Table 1. Up- and Down-regulated Genes in Confluent and Serum-Starved Cells

UniGene	Gene name	Category	Confluent cells	Serum-starved cells	Mean (fold)
Up-regulated					>1.8
Hs.74561	Alpha-2-macroglobulin	Serum protein	12.87 ± 2.13	5.13 ± 0.10	9.00 ± 2.40
Hs.75442	Albumin	Serum protein	6.07 ± 1.07	3.26 ± 0.37	4.67 ± 0.93
Hs.77054	B-cell translocation gene 1	Antiproliferative gene	4.73 ± 1.76	2.97 ± 0.83	3.85 ± 0.94
Hs.82004	Cadherin 1, E-cadherin (epithelial)	Cell adhesion	5.14 ± 2.46	2.36 ± 0.30	3.75 ± 1.29
Hs.2257	Vitronectin	Cell adhesion	3.97 ± 0.68	2.11 ± 0.22	3.04 ± 0.61
Hs.277477	MHC class IC	Cell adhesion	3.26 ± 0.82	2.17 ± 0.09	2.71 ± 0.46
Hs.77961	MHC class IB	Cell adhesion	3.33 ± 0.23	2.08 ± 0.24	2.71 ± 0.39
Hs.1119	TR3 orphan receptor	Receptor	3.38 ± 1.23	1.95 ± 0.23	2.66 ± 0.66
Hs.1665	Zinc finger transcriptional regulator	Transcription factor	2.66 ± 0.40	2.53 ± 0.40	2.60 ± 0.23
Hs.287820	Fibronectin gene	Cell adhesion	2.40 ± 0.77	2.11 ± 0.34	2.26 ± 0.36
Hs.2780	JunD	Oncogene	2.23 ± 0.39	1.99 ± 0.54	2.11 ± 0.28
Down-regulated					<0.55
Hs.89525	Hepatoma-derived growth factor	Cell growth	0.67 ± 0.27	0.42 ± 0.08	0.55 ± 0.13
Hs.7943	RMP	RNA polymerase II binding	0.44 ± 0.09	0.64 ± 0.07	0.54 ± 0.07
Hs.23960	Cyclin B	Cell cycle	0.44 ± 0.25	0.62 ± 0.17	0.53 ± 0.13
Hs.118638	Nm23A	Cell growth	0.52 ± 0.01	0.53 ± 0.11	0.52 ± 0.05
Hs.83715	Autoantigen La	RNA polymerase III synthesis	0.43 ± 0.06	0.61 ± 0.01	0.52 ± 0.06
Hs.16297	COX17	Cytochrome-*c*-oxidase	0.47 ± 0.14	0.55 ± 0.06	0.51 ± 0.07
Hs.95577	CDK1	Cell cycle	0.36 ± 0.02	0.65 ± 0.07	0.50 ± 0.09
Hs.174017	Topoisomerase (DNA) II alpha	RNA polymerase II holoenzyme	0.44 ± 0.11	0.55 ± 0.08	0.50 ± 0.05
Hs.85137	Cyclin A	Cell cycle	0.42 ± 0.03	0.56 ± 0.17	0.49 ± 0.08
Hs.9235	Nucleoside-diphosphate kinase	Cell growth	0.51 ± 0.08	0.46 ± 0.05	0.49 ± 0.04
Hs.75133	Transcription factor 6–like 1	Transcription factor	0.40 ± 0.07	0.57 ± 0.18	0.48 ± 0.09
Hs.69563	Cell division cycle 18	Cell cycle	0.45 ± 0.04	0.51 ± 0.09	0.48 ± 0.04
Hs.58593	RAP30	Transcription factor	0.37 ± 0.02	0.58 ± 0.00	0.48 ± 0.06
Hs.75323	Prohibitin	Antiproliferative gene	0.50 ± 0.01	0.39 ± 0.01	0.44 ± 0.03
Hs.1846	p53	Cell cycle	0.66 ± 0.18	0.22 ± 0.04	0.44 ± 0.15
Hs.111758	Keratin 6	Housekeeping	0.24 ± 0.02	0.63 ± 0.07	0.43 ± 0.12
Hs.78271	Keratin 8	Housekeeping	0.33 ± 0.18	0.53 ± 0.08	0.43 ± 0.10
Hs.748	Fibroblast growth factor receptor 1	Cell growth	0.39 ± 0.12	0.30 ± 0.05	0.34 ± 0.06

Hepatitis C Virus Internal Ribosome Entry Site Activity at Different Phases of the Cell Cycle

We examined the relationship between HCV IRES activity and cell division in more detail. We synchronized cell-cycle progression and compared the production of RL and FL reporter proteins during different phases of the cell cycle. Cells were blocked at the G_1/S interface by adding aphidicolin to the culture medium and were then released from the aphidicolin

Table 2. Up- and Down-regulated Initiation Factors in Confluent and Serum-Starved Cells

UniGene	Gene name	Confluent cells	Serum-starved cells	Mean (fold)
Hs.180920	Ribosomal protein S9	1.92 ± 0.01	1.11 ± 0.18	1.51 ± 0.24
Hs.172550	Polypyrimidine tract binding protein (PTB)	0.62 ± 0.17	0.62 ± 0.03	0.62 ± 0.07
Hs.83715	La protein	0.43 ± 0.06	0.61 ± 0.01	0.52 ± 0.06
Hs.2730	Heterogeneous nuclear ribonucleoprotein L (RNPL)	1.23 ± 0.33	1.02 ± 0.13	1.13 ± 0.16
Hs.63525	Poly(rC)-binding protein 2 (PCBP2)	1.20 ± 0.11	0.95 ± 0.10	1.07 ± 0.09
Hs.172182	Poly(A)-binding protein, cytoplasmic 1	1.87 ± 0.62	1.19 ± 0.05	1.53 ± 0.32
Hs.183418	Cell division cycle 2–like 1 (PITSLRE proteins)	0.85 ± 0.10	0.70 ± 0.07	0.78 ± 0.07
Hs.198899	eIF3-p170	0.47 ± 0.28	1.10 ± 0.36	0.79 ± 0.26
Hs.57783	eIF3-p116	0.58 ± 0.12	0.70 ± 0.02	0.64 ± 0.06
Hs.4310	eIF1A	0.84 ± 0.06	0.89 ± 0.17	0.86 ± 0.07
Hs.151777	eIF2A	1.37 ± 0.20	1.59 ± 0.20	1.48 ± 0.13
Hs.12163	eIF2β	0.88 ± 0.10	0.94 ± 0.02	0.91 ± 0.04
Hs.211539	eIF2γ	1.31 ± 0.03	0.61 ± 0.17	0.96 ± 0.22
Hs.129673	eIF4A	1.05 ± 0.09	0.81 ± 0.07	0.93 ± 0.08
Hs.93379	eIF4B	1.38 ± 0.48	0.94 ± 0.20	1.16 ± 0.25
Hs.79306	eIF4E	0.86 ± 0.14	0.83 ± 0.01	0.84 ± 0.06
Hs.286236	eIF5	0.91 ± 0.02	1.23 ± 0.35	1.07 ± 0.17

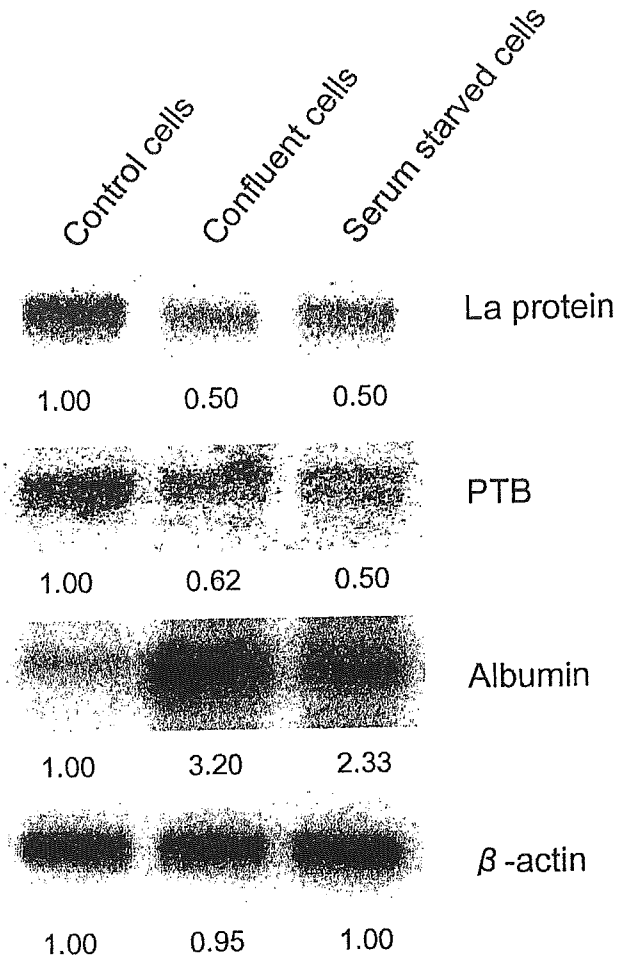

Figure 3. Northern blotting of La protein, PTB, and albumin in RCF-26. Expression of La protein and PTB is repressed in confluent and serum-starved cells, whereas albumin expression is significantly up-regulated.

block. Synchronized cells subsequently moved into the S and G_2/M phases of the cell cycle and then returned to G_1/S phase at approximately 27 hours, as determined by the cellular DNA content measured by flow cytometry (Figure 4*A*). RL activities increased proportionally, reflecting the increased number of cells after division. The cell number doubled at approximately 27 hours after 1 round of the cycle was completed. Conversely, HCV IRES activity varied with cell cycle, and the ratio of FL to RL (relative HCV IRES activity) increased during and immediately after G_2/M phase (12–18 hours after release from aphidicolin). The relative HCV IRES activity decreased by 36 hours after release (Figure 4*C*), corresponding to reentry into the G_0 and G_1 phases. However, the HCV IRES activity increased again, starting at approximately 39 hours, probably because many cells continued into a second cycle (Figure 4*A* and *C*). No significant differences in relative HCV IRES activity were found in ΔRCF-9 cells up to 30 hours after release (Figure 4*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, we evaluated gene-expression profiles in cells undergoing cell-cycle progression. Total RNA was extracted from synchronized cells at 3, 9, 15, 18, 24, 30,

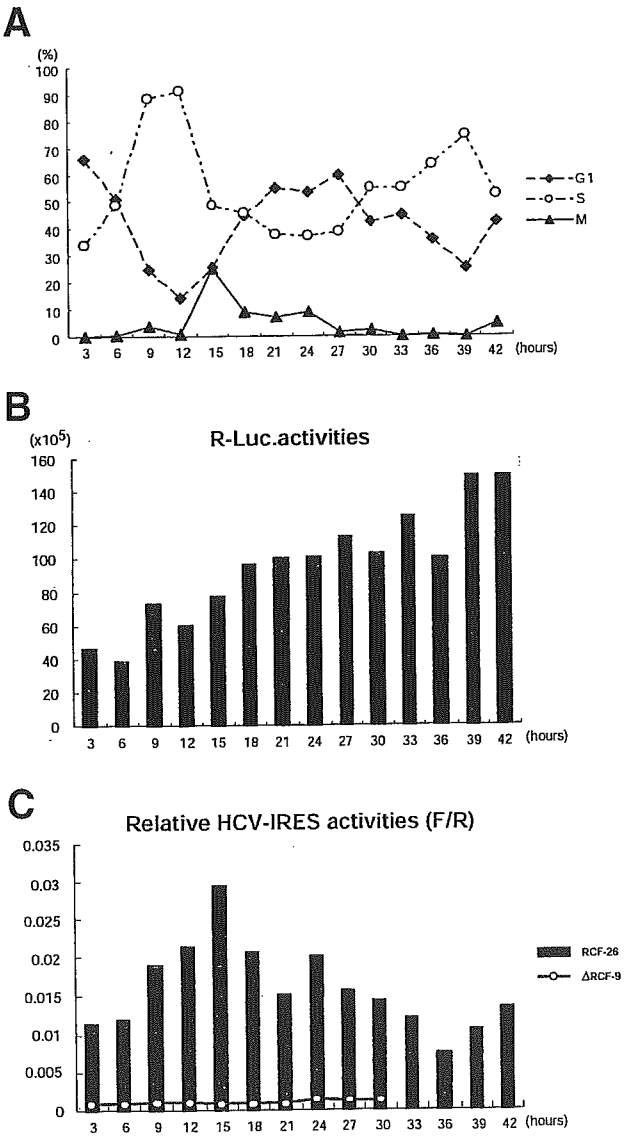

Figure 4. HCV IRES activity and cell-cycle progression. (*A*) Changes in distribution with cell-cycle progression. Proportions of G_1, S, and G_2/M are individually shown. (*B*) Changes of *Renilla* luciferase activities (cap-dependent translation) with cell-cycle progression. (*C*) Changes in relative HCV IRES activities (firefly to *Renilla* luciferase activities; FL/RL) with cell-cycle progression. HCV IRES activity varied with cell cycle in RCF-26 cells but did not change in ΔRCF-9 cells.

36, and 42 hours after release from the aphidicolin block (G_1/S border) and was analyzed with the cDNA microarray. We constructed a 1-dimensional SOM to evaluate changes in gene expression (Cluster and Tree view; http://www.microarrays.org/software/html) (Figure 5). We identified 3 large gene clusters as the cell cycle progressed. The first cluster of genes was induced at S phase (at 3 to 9 hours). The second and third clusters were induced at G_2/M (at 15–18 hours) and at G_1 (at 24 to 36 hours), respectively. Most of the HCV IRES–related canonical and noncanonical initiation factors were induced during S and G_2/M phases. PCBP-2, PTB, eIF3 (p110 and p170), eIF2γ, and eIF2β were induced during S phase, whereas La protein and RNPL were induced during G_2/M. These factors bind HCV IRES structure or have functional relevance to HCV IRES activity. Conversely, PABPC-1, eIF4A, and eIF4B were induced during G_1 phase. These factors are not required for HCV IRES–directed translation but are necessary for cap-dependent translation.[39] The induction of the ribosomal protein S9 in G_1 phase was a controversial finding because S9 was reported to bind stem loop IIId of HCV IRES. The functional role of the ribosomal protein S9 is discussed later. In cells, translation takes place immediately in the presence of mRNA, and luciferase activity could be detected within 30 seconds from the initiation of the translation. Thus, the induction of canonical and noncanonical initiation factors related to HCV IRES during S and G_2/M phases contributed to cell cycle–dependent regulation of translation directed by HCV IRES (Figure 5).

We evaluated changes in La protein expression determined by the cDNA microarray by using RTD-PCR (Figure 6). The changes in HCV IRES–directed translation and in La protein expression closely correlated (Figure 6).

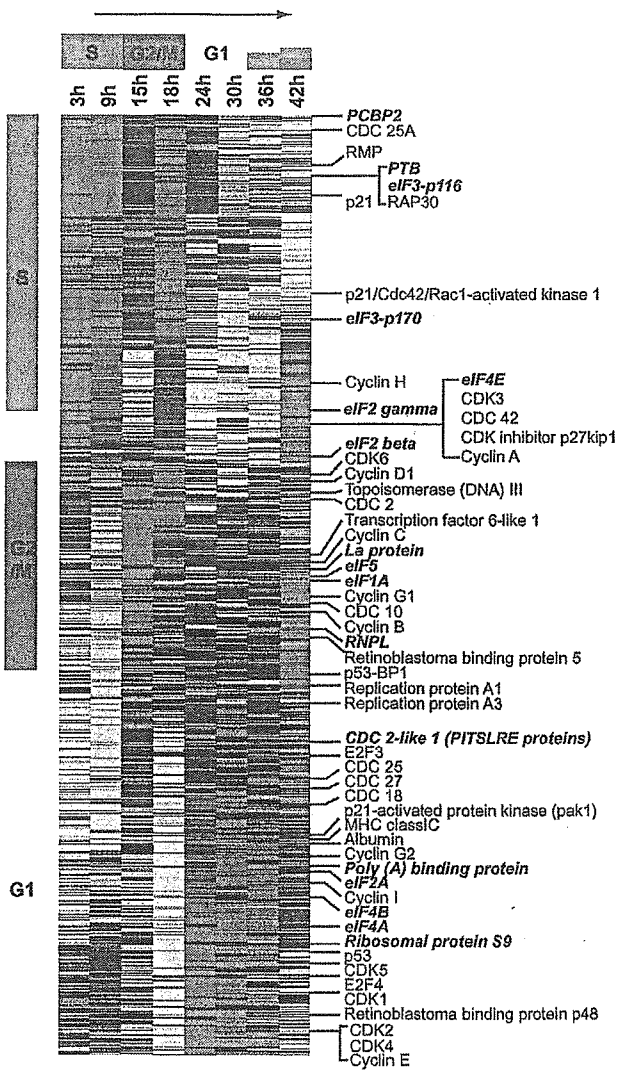

Figure 5. Gene-expression profiling in RCF-26 cells undergoing cell-cycle progression. RCF-26 cells were synchronized at the G_1/S border with aphidicolin. After release from aphidicolin block, the cell cycle progressed to S phase at 3–6 hours and G_2/M phase at 15–18 hours and returned to G_1 phase at 24–30 hours. Cells were harvested at 3, 9, 15, 18, 24, 30, 36, and 42 hours and analyzed with cDNA microarray, and then an SOM was constructed by using Cluster (Stanford University). Gene clusters up-regulated in the S, G_2/M, and G_1 phases (*red*) were detected with cell-cycle progression. Canonical and noncanonical initiation factors and cell cycle–related genes are listed (*right*).

Functional Analysis of the Effect of HCV IRES–Related Canonical and Noncanonical Initiation Factors on Translation Directed by HCV IRES

To prove that the induction of the canonical and noncanonical initiation factors during S and G_2/M phases contributes to cell cycle–dependent translation of HCV, antisense phosphorothioate oligos were designed for La protein, PTB, eIF3 p170, eIF2γ, RNPL, PABPC-1, PCBP-2, and ribosomal protein S9, and HCV IRES activity was evaluated under the suppression of these factors. RT-PCR showed that expression of the targeted factors was significantly reduced by the antisense oligos, whereas that of β-actin did not significantly change (Figure 7*B*). Reduced expression of these factors was also evaluated by Western blotting (Figure 7*C*). The suppression of La protein, PTB, and eIF2γ 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, suppression of eIF3 p170, RNPL, PABPC-1, PCBP-2, and ribosomal protein S9 did not reduce HCV IRES activity (Figure 7*A*). To rule out the

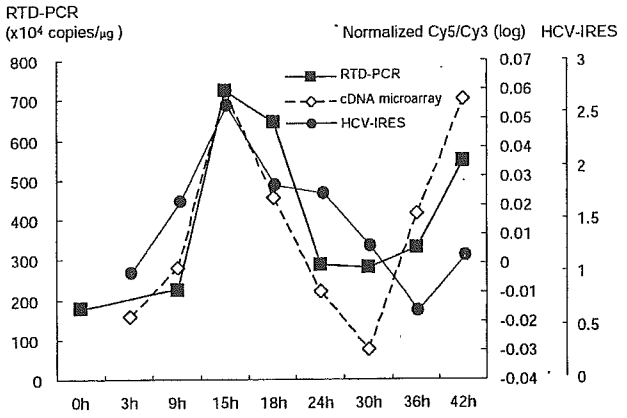

Figure 6. La protein expression in RCF-26 under cell-cycle progression determined by RTD-PCR. Normalized Cy5/Cy3 of mRNA expression of La protein and HCV IRES activities are shown in same dimension.

possibility that these reduced HCV IRES activities were not due to nonspecific suppression by the antisense oligos, antisense oligos to La protein, PTB, eIF3 p170, and ribosomal protein S9 were applied to ΔRCF-9 in which the functional HCV IRES element had been deleted. Two antisense oligos to La protein and PTB, which reduced HCV IRES activity in RCF-26, did not change HCV IRES activity in ΔRCF-9. Similarly, 2 antisense oligos to eIF3 p170 and ribosomal protein S9, which had no effect on HCV IRES activity, did not have any effect on HCV IRES activity in ΔRCF-9 (Figure 7*A*). Conversely, overexpression of La protein, PTB, and eIF3 p170 significantly enhanced HCV IRES activity in a dose-dependent manner, whereas the overexpression of eIF2γ, RNPL, PCBP-1, PCBP-2, and ribosomal protein S9 had no effect (Figure 8). The overexpression of La protein, PTB, and eIF3 p170 in ΔRCF-9 did not have any effect on HCV IRES activity (Figure 8). We also confirmed these findings in rabbit reticulocyte lysates by co-translating pRL-HL (HCV IRES reporter) and La protein, PTB, and eIF3 p170 (data not shown). Thus, of these HCV IRES–related canonical and noncanonical initiation factors, La protein and PTB significantly changed HCV IRES activity in both the 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, Polypyrimidine Tract Binding Protein, and Eukaryotic Initiation Factor 3 in Lesions of Chronic Hepatitis C

To examine the functional role of these factors on HCV replication in the lesions of chronic hepatitis C, we evaluated their expression in 26 liver samples from patients with chronic hepatitis C and in 8 normal liver samples by RTD-PCR. Tables 3 and 4 list the clinical characteristics of the patients. The expression level of La protein in the specimens of the patients with chronic hepatitis C was significantly higher than that of the normal livers, whereas the expression of PTB and eIF3 p170 was not statistically different (Table 3). Some of these samples were also reevaluated by Northern blotting, and the results were similar (data not shown). Up-regulation of the La protein was related to neither the histological stage nor the activity of liver disease (Table 4). However, the expression of La protein was significantly correlated with the amount of HCV RNA in the liver (Figure 9). Moreover, HCV RNA replication was significantly higher in liver with high La protein expres-

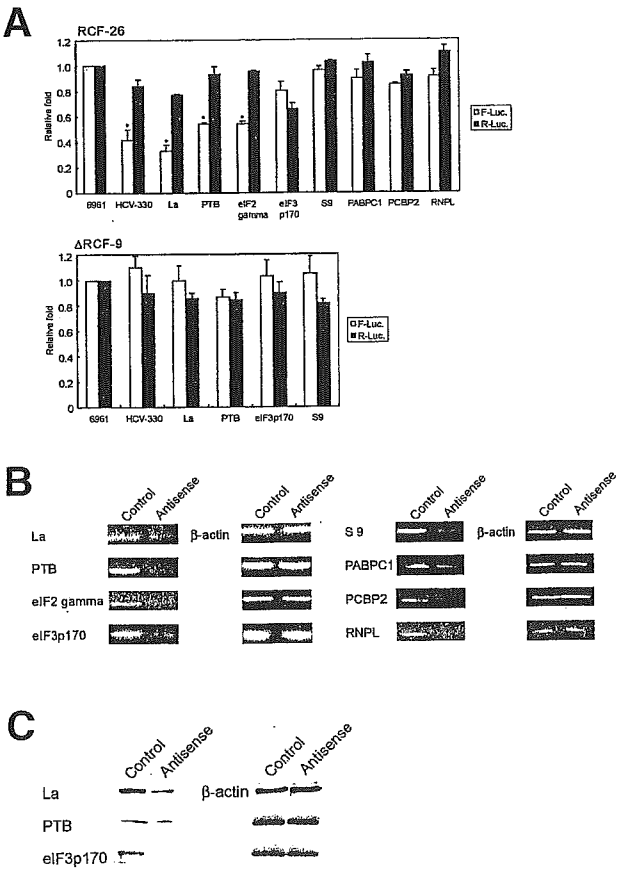

Figure 7. Suppression of HCV IRES–related canonical and noncanonical initiation factors (La protein, PTB, eIF3 [p170], eIF2γ, RNPL, PABPC-1, PCBP-2, and ribosomal protein S9) by specific antisense phosphorothioate oligos and HCV IRES activity in RCF-26 and ΔRCF-9. (*A*) Changes in *Renilla* (cap-dependent translation) and firefly luciferase (HCV IRES–directed translation) activities caused by suppression of these factors by antisense phosphorothioate oligos. $*P < .05$. (*B*) Suppression of factors confirmed by RT-PCR. (*C*) Suppression of factors confirmed by Western blotting.

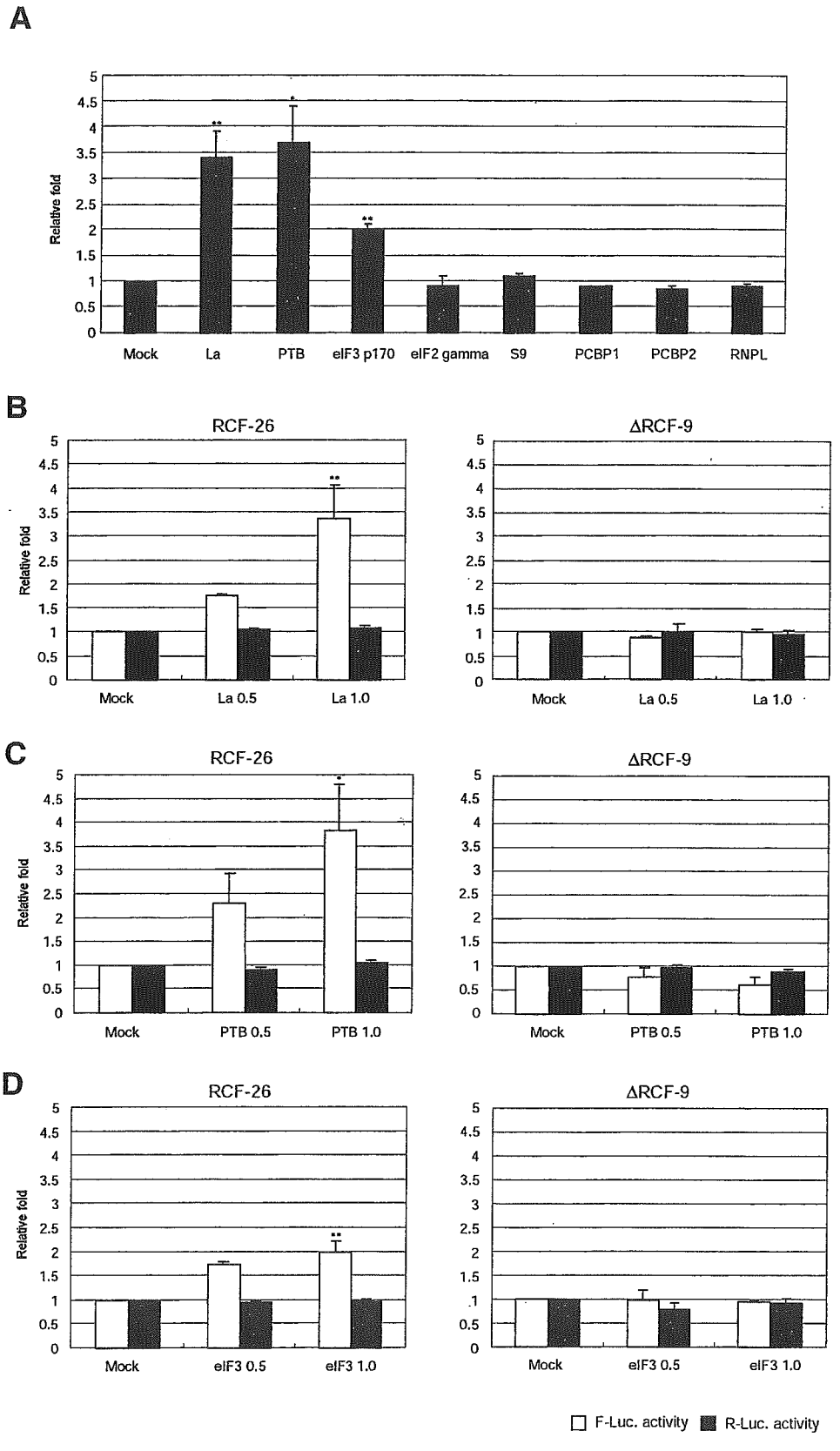

Figure 8. (*A*) Overexpression of HCV IRES–related canonical and noncanonical initiation factors (La protein, PTB, eIF3 [p170], eIF2γ, RNPL, PCBP-1, PCBP-2, and ribosomal protein S9) in RCF-26 and HCV IRES activity. (*B–D*) Dose-dependent overexpression of La protein, PTB, and eIF3 p170 in RCF-26 and ΔRCF-9. $*P < .05$; $**P < .01$.

Table 3. Expression of La Protein, PTB, and eIF3 (p170) in Liver Detected by RTD-PCR

Diagnosis	n	Age (y)	Sex (M:F)	ALT (*IU/L*)	Serological HCV type (group 1:group 2)	La (×10^6 *copies/μg*)	PTB-1 (×10^5 *copies/μg*)	eIF3 (p170) (×10^6 *copies/μg*)
Normal	8	64.5 ± 4.10	5:3	17.0 ± 9.86	ND	1.08 ± 0.11	1.34 ± 0.15	3.32 ± 0.46
Chronic hepatitis C	26	62.8 ± 2.27	20:6	67.7 ± 10.2[a]	21:3[b]	2.75 ± 0.26[c]	1.40 ± 0.14	2.21 ± 0.31

ALT, alanine aminotransferase; ND, not done.
[a]$P < .05$.
[b]Two patients were unclassified.
[c]$P < .01$.

sion (Figure 10). These findings indicate that La protein plays an important role in the replication of HCV in the livers of patients infected with chronic hepatitis C.

Discussion

Although extensive studies have examined the molecular biology of HCV, the responsible host factors that regulate HCV replication in patients with chronic hepatitis C have not yet been elucidated. Patients with a high viral load are refractory to interferon therapy, even when it is combined with ribavirin.[3–6] Recent advances in the HCV replicon system have shown some adaptive mutations in the HCV genome (NS5A or NS3) for efficient replication of cellular factors that inhibit HCV replication, such as PKR and interferon-regulatory protein 1.[40] However, there has been no clear evidence that these factors are truly determinant of HCV replication in patients with chronic hepatitis C. The identification of host factors that regulate HCV replication in vivo should show the underlying mechanism of high viral load in patients with chronic hepatitis C. Moreover, it could provide a basis for the development of a new antiviral treatment strategy.

The translation of viral polyprotein is an important step in viral replication and could thus present a target for a novel antiviral therapy. Translation of the HCV RNA genome is initiated by a highly structured RNA segment, the IRES, that occupies most of the 5′-NTR RNA.[7–15] We showed that HCV IRES activity varies during different phases of the cell cycle: it is highest during the S and M phases and lowest during the G_0 phase of the cell cycle.[22] These findings have important clinical relevance because viral translation might be enhanced by factors that stimulate the regeneration of hepatocytes in patients with chronic hepatitis C. We investigated the molecular basis of these findings and found host factors that regulate HCV IRES.

The expression of La protein, PTB, eIF3, and eIF2γ was repressed in confluent and serum-starved cells, but eIF3 and eIF2γ were reduced in confluent or serum-starved cells. Analysis of cell-cycle progression more precisely showed the interaction of these initiation factors with the cell cycle–dependent regulation of HCV IRES activity. Most of the HCV IRES–related canonical and noncanonical initiation factors (PCBP-2, PTB, eIF3, eIF2γ, eIF2β, La protein, and RNLPL) were induced during the S and G_2/M phases of the cell cycle. Conversely, eIF4A, eIF4B, and PABPC-1, which are not supposed to be a requirement for HCV IRES activity,[33,39] were induced during G_1. In cells, because protein translation takes place immediately in the presence of mRNA, dynamism of expression profiles might directly link to HCV IRES activity, although some protein levels were also regulated by the posttranslational modification. The finding that HCV uses host factors induced during cell division (S and G_2/M), but not during quiescence (G_0/G_1), is of interest. In this respect, HCV IRES–directed translation differed from either cap-dependent or IRES-directed translation by encephalomyocarditis virus and the picornavirus-like group. Reports indicate that eIF4B and PABPC-1 are required for encephalomyocarditis virus and poliovirus, but not for HCV translation.[39] G_1 induction of the ribosomal protein S9, which supposedly binds the secondary structure of HCV IRES,[7] seems controversial. To further investigate these findings, we evaluated the functional roles of these factors in HCV IRES activity. Among the canonical and noncanonical HCV IRES–related initiation factors, the suppression of PTB, La protein, and eIF2γ by using antisense oligos reduced HCV IRES activity, and the overexpression of PTB, La, and eIF3 stimulated HCV IRES activity. The La protein and PTB changed HCV IRES activity in both

Table 4. Histological Findings and La Protein Expression

Histology	n	La (×10^6 *copies/μg*)
F1	2	1.98 ± 0.03
F2	6	3.31 ± 0.80
F3	7	3.04 ± 0.48
F4	11	2.40 ± 0.30
A1	10	2.72 ± 0.52
A2	15	2.82 ± 0.30
A3	1	1.86

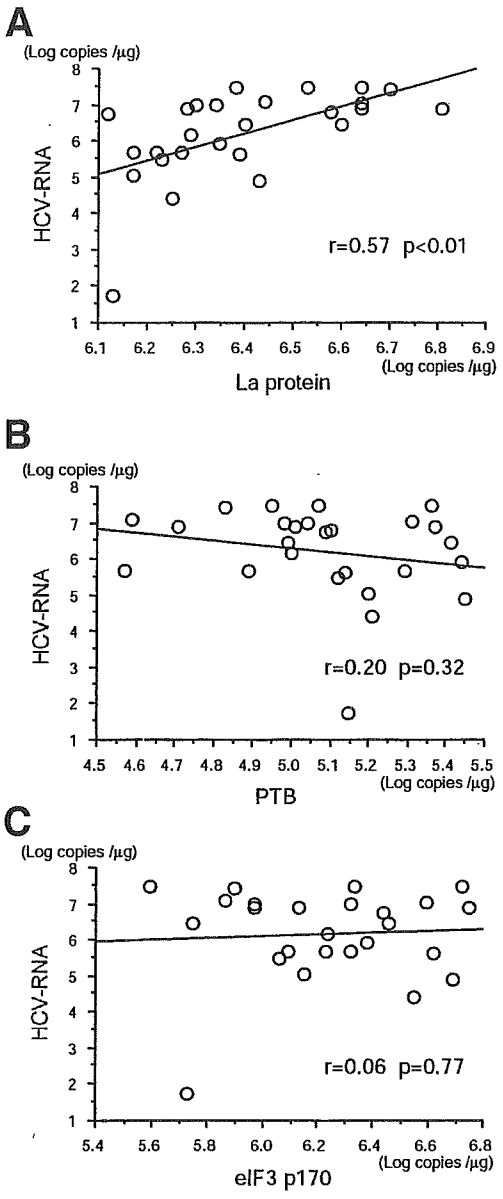

Figure 9. Correlation of La protein (*A*), PTB (*B*), and eIF3 p170 (*C*) with the amount of HCV RNA in tissue lesions of chronic hepatitis C.

the suppressed and overexpressed states. However, neither suppression nor overexpression of ribosomal protein S9 affected HCV IRES activity. Thus, this study could not identify the functional significance of ribosomal protein S9 for HCV IRES activity.

The definition of these initiation factors has very important clinical relevance to HCV replication. We therefore investigated the expression of La protein, PTB, and eIF3 in tissue lesions from patients with chronic hepatitis C. Expression of La protein was significantly increased in the liver of patients, whereas that of PTB and eIF3 did not significantly increase. Neither histological activity nor stage was associated, but the amount of liver HCV RNA was significantly correlated with the level of La protein expression. Patients that expressed high levels of La protein in the liver were infected with more HCV (Figure 10). Thus, La protein plays an important role in HCV replication in livers of patients with chronic hepatitis C. Two possible mechanisms might explain the induction of La protein in the livers of chronic hepatitis C patients. First, significant proportions of cells undergo division during hepatocyte regeneration, and the proportion of cells in M phase that lead to the induction of the La protein increases. Second, HCV induces La protein. Because the expression of PTB and eIF3 was not significantly induced in the tissue lesions of chronic hepatitis C patients in this study, there must be unknown mechanisms by which HCV infection induces La protein. Our preliminary results showed that HCV proteins increased La protein in Huh-7 cells (data not shown). Further analysis is needed to show the interaction between La protein induction and HCV replication in chronic hepatitis C.

In conclusion, we discovered host factors that regulate HCV translation and replication in the liver. The implication of these findings with regard to the HCV life cycle is shown in Figure 11. 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 antiviral therapy. Although La protein and PTB have been shown to be involved in the cell-cycle regulation of HCV IRES activity, many other host factors might also be involved.

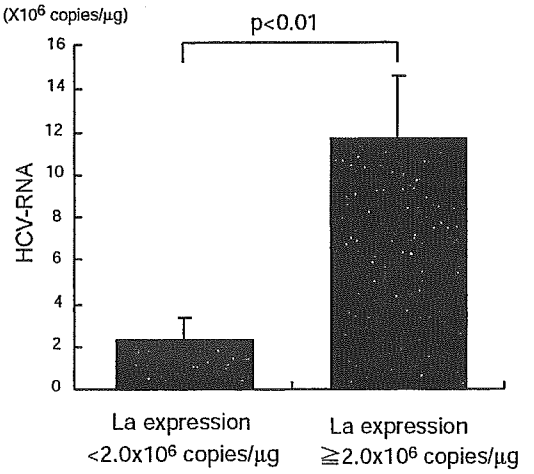

Figure 10. La protein expression and HCV RNA in liver.

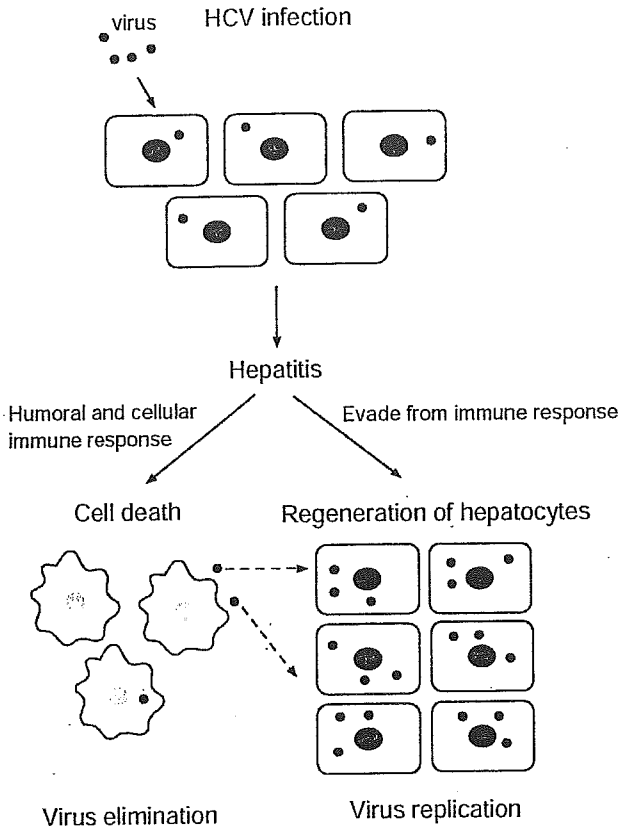

Figure 11. Hepatitis and HCV life cycle.

We are extending these analyses to other initiation factors and investigating the functional role on HCV IRES activity and replication of HCV.

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Hepatitis B virus-related insertional mutagenesis in chronic hepatitis B patients as an early drastic genetic change leading to hepatocarcinogenesis

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Growing evidence demonstrates that hepatitis B virus (HBV) integration and resulting insertional mutagenesis play an important role in cell growth or maintenance in hepatocellular carcinomas (HCCs). To determine if HBV integration occurs and affects cellular genes at such a stage of infection, we analysed viral–host junctions in chronic hepatitis tissues without HCC using PCR amplification with primers specific to human *Alu*-repeat and HBV. We obtained 42 independent viral–host junctions from six patients examined and identified chromosomal locations for 20 of the 42 junctions. In six clones, each integration apparently affected a single gene. These six candidate genes included one known tumor suppressor gene, three human homologs of drosophila genes that are critical for organ development, one putative oncogene and one recently found chemokine. Our data, together with previously reported HBV integrants in HCCs, suggested preferential HBV integration into chromosome 3 ($P=0.022$). Our virus-tagging approach provided (a) firm evidence of HBV integration in hepatocytes at an early stage of chronic infection and (b) revealed cellular genes possibly affected by HBV integration and potentially involved in early steps of the process leading to carcinogenesis.
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Introduction

Epidemiologic data provide compelling evidence (Beasley *et al.*, 1981; Yu *et al.*, 2000) for a role of hepatitis B virus (HBV) in the development of hepatocellular carcinoma (HCC). HBV is thought to be one of the DNA tumor viruses, such as human papillomavirus (HPV) and Epstein–Barr virus (Nevins and Vogt, 1996). HBV also shares with oncogenic retroviruses, a unique replication strategy through reverse transcription and a characteristic lifecycle that includes integration into the host genome (Bréchot *et al.*, 2000). Despite extensive research, the precise mechanism whereby HBV infection contributes to hepatocarcinogenesis remains unclear. One complication is that human tumorigenesis is a multistep process and several viral mechanisms are possibly involved in each step, including cis- and trans-activation of cellular genes by viral proteins, antiapoptotic action, induction of genomic instability and insertional mutagenesis (Nevins and Vogt, 1996).

Several groups have independently reported HBV integration into the human telomerase reverse transcriptase (hTERT) gene in HCCs, as well as in hepatoma derived cell lines, proving that viral insertion activates hTERT expression and, possibly, maintenance of telomere length (Gozuacik *et al.*, 2001; Horikawa and Barrett, 2001; Ferber *et al.*, 2003a). Insertional mutagenesis is an important mechanism common to both RNA and DNA virus-related oncogenesis. HPV integration into the hTERT gene has recently been reported (Ferber *et al.*, 2003a) and others have reported clonal T-cell proliferation induced by integration of retrovirus into a human proto-oncogene (Hacein-Bey-Abina *et al.*, 2003). Furthermore, the integration of woodchuck hepatitis virus (WHV), an animal virus resembling HBV, often occurs in c-Myc or N-Myc genes. Direct transforming activity of WHV/Myc integrants is thought to lead to the development of animal HCCs (Hsu *et al.*, 1988; Fourel *et al.*, 1990). Other reports describe genes with important roles in cell growth, such as retinoic acid receptor beta, cyclin A2, mevalonate kinase, sarco/endoplasmic reticulum calcium ATPase 1 and human minichromosome maintenance protein 8, undergoing deregulation by HBV insertion (for reviews, see Bréchot *et al.*, 2000; Paterlini-Bréchot *et al.*, 2003). Cumulatively, these data strongly suggest that HBV insertional mutagenesis plays an important role in the human oncogenic process.

We previously reported that HBV integration occurs early during HBV infection, even after acute self-limiting hepatitis (Murakami *et al.*, 2004). Insertional mutagenesis may represent the first drastic genetic change during a long-lasting chronic viral carrier state, preceding the development of HCC by a few decades. Furthermore, we may be able to investigate HBV

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integration with little influence of genomic recombination and chromosomal losses or gains during the early phase of chronic infection.

There have been few investigations on HBV integration in chronic hepatitis tissues. A few reports have described host sequences adjacent to viral integration in chronic hepatitis (Yaginuma *et al.*, 1987; Takada *et al.*, 1990). This paucity of data reflects limited human genome information as well as the lack of a suitable cloning method. We investigated integrated HBV–host junctions in liver tissues with chronic hepatitis B without HCC using HBV-*Alu* PCR (Minami *et al.*, 1995). We definitively identified host nucleotide sequences and chromosomal locations of HBV integration sites in chronic hepatitis tissues. This virus-tagging approach also identified genes possibly affected by HBV integrations at an early stage of chronic HBV infection. Some of these genes are aberrantly expressed depending on the hepatoma cell lines or HCC and surrounding nontumor tissues, suggesting a role in hepatocarcinogenesis. Our study also provided additional data on the chromosomal location of 20 HBV integrants. These data suggested preferential HBV integration on chromosome 3 in contrast to the previous contention that HBV integration is a random event (Tokino and Matsubara, 1991; Bréchot *et al.*, 2000).

Results

Amplification and identification of viral–host junctions using HBV-Alu PCR and sequencing

HBV-*Alu* PCR was performed using liver DNA from six patients with chronic hepatitis type B. Electrophoresis and subsequent hybridization of the PCR product with an HBV probe showed smearing signals in all six patients, suggesting multiple polyclonal HBV integrations in chronically HBV infected liver (Figure 1). The PCR products were inserted into a plasmid vector, a total of 160 clones that hybridized to an HBV probe were selected and their nucleic acid sequences were determined. Of these, 45 clones consisted of only HBV sequences but the remaining 115 clones contained viral–host junctions as intended. There were duplications of clones and, as a result, we obtained a total of 42 different viral–host junctions.

Chromosomal location of isolated viral–host junctions

A database homology search of host sequences on Genbank and UCSC revealed the chromosomal locations of viral–host junctions in 20 of 42 clones (Table 1). We could not determine the chromosomal locations in the remaining 22 clones because they consisted of (a) extremely short cellular sequences or (b) only *Alu*-repeats. These inserts contained an *Alu* primer sequence, unidentified short sequences, probably of host origin, adjacent HBV sequences and an HBV primer sequence. They presumably reflect viral integrations into *Alu*-repeats or *Alu*-clusters.

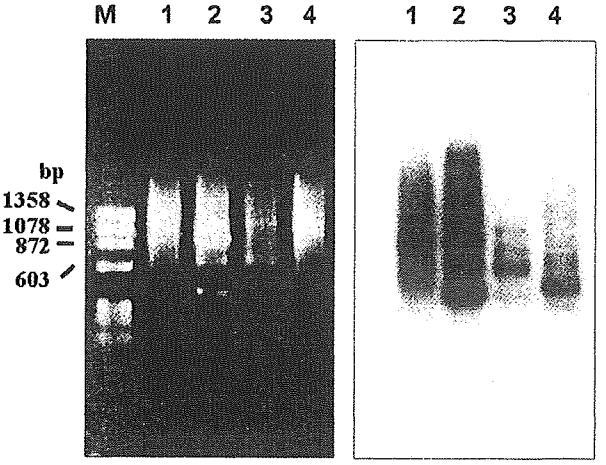

Figure 1 1.0% agarose gel electrophoresis of HBV-*Alu* PCR product and corresponding Southern hybridization with a total HBV probe of two representative cases. Smearing signals indicate polyclonal HBV integrations in chronic hepatitis tissues. Lanes 1 and 3: PCR with a HBx primer and a 5′-*Alu* primer. Lanes 2 and 4: PCR with a HBx primer and a 3′-*Alu* primer

HBV integration has been considered a random event and until now there has been no known chromosomal preference (Bréchot *et al.*, 2000). Assuming that HBV integration is random and the chromosomal location of the viral–host junction depends on the size of each chromosome, we calculated the expected rate of HBV integration into each chromosome. We found three integrations in chromosome 3, a value two-fold higher than expected (1.34), but not statistically significant ($P=0.15$). Two other groups have recently reported consecutive analyses of HBV integration in HCCs and identified chromosomal localizations of 15 and 17 viral–host junctions (Gozuacik *et al.*, 2001; Wang *et al.*, 2004). We performed statistical analysis using 52 viral–host junctions observed either in our laboratory or reported by others (Table 2). This analysis of data from three independent laboratories demonstrated that integrations in chromosome 3 were always higher than anticipated and the overall frequency (8/52) was statistically significant ($P=0.022$). There were no significant preferences in the other chromosomes.

Genes possibly affected by HBV integration

HBV integration can affect human gene expression by interrupting an open reading frame or by activating genomic transcription through viral enhancer activity (Nevins and Vogt, 1996; Bréchot *et al.*, 2000). As the gene-poor regions greater than 500 kb are termed gene deserts (Nobrega *et al.*, 2003), we listed human genes located within 250 kb from or interrupted by HBV integration in the human genome databases.

Of the 20 integrants (Table 1), 10 were found within intronic sequences of human genes with confirmed protein production (Genbank and/or UCSC) and two were situated in intronic sequences of genes with hypothetical protein products (Genbank). Three integrants were located within 'gene deserts' and no genes were apparently

Table 1 Clones containing viral–host junctions

		No. of clone	*Length of cellular sequence (bp)*	*Ch. location*	*Interrupted gene*	*Near gene 1*	*Near gene 2*
TK	1	17	5	NI			
	2	4	681	11p12			
	3	4	114	16q13.3	AXIN1 int2		
	4	3	391	7q21.11			
	5	3	183	7q22.3	SRPK2 int17	LHFPL3; 8.6 kb DN	MLL5; 98 kb UP
	6	2	4	NI			
	7	1	817	15q13.3+2q36.3	KLF13 int1	LOC283711; 47 kb UP	
	8	1	76	6p22.3			
	9	1	78	NI			
	10	1	9	NI			
	11	1	12	NI			
	12	1	6	NI			
	13	1	11	NI			
HS	1	6	508	2q31.2	PDE11A int17	FLJ13946; 150 kb UP	
	2	3	8	NI			
	3	1	373	3p22.3		STAC; 114 kb UP	
	4	1	549	8q21.11	KCNB2 int5	TERF1; 168 kb UP	
	5	1	359	3q13.12	BBX int3		
	6	1	64	NI			
	7	1	6	NI			
	8	1	20	NI			
	9	1	1	NI			
	10	1	27	NI			
	11	1	376	4p12		OCIA; 6 kb UP	
TN	1	8	150	1p36.23		PARK7; 22 kb DN	MIG6; 4 kb DN
	2	2	127	3p14.1	AL713702 int		
	3	1	82	19q13.42		CDC42EP5; 1.5 kb UP	LAIR2; 28 kb UP
	4	1	473	5p15.2	CTNND2 int20		
	5	1	11	NI			
OR	1	1	189	17q24	PITPNC1 int14	FALZ; 148 kb UP	
	2	1	11	NI			
IW	1	17	6	NI			
	2	8	472	1p35.3	EYA3 int14		
	3	2	305	11q21	BC026191 int1	SRP46; 28 kb DN	SEST3; 75 kb DN
KM	1	4	8	NI			
	2	3	3	NI			
	3	3	6	NI			
	4	1	642	18q22.2		SOCS4; 61 kb DN	
	5	1	109	NI			
	6	1	167	5q34	ODZ2 int7		
	7	1	7	NI			
	8	1	49	NI			
Total		115					

No. = number; NI = not identified; int = intron and number; DN = from 3′ end of the gene; UP = from 5′ end of the gene

affected. In the other five clones, integrations occurred within 1.5 to 168 kb of the viral–host junctions. Therefore, we listed 27 cellular genes that might be affected by HBV integration from 17 of 20 clones (Table 1).

We observed HBV integrations close to multiple genes in eight of the clones, making it difficult to determine which genes are actually affected. We were also prudent in identifying HBV integrations situated upstream or downstream of the genes. Therefore, we chose genes whose open reading frames were interrupted by HBV integration. In six clones only a single gene was interrupted and there was apparently no other gene within 250 kb of the viral–host junction. These genes are candidates for genes affected by HBV and, as a result, they are possibly related to cell proliferation and survival. They include axis inhibitor 1 (AXIN1), homolog of *bobby sox* (BBX), AL713702 (hypothetical protein), catenin delta-2 (CTNND2), eyes absent 3 (EYA3), and homolog of odd Oz 2 (ODZ2). In order to characterize these six genes we examined their expression in hepatic cell lines and HCC tissues.

mRNA expression of cellular genes interrupted by HBV integration in cell lines, HCC and surrounding nontumorous tissues

With the exception of AXIN1 in human liver, the function and expression of the genes interrupted by

Table 2 Chromosomal location of HBV–host junctions identified in this study, by Gozuacik *et al.* and by Wang *et al.*

		Frequency					
Chr.	*Size (Mb)*	*This study*	*Gozuacik*	*Wang*	*Total*	*Expected freq. to XX*	*Expected freq. to XY*
1	246.1	2	1	1	4	4.24	4.31
2	243.6	1	3		4	4.19	4.27
3	199.3	3	2	3	8	3.43	3.49
4	191.7	1		1	2	3.30	3.36
5	181.0	2	1	2	5	3.12	3.17
6	170.9	1		1	2	2.94	2.99
7	158.5	2		1	3	2.73	2.78
8	146.3	1		2	3	2.52	2.56
9	136.4		3		3	2.35	2.39
10	135.0			1	1	2.32	2.37
11	134.5	2		1	3	2.32	2.36
12	132.1		1		1	2.27	2.31
13	113.0			1	1	1.95	1.98
14	105.3		1		1	1.81	1.84
15	100.3	1			1	1.73	1.76
16	90.0	1	1		2	1.55	1.58
17	81.9	1			1	1.41	1.43
18	76.1	1	1	1	3	1.31	1.33
19	63.8	1		1	2	1.10	1.12
20	63.7		1	1	2	1.10	1.12
21	47.0					0.81	0.82
22	49.4					0.85	0.87
X	153.7					2.65	1.35
Y	50.3					0.00	0.44
Total		20	15	17	52		
46XX	6039.2						
46XY	5935.8						

Chr = chromosome number; freq = frequency

HBV integration have not been fully investigated. Therefore, we semiquantitatively analysed mRNA expression of AXIN1, BBX, AL713702, CTNND2, EYA3, and ODZ2 in five human hepatoma-derived cell lines. These six genes were expressed, although not abundantly, in some or all of these cells (Figure 2). It is noteworthy that three of these genes, ODZ2, CTNND2, and AL713702, showed marked differences in mRNA expression levels dependent on the cell lines. CTNND2 was not expressed in HLE or HLF cells, whereas AL713702 was not expressed in HLE, HLF, and HepG2 cells, even after two rounds of PCR using nested primers (data not shown). ODZ2 was scarcely expressed in HepG2 cells, but was abundant in HLE and HLF cells.

In order to further characterize the expression patterns of these six genes, we analysed mRNA expression levels in seven human HCCs and corresponding nontumor tissues (Figure 3). EYA3 and BBX showed relatively homogenous expression levels in tumor and nontumor tissues. In contrast, AXIN1, ODZ2, and AL713702 demonstrated aberrant expression patterns. AXIN1 was not expressed in one tumor tissue and predominantly expressed in nontumor tissues in three of seven cases. In accordance with aberrant expression in hepatoma cell lines, ODZ2 was not expressed in one tumor tissue and showed lower expression in tumor tissues in four of seven cases. AL713702 was expressed in only one tumor tissue and three nontumor tissues. CTNND2 was expressed in all tissues and showed predominant expression in three tumor tissues, but showed lower expression in one nontumor tissue.

Discussion

We and others previously developed a PCR-based method to rapidly and specifically amplify HBV–host junctions (Minami *et al.*, 1995). This technique, coupled with the availability of the human genomic database, permitted the identification of as many HBV integration-related genes as have been reported in the past two decades (Bréchot *et al.*, 2000; Gozuacik *et al.*, 2001; Paterlini-Bréchot *et al.*, 2003). We here employed this HBV-*Alu* PCR because of its advantage in amplifying viral–host junctions that coexist with free viral sequences. Attempts to amplify viral–host junctions are often disrupted by free HBV-derived amplicons. This is particularly evident in chronic hepatitis tissues since free viral loads are usually greater in chronic hepatitis than in HCC (personal observation). In this study, 115 viral–host junctions and no more than 45 clones consisting only of HBV sequences were identified by screening 160 HBV-containing clones.

Our data, together with those of two other groups, have identified preferential HBV integrations into chromosome 3. We, as well as Gozuacik *et al.*, employed an HBV-*Alu* PCR method to identify viral–host junctions, but Wang *et al.* used a conventional cloning

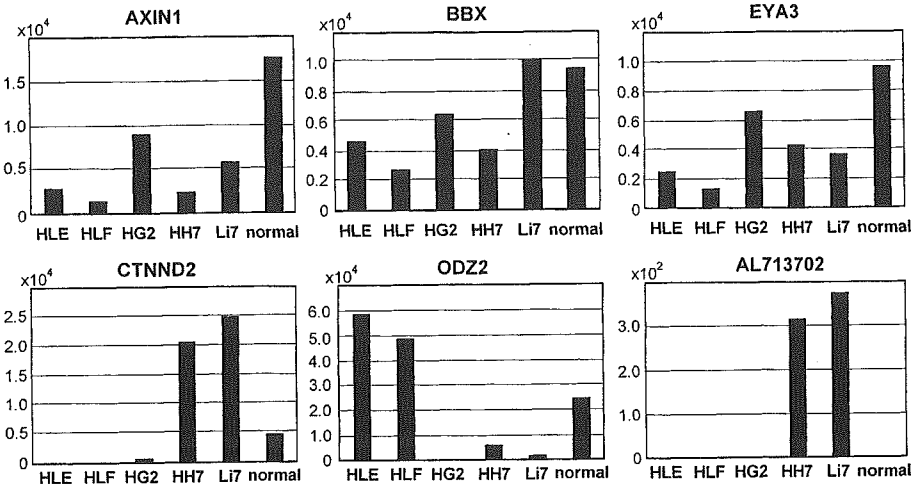

Figure 2 mRNA expression levels of six genes interrupted by HBV integration in hepatoma-derived cell lines. Copy number of mRNA is standardized and expressed as compared to 1.0×10^6 copies of beta-actin mRNA expression level. HG2, HepG2; HH7, Huh7; normal, data from normal liver

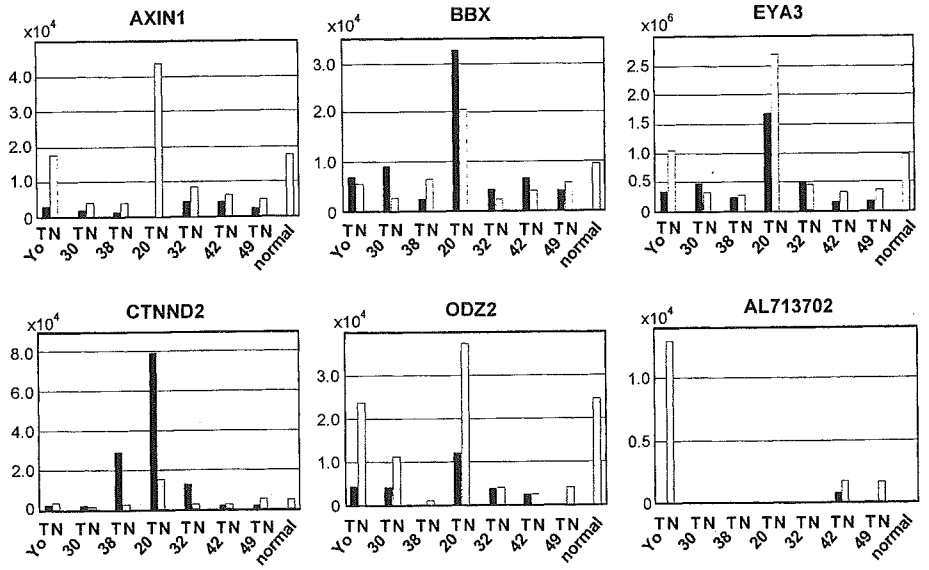

Figure 3 mRNA expression levels of six genes interrupted by HBV integration in HCC (T) and surrounding nontumor tissues (N). Copy number of mRNA is standardized and expressed as compared to 1.0×10^6 copies of beta-actin mRNA expression level in each tissue

procedure. Therefore, the identities of chromosomal preferences are independent of a cloning strategy bias. We also performed a database search for integrated HBV sequences in Genbank and identified 17 chromosomal locations in hepatoma-derived cell lines, HCCs, and chronic hepatitis tissues (data not shown). Integration in chromosome 3 was found in two of the 17 cases. Although these reports may contain intentional bias, the overall frequency of HBV integrations found in chromosome 3, including those in the database search, yielded a statistically significant ratio of 0.145 ($P=0.016$). We listed the integration sites in chromosome 3 that have been reported in the literature (Table 3). There were no common genes affected by HBV, but 3p14 and 3q25, which correspond to common fragile sites, FRA3B and FRA3D, respectively, may be preferential targets. It remains to be resolved whether or not this chromosomal preference is related to a viral integration process or to genomic recombination events during hepatocarcinogenesis or both.

Viral integration events often show preferential or common sites. For example, WHV preferentially integrates into Myc oncogene families (Hsu *et al*., 1988; Fourel *et al*., 1990), whereas HPV integration in cervical carcinoma frequently occurs near the c-Myc locus (Ferber *et al*., 2003b) and recurrently in the hTERT gene (Ferber *et al*., 2003a). Retroviral tagging has identified several common genes affected by integration in mouse hematopoietic tumors (Shen *et al*., 2003). As for HBV, common integration into hTERT has recently

Table 3 HBV integrations at chromosome 3 found in the literature and this study

	Location	*Near gene*	*Note*
Gozuacik	3p26	Inositol 1,4,5-triphosphate receptor type 1 (IP3R type 1)	
Dejean	3p24.2	Retinoic acid receptor beta (RARB)	Genbank X04014
Wang	3p23	Not indicated	Rearranged
This study	3p22.33	SRC homology 3 and cystein-rich domain (STAC)	
Wang	3p14–3p21	Not indicated	Rearranged
This study	3p14.1	TAFA-1	
		Centromere	
Gozuacik	3q11.2	Alpha 2,3 sialyltransferase (ST3GAL VI)	
This study	3q13.12	Human homologue of bobby sox (BBX)	
Koshy	3q22	Mitochondrial ribosomal protein L3 (MRPL3)	Genbank K01659
Wang	3q25	Not indicated	Rearranged
Paterlini-Bréchot	3q25.3	IL-1R-associated kinase 2 (IRAK2)	

been reported (Gozuacik *et al.*, 2001; Horikawa and Barrett, 2001; Ferber *et al.*, 2003a; Paterlini-Bréchot *et al.*, 2003). This observation, together with our finding of preferential HBV integration into chromosome 3, strongly suggests that HBV integration is not a random event and that there may be selective pressure. As HBV integration is not essential to its proliferative lifecycle, such selection would enhance viral survival in host cells. This mechanism could be related to the stimulation of host cell cycle, inhibition of cellular apoptosis, cell growth, and escape from the host immune system, leading to cellular proliferation in multistep tumorigenesis.

It has also been reported that HPV integration occurs in chromosomal fragile sites (Thorland *et al.*, 2003). Common fragile sites are thought to be highly unstable and preferential sites for translocations, deletions, intrachromosomal gene amplification, and integration of plasmid DNA and tumor viruses, such as SV40 (Smith *et al.*, 1998). They are considered to be related to carcinogenesis owing to such genomic alterations. In our study, HBV integrations were found within common fragile sites in four of 20 (20%) clones (data not shown). However, most of the common fragile sites are not precisely mapped and additional studies are needed to define the relationship between integrated sequences and common fragile sites by molecular mapping.

All of the viral sequences that we identified were situated in either intronic or intergenic sequences, but not in the exons. Viral insertional mutagenesis can affect cellular gene expression by regulating enhancer and promoter activities or by altering the exon–intron junction, possibly forming chimeric transcripts or disrupting transcription. Human intergenic sequences can regulate transcription in genes residing as far away as 1 Mb (Lettice *et al.*, 2002). Retroviral integrations apparently affect expression of genes located over hundreds of kilobases from the integration site (Fourel *et al.*, 1994). Although it is difficult to definitively identify genes affected by viral insertion, we have listed genes within 250 kb of HBV integrations. Nevertheless, in six cases, integrations occurred within the gene coding regions and no other gene existed within 250 kb. Therefore, these genes are excellent targets for insertional mutagenesis.

Among these six genes, AXIN1 is well characterized in the mouse during development and in human cancer. AXIN1 is a regulatory protein of a Wnt signaling pathway, which is crucial for the vertebral dorsal–ventral axis formation (Zeng *et al.*, 1997) and is also related to colorectal and hepatic carcinogenesis in humans. Satoh *et al.* (2000) reported the simultaneous occurrence of AXIN1 mutation and the loss of heterozygosity in the responsible region in some human HCCs. On the basis of these data, AXIN1 is believed to function as a tumor suppressor.

EYA3, ODZ2, and BBX are human homolog of drosophila genes. EYA3 is a human homolog of the drosophila *eyes absent* gene, which is essential for eye development. In humans, EYA3 gene exhibits phosphatase activity and uses this enzymatic function to switch transcriptional activation of cofactors (Li *et al.*, 2003). ODZ2 is a human homolog of the drosophila *tenascin major* (ten-m) gene, an example of a pair-rule gene, that is, every odd-numbered body segment is deleted in ten-m mutant drosophila embryos. In mammals it encodes a transmembrane protein, putatively acting as a transcriptional regulator (Bagutti *et al.*, 2003). BBX is a human homolog of the drosophila *bobby sox* gene located in a *flightless* region and encoding a product with transcription factor activity (Maleszka *et al.*, 1998). All three human homologs of drosophila genes are essential to the developments of drosophila, but their functions in humans are still unknown.

CTNND2 is a component of the adherens junction complex and its overexpression alters cell morphology and motility (Lu *et al.*, 1999). It is expressed primarily in the central nervous system (Paffenholz and Franke, 1997), but it is also found in cancerous tissues in the prostate (Burger *et al.*, 2002).

AL713702 is a spliced mRNA with a hypothetical protein product. However, very recently, Tom Tang *et al* showed that AL713702 encodes a chemokine-like protein and they named it TAFA-1. TAFA-1 is almost exclusively expressed in the brain, but its function remains unresolved (Tom Tang *et al.*, 2004).

With the exception of AXIN1, the expression of these six genes in the liver, has not been studied extensively. Our study showed that all of these genes are expressed in hepatocytes at various levels and frequencies. Consistent with previous reports, AXIN1, a tumor suppressor protein, is markedly suppressed in three tumor tissues in comparison to corresponding nontumor tissues. Both

EYA3 and BBX exhibit homogenous expression in cell lines and HCC tissues. Inasmuch as these two proteins are presumably transcriptional regulators and are expressed in the liver, additional study is needed to elucidate their roles in normal liver, chronic hepatitis and HCC tissues. ODZ2 and CTNND2 exhibit aberrant expression depending on the cell lines; for example, ODZ2 expression was higher in nontumorous tissues than in tumors in four of the seven cases, suggesting a tumor suppressive role. It is important to note that ODZ2 also colocalized with a tumor suppressor protein, PML, in the nucleus (Bagutti *et al.*, 2003). In contrast, CTNND2 expression was much higher in tumors than in nontumorous tissues in three of the seven cases. This expression pattern is consistent with a report on CTNND2 expression in prostate cancers (Burger *et al.*, 2002) and suggests a tumor-promoting role of CTNND2. AL713702/TAFA-1 was expressed in two of five cell lines and in three of seven HCC cases, preferentially in nontumorous tissues.

In summary, six genes interrupted by HBV integration included one known tumor suppressor gene, three human homologs of drosophila genes that are essential for development, one putative oncogene, and one newly found chemokine. Our observations suggest that HBV-related insertional mutagenesis in chronic infected livers may be important in regulating cell growth and in the maintenance of viral proliferation in host cells. Interestingly, two genes, TAFA-1 and CTNND2, are exclusively expressed in the central nervous system in humans, but we identified their expression in HCCs, in surrounding cirrhotic liver and in hepatoma-derived cell lines. Their role in hepatocytes undergoing carcinogenic or inflammatory transition needs further investigation.

We listed 27 genes located within 250 kb of and possibly affected by HBV integrations, including several genes related to cell growth and survival. SRPK2 is a serine/threonine kinase expressed in mouse brain, lung, and testis. It phosphorylates the HBV core protein and is a possible molecular target for inhibiting HBV replication (Daub *et al.*, 2002). TERF2 is a key component of vertebrate telomeres and plays a protective role in cellular senescence (van Steensel *et al.*, 1998). OCIA is a novel protein found by screening cDNA libraries of ovarian cancer (Luo *et al.*, 2001). However, which gene is really affected is difficult to determine. In fact, we found that cells with HBV integration in the EYA3 (clone IW 2, Table 1) yielded 46 copies per 5000 cells whereas those with integration in the 11q21 (clone IW 3, Table 1) yielded 71 copies per 5000 cells (data not shown), suggesting that the extent of clonal expansion was comparable between these two integrated clones. It is also noteworthy that noncoding intergenic sequences sometimes regulate other gene expressions (Nobrega *et al.*, 2003). Sequences that appear unimportant at the moment may have critical roles in cell growth or viral proliferation. The accumulation of viral-tagging information will narrow the genomic region repeatedly affected and lead to the identity of affected genes and regions.

In conclusion, our results provide the first extensive description of HBV integration in chronic hepatitis tissues. These integrations are potentially susceptible to deregulate gene expression and lead to cell death or cell survival and uncontrolled growth. Our data have also suggested the utility of the viral-tagging approach to identify cellular genes related to cell growth and survival. Additional studies are needed to determine the function of the genes identified in this study and to elucidate the mechanisms regulating viral survival in host cells and leading to carcinogenesis.

Materials and methods

Liver tissues and DNA preparation

Liver tissues with chronic active hepatitis were obtained by needle biopsy from six patients diagnosed with chronic HBV infection. HCC and surrounding nontumor tissues were surgically obtained from seven patients, including four with HCV infection, two with HBV infection, and one with both HBV and HCV. These procedures were performed during the patients' clinical management. All of the patients provided written informed consent and the Ethics Committee of Kyoto Prefectural University of Medicine approved all aspects of the study. DNA was extracted using a G'NOME DNA isolation kit (BIO 101, Joshua Way, CA, USA) according to the manufacturer's instructions.

Cell lines and RNA preparation

Cell lines, HLE, HLF, HepG2, Huh7, and Li7, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA from normal liver was purchased from BD Biosciences (CA, USA).

Amplification of viral–host junction

Viral–host junctions were amplified by using primers specific to human *Alu* repeat and to HBV X region as previously reported (Murakami *et al.*, 2004). The amplified products were electrophoretically separated on 1.0% agarose gel, transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia) and the HBV-specific products were visualized by hybridization using a digoxigenin-labeled total HBV probe (DIG DNA Labeling and Detection kit, Roche Diagnostics).

Cloning and sequencing of PCR products

The PCR products were blunted by T4 DNA polymerase treatment and ligated into a plasmid pCAPs, using a PCR Cloning kit (Roche Diagnostics). Plasmid DNA was obtained by using a standard protocol. Insert DNA was separated on 1.0% agarose after enzyme digestion, blotted on a Nylon membrane and screened for HBV sequence by hybridization. HBV positive clones were sequenced by a 377 Prism DNA sequencer (Applied Biosystems Inc.).

Identification of chromosomal location of viral–host junction

The sequences were searched for homologies to HBV and pCAPs using the BLAST2 program on the NCBI homepage (http//www.ncbi.nih.gov/BLAST/). Sequences other than HBV and pCAPs were examined by the BLAST and MegaBLAST programs on the NCBI homepage and by the BLAT program on the UCSC genome browser

(http//www.genome.ucsc.edu/cgi-bin/hgBlat, July 2003 freeze) to search for homologies to the human genome.

RT–PCR and quantification of RNA expression

In total, 1 μg of RNA from each cell line or liver tissue was transcribed into cDNA using an AMV reverse transcriptase and a random nine-mer primer. One fortieth of the cDNA was subjected to a semiquantitative 50 cycle RT–PCR using a LightCycler and a LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics) as per the Touch-Down PCR protocol. In order to deny possible amplification from pseudogenes, nontranscribed RNA was included and the amplified PCR product for each primer set was confirmed by direct sequencing. The specificity and quality of amplified PCR products were verified by electrophoresis on 1.5% agarose. The quantity of each cDNA was standardized using beta-actin; the detection limit was defined as 1.0×10^2 copies to 1.0×10^6 beta-actin expression level by using a LightCycler Control kit and a human beta-actin primer set (Roche Diagnostics). Each quantification was performed in duplicate. The primer sequences are listed (Table 4).

Statistical analysis

A binomial test was employed to analyse the chromosomal frequency of HBV integration. Values of $P<0.05$ were considered to be statistically significant.

Table 4 Primer sequences used for RT–PCR

Gene	*Direction*	*Sequence (5' to 3')*
Beta-actin	Sense	CAAGAGATGGCCACGGCTGCT
	Antisense	TCCTTCTGCATCCTGTCGGCA
AXIN1	Sense	CAGGCCACTATGGAGGAAAA
	Antisense	AGGGACAGGGTGTCTGCAT
BBX	Sense	CTCTCCGGGTTGCATGTACT
	Antisense	TGCCACTGAAGACACTTTCG
ODZ2	Sense	CTCTATGACCCCCTGACCAA
	Antisense	GACCTGCTTTCTCTCGGATG
CTNND2	Sense	AAAGGGATCCAGATGCTGTG
	Antisense	AATCACTTCGTGCAGTGTGC
EYA3	Sense	CCAGCATCTCAAACCAGGAT
	Antisense	TCTTGGGAAGAAGTGGCATC
AL713702 (TAFA-1)	Sense	GGAGGGACGTGTGAAGTGAT
	Antisense	CTTGGGTGAATTCTCGTGGT

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• *CASE REPORT* •

Multifocal intraportal invasion of breast carcinoma diagnosed by laparoscopy-assisted liver biopsy

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Abstract

Hepar lobatum carcinomatosum (HLC) is defined as an acquired hepatic deformity consisting of an irregularly lobulated hepatic contour caused by intravascular infiltration of metastatic carcinoma. To date, only nine cases of HLC have been reported in the literature. We report a case of a 68-year-old woman showing hepatic metastasis of breast carcinoma in radiologically unidentified form. Initially, she received left partial mastectomy for breast cancer but solid hepatic metastases were identified in S_2 and S_6, 9 mo after surgery. Then, they responded to chemotherapy and radiologically disappeared. After radiological disappearance of the liver tumors, the patient's blood chemistry showed abnormal liver function. A CT scan demonstrated heterogeneous enhancement effect in the liver in the late phase, suggesting uneven hepatic blood supply. Hepatic deformity was not obvious. Laparoscopy revealed a slightly deformed liver surface with multiple indentations and shallow linear depressions. Furthermore, a wide scar was observed on the surface of S_2 possibly at the site where the metastatic tumor existed before chemotherapy. Liver biopsy from the wide scar lesion showed intraportal tumor thrombi with desmoplastic change. Because of its similarity to the histology of the original breast cancer, we concluded that the hepatic functional abnormalities and slightly deformed liver surface were derived from the circulatory disturbance caused by microscopic tumor thrombi. Besides, since the wide scar was located at the site of the pre-existing tumor, it is probable that chemotherapy was an important cause of fibrous scarring as a result of tumor regression. These morphologic findings are compatible with those of HLC. Laparoscopy-assisted liver biopsy was useful to make definite diagnosis, even though the hepatic deformity was radiologically undetectable.

Key words: Metastatic breast cancer; Hepar lobatum carcinomatosum; Laparoscopy

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INTRODUCTION

Liver metastasis of breast cancer usually presents cancer nodules scattered in the liver and the radiological diagnosis is not difficult. However, rarely, it does not form space-occupying lesions and manifests itself as multifocal occlusion of intrahepatic branches of the portal and/or hepatic veins by tumor thrombi. Such circulatory disturbances can result in deformity of the liver, mimicking cirrhosis[1-4]. In these cases, since radiological findings are uneven hepatic circulation and hepatic deformity, the diagnosis is difficult and sometimes misleading[5-8]. We report a case of hepatic metastasis of breast carcinoma, which diffusely spread in the liver without radiologically detectable lesions or obvious hepatic deformity. It was successfully diagnosed by laparoscopy-assisted liver biopsy. We have discussed its pathogenesis based on the previous reports.

CASE REPORT

A 68-year-old woman was admitted to our hospital complaining of general malaise. She had received left partial mastectomy on August 2, 2002. The tumor was in stage I and was histologically invasive ductal carcinoma of scirrhous type. An enhanced computed tomography (CT) scan on April 2, 2003 revealed a metastatic lesion in liver S_2 (Figure 1A), and an enhanced magnetic resonance image with Ferridex on May 19, 2003 showed metastatic lesions in S_2 and S_6 (Figures 1B and C). From April to December 2003, combination chemotherapy was administered every month, using 900 mg of cyclophosphamide and 110 mg of epirubicin for the first 4 mo and then reducing cyclophosphamide to 500 mg and epirubicin to 60 mg. In February 2004, the

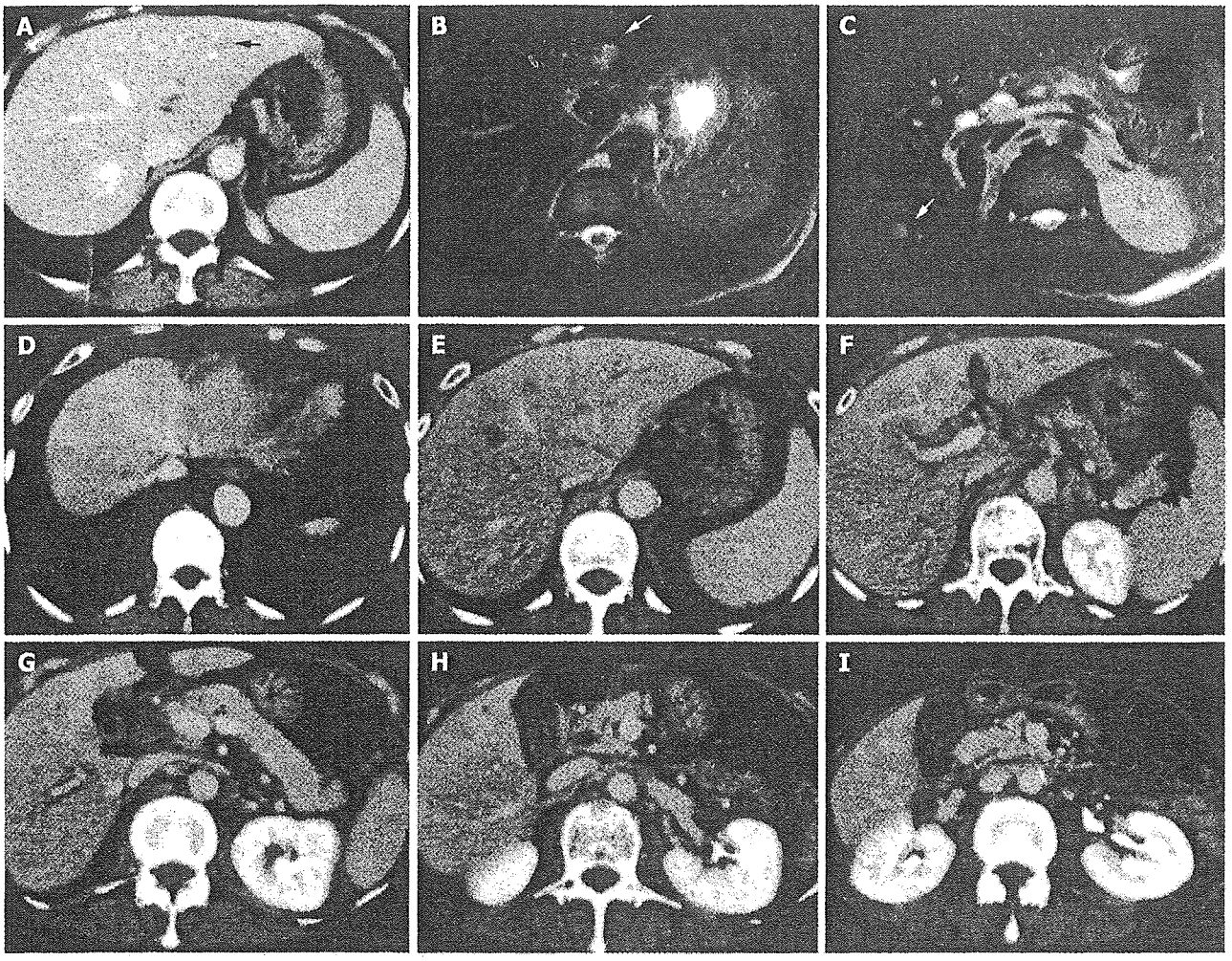

Figure 1 **A:** Enhanced CT scan on April 2, 2003. A metastatic lesion in liver S_2 (arrowhead). **B** and **C:** Enhanced magnetic resonance image with Ferridex on May 19, 2003. Metastatic lesions in S_2 (**B**, arrowhead) and S_6 (**C**, arrowhead). **D-I:** Dynamic study by CT scan on admission. Uneven enhancement effect of the liver in late phase.

patient was admitted to our hospital, complaining of general fatigue and appetite loss and showing abnormal liver function test results. After admission, she also manifested drowsiness, disorientation and insomnia. The patient was 154 cm in height and weighed 54 kg. Visible mucosa showed slight jaundice and no anemia. There was no sign of palmar erythema or vascular spider. Superficial lymph nodes were not palpable. There were two sites of skin eruption at the left anterior chest, which had already been diagnosed as skin metastasis by biopsy. There was leg edema but no hepatosplenomegaly. The blood test showed abnormalities in liver function, coagulation disturbance, and thrombocytopenia. Specifically, the results were as follows: platelet count, $9.3\times10^4/\mu L$; prothrombin time, 82%; fibrin degradation product, 15.4 μg/mL; C-reactive protein, 5.7 mg/dL; lactate dehydrogenase, 810 IU/L; aspartate aminotransferase, 219 IU/L; alanine aminotransferase, 240 IU/L; alkaline phosphatase, 1 826 IU/L; γ-glutamyltranspeptidase, 960 IU/L; total bilirubin 4.97 mg/dL; ammonia, 252 μg/dL; total protein, 5.6 g/dL; albumin, 3.1 g/dL. Dynamic study with CT scan demonstrated uneven enhancement effect in the liver in late phase, suggesting uneven hepatic blood supply. At this time, the metastatic tumors had become undetectable (Figures 1D-I) and hepatic deformity was not obvious. To clarify the exact cause of liver function abnormalities, laparoscopy was performed. It showed an irregular and deformed liver surface with multiple indentations and shallow linear depressions (Figures 2A-D). A wide scar was observed on the surface of S_2 possibly at the site where the metastatic tumor existed before chemotherapy (Figure 2E, arrowheads). Laparoscopic liver biopsy from a wide scar lesion showed residual cancer cells scattered in a wide fibrous band in one area (Figure 3A). In another area intraportal tumor thrombi were clearly demonstrated by CD31 immun-ohistochemical staining (Figures 3B and C). Cancer cells showed desmoplastic change around them (Figure 3D), which extended toward the sinusoids (Figure 3E). Because of its similarity to the histology of original breast cancer (Figure 3F), we concluded that the hepatic functional abnormalities were due to the uneven blood supply caused by intraportal invasion of the breast cancer. Drowsiness, disorientation and insomnia, along with high level of serum ammonia, were suggestive of hepatic encephalopathy. Lactulose and branched-chain amino acid supplement were given but failed to improve the symptoms, and serum ammonia level stayed