Engineered Long Terminal Repeats of Retroviral Vectors Enhance Transgene Expression in Hepatocytes in Vitro and in Vivo

Kanji Yamaguchi,1,2 Katsuhiko Itoh,1,* Naoki Ohnishi,1 Yoshito Itoh,2 Christopher Baum,3 Takashi Tsuji,4 Toshikazu Nagao,1 Hiroaki Higashitsujii,1 Takeshi Okanoue,2 and Jun Fujita1

1Department of Clinical Molecular Biology, Faculty of Medicine, Kyoto University, Kyoto 606-8507, Japan
2Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Graduate School of Medicine, Kyoto 602-0824, Japan
3Department of Hematology/Oncology, Hannover Medical School, 30625 Hannover, Germany
4Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, Noda 278-8510, Japan

*To whom correspondence and reprint requests should be addressed at the Department of Clinical Molecular Biology, Faculty of Medicine, Kyoto University, Shogoin-Kawaharacho 54, Sakyo, Kyoto 606-8507, Japan. Fax: +81-75-751-3750. E-mail: katsu@virus.kyoto-u.ac.jp.

To analyze the important elements for retroviral expression in hepatocytes, cis-acting elements in the U3 region of the long terminal repeat (LTR) of the polycythemic strain of spleen focus-forming virus (SFFVp) were analyzed in a hepatocellular carcinoma cell line. Two cis-acting elements located within the upstream region of the direct repeat, which positively regulated retroviral expression, were identified. Transcription factors NFAT5 and Sp1, which are ubiquitously expressed in a variety of tissues, bound to these elements. To increase specificity without lowering the potency of retroviral expression in hepatocytes, these elements were replaced by a sequence derived from the hepatitis B virus enhancer II region. Novel vectors, SF-Hep3 and SF-Hep5 (SFFVp-based vector for hepatocytes 3 and 5), were developed with these engineered LTRs. The engineered LTRs of these vectors enhanced the retroviral expression only in hepatocellular carcinoma cell lines in vitro. These vectors also increased transgene expression 4- to 9-fold or 3.5- to 5-fold in comparison with a Moloney murine leukemia virus-based vector or a vector containing the wild-type LTR of SFFVp, respectively, in murine hepatocytes in vivo.

Key Words: hepatocyte, liver, retrovirus, vector, SFFV, LTR

INTRODUCTION

Retroviral vectors are the most commonly used vehicles for stable gene transfer into various cell types. These vectors can integrate into the host genome to provide long-term transgene expression in the target cells [1,2]. Hepatocytes are attractive targets for these vectors, since they synthesize a myriad of proteins that play pivotal roles in metabolism or hemostasis. One of the major limitations to the use of retroviral vectors in hepatocytes is the low level of transgene expression [3,4].

cis-acting elements located in the U3 region of the retroviral long terminal repeats (LTRs) determine mainly the transcriptional level of newly introduced genes in target cells. Most of the cis-acting elements are found within the direct repeat (DR) [5–10]. In addition to the DR, the elements were also identified in the upstream control region (UCR; the upstream region of the DR) [11–14]. Although these elements were proven to regulate retroviral expression, the activities of these elements have been analyzed in detail mainly in hematopoietic or embryonic cells and not in hepatocytes.

The polycythemic strain of spleen focus-forming virus (SFFVp) is a replication-incompetent virus related to Friend mink cell focus-forming viruses [15]. Its LTR provides high transgene expression in primitive hematopoietic cells [16,17] and spermatogenic cells [18]. Recently, we have demonstrated that the SFFVp U3 also provides about fourfold stronger retroviral enhancer activity than that of the Moloney murine leukemia virus (MoMLV) in hepatocytes in vivo [19]. In the present study, we have analyzed the regulatory elements of the SFFVp LTR in a hepatocellular carcinoma cell line and an attempt has been made to enhance its promoter activity especially in hepatocytes.

RESULTS AND DISCUSSION

Strong cis-Acting Elements Are Located in the Upstream Region of the DR in SFFVp

To localize the region important for high retroviral expression within the SFFVp U3, we constructed a series of reporter gene plasmids containing the sequence from the
SFFVp U3 (Fig. 1A). We used a well-differentiated human hepatocellular carcinoma cell line, PLC/PRF/5 [20], for transient reporter assays, since the results of reporter assays in cultured PLC/PRF/5 cells correlated well with those in hepatocytes in vivo [19]. pSF418luc contains the sequence from position −4 (4 bp upstream of the cap site) to +418. Deletion of more than 56 bp from the 5′ end significantly reduced the activities (pSF352luc, pSF322luc, or pSF289luc in Fig. 1A), indicating the presence of cis-acting elements in the upstream region of the DR (the UCR). We constructed another series of reporter gene plasmids, which contain a series of 3′ to 5′ deletions from position −178 (Fig. 1B). Deletion of the UCR (the region between −371 and −303) reduced the activities to half of that of pSF418luc (pSF371-311/178luc or pSF371-320/178luc in Fig. 1B), confirming the enhancer activity in the UCR.

To analyze the important elements, we cloned the fragment of the U3 from position −371 to −303 into another reporter gene plasmid, pTKluc, containing a thymidine kinase promoter of the herpes simplex virus. This fragment showed enhancer activity regardless of orientation (Fig. 1C, p371-303TKluc and p303-371TKluc). Further analysis showed that the sequences from −362 to −343 and from −320 to −303 were sufficient to enhance the activity (Fig. 1D).

Taken together, these results clarified the enhancer activity of the UCR in SFFVp U3 and that the activity was localized to two regions, spanning from −362 to −343 and from −320 to −303.

Deletion of the Enhancer Elements from Retroviral Vectors Reduces Transgene Expression
To test whether these regions are also crucial for retroviral expression after integration into the genome, we generated a series of retroviral vectors that carry a chloramphenicol acetyltransferase (CAT) gene as a reporter. We produced retrovirus vectors using Phoenix-ampho cells and infected PLC/PRF/5 cells with these vectors. We selected cells in the presence of G418 and measured expression of CAT protein. Although the average numbers of proviral DNAs of SF/CATwt, SF/CATΔ320-303, and SF/CATΔ362-320-303 in the pooled cells were not significantly different (1.29, 1.27, 1.25, and 1.46, respectively), the expression levels from the enhancer-deleted vectors (SF/CATΔ362-320-303) were almost equal to one another and were about half of that from SF/CATwt, which contains the full-length LTR (Fig. 2B). These results confirmed the importance of these two regions for retroviral expression.

Mutation Analysis of the cis-Regulatory Elements in the UCR
We performed substitution mutation analysis to identify important regulatory elements. We scanned the regions from −362 to −343 and from −320 to −303 using a series of reporter gene plasmids, pMut/1 to pMut/10 (Fig. 3A). Luciferase activities were significantly reduced by the mutation within the region from −358 to −347 or from −313 to −306, while no significant effect was observed.
with the flanking mutations (Fig. 3A), indicating that the sequences of GGCATGGAAAAA and GGGCGGGT were crucial for the cis activity.

The first sequence, GGCATGGAAAAA, is well conserved among murine retroviruses and is located in the 3’ flanking region of the binding motif for YY-1 (Fig. 3B). The other sequence, GGGCGGGT, is located in the 5’ flanking site of the DR, overlapping the Sp1 binding site [11], and is not conserved between viruses (Fig. 3B).

We have identified these elements located in the UCR and these elements have been proved to be crucial for retroviral expression. On the other hand, there might be some other elements located in regions other than the UCR (i.e., the DR or the 3’ flanking region of the DR) that are also crucial for retroviral expression. Such elements remain to be clarified for our further understanding of high promoter activity of the SFFVp LTR in hepatocytes.

The Nuclear Factor of Activated T Cells 5 (NFAT5) Transcription Factor Binds to the UCR and Enhances Reporter Gene Expression

One of the identified regions (the position from −358 to −347) contains the GGA core motif for NFAT [21] and the substitution mutation analysis showed a clear preference for the sequence longer than the core motif, which contains the target motif for NFAT5 (PuTGGAAAnA) [24]. Since the expression of NFAT5, but not NFAT1, NFAT2, nor NFAT3, was detected by RT-PCR in PLC/PRF/5 cells (data not shown), we analyzed the binding ability of NFAT5 to this region. We transfected pCMV/flag-NFAT5, which contains human NFAT5 cDNA tagged by flag sequences (Fig. 4A), into HEK293T cells and used nuclear extracts for electrophoretic mobility shift assays. One of

FIG. 2. Expression of the CAT reporter gene in PLC/PRF/5 cells transduced by retroviral vectors containing the truncated UCR. (A) Retroviral vector plasmids containing the deletion within the UCR are schematically shown. (B) Retroviral vector plasmids were transiently transfected into Phoenix-ampho cells and the supernatants were used to transduce PLC/PRF/5 cells. After selection, concentration of CAT protein was measured. Each column and bar represents the mean value and standard deviation of three assays, respectively. *Significantly different from the CAT value mediated by the SF/CATwt vector (P < 0.005).

FIG. 3. Substitution mutation analysis of the cis-regulatory elements. (A) Transient expression of luciferase mediated by the constructs containing three to four base pair substitution mutations. Each column and bar represents the mean value and standard deviation of three assays, respectively. *Significantly different from the value mediated by p362-343/320-303TKluc (P < 0.05). (B) Comparison of the sequences of the upstream region of the DR among murine retroviruses and summary of the mutation analysis. Boxed regions have been identified as crucial cis-regulatory sequences in the present study. Doubled underlines indicate the motifs for YY-1, ELP, or Sp1. Dotted sequences are different from the sequences of MoMLV.
the bands detected with oligonucleotides (GGCATG-GAAAAA) (Fig. 4B, lane 2; indicated by an arrowhead) was abolished (lane 3) or supershifted and diminished (lane 4) by adding 100-times excess of unlabeled oligonucleotides or an anti-flag antibody, respectively, indicating that NFAT5 protein bound to this sequence.

Next, we examined whether the exogenously expressed NFAT5 increased expression of a reporter gene. Expression of NFAT5 increased the promoter activity of the SFFVp U3 in HEK293T cells (Fig. 4C). Another reporter plasmid, p362-343x4TKluc, which contained four repeats of the sequence corresponding to position 362 to 343, was also transfected with pCMV/flag-NFAT5 (Fig. 4D). The transfection of pCMV/flag-NFAT5 increased the luciferase activity in a dose-dependent manner, indicating that NFAT5 could enhance the retroviral expression.

NFAT5 belongs to the NFAT family of transcription factors and has also been designated as a tonicity enhancer binding protein, TonEBP [22]. Functional analysis of this protein, TonEBP/NFAT5, has clarified that it is induced upon hyposmotic stimuli and is a target of integrin signaling [23]. Here we showed its potential to bind to the UCR of murine retroviruses and positively regulate their expression.

**Engineering of the UCR Enhances the Reporter Gene Expression in Hepatocellular Carcinoma Cell Lines in Vitro**

We have identified two regions important for retroviral expression. The first one contains a target of NFAT5 and the second one is known to be a target of Sp1 [11]. Since these transcription factors are expressed ubiquitously in a variety of tissues, but not restricted to hepatocytes [24,25], we made an attempt to examine whether the replacement of these target sequences increases specificity without lowering potency of retroviral expression in hepatocytes.

Hepatitis B virus (HBV) predominantly infects hepatocytes and the HBV genes are expressed specifically in liver, controlled by the combined action of the promoters and enhancers. The transcription of the viral mRNAs is regulated by two HBV enhancers, Enhancer I (EnI) and EnII, and the latter shows strong hepatocyte specificity [26,27]. We replaced two regions within the SFFVp U3 with the sequences from HBV EnII (designated as “a” or “b” in Fig. 5A), which determine the strong hepatocyte specificity and contain the targets of C/EBP, HNF4, HNF3, and HNF1. As shown in Fig. 5B, LTRs of pSF/Hep3-luc and pSF/Hep5-luc especially showed significantly higher promoter activity than the SFFVp LTR in PLC/PRF/5 cells (P < 0.005, Fig. 5B).

We generated two retroviral vectors, SF-Hep3/CAT and SF-Hep5/CAT, which contained the LTRs from pSF/Hep3-luc and pSF/Hep5-luc respectively (Fig. 6A). First, we examined the efficiencies of the viral production of these novel vectors. Using Phoenix-eco packaging cells, titers of SF-Hep3/CAT and SF-Hep5/CAT were 5.7 × 10^5 and 6.0 × 10^5 G418-resistant colony-forming units per milliliter on NIH/3T3 cells (CFU-G418/ml), respectively, and were comparable with that of SF/CATwt (6.7 × 10^5 CFU-G418/ml). Next, we examined the activities of these engineered enhancers upon stable integration into the genome in...
The Novel Vectors SF-Hep3 and SF-Hep5 Efficiently Express a Transgene in Murine Hepatocytes in Vivo

To test whether the engineered LTRs provide a high transgene expression in vivo, we infused mice with SF/CATwt, SF-Hep3/CAT, SF-Hep5/CAT, or a MoMLV-based vector, MO3/CAT [19] (Fig. 6B), after partial hepatectomy. First, transgene expression was compared among these vectors 4 days after infusion. LTRs of SF/CATwt, SF-Hep3/CAT, and SF-Hep5/CAT were from SFFVp, pSF/Hep3-luc, and pSF/Hep5-luc, respectively. Expression of CAT by SF/CATwt, SF-Hep3/CAT, or SF-Hep5/CAT was about 2.5-, 4-, or 9-fold higher, respectively, than that by the MoMLV-based vector, MO3/CAT, and SF-Hep5/CAT provided about 3.5-fold higher expression than SF/CATwt (Fig. 6C). Since a partial hepatectomy induces liver cell replication, we additionally compared transgene expression among these vectors after 1 month of infusion to evaluate the expression in quiescent hepatocytes. Expression of CAT by SF/CATwt, SF-Hep3/CAT, or SF-Hep5/CAT was about 1.6-, 4.5-, or 5-fold higher, respectively, than that by MO3/CAT, and both SF-Hep3/CAT and SF-Hep5/CAT provided about 3-fold higher expression than SF/CATwt (Fig. 6D).

These results indicated strongly improved expression of a transgene by engineered LTRs in hepatocytes in vivo. To increase the transgene expression in hepatocytes in vivo, we have compared the promoter activities of murine retroviruses [19] and engineered the LTR from SFFVp in this study. An alternative approach to increasing the expression is to use hepatocyte-specific internal promoters. Although both positive and negative influences can be transmitted between the LTR and such an internal promoter [28], it has been proven that the use of the α1-antitrypsin promoter or the ApoE enhancer–α1-antitrypsin promoter increased transgene expression in hepatocytes in vivo [29,30]. Intensive effort has also been put into the development of viral vectors based on adeno-associated virus [31] and lentiviruses [32]. Together with the development of these vectors, the novel vectors designated SF-Hep3 and SF-Hep5 will provide a useful tool in the field of experimental hepatology, as well as important mechanistic information for the development of vectors for liver-targeted gene therapy.

MATERIALS AND METHODS

Cell lines. The human hepatocellular carcinoma cell lines PLC/PRF/5 [20], HLE [33], and HuH-7 [34]; fibroblastic cell lines HEK293 and HEK293T; and hematopoietic cell lines K562 and Jurkat were maintained in DMEM supplemented with 10% fetal calf serum. A murine fibroblastic cell line, NIH/3T3, was maintained in DMEM with 10% calf serum. Amphotropic and ecotropic retrovirus packaging cell lines Phoenix-ampho and Phoenix-eco (kindly provided by Dr. G. P. Nolan of Stanford University Medical
A fragment containing the SFFVp U3 (position from 3 to 371) was amplified, ligated into pBS (pBS362-303), excised as an Xbal-Xhol fragment, and ligated into Nhel-Xhol sites of pTKluc to obtain p351-320TKluc. Using the same strategy, p371-311TKluc and p371-303TKluc were obtained, which contain the sequences from position −371 to −311, from −371 to −303, and from −352 to −303, respectively. Plasmid p303-371TKluc was obtained from a fragment containing the sequence from −303 to −371 in the direction opposite to p371-303TKluc. A series of reporter gene plasmids, schematically shown in Fig. 1D, was constructed as follows. Plasmid DNA of pBS362-303 was amplified by PCR, phosphorlated, self-ligated (pBS362-343/320-303), excised as an Xbal–Xhol fragment, and ligated into Nhel–Xhol sites of pTKluc to obtain p362-343/320-303TKluc. Using the same strategy, p371-311TKluc and p371-343/320-303TKluc were obtained, in which the sequence from position −371 to −311, from −371 to −303, and from −352 to −303, respectively, was deleted from p371-352/320-303TKluc or p362-343/320-303TKluc to obtain p320-303TKluc or p362-343TKluc, respectively. All these plasmids were sequenced before the experiments. A reporter gene plasmid containing tandem repeats of the cis-regulatory element is schematically shown in Fig. 4D. The following pair of oligonucleotides was annealed, ligated into the EcoRV site of pBS, excised as an Xbal–Xhol fragment, and ligated into Nhel–Xhol sites of pTKluc to obtain p362-343/4TKluc, which contains four tandem repeats of the sequence from position −343 to −362: 5′-GGAAGCCATGGATAAACAAGCAGGGCCATGGAAAAATACCGCAAGGCATGGAAAAATACC-3′ and 5′-GGGTACTTCTCATGCTCCCTGGTTTTCTCTGCTGGTATTCTCCATGCCTGCTGGG-3′.
Construction of reporter gene plasmids for mutation analysis of the cis-regulatory elements. A series of reporter gene plasmids, schematically shown in Fig. 3A, was constructed as follows. The three to four base pair substitution mutations (A → G, T → C, G → T, G → A) were sequentially introduced from 3’ to 5’ into pBS62-343/320-303 using the Quick Change mutagenesis kit. After sequencing, an XhoI–Xhol fragment was excised and ligated into NheI–Xhol sites of pHTkuC to obtain pMut1 to pMut10.

Construction of a plasmid for the expression of NFAT5. A human NFAT5 cDNA was amplified by reverse transcriptase-polymerase chain reaction from human liver cDNA and ligated into BamHI–Xhol sites of a mamma- lian expression vector, pCMV-Tag2 (Stratagene), to obtain pCMV/flag-NFAT5.

Construction of retroviral vector plasmids. The U3 region of the 5’ LTR of p5FN was replaced by a human cytomegalovirus promoter to obtain p5FN/CMV. A plasmid DNA of p5FS/LTR was amplified, self-ligated, ex- cited, and ligated into pCMV to generate p5FN/Δ326-343, p5FS1N/Δ320-320, or p5FS/Δ362-343/320-303. A NotI–Nof fragment containing a CAT gene at the 5’ position and internal ribosomal entry site from poxivirus was ligated into a NotI site of pSF/CMV, p5FS1N/Δ326-343, p5FS1N/Δ320-320, or p5FS/Δ362-343/320-303 to obtain pSF/CATw, pSF/CATΔ326-343, pSF/CATΔ3120-320, or pSF/CATΔ162-362/320-303, respectively. Construction of pMOI/CAT was described elsewhere [19].

Construction of plasmids containing engineered LTRs. A series of reporter gene plasmids or retroviral plasmids containing the engineered LTRs is schematically shown in Fig. 5B or 6A, respectively. A BamHI–Xhol fragment containing the 3’ LTR was ligated into p5FS and plasmid DNA was amplified by PCR and ligated with annealed oligonucleotides (a) 5′-TTACTAGGAGGACTCTTGGACTCA-3′ and 5′-TGAGTTCTGTGTGGATTGTTGATGATG-3′ to generate p5FS/Δ362-343/320-303. NFAT5–I sites of pSF/CATw to obtain pSF/CATΔ362-343/320-303.

Transient transfection and reporter gene assays. For transient reporter assays, 10 μg of firefly luciferase reporter gene vector DNA was cotransfected with pRL-CMV or pRL-TK (a Renilla luciferase vector; Promega Japan, Madison, WI) into HEK293T cells or pSF/CMV to generate p5FS/Δ326-343. Transfected cells were harvested after 48 h of incubation and lucife- erase assays were measured according to the Promega protocols and applications guide by using the Dual-Luciferase Reporter Assay System (Promega Japan) with the Turner Designs luminometer Model TD-20/20 (Promega Japan). The firefly luciferase activity of each lysate was normalized by the activity relative to that of pSF14Bluc, pTKluc, or p5FS/326-343/320-303. In some experiments, plasmids for the expression of transcription factors were additionally transfected into cells.

Production of retroviral vectors. Phoenix-ampho or Phoenix-eco cells were transfected with retroviral vector plasmids. After 2–5 days of culture, supernatant containing viral vectors was used in this study. Viral vectors were termed SF/CATwT, SF/CATAΔ326-343, SF/CATΔ320-320, SF/CATΔ362-343/320-303, MOI/CAT, SF-Hept/CAT, or SF-Hept/CAT to distinguish them from the corresponding plasmids.

Retroviral transduction and measurement of CAT protein and copy numbers of integrated proviral DNAs. Cells were infected with SF/CATwT, SF/CATAΔ362-343, SF/CATΔ320-320, SF/CATΔ362-343/320-303, SF-Hept/CAT, or SF-Hept/CAT and were selected in the presence of G418 for 2 weeks. After selection, cells consisting of more than 100 different clones were lysed and the concentration of CAT protein of each lysate was measured by using a CAT enzyme-linked immunosorbent assay kit (Boeh- ringer Mannheim GmbH, Mannheim, Germany). The concentration of CAT protein in each lysate was normalized to the concentration of total proteins (pg/mg) and was represented as CAT activity relative to that of SF/CATwT.

In pooled cells were not significantly different among the vectors, TaqMan real-time PCR was performed after genomic DNAs were isolated from the samples. Genomic DNAs from clones containing one or two proviral integrations were used as standards. The estimated numbers were further normalized to β-actin using the following primers and probes: CAT prim- ers, 5′-GTTTCACATGGGCTGAGTGA-3′, 5′-CAAGGCACATCTGC- GAAATA-3′; CAT probe, 5′-CCAGCAAGGTTCCGTCTCACTAC-3′; mouse β-actin primers, 5′-AAGGCTGATGACTGCTGACG-3′ and 5′- CACAGAAGGATGGAAAGAA-3′; mouse β-actin probe, 5′-GCCCTC- CACGTTAAGGCGTCCTGAGG-3′; human β-actin primers; and a probe, TaqMan β-actin detection reagents kit (PE Applied Biosystems Japan, Tokyo).

In vivo experiments were performed as described previously [19]. In brief, virus particles were produced using Phoenix-eco packaging cells and were concentrated [36]. Forty-eight hours after partial hepatectomy, C57/Bl6 mice were infused via the tail vein with viruses representing infusion of 3–5 × 10⁹ G418-resistant colony-forming units on NIH/3T3 cells. Four days or 1 month after infusion, the liver was removed and the concentra- tion of CAT protein in each lysate was normalized to the concentration of total proteins (pg/mg). Genomic DNAs from the samples were isolated and the proportions of the genomic integration of proviral DNAs were esti- mated by the TaqMan real-time PCR method. A genomic DNA from a NIH/3T3 cell containing a single integrated copy and that from an uninfected clone were mixed at a ratio ranging from 1:1000 to 1:10 and were used as standards. The estimated proportion was normalized to β-actin to determine the integration efficiencies (%). Normalized concentra- tion of CAT protein of each sample (pg/mg) was further normalized to the integration efficiency (%) and was represented as normalized CAT/ integration (pg/mg/%) in Figs. 6C and 6D.

Western blotting and gel mobility shift assays. After the transfection of pCMV/flag-NFAT5 into HEK293T cells, cell lysate was analyzed by Western blot using an antibody to flag (M2, Sigma, St. Louis, MO). Preparations of nuclear extracts and gel mobility shift assays were performed as previously described [11]. The following set of oligonucleotides was annealed and used for this study: GCCAAGGCTGAGAAAAATC and GGTATTTTCG- CATGCCTTGC. Supershift assays using an antibody to flag (M2, Sigma) were performed by incubating antibodies with nuclear extract for 1 h at 4°C.

ACKNOWLEDGMENTS
This work was partly supported by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture of Japan.


REFERENCES
important for activity of the core I element of the murine retrovirus SL3-3 in T lymphocytes. J. Virol. 72: 3129–3137.


