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# Targeting of therapeutic gene expression to the liver by using liver-type pyruvate kinase proximal promoter and the SV40 viral enhancer active in multiple cell types

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**Abstract**

To achieve the liver-directed expression in sufficient amounts of therapeutic genes for successful and safe gene therapy, natural liver-specific promoters can be used to direct the expression of therapeutic genes in the liver, whereas strong viral enhancers were used to obtain sufficient amounts of expressed therapeutic gene products. However, very often use of either the former or the latter does not guarantee both potent and liver-specific therapeutic gene expression. Here we conglomerate them and thus create a potent tissue-specific promoter by characterizing and using the liver-type pyruvate kinase proximal promoter (LPKPP) harboring its TATA box and a HNF-1α binding site. Alone it hardly activated its reporter gene expression in non-hepatocytes or hepatocytes. However, in the presence of the SV40 viral enhancer (SV40VE), which is active in multiple cell types, it was able to potently activate its reporter gene expression specifically in hepatocytes. The tissue-specific activation of the LPKPP by the viral enhancer was attributed to HNF-1α binding to the LPKPP. Taken together, these results support the idea that the constitutively active SV40VE could be used to activate the LPKPP in a tissue-specific manner in the presence of HNF-1α. To our knowledge, this is the first study to utilize HNF-1α and its binding site, in the context of the LPKPP, to generate a basal promoter that is transcriptionally activated potently in a tissue-specific manner by a viral enhancer that is active in multiple cell types.


*Keywords:* Gene therapy; L-PK proximal promoter; HNF-1α; SV40 viral enhancer; Tissue-specific gene expression

The liver performs a large number of essential metabolic functions and is thus associated with many genetic disorders including inherited liver disorders such as familial hypercholesterolemia, ornithine transcarbamylase deficiency, Wilson's disease, and inherited systemic disorders such as hemophilia A or B, and acquired disorders such as hepatitis and liver tumors [1–4]. Very recently it has also served as a surrogate organ for insulin gene therapy against type I diabetes mellitus [5,6]. Thus, the liver is a major target organ for gene therapy.

It is critical to achieve the liver-directed expression in sufficient amounts of many therapeutic genes for gene therapy to be successful and safe. One way of directing the expressions of therapeutic genes in the liver is to use natural liver-specific promoters. These include albumin, the liver-type pyruvate kinase, phosphoenolpyruvate kinase, and human α1-antitrypsin promoters [7]. However, major disadvantages of their use in gene therapy are often caused by their lower transcriptional activity compared with strong viral enhancers such as

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cytomegalovirus (CMV), Long terminal repeats (LTR), or simian virus-40 (SV40) [8–11]. Here we conglomerate these two kinds of promoters to create a potent artificial tissue-specific promoter by using minimal regulatory sequences of a liver-type pyruvate kinase promoter (LPKPP) harboring its TATA box and a HNF-1α binding site and the SV40 viral enhancer (SV40VE) that is known to be active in many cell types [12–16].

## Materials and methods

*Plasmids.* pLT and pLT-SE1 were prepared by inserting the rat LPKPP region (−96/+13) [17] amplified by using a PCR (template; pLPK-SIA [5], and primers; A and E, as described below) into pGL3-basic and pGL3-enhancer vectors (Promega, Madison, WI, USA), respectively. Deletion mutants of the LPKPP region were generated by PCR (template; pLT-SE1) and inserted into pGL3-enhancer. The primers used to produce the constructs were B and E for pLT-SE1(−65/+13), C and E for pLT-SE1(−31/+13), D and E for pLT-SE1(−19/+13), and A and F for pLT-SE1(−96/−14). (A) 5′-GAA GAT CTC TAG CTG GTT ATA CTT TAA CCA G, (B) 5′-GAA GAT CTT CAT CTG AGC CAG GCC CC, (C) 5′-GAA GAT CTC GCA GTA TAA AGC AGA CC, (D) 5′-GAA GAT CTC AGA CCC ACA GAC ACA GC, (E) 5′-CCC AAG CTT ACG TTG CTT ACC TGC TGT GTC TG, and (F) 5′-CCC AAG CTT TAC CTG CTG TGT CTG TG. PCR was used to create a double point mutation in the LF-B1 binding site (pLT-SE1(TT → CC)). In brief, two sets of primers (upper 1; 5′-CTA GCA AAA TAG GCT GTC CC and lower 1; 5′-GAT GAG TCC TGG TTG GAG TAT AAC CAG CTA G, and upper 2; 5′-CTA GCT GGT TAT ACT CCA ACC AGG ACT CAT C and lower 2; 5′-CTT TAT GTT TTT GGC GTC TTC CA) were designed in such a way that the amplified products overlapped the region containing the mutation. To generate pLFB1, pLT-SE1 was digested with *Hin*dIII and *Xba*I to remove the luciferase coding sequence, where the *Hin*dIII/*Xba*I-digested fragment of the annealed oligonucleotides was inserted (upper; 5′-CCC AAG CTT GAA TTC AGC TGA TAT CTG C and lower; 5′-TGC TCT AGA CTG CAG ATA TCA GCT GAA T). The β-galactosidase coding sequence derived from pAAV-LacZ (Stratagene, La Jolla, CA, USA) was inserted into pLFB1 to generate pLFB1-LacZ.

*Cell culture and transfections.* HepG2 and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (200 IU/ml), and streptomycin (100 μg/ml). Transfections by using Tfx-50 and luciferase/β-galactosidase assays were carried out according to the manufacturer's protocol (Promega, Madison, WI, USA). Briefly, before transfection, the medium was aspirated and the remaining mono-layer cells were then exposed to the cocktail solution containing 200 μl DMEM, 4 μl plasmids (2 μg), and 4 μl Tfx-50 reagent for 1 h, followed by the addition of 1 ml of the culture DMEM. The transfected cells were grown further for 24–48 h. For luciferase and β-galactosidase assays, cell extracts were prepared according to the manufacturer's protocol (Promega, Madison, WI, USA). Briefly the transfected cells were exposed to lysis buffer for 10 min and the lysed cell extracts were saved after a brief centrifugation. The firefly luciferase assays were carried out: 10 μl (5–50 μg protein) of the extracts was added to 40 μl firefly luciferase assay buffer. The samples were placed in a luminometer (MicroLumat, LB96P, EG&G BERTHOLD) and light output was determined. For an internal control, the β-galactosidase assays were carried out: 10 μl (5–50 μg protein) of the extracts was added to 40 μl assay buffer and incubated for 30 min–24 h at 37 °C. The samples were placed in an UV spectrophotometer (UV-1601 PC, SHIMADZU, Japan) and optical density was determined at 420 nm.

*Electrophoretic mobility shift assay.* Electrophoretic mobility shift assays (EMSAs) were performed according to the methods previously described [18]. In brief, the following oligonucleotides were annealed and labeled with [γ-$^{32}$P]ATP (DuPont NEN); the wild type LF-B1 binding site (upper strand: 5-CTA GCT GGT TAT ACT TTA ACC AGG ACT CAT C and lower strand: 5-GAT GAG TCC TGG TTA AAG TAT AAC CAG CTA G) and the mutated LF-B1 binding site (upper strand: 5-CTA GCT GGT TAT ACT CCA ACC AGG ACT CAT C, and lower strand: 5-GAT GAG TCC TGG TTG GAG TAT AAC CAG CTA G). The binding reaction was preincubated for 10 min on ice in a final volume of 20 μl containing binding buffer (10 mM Hepes, 60 mM KCl, 1 mM DTT, 1 mM EDTA, and 7% glycerol, pH 7.6), about 20 fmol of labeled probe (>10,000 cpm), 20–50 μg nuclear protein, and 1–2 μg poly(dI–dC), and followed by addition of nuclear extracts and incubation for 30 min on ice. Incubated samples were loaded on a 6% nondenaturing polyacrylamide gel (37.5:1 acrylamide/bisacrylamide) and fractionated in 45 mM Tris–borate, 1 mM EDTA (0.5× TBE) at 180 V for 2 h at 4 °C. The dried gel was exposed to Kodak XR5 film (Eastman Kodak) on an intensifying screen overnight at −70 °C. For competition assays, unlabeled oligonucleotides were added in the preincubation mixture in 1–100-fold molar excess. For supershift assays, antibody either to HNF-1α (sc-6548, Santa Cruz, CA, USA) or to insulin (Guinea Pig anti Insulin, DAKO, Glostrup, Denmark) was included in the preincubation mixture.

*Recombinant adenoviruses.* For generation of adenoviral constructs of pLFB1-insulin analog (Ad-pLFB1-insulin analog), AdEasy Adenoviral vector system (Stratagene, La Jolla, CA, USA) was utilized and all the procedures were performed according to the manufacturer's instruction. Briefly, the *Xho*I/*Sal*I-digested fragment of pLFB1-insulin analog was inserted into pShuttle (*Xho*I/*Sal*I) to produce pShuttle-pLFB1-insulin analog. This resulting plasmid was linearized with *Pme*I and cotransformed into bacteria BJ5183 together with pAdEasy-1. Transformants were selected for kanamycin resistance to obtain Ad-pLFB1-insulin analog plasmid. Recombinant adenoviruses were purified from the cell lysate of Ad-pLFB1-insulin analog transfected HEK293 cells by two consecutive CsCl density gradient ultracentrifugations on preformed gradients.

*X-gal staining and immunohistochemistry.* For visualization of β-galactosidase activity of cultured cells with X-gal, transfected cells were washed with PBS, fixed with 4% paraformaldehyde (10 min, room temperature), and washed twice with PBS. Subsequently the cells were incubated in the staining solution containing 1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN_6)$–$^3H_2O$, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 for 3–5 h at 37 °C. The activity of β-galactosidase was visualized as the blue color of the 3,5′-dichromo-4,4′-dichloroindigo molecule resulting from cleaving the X-gal substrate by the β-galactosidase in the cells. After X-gal staining procedure, the cells were washed with PBS and photographed with a Nikon Diaphot 300 microscope and Nikon F-601 camera. For immunohistochemistry study, immunohistochemistry kit AEC (Innogenex, San Ramon, CA, USA) was used and all the procedures were carried out according to the manufacturer's instruction. Briefly, pepsin (Biogenex, San Ramon, CA, USA) was utilized for antigen removal. The endogenous peroxidase activity in cells was blocked by treatment with 3% hydrogen peroxide. Prior to antibody labeling, 10% goat serum was applied to the samples to prevent non-specific antibody labeling. The samples were incubated in the absence or presence of the primary antibody (guinea pig anti-insulin antibody (1:100, DAKO, Glostrup, Denmark) 1 h at 37 °C. After washing in PBS including 0.1% Tween 20, the samples were allowed to react with the secondary antibody (rabbit anti-guinea pig immunoglobulin (1:500, DAKO, Glostrup, Denmark) for 5 min at 37 °C. After washing the samples three times in PBS, biotinylated anti-rabbit immunoglobulin and streptavidin peroxidase conjugate were added and specific binding was visualized by staining with 3-amino-9-ethyl carbazole (red). The nuclei of immuno-stained cells were counterstained with Mayer's hematoxylin (Sigma–Aldrich, St. Louis, MO, USA).

*Northern blot analysis.* Hybridizations were performed using a kit purchased from Amersham Biosicence (St. Gail, UK) and all the procedures were performed according to the manufacturer's instruction. In brief, UV cross-linked blots were pre-hybridized in 10 ml rapid hybridization buffer at 65 °C for 15 min and hybridized at 65 °C for 2 h in the presence of human insulin cDNA probe labeled with [α-$^{32}$P]dCTP by random priming. Then blots were washed with 2× SSC, 0.1% SDS at room temperature for 10 min twice, 1× SSC, 0.1% SDS at 65 °C for 15 min, and 0.1× SSC, 0.1% SDS at 65 °C for 10 min twice and exposed to BioMax MS film (Kodak) between two intensifying screens at −80 °C.

## Results and discussion

### *Cell-specific activation of the LPKPP by the SV40VE active in multiple cell types requires HNF-1α binding to the LPKPP*

The liver-type pyruvate kinase (L-PK) promoter (∼4000 bp in size) directs the expression in the liver of the gene encoding pyruvate kinase that is critical for glycolysis [19]. As shown in Figs. 1A and B, the LPKPP spanning the TATA box and its some flanking region (∼100 bp in size) alone hardly activated the expression of a reporter gene in HEK293 cells, a non-hepatic cell line, or in HepG2 cells, a hepatic cell line, but did significantly enhance its expression in HepG2 cells in the presence of the SV40VE (∼250 bp in size) [12,19]. This indicates that a *cis*-element(s) residing in the LPKPP is needed for the cell-specific activation of the LPKPP by the SV40VE. To identify such regulatory elements using transfection, we prepared plasmids harboring deletion mutants of the LPKPP in the presence of the SV40VE (Fig. 1C). As shown in Fig. 1D, LPKPP mutants absent from the region spanning −96/−66 hardly activated its reporter gene (lanes 6, 8, and 10), while the mutants retaining the region did activate the gene (lanes 4 and 12). On the other hand, neither of the deletion mutants produced significant activation in HEK293 cells (lanes 3, 5, 7, 9, and 11). These results indicate that a positive regulatory factor(s) rather than a negative factor(s) in HEK293 cells may bind to the region spanning −96/−66 to modulate HepG2 cell-specific activation of the LPKPP by the SV40VE.

Based on the consensus sequence information on HNF-1α binding site, the region spanning −96/−66 has been suggested to harbor a proposed binding site (LF-B1 binding site) of HNF-1α [17,19], suggesting that HNF-1α may be required for the phenomenon. To

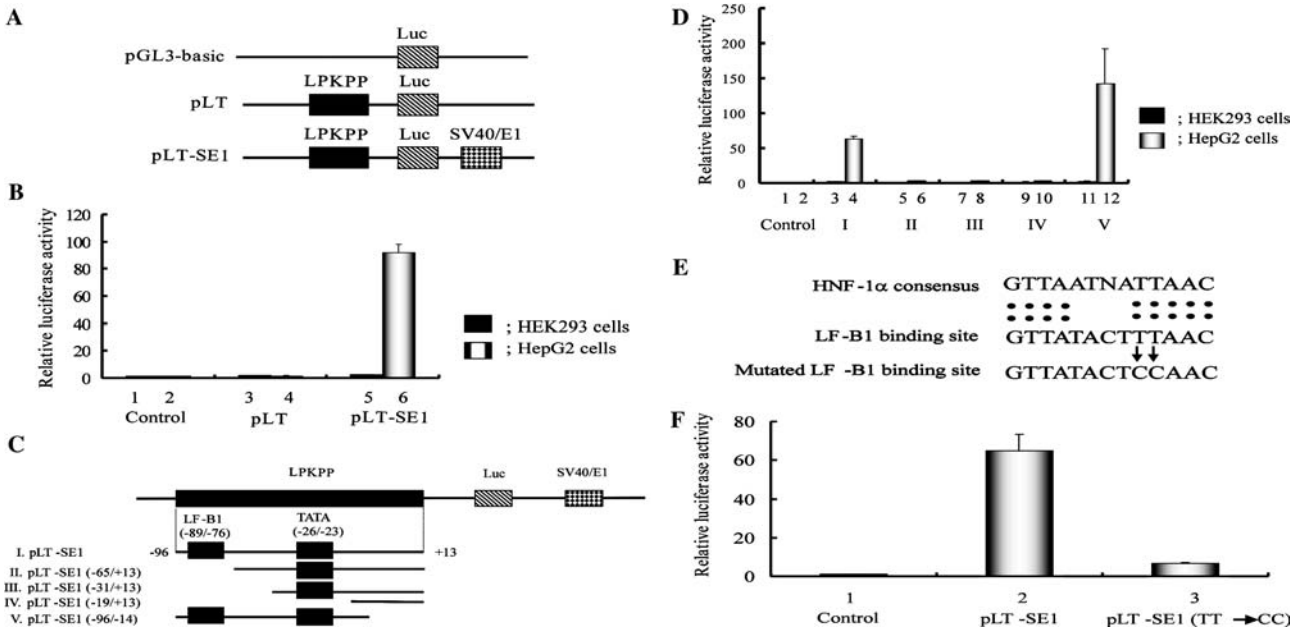


Fig. 1. Simplified structure of the plasmids harboring deletion or point mutations of the LPKPP, and their effects on transcriptional activation either in the absence or in the presence of the SV40VE. (A) Structure of the plasmids containing the LPKPP: LPKPP, the L-PK proximal promoter; Luc, the luciferase coding sequence, and SV40/E1, a copy of the SV40VE. (B) HEK293 cells (lanes 1, 3, and 5) and HepG2 cells (lanes 2, 4, and 6) were transfected with pGL3-basic as a control (lanes 1–2), pLT (lanes 3–4), or pLT-SE1 (lanes 5–6). Results are means ± SD of at least three independent experiments. (C) Structures of the plasmids containing deletion mutants of the LPKPP. (D) HEK293 cells (lanes 1, 3, 5, 7, 9, and 11) and HepG2 (lanes 2, 4, 6, 8, 10, and 12) cells were transfected with pGL3-basic (lane 1) and pGL3-enhancer (lane 2) as a control, pLT-SE1 (lanes 3–4), pLT-SE1(−65/+13) (lanes 5–6), pLT-SE1(−31/+13) (lanes 7–8), pLT-SE1(−19/+13) (lanes 9–10), or pLT-SE1(−96/−14) (lanes 11–12). Results are means ± SD of three independent transfection experiments. (E) Comparisons of HNF-1α binding consensus sequence [21] and wild/mutated type LF-B1 binding sites. Arrows indicate the sites of mutation. Dotted lines indicate 100% homology and N indicates any nucleotide. (F) HepG2 cells were transfected with pLT as a control (lane 1), pLT-SE1 (lane 2), or pLT-SE1(TT → CC) (lane 3). Results are means ± SD of three independent transfection experiments.

characterize this further, we double mutated the binding site (Fig. 1E). As shown in Fig. 1F, as we expected, the LPKPP containing the intact LF-B1 binding site was active (lane 2), however, the LF-B1 binding site containing the double mutation (TT → CC) showed a dramatic decrease (lane 3). These results suggest that the regulatory factor binding to the LF-B1 binding site is sensitive to the mutation and requires cell-specific activation of the LPKPP by the SV40VE.

We next tested our hypothesis that HNF-1α is absent in HEK293 cells and thus that the LPKPP is not able to activate luciferase reporter gene expression even in the presence of the SV40VE in these cells. This suggests that upon ectopic expression of HNF-1α cDNA in HEK293 cells, the activation of the LPKPP by the viral enhancer may be feasible. To test this idea, we cotransfected HEK293 cells with pLT-SE1 and HNF-1α cDNA. As shown in Fig. 2, the LPKPP was active in the presence of HNF-1α (lane 3) but not in control DNA (lane 2). On the other hand, HNF-1α ectopic expression did not help significantly the mutant (TT → CC) activate its reporter gene expression (lanes 4–5). These results indicate that transfected HNF-1α binds to the LF-B1 binding site and that the bound HNF-1α mediates the activation of the LPKPP by the SV40VE in HEK293 cells.

To survey and confirm whether the complex binding to the LF-B1 binding site contains bona fide HNF-1α, we performed EMSAs using nuclear extracts of HepG2 cells. An additional complex indicated as complex 1 was seen only in the presence of the intact LF-B1 binding site (Fig. 3A, lane 1). Its DNA binding activity was sequence-specific, since it hardly appeared in the presence of the mutated form (lane 2), where complexes 2 and 3 appeared. As a further test of its sequence-specific binding characteristics, competition assays were performed in the presence of unlabeled intact or mutated LF-B1 binding site as competitors (Fig. 3B). Complex 1 only (compare with those of complexes 2 and 3) began to fade away in the presence of a 10-fold molar excess of the intact form (lane 3) and was hardly detected in a 100-fold molar excess (lane 4), while it appeared even in a 100 fold molar excess of the mutated form (lane 8). This result strongly supports the notion that the DNA binding activity of the regulatory factor in complex 1 is sequence-specific. Finally, using antibodies to HNF-1α, we tested whether the sequence-specific DNA binding complex 1 contains HNF-1α. As shown in Fig. 3C, only HNF-1α antibody, but not insulin antibody, recognized complex 1 (lanes 2 and 3, resp.), as manifested by the supershifted complex.

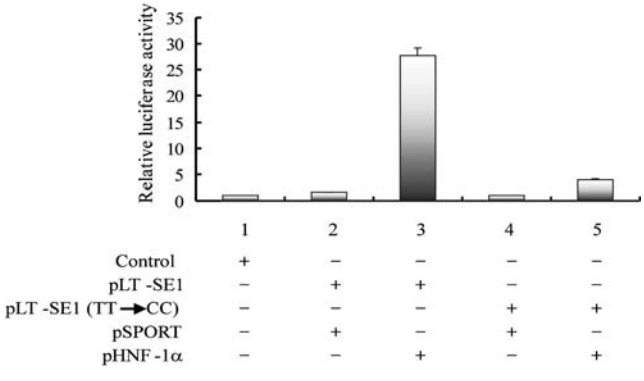


Fig. 2. The effect of the ectopic expression of HNF-1α cDNA in HEK293 cells on the activation of the LPKPP by the SV40VE. HEK293 cells were transfected with pLT as a control (lane 1), pLT-SE1 (lanes 2–3), or pLT-SE1(TT → CC) (lanes 4–5) in the presence of pSPORT1 (Gibco-BRL, USA) control DNA lacking HNF-1α cDNA (lanes 2 and 4), or HNF-1α cDNA (lanes 3 and 5) [22]. Results are means ± SD of three independent transfection experiments.

*Comparison of the cell-specificities and the potencies of the LPKPP and SV40 viral promoter induced by the SV40VE*

We next compared the potency and the cell-specificity of the LPKPP activity induced by the SV40VE to those of the SV40 viral promoter/enhancer. As shown in Table 1, the SV40 viral promoter was activated up to 71.9-fold and the LPKPP up to 63.4-fold in HepG2 cells, whereas the former was activated up to 21.6-fold and the latter up to 1.8-fold in HEK293 cells. These results indicate that the LPKPP activity is quite potent and cell-specific, since it yields almost 90% that of the SV40 viral promoter and its cell-specificity is 10 times that of the SV40 viral promoter.

*A newly generated expression vector harboring the LPKPP and the SV40VE directs expression of its reporter genes in cell- and tissue-specific manners*

For generation of a tissue-specific expression vector, we hoped to utilize our findings that the SV40VE active even in multiple cell types is able to potently activate the LPKPP in a tissue-specific manner with help of HNF-1α. Thus, the luciferase-coding region was removed from pLT-SE1, where a polylinker site was inserted to allow insertion of the genes of interest (Figs. 4A and B). To test whether the modification of the vector allowed the inserted genes to be expressed properly, we inserted the gene encoding β-galactosidase (β-gal) into the modified plasmid and transfected the resulting plasmid into HepG2 and HEK293 cells. The observed β-gal gene expression pattern is shown in Fig. 4C. β-gal gene expression appeared only in HepG2 cells transfected with pLFB1-LacZ, but not in cells transfected with pLFB1 under our conditions, while the β-gal expression driven by the CMV enhancer/promoter appeared in both cell types. These results suggest that the newly engineered vector is able to drive its inserted gene expression in a cell-specific manner. To test whether its expression capability and specificity are retained in animal tissues,

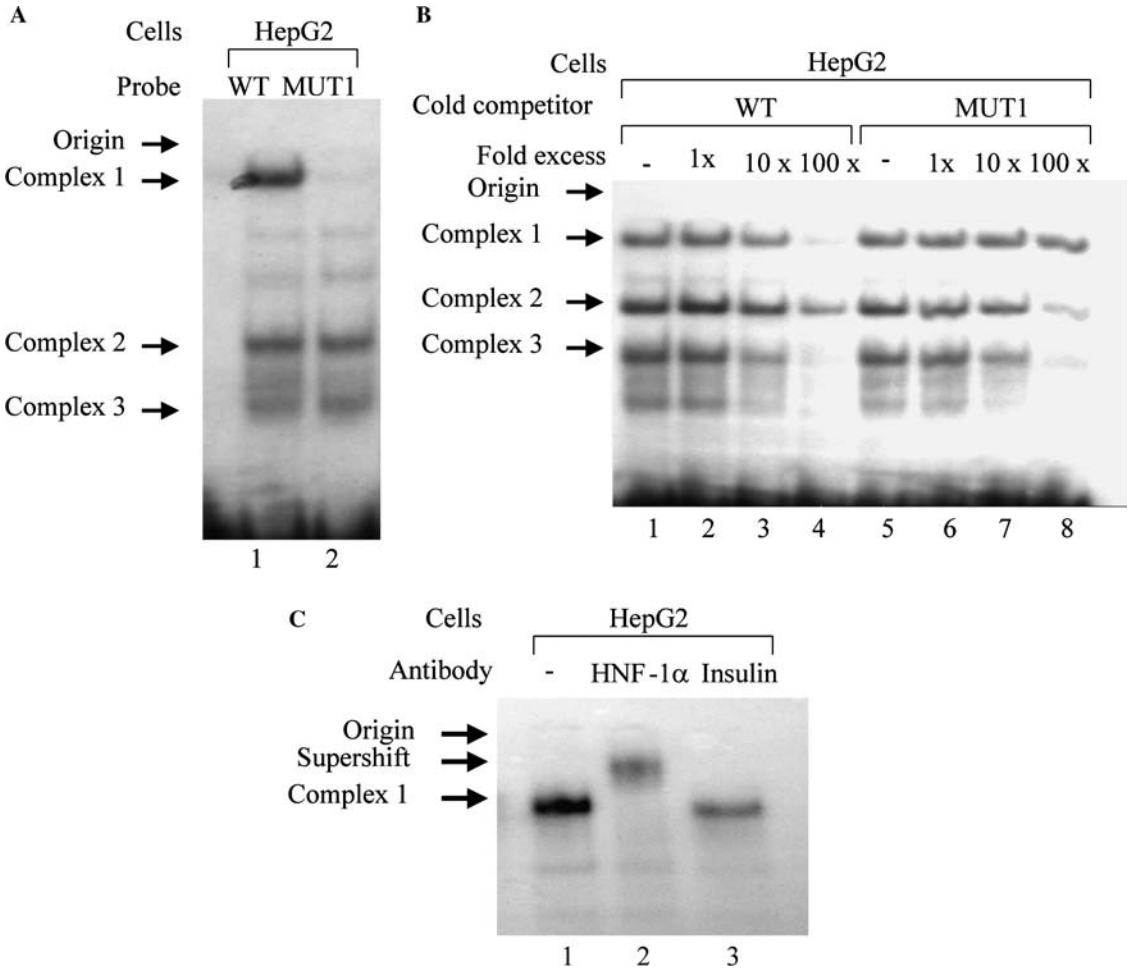


Fig. 3. Electrophoretic mobility shift assay (EMSA) of nuclear extracts prepared from HepG2 cells in the presence of the intact/mutated LF-B1 binding sites as $^{32}$P-radiolabeled probes. (A) The binding reaction was performed as described in Materials and methods. $^{32}$P-radiolabeled intact (WT, lane 1) or mutated (MUT1, lane 2) LF-B1 binding sites were used as a probe: Origin indicates place for sample loading. (B) For competition assays, no competitors (lanes 1 and 5), unlabeled intact (WT, lanes 2–4) or mutated (MUT1, lanes 6–8) LF-B1 binding sites were added to the preincubation mixture at a 1–100-fold molar excess in the presence of $^{32}$P-radiolabeled intact LF-B1 binding sites as a probe. (C) For supershift assays, no antibody (lane 1), antibody to HNF-1a (lane 2), or to insulin (lane 3) was included in the preincubation mixture.

Table 1
Comparison of the cell-specificities and the potencies of the LPKPP and SV40 viral promoter induced by the SV40VE

| Promoter | Enhancer | HepG2 | HEK293 | HepG2 activity comparison (%) | Cell specificity HepG2/HEK293 |
|---|---|---|---|---|---|
| SV40 | SV40 | 71.9 ± 14.6 | 21.6 ± 0.2 | 100 | 3.3 |
| LPKPP | SV40 | 63.4 ± 4.4 | 1.8 ± 0.2 | 87 | 35 |

Results are means ± SD of three independent transfection experiments.

we prepared the recombinant adenovirus of pLFB1 harboring the insulin analog gene as a reporter and then infused the recombinant virus via a tail vein of rats. As shown in Fig. 4D, immunohistochemical studies indicate that pLFB1 is able to express its reporter gene in the liver tissue. On the other hand, Northern blot analyses using total RNA from a variety of tissues derived from the recombinant virus-treated rats show that the transcript of the insulin analog gene was expressed in the liver tissue (lane 2), but not significantly in the other tissues examined (lanes 1, lanes 3–9 in Fig. 4E). The ethidium bromide-stained signal of 28S ribosomal RNA in the lower panel excludes the possibility of biased loading. Thus, these results suggest that pLFB1 could be used to activate the expression of genes of interest in a tissue-specific manner in animal tissues.

Although we show clearly here that HNF-1α is required for the constitutively active SV40VE to potently upregulate the activity of the LPKPP in a tissue-specific manner, we do not fully understand the mechanisms

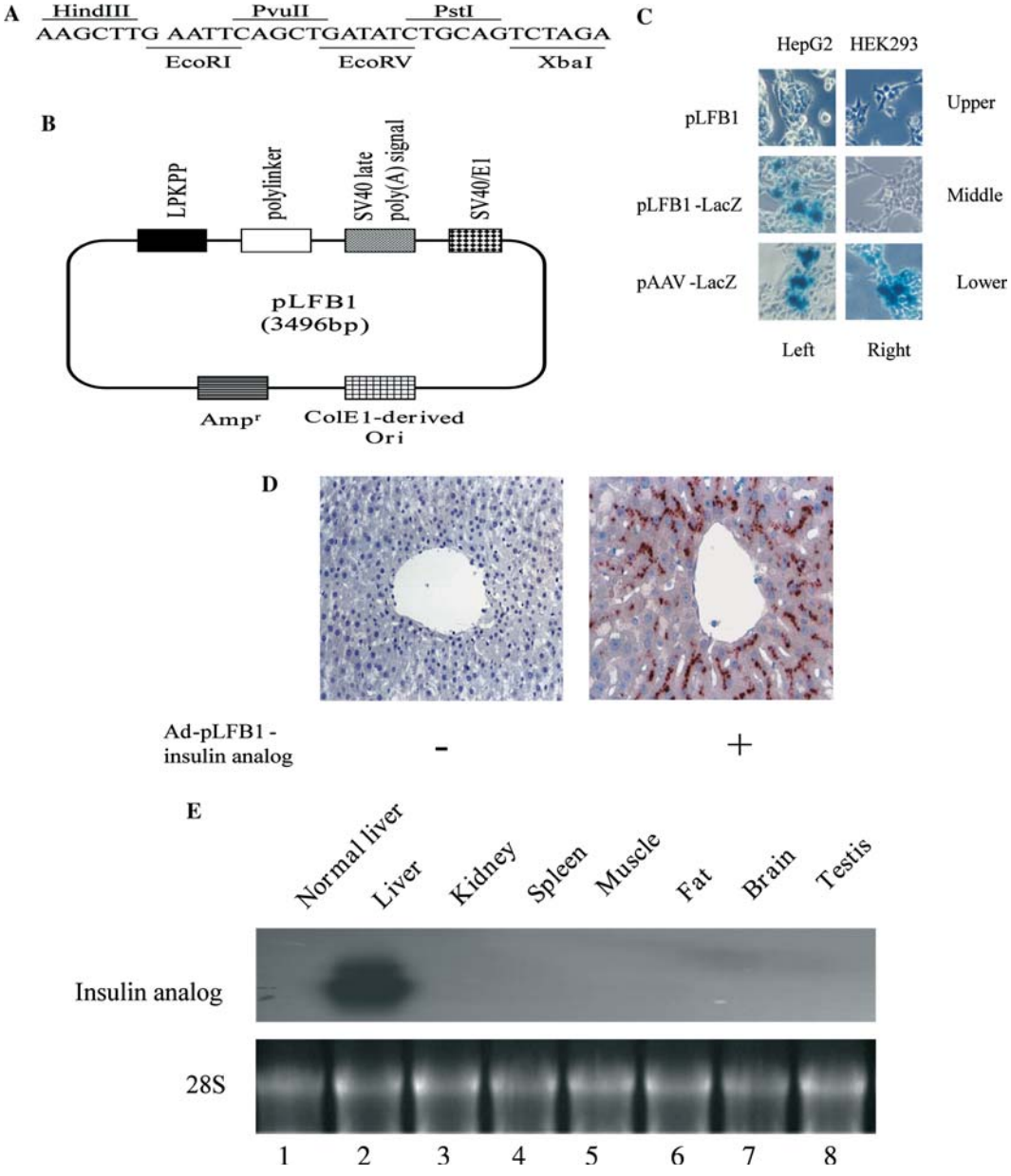


Fig. 4. Generation of a tissue-specific expression vector (pLFB1) and the expression patterns of the genes inserted in pLFB1 in cell lines and animal tissues. (A) Sequences of the polylinker and of the unique restriction enzyme sites are shown. (B) Simplified structure of pLFB1: Amp$^r$; β-lactamase encoding gene, Ori; bacterial replication origin. (C) X-gal staining of HepG2 cells in the left panel and HEK293 cells in the right panel, transfected with pLFB1 as a control (upper), pLFB1-LacZ (middle), or pAAV-LacZ (lower) where the CMV promoter controls expression of the β-galactosidase gene. Magnification is 100×. (D) Immunohistochemical staining in the presence of guinea pig anti-insulin of the liver tissues derived from either control (left) or the recombinant virus-treated rats (right). Sprague–Dawley rats were infused via a tail vein with $7.5 \times 10^{12}$ recombinant adenoviral particles containing Ad-pLFB1-insulin analog. Two days later, the liver tissues were prepared for immunohistochemical staining. Magnification is 200×. (E) Expression pattern of the insulin analog-encoding gene inserted in pLFB1 carried by recombinant adenoviral vectors in rat tissues: Upper panel; Northern blot analysis using total RNA from the liver of no recombinant virus-treated rats (lane 1) and tissues (lanes 2–8) of the recombinant virus-treated rats was carried out in the presence of human insulin analog gene as a probe [23] and lower panel; The ethidium bromide-stained pattern of 28S ribosomal RNA expressed in the tissues used.

involved in the following observations: First even though HNF-1α is a potent transcription factor [20], it hardly transactivated the L-PK TATA box residing in the LPKPP alone, but was required for the tissue-specific activation of the L-PK TATA box by the SV40VE. Second, the SV40VE failed to activate the L-PK TATA box (e.g., the LPKPP harboring the mutated form of the LF-B1 binding site) efficiently in either cell type. Although better understanding of the mechanisms requires further study, it is possible that the L-PK TATA box region might be somehow locked (e.g., by a chromatin structure) in both cell types, and thus

regulatory factors binding to the SV40VE may not have access. However, if HNF-1α is available (e.g., in HepG2 cells), it could bind to the LF-B1 binding site and unlock the TATA box region allowing the SV40VE to activate the L-PK TATA box. If this is the case, other enhancers, regardless of their being inducible or constitutive, could be used instead of the SV40VE to better modulate the tissue-specific activation of the LPKPP in the liver.

In conclusion, we describe a novel way of generating a potent artificial tissue-specific promoter: The SV40VE, although constitutively active, could be used to activate the LPKPP in a tissue-specific manner in the presence of HNF-1α. To our knowledge, this is the first example of using HNF-1α and its binding site, in the context of the LPKPP, to generate a basal promoter that is transcriptionally activated potently in a tissue-specific manner by the SV40VE. Moreover, this basal promoter might be further activated by other enhancers to modulate therapeutic gene expression in a tissue-specific manner. The feasibility of this is under an intensive investigation.

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## References

[1] C. Guha, N. Roy-Chowdhury, H. Jauregui, J. Roy-Chowdhury, Hepatocyte-based gene therapy, J. Hepatobiliary Pancreat. Surg. 8 (2001) 51–57.
[2] N. Ferry, Gene therapy and liver diseases, Gastroenterol. Clin. Biol. 27 (2003) 288–290.
[3] Y. Ilan, H. Saito, N.R. Thummala, N.R. Chowdhury, Adenovirus-mediated gene therapy of liver diseases, Semin. Liver Dis. 19 (1999) 49–59.
[4] G.V. Kalpana, Retroviral vectors for liver-directed gene therapy, Semin. Liver Dis. 19 (1999) 27–37.
[5] H.C. Lee, S.J. Kim, K.S. Kim, H.C. Shin, J.W. Yoon, Remission in models of type 1 diabetes by gene therapy using a single-chain insulin analogue, Nature 408 (2000) 483–488.
[6] H. Dong, N. Morral, R. McEvoy, M. Meseck, S.N. Thung, S.L. Woo, Hepatic insulin expression improves glycemic control in type 1 diabetic rats, Diabetes Res. Clin. Pract. 52 (2001) 153–1631.
[7] W. Walther, U. Stein, Cell type specific and inducible promoters for vectors in gene therapy as an approach for cell targeting, J. Mol. Med. 74 (1996) 379–392.
[8] D.G. Hafenrichter, K.P. Ponder, S.D. Rettinger, S.C. Kennedy, X. Wu, R.S. Saylors, M.W. Flye, Liver-directed gene therapy: evaluation of liver specific promoter elements, J. Surg. Res. 56 (1994) 510–517.
[9] J.Y. Dong, P.D. Fan, R.A. Frizzell, Quantitative analysis of the packaging capacity of recombinant adeno-associated virus, Hum. Gene Ther. 7 (1996) 2101–2112.
[10] P.D. Robbins, S.C. Ghivizzani, Viral vectors for gene therapy, Pharmacol. Ther. 80 (1998) 35–47.
[11] C. Kimchi-Sarfaty, M. Arora, Z. Sandalon, A. Oppenheim, M.M. Gottesman, High cloning capacity of in vitro packaged SV40 vectors with no SV40 virus sequences, Hum. Gene Ther. 14 (2003) 167–177.
[12] H. Barrera-Saldana, K. Takahashi, M. Vigneron, A. Wildeman, I. Davidson, P. Chambon, All six GC motifs of the SV40 early upstream element contribute to promoter activity in vivo and in vitro, EMBO J. 4 (1985) 3839–3849.
[13] G.R. Albrecht, B. Cavallini, I. Davidson, Detection of specific protein binding to the SV40 early promoter in vivo, Nucleic Acids Res. 17 (1989) 7945–7963.
[14] M. Vigneron, H.A. Barrera-Saldana, D. Baty, R.E. Everett, P. Chambon, Effect of the 21-bp repeat upstream element on in vitro transcription from the early and late SV40 promoters, EMBO J. 3 (1984) 2373–2382.
[15] U. Hansen, P.A. Sharp, Sequences controlling in vitro transcription of SV40 promoters, EMBO J. 2 (1983) 2293–2303.
[16] R.D. Everett, D. Baty, P. Chambon, The repeated GC-rich motifs upstream from the TATA box are important elements of the SV40 early promoter, Nucleic Acids Res. 11 (1983) 2447–2464.
[17] M. Cognet, Y.C. Lone, S. Vaulont, A. Kahn, J. Marie, Structure of the rat L-type pyruvate kinase gene, J. Mol. Biol. 196 (1987) 11–25.
[18] C.W. Park, M.D. Walker, Subunit structure of cell-specific E box-binding proteins analyzed by quantitation of electrophoretic mobility shift, J. Biol. Chem. 267 (1992) 15642–15649.
[19] M. Cognet, M.O. Bergot, A. Kahn, cis-acting DNA elements regulating expression of the liver pyruvate kinase gene in hepatocytes and hepatoma cells. Evidence for tissue-specific activators and extinguisher, J. Biol. Chem. 266 (1991) 7368–7375.
[20] E. Soutoglou, G. Papafotiou, N. Katrakili, I. Talianidis, Transcriptional activation by hepatocyte nuclear factor-1 requires synergism between multiple coactivator proteins, J. Biol. Chem. 275 (2000) 12515–12520.
[21] T. Ozeki, Y. Takahashi, T. Kume, K. Nakayama, T. Yokoi, K. Nunoya, A. Hara, T. Kamataki, Co-operative regulation of the transcription of human dihydrodiol dehydrogenase (DD)4/aldo-keto reductase (AKR)1C4 gene by hepatocyte nuclear factor (HNF)-4alpha/gamma and HNF-1alpha, Biochem. J. 355 (2001) 537–544.
[22] J.Y. Cha, H.I. Kim, S.S. Im, T.Z. Li, Y.H. Ahn, HNF1 and/or HNF3 may contribute to the tissue specific expression of glucokinase gene, Exp. Mol. Med. 33 (2001) 59–63.
[23] D.J. Groskreutz, M.X. Sliwkowski, C.M. Gorman, Genetically engineered proinsulin constitutively processed and secreted as mature, active insulin, J. Biol. Chem. 269 (1994) 6241–6245.