Identification of a Specific Pseudo *attP* Site for Phage phiC3 Integrase in the Genome of Chinese Hamster in CHO-K1 Cell Line

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ARTICLE INFO	ABSTRACT
Article type: Brief Report *Article history:* Received: 14 Apr 2012 Revised: 31 Jul 2012 Accepted: 05 Sep 2012 *Keywords:* Inverse PCR phiC31 Integrase Pseudo *attP* Site	***Background:*** PhiC31 integrase is a DNA site-specific recombinase integrates DNA into the chromosomes between the two sites of *attB* and *attP*. Several pseudo *attPs* have been identified in mammalian genomes with critical features for long-term expression of transgene. In this manuscript, we report a novel intrinsic pseudo *attP* site named CHOL1 in the Chinese hamster genome implementing an inverse Polymerase Chain Reaction (IPCR). ***Objectives:*** Identification of pseudo *attP* site(s) of Tenecteplase cDNA integration in the genome of stable transformed CHO cell line. ***Materials and Methods:*** First, genome was extracted from a stable transformed CHO cell line expressing Tenecteplase. By creating of minicircle DNA in the last step, sequencing was performed. ***Results:*** We obtained one band. BLAST analysis of the respective sequence of inverse PCR band identified a pseudo *attP* site. ***Conclusions:*** Data demonstrated that the phiC31 integrase provides a suitable insertion site in the genome to express the gene of interest. Published by *Kowsar Corp*, 2013. cc 3.0.

▶ *Implication for health policy/practice/research/medical education:*
This study has implication on Biotechnology researchers.

1. Background

Nowadays, recombinant proteins have been produced in basic and applied studies. Several systems have been introduced for the efficient production of recombinant proteins in a large scale. The simplest one is overexpression of recombinant proteins in bacterial systems. However, one of the main disadvantages is the requirement of

proper folding environment or posttranslational modification for accurate biological activity of recombinant proteins. In spite of this, using eukaryotic expression system could bypass this problem (1). To obtain robust and long-term expression in the eukaryotic systems, integration of the gene of interest should be in appropriate location in the genome of target cell. However, most of current methods for stable integration of exogenous DNA into genome involve random integration of DNA (2, 3). In random integration, the expression of recombinant protein mainly depends on the positional effects of the adjacent sequences in target chromatin (4). Therefore, painstaking screenings are required to find best cellular colonies for desired level of protein expression of the target gene. Recently, PhiC31 integrase, as a site specific recombinase, has been proven to minimize the random integration and provides a long-term expression with efficient functionality in mammal cells (5, 6) PhiC31 integrase mediates recombination between two sequences of *attB* and *attP*. If an *attB* site is located at the exogenous plasmid carrying the gene of interest, phiC31 integrase would be capable of integrating whole sequence of the plasmid at similar native *attP* sites so called pseudo *attP* sites in the mammalian genome. This style of site specific integration leads to robust expression levels in the cell (7, 8). This feature minimizes position effects of nearby sequences around pseudo *attP* sites (9). In our previous study, we used this system for the long-term overexpression of Tenecteplase protein in Chinese hamster ovary (CHO) cells (10).

2. Objectives

Since determination of integrated site in transgenic stable cell lines is essential, we tried to identify pseudo attP site in Chinese hamster genome using an inverse PCR (IPCR) in this paper.

3. Materials and Methods

Inverse PCR has been employed successfully for amplification of unknown regions of the genomic DNA, outside the boundaries of identified sequences (11, 12). This technique involves digestion of genomic DNA with appropriate enzyme(s) (Figure 1A), creation of mini-circle DNA using self-ligation and appropriate primers to amplify known sequences at both sides of a DNA core region (12).

3.1. Cell Line

Tenecteplase producing CHO cell line (CHO+) was used in this study as was previously described (10).

3.2. Culture of CHO Cells and Genomic DNA Extraction

CHO+ and untransfected CHO cells (CHO-) were cultivated in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (Sigma) supplemented with 10% fetal bovine serum (Gibco) and 10% CO2 at 37°C in a hu-

mid atmosphere. After 24 hours, CHO+ cells were treated with G418 (600 μg/mL, Sigma) and cultured for 3 days. Approximately 5×10^6 cells from each group (CHO+ and CHO-) were harvested and genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen) (Figure 1B). Restriction enzymes digestion and ligation were performed as follows: Five μg genomic DNA of each sample was digested with a pair of restriction enzymes, NheI and XbaI (Fermentas) (Figure 1C). For self-circularization of digested DNA in an inverse PCR (IPCR), low amounts of DNA (1, 2, 3 ng/μL) were used in 10 μL of the ligation reaction (TaKaRa) (Figure 1D).

3.3. PCR and Nested PCR

To detect site specific integration before starting nested PCR, a normal PCR was performed. In case of random exogenous plasmid integration in Chinese hamster genome, intact *attB* arm should be amplified using attR and attB-F3 primers. Otherwise site specific integration causes breakage. Sequences of the primers are listed in Table 1. The PCR program used for amplification was as follow: 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 45 sec and a final extension period of 72°C for 10 min. Then PCR products were subjected to 1% agarose gel (CinnaGen) in TBE buffer (90 mM Tris-HCl, 64.6 mM boric acid, 2.5 mM EDTA, pH 8.3). Nested PCR was performed for amplifying unknown regions of DNA. Five μL of ligation mixture was diluted with 15 μL dH_2O, which was used as a template in the first round of PCR (PCRI) using TNK103-F and attB-F3 primers. In the second round of PCR, 1 μL of PCRI product was used as template using USP296-9F and attB-F3 primers (10, 13). The PCR program was as follows: 94°C for 10 min, followed by 35cycles of 94°C for 45 s, 56°C for 45 sec, and 72°C for 4 min and a final extension period of 72°C for 10 min. Cloning and sequencing were performed as follows: One product band was obtained from IPCR. This band was purified and ligated into a T-vector (InsTAcloneTM PCR Cloning Kit, Thermo Scientific) and was sequenced. Sequence analysis was performed by BLAST with RefSeq-Gene sequence databases. We have already published the isolation and characterization of a stable transformant CHO cells producing Tenecteplase using phiC31 integrase (10). To evaluate site specific recombination with phiC31 integrase, a normal PCR was designed based on the breakage of *attB* arms in exogenous plasmid encompassing Tenecteplase CDS, as a positive control (Figure 2A, Lane 1).

4. Results
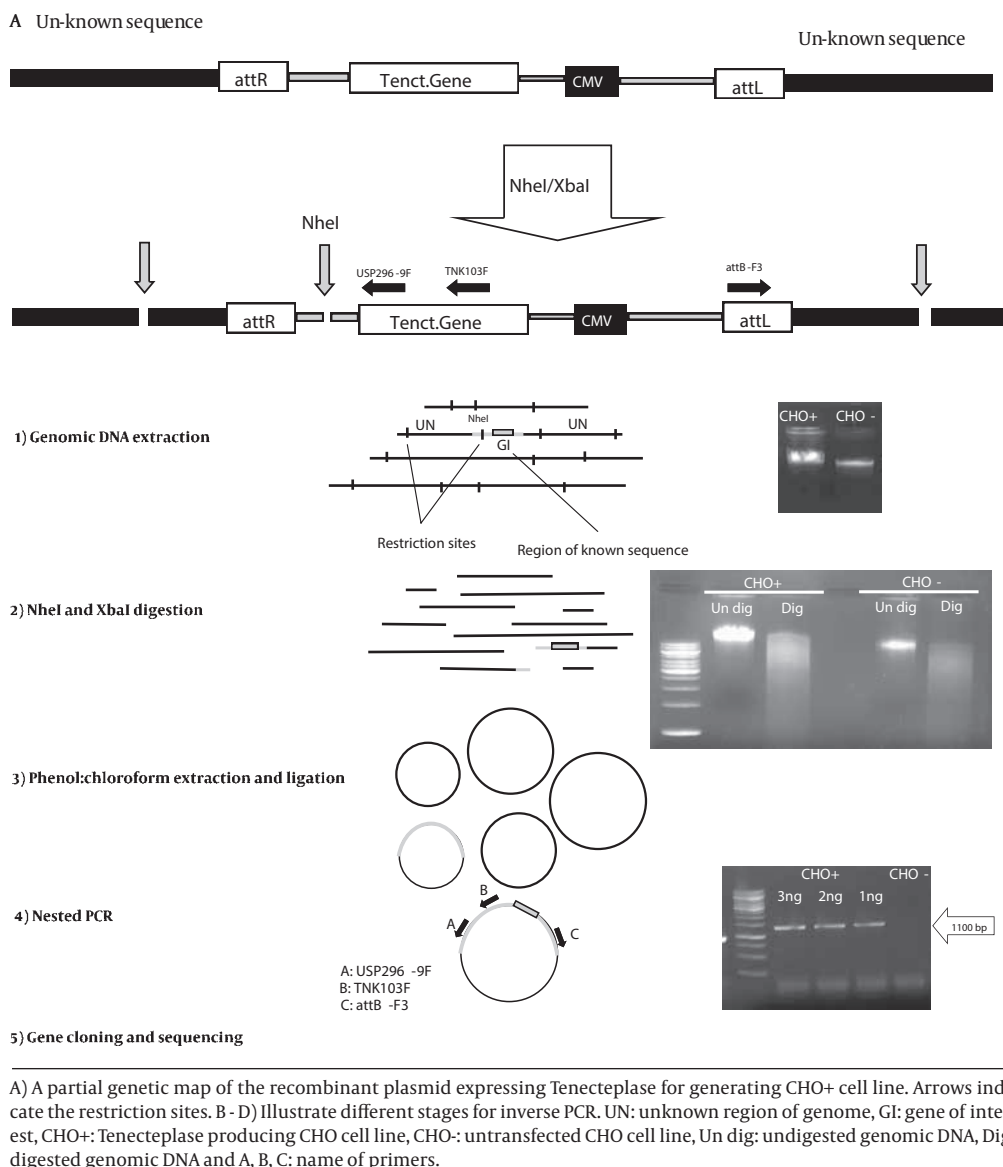
Polymerase chain reaction on CHO+ genome illustrated that *attB* arm was ruptured with the phiC31 integrase and the specific primers for this fragment did not amplify the expected size, (Figure 2A, Lane 3) confirming the site specific recombination occurred by phiC31 integrase in CHO+ cells. Thus, we tried to determine the pseudo *attP* site in the genome of CHO+ cells with an inverse PCR.
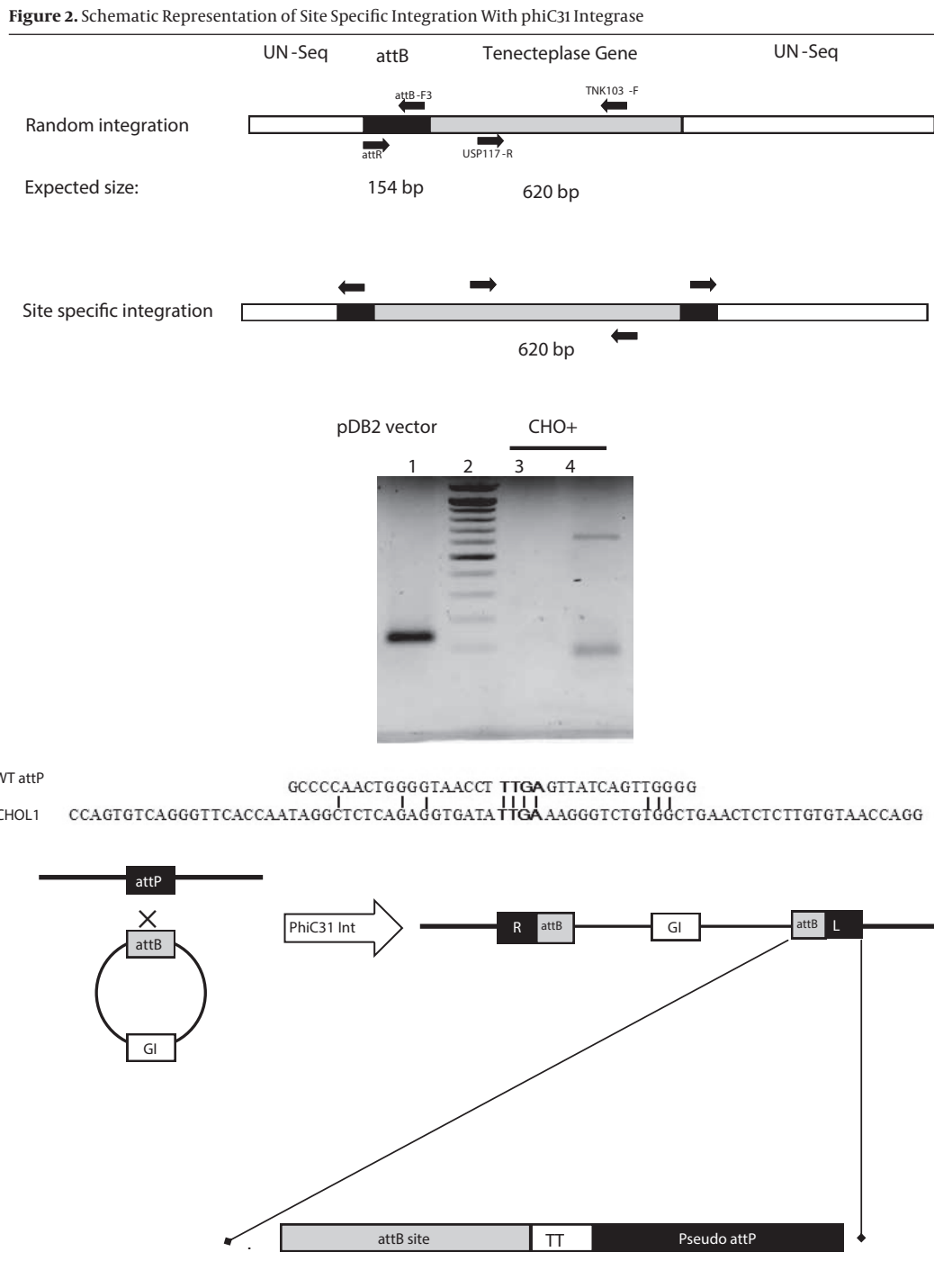
Table 1. The List of Primers

Name	Sequence 5´-3´
attB-F3 (13)	GTAGGTCACGGTCTCGAAGC
attR (13)	GGATCAACTACCGCCACCT
TNK103-F (10)	GGCAACTGGAGCACAGCGGAGA
USP296-9F (10)	CGCGGCGTCGCCCGGAGAGCGG
USP117R (10)	CGCGCTGCTTTGCCAGTTGGTGCA

First, compatible cohesive site producing restriction enzymes was used (Figure 1A and Figure C). These enzymes cut the plasmid at least in one position. Thereby, the right site of the recombination could be amplified in nested PCR (Figure 1A). After creating self-minicircle DNA in the last step of inverse PCR, we obtained one band (Figure 2A, Lane 4). BLAST analysis of the inverse PCR band sequence showed that the junctions at the site of specific recombination contained half of the *attB* site fused with the genomic sequence of Chinese hamster (GenBank accession number: NW_003613759.1).

Figure 1. Schematic Representation of Inverse PCR Stages

A) A partial genetic map of the recombinant plasmid expressing Tenecteplase for generating CHO+ cell line. Arrows indicate the restriction sites. B - D) Illustrate different stages for inverse PCR. UN: unknown region of genome, GI: gene of interest, CHO+: Tenecteplase producing CHO cell line, CHO-: untransfected CHO cell line, Un dig: undigested genomic DNA, Dig: digested genomic DNA and A, B, C: name of primers.

Figure 2. Schematic Representation of Site Specific Integration With phiC31 Integrase

A) Site specific and random integrations were testified using PCR. Lane1: PCR product of the intact attB site in the pDB2 vector (positive control); Lane2: ladder 100bp (Fermentas); Lane 3: expected product (154bp) of the attB site in CHO+ regarding random integration was not observed; Lane 4: partial product of Tenecteplase CDS amplification in CHO+ delineating specific integration into the genome. Star: primer dimer. UNSeq: unknown sequence. CHO+: Tenecteplase producing CHO cell line. B) The sequence of identified pseudo attP (CHOL1). WT attP: native attP sequence, GI: gene of interest (Tenecteplase CDS), TT: double Thymidine core at the point of crossover was recognized with phC31 integrase.

5. Discussion

We assigned the term of CHOL1 to this newly recognized pseudo *attP* site (Figure 2B). It has been proven that phiC31 enzyme can integrate a donor plasmid into the mammalian genome (6). Several studies have indicated that phiC31 integrase prefers identified sites that are named pseudo *attP* in several species including human (6, 14-17). Integration into these sites results in long-term expression of transgene in host cells (5, 6). Therefore, these sites could be assigned as a marker of site specific recombination in phiC31 integrase system. This system is feasible to produce desired proteins in CHO cells (7). In this study we tried to identify a pseudo *attP* site in Chinese hamster genome using inverse PCR named CHOL1. BLAST analysis indicated that CHOL1 sequence was similar to WT *attP* sequence reported in previous studies (18). The most identical sequence was observed in core region of the *attB* (TTGA) which is critical for phiC31 integrase site specific recombination (Figure 2B). This site has a homology (77%) with mouse genome sequence in chromosome 10 (NC_000076.5).

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Authors' Contribution

Mohammad Hadi Sekhavati: Conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing. Kianoush Dormiani: Conception and design, data analysis and interpretation. Kamran Ghaedi: Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript. Yahaya Khazaie: Conception and design, data analysis and interpretation. Morteza Hosseini: Conception and design, data analysis and interpretation. Mojtaba Tahmoorespur: Data analysis and interpretation. Mohammad Reza Nasiri: Data analysis and interpretation. Mahboubeh Foruzanfar: Technical assistance. Mohammad Hossein Nasr Esfahani: Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

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