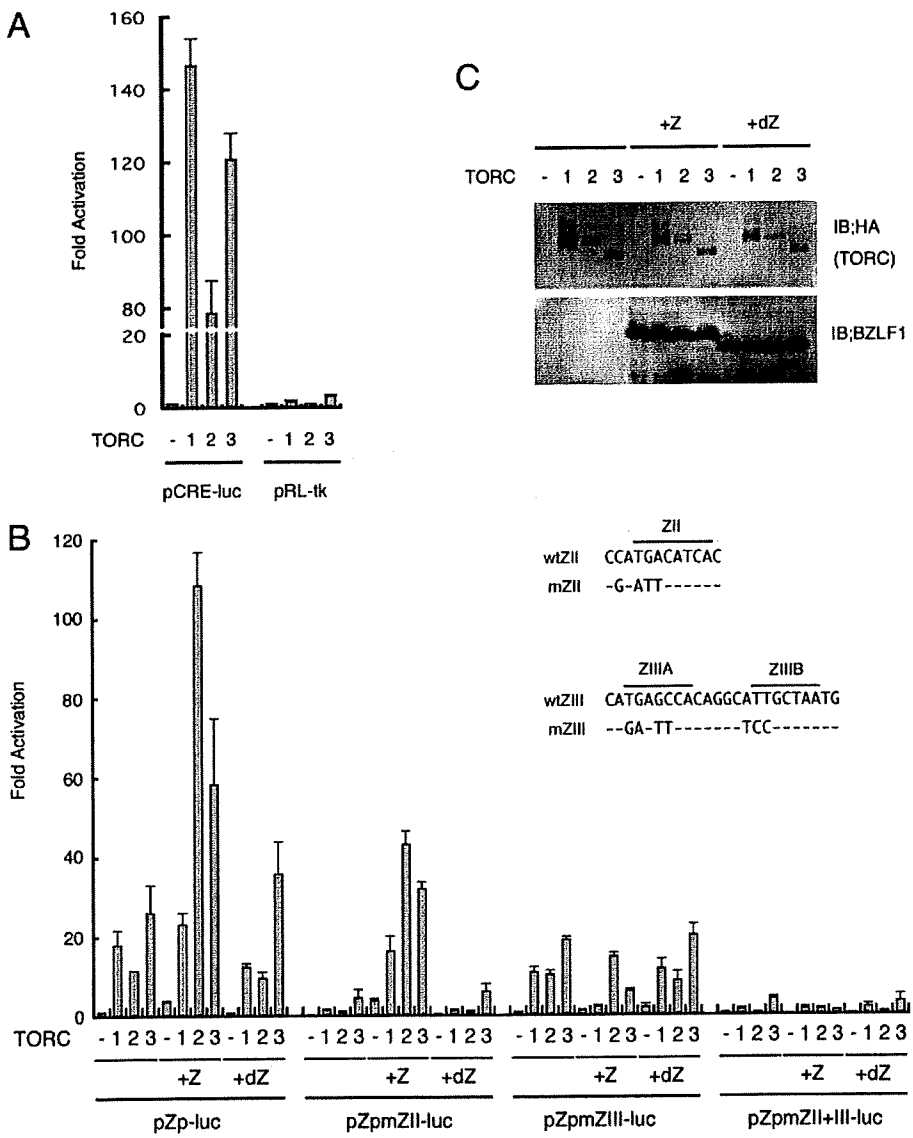

FIGURE 1. **TORCs activate transcription from Zp.** *A*, TORCs enhance CRE-dependent transcription. HEK293T cells were transfected with 10 ng of pCRE-luc or pRL-TK reporter plasmid and 50 ng of expression plasmids for TORC1, -2, or -3. Luciferase assays were carried out as described under "Experimental Procedures." The luciferase activity is shown as –fold activation of that without TORC for each reporter. *B*, effects of TORC proteins on Zp. HEK293 cells were transfected with 10 ng of reporter plasmid, pZp-luc, or its derivatives and 50 ng of expression plasmids for TORC1, -2, or -3 with 10 ng pcDNABZLF1 (+*Z*) or pcDNAdBZLF1 (+*dZ*) lacking b-Zip domain. The pZp-luc reporter contains the minimal Zp for the EBV B95-8 strain. For derivatives, specific mutations were introduced for mZII or/and mZIII as indicated. Luciferase assays were carried out as described under "Experimental Procedures." The luciferase activity is shown as -fold activation of that with neither BZLF1 nor TORC for pZp-luc (*leftmost bar*). Each *bar* represents the mean and S.D. of three independent transfections. *wt*, wild type. *C*, the expression levels of TORC and BZLF1 proteins in *B* were measured by immunoblotting.

by agarose gel electrophoresis and visualized with ethidium bromide staining.

Immunofluorescence Assay—For HEK293 cells, cells were fixed with 1% formaldehyde and permeabilized with 0.1% Nonidet P-40 in phosphate-buffered saline. The cells were washed and blocked in 1% bovine serum albumin in phosphate-buffered saline and then incubated with anti-FLAG antibody. Samples were then incubated with the secondary goat anti-mouse IgG antibody conjugated with Alexa Fluor 488. For Tet-BZLF1/B95-8, staining was carried out as described (25). Briefly, cells were washed with phosphate-buffered saline and lysed in ice-cold 0.5% Triton X-100-mCSK buffer (10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Triton X-100, and protease inhibitors) and fixed with 70% ethanol. The cells were blocked and then incubated overnight with primary antibodies. The samples were then incubated for 2 h with the secondary goat anti-mouse and rabbit IgG antibodies conjugated with Alexa Fluor 488 and 594, respectively. After immunostaining, cells were then mounted and stained with 4′,6-diamidino-2-phenylindole (DAPI) using ProLong Gold antifade reagent with DAPI (Invitrogen). Image acquisition was performed with a Bio-Rad Radiance 2000 confocal laser-scanning microscope equipped with a PlainApo 100 × 1.4-numerical-aperture oil immersion objective lens.

Small Interfering RNA (siRNA) and RT-PCR—Duplexes of 21-nucleotide (siRNA) specific to TORC2 mRNA, including two nucleotides of deoxythymidine at the 3′ end, were synthesized and annealed (Sigma Genosys). The sense and antisense sequences of the duplex were 5′-CUGCGACUGGCAUACACAAdTdT-3′ and 5′-UUGUGUAUGCCAGUCGCAGdTdT-3′, respectively. GTC-4 or Akata cells (1×10^5) were transfected with 50 pmol of the duplex RNA per well of a 24-well plate using a Microporator (Digital Bio). Twenty-four hours after transfection, TPA and A23187 or IgG were added and then incubated for another 24 h. Cells were then harvested for RT-PCR and IB. Primers used for the RT-PCR were as follows: for TORC2 mRNA, 5′-AAAGAATTCTACACAAGGAGCTCTCATTATG-3′ and 5′-GCTTGTCCTGTTAAGTGCAG-3′; for GAPDH mRNA, 5′-GGGAAGGTGAAGGTCGGAGT-3′ and 5′-AAGACGCCAGTGGACTCCAC-3′.

RESULTS

TORC Activates Transcription from EBV Zp—To confirm that TORC proteins activate CRE-dependent transcription, luciferase assays using pCRE-luc and pRL-TK reporter vectors were performed (Fig. 1*A*). Expression of TORC1, -2, or -3

enhanced the CRE-mediated transcription by 147-, 79-, or 121-fold, respectively, whereas no obvious transcriptional stimulation from the herpes simplex virus thymidine kinase promoter was observed, demonstrating the specificity of TORCs for CRE-dependent transcription.

To test the effect of TORC proteins on Zp, a pZp-luc reporter plasmid containing the minimal sequence (4) sufficient for transcriptional activation by TPA/ionophore or IgG was used for the assay. It was found that TORC1, -2, and -3 alone enhanced transcription from Zp by 18-, 12-, and 26-fold, respectively (Fig. 1*B*). Expression of the BZLF1 protein elevated the transcription in the absence of TORC up to 3.9-fold. Co-expression of the BZLF1 protein and TORC1, -2, and -3 further enhanced pZp-luc activity, reaching 23-, 108-, and 58-fold, respectively.

Levels of BZLF1 as well as TORC proteins were checked in Fig. 1*C* because we used the CMV promoter for BZLF1 expression, and TORC proteins might enhance the CMV promoter activity. Despite the fact that the CMV promoter has a CREB binding motif (26), levels of BZLF1 were comparable (Fig. 1*C*). A reporter assay also indicated that the CMV promoter was only marginally affected by TORC proteins (only 1.5–2.5-fold increase), at least under this condition, by TORC proteins (data not shown).

Analogous experiments were carried out in a B cell line as well (data not shown). In B cells, a single TORC2 expression caused a 9.9-fold enhancement, and with wild-type BZLF1, the activity reached to 48-fold (data not shown).

Taken together, although TORC alone can enhance transcription from the Zp, the BZLF1 protein is somehow able to further increase the transcription levels synergistically. Interestingly, although the activation of Zp by TORC2 alone was less potent than that by TORC1 or -3, co-expression of the BZLF1 protein dramatically enhanced the TORC2-mediated transcriptional activation. We also tested the effect of the BZLF1 deletion mutant dZ, which lacks the b-Zip domain, on the reporter gene as negative controls. The levels of luciferase activity were almost equal to those without the wild-type BZLF1 protein.

To further analyze the synergistic enforcement of Zp by TORCs, a pZpmZII-luc plasmid was made, the ZII element being mutated as shown in Fig. 1*B*. This mutation disrupts the CRE/activation transcription factor motif and abrogates the induction from the promoter by TPA/ionophore or IgG (4). The basal luciferase activity from this reporter plasmid became very low (only 25% of wt pZp-luc), and the activity did not appreciably increase even with TORC proteins (Fig. 1*B*), showing that the Zp activation by TORCs in the absence of the BZLF1 protein is caused through the ZII domain containing the CRE/activation transcription factor motif. However, this reporter still responded to the BZLF1 expression, reaching the same levels of transcriptional activity as with wt pZp-luc because it still contained ZIII, the BZLF1 protein binding sites. When TORC1, -2, or -3 were co-expressed with the BZLF1 protein, pZpmZII-luc exhibited significant enhancement of the transcriptional activity to around 20–50-fold. Because TORCs could not enhance this mutant promoter activity without expression of the BZLF1 protein, this activation might be due to co-operation between TORC and the BZLF1 protein. It is also noteworthy that TORC2, with the BZLF1 protein, was the most efficient, suggesting again that TORC2 is particularly compatible with the BZLF1 protein regarding synergism of the transcription activation of Zp.

To extend this analysis, we constructed pZpmZIII-luc, in which ZIIIA and ZIIIB sites are mutated. This mutation almost completely disrupts the BZLF1 protein binding (27), thereby abolishing the response to the BZLF1 protein. As pZpmZIII-luc still bears the ZII element, the reaction to TORCs was almost comparable with wt pZp-luc in the absence of the BZLF1 protein. However, even in the presence of the BZLF1 and TORC proteins, the activity remained low.

Last, when both the ZII and ZIII elements were mutated, we could not observe any enhancement of the transcription, even with or without TORCs and/or the BZLF1 protein. Overall, these results imply that activation of transcription from Zp by TORCs is mainly mediated by ZII and ZIII elements but not via ZI or other elements.

TORCs Interact with the BZLF1 Protein and Function as Coactivators for the BZLF1-mediated Transcriptional Activation—Because the above results strongly suggest that TORC proteins co-operate with the BZLF1 protein to enhance the Zp promoter activity, we next analyzed protein-protein interactions by co-IP experiments. HEK293T cells were co-transfected with expression plasmids encoding the BZLF1 protein (+Z) and FLAG-tagged TORC1, -2, or -3. Complexes immunoprecipitated with anti-FLAG antibody were resolved by SDS-PAGE and analyzed by IB using anti-BZLF1antibody. As shown in Fig. 2*A*, a 36-kDa band corresponding to the expected size of the BZLF1 protein was immunoprecipitated and identified as the BZLF1 protein (Fig. 2*A*). When we used the mutant BZLF1 protein lacking b-Zip sequence (+dZ), the interaction became less clear (Fig. 2*A*). Furthermore, because the N-terminal region of TORC proteins possesses a coiled-coil domain, a motif that has been implicated in protein-protein interactions (11), we tested if the region is involved in the interaction with the BZLF1 protein using the N-terminal deletion mutants of the TORC proteins (d1, d2, and d3). We found little or no association of the TORC mutants with the BZLF1 protein in the absence of the coiled-coil domain (Fig. 2*A*).

To examine whether TORC proteins have effects on BZLF1 protein-dependent transcriptional activity, we prepared an expression plasmid encoding a Gal4 DNA binding domain-BZLF1 fusion protein (Gal4-Z) and pGal4-luc, which contains five Gal4 binding sites and an SV40 minimal promoter. Expression of Gal4-BZLF1 fusion protein alone was able to activate pGal4-luc 3.6-fold (Fig. 2*B*). Because binding between the polypeptide of Gal4 DNA binding domain and the Gal4 binding sites in the promoter of the reporter construct is highly specific and exclusive, only the Gal4-BZLF1 fusion protein can be recruited onto the promoter, indicating that the increase reflects the net transcriptional activity of the BZLF1 protein but not of any other factors. Co-expression of Gal4-BZLF1 and TORC1, -2, and -3 proteins resulted in an 14-, 8.0-, and 9.9-fold increase, respectively, in the transcriptional activity as compared with Gal4-BZLF1 alone, whereas deletion mutants of the TORC proteins (d1, d2, and d3) failed to increase the levels

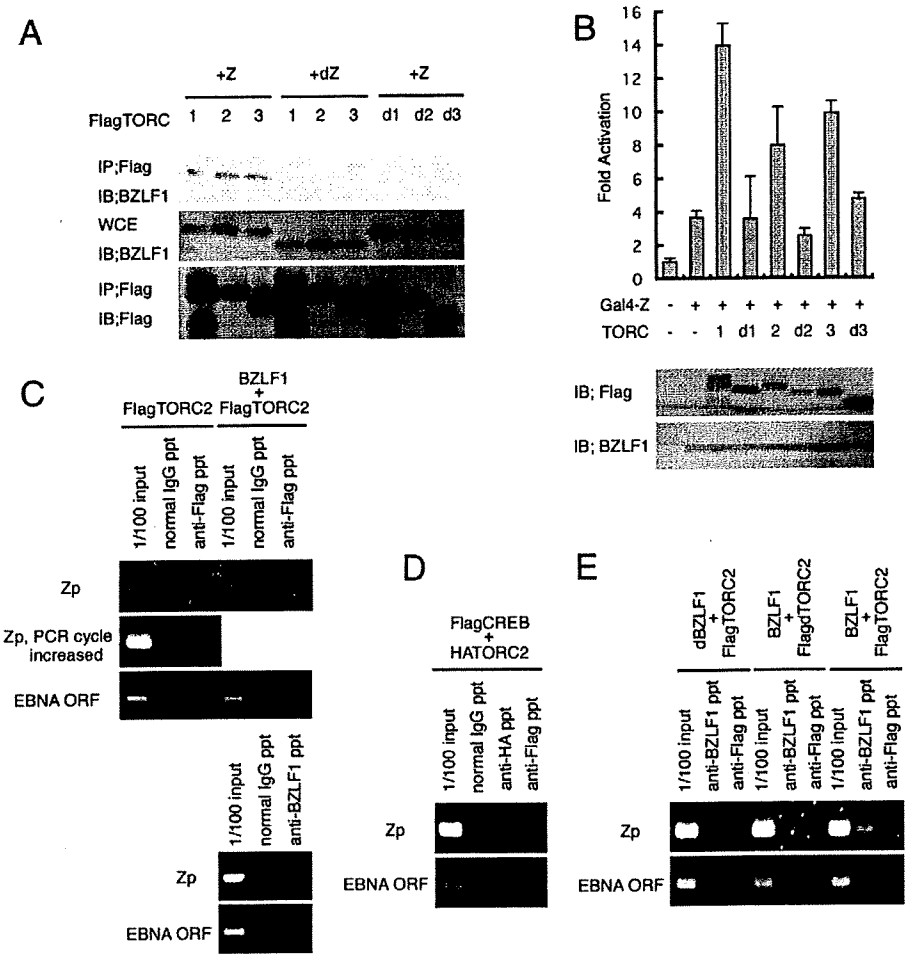

FIGURE 2. **TORCs associate with the BZLF1 protein to enhance transcription from Zp.** *A*, TORCs coimmunoprecipitated with the BZLF1 protein. FLAG-tagged TORC1, 2-, -3 or FLAG-tagged TORC with deletion at the coiled-coil domain of the protein (*d1, d2, d3*) expression vectors were co-transfected with wt BZLF1 (+*Z*) or BZLF1 without the b-Zip (+*dZ*) expression vector. IP was carried out using anti-FLAG antibody and immunoblotted with anti-BZLF1 antibody (*top*), then stripped and reprobed with anti-FLAG antibody (*bottom*). As a control, whole cell extracts (*WCE*) from the samples were also stained with anti-BZLF1 antibody (*middle*). *B*, BZLF1protein-dependent transcription is enhanced by TORCs. HEK293T cells were transfected with 25 ng of Gal4-luciferase reporter plasmid and 25 ng of the plasmid expressing the Gal4 DNA binding domain-BZLF1 fusion protein (+*Gal4-Z*) together with 100 ng of plasmids expressing TORC1, 2-, -3 or the deletion mutant at the coiled-coil domain (*d1, d2, d3*). Luciferase assays were carried out as described under "Experimental Procedures." The luciferase activity is shown as -fold activation of that with neither Gal4-Z nor TORC. Each *bar* represents the mean and S.D. of three independent transfections. Expression levels of FLAG-tagged TORC proteins and BZLF1 were also analyzed. *C*, ChIP assays were performed to evaluate the association of TORC2 with Zp. EBV-293cells were transfected with a FLAG-tagged TORC2 expression plasmid with or without the BZLF1 expression plasmid. After fixation and sonication, protein-DNA complexes were immunoprecipitated with anti-FLAG (*upper panels*) or anti-BZLF1 (*lower panels*) antibody or normal IgG followed by uncoupling of the cross-linking and PCR reactions using primers for Zp or the coding region of EBNA-1. *ORF*, open reading frame; *ppt*, precipitate. *D*, ChIP assays were carried out to show that both CREB and TORC2 were recruited to the Zp even without BZLF1. EBV-293cells transfected with FLAG-tagged CREB and hemagglutinin-tagged TORC2 expression vectors were subjected to ChIP assay with normal IgG, anti-hemagglutinin (*HA*), or -FLAG antibody followed by PCR. *E*, ChIP assays showing the importance of b-Zip of BZLF1 and coiled-coil domain of TORC2 for their recruitment to the promoter. Wild-type or deletion mutant of BZLF1 and FLAG-tagged TORC2 were transfected in pairs as noted. Precipitations were done using the anti-BZLF1 or -FLAG antibody followed by detection.

(3.5-, 2.5-, and 4.7-fold, respectively). This induction of the luciferase activity means that TORC functions through the BZLF1 protein but not through any other factors, as the only protein on the promoter is Gal4-BZLF1.

To further analyze the behavior of TORC on the Zp promoter, we checked whether TORC was recruited onto the Zp *in vivo* by ChIP assays (Fig. 2*C*). We used TORC2 because it has been studied most extensively and was demonstrated to be the most crucial factor, at least *in vivo* (28–31) (also see Figs. 1, 4, and 5). EBV-293 cells harboring EBV Bac DNA were transfected with the expression plasmid of FLAG-tagged TORC2 with or without the BZLF1 expression vector, and then ChIP assays were performed using the anti-FLAG antibody and normal IgG as a negative control. With expression of the BZLF1 protein, FLAG-tagged TORC2 was recruited to the Zp of the EBV genome in cells (Fig. 2*C*, *top panel, right*). In contrast, TORC2 was hardly detected without BZLF1 on the promoter (Fig. 2*C*, *top panel, left*) unless the PCR cycle was increased (Fig. 2*C*, *second panel*). A primer set for the EBNA-1 coding region was included (Fig. 2*C*, *third panel*) as a negative control to prove that the signal for the Zp was specific. We also confirmed that the BZLF1 protein was also recruited to the Zp when both the BZLF1 protein and FLAG-tagged TORC2 were expressed (Fig. 2*C*, *fourth panel*).

In the *second panel* in Fig. 2*C*, TORC2 was shown to be recruited onto Zp without BZLF1. Speculating that TORC2 is on the promoter through the interaction with CREB, the association of CREB protein with the promoter was also tested in Fig. 2*D*. As expected, both CREB and TORC2 came onto Zp even without BZLF1 protein.

To further confirm the importance of the interaction between BZLF1 and TORC2, we also tested dBZLF1, which lacks the b-Zip motif, and dTORC2, which is devoid of coiled-coil motif (Fig. 2*E*). When wild-type BZLF1 and wild-type TORC2 were present, both were detected bound to Zp (Fig. 2*E*, *right*). Deletion of the coiled-coil domain in TORC2 caused significant loss of its binding (Fig. 2*E*, *middle*), and truncation of BZLF1 b-Zip also harmed its association with Zp (Fig. 2*E*, *left*).

Activation of TORC2 by Dephosphorylation and Nuclear Transport—It has been demonstrated that the phosphorylation state of TORC2 regulates its activity (12, 13). Under normal conditions, TORC2 is sequestered by cytoplasmic 14-3-3 proteins, which recognize phosphorylated proteins. In the pres-

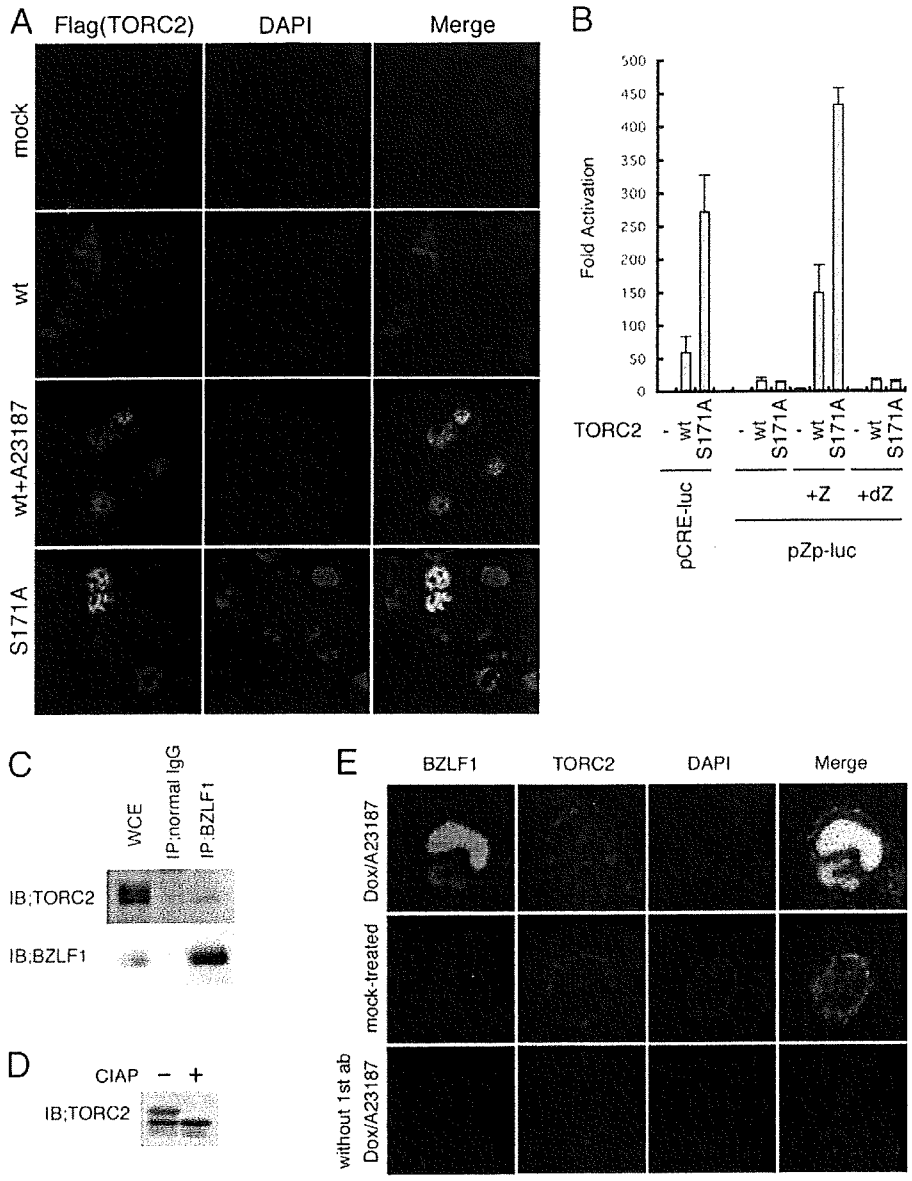

FIGURE 3. **Dephosphorylation of TORC2 at Ser171 promotes nuclear localization and transcriptional activation for Zp.** *A*, immunocytochemical analysis showing the effects of ionophore (A23187) or the S171A mutation of TORC2 on its subcellular localization. Cells were stained with anti-FLAG antibody (*green*) and 4′,6-diamidino-2-phenylindole (*DAPI*; *blue*). *wt*, wild type. *B*, transient reporter assay results for the CRE-mediated promoter (*left*) and Zp (*right*). HEK293T cells were transfected with pCRE-luc or pZp-luc together with expression vectors for the wild-type or the S171A mutant of TORC2. For pZp-luc, plasmids with wt BZLF1 (+*Z*) or a mutant BZLF1 lacking the b-Zip domain (+*dZ*) were also transfected. Luciferase assays were carried out as described under "Experimental Procedures." The luciferase activity is shown as -fold activation of that without TORC2 for pCRE-luc (*left part*) and that with neither BZLF1 nor TORC2 for pZp-luc (*right*). Each *bar* represents the mean and S.D. of three independent transfections. *C*, dephosphorylated TORC2 interacts with BZLF1. Protein lysates from B95-8 cells treated with TPA and A23187 for 24 h were subjected to IP using normal IgG or anti-BZLF1 antibody followed by SDS-PAGE and IB with anti-TORC2 (*upper panel*) and -BZLF1 (*lower panel*) antibodies. *WCE*, whole cell extracts. *D*, phosphorylation of TORC2. Proteins from B95-8 cells treated with TPA and A23187 were lysed in calf intestine alkaline phosphatase buffer and incubated with or without calf intestine alkaline phosphatase for 1h followed by SDS-PAGE and IB with anti-TORC2 antibody. *E*, localization of endogenous TORC2 (*red*) and BZLF1 protein (*green*) in Tet-BZLF1/B95-8 cells. Cells were mock-treated (*middle panels*) or treated with doxycycline and A23187 (*top* and *bottom panels*) and analyzed by immunofluorescence assay using confocal microscopy. 4′,6-Diamidino-2-phenylindole (*blue*) staining was also carried out. As a negative control, treatment with first antibodies was omitted for the *bottom panels*.

ence of calcium signaling, TORC2 is dephosphorylated by calcineurin at phosphoserine 171, triggering disruption of the interaction with 14-3-3 and import into the nucleus where it can activate CRE-mediated transcription. We could confirm that the addition of A23187, a calcium ionophore, enhanced the translocation of FLAG-tagged TORC2 to nuclei as well as an alanine-substituted TORC2 mutant at Ser-171 (S171A) (Fig. 3*A*).

Using the construct, we examined the effect of the S171A mutation of TORC2 on the Zp of EBV (Fig. 3*B*). Wild-type TORC2 induced CRE-dependent reporter gene expression 59-fold and its S171A mutant 272-fold when compared with the luciferase activity without TORC. The result was quite similar to a previous report (12) indicating the reliability of this system. When both wt BZLF1 protein (+Z) and wild-type TORC2 were expressed, Zp was activated 150-fold as compared with the activity with neither wt BZLF1 protein nor TORC2. Co-expression of wt BZLF1 protein (+Z) and the S171A TORC2 exhibited 433-fold activation, whereas co-expression of the mutant BZLF1 protein lacking b-Zip sequence (+dZ) and the S171A TORC2 were without effect.

In Fig. 3*C*, coimmunoprecipitation assay not only confirmed the interaction between endogenous TORC2 and BZLF1 but also revealed that BZLF1 protein preferentially associates with faster-migrating species of TORC2, which are dephosphorylated forms of the protein (Fig. 3*D*) (12, 31).

In the EBV lytic replication, it was previously demonstrated that the BZLF1 protein localizes to replication compartments, the sites of viral genome replication and transcription, in the nuclei (3, 32). Immunofluorescence analysis showed that upon induction, TORC2 was recruited to the replication compartments and colocalized with BZLF1 protein in the nucleus in the lytic phase, whereas the protein was localized in the cytoplasm in the latent phase (Fig. 3*E*).

In addition, we confirmed that cyclosporin A, an inhibitor of calcineurin signaling pathway, clearly blocks the BZLF1 expression in B95-8 cells treated with TPA and calcium ionophore (supplemental Fig. S1). These results

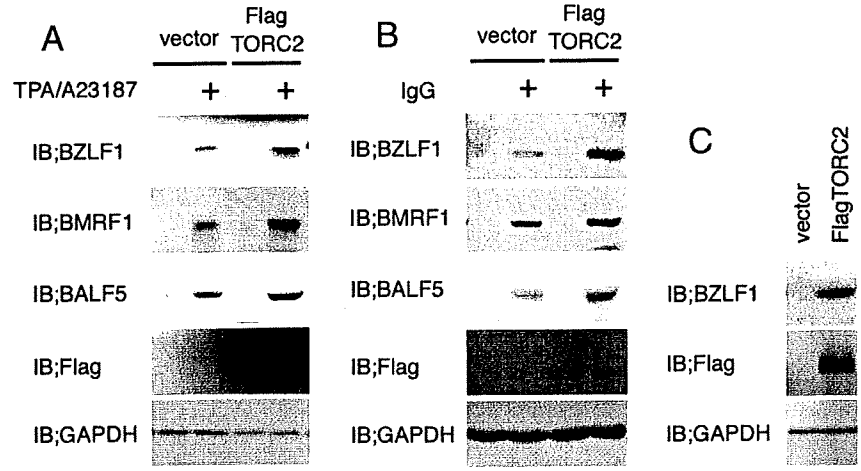

FIGURE 4. **Increased expression of the BZLF1 protein on exogenous expression of TORC2.** *A* and *B*, GTC-4 (*A*) cells or Akata (*B*) cells were transfected with the TORC2 expression vector, and 24 h thereafter, TPA(20 ng/ml) and A23187 (0.5 μM) or IgG was added to the culture followed by incubation for another 24 h and IB with anti-BZLF1, -BMRF1, -BALF5, -FLAG, and -GAPDH antibodies. *C*, EBV-293 cells transfected with the TORC2 expression vector were incubated for 24 h followed by IB with anti-BZLF1, -FLAG, and -GAPDH antibodies.

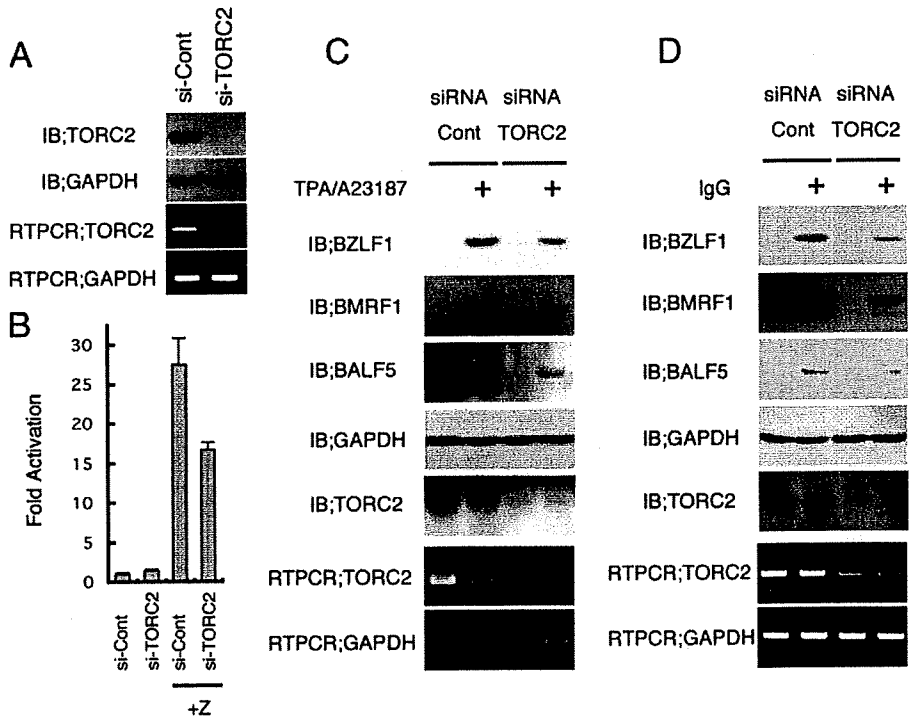

FIGURE 5. **BZLF1-mediated transcription depends on endogenous TORC2 expression.** *A* and *B*, HEK293T cells were transfected with duplexes of 21-nucleotide siRNA against TORC2 (*si-TORC2*) or control (*si-Cont*) siRNA together with 10 ng of pZp-luc with or without 10 ng pcDNABZLF1 (+*Z*). IB and RT-PCR assays (*A*) and luciferase assays (*B*) were carried out as described under "Experimental Procedures." The luciferase activity is shown as -fold activation of that with control siRNA without BZLF1. Each *bar* represents the mean and S.D. of three independent transfections. *C* and *D*, knock-down of TORC2 mRNA in GTC-4 (*C*) and Akata (*D*) cells. Cells transfected with siRNA against TORC2 (*siRNA TORC2*) or the Control (*siRNA Cont*) were cultured with TPA (20 ng/ml) and A23187 (0.5 μM) or IgG for 24 h. Protein or mRNA levels of BZLF1, BMRF1, BALF5, TORC2, and GAPDH were examined by IB or RT-PCR.

suggest a cooperative influence of the BZLF1 protein and dephosphorylated TORC2 in the presence of calcineurin signaling activation.

Role of TORC2 in EBV Reactivation from Latency—To examine the role of TORC2 in EBV reactivation from latency, GTC-4 cells, in which EBV is latently infected, were transfected with the TORC2 expression vector and incubated with or without TPA/A23187 (Fig. 4*A*). The cells expressed the BZLF1, BMRF1, and BALF5 proteins in response to TPA/A23187 treatment, and further exogenous expression of TORC2 increased the levels of the proteins. A similar result was obtained in Akata cells (Fig. 4*B*). Expression of S171A mutant of TORC2 appears to impact on BZLF1 levels significantly (data not shown). We also tested EBV-293 cells, in which levels of exogenous gene expression are very efficient (Fig. 4*C*). Even in the absence of TPA/A23187, overexpression of TORC2 clearly enhanced BZLF1 protein levels.

To examine the function of TORC2 under physiological conditions, we employed siRNA technology using a synthetic oligonucleotide that forms a duplex RNA encoding partial nucleotides from TORC2. As shown in Fig. 5*A*, treatment with siRNA against TORC2 reduced the level of TORC2 mRNA in HEK293T cells, whereas the level of GAPDH remained unchanged. TORC2 siRNA treatment also resulted in a decrease in the BZLF1-mediated transcription (Fig. 5*B*; +*Z*, *si-TORC2*) when compared with control siRNA treatment (+*Z*, *si-Cont*).

In addition, the effect of siRNA against TORC2 was also examined in GTC-4 and Akata cells, as shown in Fig. 5, *C* and *D*, respectively. Treatment with TORC2 siRNA suppressed the mRNA expression of TORC2, whereas the GAPDH gene was unaffected. The treatment also reduced the levels of viral lytic proteins including BZLF1.

To eliminate the possibility that the siRNA against TORC2 might elicit interferon signaling pathway, we analyzed interferon-β expression by RT-PCR (33) because activation of the signaling pathway provoked by double-stranded RNA causes the promoter activation. Treatment with TORC2 siRNA did not induce the levels of interferon-β (supplemental Fig. S2), indicating that interferon signaling is not activated by si-TORC2.

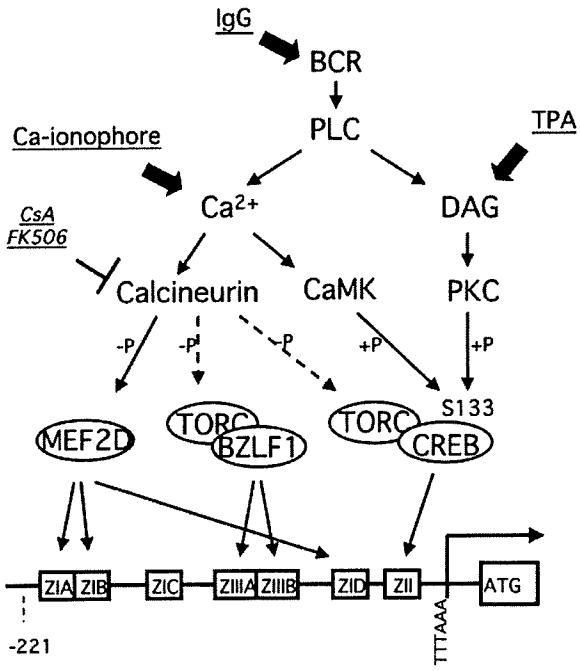

FIGURE 6. **Proposed model for EBV Zp activation.** TORC2, as well as myocyte enhancer factor 2D (*MEF2D*), is activated by calcineurin, a phosphatase that can be inhibited by CsA or FK506. TORC2 is able to associate with CREB or the BZLF1 protein and enhances Zp activity through binding to ZII and ZIII *cis* elements. *PLC*, phospholipase C; *PKC*, protein kinase C; *DAG*, diacylglycerol; *CaM*, calmodulin; BCR, B-cell receptor.

To deny the possibility that the TORC2 siRNA used in Fig. 5 might act through unknown off-target effects, we tested another TORC2 siRNA in supplemental Fig. S3. It also suppressed the expression level of the BZLF1 protein.

To test if not only TORC2 but also other TORCs might be involved in this process, all the members of TORC proteins were silenced simultaneously. In that case, however, reduction of the BZLF1 level was no stronger than that by si-TORC2 only (Fig. supplemental S4), suggesting the dominant role of TORC2 in this transcriptional activation. These results strongly suggest the importance of TORC2 in BZLF1 production and EBV reactivation from latency.

DISCUSSION

In this report we document evidence that TORC is able to enhance transcription from Zp and, more interestingly, that TORC interacts with the BZLF1 protein to activate the promoter very strongly. Fig. 6 shows our working model for Zp induction. Previous studies have demonstrated that both ZI and ZII elements are necessary for the initial activation (2, 34). It has been reported that myocyte enhancer factor 2D plays a crucial role in virus reactivation from latency (5), being dephosphorylated by calcineurin and enhancing its binding to ZI. CREB family transcription factors bind to ZII when phosphorylated by protein kinase C, calmodulin kinase, or possibly mitogen-activated protein kinases. In addition to the activation by phosphorylation, our reporter assays indicated that CREB is activated by TORC in a CREB phosphorylation-independent manner. Furthermore, our study strongly suggests that TORC also potentiates the promoter activity by binding to the ZIII element through the BZLF1 protein. Calcineurin, a serine/threonine-phosphatase sensitive to cyclosporin A is responsible for the dephosphorylation and the activation of TORC. In turn, cyclosporin A and FK506 are very effective for suppressing EBV.

A number of cellular proteins have been reported to interact with the BZLF1 protein, including p53 (35, 36), C/EBPα (37), NF-κB (38), basic transcriptional machinery TFII components (39), and CREB-binding protein (CBP) (40, 41). Among these, CBP has histone acetyltransferase activity and cooperates with the BZLF1 protein to transactivate BZLF1-dependent transcription, inducing the viral lytic cycle. Mutation analysis revealed that at least the homodimerization domain (b-Zip) of the BZLF1 protein is required for its interaction with CBP, but other parts of the protein also must be involved in the association (40, 41). The BZLF1 protein also interacts with TFII components mainly through the transactivation domain and stabilizes the association of initiation complexes on DNA. Stable assembly of general transcriptional machinery might promote transcription from BZLF1-responsive promoters. Interestingly, TORC enhances the interaction of CREB with the $TAF_{II}130$ component of TFIID (11), and at least TORC2 mediates target gene activation by associating with CBP/p300 and increasing its recruitment to CREB-responsive promoters (31). From these studies and our own results, the BZLF1 protein may not only directly recruit CBP/p300 and basic transcriptional machinery but also be able to recruit them through TORC2.

Besides EBV, transcription from human T-cell leukemia virus type 1 long terminal repeats is also affected by TORC proteins (21, 42). TORC activates long terminal repeats through interaction with the viral transcriptional factor Tax as well as CREB. So this mode of the action is quite parallel to the situation with EBV. Because both human T-cell leukemia virus type 1 and EBV are lymphotropic viruses, there is a possibility that other lymphotropic viruses such as the human immunodeficiency virus might also be controlled by TORC proteins.

Curiously, although CMV immediate-early promoter has a CREB binding motif (26), we here observe that transcription from the promoter is less affected by TORC proteins (Fig. 1*C*) when compared with the BZLF1 promoter, an EBV immediate-early gene. Others also have used expression vectors driven by the CMV immediate-early promoter and shown that the promoter activity is relatively unaffected (22, 42). It is speculated that this might be because the activation by TORC is dependent on the promoter context (11, 43). Because immediate-early genes of herpesviruses are crucial for lytic infection, distinct dependence of the promoters on TORC proteins may reflect differences in the characters of those herpesviruses.

Although TORC proteins could enhance Zp 100-fold in reporter assays, overexpression or ablation of TORC2 had only a relatively small impact on BZLF1 production under physiological conditions. It is likely that transcriptional suppressors of the promoter such as YY1 (44) might inhibit transcription. Another intriguing possibility is that there might be epigenetic regulation such as DNA methylation or histone deacetylation. Interestingly, Gruffat *et al.* (45) reported that myocyte enhancer factor 2 family protein, a crucial transactivator for the

Zp, recruits class II histone deacetylases to suppress transcription from Zp. They argued that the switch from latency to the productive cycle is dependent at least in part on the post-translational modification of myocyte enhancer factor 2 and local acetylation state of histones around the Zp. Likewise, the transcriptional co-activator TORC can associate with BCL-3, which recruits histone deacetylases to inhibit transcription (22). These results and the cited reports suggest that the molecular mechanism regulating EBV reactivation from latency is not quite as simple as expected, and further clarification of the mechanism of BZLF1-mediated transcription is necessary. Elucidation of associating factors and chromosomal environment of the Zp proximity may contribute to the development of anti-EBV compounds.

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REFERENCES

1. Speck, S. H., Chatila, T., and Flemington, E. (1997) *Trends Microbiol.* **5,** 399–405
2. Amon, W., and Farrell, P. J. (2005) *Rev. Med. Virol.* **15,** 149–156
3. Tsurumi, T., Fujita, M., and Kudoh, A. (2005) *Rev. Med. Virol.* **15,** 3–15
4. Flemington, E., and Speck, S. H. (1990) *J. Virol.* **64,** 1217–1226
5. Liu, S., Liu, P., Borras, A., Chatila, T., and Speck, S. H. (1997) *EMBO J.* **16,** 143–153
6. Liu, S., Borras, A. M., Liu, P., Suske, G., and Speck, S. H. (1997) *Virology* **228,** 11–18
7. Liu, P., Liu, S., and Speck, S. H. (1998) *J. Virol.* **72,** 8230–8239
8. Ruf, I. K., and Rawlins, D. R. (1995) *J. Virol.* **69,** 7648–7657
9. Flemington, E., and Speck, S. H. (1990) *J. Virol.* **64,** 1227–1232
10. Iourgenko, V., Zhang, W., Mickanin, C., Daly, I., Jiang, C., Hexham, J. M., Orth, A. P., Miraglia, L., Meltzer, J., Garza, D., Chirn, G. W., McWhinnie, E., Cohen, D., Skelton, J., Terry, R., Yu, Y., Bodian, D., Buxton, F. P., Zhu, J., Song, C., and Labow, M. A. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 12147–12152
11. Conkright, M. D., Canettieri, G., Screaton, R., Guzman, E., Miraglia, L., Hogenesch, J. B., and Montminy, M. (2003) *Mol. Cell* **12,** 413–423
12. Screaton, R. A., Conkright, M. D., Katoh, Y., Best, J. L., Canettieri, G., Jeffries, S., Guzman, E., Niessen, S., Yates, J. R., III, Takemori, H., Okamoto, M., and Montminy, M. (2004) *Cell* **119,** 61–74
13. Bittinger, M. A., McWhinnie, E., Meltzer, J., Iourgenko, V., Latario, B., Liu, X., Chen, C. H., Song, C., Garza, D., and Labow, M. (2004) *Curr. Biol.* **14,** 2156–2161
14. Goldfeld, A. E., Liu, P., Liu, S., Flemington, E. K., Strominger, J. L., and Speck, S. H. (1995) *Virology* **209,** 225–229
15. Delecluse, H. J., Hilsendegen, T., Pich, D., Zeidler, R., and Hammerschmidt, W. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 8245–8250
16. Tatsumi, Y., Sugimoto, N., Yugawa, T., Narisawa-Saito, M., Kiyono, T., and Fujita, M. (2006) *J. Cell Sci.* **119,** 3128–3140
17. Kanamori, M., Tajima, M., Satoh, Y., Hoshikawa, Y., Miyazawa, Y., Okinaga, K., Kurata, T., and Sairenji, T. (2000) *Virus Genes* **20,** 117–125
18. Kudoh, A., Fujita, M., Kiyono, T., Kuzushima, K., Sugaya, Y., Izuta, S., Nishiyama, Y., and Tsurumi, T. (2003) *J. Virol.* **77,** 851–861
19. Takada, K., Horinouchi, K., Ono, Y., Aya, T., Osato, T., Takahashi, M., and Hayasaka, S. (1991) *Virus Genes* **5,** 147–156
20. Daikoku, T., Kudoh, A., Sugaya, Y., Iwahori, S., Shirata, N., Isomura, H., and Tsurumi, T. (2006) *J. Biol. Chem.* **281,** 11422–11430
21. Koga, H., Ohshima, T., and Shimotohno, K. (2004) *J. Biol. Chem.* **279,** 52978–52983
22. Hishiki, T., Ohshima, T., Ego, T., and Shimotohno, K. (2007) *J. Biol. Chem.* **282,** 28335–28343
23. Ego, T., Tanaka, Y., and Shimotohno, K. (2005) *Oncogene* **24,** 1914–1923
24. Murata, T., and Shimotohno, K. (2006) *J. Biol. Chem.* **281,** 20788–20800
25. Daikoku, T., Kudoh, A., Fujita, M., Sugaya, Y., Isomura, H., and Tsurumi, T. (2004) *J. Biol. Chem.* **279,** 54817–54825
26. Lang, D., Fickenscher, H., and Stamminger, T. (1992) *Nucleic Acids Res.* **20,** 3287–3295
27. Taylor, N., Flemington, E., Kolman, J. L., Baumann, R. P., Speck, S. H., and Miller, G. (1991) *J. Virol.* **65,** 4033–4041
28. Koo, S. H., Flechner, L., Qi, L., Zhang, X., Screaton, R. A., Jeffries, S., Hedrick, S., Xu, W., Boussouar, F., Brindle, P., Takemori, H., and Montminy, M. (2005) *Nature* **437,** 1109–1111
29. Kuraishy, A. I., French, S. W., Sherman, M., Herling, M., Jones, D., Wall, R., and Teitell, M. A. (2007) *Proc. Natl. Acad. Sci. U. S. A.* **104,** 10175–10180
30. Dentin, R., Liu, Y., Koo, S. H., Hedrick, S., Vargas, T., Heredia, J., Yates, J., III, and Montminy, M. (2007) *Nature* **449,** 366–369
31. Ravnskjaer, K., Kester, H., Liu, Y., Zhang, X., Lee, D., Yates, J. R., III, and Montminy, M. (2007) *EMBO J.* **26,** 2880–2889
32. Daikoku, T., Kudoh, A., Fujita, M., Sugaya, Y., Isomura, H., Shirata, N., and Tsurumi, T. (2005) *J. Virol.* **79,** 3409–3418
33. Arimoto, K., Takahashi, H., Hishiki, T., Konishi, H., Fujita, T., and Shimotohno, K. (2007) *Proc. Natl. Acad. Sci. U. S. A.* **104,** 7500–7505
34. Bryant, H., and Farrell, P. J. (2002) *J. Virol.* **76,** 10290–10298
35. Zhang, Q., Gutsch, D., and Kenney, S. (1994) *Mol. Cell. Biol.* **14,** 1929–1938
36. Kudoh, A., Fujita, M., Zhang, L., Shirata, N., Daikoku, T., Sugaya, Y., Isomura, H., Nishiyama, Y., and Tsurumi, T. (2005) *J. Biol. Chem.* **280,** 8156–8163
37. Wu, F. Y., Chen, H., Wang, S. E., ApRhys, C. M., Liao, G., Fujimuro, M., Farrell, C. J., Huang, J., Hayward, S. D., and Hayward, G. S. (2003) *J. Virol.* **77,** 1481–1500
38. Morrison, T. E., and Kenney, S. C. (2004) *Virology* **328,** 219–232
39. Deng, Z., Chen, C. J., Zerby, D., Delecluse, H. J., and Lieberman, P. M. (2001) *J. Virol.* **75,** 10334–10347
40. Adamson, A. L., and Kenney, S. (1999) *J. Virol.* **73,** 6551–6558
41. Zerby, D., Chen, C. J., Poon, E., Lee, D., Shiekhattar, R., and Lieberman, P. M. (1999) *Mol. Cell. Biol.* **19,** 1617–1626
42. Siu, Y. T., Chin, K. T., Siu, K. L., Yee Wai Choy, E., Jeang, K. T., and Jin, D. Y. (2006) *J. Virol.* **80,** 7052–7059
43. Xu, W., Kasper, L. H., Lerach, S., Jeevan, T., and Brindle, P. K. (2007) *EMBO J.* **26,** 2890–2903
44. Montalvo, E. A., Cottam, M., Hill, S., and Wang, Y. J. (1995) *J. Virol.* **69,** 4158–4165
45. Gruffat, H., Manet, E., and Sergeant, A. (2002) *EMBO Rep.* **3,** 141–146

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Genetic Analysis of Hepatitis C Virus with Defective Genome and Its Infectivity in Vitro▽

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Replication and infectivity of hepatitis C virus (HCV) with a defective genome is ambiguous. We molecularly cloned 38 HCV isolates with defective genomes from 18 patient sera. The structural regions were widely deleted, with the 5′ untranslated, core, and NS3-NS5B regions preserved. All of the deletions were in frame, indicating that they are translatable to the authentic terminus. Phylogenetic analyses showed self-replication of the defective genomes independent of full genomes. We generated a defective genome of chimeric HCV to mimic the defective isolate in the serum. By using this, we demonstrated for the first time that the defective genome, as it is circulating in the blood, can be encapsidated as an infectious particle by *trans* complementation of the structural proteins.

Viruses with a deletion mutation in their genome have been identified as defective interfering (DI) particles for many virus species (1, 3, 9, 16). Part of the DI virus genome is deleted, but regions indispensable for replication and packaging are preserved. Most DI viruses occur spontaneously in the course of cell culture infected with a high titer of wild-type viruses. Hepatitis C virus (HCV) with a defective genome has been found in liver and serum specimens of some HCV patients (4, 8, 15). HCV has a plus-strand RNA genome that encodes the viral core, E1, E2, and p7 structural proteins and NS2, NS3, NS4A, NS4B, NS5A, and NS5B nonstructural proteins (10). According to the reports, the deletions have been found mainly in the structural region and most of the deletions are in frame, but some deletions are out of frame (4), raising questions about whether the defective HCV genome is merely a by-product of a full genome or a self-replicating genome and whether it can be encapsidated into an infectious virus particle.

In the present study, we molecularly cloned 38 HCV isolates with defective genomes from HCV patient sera to address these questions by genetic analyses and infection experiments. As long as we explored, all of the deletions were in frame, indicating the potential to support translation from the authentic initiation codon to the termination codon, although the structural region was widely deleted, as reported previously. Phylogenetic analyses evidenced self-replication of the defective genomes independent of full genomes. We demonstrated for the first time, by *trans* complementation experiments, that the defective genome, as it is circulating in the blood, can be encapsidated as an infectious particle, designated HCV_{CCD}.

First, to amplify HCV cDNAs in 21 serum specimens from 18 HCV patients (genotype 1b), we performed three sets of long-distance reverse transcription (RT)-PCRs flanking (i) the 5′ untranslated region (UTR) to the 5′ part of the NS3 region, (ii) the remaining part of the NS3 region to the end of NS5B, and (iii) the 5′ UTR to the end of the NS5B region (Fig. 1A). The specimens were collected with informed consent. cDNA was synthesized with RNase H-deficient reverse transcriptase Superscript III (Invitrogen, Carlsbad, CA) at a higher temperature (55°C) to reduce template switching and mispriming. PCRs were performed in a (hemi)nested manner with high-fidelity polymerase KOD plus or KOD FX (Toyobo, Osaka, Japan) as described previously (5). For some target nucleotide positions, a mixture of two or three primers was used to reduce mismatches due to sequence heterogeneity (Table 1). Of the 21 specimens examined, representative results are shown in Fig. 1. An amplicon of the 5′ UTR-NS3 region of the predicted size (ca. 3.7 kb) was detected in all specimens (18/18), and representative results are shown in Fig. 1B. In addition, a shorter amplicon suggestive of a defective HCV genome was simultaneously present in four specimens from 1 (R4) of 12 cases of clinically mild hepatitis and from 3 (T5, K3, and K4-pre) of 6 cases of active hepatitis (clinical data not shown). Defective genomes were found in the patients with relatively higher copy numbers of HCV RNA ($>8.1 \times 10^5$ copies/ml in the 5′ UTR, Table 2), suggesting that the coexistence of a defective genome is related to hepatitis severity. The authentic-size amplicon was poorly detected when coexisting with a defective HCV genome shorter than 2 kb (T5 and K3), presumably because of preferential amplification of the shorter amplicon. A shorter amplicon was not detected for the NS3-

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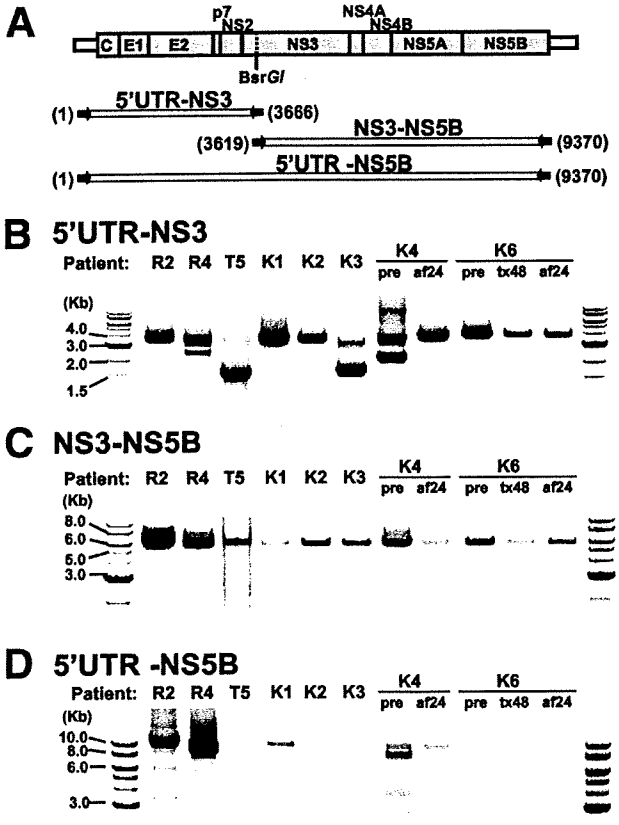

FIG. 1. Representative results of long-distance RT-PCRs for serum HCV. (A) The three sets of long-distance RT-PCR used: 5′ UTR-NS3 (5′ UTR to the 5′ part of the NS3 region), NS3-NS5B (the remaining part of the NS3 region to the end of NS5B), and 5′ UTR-NS5B (5′ UTR to the end of the NS5B region). The nucleotide positions of the 5′ and 3′ ends of each amplicon are indicated in parentheses. PCR products were electrophoresed and stained with ethidium bromide. Results of the representative 11 specimens (eight patients) are shown for 5′ UTR-NS3 (B), NS3-NS5B (C), and 5′ UTR-NS5B (D). Serum specimens were collected from patients K4 and K6 before interferon treatment (pre), at the end of the full 48-week treatment period (tx48), and 24 weeks after the full treatment period (af24). DNA molecular size markers are at both sides of panels B to D.

NS5B region, while the amplicon of the predicted size (ca. 5.6 kb) was detected in all of the specimens, albeit with various efficiencies (Fig. 1C). The 5′ UTR-NS5B region, which covers almost the whole genome, was then amplified, and an amplicon of the predicted size (9.4 kb) was detected in 10 specimens from nine patients (5 specimens in Fig. 1D). Of the successfully amplified specimens, two (R4 and K4-pre) also contained a shorter amplicon, in accordance with the results of the 5′ UTR-NS3 PCR. The NS3-NS5B region is essential for autonomous replication of HCV as an RNA replicon in vitro (5–7). It has been shown that NS5A is the only nonstructural protein that can *trans* complement HCV replication (13). They used a nonadaptive mutation of NS5A as a replication-incompetent NS5A protein instead of a deletion mutant protein. Thus, we speculate that deletion of the NS3-NS5B region cannot be complemented in *trans*. Intriguingly, the shorter amplicon was not detected after full-term interferon treatment in patient K4 (K4-af24), although it was detected prior to treatment (K4-pre) (Fig. 1B and D). The possible reasons for this are that (i) the defective genome disappeared naturally, (ii) packaging of the defective genome by the helper virus was impeded by an unknown mechanism of interferon, or (iii) replication of the defective genome is preferentially inhibited by the interferon pathway. Further studies are needed to reveal the effect of a defective HCV genome on the pathogenesis and treatment of HCV.

A total of 38 isolates with defective HCV genomes were molecularly cloned into plasmid vector pASGT (unpublished data) from the shorter amplicons of the 5′ UTR-NS3 PCR from four serum specimens (R4, T5, K3, and K4-pre in Fig. 1B) at the AscI and BsrGI restriction sites. The nucleotide sequences were determined with an autosequencer (3730 DNA analyzer; Applied Biosystems, Foster City, CA). Sequence analyses revealed that the structural region was widely deleted in all of the defective isolates and that the deletion ranges were quite diverse among the isolates (extending up to the NS2 region) (Fig. 2A). In contrast, the 5′ UTR and core regions were constantly preserved, suggesting that these regions, as well as the NS3-NS5B region, are indispensable for the production of HCV with a defective genome. Intriguingly, defective genomes with different deletion patterns coexisted in single specimens from two patients (three patterns in patient K3 and four patterns in patient K4-pre). Moreover, two deletions in a single genome were observed in five isolates from patient R4 (isolate R4S-5). As many as three deletions in a single genome were observed in the isolate from patient K3 (e.g., isolate K3S-15), in which two small deletions resulted in two tiny residual fragments. Such diversity in deletion ranges indicates flexibility of the remaining structural region for the replication of defective HCV genomes. Nevertheless, all of the deletions identified in the 38 isolates were in frame (Fig. 2B), implying that these defective HCV genomes have the potential for translation from the core to the authentic end of NS5B without a frameshift.

To determine the ratio of defective to full genomes, we performed quantitative PCRs targeting a relatively conserved E2 sequence, which is commonly deleted in the defective genomes, with primers listed in Table 1. Calculation of the 5′ UTR/E2 ratio, which must theoretically be 1 without the existence of the defective genome, showed higher values (1.7 to 2.45) in specimens containing the defective genomes (R4, K3, and K4pre in Table 2), indicating that the defective genome level in serum is 0.7 to 1.45 times the full genome level. However, to clarify the impact of defective genomes on pathogenesis and their effect on the treatment of HCV, accumulation of more data is needed.

The nucleotide sequence comparison of 38 defective HCV isolates showed sequence diversity. Such diversity was observed even among isolates obtained from the same specimen. Perhaps such diversity is a result of self-replication and the subsequent evolution of the defective HCV genome. To explore this possibility, phylogenetic analyses were performed on the nucleotide sequence data from patient K4. Sequences at the 5′ and 3′ maximum overlapping regions located outside the deletions were separately compared (Fig. 2A), and phylogenetic trees were created by the neighbor-joining method with GENETYX software (Genetyx Inc., Tokyo, Japan). As a re-

TABLE 1. Primers used for long-distance and quantitative RT-PCRs in this study

Test and region(s)	Direction	Primer(s)[a]	Sequence[b]	Position[c]
Long-distance PCR				
5′ UTR-NS3	RT	606R/712R	GTTTCCATAGACTC(A/G)ACGGG	3930–3949
5′ UTR-NS3, 5′ UTR-NS5B	1st forward	420	GGCGACACTCCACCATAGATCACTC	1–42
5′ UTR-NS3	1st reverse	605R/713R	ACCGGAATGACATCAGCATG(T/C)CTCGT	3741–3766
5′ UTR-NS3, 5′ UTR-NS5B	2nd forward	AscT7-420	ATCGTAGGCGCGCCTC*TAATACGACTCACTATA*GC CAGCCCCCGATTGGGGGCGACACTCCACCATAGATCACTC	1–42
5′ UTR-NS3	2nd reverse	604R/714R	CGAGGTCCTGGTCTACATT(G/A)GTGTACAT	3639–3666
NS3-NS5B, 5′ UTR-NS5B	RT	386R	AATGGCCTATTGGCCTGGAG	9390–9392
NS3-NS5B	1st forward	602/723	CCACCGCAACACAATCTTTCCT(G/A)GCGAC	3529–3556
NS3-NS5B, 5′ UTR-NS5B	1st reverse	719R/720R/721R	GAGTGTTTAGCTCCCCGTTCA(T/C/G)CGGTTGGG	9363–9392
NS3-NS5B	2nd forward	603/724	CAAAGGGTCCAATCACCCA(A/G)ATGTACAC	3619–3646
NS3-NS5B, 5′ UTR-NS5B	2nd reverse	607R/654R/722R	CGGTTGGGGAGCAGGTA(G/A/G)A(T/T/C)GCCTAC	9345–9370
Quantitative PCR				
5′ UTR	RT	738RH	ACTCGCAAGCACCCTATCAGGC	291–312
5′ UTR	Forward	736	AAGCGTCTAGCCATGGCGTTAGTA	73–96
5′ UTR	Reverse	737R	GGCAGTACCACAAGGCCTTTCG	272–293
5′ UTR	Probe	733FB	FAM-TCTGCGGAACCGGTGAGTACAC-BHQ1	147–168
E2	RT	743RH/744RH/753RH/753RH	CAACGCTCTCCTCG(A/A/G/G)GTCCA(A/G/A/G)TTGCA	2271–2296
E2	Forward[d]	751/752	GGCCTCCACATGGCAA(C/T)TGGTTCGG	1972–1993
E2	Forward[d]	739/740	CCGCCGCAAGGCAACTGGTT(C/T)GG	1974–1993
E2	Reverse	741R/742R	GCCTCGGGGTGCTTCCGGAAGCA(G/A)TCCGT	2088–2116
E2	Probe	734FB/735FB	FAM-TGGATGAA(T/C)AGCACTGGGTTCACCAAGAC-BHQ1	2001–2029

[a] Primers separated by slashes harbor a nucleotide substitution(s) (in parentheses) in the sequence in the same order.
[b] An underline and a double underline indicate recognition sequences for AscI and BsrGI, respectively, with which the PCR products were subcloned into plasmid vector pASGT5. Italics denote the T7 promoter, which was used to synthesize RNA in vitro from the T5S2 isolate (Fig. 4A).
[c] Nucleotide positions correspond to the HCV-JS sequence (12).
[d] Forward primers for E2 were mixed in the reaction mixture.

sult, isolates with the same deletion pattern formed genetic clusters that were distinct from each other, as well as from those of nondefective HCV isolates (Fig. 3A and B). Similar results were obtained for the other patients with defective HCV genomes (data not shown). These results suggest that a defective HCV genome is capable of replication to accumulate mutations and to evolve independently of the nondefective HCV genome.

TABLE 2. Quantitative PCRs for the 5′ UTR and E2 regions of HCV[a]

Region for quantification	No. of copies/ml		5′ UTR/E2 ratio
	5′ UTR	E2	
R2	2.0×10^6	1.7×10^6	1.17
R4	5.3×10^6	2.2×10^6	2.44
T5	ND[b]	ND[b]	
K1	8.3×10^5	8.4×10^5	0.99
K2	3.6×10^5	3.5×10^5	1.01
K3	8.6×10^5	5.1×10^5	1.7
K4pre	8.1×10^5	3.3×10^5	2.45
K4af24	4.5×10^5	4.4×10^5	1.01

[a] For quantification of the 5′ UTR and E2 regions, the TaqMan Fast PCR Universal mixture and the 7500 Fast Real-Time PCR system (Applied Biosystems) were used in a two-step method with the primers and probes shown in Table 1 according to the manufacturer's protocol. The copy number of HCV was determined by the standard-curve method with serial dilutions of the synthesized full-length HCV RNA.
[b] ND, not determined due to sample shortage.

Next, the ability of the defective HCV genome to be encapsidated and released from cells as HCV_{CCD} was examined. A genotype 1b replicon RNA lacking the structural region was synthesized by using defective isolate T5S-2 from patient T5 (Fig. 2 and 4A) as the template in an in vitro transcription system (MEGAscript T7 kit; Ambion, Inc., Austin, TX) under the control of the T7 promoter. Also, capped mRNA encoding the genotype 1b structural proteins from the same patient (designated C-NS2 in Fig. 4A) was synthesized in vitro with the mMessage mMachine T7 kit (Ambion). Both synthesized RNAs were cotransfected into Huh7.5 hepatoma cells. However, HCV_{CCD} was not obtained, presumably because of low replication or virus productivity of genotype 1b HCV per se. In fact, we transfected the defective RNA alone and observed the replication and protein expression of HCV, but with low efficiency (data not shown). Thus, to augment virus productivity, a JFH1-based chimeric HCV genome (genotype 1b/2a) and its deletion mutant were generated to mimic isolate T5S-2 (designated TNS2J1 and TNS2J1ΔS, respectively, Fig. 4A). JFH1 is genotype 2a HCV isolate that can produce high levels of infectious virus (14). To verify the virus productivity of TNS2J1, Huh7.5 cells (10-cm plate) were transfected with 10 μg of in vitro-synthesized RNA from TNS2J1 or JFH1 by lipofection with TransMessenger transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. Two days later, the culture medium was concentrated 10-fold and inoculated into naïve Huh7.5 cells (four-well chamber

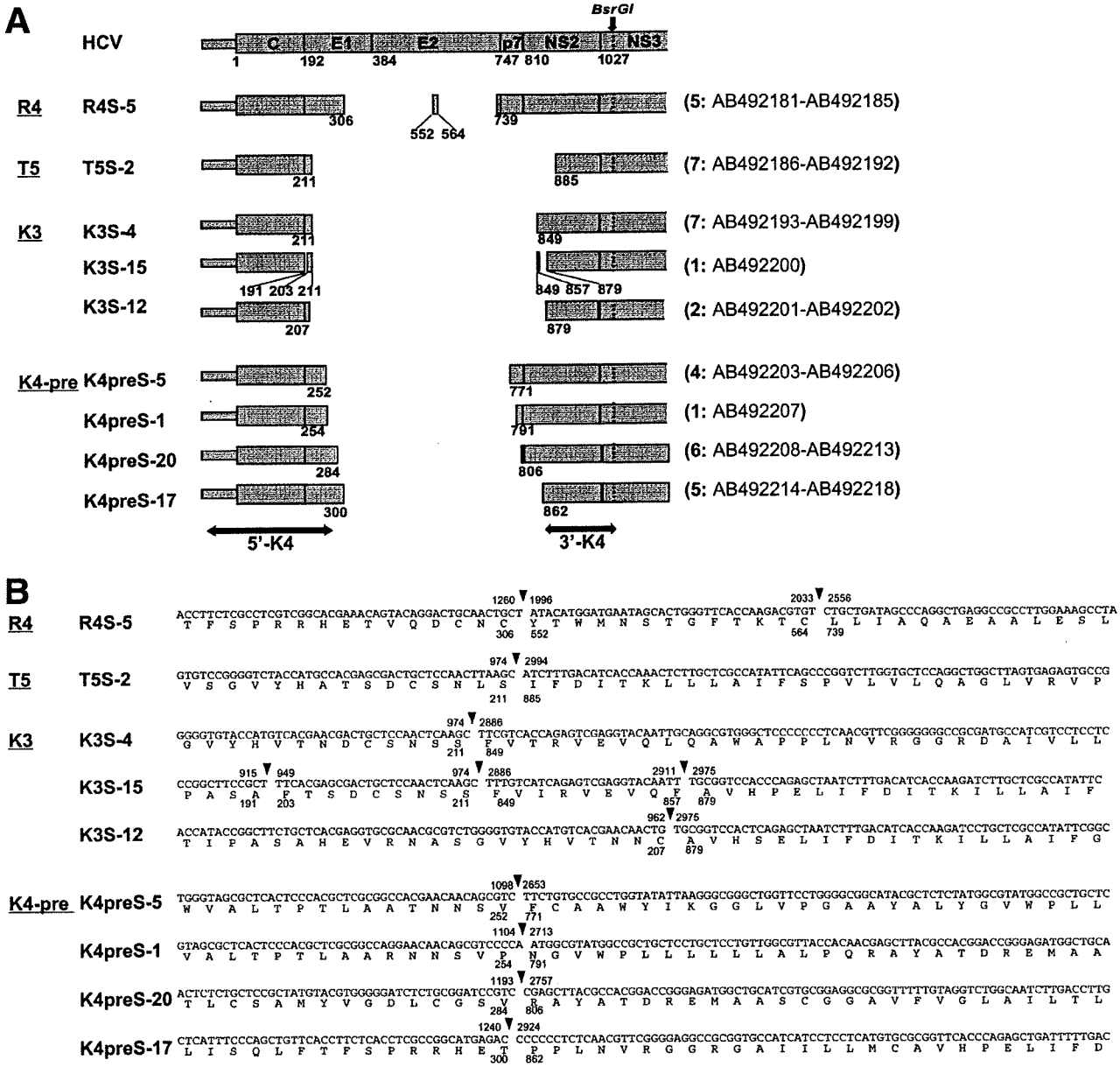

FIG. 2. Sequence analysis of the defective HCV genomes. A total of 38 isolates were molecularly cloned into a plasmid vector and sequenced. Data from representative isolates are presented. Nucleotide positions and deduced amino acid positions correspond to those of genotype 1b strain HCV-JS (12). (A) Defects located in the structural region were compared. The remaining regions are illustrated as shaded boxes. Below the boxes are numbers indicating amino acid positions at the end of each remaining region. At the top of the panel is the HCV genome with the amino acid position at the N terminus of each HCV protein below. The BsrGI restriction site that was used to clone the PCR products is shown as a dotted line. Each value in parentheses at the right is the number of isolates showing the same deletion pattern, followed by the GenBank accession number(s). The two-headed arrows indicate the 5′ and 3′ maximum overlapping regions among the defective HCV isolates in the K4-pre specimen that are compared in the following phylogenetic analyses (5′-K4 and 3′-K4; see Fig. 3). (B) Deletion breakpoints and their adjacent nucleotides and deduced amino acid sequences are indicated. Solid triangles denote breakpoints, and numbers indicate the nucleotide positions (above) and amino acid positions (below) at the junctions.

slide). Cells inoculated with the culture medium from TNS2J1 RNA-transfected cells markedly expressed HCV protein, as shown by immunofluorescent staining (Fig. 4B). The percentage of HCV-positive cells in chimera-infected cells, 40% (565/1,240), was greater than that of JFH1, 3% (37/1,210), demonstrating that the chimeric genome TNS2J1 can produce infectious HCV more robustly than JFH1 can ($P < 0.0001$).

Taking advantage of this chimeric genome, we conducted *trans* complementation experiments. To mimic the T5S-2 iso-

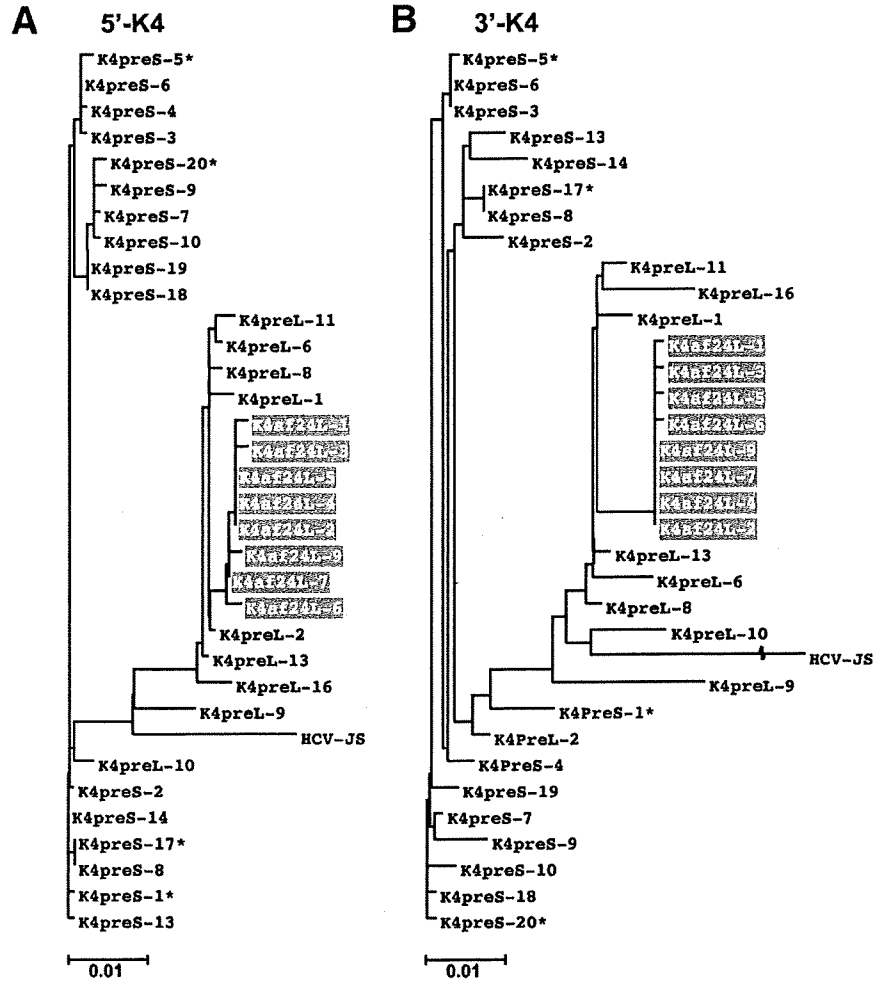

FIG. 3. Phylogenetic analyses of defective HCV genomes. Nucleotide sequence data from 33 isolates from patient K4 were used for phylogenetic analyses. The defective HCV genome (16 isolates) and the nondefective HCV genome coexisting before interferon treatment (9 isolates; GenBank accession no. AB492219 to AB492227) and those after treatment (8 isolates; GenBank accession no. AB492228 to AB492235) were compared in the 5′ and 3′ maximum overlapping regions separately (5′-K4 and 3′-K4 in Fig. 2A). Phylogenetic trees were created for the respective regions (A and B). In the isolate designations, pre and af24 stand for before and after interferon treatment and S and L stand for defective and nondefective HCV genomes, respectively. Isolates with the same deletion pattern (according to K4-pre in Fig. 2) are shaded in the same color. Asterisks denote the representative isolates illustrated in Fig. 2.

late, the region corresponding to the defect found in T5S-2 was identically deleted from the TNS2J1 genome (designated TN2J1ΔS, Fig. 4A). Ten micrograms of synthesized RNA of TN2J1ΔS was cotransfected into Huh7.5 cells (10-cm plate) together with 10 μg of synthesized capped mRNA encoding the structural region, including part of the nonstructural region of TNS2J1, designated C-NS2 or C-NS3P (Fig. 4A). Two days later, the culture medium was concentrated and inoculated into naïve Huh7.5 cells as previously described. HCV protein was expressed when cells were inoculated with the medium of cells cotransfected with TN2J1ΔS RNA and C-NS2 or C-NS3P mRNA, whereas no expression was observed in the case of TN2J1ΔS RNA alone (Fig. 4C). To stably provide the structural proteins in *trans*, packaging cell lines were established by retroviral transduction (2) of Huh7.5 cells with genes encoding the C-NS2 or C-NS3P region (Fig. 4A). These packaging cell lines were transfected with TN2J1ΔS RNA, and HCV protein was expressed in cells inoculated with the culture medium from the RNA-transfected packaging cells (Fig. 4D). Notably, the construct C-NS2 helped to produce HCV_{CCD} more efficiently than C-NS3P did (Fig. 4C). We observed less expression of the structural proteins with the C-NS3 construct than with the C-NS2 construct in a transient expression experiment (data not shown). One possible reason for this is that the C-NS3 construct needs one additional process, i.e., cleavage between NS2 and NS3, to produce NS2 and may affect the other proteins. Otherwise, it is simply because of the difference in the lengths of the constructs. These results indicate that a defective HCV genome lacking the structural region can be encapsidated by *trans* complementation of the structural proteins, thus conferring infectivity in vivo. Recently, a *trans*-packaging system consisting of an HCV subgenomic replicon and a reporter gene

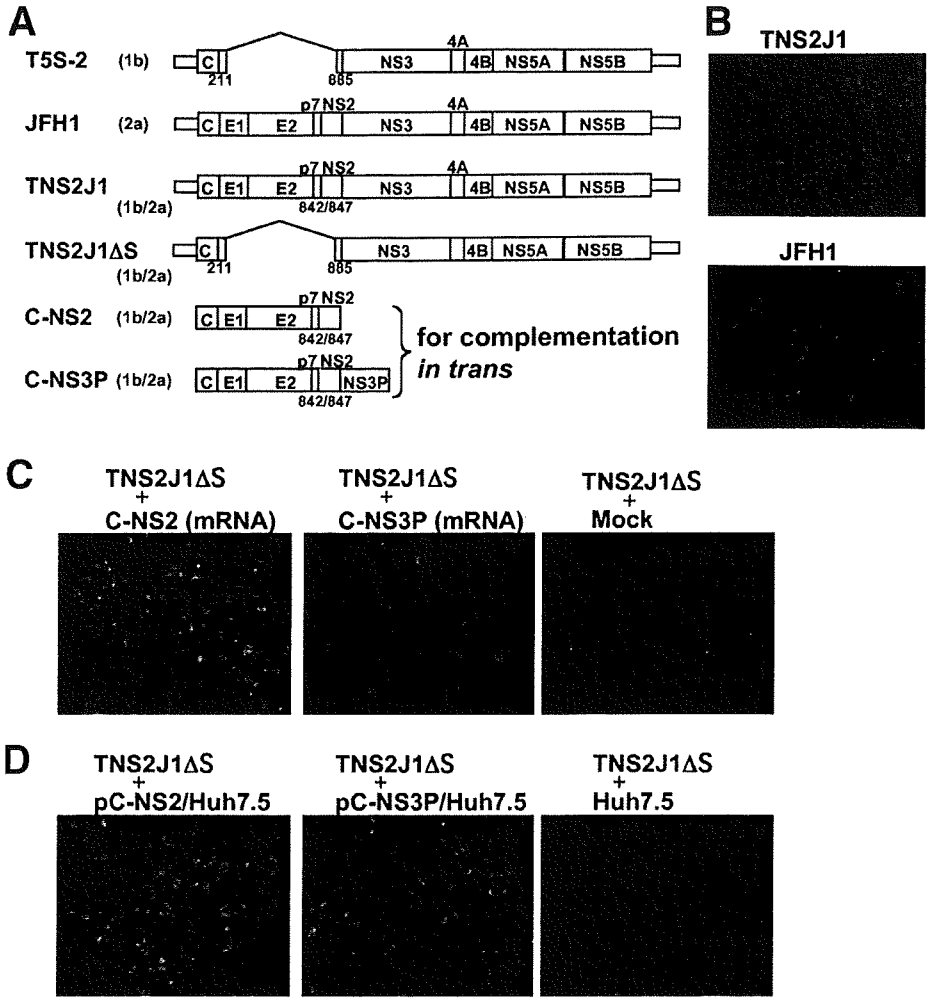

FIG. 4. In vitro infectivity of deletion mutant of chimeric HCV conferred by *trans* complementation of structural proteins. (A) Schematics of the following HCV genomic constructs: the defective HCV isolate (T5S-2), JFH1, chimeric virus of genotypes 1b and 2a (TNS2J1), and its deletion mutant (TNS2J1ΔS). C-NS2 and C-NS3 are fragments encoding the region from the core to the C terminus of the NS2 region and to the C terminus of the serine protease moiety in NS3, respectively. For the *trans* complementation experiments, the latter two constructs were inserted into pcDNA3.1 (Invitrogen) to synthesize capped mRNAs or into retroviral vector pCX4bsr (GenBank accession no. AB086384) to establish packaging cell lines stably expressing the proteins. Shaded and open boxes represent genotypes 1b (isolate from patient T5) and 2a (JFH1), respectively. The numbers below the boxes are amino acid positions at deletion breakpoints or at PCR-based recombination junctions. Naïve Huh7.5 cells were inoculated with the culture medium from cells transfected with JFH1 or TNS2J1 RNA (B), from cells cotransfected with TNS2J1ΔS RNA together with the structural region mRNA (C-NS2 or C-NS3P) or TNS2J1ΔS RNA alone (C), and from the packaging cell line (C-NS2/Huh7.5 or C-NS3P/Huh7.5) transfected with TNS2J1ΔS RNA and parental Huh7.5 cells transfected with TNS2J1ΔS RNA (D). HCV protein was detected by human HCV serum (1:500) by the indirect immunofluorescent method with Alexa Fluor 568 goat anti-human immunoglobulin G (1:200; red; Invitrogen). Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI; blue).

was also reported in which an intragenotypic chimera (2a/2a) was used as the most efficient packaging construct (11). Our packaging system used an efficient intergenotypic chimera (1b/2a) to encapsidate a genome mimicking a naturally occurring deletion (1b). Thus, although its efficiency may be different, our system could be a useful tool for the study of HCV_{CCD} of chimeric genome 1b/2a or genotype 1b.

Taken together, genetic analyses of the defective HCV genome showed the potential of its translation and self-replication. These defective genomes can be encapsidated into infectious virus-like particles by *trans* complementation of the structural proteins in vitro. The 5′ UTR and core regions, which are preserved in defective HCV genomes, are targets for the clinical quantification of HCV. Therefore, measured values may represent additive values for defective and nondefective HCVs and the method used for HCV quantification should be reevaluated.

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REFERENCES

1. **Brinton, M. A.** 1983. Analysis of extracellular West Nile virus particles produced by cell cultures from genetically resistant and susceptible mice indicates enhanced amplification of defective interfering particles by resistant cultures. J. Virol. **46**:860–870.
2. **Chen, C. J., K. Sugiyama, H. Kubo, C. Huang, and S. Makino.** 2004. Murine coronavirus nonstructural protein p28 arrests cell cycle in G_0/G_1 phase. J. Virol. **78**:10410–10419.
3. **Huang, A. S., and D. Baltimore.** 1970. Defective viral particles and viral disease processes. Nature **226**:325–327.
4. **Iwai, A., H. Marusawa, Y. Takada, H. Egawa, K. Ikeda, M. Nabeshima, S. Uemoto, and T. Chiba.** 2006. Identification of novel defective HCV clones in liver transplant recipients with recurrent HCV infection. J. Viral Hepat. **13**:523–531.
5. **Kato, N., K. Sugiyama, K. Namba, H. Dansako, T. Nakamura, M. Takami, K. Naka, A. Nozaki, and K. Shimotohno.** 2003. Establishment of a hepatitis C virus subgenomic replicon derived from human hepatocytes infected in vitro. Biochem. Biophys. Res. Commun. **306**:756–766.
6. **Kishine, H., K. Sugiyama, M. Hijikata, N. Kato, H. Takahashi, T. Noshi, Y. Nio, M. Hosaka, Y. Miyanari, and K. Shimotohno.** 2002. Subgenomic replicon derived from a cell line infected with the hepatitis C virus. Biochem. Biophys. Res. Commun. **293**:993–999.
7. **Lohmann, V., F. Körner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager.** 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science **285**:110–113.
8. **Noppornpanth, S., S. L. Smits, T. X. Lien, Y. Poovorawan, A. D. Osterhaus, and B. L. Haagmans.** 2007. Characterization of hepatitis C virus deletion mutants circulating in chronically infected patients. J. Virol. **81**:12496–12503.
9. **Poidinger, M., R. J. Coelen, and J. S. Mackenzie.** 1991. Persistent infection of Vero cells by the flavivirus Murray Valley encephalitis virus. J. Gen. Virol. **72**(Pt. 3):573–578.
10. **Shimotohno, K.** 1995. Hepatitis C virus as a causative agent of hepatocellular carcinoma. Intervirology **38**:162–169.
11. **Steinmann, E., C. Brohm, S. Kallis, R. Bartenschlager, and T. Pietschmann.** 2008. Efficient *trans*-encapsidation of hepatitis C virus RNAs into infectious virus-like particles. J. Virol. **82**:7034–7046.
12. **Sugiyama, K., N. Kato, T. Mizutani, M. Ikeda, T. Tanaka, and K. Shimotohno.** 1997. Genetic analysis of the hepatitis C virus (HCV) genome from HCV-infected human T cells. J. Gen. Virol. **78**(Pt. 2):329–336.
13. **Tong, X., and B. A. Malcolm.** 2006. Trans-complementation of HCV replication by non-structural protein 5A. Virus Res. **115**:122–130.
14. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11**:791–796.
15. **Yagi, S., K. Mori, E. Tanaka, A. Matsumoto, F. Sunaga, K. Kiyosawa, and K. Yamaguchi.** 2005. Identification of novel HCV subgenome replicating persistently in chronic active hepatitis C patients. J. Med. Virol. **77**:399–413.
16. **Yoon, S. W., S. Y. Lee, S. Y. Won, S. H. Park, S. Y. Park, and Y. S. Jeong.** 2006. Characterization of homologous defective interfering RNA during persistent infection of Vero cells with Japanese encephalitis virus. Mol. Cells **21**:112–120.

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Original Article

Case–control study for the identification of virological factors associated with fulminant hepatitis B

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Background: Host and viral factors can promote the development of fulminant hepatitis B (FHB), but there have been no case–control studies for figuring out virological parameters that can distinguish FHB.

Methods: In a case–control study, virological factors associated with the development of FHB were sought in 50 patients with FH developed by transient hepatitis B virus (HBV) infection (FH-T) and 50 with acute self-limited hepatitis B (AHB) who were matched for sex and age. In addition, 12 patients with FH developed by acute exacerbation (AE) of asymptomatic HBV carrier (ASC) (FH-C) were also compared with 12 patients without FH by AE of chronic hepatitis B (AE-C).

Results: Higher HBV DNA levels, subgenotype B1/Bj, A1762T/G1764A, G1896A, G1899A and A2339G mutation were significantly more frequent ($P < 0.05$), while hepatitis B e-antigen was less frequent in the FH-T patients than AHB. In multivariate analysis, G1896A mutation (odds ratio [OR], 13.53; 95% confidence interval [CI], 2.75–66.64), serum HBV DNA more than 5.23 log copies/mL (OR, 5.14; 95% CI, 1.10–24.15) and total bilirubin more than 10.35 mg/mL (OR, 7.81; 95% CI, 1.77–34.51) were independently associated with a fulminant outcome by transient HBV infection. On the other hand, in comparison with the patients between FH-C and AE-C groups, there was no significant difference of virological factors associated with the development of FHB.

Conclusion: A number of virological factors have been defined that may distinguish FH-T from AHB in a case–control study. The pathogenic mechanism of FHB between transient HBV infection and AE of ASC would be different.

Key words: acute exacerbation of asymptomatic hepatitis B virus carrier, fulminant hepatitis, genotypes, transient hepatitis B virus infection

INTRODUCTION

IN JAPAN, 634 patients with fulminant hepatitis (FH) were registered from 1998–2003. Of them, 41.8% were infected with hepatitis B virus (HBV) that is the most frequent cause of FH there.[1] HBV is classified into eight genotypes (A–H) based on a sequence divergence of more than 8% in the entire genome of approximately

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3200 nucleotides.[2–5] They have distinct geographical distributions and are associated with the severity of liver disease.[6,7] Furthermore, subgenotypes have been reported for HBV/A, B and C, and they are named A1/Aa (Asian/African type) and A2/Ae (European type),[8] B1/Bj (Japanese type) and B2/Ba (Asian type),[9] and C1/Cs (Southeast Asian type) and C2/Ce (East Asian type).[10,11] HBV genotypes/subgenotypes and mutations in the pre-core region and the core promoter can influence the viral replication and expression of hepatitis B e-antigen (HBeAg).[6,12]

Acute HBV infection in adulthood resolves in the most cases by far, but can induce FH or go on to become chronic in some. It has been reported that host and viral factors may influence the development of fulminant hepatitis B (FHB), but the pathogenesis of FHB remains unclear. As for virological factors associated with FHB, mutations in the core promoter (A1762T/G1764A)[13] and the pre-core region (G1896A)[14–16] have been reported in association with the development of FHB in Asia and the Middle East. Additional mutations, including T1753V, T1754V and A2339G in the core gene are implicated, also.[17,18] In regard of HBV genotypes, subgenotype B1/Bj is highly associated with the development of FHB in Japan.[15] In contrast, an association of HBV genotypes with the fulminant outcome has not been reproduced in patients from the USA and Europe.[19–22] Such a discrepancy would be attributed, at least in part, to distinct geographical distributions of HBV genotypes/subgenotypes over the world.

The original definition by Trey *et al.*[23] about fulminant hepatic failure is widely used all over the world. On the other hand, in Japan, the diagnosis of FH was contingent on a slight modification of Trey's original definition by the Inuyama Symposium (Aichi, Japan in 1981). Furthermore, the Intractable Liver Diseases Study Group of Japan modified the criteria for the etiology of FH and late-onset hepatic failure in 2002. According to the criteria of the Intractable Liver Diseases Study Group of Japan, there are two clinical entities of FHB that are induced, respectively, by transient HBV infection and acute exacerbation (AE) of an asymptomatic HBV carrier (ASC).[1]

Recently, FH developing in ASC who undergo AE is increasing in Japan.[1] In patients with hematological malignancy, in particular, rituximab and/or glucocorticoid, can reactivate HBV for the development of FHB.[24] The outcome is poor for FHB precipitating in ASC who undergo acute exacerbation,[1] but it has been difficult to identify it by clinical examinations.

As there have been no case–control studies for figuring out virological parameters that can distinguish FHB, a case–control study was conducted on the patients with FH by transient HBV infection and acute self-limited hepatitis B (AHB) in this study, for the identification of virological factors that influence a fulminant outcome. In addition, the patients with FH by AE of ASC, which is assumed as a different clinical condition from transient HBV infection, were also compared with the patients without FH by AE of chronic hepatitis B (CHB) in a case–control study.

METHODS

Patients

DURING 9 YEARS from 1998 to 2006, in twenty-six hospitals all over Japan, sera were obtained from the 50 FH patients by transient HBV infection (the FH-T group) and the 50 patients with AHB (the AHB group) who were controlled for age and sex. As the elder patients with FHB were enrolled in this study (mean age, 42.8 years), the mean age of AHB patients became relatively high (42.9 years, Table 1). Furthermore, the 12 FH patients developed by AE of ASC (the FH-C group) were also compared with the 12 patients without FH by AE of CHB who were matched by age and sex (the AE-C group).

All the serum samples tested for this study were collected at hospitalization. All 124 patients had hepatitis B surface antigen (HBsAg) in serum. Infection with hepatitis A virus and hepatitis C virus, as well as alcoholic hepatitis, were excluded in them.

The diagnosis of acute hepatitis B was based on sudden manifestation of clinical symptoms of hepatitis and detection of high-titered immunoglobulin (Ig)M anti-hepatitis B core (HBC). Patients with initial high-titered anti-HBC (>90% inhibition by a 1:200 diluted serum) were excluded. The diagnosis of FH was contingent on a slight modification by Inuyama Symposium (Aichi, Japan in 1981) of the original definition by Trey *et al.*:[23] (i) coma of grade II or higher; and (ii) a prothorombin time less than 40% developing within 8 weeks after the onset of hepatitis. To exclude AE of ASC in FH-T and AHB groups, we confirmed the negativity of HBsAg before onset of FHB or AHB and no family histories of hepatitis were found among all the patients. Furthermore, serum HBsAg in all patients with FH-T or AHB became naturally seronegative within 24 weeks. AE of ASC or CHB was defined as the elevation of alanine aminotransferase (ALT >300 IU/L) or total bilirubin (T.bil >3.0 mg/dL).[25] All 24 patients with AE of ASC or CHB could be confirmed positive for serum HBsAg before the onset of acute liver injury.

Table 1 Baseline characteristics between fulminant hepatitis B patients by transient infection (FH-T) and acute self-limited hepatitis B (AHB) patients

Features	FH-T ($n = 50$)	AHB ($n = 50$)	Differences *P*-value
Age (years)	42.8 ± 16.1	42.9 ± 14.6	Matched
Men	25 (50%)	25 (50%)	Matched
ALT (IU/L)	3788 ± 2856	2170 ± 1350	<0.001
AST (IU/L)	3131 ± 3673	1676 ± 1851	<0.05
Total bilirubin (mg/dL)	14.8 ± 8.6	9.5 ± 9.8	<0.01
Prothrombin time (%)	16.9 ± 11.2	72.8 ± 26.0	<0.001
HBeAg positive	15 (30%)	28 (56%)	<0.01
Core protein (log U/mL)	3.21 ± 1.28	3.01 ± 1.00	NS
HBcrAg (log U/mL)	5.30 ± 1.32	5.95 ± 1.13	<0.01
HBV DNA (log copies/mL)	5.97 ± 1.87	4.98 ± 1.17	<0.005
Deceased	19 (38%)	0 (0%)	<0.001

AHB, acute self-limited hepatitis B; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FH-T, fulminant hepatitis B by transient HBV infection; HBcrAg, hepatitis B core related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NS, not significant.

Serological markers of HBV infection

Hepatitis B surface antigen, HBeAg and the corresponding antibody (anti-HBe) were determined by enzyme immunoassay (EIA) (AxSYM; Abbott Japan, Tokyo, Japan) or chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio, Tokyo, Japan). Anti-HBC of IgM and IgG classes were determined by radioimmunoassay (Abbott Japan). Core protein constituting the viral nucleocapsid and HBV core-related antigen (HBcrAg), both of which correlate with HBV DNA in serum, were measured by CLEIA as described elsewhere.[26,27]

Quantification of serum HBV DNA

Hepatitis B virus DNA sequences spanning the S gene were amplified by real-time detection polymerase chain reaction (RTD-PCR) in accordance with the previously described protocol[28] with a slight modification;[8] it has a detection limit of 100 copies/mL.

Sequencing and molecular evolutionary analysis of HBV

Nucleic acids were extracted from serum samples (100 μL) using the QIAamp DNA extraction kit (Qiagen, Hilden, Germany) and subjected to PCR for amplifying genomic areas bearing enhancer II/core promoter/pre-core/core regions [nt 1628–2364], as described previously.[29] The target of PCR covered several mutations which were associated with FHB. Amplicons were sequenced directly with use of the ABI Prism Big Dye ver. 3.0 kit in the AMI 3100 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA). All sequences were analyzed in both forward and backward directions.

Hepatitis B virus genotypes were determined by molecular evolutionary analysis. Reference HBV sequences were retrieved from the DDBJ/EMBL/GenBank database and aligned by CLUSTAL X, then genetic distances were estimated with the 6-parameter method in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/).[30] Based on obtained distances, phylogenetic trees were constructed by the neighbor-joining (NJ) method with the mid-point rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1000 times.

Statistical analysis

Statistical differences were evaluated by the Mann–Whitney *U*-test, Fisher's exact probability test and χ^2-test, where appropriate. Differences were considered to be statistically significant at $P < 0.05$. Multivariate analyses with logistic regression were utilized to sort out independent risk factors for FHB. STATA Software ver. 8.0 was employed for all analyses.

RESULTS

Baseline characteristics of the patients with FHB by transient HBV infection and AHB

TABLE 1 COMPARES baseline clinical characteristics of the 50 FH-T patients and the 50 AHB who

were matched for age and sex. The peak ALT, AST and T.bil levels were significantly higher (3788 ± 2856 vs 2170 ± 1350 IU/L, $P < 0.001$; 3131 ± 3673 vs 1676 ± 1851 IU/L, $P < 0.05$; and 14.8 ± 8.6 vs 9.5 ± 9.8 mg/dL, $P < 0.01$, respectively), while HBeAg was less frequent (30% vs 56%, $P < 0.01$) in the FH-T patients than AHB. The level of HBcrAg was significantly lower (5.30 ± 1.32 vs 5.95 ± 1.13 log U/mL, $P < 0.01$), while HBV DNA loads were higher (5.97 ± 1.87 vs 4.98 ± 1.17 log copies/mL, $P < 0.005$), in the FH-T patients than AHB. The level of core protein in sera tended to be higher in the FH-T patients than AHB (3.21 ± 1.28 vs 3.01 ± 1.00 log U/mL). Death occurred more often in the FH-T patients than AHB (38% vs 0%, $P < 0.001$).

HBV Genotypes and enhancer II/core promoter/pre-core/core Mutations in Patients with FHB by transient HBV infection and AHB

Figure 1(a) compares the distribution of HBV genotypes/subgenotypes between the FH-T and the AHB patients. The subgenotype C2/Ce was most prevalent in both patients with FH-T and AHB (66% and 62%, respectively), whereas B1/Bj was more frequent in the FH-T patients than AHB (22% vs 6%, $P < 0.05$). Likewise, mutations in enhancer II/core promoter/pre-core/core regions are compared between the FH-T and AHB patients in Figure 1(b). A1762T/G1764A, G1896A, G1899A and A2339G mutation were more frequent in the FH-T patients than AHB (48% vs 16%, $P < 0.001$; 62% vs 6%, $P < 0.001$; 24% vs 4%, $P < 0.001$; and 8% vs 0%, $P < 0.05$, respectively).

Figure 2(a) compares various mutations between the 11 FH-T patients and the three AHB patients who were infected with B1/Bj. Only G1896A was significantly more frequent (73% vs 0%, $P < 0.05$), while the lack of any mutations was less common (0% vs 33%, $P < 0.05$) in the FH-T patients than AHB. In comparison with the 33 FH-T patients and the 31 AHB patients who were infected with C2/Ce (Fig. 2b), A1762T/G1764A (70% vs 19%, $P < 0.001$), G1896A (61% vs 6%, $P < 0.001$) and the combination of all three mutations (A1762T/G1764A and G1896A) (45% vs 6%, $P < 0.001$) were significantly more frequent, while the lack of any mutations was less common (9% vs 70%, $P < 0.001$) in the FH-T patients than AHB. Interestingly, all the AHB patients with both G1896A and A1762T/G1764A mutations suffered acute severe hepatitis B that was defined by prothrombin time less than 40% but without coma of grade II or higher.

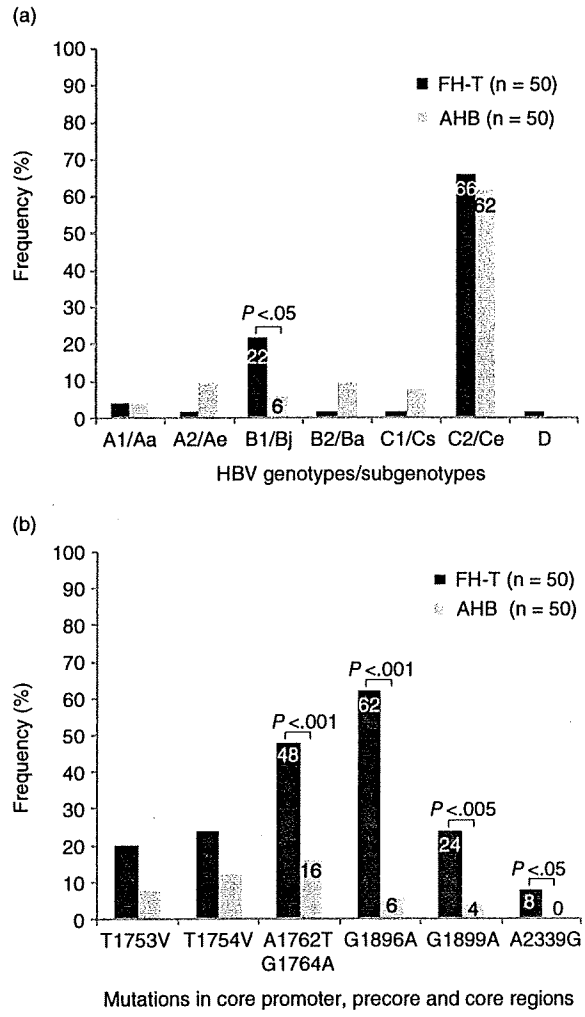

Figure 1 Genotypes/subgenotypes (a) and mutations in core promoter, pre-core and core regions (b) between the 50 transient hepatitis B virus infection (FH-T) and the 50 acute self-limited hepatitis B (AHB) patients.

Factors independently associated with the development of FHB by transient HBV infection

The following independent factors, promoting the development of FHB, were evaluated by multivariate analysis: ALT, AST, T.bil, HBeAg, HBV DNA, core protein, HBcrAg, genotypes/subgenotypes (B1/Bj or not) and mutations (T1753V, T1754V, A1762T/G1764A, G1896A, G1899A and A2339G). T.bil more than 10.35 mg/dL (OR, 7.81 [95% CI, 1.77–34.51], $P = 0.0067$), G1896A mutation (OR, 13.53 [95% CI,

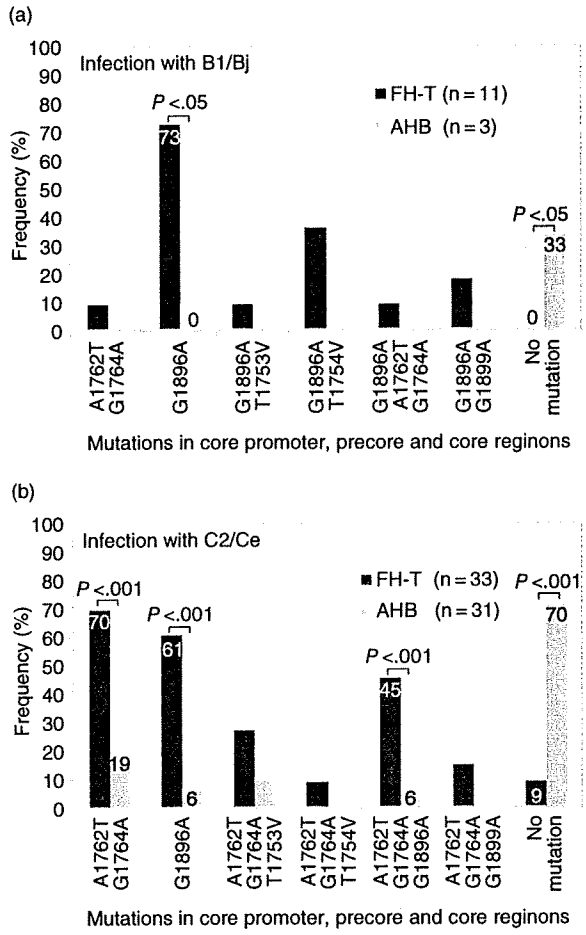

Figure 2 Frequencies of core promoter, pre-core and core mutations compared between the transient hepatitis B virus infection (FH-T) and the acute self-limited hepatitis B (AHB) patients who were infected with HBV of subgenotype B1/Bj (a) or C2/Ce (b).

2.75–66.64], $P = 0.0014$) and serum HBV DNA more than 5.23 log copies/mL (OR, 5.14 [95% CI, 1.10–24.15], $P = 0.0379$) were independent risk factors for the development of FHB by transient HBV infection (Table 2). Other mutations (T1753V, T1754V, A1762T/G1764A, G1899A and A2339G) were not significantly associated with the development of FHB by transient HBV infection, however.

Baseline clinical characteristics for distinguishing between the patients with FHB by AE of ASC (FH-C) and those without FHB by AE of CHB (AE-C)

Table 3 compares baseline clinical characteristics between the 12 FH-C patients and the 12 AE-C patients who were matched for age and sex. The levels of T.bil were significantly higher in the FH-C patients (15.0 ± 7.3 vs 7.3 ± 8.8 mg/dL, $P < 0.05$), but the peak ALT and AST levels tended to be slightly higher in the FH-C patients than AE-C (887 ± 681 vs. 641 ± 620 IU/L and 701 ± 451 vs 601 ± 753 IU/L, respectively). There were also no significant differences in levels of sera HBV DNA, core protein and HBcrAg between these two groups (7.44 ± 1.51 vs 6.60 ± 1.10 log copies/mL, 5.04 ± 1.45 vs 5.07 ± 1.07 log U/mL, and 6.35 ± 1.70 vs 6.29 ± 1.95 log U/mL, respectively).

HBV genotypes and enhancer II/core promoter/pre-core/core mutations between the patients with FH-C and those with AE-C

There were no significant differences in the frequencies of any HBV genotypes between the 12 FH-C patients and the 12 AE-C patients (Fig. 3a). In addition, there were also no significant differences in the frequencies

Table 2 Multivariate analysis for factors independently associated with fulminant hepatitis by transient HBV infection

Factors	Odds ratio	95% confidence interval	*P*-value
Total bilirubin (mg/dL)†			
<10.35	1		
≥10.35	7.81	1.77–34.51	0.0067
G1896A mutation			
Absent	1		
Present	13.53	2.75–66.64	0.0014
HBV DNA (log copies/mL)†			
<5.23	1		
≥5.23	5.14	1.10–24.15	0.0379

†Median values. HBV, hepatitis B virus.

Table 3 Baseline characteristics between patients with FH by AE of ASC (FH-C) and those without FH by AE of CHB (AE-C)

Features	FH-C ($n = 12$)	AE-C ($n = 12$)	Differences *P*-value
Age (years)	51.7 ± 14.7	49.9 ± 5.6	Matched
Male	10 (83%)	9 (75%)	Matched
ALT (IU/L)	887 ± 681	641 ± 620	NS
AST (IU/L)	701 ± 451	601 ± 753	NS
Total bilirubin (mg/dL)	15.0 ± 7.3	7.3 ± 8.8	<0.05
Prothrombin time (%)	25.8 ± 6.6	48.4 ± 21.5	<0.005
HBeAg positive	4 (33%)	3 (25%)	NS
Core protein (log U/mL)	5.04 ± 1.45	5.07 ± 1.07	NS
HBcrAg (log U/mL)	6.35 ± 1.70	6.29 ± 1.95	NS
HBV DNA (log copies/mL)	7.44 ± 1.51	6.60 ± 1.10	NS

AE, acute exacerbation; ALT, alanine aminotransferase; ASC, asymptomatic HBV carrier; AST, aspartate aminotransferase; CHB, chronic hepatitis B; HBcrAg, hepatitis B core related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NS, not significant.

of any specific mutations between these two groups (Fig. 3b).

DISCUSSION

THE MAGNITUDE OF liver injuries depends on the replication level of HBV and cytotoxic immune responses of the host raised against viral epitopes in general.[31] Various viral factors have been proposed that promote the development of FHB, represented by pre-core (G1896A) and core promoter (A1762T/G1764A) mutations.[13–16] Impact of virological factors on the development of FHB has remained controversial, however, especially because these mutations are rarely detected in the patients from the USA and France.[19–21] It has been argued that the development of FHB is not promoted by these mutations and is dependent on host factors including the human leukocyte antigen (HLA) environment.[22]

The expression of HBeAg is terminated by G1896A mutation in the pre-core region at the translation level,[32] and downregulated by the A1762T/G1764A double mutation at the transcription level.[33,34] Lamberts *et al.* are the first to implicate a negative influence of HBeAg on the replication of HBV.[35] Should HBeAg suppress the replication of HBV, presumably by inhibiting the encapsidation of pre-genome,[35] the lack or decrease of HBeAg would enhance the reproduction of HBV. Furthermore, HBeAg acts as a tollerogen to T cells recognizing epitopes on core protein, thereby, obviating immune injury of hepatocytes.[36,37] In the absence or decrease of HBeAg, therefore, hosts would mount vigor cytotoxic T-cell responses to core epitopes excessively presented on hepatocytes, and develop severe liver injuries culminating in FHB.[38]

There is a possibility that influence of viral factors such as HBV mutants with a HBeAg-negative phenotype, on the induction of FHB, may have been confounded by host factors and created disagreement. Therefore, the sheer influence of virological factors on FHB would need to be evaluated in case–control studies, as has been attempted to sort out the influence of HBV genotypes on development of cirrhosis and hepatocellular carcinoma.[8] These backgrounds have instigated us to identify virological factors accelerating the severity of liver disease in the 50 FHB patients by transient HBV infection and the 50 AHB patients who were of the same ethnicity and matched for age as well as sex.

In this case controlled study, A1762T/G1764A, G1896A, G1899A and A2339G mutation were significantly more frequent in the patients with FH-T than AHB, providing further corroboration of previous studies;[13–16] these mutations could enhance viral replication. Interestingly, our recent study using an *in vitro* replication model, showed that A2339G mutation in the core region enhanced viral replication and the effect of A2339G mutation may be associated with inhibition of the cleavage of the core protein by a furin-like protease, resulting in the high expression of the complete core protein.[18] Such enhanced HBV would induce significant immune response, resulting in development of FHB.

In multivariate analysis, higher levels of serum HBV DNA and G1896A mutation were independent virological risk factors for the development of FHB by transient