

## HEPATITIS C VIRUS TRANSLATION AND CELL CYCLE

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*The 5' nontranslated region (NTR) of hepatitis C virus (HCV) contains highly structured segments which form internal ribosome entry site (IRES). This cis-active RNA element directs the cap-independent initiation of translation of the viral polyprotein in association with trans-acting cellular proteins. Thus HCV IRES plays a key role in the replication of virus, especially in liver in which various cellular proteins changes in their expression levels under the condition of chronic hepatitis. To construct a system in which HCV IRES activity could be continuously monitored in vivo, 2 stably transformed cell lines from Huh-7 cells (human hepatocellular carcinoma cells) that express a reporter protein (firefly luciferase) under the translational control of the IRES were established. The activity of the HCV IRES varies in different phases of the cell cycle and HCV IRES activity is highest during the mitotic (G2/M) phases of the cell cycle and lower in the quiescent (G0) phases. The gene expression dynamics of host factors and kinetics of HCV IRES activity in cells at various points during the cell cycle were evaluated using a cDNA microarray. HCV IRES activity correlated with a gene cluster induced in S and G2/M phases. Interestingly, most initiation factors known to bind or interact with HCV IRES (PCBP2, PTB, eIF3, eIF2 gamma, eIF2 beta, La protein and RNLPL) were induced during S and G2/M phases. Of these factors, the expression of La protein, PTB and eIF3 (p116, 170) were predominantly repressed in quiescent (G0) and induced in S and G2/M phases. Suppression or overexpression of La protein and PTB in cells significantly changed HCV IRES activity. In the livers of patients with chronic hepatitis C, the expression of La protein was significantly increased and correlated with the amount of HCV-RNA. Collectively, these data indicated that HCV utilizes host factors induced during cell division but not during quiescence for replication. Of these, La protein is a potent regulator and enhances HCV replication in regenerating hepatocytes in patients with chronic hepatitis C. **Biomed Rev 2004; 15: 37-46.***

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### INTRODUCTION

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Hepatitis C virus (HCV), a positive-strand, enveloped RNA virus is classified within the genus *Hepacivirus* of the family *Flaviviridae* (1). The capacity of this hepatotropic virus to establish a persistent infection in most infected persons underlies its public health importance, as persistent infection with HCV may be associated with the development of chronic hepatitis, cirrhosis and/or hepatocellular carcinoma (1, 2).

The translation of HCV genome is a critical step in viral replication and is initiated by an unusual process that

is very distinct from normal cellular mechanisms (3,4). The translation of the viral RNA is controlled by a highly structured RNA segment, the internal ribosome entry site (IRES), that occupies most of the 5' nontranslated (5'NTR) RNA, extending from nucleotide (nt) ~42-345 of the HCV genome (5-8). IRES activity is highly dependent upon both the primary sequence of this segment, as well as its ability to form complex secondary and tertiary RNA structure (9-13). Recent investigations suggest that HCV translation is regulated by cellular proteins and that viral replication is enhanced by factors that stimulate the regeneration of hepatocytes in

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patients with chronic hepatitis C. In this review, the unique feature of HCV IRES activity, the interaction with cellular proteins and relationship to the pathogenesis of chronic hepatitis C are summarized.

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### SECONDARY AND TERTIARY STRUCTURE OF 5' NTR OF HCV

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Functional and structural studies of the HCV IRES have been carried out in a number of laboratories (3,11,12,14-17). Most of these studies have drawn on a model of the secondary structure of the 5'NTR of HCV that was proposed by Brown *et al* in 1992 (18). This model was modified by Wang *et al* in 1995 (10) following the demonstration of a pseudoknot within the 5'NTR that is required for translation, and it was further refined by Smith *et al* (19) in 1995 and Honda *et al* (12) in 1996.

A significantly different prediction of the secondary structure of this segment of the HCV 5'NTR was reported based on a comparison of the HCV sequence with that of a newly discovered, hepatotropic mammalian virus, GB virus B (GBV-B) (12,20). The computer-based, thermodynamic predictions suggest that a more stable structure may be formed by alternative interactions between bases predicted previously to form hairpin IIa and those in the opposing bulge loop (Fig. 1). By a mutational analysis and nuclease mapping studies, finally secondary and tertiary RNA structure within the complete 5'NTR of HCV and immediately downstream open reading frame were proposed and used everywhere (Fig. 1).

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### CANONICAL AND NON-CANONICAL INITIATION FACTORS THAT REGULATE HCV IRES ACTIVITY

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The machineries of cap-dependent and HCV IRES-directed translation can be differentiated in terms of requirements for canonical and non-canonical initiation factors. The translation machinery of HCV is simple and, being a prokaryote, requires only the ribosomal 40s subunit, eIF2/GTP/Met-tRNA complex, and eukaryotic initiation factor 3 (eIF3) to initiate translation (16). In contrast, cap dependent translation is more complex and requires additional canonical initiation factors such as eIF4E, eIF4G, eIF4A and eIF4B (16). Many other non-canonical translation initiation factors such as La protein (14,21), polypyrimidine tract binding protein (PTB) (22), heterogeneous nuclear ribonucleoprotein L (RNPL) (23), poly(rC)-binding protein 2 (PCBP2) (24), and ribosomal protein S9 (16), interact with HCV IRES and might regulate HCV translation. Despite these observations, there is little understanding of how the process of HCV translation may be regulated *in vivo*. The translation of polyprotein is a critical step in viral replication, and the unique mechanism by

which it is initiated suggests that it might be a fruitful target for antiviral drug development.

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### STABLY TRANSFORMED IRES REPORTER CELL LINES

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Cellular proteins are usually translated by a process that typically involves recognition of the 5' cap structure of a messenger RNA (mRNA) molecule by the 40S ribosome subunit. This is followed by 3' scanning of the 40S subunit in search of an AUG codon that is in the proper context for translation initiation. The 40S subunit pauses at such an AUG, is joined by a 60S subunit to form a translationally competent ribosome, and then begins the process of polypeptide synthesis (25,26). A considerable number of cellular initiation and elongation factors contribute to this process. However, ribosomes do not continue to efficiently scan the RNA once they have completed the synthesis of the protein encoded by a reading frame, and reinitiation of translation on a second reading frame is a very unusual event. Thus, eukaryotic mRNAs are typically monocistronic. In contrast, the plasmid pRL-HL contains a dicistronic transcriptional unit under control of a composite CMV/T7 promoter (Fig. 2A). Transcripts produced from this plasmid contain 2 open reading frames, the most 5' of which encodes *Renilla* luciferase, whereas the 3' reading frame encodes a fusion of the N-terminal HCV core protein (22 amino acid residues) with firefly luciferase. These coding sequences are separated by the 5'NTR sequence of HCV, which contains an IRES that is capable of directly binding the 40S subunit and initiating translation internally on this RNA in a non-canonical fashion that is independent of the 5' end of the RNA (Fig. 2B).

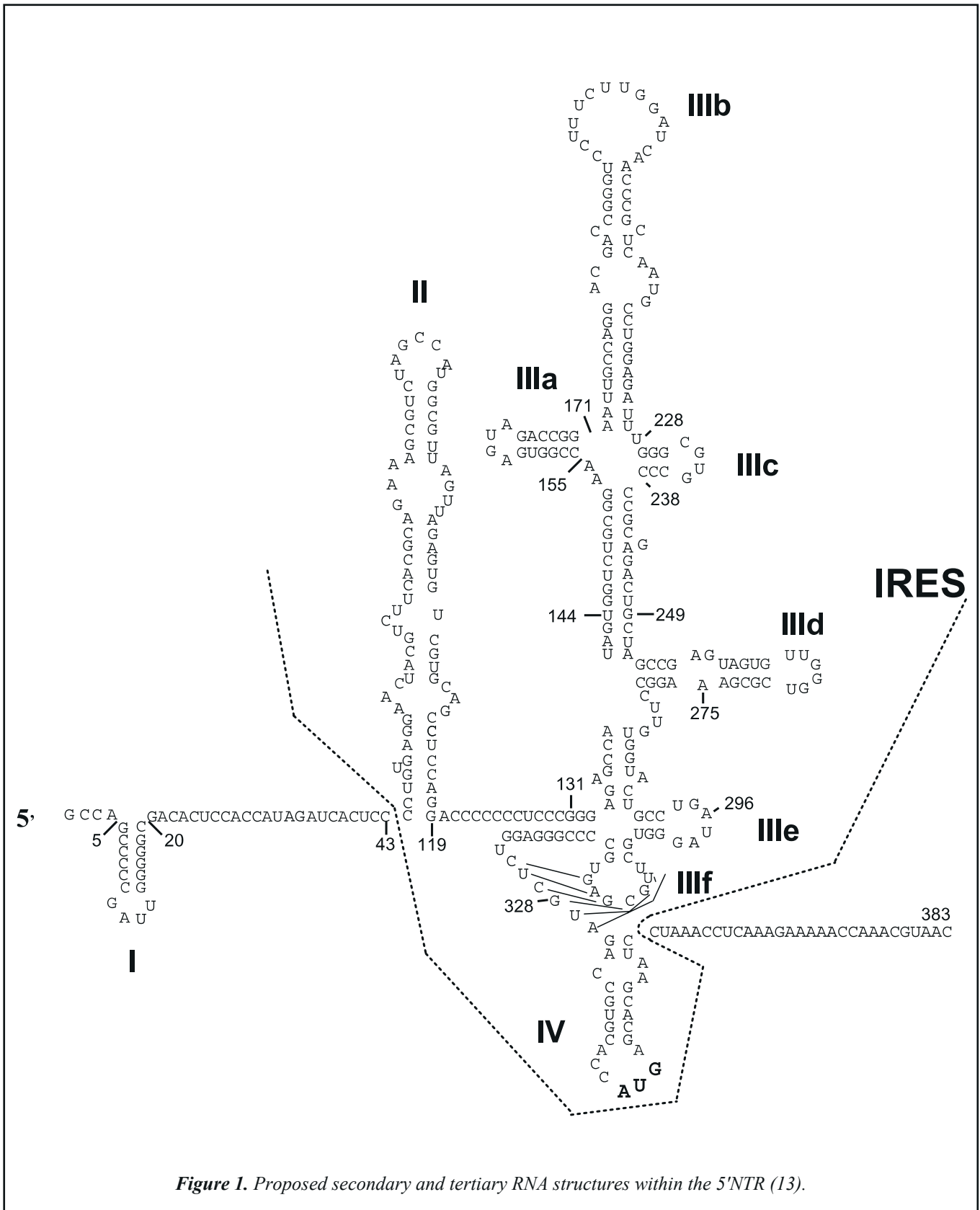
The pRL-HL plasmid was transfected into Huh-7 cells, and several stably transformed cell lines were clonally isolated under *neo* selection. Two independently isolated clonal cell lines, RCF-1 and RCF-26, were shown to constitutively express both the *Renilla* luciferase and firefly luciferase proteins.

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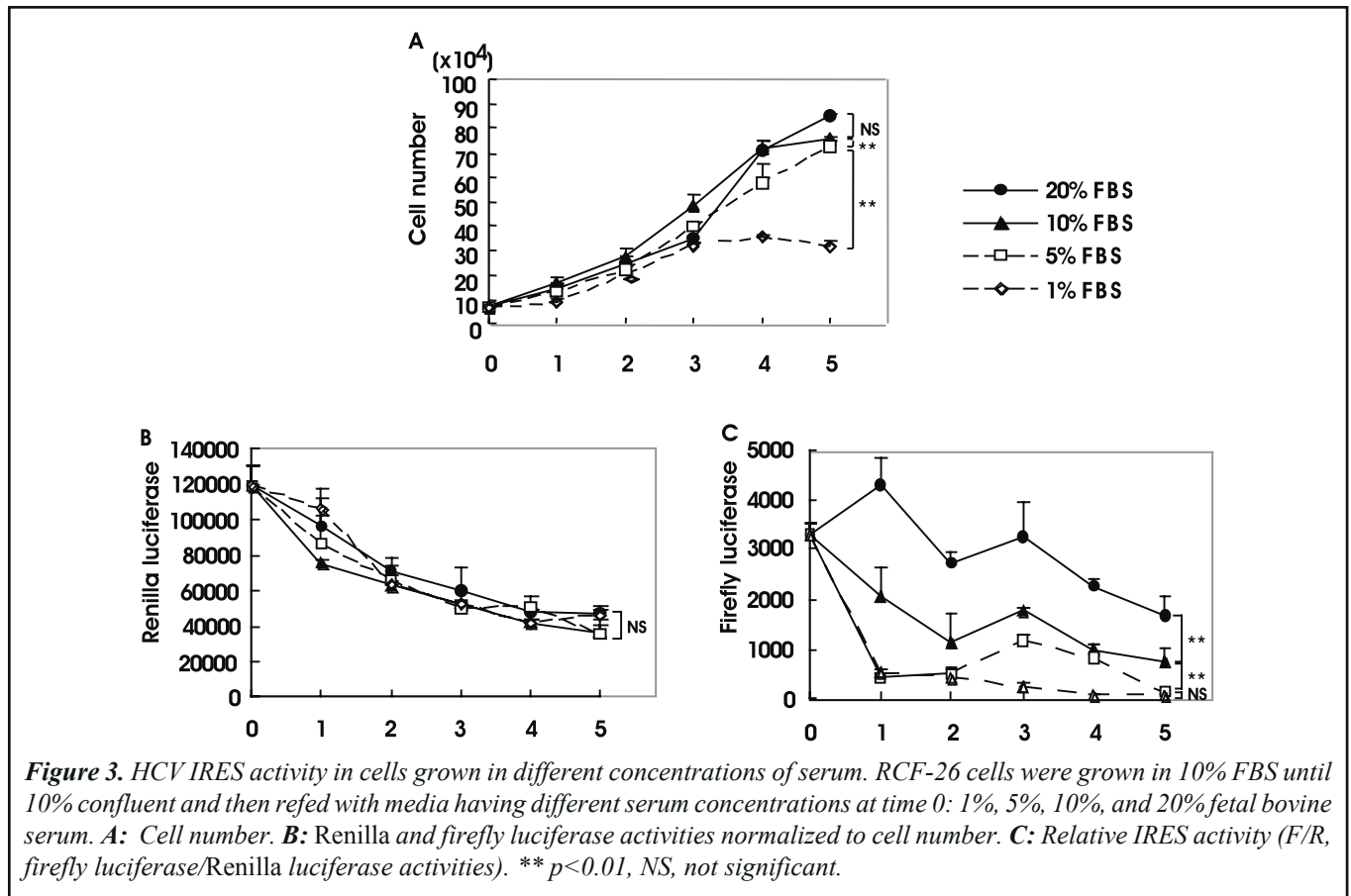
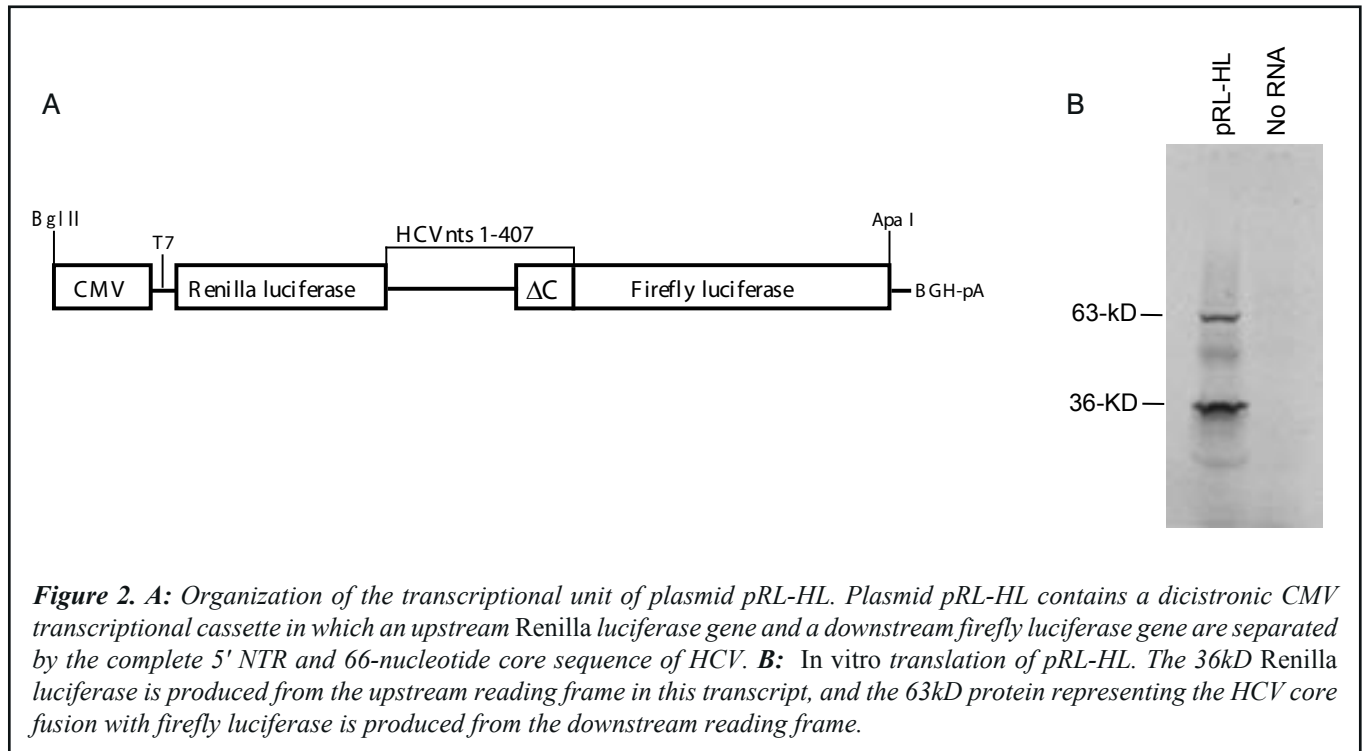
### CELL GROWTH AND HCV IRES-DIRECTED TRANSLATION

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RCF-26 cells were seeded at 10% confluency and fed with media containing different concentrations of serum (1%, 5%, 10% and 20% FBS)(27). The number of cells and the luciferase content of the cultures were monitored over a period of 5 days (Fig. 3). The cell confluency increased from about 10% at day 0 to 90% at day 5 in 10% and 20% FBS conditions. There was a modest decline in *Renilla* luciferase units/cell over the 5 day period but, importantly, the cellular *Renilla* luciferase content was not influenced by the concentration of serum (Fig. 3B). On the other hand, firefly luciferase activities varied markedly with the serum concentration (Fig. 3C), being highest in 20% FBS and lowest at 1-5% FBS. The ratio of firefly to *Renilla* luciferase activity was 4- to 5-fold greater in cells cultured in



**Figure 1.** Proposed secondary and tertiary RNA structures within the 5'NTR (13).



media containing 20% FBS than in cells cultured in media containing only 1% FBS (Fig. 3C), conditions which slow the rate of cell division and/or decrease cell survival (Fig. 3A). Thus, these data show that the activity of the HCV IRES is closely tied to cellular metabolism, and that it is significantly greater in healthy, rapidly dividing cells than in stationary or starved cells.

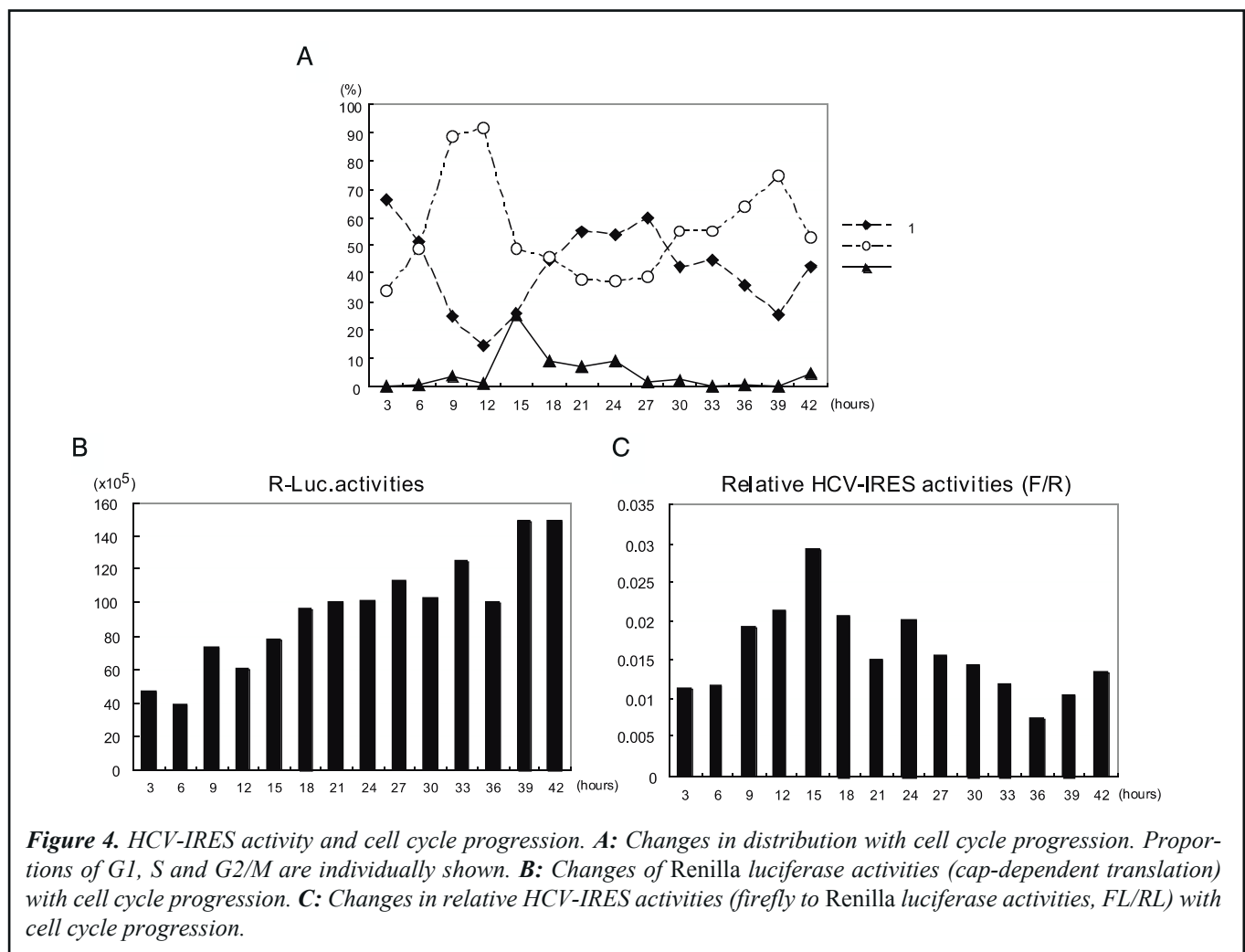
### HCV IRES ACTIVITY IN DIFFERENT PHASES OF THE CELL CYCLE

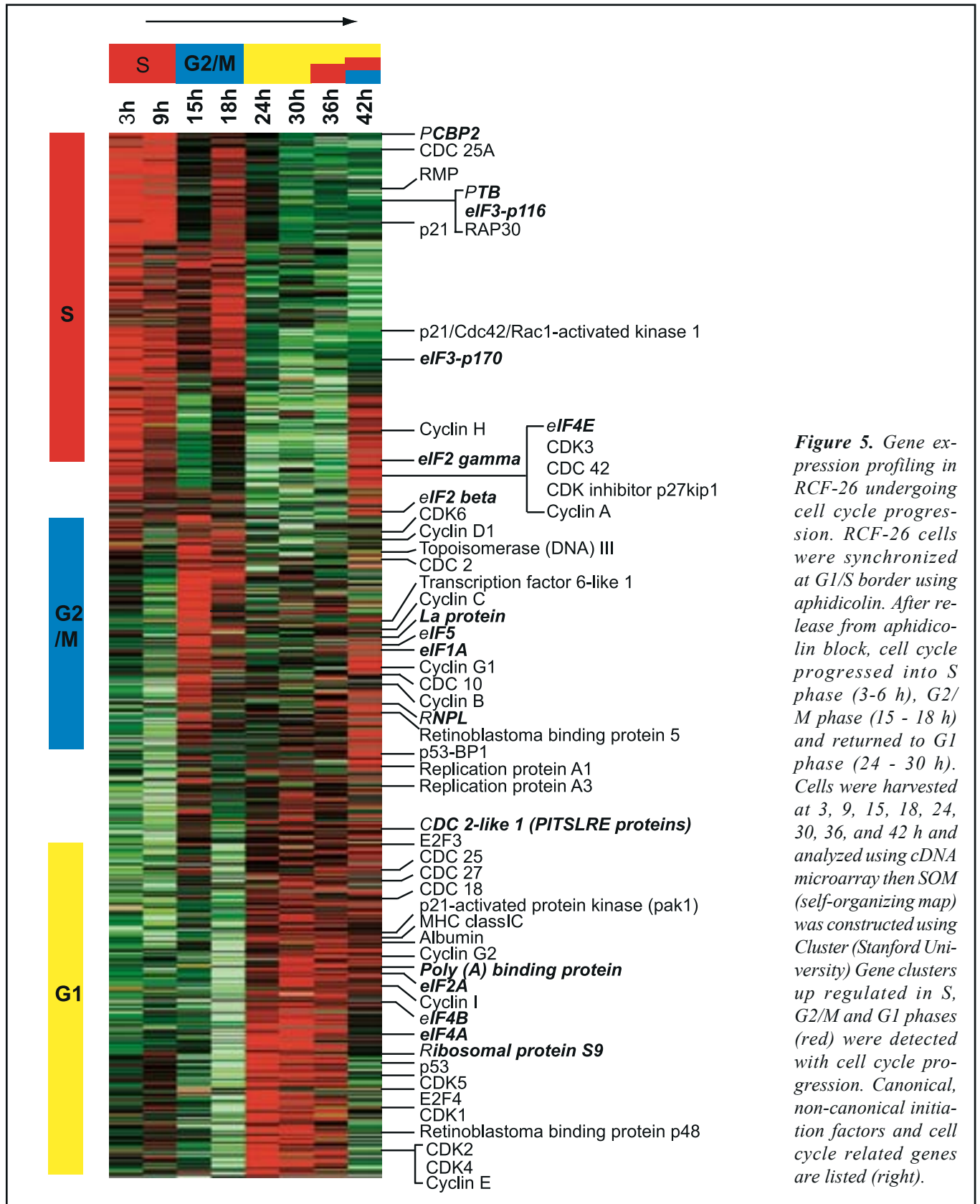
The cell cycle progression was synchronized and compared the production of *Renilla* and firefly luciferase reporter proteins during different phases of the cell cycle. Cells were blocked at the G1/S interface by adding aphidicolin to the culture medium, and then released from the aphidicolin block. Synchronized cells subsequently moved into S and G2/M phases of the cell cycle and then returned to G1/S phase at around 27 h as determined by the cellular DNA

content measured by flow cytometry (Fig. 4A). *Renilla* luciferase activities increased proportionally, reflecting the increased numbers of cells after division (Fig. 4B). The cell number doubled at around 27 h after completing one round of the cycle. On the other hand, HCV-IRES activity varied with cell cycle, and the ratio of firefly luciferase to *Renilla* luciferase (relative HCV-IRES activity = F/R) increased during and immediately following G2/M phase (12-18 h post release from aphidicolin). The relative HCV-IRES activity decreased by 36 h post release (Fig. 4C), corresponding to reentry into G0 and G1 phases. However, the HCV-IRES activity increased again, starting at about 39 h, probably due to many cells continuing into a second cycle (Fig. 4A,C).

### GENE EXPRESSION PROFILES IN CELLS UNDERGOING CELL CYCLE PROGRESSION

To determine which host factors are involved in this cell cycle dependent regulation of HCV-IRES activity, gene





**Figure 5.** Gene expression profiling in RCF-26 undergoing cell cycle progression. RCF-26 cells were synchronized at G1/S border using aphidicolin. After release from aphidicolin block, cell cycle progressed into S phase (3-6 h), G2/M phase (15 - 18 h) and returned to G1 phase (24 - 30 h). Cells were harvested at 3, 9, 15, 18, 24, 30, 36, and 42 h and analyzed using cDNA microarray then SOM (self-organizing map) was constructed using Cluster (Stanford University) Gene clusters up regulated in S, G2/M and G1 phases (red) were detected with cell cycle progression. Canonical, non-canonical initiation factors and cell cycle related genes are listed (right).

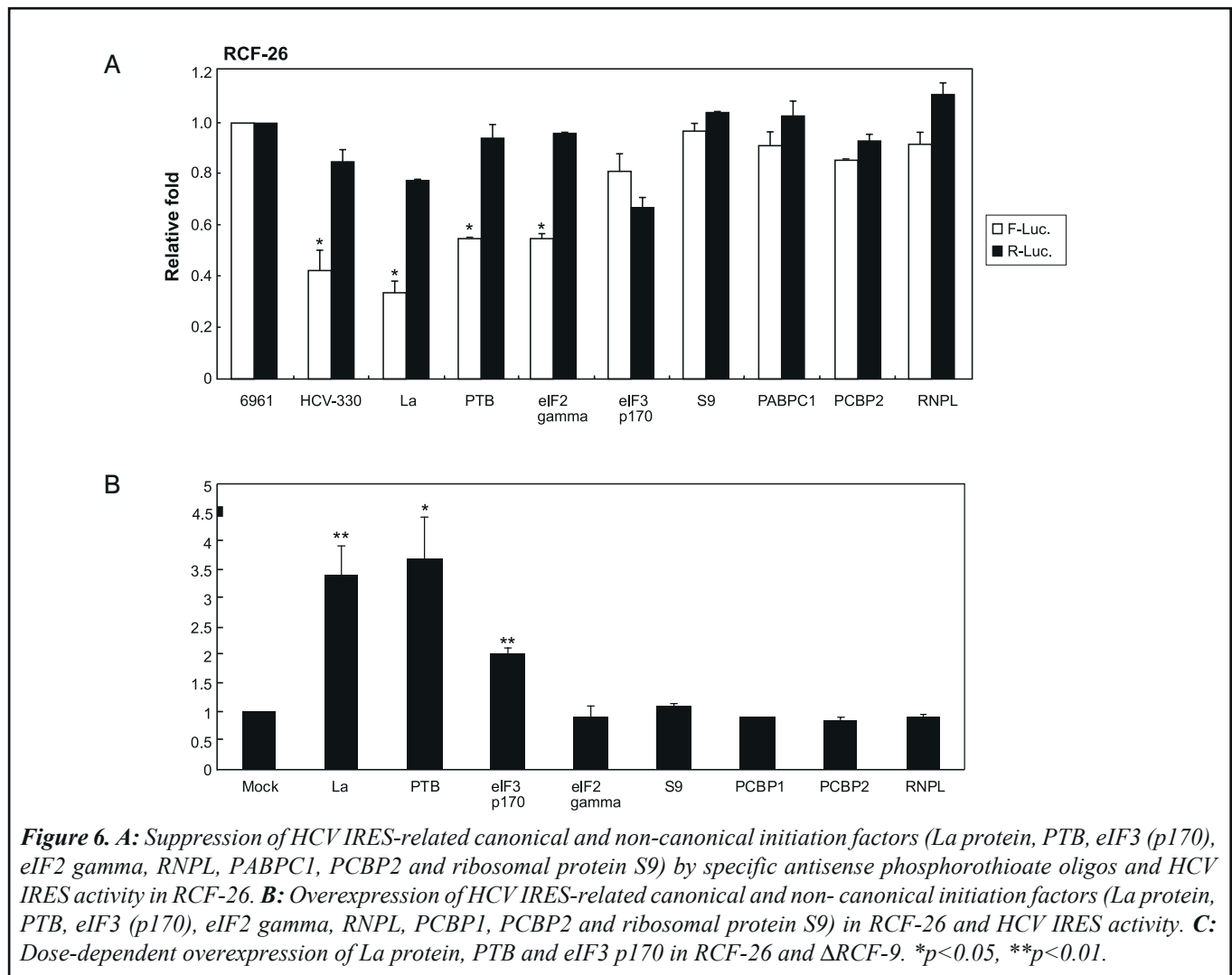


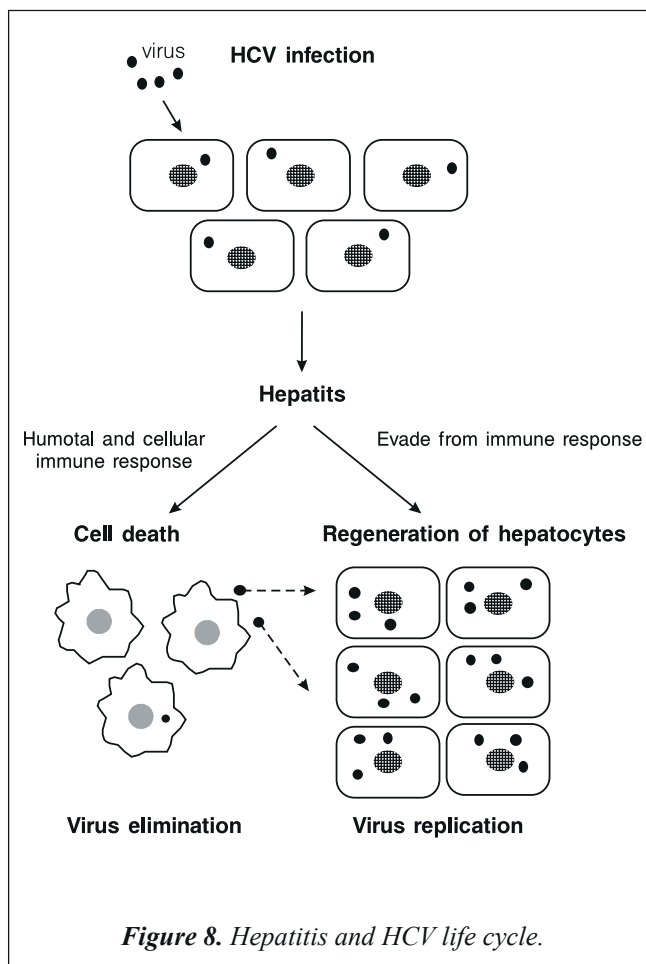
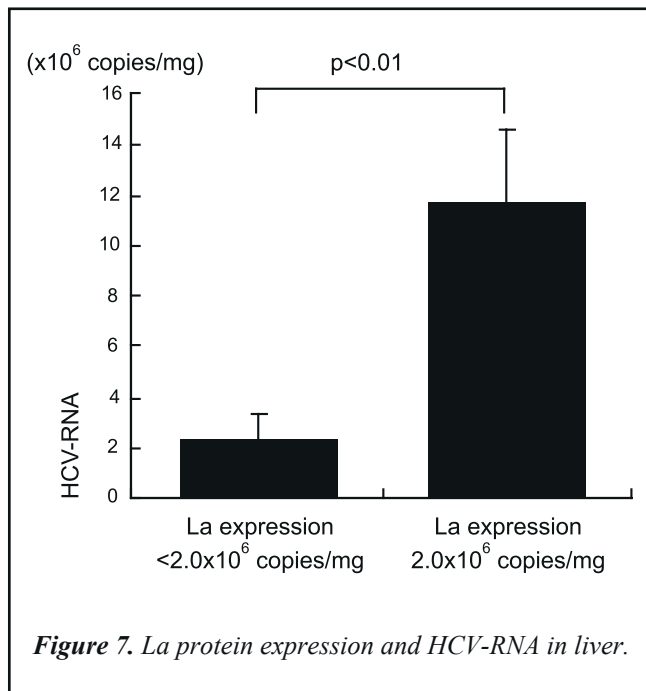
expression profiles in cells undergoing cell cycle progression were evaluated. Total RNA was extracted from synchronized cells at 3, 9, 15, 18, 24, 30, 36, 42 h, after release from the aphidicolin block (G1/S border), and analyzed using the cDNA microarray. Three large gene clusters were identified as the cell cycle progressed (Fig. 5). The first cluster of genes was induced at S phase (at 3 to 9 h). The second and third clusters were induced at G2/M (at 15 to 18 h), and at G1 (at 24 to 36 h), respectively. Most of the HCV-IRES related canonical and non-canonical initiation factors were induced during S and G2/M phases. PCBP2, PTB, eIF3 (p110 and p170), eIF2 gamma and eIF2 beta were induced during S phase, whereas La protein and RNPL were induced during G2/M. These factors bind HCV-IRES structure or have functional relevance to HCV-IRES activity. On the other hand, PABPC1, eIF4A and eIF4B were induced during G1 phase. These factors were not required for HCV-IRES

directed translation, but are necessary for cap-dependent translation.

#### FUNCTIONAL ANALYSIS OF THE EFFECT OF HCV IRES RELATED CANONICAL AND NON-CANONICAL INITIATION FACTORS ON TRANSLATION DIRECTED BY HCV IRES

To prove that the induction of the canonical and non-canonical initiation factors during S and G2/M phases contributes to cell cycle dependent translation of HCV, antisense phosphorothioate oligos were designed for La protein, PTB, eIF3 p170, eIF2 gamma, RNPL, PABPC1, PCBP2 and ribosomal protein S9 respectively, and HCV IRES activity was evaluated under the suppression of these factors. The suppression of La protein, PTB and eIF2 gamma specifically reduced HCV IRES activity to 40%, 50% and 53% of the control level, respectively. The effect





of inhibiting HCV IRES activity was equal to or greater than that exerted by an antisense oligo against 5' NTR of HCV (nt 330-350). However, the suppression of eIF3 p170, RNPL, PABPC1, PCBP2 and ribosomal protein S9 did not reduce HCV IRES activity (Fig. 6A). Conversely, overexpression of La protein, PTB and eIF3 p170 significantly enhanced HCV IRES activity in a dose-dependent manner, while the overexpression of eIF2-gamma, RNPL, PCBP1 and PCBP2 and ribosomal protein S9 had no effect (Fig. 6B). Thus, out of these HCV IRES-related canonical and non-canonical initiation factors, La protein and PTB significantly changed HCV IRES activity in both suppressed and overexpressed states. Thus, changes in the expression of these factors alter HCV IRES activity in a cell cycle dependent manner.

### EXPRESSION OF LA PROTEIN, PTB AND EIF3 IN LESIONS OF CHRONIC HEPATITIS C

To examine the functional role of these factors on HCV replication in the lesions of chronic hepatitis C, the expression of La protein, PTB and eIF3 in 26 liver samples from patients with chronic hepatitis C and in 8 normal liver samples were evaluated by RTD-PCR. The expression level of La protein in the specimens of the patients with chronic hepatitis C was significantly higher than that of the normal liver, whereas the expression PTB and eIF3 p170 was not statistically different. The expression of La protein was significantly correlated with the amount of HCV-RNA in the liver. Moreover, HCV-RNA replication was significantly higher in the liver with high La protein expression (Fig. 7). These findings indicate that La protein plays an important role in the replication of HCV in the liver of patients infected with chronic hepatitis C.

### CONCLUSION

The implication of these findings with regards to the HCV life cycle is shown in Figure 8 (28). Hepatitis and the resulting increased regeneration of hepatocytes increase IRES activity and enhance HCV replication. This may be an important mechanism by which HCV maintains its viral load under host defense immune pressure. These findings shed new light on the mechanism of HCV replication and could be the basis for developing a novel anti viral therapy. Although La protein and PTB have been revealed to be involved in the cell cycle regulation of HCV IRES activity, many other host factors might also be involved. Extended analyses to other initiation factors and the functional role on HCV IRES activity and replication of HCV need to be investigated furthermore.



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