制すると感染性HCV粒子放出も減少することから，HCVは超低比重リポ蛋白（VLDL）分泌系を利用している可能性が考えられている[9]。HCVゲノムの囲むようにヌクレオキャプシドが形成され，その周りにヘテロダイマーの形成したE1とE2蛋白が覆っている。最近，さらにこの粒子がVLDLや低比重リポ蛋白（LDL）に包まれていることを示す報告が集積してきている。E1とE2にHDL，LDL，

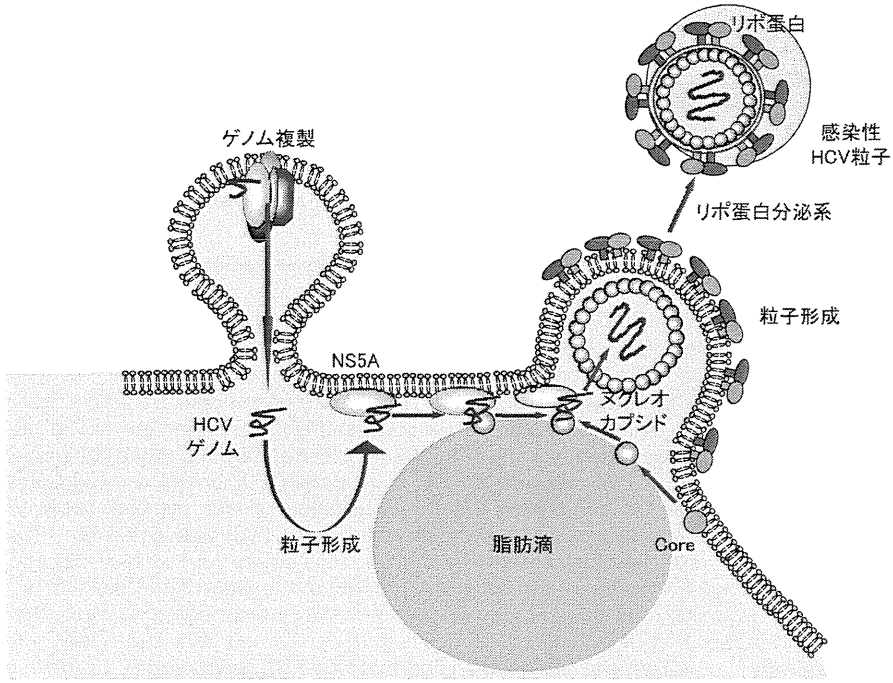

図３　脂質ラフトを利用したHCV粒子形成のモデル

VLDLと結合する性質があり，リポ蛋白に含まれるApolipoprptein E（ApoE），ApoB，ApoC1などが感染性に重要ということが報告されている。

おわりに

HCVはゲノム配列が多様で，大変変異しやすいウイルスであり，IFNやリバビリンなどの薬剤に対しても耐性を持つウイルスが出現しやすいことが知られている。新たな抗HCV薬として，ウイルスプロテアーゼやポリメラーゼなどのウイルス複製に関与する酵素を標的とした薬剤の開発研究が盛んに行われている。HIVと同様にこれらの薬剤についてもHCVは耐性変異を獲得することが報告されている。本稿で報告した宿主の脂質産生系[10]（図3）などウイルス生活環に関与する宿主因子を標的とし，感染した細胞側の働きを抑えてウイルス増殖を抑制する抗HCV薬の開発は耐性ウイルスが出現しにくい薬剤につながる期待がある。

謝辞

本研究は下記の多くの研究者のご協力を得て遂行できたものであり，ここに謝意を表す：松浦善治（大阪大学微生物病研究所・分子ウイルス分野），深澤征義，花田賢太郎（国立感染症研究所・細胞化学部），西島正弘（国立医薬品食品衛生研究所），マイケル・ライ（南加大・微生物学免疫学研究室），脇田隆字，鈴木哲朗，宮村達男（国立感染症研究所・ウイルス第二部）。

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PLOS ONE

Hepatitis C Virus Translation Preferentially Depends on Active RNA Replication

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Abstract

Hepatitis C virus (HCV) RNA initiates its replication on a detergent-resistant membrane structure derived from the endoplasmic reticulum (ER) in the HCV replicon cells. By performing a pulse-chase study of BrU-labeled HCV RNA, we found that the newly-synthesized HCV RNA traveled along the anterograde-membrane traffic and moved away from the ER. Presumably, the RNA moved to the site of translation or virion assembly in the later steps of viral life cycle. In this study, we further addressed how HCV RNA translation was regulated by HCV RNA trafficking. When the movement of HCV RNA from the site of RNA synthesis to the Golgi complex was blocked by nocodazole, an inhibitor of ER-Golgi transport, HCV protein translation was surprisingly enhanced, suggesting that the translation of viral proteins occurred near the site of RNA synthesis. We also found that the translation of HCV proteins was dependent on active RNA synthesis: inhibition of viral RNA synthesis by an NS5B inhibitor resulted in decreased HCV viral protein synthesis even when the total amount of intracellular HCV RNA remained unchanged. Furthermore, the translation activity of the replication-defective HCV replicons or viral RNA with an NS5B mutation was greatly reduced as compared to that of the corresponding wildtype RNA. By performing live cell labeling of newly synthesized HCV RNA and proteins, we further showed that the newly synthesized HCV proteins colocalized with the newly synthesized viral RNA, suggesting that HCV RNA replication and protein translation take place at or near the same site. Our findings together indicate that the translation of HCV RNA is coupled to RNA replication and that the both processes may occur at the same subcellular membrane compartments, which we term the replicasome.

Citation: Liu HM, Aizaki H, Machida K, Ou J-HJ, Lai MMC (2012) Hepatitis C Virus Translation Preferentially Depends on Active RNA Replication. PLoS ONE 7(8): e43600. doi:10.1371/journal.pone.0043600

Editor: Jinah Choi, University of California Merced, United States of America

Received May 30, 2012; **Accepted** July 26, 2012; **Published** August 24, 2012

Funding: This work was supported by National Institutes of Health grants AI40038, CA108302, and Academia Sinica Institutional Fund. No additional external funding received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Hepatitis C virus (HCV) is a positive-sense RNA virus that is estimated to chronically infect as many as 3% of the world's population. As a member of Flaviviridae, HCV is an enveloped virus with a single, positive-stranded RNA around 9.6 kb in length [1]. The viral genome encodes a large viral polyprotein, which is proteolytically processed by cellular signal peptidases and viral proteases into structural (C, E1, E2, and p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins [2]. Membrane association of the viral proteins is essential for HCV replication, at both steps of RNA transcription and translation [3–5]. To decipher the mechanisms by which HCV navigates these steps may necessitate an understanding of the cell biological processes as diverse as cytoplasmic organelle structure and membrane biogenesis and trafficking in the secretory pathway.

Using the HCV subgenomic replicon system as well as infectious virus system, many host factors have been identified to be involved in HCV RNA replication, including the human homologue of the 33-kDa vesicle-associated membrane protein-associated protein (hVAP-33) [6], Golgi-specific brefeldin A resistant guanine nucleotide exchange factor 1(GBF1) [7], Endocytic Rab proteins [8], polypyrimidine-tract-binding protein (PTB) [9,10], La autoantigen [10], SYNCRIP [11], and host geranylgeranylated proteins and fatty acids [12]. These host proteins that are identified to be in the HCV RNA replication complexes are important in either membrane sorting and trafficking or RNA binding and processing. Some of these host factors, such as PTB and La autoantigen, have been found to regulate HCV translation as well_ENREF_13 by virtue of their binding to the 5′ or 3′ UTR of HCV RNA [13–15]. The identification of host proteins with dual-functions in regulating both translation and transcription implies the possibility of coupled transcription/translation of HCV RNA.

The balance between viral RNA transcription and translation is critical for the replication of positive-stranded RNA viruses, since the same RNA is used both for translation and as the template for negative-strand RNA synthesis. Transcription of poliovirus has been reported to be dependent on the translational activity of the viral RNA [16]. On the other hand, the translation of Sindbis virus and vesicular stomatitis virus has been reported to be transcriptionally dependent [17]. Such coupling of transcription-translation has been well documented to confer advantage in maintaining the stability of the RNA molecule in bacteria [18,19] and also to respond to regulatory signals coordinately.

In this study, we observed that HCV RNA exit from the site of RNA synthesis to the Golgi complex, a process that can be blocked by nocodazole, an inhibitor of the ER-Golgi transport pathway. Surprisingly, HCV protein translation was enhanced when HCV RNA movement was blocked, suggesting that the translation of

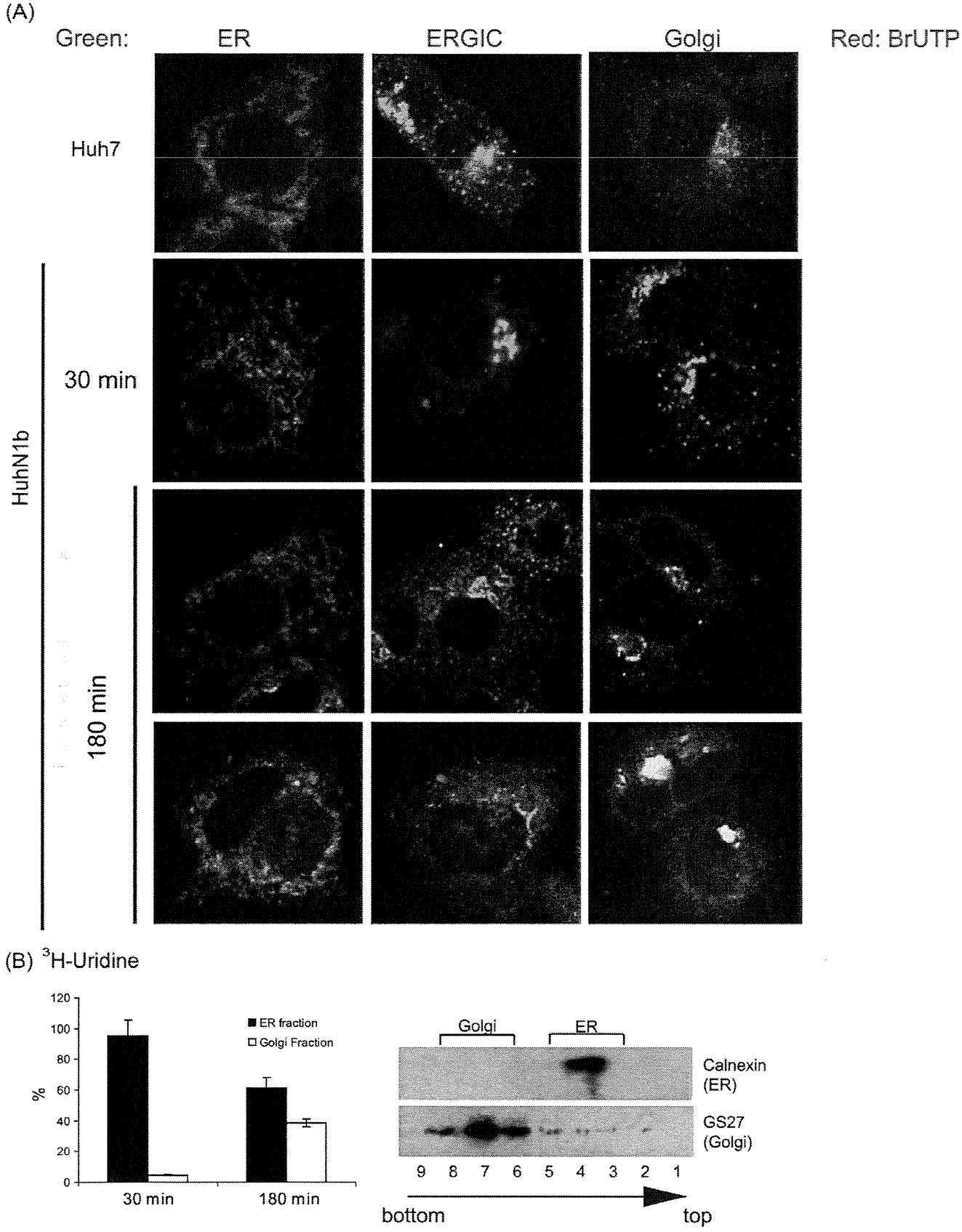


Figure 1. The translocation of newly-synthesized HCV RNA. HCV replicon cells were labeled with BrUTP (A) or ^{3}H-Uridine (B) in the presence of actinomycin D and chased for up to 180 minutes. (A) Immunofluorescence staining with anti-BrdU and other organelle antibodies shows the colocalization of BrU-labeled HCV RNA with ER initially (30 min) and then with Golgi (180 min). (B) Fractionation of ER and Golgi by sucrose gradient.

Fraction numbers and their gradient positions are noted at the bottom. ^{3}H-Uridine-labeled RNA in the ER (fraction 4) and the Golgi (fraction 6–8) fractions were collected, and the radioactivity of ^{3}H-Uridine-labeled RNA was counted. Immunoblotting of ER and Golgi makers demonstrates the separation of ER and Golgi by sucrose gradient fractionation.
doi:10.1371/journal.pone.0043600.g001

viral proteins occurred near the site of RNA synthesis. We also found that the translation of HCV proteins was dependent on active RNA synthesis: inhibition of RNA synthesis resulted in decreased HCV viral protein synthesis before there was significant decrease in the total amount of HCV RNA, and that the replication-defective HCV RNA could not be translated efficiently *in vivo*. Finally, we found that at least most of the newly synthesized HCV proteins colocalized with the newly synthesized viral proteins. These findings together thus indicate that HCV replication and translation are coupled, in the sense that replication of viral RNA is linked to translation of viral RNA in situ.

Materials and Methods

Cell Lines, HCV Full-length and Subgenomic Constructs

Huh7 or Huh7.5 cells were grown at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and nonessential amino acids. Huh7 cells were obtained from Dr. Sato's lab [20], and Huh7.5 cells were obtained from Dr. Rice' lab [21]. Bicistronic HCV-N1b replicon was derived from the HCV-N strain with a neomycin-phosphotransferase (NPT) gene for selection as described [22]. Huh-Noe cells are stable cells derived from Huh7 cells with NPT expression as described previously [23,24]. Huh-N1b replicon cell line and Huh-Neo cells containing an NPT gene were grown under the same conditions as Huh7 cells using the same media containing 0.5 mg/ml G418.

In vitro Transcription and Electroporation of HCV Full-length and Subgenomic RNA

HCV JFH1 and JFH-GND constructs were obtained from Dr. Wakita's lab (NIID, Japan) [25]. Bicistronic replicon with either firefly luciferase (FFLuc) or Renilla luciferase (RLuc) gene was derived from HCV1bneo [22] by replacing NPT with either FFLuc or RLuc reporter gene. To prepare the template for *in vitro* transcription, the plasmids were digested by Xba I and Mungbean nuclease and gel-purified. For electroporation, Huh7 or Huh7.5 cells were trypsinized, washed and resuspended in serum-free DMEM. HCV replicon RNA or JFH full-length RNA were transcribed *in vitro* by T7 MegaScript (Ambion). A total of 6 to 10 μg of RNA and 10^{7} Huh7 cells were mixed and incubated on ice for 5 minutes and subjected to an electric pulse at 975 μF and 220 V. Cells were immediately transferred to 8 ml of DMEM containing 10% FBS for incubation.

Labeling and Immunofluorescene Staining of De Novo-synthesized Viral RNA and Newly-translated Peptides

Labeling of de novo-synthesized viral RNA, immunofluorescence staining and confocal microscopy were modified from the previously described methods [26]. Briefly, Huh7, Huh7.5 or replicon cells were plated on 8-well chamber slides at density of 1×10^{4} cells per well. Two days after seeding, cells were incubated with actinomycin D (10 μg/ml) for 1 hour to inhibit cellular RNA synthesis, and in some experiments, also with 20 nM of hippuristanol [27] for 1 hour to inhibit eIF4A-dependent protein synthesis, which represents most of the host cell protein synthesis. For immunofluorescence detection of de novo synthesized viral RNA, 2 mM of bromouridine triphosphate (BrUTP) was subsequently transfected into cells at 4°C for 15 min using Fugene 6 transfection reagent according to the manufacturer's instructions (Roche). For live-cell imaging of both RNA and proteins, Cy5-UTP and BODIPY-FL-Lys-tRNA were cotransfected to label nascent HCV RNA and peptides, respectively. For immunofluorescence staining of both nascent viral RNA and proteins, Transcend biotinyl-Lys-RNA (Promega) [28] and BrUTP were instead used. The transfected cells were washed with phosphate-buffered saline (PBS) twice and incubated at 37°C with DMEM supplemented with 10% FBS for different periods of time. After incubation, cells were washed twice with PBS and subsequently fixed with 4% formaldehyde for 1 hr at 4°C. For permeabilization, the cells were treated with 0.1% Triton X-100 in PBS supplemented with 1% FBS for 30 min at RT. Primary antibodies were diluted in PBS containing 1% bovine serum albumin (BSA) and incubated with cells for 1 hr at RT. After three washes in PBS, the cells were incubated with fluorescein-isothiocyanate (FITC)-conjugated or Rhodamine-conjugated secondary antibodies, or Texas-red-conjugated streptoavidin diluted at a 1:100 with PBS containing 5% BSA for 1 hr at RT. Then the cells were washed three times in PBS and mounted in Vectashield (Vector Laboratories).

Analysis of Intracellular Viral RNA by Northern Blotting and Real-time RT PCR

To determine the quantity of RNA by real-time PCR, a single-tube reaction was performed by using the TaqMan EZ RT-PCR Core Reagents (Applied Biosystems). Duplicate reactions for RNA standards and the samples were performed in 20-μl volume using 1 μl of HCV RNA, primers from HCV 5′ non-coding region (5′ GAG TGT CGT GCA GCC TCC A 3′ and 5′ CAC TCG CAA GCA CCC TAT CA 3′) of the HCV 1b sequence [29], and a fluorescent probe [5′ (FAM) CCC GCA AGA CTG CTA GCC GAG TAG TGT TGG (TAMRA) 3′] spanning these two regions. The RT step was performed at 60°C for 50 min, followed by 1 min at 50°C. The amplification condition was as follows: 95°C for 5 min and 50 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 10 sec, and extension at 69°C for 1 min.

Using the ABI Prism 7900 program, standard curves of the assays were obtained automatically by plotting the three hold values against each standard dilution of known concentration (10^{1}–10^{6} copies per reaction) of HCV genotype 1b transcript. The same software was used to calculate the coefficients of regression. Values were normalized to that of GAPDH (Applied Biosystems). Each test was done in triplicate and averages were obtained.

Fractionation of ER and Golgi Membrane

The procedure was based on the published method [30]. Cell lysates were applied to a discontinuous sucrose gradient composed of layers of 2 M, 1.3 M, 1.0 M and 0.6 M sucrose. The ER fraction was concentrated at the interface between 0.6 M and 1.0 M sucrose, and the Golgi fraction was concentrated at the interface between 2 M and 1.3 M. To determine the signal of ^{3}H-Uridine-labeled RNA, the fractions were passed through DE81 membranes to concentrate the labeled RNA. The membranes were then counted by scintillation counter, and the ratio of signals from the ER and Golgi fractions were calculated.

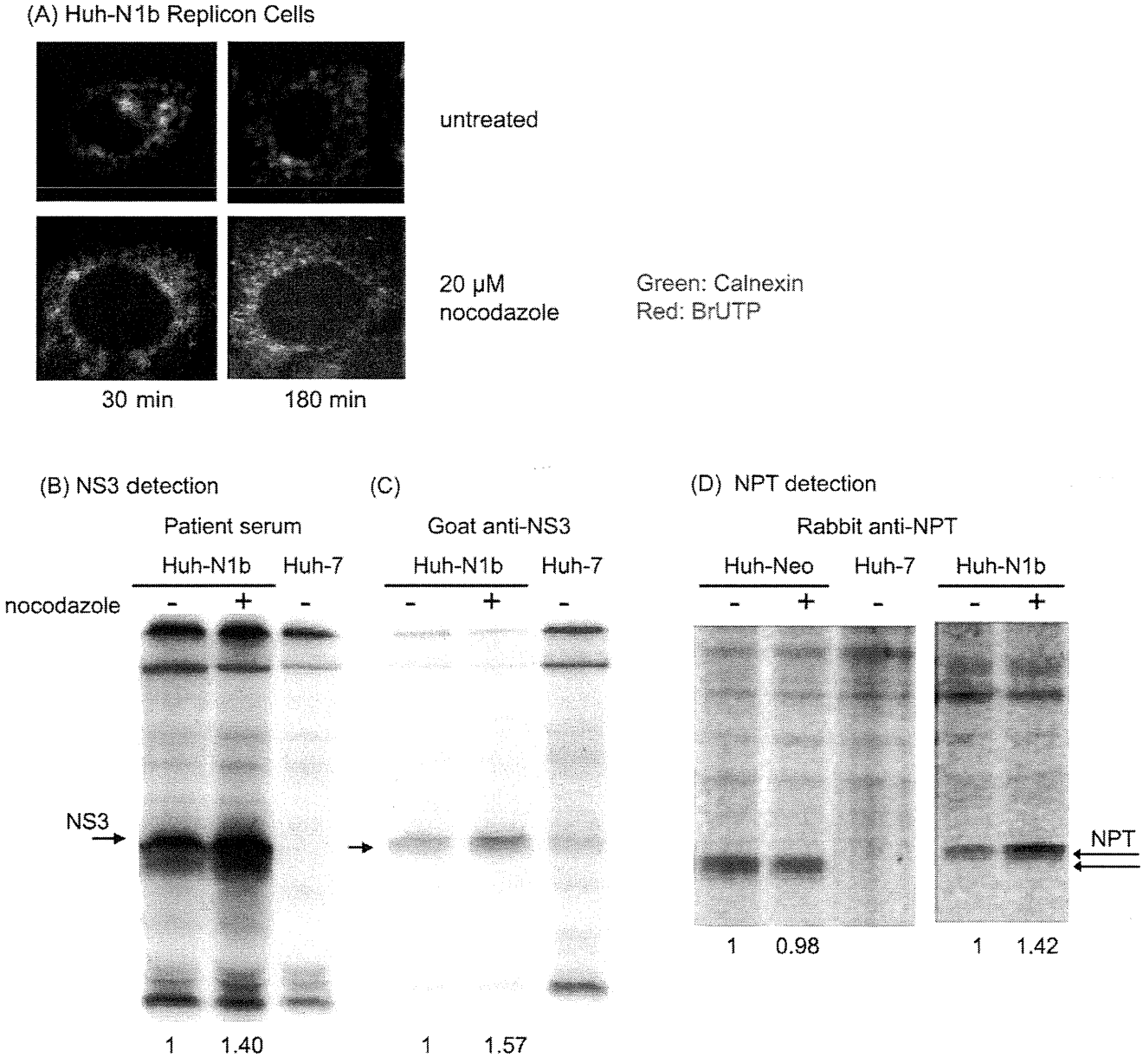


Figure 2. Increase in replicon RNA translation in nocodazole-treated HCV replicon cells. Huh-N1b cells was pre-treated with 20 μM nocodazole for 4 hours and then labeled with BrUTP or with ^{35}S-Methionine. (A) The BrU-labeled RNA remained colocalized with calnexin (an ER amrker) even after 180 min. (B–D) Proteins were immunoprecipitated with (B) HCV patient serum, (C) Goat anti-NS3 antibody, or (D) Rabbit anti-NPT antibody. The immunoprecipitated products were detected by autoradiography. The nocodazole-pretreated Huh-N1b cells showed about 50% increase in NS3 and NPT translated from replicon RNA, whereas NPT translation in the Huh-Neo control cells was not affected by the nocodazole treatment.
doi:10.1371/journal.pone.0043600.g002

Results

The Newly-synthesized HCV RNA Localizes to the ER and Moves Along with Anterograde Vesicle Trafficking

To visualize the replication of HCV RNA, we performed pulse BrUTP labeling in the actinomycin D-treated Huh-N1b replicon cells; under such conditions, only the viral RNA, which depends on RNA-dependent RNA polymerase, is labeled. After 15-minute labeling, the labeled RNA was chased in non-labeled media for 30 minutes to 3 hours, and the subcellular localization of BrU-labeled RNA was detected with anti-BrdU antibody and co-stained with individual organelle markers for ER (Calnexin), ERGIC (ER-GIC53), and Golgi apparatus (GS27), respectively (Fig. 1A). Consistent with our previous report, the newly synthesized HCV RNA was initially colocalized with the ER marker (Fig. 1A). However, after 3 hours of chase, the majority of the labeled HCV RNA did not colocalize with the ER marker, but colocalized with the Golgi marker instead (Fig. 1A, bottom panels). We did not observe any significant colocalization between BrU-labeled HCV RNA and ERGIC53. As a control, the BrUTP signals could not be detected in the actinomycin D-treated Huh7 cells, while the immunofluorescence-staining patterns of the ER, ERGIC, and Golgi apparatus appeared similar between Huh7 and Huh-N1b cells (Fig. 1A).

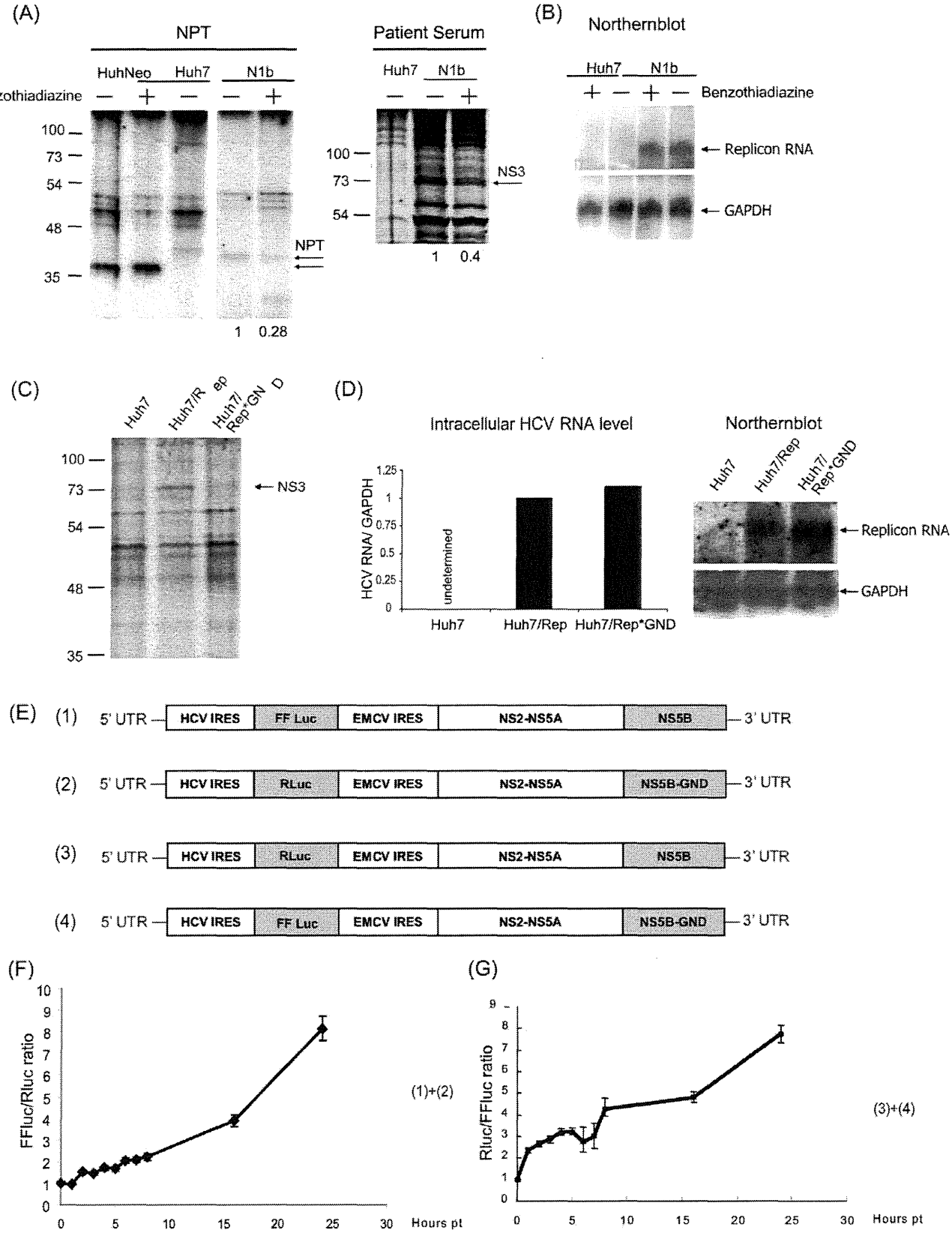


Figure 3. HCV RNA translation is dependent on the RNA transcription. (A), Mock- or Benzothiadiazine-treated Huh-N1b and Huh-Neo cells were labeled by ^{35}S-Methionine for 4 hours, and followed by immunoprecipitation with anti-NPT or anti-NS3 antibodies or sera from hepatitis C patients. The immunoprecipitates were separated by SDS-PAGE and detected by autoradiography. (B) The intracellular replicon RNA was detected by Northern blotting. (C) Huh7 cells transfected with *in vitro* transcribed Rep or the replication-defective Rep*GND RNA were metabolically labeled with ^{35}S-Methionine for 14 hours and followed by immunoprecipitation with anti-NS3 antibody. (D) The amounts of the intracellular HCV RNA in panel (C)

were determined by realtime RT-PCR and Nothern blotting. The relative ratios of the HCV RNA/GAPDH mRNA in the different cells are presented. E) Structures of the bi-cistronic replicon reporter constructs used. Time course studies of the luciferase activity in cells transfected with constructs 1 and 2 (panel (F)) and constructs 3 and 4 (panel (G)) were measured by dual luciferase assay at various time points after transfection. The ratios of the FFluc/Rluc (F) or Rluc/FFluc (G) are presented. Error bars represent +/− standard deviation.
doi:10.1371/journal.pone.0043600.g003

The ER-to-Golgi trafficking of the newly synthesized RNA was further confirmed by biochemical analysis. The actinomycin D-treated Huh-N1b cells were labeled with ^{3}H-uridine for 30 minutes and chased for 30 minutes to 3 hours. The labeled cell lysates were separated into ER and Golgi fractions by ultracentrifugation. Immunoblotting studies showed that the ER and the Golgi apparatus were efficiently separated by this procedure (Fig. 1B, right panel). The relative ratio of ^{3}H-uridine-labeled RNA in the Golgi and the ER significantly increased over time (Fig. 1B). The result suggested that the newly-synthesized HCV RNA was transported from the ER-derived to the Golgi apparatus-derived membranes.

HCV RNA Translation is Increased when Anterograde Vesicle Trafficking is Blocked

The ER-to-Golgi apparatus trafficking is known as the anterograde vesicle trafficking pathway, and can be blocked by nocodazole, which depolymerized microtubules and disrupts Golgi apparatus [31,32]. When BrUTP labeling was performed in the presence of nocodazole, BrU-labeled RNA colocalized with ER (calnexin) even after 3 hours of chase (Fig. 2A). Under these conditions, the morphology of cells was not altered by the treatment. These results suggest that the anterograde vesicle trafficking is involved in the transport of HCV RNA after its synthesis. We then further investigated if this transportation is required for certain steps of the HCV life cycle. Previously it has been shown that prolonged (more than 24 hours) nocodazole treatment inhibits HCV replication and viral production [33]; we thus tested if the nocodazole treatment could affect HCV translation. Huh7 or Huh-N1b (HCV replicon) cells were pre-treated with nocodazole for 4 hours, and then labeled with ^{35}S-Methionine for 4 hours to determine HCV translation activity (Fig. 2B). We determined the amounts of ^{35}S-Methionine-labeled Neomycin-phospho-transferase (NPT) and HCV NS3 proteins, both of which are encoded from the HCV replicon RNA but under the control of separate IRES elements. We found that after a 4-hour nocodazole treatment, the total amount of the labeled NS3 protein, as detected by anti-NS3 or HCV patients' sera, was significantly increased (Fig. 2, B and C). Similar observation was made for the NPT protein translated from the HCV replicon (Fig. 2D). Quantitation of the proteins showed a 40%–50% increase in both NPT and NS3 protein synthesis after a 4-hour nocodazole treatment. Since the translation of these two proteins was under the regulation of different sequence elements, these results suggest that the increase in HCV protein translation was not due to specific enhancement of HCV IRES-mediated translational activity. We also tested the effects of the nocodazole treatment on the NPT synthesis in the neomycin-resistant Huh-Neo cells, in which NPT is expressed from an integrated plasmid DNA. In contrast to HCV replicon cells, NPT synthesis in Huh-Neo cells was not affected by the nocodazole treatment (Fig. 2D,), indicating that the increase in HCV protein synthesis by the nocodazole treatment in HCV replicon cells was not due to enhancement of global translation.

These results are unexpected, raising a possibility that the newly-synthesized HCV RNA may be used for RNA translation in situ, without being transported away from the site of RNA synthesis. This result brought up an intriguing possibility that HCV RNA replication and translation are coupled and take place in the same replication complex.

HCV RNA Translation is Dependent on the Transcriptional Activity of the RNA

To test the idea that the newly synthesized HCV RNA is used for translation in situ, we then investigated whether translation was dependent on active RNA synthesis. We used a specific NS5B polymerase inhibitor, Benzothiadiazine [34,35], to inhibit HCV RNA synthesis and then examined the possible effects, if any, on HCV translation.

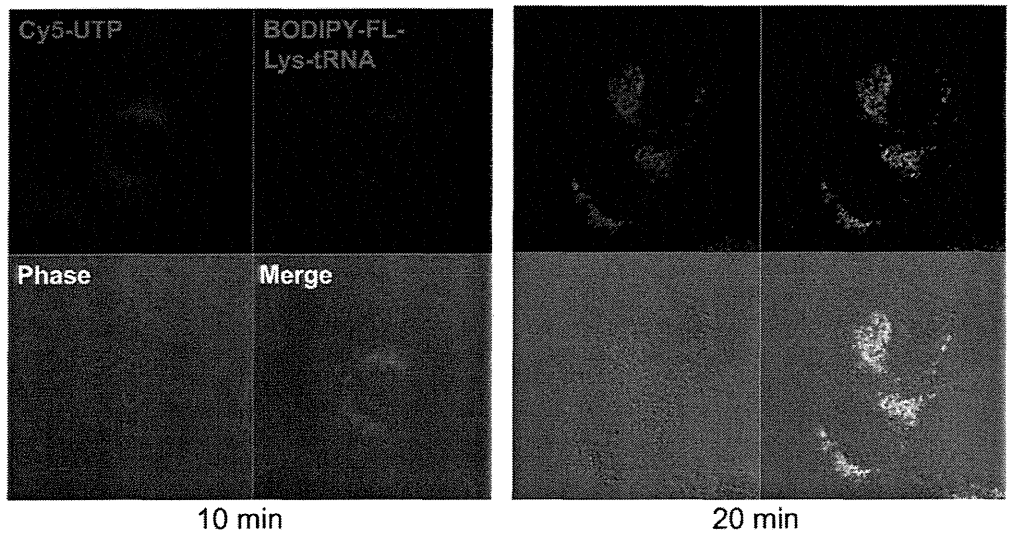
We first determined the efficiency and specificity of the inhibitor on ^{3}H-uridine incorporation (Fig. S1A). Huh7-N1b replicon cells were pretreated with or without Benzothiadiazine for 16 hours and then with actinomycin D for an additional 1 hour prior to ^{3}H-uridine labeling. Under this condition, ^{3}H-uridine is expected to be incorporated into HCV RNA only, but not cellular RNA [30]. The data showed that, in Huh7 cells, actinomycin D almost completely inhibited uridine incorporation. However, in Huh-N1b cells, actinomycin D did not completely inhibit ^{3}H-uridine incorporation; the residual incorporation likely represents HCV RNA synthesis, as confirmed by the autoradiography of the RNA products (Fig. S1A, lower panel). This residual RNA synthesis was inhibited by Benzothiadiazine. Furthermore, the Br-UTP label in Huh-N1b cells was detected as speckles in the perinuclear region; these speckles were not visible when the cells were treated with Benzothiadiazine (Fig. S1B). These results together indicate that Benzothiadiazine inhibits viral RNA synthesis specifically.

We also studied the effects of Benzothiadiazine on the steady-state level of replicon RNA by realtime RT-PCR analysis. The data showed that even after 16 hours of treatment, the total amount of replicon RNA in the cells was not significantly affected (Fig. S1C). Furthermore, the size of HCV RNA remained the same even after 16 hours of Benzothiadiazine treatment. After 2 days of treatment, however, the amounts of the replicon RNA decreased by about 50%. After 5 days, the RNA level dropped to 10% that of the control cells (Fig. S1D). As a comparison, nocodazole, which was reported to inhibit HCV RNA replication [33], had a smaller effect on the amounts of HCV RNA.

Having established the specificity of Benzothiadiazine on HCV RNA synthesis, we then labeled the Benzothiadiazine-treated cells with ^{35}S-Met and immunoprecipitated HCV proteins from the cell extracts (Fig. 3A). At 4 hour post-treatment, the amounts of newly synthesized HCV NS3 and NPT proteins were significantly decreased after the Benzothiadiazine treatment (Fig. 3A), while the amount and size of HCV RNA were not significantly affected, as determined by realtime RT-PCR and Northern blot (Fig. 3B). In contrast, in Huh-Neo cells, the Benzothiadiazine treatment did not affect the translation of NPT (Fig. 3A), indicating that Benzothiadiazine specifically inhibited translation of HCV RNA.

We further used a replication-defective replicon RNA (GND mutation in the NS5B region) to assess if active RNA replication of HCV is required for HCV protein translation. A separate experiment using an *in vitro* translation system showed that both wild-type HCV replicon and its GND mutant RNAs could produce equivalent amounts of NS2 to NS5B proteins (data not shown), indicating that the open reading frames in these RNAs are intact. The wild-type HCV replicon (Rep) and its GND mutant

HCV(JFH1)-infected Huh7.5 cells



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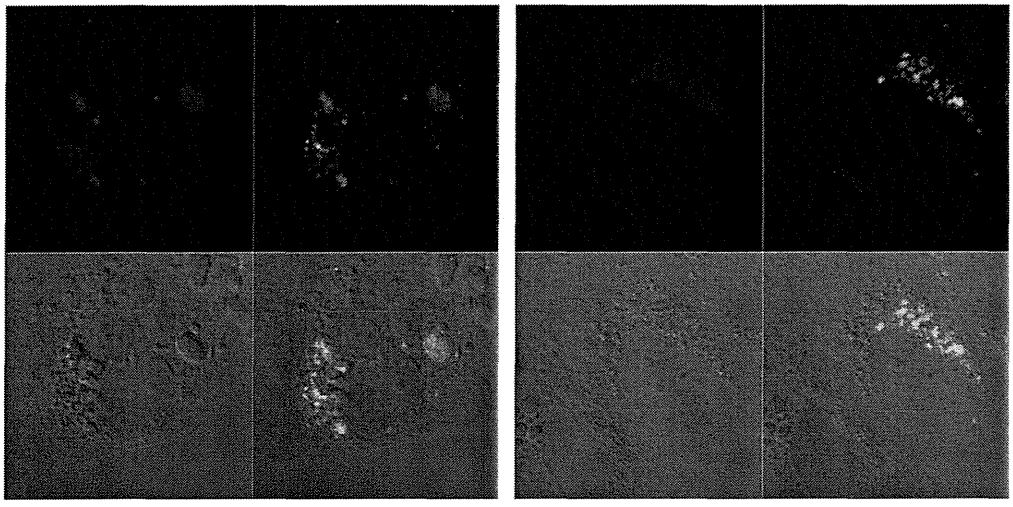

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Huh7.5 cells

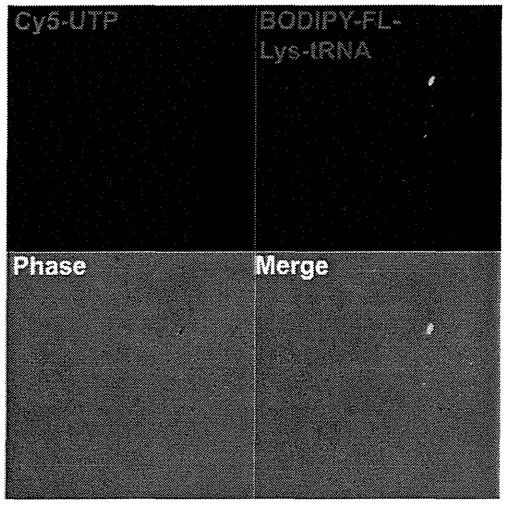

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Figure 4. Double-labeling of newly-synthesized HCV RNA and newly synthesized viral peptides in JFH1-infected Huh-7.5 cells. Huh7.5 cells were infected with HCV JFH-1 strain for 2 days, and then were labeled with Cy5-UTP and BODIPY-FL-Lys-tRNA in the presence of actinomycin D and hippuristanol, which inhibit host RNA and protein synthesis, respectively. The cells were kept in 37°C chamber supplied with CO_2 for live cell imaging on a Zeiss LSM 510 laser scanning confocal microscope. Images were taken after 10–40 minutes of chase. Newly-synthesized HCV RNA was the first to be detected (as shown in red) and was in a perinuclear pattern. Newly-translated HCV viral peptides (as shown in green) were detected at later time points, completely co-localized with the sites of RNA synthesis. No significant amount of Cy5-UTP and BODIPY-FL-Lys-tRNA labeling could be detected in naïve Huh7.5 cells (as a negative control) in the presence of actinomycin D and hippuristanol.
doi:10.1371/journal.pone.0043600.g004

(Rep*GND) RNAs were then transfected into Huh7 cells, and the transfected cells were metabolically labeled with ^{35}S-Met for 14 hours to detect protein syntheses by immunoprecipitation with anti-NS3 or -NPT antibodies. The result showed that, the GND mutant yielded very little NS3 as compared with the corresponding wildtype replicon RNA (Fig. 3C). The amounts of wildtype and mutant RNAs were equivalent at 14 hours post RNA electroporation (Fig. 3D). This result suggested that HCV RNA replication enhanced the efficiency of HCV RNA translation, but could not rule out the possibilities that this enhanced translation was due to quicker degradation of the Rep*GND mutant and/or a higher copy number of the Rep RNA as a result of RNA replication. To further investigate if replication-competent HCV replicon RNA was preferentially translated, we compared the translation activity of a bicistronic Firefly luciferase replicon RNA (Luc-Rep) and the comparable but replication-defective Renilla luciferase replicon GND mutant (RLuc-RepGND) (Fig. 3E). Both constructs were first tested by *in vitro* translation assay to ensure that the both reporters were functional (data not shown). Immediately after transfection into Huh7 cells, both luciferase activities were equivalent initially; however, the FFLuc/RLuc ratio increased over time (Fig. 3F). The reverse paired replicons, RLuc-Rep and Luc-GND, also gave a similar result (Fig. 3G). We observed 2–4 folds more luciferase activities translated from the replication-competent replicon RNA than those from the GND mutants at 8 hr post-transfection (Fig. 3F & G), at which time the incoming RNAs had not yet been degraded, indicating that the replication-competent HCV RNA was preferentially translated.

We further performed a similar study using the infectious HCV clone JFH1 and its replication-defective mutant, JFH1-GND, in a time-course study of HCV protein translation. Huh7 cells were transfected with JFH1 or JFH1/GND RNA, labeled with ^{35}S-Met during the 0–8, 8–16, or 16–24 hours post-transfection, and followed by immunoprecipitation. The amounts of these two RNAs at 24 hours post-transfection were almost the same (Fig. S2B). However, only the infectious JFH RNA, but not its GND

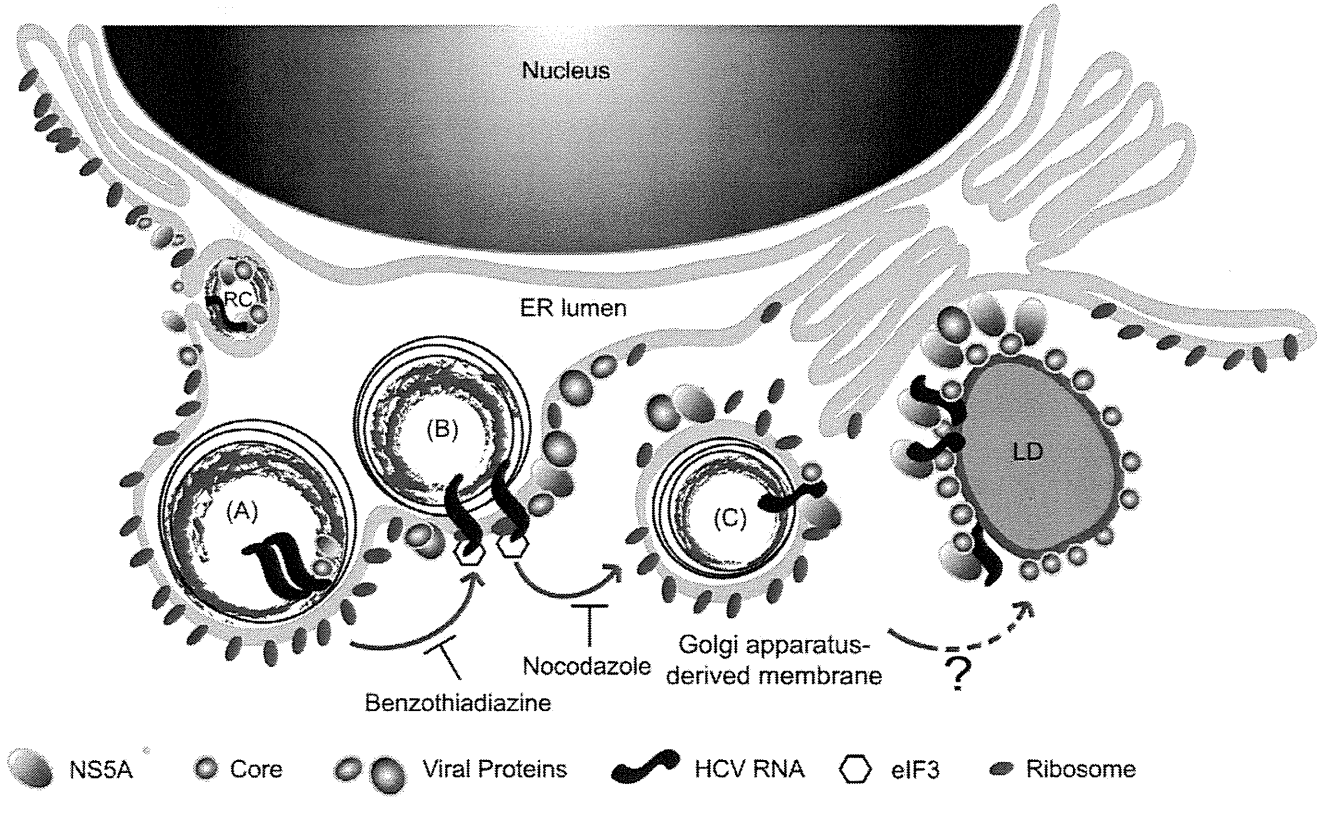

Figure 5. The proposed model of coupled replication/translation of HCV RNA. A proposed model of HCV replication-translation complex "replicasome". As reported, HCV replication complexes are assembled at ER, and then bud into the ER lumen. Consequently, (A) HCV RNA replication is first initiated in the multi-layered vesicle structure derived from the ER membrane. (B) The newly synthesized HCV RNA is translated around the ER-derived vesicle [39], where there are membrane-associated ribosomes. Benzothiadiazine blocks HCV RNA transcription and therefore decreases translation. (C) The newly-synthesized HCV RNA is later transported away from ER; nocodazole inhibits this transportation. HCV RNA is then transported to Golgi-derived membrane and/or then the lipid droplet (LD) for packaging and assembly of virus particles.
doi:10.1371/journal.pone.0043600.g005

mutant, yielded detectable amounts of NS3 and NS5A at 16–24 hours posttransfection (Fig. S2A). The kinetics of HCV protein synthesis corresponded well with the previously reported kinetics of HCV RNA synthesis following HCV (JFH1) RNA transfection [36]. The result further supported the conclusion that the replication of HCV RNA is required for competent HCV RNA translation.

We next assessed if the replication and translation of HCV RNA occurred in the same subcellular localizations. Huh7.5 cells were infected with HCV (JFH1); at 2 days after infection, the cells were labeled with Cy5-UTP and BODIPY-FL-lys-tRNA in the presence of hippuristanol and actinomycin D, which blocked eIF4A-dependent translation and host RNA transcription, respectively [27,37]. Under such a condition, Cy5-UTP will label newly synthesized HCV RNA, while BODIPY-FL-lys-tRNA will label only the newly synthesized HCV proteins since HCV translational initiation does not require eIF4A, which is blocked by hippuristanol [38]. A control experiment showed that neither dyes labeled the uninfected Huh7.5 cells (Fig. 4, lower panel). JHF-1-infected Huh7.5 cells were labeled with Cy5-UTP and BODIPY-FL-lys-tRNA for 15 minutes and then chased in unlabeled media from 10 to 40 minutes. The Cy5-U label could be detected at the early time point (10 min.) in the perinuclear region; BODIPY-FL-Lys-tRNA-labeled peptides were detected sparingly at this time, but gradually increased in intensity at later time points (Fig. 4). The earlier detection of Cy5-U label than BODIPY-FL-lys-tRNA label may have been due to the more efficient incorporation and more sensitive detection of the former label. Strong BODIPY-FL-lys-tRNA labeling signals were detected after 30 minutes of chase. Significantly, all of the BODIPY-FL-lys-tRNA labeled peptide colocalized with Cy5-U-labeled RNA at all the time points studied (Fig. 4). These findings suggest that HCV protein translation occurs on the newly synthesized RNA, and thus is likely coupled with RNA synthesis. From all the results above, we conclude that HCV RNA replication activity is a prerequisite to the efficient translation of HCV RNA.

Discussion

The mechanisms of replication and translation of HCV RNA have been extensively studied in the past few years. However, the exact subcellular localization of HCV RNA replication and translation is still unclear. Evidence has previously been presented that HCV RNA replication occurs on the detergent-resistant membrane (DRM) possibly derived from the ER [23,39]. In this report, the newly synthesized RNA was shown to be transported by the anterograde vesicle transport pathway. The microtubule-dependent mobility of newly-synthesized HCV RNA or the replication complex has also been described elsewhere [40]. Our data in this study further showed that the nocodazole treatment inhibited the transportation of the newly-synthesized RNA from the ER-derived replication complex to Golgi but did not inhibit the initiation of HCV RNA replication, since BrUTP labeling of HCV RNA occurred normally in the presence of nocodazole (Fig. 2). Intuitively, the newly-synthesized HCV RNA is expected to be transported to the site of the cellular translation machinery, similar to the case for cellular mRNAs, which are synthesized in the nucleus and transported to the cytoplasmic translation machinery for protein synthesis. However, we instead found that the movement of the HCV RNA from the ER to Golgi was not required for HCV translation, suggesting that the newly synthesized HCV RNA is used for translation near the site of HCV RNA synthesis before being transported away. Furthermore, we showed that active RNA replication was a prerequisite for efficient HCV translation. This conclusion was demonstrated using four different approaches, including studying the effects of an HCV RNA polymerase inhibitor on HCV protein translation (Fig. 3A), comparing the translation efficiencies of wildtype and replication-defective replicons (Fig. 3C) and those of infectious and non replicating JFH strain of HCV (Fig. S2A), and also by determining the relative translation efficiencies of the replicating and non-replicating dual luciferase reporter plasmids (Figs. 3F–G). Finally, we showed that the newly synthesized viral proteins almost completely colocalized with the newly synthesized viral RNA, suggesting that the sites of HCV RNA replication and protein translation nearly overlap. This mechanism of coupled RNA replication and translation may explain the previous findings that many cellular proteins, such as PTB [9,26], La antigen [10,13] and SYNCRIP [11,14], are involved in both the replication and translation in the HCV life cycle. The close proximity of these two machineries will allow for ready switches between translation and replication.

Although coupling of translation and RNA replication has been reported for many RNA viruses [16,17,41,42]_ENREF_37, the HCV case appears to be unique. For example, translation and replication of poliovirus RNA are coupled, but in the sense that RNA transcription is dependent on viral translation in *cis* [16]. Insertion of an early termination codon resulted in lower efficiency of poliovirus RNA replication. The translation and replication are regulated by the binding of different cellular or viral proteins to the 5? UTR of poliovirus RNA [43–45]. Also, the microtubule-dependent movement of poliovirus viral RNA is associated with the replication activity of viral RNA [46]. While the inactive replication complexes reside at microtubule-organizing center (MTOC), the replicating viral RNA is localized at the perinuclear sites [46]. Thus, the RNA movement is required for poliovirus replication, in contrast to the situation with HCV. In HCV, nocodazole did not inhibit viral RNA replication; also, the newly-synthesized HCV RNA failed to exit from ER after the nocodazole treatment and yet, protein translation increased; thus, the cytoskeleton-assisted movement of the newly-synthesized HCV RNA is not required for RNA translation. Thus, in HCV, the observed movement of the viral RNA from the ER-derived to the Golgi-derived membrane appears to be required for other steps of HCV replication, rather than protein translation. Due to the fact that the viral structural proteins are absent in the HCV replicon cells, this RNA movement is likely mediated by viral NS proteins, such as NS5A, which has been reported to target Golgi apparatus [47]. In a kinetics study examining the appearance of the newly synthesized HCV RNA and HCV proteins in the HCV (JFH-1)-infected cell, we also found that all of the newly synthesized proteins were at the site of newly synthesized RNA (Fig. 4). Thus, there appears to be a replication complex that carries out both replication and translation. This concept is novel to the known mechanisms of RNA virus translation and transcription.

These findings raised an important issue, namely, how the initial viral translation is carried out, since, as a positive-strand RNA virus, the very initial round of translation from the incoming HCV viral genome has to take place before viral RNA replication can occur. Conceivably, the free viral RNA genome generated by uncoating of the incoming virion in the endosome (or from the transfected viral RNA or replicons) can associate with ribosomes on the rough ER and be translated in an RNA replication-independent manner. Such translation is likely of low efficiency, but is sufficient to support first round of HCV RNA translation. These initial viral protein products and RNAs will then be encased into the membranous replication complex and become part of the