Endocrine Journal 2008, **55** (6), 1033–1041

Experimental Parathyroid Hormone Gene Therapy using ØC31 Integrase

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Abstract. ØC31 integrase can integrate targeted plasmid DNA into preferred locations in mammalian genomes, resulting in robust, long-term expression of the integrated transgene. This system represents an effective tool that opens up promising possibilities for gene therapy. The classical treatment for hypoparathyroidism was calcium and vitamin D replacement. Recently, parathyroid hormone (PTH) replacement was reported to be a more potentially physiologic treatment option. However, PTH synthesis is technically difficult and costly. These issues may be minimized by using PTH gene therapy. We attempted to achieve site-specific genomic integration of the PTH gene into a human cell line and mice using this system. We cotransfected 293 HEK cells with PTH-*attB* plasmid with or without ØC31 integrase plasmid. Expression and secretion of PTH into culture supernatants and site-specific genomic integration of PTH cDNA were assessed by immunoradiometric assays and pseudo-site analysis, respectively. In *in vivo* experiments, we injected the PTH-*attB* plasmid with or without ØC31 integrase plasmid into a mouse tail vein using the hydrodynamic method. Plasma PTH concentrations were serially measured, and site-specific integration of PTH cDNA into the mouse genome was confirmed by examining hepatic genomic DNA. PTH was expressed and secreted from 293 HEK cells and mouse hepatocytes, and pseudo-site analysis confirmed the site-specific integration of PTH cDNA into the host genomes. The site-specificity and efficiency of this system are advantageous in many areas, including, potentially, gene therapy. PTH gene therapy is one candidate; however, for clinical applications, we need to regulate PTH expression and secretion in the future.

Key words: Gene therapy, Parathyroid hormone, Hypoparathyroidism, ØC31 integrase

(*Endocrine Journal* **55**: 1033–1041, 2008)

A safe and effective gene delivery system is a prerequisite for clinical gene therapy. Gene therapy has been successfully used in several genetic diseases [1, 2], nevertheless, it is far from being popular. A major reason why it has not been established as a general treatment is the lack of a safe and effective gene delivery system. These systems are largely divided into 2 categories: viral and non-viral delivery systems. The former can effectively and efficiently deliver a relatively large gene into the genome; however, the intrinsic biological hazard raises medical concerns about infections and mutations. The non-viral gene delivery

Received: February 1, 2008
Accepted: August 1, 2008
This abstract was presented at the 87th Annual Meeting of the Endocrine Society in San Diego, CA, USA.
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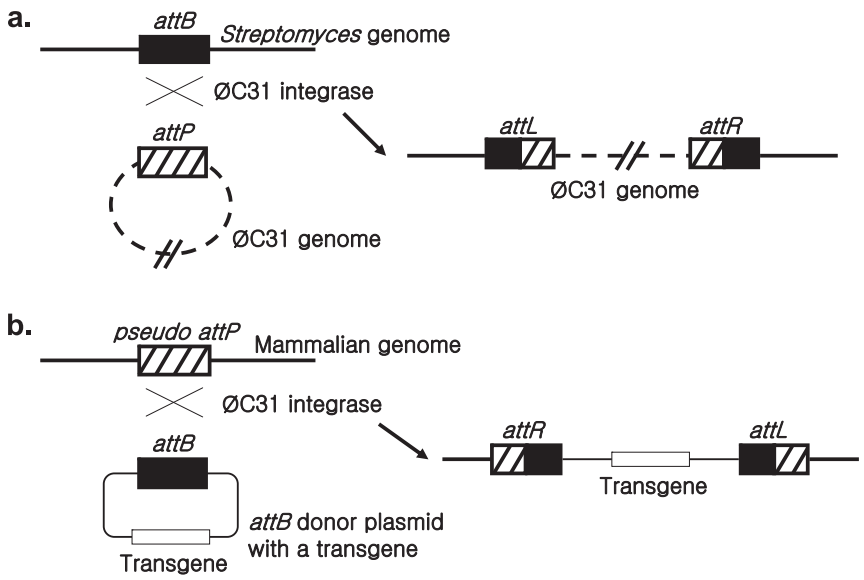

Fig. 1. Schematic diagram of ØC31 integrase-mediated recombination. a. In nature, ØC31 integrase performs precise recombination between an *attB* site located in the *Streptomyces* genome and an *attP* site located on the ØC31 phage genome. b. In mammalian cells, the ØC31 integrase system works optimally if the plasmid to be integrased carries an *attB* site, rather than *attP*. ØC31 integrase performs recombination between *attB* and naturally-occurring genomic sequences related to *attP*.

system circumvents the biological hazard of the viral delivery system but the efficiency of gene delivery is severely restricted compared with the virus-based system. The establishment of a new gene delivery system that is safe and effective is urgently required. Therefore, we have assayed the use of the bacteriophage ØC31 integrase for its applicability in non-viral gene therapy [3, 4]. Through lysogeny, certain bacteriophages can site-specifically integrate into the host genome using the phage-encoded enzymes termed integrases. These integrases make unidirectional and site-specific integration through specific phage and bacterial sequences, *attP* and *attB*, respectively. Using integrases, *attB*-containing plasmids can be inserted into pseudo-*attP* sites in mammalian cells, thus raising the possibility of developing an effective, site-specific gene delivery system (Fig. 1) [5].

Hypoparathyroidism is a rare complication seen most frequently after thyroidectomy, though idiopathic hypoparathyroidism does occur [6]. Although the incidence of hypoparathyroidism has decreased substantially due to the progress of surgical techniques, it is still frequently encountered in clinics [7]. The primary symptom of hypoparathyroidism is spasm due to hypocalcemia, which until recently was treated with calcium and vitamin D. However, PTH replacement could be a more effective and physiologic treatment [8]. Replacing PTH would be ideal; however, it must be administered parenterally for the remainder of the patient's life. Therefore, autologous parathyroid transplants and other treatment methods have been developed. To improve upon these methods, we evaluated the use of ØC31 integrase in PTH gene therapy. Consequently, the aim of this study was to provide the experimental basis for PTH gene therapy, which is a more physiological and permanent treatment for hypoparathyroidism.

Materials and Methods

Plasmid preparation

The 285-bp ØC31 *attB* sequence obtained from pTA-*attB* plasmid, kindly provided by Dr MP Calos, was inserted as a *Bgl*II fragment into the *Bgl*II sites of the pcDNA3.0/neo, creating the plasmid pcDNA*attB*. The hPTH (1-84) sequences were PCR-amplified from pCDM8-PTH-1 [9], kindly provided by Dr HM Kronenberg, as a blunted *Hind*III/*BamH*I fragment, and inserted into *Hind*III/*BamH*I sites of pcDNA*attB*, creating the pcDNA*attB*PTH. Subsquently, internal ribosome entry site (IRES) and puromycin resistant sequences were removed from the MFG.hsp70.ires.puro

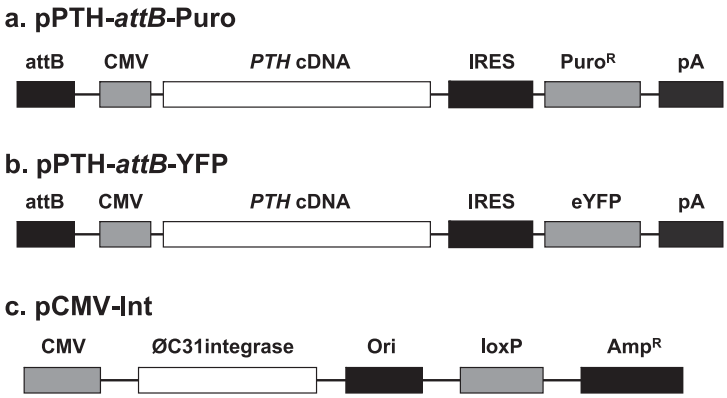

Fig. 2. Plasmid constructions. **a.** ØC31 *attB* sequence was inserted as a *Bgl*II fragment into the *Bgl*II sites of the backbone vector pcDNA3.0/neo, creating pcDNA*attB*. The hPTH (1-84) sequence was inserted into the *Hind*III/*BamH*I sites of pcDNA*attB*, creating pcDNA*attB*PTH. Subsquently, IRES and puromycin resistant sequences were inserted into the *BamH*I/*Eco*RV sites of pcDNA*attB*PTH, creating the transfer plasmid, pcDNA3.0-*attB*-hPTH-IRES-Puro (pPTH-*attB*-Puro). **b.** IRES-EYFP sequences were inserted into the BamHI/XhoI site of pcDNA*attB*PTH, creating another transfer plasmid, pcDNA3.0-*attB*-hPTH-IRES-EYFP (pPTH-*attB*-YFP). c. pCMV-Int, expressing ØC31 integrase, was obtained. *CMV, promoter sequence of CMV; IRES, internal ribosome entry site; Puro, puromycin resistance gene sequence; pA, poly A sequence; eYFP, enhance yellow fluoroscence protein gene sequence; Amp,^{R} amphicillin resistance gene sequence.*

vector, kindly provided by Dr HY Chung, and inserted into the *BamH*I/*Eco*RV sites of pcDNA*attB*PTH, creating the transfer plasmid pcDNA3.0-*attB*-hPTH-IRES-Puro (pPTH-*attB*-Puro; Fig. 2a). IRES-EYFP sequences were inserted into the BamHI/XhoI site of pcDNA*attB*PTH, creating another transfer plasmid, pcDNA3.0-*attB*-hPTH-IRES-EYFP (pPTH-*attB*-YFP; Fig. 2b). pCMV-Int, expressing ØC31 integrase was kindly provided by Dr Calos (Fig. 2c) [10].

In vitro cell culture experiment

293 HEK cell culture and gene transfer

293 HEK cells were grown in culture using DMEM containing 10% fetal bovine serum at 37°C with 5% carbon dioxide. pPTH-*attB*-Puro and pCMV-Int were cotransfected into 293 HEK cells with LipofecAMINE according to the manufactuer's instructions (Invitrogen, Carlsbad, CA, USA). Simultaneously, as a control, pPTH-*attB*-Puro and the same amount of pCMV empty vectors were transfected. Six-well plates and total of 2 μg of DNA (1 μg of pPTH-*attB*-Puro and 1 μg of pCMV-Int) were used in the transfection. The cells were approximately 80% confluent. For selection, 3 days after transfection, the cells were subjected to 10 day-treatment with puromycin at 4 μg/mL.

Measurement of PTH in culture medium and assessment of integrants at pseudo-attP sites

Seven; 15; 27; 39; 50; and 60 days after transfection, the concentration of PTH in the culture medium was measured using intact PTH IRMA kits (Nicholas Institute Diagnostics, San Clemente, CA, USA). Genomic DNA from 293 HEK cells cotransfected pPTH-*attB*-Puro and pCMV-Int were extracted using DNeasy Kits (QIAGEN, Valencia, CA, USA). A primer set recognizing both the 8p22 site, one of the human pseudo-*attP* sites, and the inserted gene area was prepared, and PCR was performed to assess whether the constructs integrated into the desired site (Fig. 3a, Table 1) [11]. PCR was conducted in 50 μl reaction mixtures with 300 ng of gDNA, 5 μl 10× PCR buffer, 4 μl 2.5 mM dNTP Mix, 10 pmole of each oligonucleotide primer pair, and 1.5 units BioTherm DNA polymerase (Genecraft, Munster, Germany) as follows: 94°C, 5 minutes; 35 cycles of 94°C for 30 seconds; 57°C for 35 seconds; 72°C for 35 seconds; and a final 10-minute extension at 72°C.

In vivo animal experiments

In vivo transfection by the hydrodynamic method

Six to 8-week-old male mice [Crj:CD-1 (ICR), Charles River Japan Co., Tokyo, Japan] were purchased and maintained in a specific pathogen-free environment. Twenty-five μg of pPTH-*attB*-YFP and

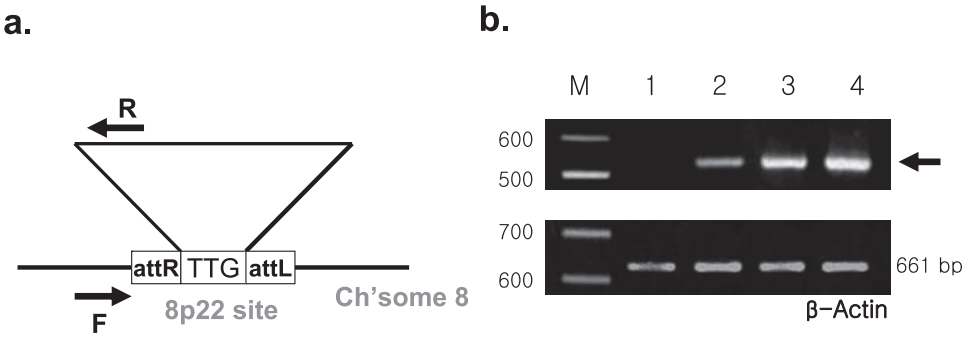

Fig. 3. PCR analysis of integrants at a pseudo-*attP* site. **a.** In 293 HEK cells cotransfected pPTH-*attB*-Puro and pCMV-Int, a primer pair recognizing 1 of the human pseudo-*attP* sites, 8p22, and the inserted gene area was used simultaneously to assess whether PTH was inserted into the desired site. **b.** As expected, 506 bp bands were detected in cells containing both pPTH-*attB*-Puro and pCMV-Int. PCR was validated by the detection of the housekeeping gene, beta actin. *F, forward primer; R, reverse primer; lane 1, transfected with pPTH-attB-Puro and pCMV empty vector; lane 2-4, transfected with pPTH-attB-Puro and pCMV-Int together; TTG, the 3-base pair overlap regional sequence where crossing-over occurs.*

Table 1. Primers used in plasmid rescue sequence analysis of integrants at pseudo-*attP* site

	Sequence	Product Size (bp)
8p22	F 5'-GAG GTG TTT GGG TGC TTC TG-3'	506
	R 5'-CCG TGA CCG TCG AGA ACC-3'	
MpsL1	F 5'-GTA GGT CAC GGT CTC GAA GC-3'	529
	R 5'-GGA GCC TTA GCA ACA AAT GC-3'	
Beta Actin	F 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3'	661
	R 5'-CTA GAA GCA TTG GCG GTG GAC GAT GGA GGG-3'	

25 ug of pCMV-Int were dissolved in 1.8 mL of 0.9% saline and injected into the tail vein in 5–8 seconds. As controls, the first group was injected with 25 μg of pPTH-*attB*-YFP and 25 μg of pCMV empty vector, the second group with 25 μg of pCMV-Int and 25 μg of pCMV empty vector, the third group with 25 μg of pTA-*attB* and 25 μg of pCMV empty vector, the fourth group with 25 μg of pIRES-EYFP and 25 μg of pCMV empty vector, and the last group with same amount of saline. This animal experiment was approved by the IRB for animal experiments at the University of Tokyo in Tokyo, Japan.

Measurement of serum parathyroid hormone and calcium

Hypoparathyroidism model such as parathyroidectomized mice could not be developed due to technical difficulty. Therefore, PTH levels in mice with intact parathyroid function could only be measured. Venous blood was regularly obtained by the retro-orbital approach. Serum total calcium concentrations were measured (Eagle Diagnostics, DeSoto, TX, USA) using the *o*-cresolphthalein-complexone method. Serum PTH concentrations were measured using the mouse intact PTH enzyme-linked immunosorbent assay (Immutopics, San Clemente, CA, USA).

Microscopic examination of liver samples

Direct fluorescence from freshly dissected liver samples was detected using a confocal microscope (Zeiss LSM510). Immunohistochemical stain for PTH were performed using mouse monoclonal antibody of PTH (Clone 105G7, 1 : 100, Mouse monoclonal, Novocastra, Newcastle, UK). Four micrometer-thick liver tissue sections were heated by microwave with citrate buffer pH 6.0 for 10 minutes. After blocking endogenous peroxidase and non-specific binding, the primary antibody was incubated at room temperature for 2 hrs, followed by detection with the ultrastreptavidin system.

Assessment of integrants at pseudo-attP sites

Genomic DNA was extracted from mouse liver samples using DNeasy Tissue Kits (QIAGEN, Valen-

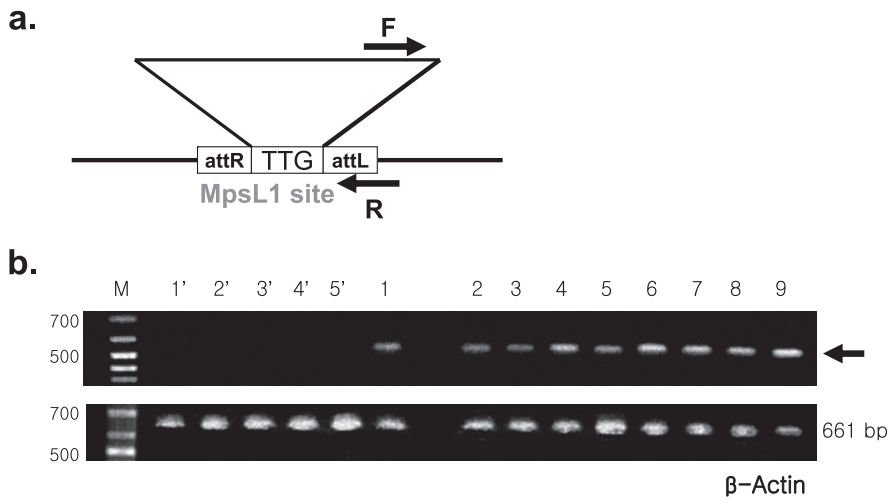

Fig. 4. PCR analysis of integrants at a pseudo-*attP* site. **a.** A primer pair simultaneously recognizing 1 of the murine pseudo-attP sites, MpsL1, and the inserted gene was used to assay for site-specific integration. **b.** In PCR using genomic DNA extracted from the cells transfected with pPTH-*attB*-YFP and pCMV-Int together, bands with the desired size were detected. PCR was validated by the detection of the housekeeping gene beta actin. *F, forward primer; R, reverse primer; mice numbers 1 to 9, transfected with pPTH-attB-YFP and pCMV-Int together; mice numbers 1' to 5', transfected with pPTH-attB-YFP and pCMV empty vector; TTG, the 3-base pair overlap regional sequence where crossing-over occurs.*

cia, CA, USA). A primer set recognizing both the MpsL1 site, one of the murine pseudo-*attP* sites, and the inserted gene area was prepared. PCR was performed to assess whether site-specific integration had occurred (Fig. 4a, Table 1) [12]. PCR was conducted in 20 µl reaction mixtures with 200 ng of gDNA, 2 µl 10× PCR buffer, 1.6 µl 2.5 mM dNTP Mix, 10 pmole of each oligonucleotide primer pair, and 1 unit Bio-Therm DNA polymerase (Genecraft, Munster, Germany) as follows: 94°C for 5 minutes; 35 cycles of 94°C for 30 seconds; 53°C for 35 seconds; 72°C for 35 seconds; and a 7-minute extension at 72°C.

Results

In vitro cell culture experiment

Measurement of PTH concentrations in culture medium

The concentration of PTH in the culture medium was measured at 7; 15; 27; 39; 50; and 60 days after transfection. In cells cotransfected with pPTH-*attB*-Puro and pCMV empty vector, PTH levels were 305 pg/mL on the 7th day and gradually decreased thereafter. After 1 month, parathyroid hormone was not detectable. In the cells cotransfected with pPTH-*attB*-Puro and pCMV-Int, parathyroid hormone medium concentration was approximately 2,000 pg/mL for up to 2 months after transfection. The experiments were repeated 5 times and the results are expressed as mean +/– SEM (Fig. 5).

Investigation of genomic pseudo-attP integration sites in vitro

A few days after treatment of puromycin, the cells disappeared and re-emerged as forming colonies. The cells put under selection were pooled together and genomic DNA was extracted from cells cotransfected pPTH-*attB*-Puro and pCMV-Int as well as from cells cotransfected pPTH-*attB*-Puro and pCMV empty vector. Integration into the 8p22 site, one of the pseudo-*attP* sites, was assayed by PCR with a primer set recognizing both the 8p22 site and the inserted gene area. With cells containing both pPTH-*attB*-Puro and pCMV-Int, a band of the expected size was detected [11]. Three different genomic DNAs extracted from different colonies revealed bands of the same size. As expected, no bands were detected for cells cotransfected with pPTH-*attB*-Puro and the pCMV empty vector, indicating the role of integrase of mediating site-specific integration (Fig. 3b).

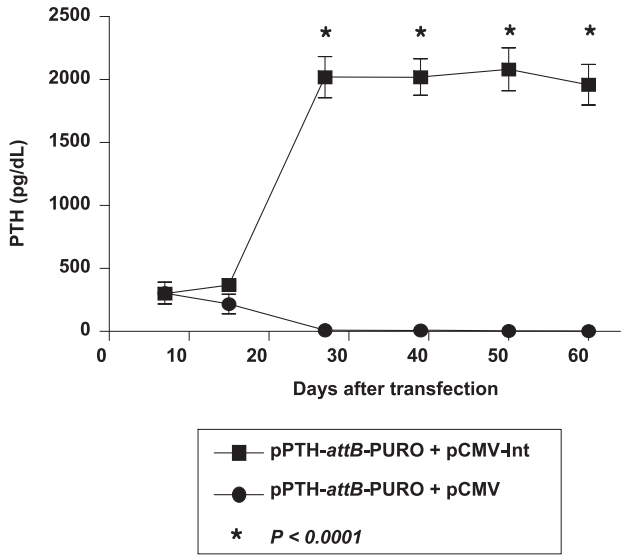

Fig. 5. PTH level after transfection with pPTH-*attB*-Puro and/or pCMV-Int in 293 HEK cell line. Seven; 15; 27; 39; 50; and 60 days after transfection, the concentration of parathyroid hormone in the culture medium was measured using intact PTH IRMA kits (Nicholas Institute Diagnostics, San Clemente, CA, USA). On day 7 after transfection, PTH concentration was 305 pg/mL in cells transfected with pPTH-*attB*-Puro and pCMV empty vector. From there it decreased gradually and after 1 month, PTH was not detected. In cells cotransfected with pPTH-*attB*-Puro and pCMV-Int, PTH concentration was 2,000 pg/mL for up to 2 months after transfection (*, PTH level in the pPTH-*attB*-Puro + pCMV-Int group compared with PTH level in the pPTH-*attB*-Puro + pCMV group; unpaired *t*-test by GraphPad Prism 4).

Animal experiments

Measurement of serum parathyroid hormone

The blood volume that could be obtained by the retro-orbital approach is limited; hence, it was diluted by 2-fold. In both the group injected with pPTH-*attB*-YFP and pCMV-Int and the group injected with pPTH-*attB*-YFP and pCMV empty vector, at 1 day after the injection, the parathyroid hormone concentration was over 10 times higher than the normal group (367.1 ± 54.2 pg/mL and 224.8 ± 62.2 pg/mL, respectively, Table 2). Serum calcium concentrations went up as high as 30 mg/dL and showed close correlations with PTH concentrations (Table 3). Although serum PTH concentrations gradually decreased, they were 88.5 ± 15.3 pg/mL on day 29, suggesting that the parathyroid hormone was being secreted continuously. The experimental group injected with pPTH-*attB*-YFP and pCMV empty vector also showed sustained high PTH concentration (Table 2). All mice died by 40 days after gene introduction. Early termination of this study makes the effects of long-term expression of the introduced gene ambiguous. Paradoxically, however, death from hypercalcemia is definite evidence for the efficient introduction of the PTH gene. To rule out the possibility that pCMV-Int, pTA-*attB* or pIRES-EYFP has effects on increasing serum PTH and calcium levels, serum PTH and calcium levels were measured in the blood obtained from the control mice that were injected with these plasmids or saline. None of the control groups have shown a significant increase in serum PTH and calcium level (calcium data not shown) (Table 2).

Microscopic finding of liver samples

Liver samples from mice cotransfected with pPTH-*attB*-YFP and pCMV-Int revealed yellow fluorescence, demonstrating integration and expression of the plasmid in the hepatocytes (Fig. 6a-1). In contrast, liver samples from mice cotransfected with pPTH-*attB*-YFP and pCMV empty vector revealed little or no yellow fluorescence, indicating that integration didn't occur in this group (Fig. 6a-2). Strong and frequent immunoreactivities of PTH were evident in the liver section cotransfected with pPTH-*attB*-YFP and pCMV-Int (Fig. 6b-1), however, focal and weak immunoreactivities of PTH were observed in the liver section cotransfected with pPTH-*attB*-YFP and pCMV empty vector (Fig. 6b-2).

Investigation of one of the genomic pseudo-attP integration sites in vivo

To assess whether the gene was inserted in the MpsL1 site, one of the pseudo-*attP* sites in mice, genomic DNA were extracted from mouse liver and PCR was performed using a primer set recognizing both the MpsL1 site and the inserted gene area. Bands of the expected size were detected in the group that was injected with pPTH-*attB*-YFP and pCMV-Int. In contrast, no bands were detected in the group that was injected with pPTH-*attB*-YFP and pCMV empty vector (Fig. 4b) [12].

Discussion

Bacteriophages infect the host bacteria by utilizing

Table 2. PTH level from mice after transfection with pPTH-*attB*-YFP and/or pCMV-Int

	Day 1	Day 4	Day 7	Day 29
PTH + Int	367.1 ± 54.2* (9)	210.5 ± 24.5* (8)	162.0 ± 26.6* (5)	88.5 ± 15.3* (4)
PTH	224.8 ± 62.2 (6)	137.5 ± 38.0 (4)	180.3 ± 68.6 (4)	97.0 ± 27.6 (3)
Int	11.4 ± 1.3 (5)	14.8 ± 1.5 (5)	11.6 ± 1.0 (5)	12.4 ± 0.8 (5)
attB	21.7 ± 7.1 (3)	15.8 ± 1.7 (3)	15.7 ± 3.2 (3)	16.8 ± 0.3 (3)
eYFP	22.8 ± 4.5 (2)	17.3 ± 0.9 (2)	32.7 ± 0.3 (2)	18.1 ± 0.3 (2)
Saline	15.8 ± 1.3 (10)	18.7 ± 0.9 (10)	14.6 ± 1.01 (10)	13.2 ± 1.4 (10)

PTH, pPTH-attB-YFP; Int, pCMV + Int; attB, pTA-attB plasmid; eYFP, pIRES-EYF.; Data are shown as mean ± SEM (number of mice).
*$p<0.0001$, PTH levels from the mice after transfection with pPTH-attB-YFP and pCMV-Int compared to those from mice injected with saline (unpaired t-test by GraphPad Prism 4.)

Table 3. Calcium level from mice after transfection with pPTH-*attB*-YFP and/or pCMV-Int

	Day 0	Day 1	Day 4	Day 7	Day 29
PTH + Int	6.3 ± 1.0 (9)	28.3 ± 2.2* (9)	24.0 ± 3.1* (8)	15.9 ± 2.5* (5)	10.9 ± 1.0 (4)
PTH	5.7 ± 1.2 (6)	23.3 ± 5.7 (6)	28.6 ± 6.6 (4)	21.6 ± 6.0 (4)	7.6 ± 0.7 (3)
Saline	7.3 ± 1.4 (6)	5.7 ± 2.1* (6)	6.7 ± 2.4* (6)	5.9 ± 1.5* (6)	6.2 ± 1.7 (6)

PTH, pPTH-attB-YFP; Int, pCMV + Int; Data are shown as mean ± SEM (number of mice).
*$p<0.001$, Calcium levels from the mice after transfection with pPTH-attB-YFP and pCMV-Int compared to those from mice injected with saline (unpaired t-test by GraphPad Prism 4.)

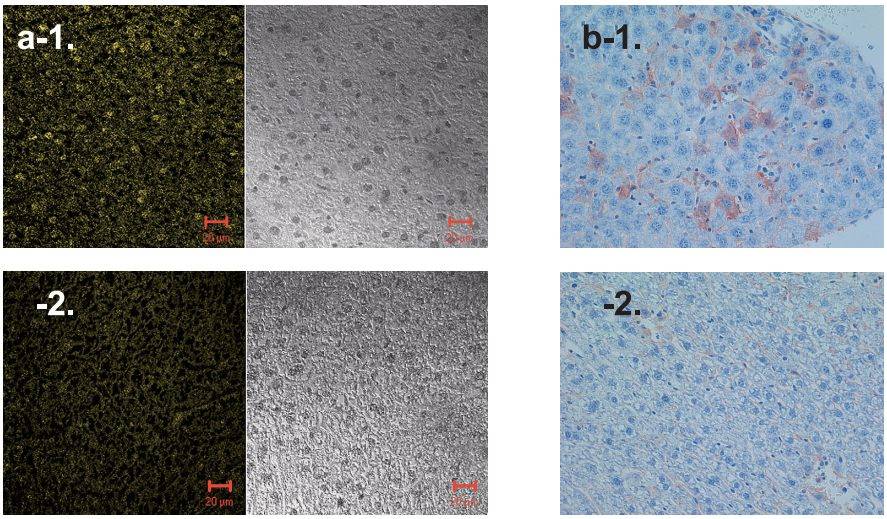

Fig. 6. Microscopic finding of liver samples transfected with either pPTH-*attB*-YFP and pCMV-Int or pPTH-*attB*-YFP and pCMV empty vector. **a-1.** Direct fluorescence from freshly dissected mice liver was detected using a confocal microscope next to contrast view for anatomical orientation (Zeiss LSM510). Mice livers transfected with pPTH-*attB*-YFP and pCMV-Int together revealed yellow fluorescence, demonstrating the integration and expression of the plasmid in hepatocytes. **a-2.** Mice livers transfected with pPTH-*attB*-YFP and pCMV empty vector revealed little or no yellow fluorescence, indicating that integration didn't occur in this group. (×400) **b-1.** Strong and frequent immunoreactivities of PTH were evident in the liver section cotransfected with pPTH-*attB*-YFP and pCMV-Int. **b-2.** Focal and weak immunoreactivities of PTH were observed in the liver section cotransfected with pPTH-*attB*-YFP and pCMV empty vector. (Immunohistochemical stain for PTH ×400)

the replicatory machinery of bacteria. As a result, more bacteriophages are produced [13]. When the growth environment is unsuitable for replication, the phage integrates into the bacterial genome and remains dormant until the environment becomes favorable for replication. Integration is accomplished with an inte-

grase, and this mechanism has been applied to gene delivery [11, 14–16]. Bacteriophage integrases mediate the ligation of genes by recognizing the bacteriophage *attP* site and the bacterial *attB* site. Integrases are classified as tyrosine or serine integrase, according to the catalytic unit. The widely used *Cre* and *Flp* recombinases are tyrosine integrases and have been extensively studied and used in mammalian cells [17]. Serine integrases recognize a relatively small attachment site and can be used in mammalian cells. Hence, we used serine ØC31 integrase derived from *Streptomyces lividans*. Extended expression of vascular endothelial growth factor in mouse hindlimb muscle mediated by ØC31 integrase also justifies its use in this experiment [18].

Calos *et al.* stably introduced the coagulation factor IX gene to a specific murine genomic site and demonstrated that the factor was expressed at therapeutic levels [12]. Furthermore, after introducing the COL7A1 gene into primary epidermal progenitor cells from patients with recessive dystrophic epidermolysis bullosa and these corrected cells were then transplanted into CB.17 scid/scid mice, a fatal blistering skin disorder caused by the congenital deficiency of type 7 collagen was improved [19]. To date, an extensive search for ØC31 integration sites has demonstrated that the risk of oncogenesis due to insertional mutagenesis is likely to be low, thus suggesting its safety [3].

In *in vitro* cell culture studies, the efficacy and site-specific characteristic of the integration was confirmed. In humans, there are several pseudo-*attP* sites other than 8p22; therefore, insertion of target genes into all of these sites will be an avenue of future tasks. To assess the action of ØC31 integrase *in vivo*, the hydrodynamic method was introduced. This is a relatively new method whose effectiveness and mechanism have been characterized [12, 20, 21]. This introduces a gene *in vivo* relatively easily. Similar to previous reports, the highest concentration of PTH was obtained on day 1 after gene introduction and it gradually decreased thereafter. Between the mice injected with pPTH-*attB*-YFP and pCMV-Int and mice injected with pPTH-*attB*-YFP and pCMV empty vector, no great difference in concentration of PTH was detected. The nearly equivalent expression of PTH in the integrase (–) group could be explained as follows: First, in the present study, all mice died of hypercalcemia by 40 days after introduction of gene, which is within the period that the differences between the 2 groups could not be seen. Similar experiments using AAT demonstrated a difference between integrase (+) and (–) groups after up to 1 month [12]. Second, in the integrase (–) group, most of the integrations of the PTH gene occurred at non-*attP* sites and in an integrase-independent fashion, which accounted for inefficient integration of the targeted gene but sustained expression of it.

Based on our results, the introduction of a gene using ØC31 integrase can occur very effectively and in a site-specific manner not only in cells but also *in vivo*. The clinical and experimental application of this method is promising; however, to be applied to the clinical treatment of hypoparathyroidism patients, difficult problems remain. One of the biggest problems is the absence of the function that recognizes serum calcium concentration and controls the secretion of parathyroid hormone. In fact, due to hypercalcemia owing to the uncontrolled secretion of the parathyroid hormone, all of the mice died. To overcome this hurdle, numerous points should be considered to understand the physiology of the normal parathyroid gland. Calcium-sensing receptors, which recognize serum calcium concentration, are expressed on the cell membrane of the principle cells in the parathyroid gland. This receptor is utilized in the intracellular signal transduction system and controls the synthesis and secretion of parathyroid hormone in the nucleus [22]. If PTH cDNA is regulated through calcium response element in the promoter and the calcium-sensing receptors are introduced, then serum calcium concentrations may be better physiologically controlled.

In conclusion, in spite of the limitation that lacks long-term effect ØC31 integrase *in vivo*, we performed an experimental gene therapy of the parathyroid hormone using ØC31 integrase. These findings may help realize ideal gene therapy.

Acknowledgements

We express our gratitude to Drs Michele P. Calos, Henry M. Kronenberg, and Hee-Yong Chung for their provision of pTA-*attB*, pCMV-Int plasmid, pCDM8-PTH-1 vector, and MFG.hsp70.ires.puro vector. We thank Drs Chae Ok Yun and Ranganath Muniyappa for their advice on the preparation of the manuscript. We thank Mr. Hyeongjae Jeong and Mr. Hyung Kwan Kim for immunohistochemical staining and animal

experiments.

This study was supported by Grant 03-PJ10-PG6-GP01-002 from the Korea Health 21 R&D project (to S-K Lim). This work was supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2008-331-E00124) (to S. Lee).

Disclosure

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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