

Short Communication

Temporal and Tight Hepatitis C Virus Gene Activation in Cultured Human Hepatoma Cells Mediated by a Cell-Permeable Cre Recombinase

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Abstract Conditional gene expression has greatly facilitated the examination of the functions of particular gene products. Using the Cre/lox P switching expression system, we plan to develop efficient conditional transgene activation of hepatitis C virus core protein (HCV-C) cDNA (nucleotide 342–914) in the transgenic mice to overcome “immune tolerance” formed during the embryonic period and “immune escape” against hepatitis virus antigen in our project. To use this system *in vivo*, the dormant transgenic construct, i.e., pApoE-SCS-EGFP-HCV-C, was generated using techniques of standard molecular biology. The liver-specific human *apoE* promoter was here used to target expression of genes of interest (EGFP and HCV-C) to murine liver. Prior to generating the transgenic mice, the availability of Cre/lox P system and construct functionality were successfully verified by a cell-free recombination system and via checking the expression of EGFP and HCV-C in the human hepatoma cells at the mRNA and protein levels. These results suggest that the Cre/lox P system could tightly control expression of EGFP and HCV-C *in vitro*, which laid a solid foundation to conditionally activate expression of target gene(s) in transgenic mice by Cre-mediated site-specific recombination.

Key words hepatitis C virus; Cre/lox P switching expression system; cell-free and intracellular recombination systems; cell-permeable Cre recombinase; EGFP; human hepatoma cells

Hepatitis C virus (HCV) is not infectious *in vivo* except for primates, so the proper HCV culture system and inbred animal model are difficult to set up, which has hampered detailed analysis on viral life cycle and pathogenesis of HCV infection [1,2]. Hepatitis B virus (HBV) and HCV transgenic mouse models have been created by micro-injecting the full or selected parts of the virus genome into the fertilized eggs of inbred immunodeficient mice to provide new insights into the pathogenesis of hepatitis and hepatocellular carcinoma (HCC) [3–6]. A disadvantage of the transgenic animals produced by the consistent gene expression system is that they aren't immunocompetent for

the transgene products. Therefore, the immune system of newborn transgenic mice can't recognize the exotic identity of viral antigens. However, in human chronic hepatitis C, hepatocyte injury is not directly caused by HCV infection, but is a consequence of the destruction of infected hepatocytes by cytotoxic lymphocytes [7]. In these HCV transgenic mice, the antigen genes express normally, but little pathological changes are observed in the liver and the serum alanine aminotransferase levels keep unchanged, indirectly suggesting that the immune system plays a rather important role in hepatitis pathogenesis [3–6].

Recently, a development in transgene technology is the control of tissue/organ-specific target gene expression at particular developmental stage, to mimic HBV or HCV infection in immunocompetent transgenic mice like that in human. Two systems successfully and frequently employed are tetracycline-inducible system (tet-off system or tet-on system) and Cre/lox P site-specific recombinase system [8–10]. The tetracycline-inducible expression system is

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not very tight in controlling [8,9,11]. Chisari's lab has produced transgenic mice expressing HBV envelope protein or HCV core protein (HCV-C) by using the tet-off system. The results showed that the transgene expression was undetectable in utero or early life, but the transgene mice were tolerant to HBV envelope protein or HCV-C immunization (Chisari, 2003, personal communication), which might be caused by the leakiness. New variants of the tetracycline-based regulatory elements that can minimize or eliminate leaky transgene expression have been tested successfully *in vitro*, but approaches that can be used to eliminate basal transgene leak *in vivo* have not been well characterized yet [11].

The Cre-mediated gene *in vivo* and *in vitro* conditional activation/inactivation have been widely employed in the different research fields [12,13] and the general strategy is fully demonstrated in Fig. 1(B). In conjunction with the inducible system controlling Cre expression [8–10], the administration of the cell-permeable Cre protein [14–16] or Cre-expressing adenovirus vector [12], the Cre/*lox* P switching expression system is a true “off/on” system. The “off/on” nature of this regulation will be very useful to express hepatitis virus antigen genes in a temporally restricted fashion and precisely define the immunological

reactions against antigens and pathogenesis of HCV infection. Wakita *et al.* [17–19] used the Cre/*lox* P system to efficiently conditionally express the core, E1 and E2 protein genes in transgenic mice, which provided useful animal models for investigating host immune response and pathogenesis of HCV infection.

Spatially and temporally expressing HCV-C gene in immunocompetent transgenic mice can be mediated by Cre/*lox* P system, so that the host immune response against HCV infection and pathogenesis of HCV infection can be fully and precisely elucidated.

In the present study, the dormant transgene vector was generated, followed by *in vitro* identifying Cre/*lox* P system for availability and construct for functionality.

Materials and Methods

Materials

Human hepatoma cell line BEL-7402 was from Cell Bank, Center of Experimental Animals, Sun Yat-sen University. Lipofectamine™ 2000, RPMI 1640 medium, fetal bovine serum (FBS), 0.25% trypsin solution, HEPES,

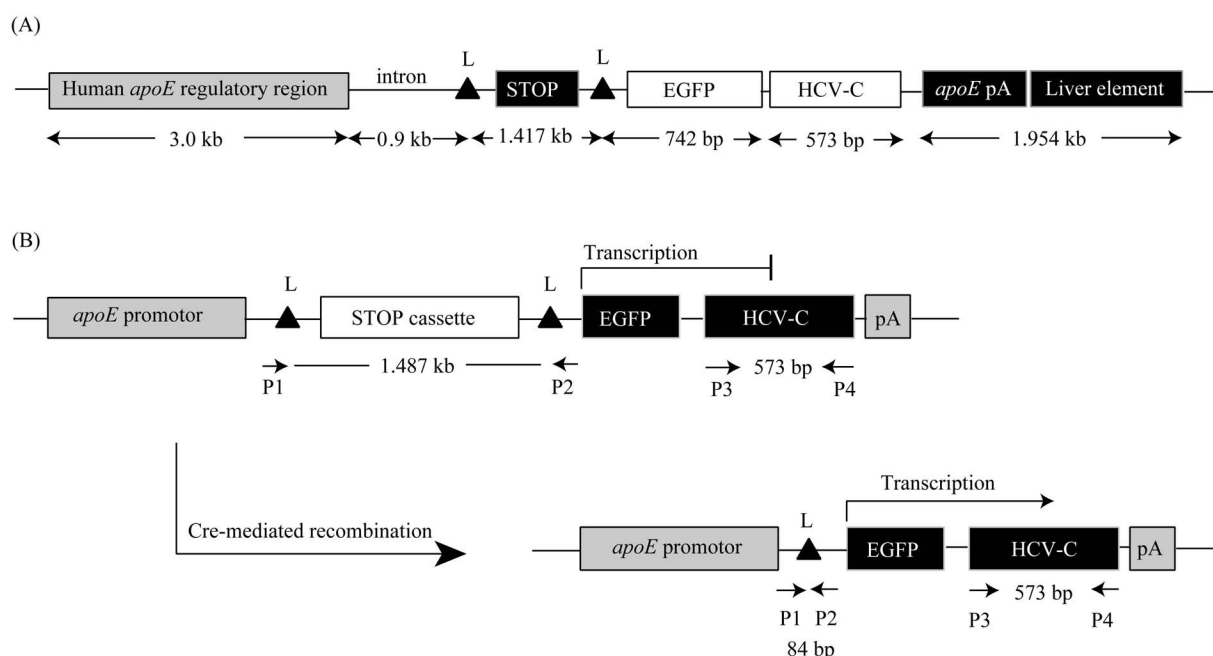


Fig. 1 Experimental design

(A) Structure of the dormant transgene construct of pApoE-SCS-EGFP-HCV-C. The human *apoE* promoter, EGFP and HCV-C genes were separated by transcriptional STOP sequence flanked by two *lox* P sites in the same direction. (B) Conditional expression of HCV-C and EGFP mediated by the Cre/*lox* P system. In the absence of Cre, EGFP and HCV-C gene expression was prevented by STOP sequence flanked by *lox* P sites. Upon recognition of the *lox* P site, Cre deleted the SCS along with one *lox* P site and ligated the *apoE* promoter to EGFP and HCV-C gene sequence. L, *lox* P site; *apoE* pA, human *apoE* poly(A) signal.

dimethyl sulfoxide (DMSO), culture flask and plate were supplied by Gibco BRL (Life Technologies Inc.). Other chemicals and materials were of analytical grade obtained from commercial sources.

The pLiv.7 vector [20] containing a 3.0 kb human apolipoprotein E (apoE) gene promoter for expression of the liver-specific gene was used to clone HCV-C genomic region. The plasmid pMA-HCV [21] containing complete coding sequence of HCV type 2b gene for polyprotein (GenBank accession No. AB030907) was a gift of Prof. Akio Nomoto (Department of Microbiology, Graduate School of Medicine, The University of Tokyo, Japan). The vector pBS302 [22] containing STOP cassette sequence (SCS) flanked by *lox* P sites, and pBS185 [22] containing Cre coding sequence were generously provided by Dr. Brian Sauer (Stowers Institute for Medical Research, Kansas City, USA).

Plasmid construction

For liver-specific expression of target gene *in vivo*, the transgene construct [Fig. 1(A)] containing enhanced green fluorescent protein (EGFP) and HCV-C gene under the control of the liver-specific *apoE* promoter was constructed as described in Sambrook *et al.* [23].

pApoE-HCV-C HCV-C cDNA (position 342–914) was amplified from pMA-HCV with primer HCV-C-FP/HCV-C-RP, and inserted into the T-vector of pMD18-T (TaKaRa) to construct pMD18-T-HCV-C. The PCR reaction was set up for 7 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 58 °C, 1 min at 72 °C, and a final cycle of 7 min at 72 °C.

Forward primer HCV-C-FP: 5'-CGGAACGCGTATGAGC-ACAAATCCTAAACCTC-3' (*Mlu*I site underlined)

Reverse primer HCV-C-RP: 5'-ATGACTCGAGCTATGA-AGACACTGGCACTGTAAC-3' (*Xho*I site underlined, and stop codon double-underlined)

HCV-C fragment (573 bp) was recovered from pMD18-T-HCV-C by digestion with *Mlu*I and *Xho*I, and directionally subcloned into the *Mlu*I and *Xho*I sites in the polylinker (5'-*Kpn*I-*Mun*I-*Hpa*I-*Mlu*I-*Cla*I-*Xho*I-3') of pLiv.7 (9.3 kb) to create pApoE-HCV-C, as verified by restriction enzyme digestion.

pApoE-EGFP-HCV-C To generate the plasmid containing EGFP reporter gene, 742 bp EGFP gene segment was excised from pBI-EGFP (Clontech) by digestion with *Eco*RV and *Spe*I, blunted at the *Spe*I site, and subsequently subcloned into pApoE-HCV-C at the dephosphorylated *Hpa*I site, designated pApoE-EGFP-HCV-C. The orientation of EGFP fragment inserted in the frame was identified with *Eco*RI.

pApoE-SCS-EGFP-HCV-C For conditional expression of HCV-C *in vitro* and *in vivo* mediated by the Cre/*lox* P system, an expression vector with SCS between the promoter and the target coding sequence was generated. SCS fragment (about 1.487 kb) was amplified by PCR with primer SCS-FP/SCS-RP using pBS302 as template, and cloned into pMD18-T to give pMD18-T-SCS. PCR reactions involved 30 cycles of denaturation for 45 s at 94 °C, annealing for 45 s at 62 °C, and extension for 2 min at 72 °C.

Forward primer SCS-FP: 5'-GCACGAATTCTCAAGC-TTAGGATCCG-3' (*Eco*RI site underlined)

Reverse primer SCS-RP: 5'-ATTAGGTACCAGTGAA-CCTCTTCGAGG-3' (*Kpn*I site underlined)

The *Kpn*I-*Eco*RI SCS fragment released from pMD18-T-SCS was orientatively subcloned into *Kpn*I and *Mun*I sites of pApoE-EGFP-HCV-C to prepare pApoE-SCS-EGFP-HCV-C. This complete sequence of the dormant transgene was further verified by sequencing.

Identification of *lox* P sites in the vector by Cre-mediated *in vitro* recombination

The pApoE-SCS-EGFP-HCV-C was digested with Cre protein (Novagen) *in vitro*, followed by plasmid DNA transformation, restriction enzyme digestion and PCR to confirm *lox* P site sequence. Cre digestion on pApoE-SCS-EGFP-HCV-C was carried out according to the manufacturer's instructions.

Intracellular confirmation for recombination system

Cell transfection BEL-7402 cells were transiently transfected with plasmids mentioned in Fig. 4 to further test the function of pApoE-SCS-EGFP-HCV-C at cell level. The cell culture and transient transfection were performed as previously reported [24].

BEL-7402 cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 10 mM HEPES, 2 mM *L*-glutamine (Sigma), 100 U/ml penicillin (Sigma), and 100 mg/ml streptomycin (Sigma), refreshed 2–3 times per week and passed when being confluent. The cells were kept at 5% CO₂ in a humidified 37 °C incubator. The day before transfection, BEL-7402 cells were added into 6-well culture plate, and transiently transfected with the corresponding plasmid (Fig. 4) according to the liposome-mediated transfection method using Lipofectamine™ 2000 once cells reached 50%–80% confluency. 6–10 h after transfection, transfecting solution was replaced with the complete medium. In pApoE-SCS-EGFP-HCV-C+Cre transfecting group, the complete medium and 10 μM His₆-NLS-Cre-MTS (recombinant cell-permeable

Cre protein) [14] were together added into the corresponding well after discarding the transfecting solution. The transfection experiment was repeated three times with 1 μ g, 5 μ g and 10 μ g of each corresponding plasmid being employed, respectively. In addition, all the plasmids used for transfection were purified using EndoFree plasmid maxi kit (Qiagen) and non-linearized.

EGFP assay 48–96 h after transfection, cells were screened for the EGFP expression under fluorescence microscopy (excitation 450–490 nm).

Analysis of RNA using RT-PCR After activation of target genes, the HCV-C RNA level in BEL-7402 cells was examined by RT-PCR. RNA was isolated using the RNeasy mini kit (Qiagen) following the manufacturer's recommendations. Isolated RNA was fully treated with amplification grade RNase free DNase (Promega) at 37 °C for 30 min to remove any contaminating DNA, and then heated at 72 °C for 30 min to inactivate the DNase. A specific system for the amplification of HCV-C mRNA used was One-step mRNA selective RT-PCR kit (Ver. 1.1, TaRaKa). RT-PCR was carried out as recommended by the manufacturer with primer pairs HCV-C-FP/RP (PCR product size: about 573 bp). RT-PCR amplification was carried out: 30 min at 50 °C for RT, denaturation for 5 min at 85 °C; then a succession of 35 cycles as follows, 1 min at 85 °C, 1 min at 58 °C, 60 s at 72 °C; a final extension at 72 °C for 10 min. Human β -actin gene was used as an internal standard with following primers (product size being 758 bp):

Sense primer: 5'-GATATCGCTGCGCTGGTCGT-3'

Antisense primer: 5'-CGGAACCGCTCGTTGCCAAT-3'

For β -actin mRNA, one-step RT-PCR was carried out: 30 min at 50 °C for RT; and then a succession of 30 cycles

as follows, 85 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min. All experiments were performed in triplicate.

Western blot analysis of HCV-C expression After activation of target genes, HCV-C expression in BEL-7402 cells was also tested by Western blot. Cell lysates were prepared and 100 mg of crude protein was processed for Western blot analysis as Sambrook *et al.* [23] described. Western blot was performed with monoclonal anti-HCV-C (1:25, Bidesign International) as primary antibody and Horseradish peroxidase-conjugated goat anti-mouse IgG (1:2000, Amersham) as secondary antibody. Hybridization reaction was developed with enhanced chemiluminescence (ECL, Amersham) following the manufacturer's instructions.

Results

Construction of pApoE-SCS-EGFP-HCV-C

pApoE-HCV-C HCV-C was amplified by PCR, and inserted into pMD18-T to prepare pMD18-T-HCV-C and confirmed by sequencing. After that, the 573 bp HCV-C cDNA was excised, isolated, purified, and subsequently directionally subcloned into the *Mlu*I and *Xho*I sites of pLiv.7 (8.3 kb) linearized with *Mlu*I and *Xho*I to give pApoE-HCV-C (8.873 kb), as confirmed by enzyme digestion (Fig. 2).

pApoE-EGFP-HCV-C The 742 bp EGFP segment was excised from pBI-EGFP with *Eco*RV and *Spe*I and blunted at *Spe*I site, and cloned into the dephosphorylated *Hpa*I site of pApoE-HCV-C to obtain pApoE-EGFP-HCV-C (9.613 kb), which was verified by enzyme digestion

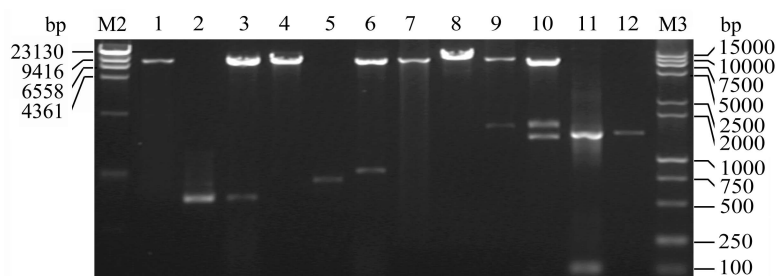


Fig. 2 Enzyme digestion identification of the resulting plasmid pApoE-SCS-EGFP-HCV-C by analysis

M2, λ -HindIII digest marker (TaKaRa); M3, DL2000+DL15000 marker (TaKaRa); 1, purified vector DNA (pLiv.7) for ligation; 2, purified insert HCV-C for ligation; 3, pApoE-HCV-C cut by *Mlu*I and *Xho*I; 4, pApoE-HCV-C digested by *Hpa*I or by *Xho*I (data not shown); 5, blunted and purified insert EGFP for ligation; 6, pApoE-EGFP-HCV-C cut by *Eco*RI; 7, pApoE-EGFP-HCV-C digested by *Kpn*I and *Mun*I or *Kpn*I (data not shown); 8, pApoE-SCS-EGFP-HCV-C digested by *Kpn*I; 9, pApoE-EGFP-HCV-C digested by *Hind*III; 10, pApoE-SCS-EGFP-HCV-C cut by *Kpn*I and *Hind*III; 11, SCS PCR product (1.487 kb) amplified from pApoE-SCS-EGFP-HCV-C with primers P1/P2 specific for SCS; 12, a 1.560 kb *Nor*I-segment of SCS excised from pBS302.

(Fig. 2). The orientation of EGFP insert in the frame was identified by *EcoRI* digestion. The positive recombinant showed 8.742 kb and 0.826 kb bands (Fig. 2), while the inverted recombinant generated two fragments of 8 kb and 1.568 kb after *EcoRI* digestion (data not shown).

pApoE-SCS-EGFP-HCV-C The 1.487 kb SCS amplified by PCR was cloned into the pMD18-T to give pMD18-T-SCS, and confirmed by sequencing. SCS fragment with *KpnI-EcoRI* site, released from pMD18-T-SCS, was targetedly subcloned into the *KpnI* and *MunI* sites of pApoE-EGFP-HCV-C to produce the final desired plasmid pApoE-SCS-EGFP-HCV-C (11.1 kb). The desired resulting pApoE-SCS-EGFP-HCV-C digested by *KpnI* showed a 11.1 kb band, while cut by *HindIII* produced 1.56 kb and 9.48 kb bands (Fig. 2). The 1.56 kb, 1.4 kb and 8 kb fragments were excised from the resultant pApoE-SCS-EGFP-HCV-C through double enzyme digestion of *KpnI* and *HindIII* (Fig. 2).

Consequently the genes of interest, such as SCS flanked by directly repeated *lox P* sequences, EGFP and HCV-C, were successively positioned downstream of *apoE* promoter/intron and upstream of the *apoE* polyadenylation sequences/liver element to prepare the desired construct pApoE-SCS-EGFP-HCV-C [Fig. 1(A)].

Confirmation of *lox P* site sequences in the vector

In pApoE-SCS-EGFP-HCV-C, SCS was flanked by two *lox P* sites (Fig. 1). The recombination event *in vitro* mediated by Cre between two *lox P* sites will led to the excision of the *lox P*-flanked ("floxed") intervening DNA sequence to create the resulting plasmid pApoE-*lox P*-EGFP-HCV-C. Under conditions used in this study, 1.487

kb of the intervening sequence between *lox P* sites was amplified efficiently by PCR using pApoE-SCS-EGFP-HCV-C as template, whereas actually Cre mediated recombination theoretically allowed amplification of an 84 bp PCR product that could not observed on the electrophoresis map (Fig. 3). That Cre could excise efficiently STOP cassette between two *lox P* sites from pApoE-SCS-EGFP-HCV-C was verified by enzyme digestion analysis (Fig. 3).

Cell-permeable Cre-mediated conditional expression in the cultured cell

In negative control I and II experiments [Fig. 4(A): a,b], the control cells had no background under fluorescence microscopy phase, as well as under phase contrast microscopy (data not shown). That EGFP fluorescence was observed 48 h after transfection in BEL-7402 cells transfected with pApoE-EGFP-HCV-C [Fig. 4(A): d] and pApoE-*lox P*-EGFP-HCV-C [Fig. 4(A): e] confirmed the construct (e.g. pApoE-EGFP-HCV-C) for functionality and that EGFP could express normally in the presence of one *lox P* site in the frame. In the absence of Cre, no background expression of EGFP could be detected in BEL-7402 cells transfected with pApoE-SCS-EGFP-HCV-C [Fig. 4(A): c], due to the strong transcriptional stop sequence inserted between the promoter and the coding sequences. However, when the cell-permeable Cre proteins (final conc: 10 μ M) were added into the well containing BEL-7402 cells transfected with pApoE-SCS-EGFP-HCV-C, the resulting transfected BEL-7402 cells displayed apparently ubiquitous expression of EGFP 72 h later [Fig. 4(A): f], indicating efficient excision of the floxed

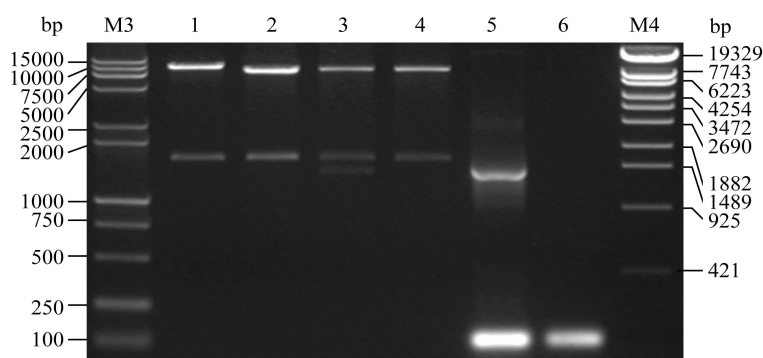


Fig. 3 Cre-mediated *in vitro* recombination

After recombination, the resulting plasmid (RP) and pApoE-SCS-EGFP-HCV-C (control) were employed to be substrate for enzyme digestion and template for PCR. M3, DL2000+DL15000 marker (TaKaRa); M4, λ -*EcoT14I* digest marker (TaKaRa); 1, pApoE-SCS-EGFP-HCV-C digested by *HindIII*; 2, RP digested by *HindIII*; 3, pApoE-SCS-EGFP-HCV-C digested by *KpnI* and *HindIII*; 4, RP digested by *KpnI* and *HindIII*; 5, pApoE-SCS-EGFP-HCV-C used as PCR template; 6, RP used as PCR template.

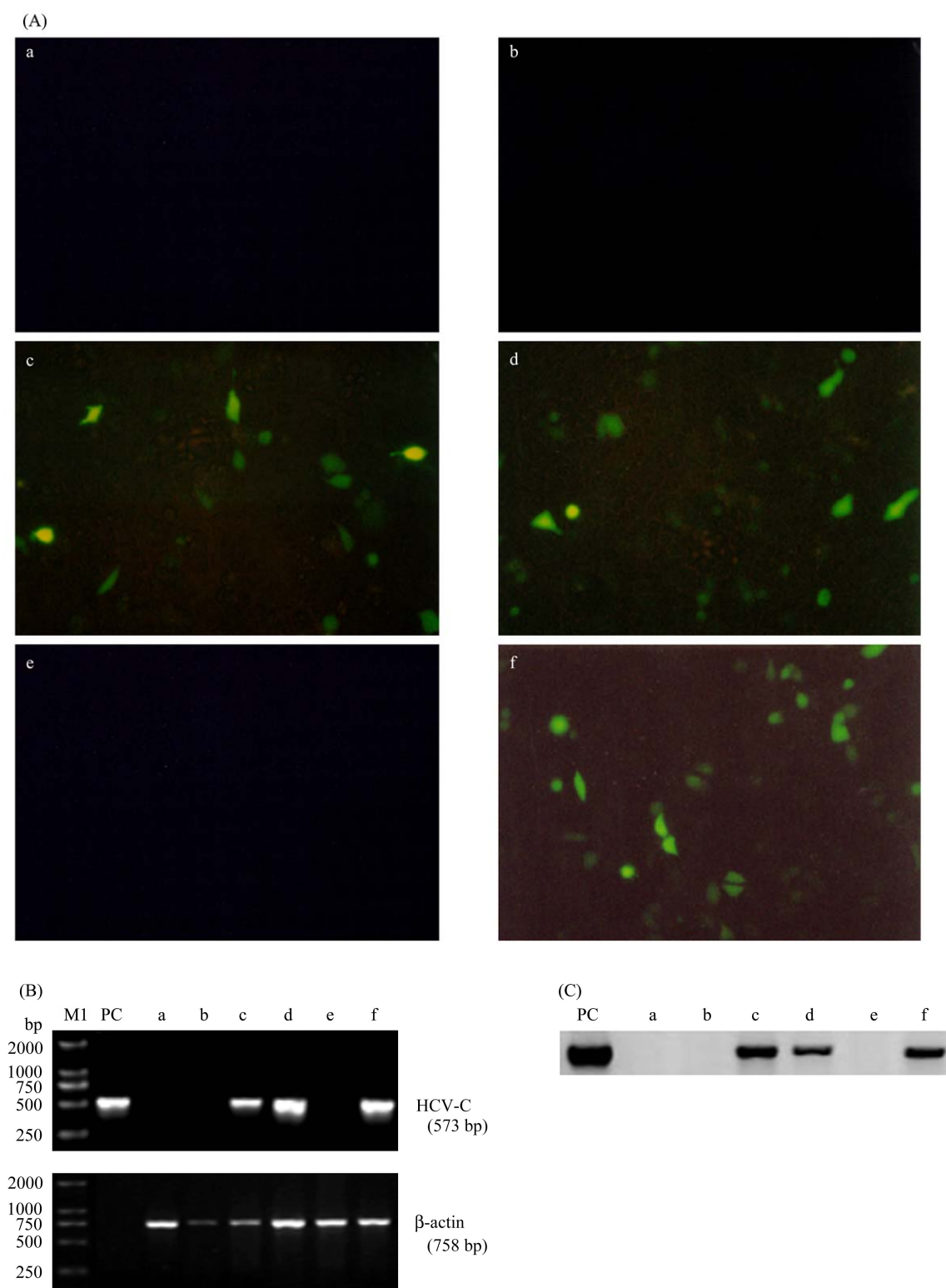


Fig. 4 Assay for the cell-permeable Cre-mediated DNA recombination in the cell level

(A) EGFP assay under fluorescence microscopy. (B) Checking Cre-mediated activation of HCV-C expression in the mRNA level. β -actin was used as an internal. The molecular weight of HCV-C and β -actin was 573 bp and 758 bp, respectively. (C) Western blot analysis of Cre-mediated HCV-C expression in BEL-7402 cells. M1, DL2000 DNA marker; PC, cells transfected with pApoE-SCS-EGFP-HCV-C expressing HCV-C; a, untransfected cells; b, cells transfected with pLiv.7; c, cells transfected with pApoE-EGFP-HCV-C; d, cells transfected with pApoE-*lox* P-EGFP-HCV-C resulted from pApoE-SCS-EGFP-HCV-C cut *in vitro* by Cre; e, cells transfected with pApoE-SCS-EGFP-HCV-C; f, 7402 cells transfected with pApoE-SCS-EGFP-HCV-C with the addition of Cre.

stop sequence. Moreover, HCV-C activation expression further confirmed the availability of Cre/*lox* P system and construct functionality [Fig. 4(B,C)].

Taken together, these data demonstrate the tight and conditional regulation of EGFP and HCV-C expression mediated by the Cre/*lox* P switching expression system in the cell.

Discussion

Since the harmful transgene product will behave as a self antigen inducing negative selection of reactive T cells in the thymus, immunologic response can only be studied through a system by which the transgene will be expressed in the transgenic animal only when being induced at desired time points [8–10]. The Cre-mediated conditional expression system developed recently can dissolve this problem [17–19]. The Cre/*lox* P conditional transgene activation system stringently controlling HCV-C transgene expression *in vivo* and *in vitro* was well elucidated in Fig. 1(B). In this system, the *lox* P² STOP cassette must locate between the desired promoter and target gene/reporter gene to give the dormant transgenic construct. Transgenic mice carrying the dormant transgene can mate Cre transgenic mice to generate bi-transgenic mice, in which Cre could be inducibly expressed to evict the STOP sequence to activate the dormant transgene. Transgenic mice carrying the dormant transgene can also be administered with cell-permeable Cre protein or Cre-expressing virus vectors by intravenous injection to induce Cre-mediated recombination.

A critical issue in using the *lox* P² STOP strategy is that after recombination the remaining *lox* P site must not interfere with expression of the target transgene [13]. Because *lox* P contains two ATG translational start codons in one orientation, but not in the other, as verified by sequencing, it is important that the *lox* P² STOP cassette be properly oriented (Fig. 2) so that there remains no false ATG codon that could interfere with downstream transgene expression after recombination [13,25]. Generally speaking, Cre-mediated activation system can tightly control the expression of dormant transgenes [12,13].

The tissue specific expression of the recombinationally activated dormant transgene is regulated by the promoter specificity of the target transgene and Cre transgene. In this study, Cre-mediated excision was realized by the intravenous injection of cell-permeable Cre protein or Cre-expressing virus vectors. The liver-specific *apoE* promoter system was employed to target the expression

of the viral proteins to the physiologically relevant site of hepatocyte after activated. Actually, when the target gene (s) is/are placed downstream of the human *apoE* promoter, and upstream of the *apoE* polyadenylation sequence [Fig. 1(A)], the high expression of target gene can be ensured [20,26–28].

The non-invasive visualization is the key for GFP to being able to monitor physiological and biochemical processes *in vitro*, *in vivo* and in real time [29]. In this study, EGFP gene served as a reporter gene to conveniently monitor the *in vitro* function of Cre/*lox* P system and the *apoE* promoter.

In summary, we conclude that the Cre/*lox* P switching expression system is able to successfully and stringently control the expression of the dormant transgene EGFP and HCV-C in the cultured human hepatoma cells, which lays a solid foundation for efficient and conditional HCV-C gene activation in transgenic mice by Cre-mediated site-specific recombination.

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