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# S Phase-preferential Cre-recombination in Mammalian Cells Revealed by HIV-TAT-PTD-mediated Protein Transduction

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**The Cre recombinase of bacteriophage P1 is a powerful tool for artificial modification of genomic function in mammalian cells. To date, many researchers have studied the enzymatic biochemistry of Cre recombinase in *loxP* site-specific cleavage and rearrangement, as well as its use in gene technology. However, the intricate mechanisms of Cre-mediated recombination are still poorly understood. For example, more knowledge is needed in order to understand Cre recombinase's dependency on cell cycle, the necessity of other factors for recombination, and the exact nuclear environment that's required at the target locus, in order for recombination to take place in eukaryotic cells. In this study, we showed that P1 Cre-mediated recombination occurred frequently during S-phase of the cell cycle. HeLa cells were synchronized in cell cycle with the thymidine-hydroxyurea block method, and recombinant Cre proteins were fused with HIV-1 TAT protein transduction domains (PTD) in every phase of the cell cycle. Results showed that the transduction of PTD-Cre gave rise to genomic recombination preferentially during the S-phase of cell cycle. These findings will contribute significantly to the development of the Cre/*loxP* recombination system *in vivo*.**

**Key words: cell cycle, Cre recombinase, GFP, protein transduction, synchronized cell culture.**

Abbreviations: PTD, protein transduction domain; HIV-1, human immunodeficiency virus-1; β-Gal, β-galactosidase; EGFP, enhanced green fluorescent protein.

Gene targeting techniques, such as homologous recombination, have become powerful tools for genetic manipulation and the creation of animal models for human disease (*1–3*). In particular, the use of Cre recombinase from the bacteriophage P1 has been indispensable for techniques, such as genetic transformation, conditional knockout mice (*4*, *5*), and human artificial chromosome (HAC) vectors (*6*, *7*). Cre recombinase represents one of the more simple tyrosine recombinases (*8*, *9*), which recognizes the *loxP* recombination site, a 34 bp nucleotide sequence (*9*, *10*). Cre-mediated recombination is initiated first at one *loxP* site to form a Holliday-intermediate, which then, through a multiple-step reaction in bacterial cells (*9*, *11*, *12*), is resolved by cleavage and exchange of the other site to yield recombinant products. The intracellular mechanism of Cre-mediated chromosomal recombination is still poorly understood in eukaryotic cells, particularly the dependence on cell cycle, cooperation of other factors and the appropriate nuclear environment of the target locus that is required for recombination. Homologous recombination that occurs during the repair of DNA damage is known to be dependent on the S phase of cell cycle (*13*, *14*); therefore, it is interesting to further explore cell cycle-dependency in Cre-mediated recombination.

Although gene delivery techniques using viral and plasmid vectors and mRNA transduction can produce high levels of Cre expression in transformed cells (*15*), it is difficult to control the quantity and stage of protein expression. The protein transduction technique has recently been developed to introduce physiologically active proteins into living mammalian cells and tissues for therapeutic purposes, in place of gene therapy. Protein transduction domains (PTDs) were found to confer membrane permeability in mammalian cells in small cationic regions of proteins, such as herpes simplex virus (HSV) viral protein VP-22 (*16*), *Drosophila melanogaster* antenna-pedia protein (ANTP) (*16*, *17*), Kaposi FGF, and the human immunodeficiency virus-1 (HIV-1) TAT and arginine/lysine rich polypeptides (*18*, *19*). Although the mechanisms that PTDs employ in order to cross biological membranes remains poorly understood, the presence of basic amino acids (Arg and Lys) is important for initial contact with the negatively charged lipids on the plasma membrane (*19–21*). PTD in the TAT protein, which is composed of the amino acid sequence RKKRRQRRR, was shown to be involved in internalization into cells when added exogenously (*22–24*).

In this study, we have constructed and purified the recombinant fusion protein of HIV-1 TAT PTD and Cre recombinase and transformed synchronized reporter cells

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with this protein. Transient transduction of PTD-Cre during S phase revealed that Cre-mediated *loxP* site-specific genome recombination was preferentially induced during S phase of the cell cycle. This finding will greatly benefit the development of animal models for human diseases, as well as construction of human artificial chromosome vectors for gene therapy.

## MATERIALS AND METHODS

*Construction of Plasmids and Purification of His-tagged Protein*—For purification of various proteins from bacterial strains, cDNAs were inserted into the pCold III expression vector (Takara, Kyoto, Japan), which expresses designed transcripts via the promoter of the *Escherichia coli* cold-shock protein gene. The vector also contained translation-enhancing elements (TEEs) for efficient translation and a six-histidine stretch for purification tags (6His) in N-terminus of the proteins. The constructed plasmids were transformed into the *E. coli* BL21 strain. Expression of these fusion proteins was induced by 0.25–0.5 mM isopropylthiogalactoside (IPTG) for several days at 15°C. The harvested cells were lysed by sonication in lysis buffer (50mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, and pH 8.0). The His-tagged proteins were then purified twice through a $Ni^{2+}$-NTA agarose column (Qiagen, Stanford, CA, USA). The proteins were eluted by stepwise increasing concentrations of imidazole (5–500 mM) in elution buffer, and were dialysized in 8% glycerol, 0.3 M NaCl and 1 mM DTT (dithiothreitol)/phosphate-buffered saline (PBS).

*Extraction of Cytoplasm and Nucleoplasm from HeLa Cells*—HeLa cells were suspended in Buffer A [10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM DTT, 0.15 mM spermine, 0.5 mM spermidine] and incubated for 15 min on ice. The cells were dissolved in 0.625% Nonidet-P40 by vortexing for 15 sec and centrifuged at 15,000 r.p.m. for 30 sec at room temperature. Supernatant was collected as cytoplasm fraction. The nuclear pellets were resuspended in Buffer C [20mM HEPES-KOH (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 0.5 mM spermidine] and centrifuged at 15,000 r.p.m. for 5 min at 4°C. Supernatant was recovered as nucleoplasm fraction.

In Vitro *Assay of loxP Site-specific Recombination*—Cre-mediated *loxP* site-specific recombination was performed in 50 μl reaction buffer containing 50 mM Tris–HCl, pH 7.5, 33 mM NaCl, 10 mM $MgCl_2$, 200 ng substrate DNA (3.5 kb linear DNA with 1 kb fragment flanked *loxP* sites) and 400 ng purified Cre recombinase at 37°C for 0–90 min, and terminated by 0.1% SDS, followed by heat treatment at 70°C for 10 min. The reaction products were then resolved on 0.8% agarose gels and stained with ethidium bromide.

*Cell Culture and Establishment of Reporter Cell Line Rep250*—Human cervical cancer cells (HeLa) and Rep250 cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 200 units/ml penicillin and 100 μg/ml streptomycin, in humidified chambers with 5% $CO_2$. The pReporter plasmid was constructed by insertion of the DsRed gene (red fluorescent protein derived from *Discosoma*. sp) (Clontech Laboratories, Inc. Mountain View, CA, USA) and SV40 polyA signal flanked by *loxP* sites into the multiple cloning site of the pEGFP-N3 vector. For determination of *loxP* site-specific recombination, the reporter cell line Rep250 was established from HeLa cells transfected with 10 μg of the pReporter plasmid by calcium phosphate precipitation method, as described previously (*25*). Positive reporter cell lines were screened with selection medium supplemented with 500 μg/ml G418 (neomycine).

*Synchronization of Cell Cycle*—The exponentially growing Rep250 cells were treated with 2.5 mM thymidine for 36 h, washed twice with PBS and subsequently cultured in growth medium for 12 h. Then, the cells were supplied with 1 mM of hydroxyurea and cultured for 24 h. As the result, the cells were synchronously arrested at G1/S boundary. For progression of the cell cycle, the cells were washed twice with serum-free medium and cultured with growth medium.

*Transduction of Cells by Purified Protein*—The cells pre-washed with serum-free medium were treated with 1.5 μM recombinant protein for 2 h at 37°C, followed by two washes with serum-free medium and cultured with growth medium.

*Flow Cytometry*—To determine cell cycle stage, cells were harvested from plates with 0.25% trypsin/EDTA at 37°C, washed twice with PBS and then fixed with ice cold 70% Ethanol/PBS. The cells were treated with 100 μg/ml of RNase/PBS for 40 min at 37°C to prevent RNA staining. Subsequently, the cells were incubated in 50 μg/ml propidium iodide (PI)/PBS for 10 min at room temperature. The cells were then washed with PBS, suspended in ice cold PBS, and further analysed by an EPICS-XL flow cytometer (Coulter Electronics, Hialeah, FL, USA).

For analysis of incorporation and expression of enhanced green fluorescent protein (EGFP) and DsRed, the cells were harvested from dishes and fixed in 4% paraformaldehyde/PBS for 15 min on ice. The cells were washed once with PBS and analysed by flow cytometry.

*Polymerase Chain Reaction (PCR) of Genomic DNA*—To detect *loxP* site-specific recombination, a forward primer (5′-GCGTGTACGGTGGGAGGTCTAT-3′) was designed at the cytomegalovirus promoter ($^{P}$CMV) region, and a reverse primer (5′-GTGAACAGCTCCTCG CCCTTGC-3′) was designed inside the EGFP gene sequence of the pReporter. PCR was performed with the following settings: 26 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. For the internal control, we employed the human leukaemia inhibitory factor-1 (LIF-1) locus, using the following primer set: forward, 5′-CAACCTGGACAAGCTATGTG-3′ and reverse, 5′-CTA GAAGGCCTGGGCCAACA-3′. The reaction products were detected using 5% polyacrylamide gel electrophoresis (PAGE).

*Western Blot Analysis*—Transduced proteins were determined by western blot analysis. Lysates (50 μg) collected from PTD-Cre-transducd cells were separated on SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. PTD-Cre was detected with rabbit polyclonal antibody against His-tag (Santa Cruz Biotechnology, Inc., CA, USA). Immunoreactive

protein bands were visualized using horseradish peroxidase-linked secondary anti-rabbit IgG antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) and ECM-peroxidase detection system (Amersham Pharmacia Biotech) according to the manufacture's instructions. To identify intracellular distribution of PTD-Cre, we employed monoclonal anti-α-tubulin mouse IgG antibody (SIGMA, SL, USA) as cytoplasm marker, and polyclonal anti-Lamin B goat IgG antibody (Santa Cruz Biotechnology, Inc. CA, USA) as nucleoplasm marker.

## RESULTS

*Purification and Characterization of Recombinant PTD-Cre Protein*—PTDs from HIV-1 TAT have been used for efficacious transport of purified proteins into various cells and tissues *in vivo* and *in vitro* (20–24). PTDs are expected to transduce Cre recombinase protein exclusive of vectors, followed by chromosomal recombination between *loxP* sites in mammalian cells. To confirm this hypothesis, we first evaluated the efficiency of Cre-recombination mediated by PTD-Cre fusion protein. We purified P1 phage Cre recombinase (Cre), *E. coli* β-galactosidase (β-Gal) and *Aequorea victoria* EGFP, and expressed these proteins under control of the *E. coli* cold-shock promoter (pCold plasmids). For proper protein transduction, these proteins contained PTD domain sequences (YGRKKRRQRRR) from HIV-1 TAT proteins at the amino (N)-terminal, as well as TEEs for efficient translation, and a six-histidine stretch at the N-terminus of the protein for purification tags (6His) (Fig. 1A).

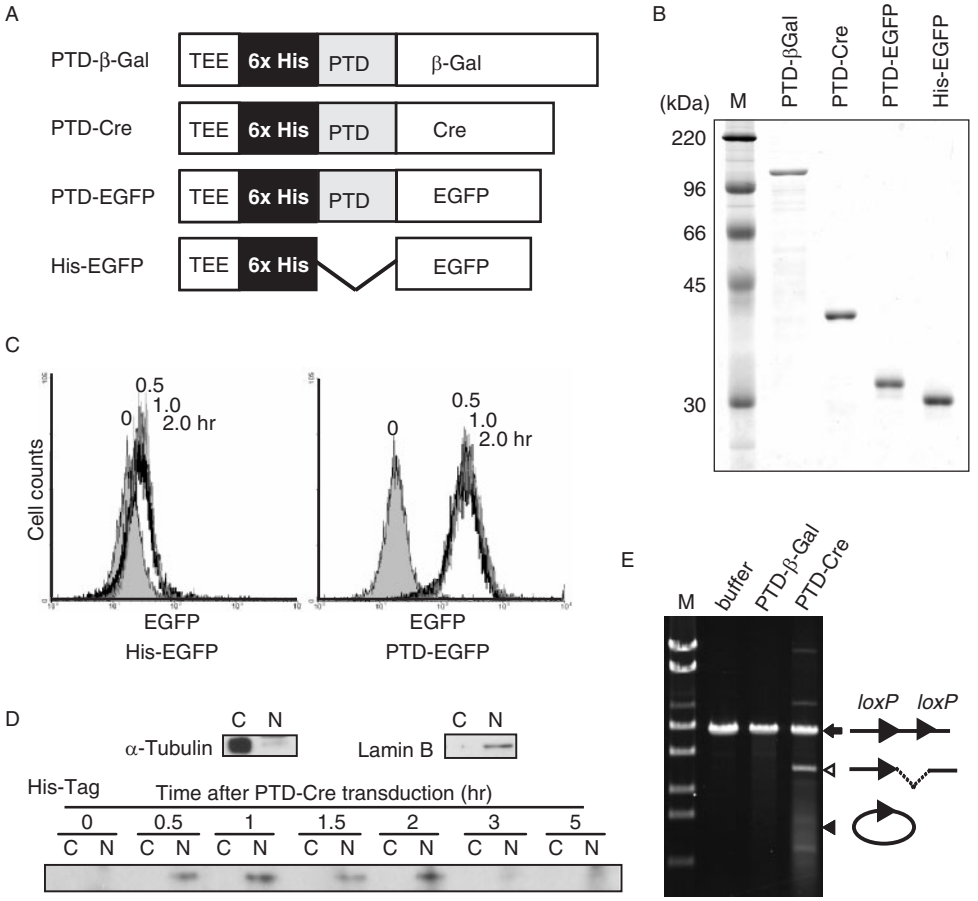


Fig. 1. **Purification of PTD-Cre fusion proteins and *in vitro* recombination.** (A) Schematic structures of the recombinant proteins. TEE indicates translation-enhancing element, composed of amino acid sequence MNHKV. '6x His' indicates 6 histidine repeats (His tag) for protein purification. Protein transduction domain, PTD (GRKKRRQRRRR), was derived from the HIV TAT protein. The *E. coli* β-Galactosidase is depicted as β-Gal; P1 phage Cre recombinase, Cre; and jellyfish green fluorescent protein, EGFP. These recombinant proteins were expressed in pCold plasmids at 15°C for several days in *E. coli* BL21. (B) His-tagged fusion proteins, purified by $Ni^{2+}$ affinity chromatography, were determined by SDS-PAGE and stained with Coomassie brilliant blue. M represents protein marker. (C) Transduction of PTD fusion proteins into HeLa cell culture. A total of 1.5 μM of His-EGFP and PTD-EGFP were administered to HeLa cells at 37°C for 0 h (filled gray), 0.5 h (black line), 1.0 h (bold line) and 2.0 h (gray line). Treated cells were fixed by cold 4% paraformaldehyde/PBS and EGFP fluorescence was detected by flow cytometry. (D) Intracellular distribution of transduced PTD-Cre. A total of 1.5 μM of PTD-Cre was administered to HeLa cells for indicated time. After washing out PTD-Cre, cells were incubated with growth medium for 3 h. Detection of PTD-Cre in cytoplasm (C) and nucleoplasm (N) fractions by western blot analysis. (E) *In vitro* recombination assay. A 200 ng DNA substrate and 400 ng PTD-Cre fusion proteins were incubated in reaction solution, followed by agarose electrophoresis and staining with EtBr. Arrow, indicates substrate DNA (3.2 kb); Open and closed arrowheads show the products, linear DNA (2.1 kb) and circular DNA (1.1 kb), respectively.

Degrees of protein purity were determined by SDS-PAGE, which resulted in a single band of each protein (Fig. 1B). To examine the validity of the TAT PTD sequence in the purified proteins, we determined the activity of internalization and uptake of PTD-EGFP and His-EGFP proteins by flow cytometry (proteins absent of PTD sequence served as controls) (Fig. 1C). Following addition of recombinant protein (1.5 μM), transduction of PTD-EGFP into HeLa cells took place within 30 min, and the level of transduction was sustained between 30 min and 2 h (Fig. 1C, right panel). In contrast, transduction of His-EGFP was scarcely detected 2 h after protein administration (Fig. 1B, left panel).

To assess intracellular distribution of PTD-Cre, we determined PTD-Cre in cytoplasm and nucleoplasm fractions by western blot analysis. Following administration of PTD-Cre protein (1.5 μM), almost all PTD-Cre rapidly migrated into nuclei at 30 min and sustained for 2 h (Fig. 1D). Furthermore, in order to determine the enzyme activity of purified PTD-Cre recombinase *in vitro*, we constructed a linear DNA substrate (3.2 kb) that had a 1.1 kb sequence flanked by *loxP* sites. Following incubation of DNA substrates (200 ng) with PTD-Cre proteins (400 ng), *loxP*-site recombination proved to be specific and produced 1.1 kb circular DNA and 2.1 kb linear DNA products (Fig. 1E, open and closed arrowhead), while PTD-β-Gal exhibited no reaction to the substrate. The linear DNA products were identified by their sensitivity to the specific restriction enzyme (data not shown). These results suggest that the purified PTD fusion proteins maintained their ability to penetrate the cells, and they displayed specific enzyme activity to induce loxP site recombination *in vitro*.

*Establishment of Reporter Cell Line Rep250*—In order to assess *loxP* site-specific recombination of PTD-Cre fusion proteins in genomic DNA, we established a reporter cell line, Rep250, which originated from HeLa cells. This epithelial cell line contained few copies of stably integrated reporter sequences in the cell's genome. As shown in Fig. 2A, the reporter sequence was constructed as follows: expression of the entire construct was driven by the $^{P}$CMV; DsRed cDNA with poly A signal was flanked by *loxP* sites and was inserted just upstream of EGFP cDNA with poly A signal. Therefore, the reporter cell line Rep250 expressed DsRed without transduction of Cre recombinase, which resulted in red fluorescence. If DsRed cDNA that was flanked by *loxP* sites was excised from the reporter sequence by Cre recombinase, the transcript from the reporter sequence should contain only the EGFP coding sequence, and the reporter cell should generate green fluorescence.

In order to confirm the Cre-recombination reporter system by induction of EGFP expression, Rep250 cells were incubated with PTD-β-Gal (1.5 μM) and PTD-Cre (1.5 μM). Through the use of confocal microscopy, we showed that the Rep250 cells switched from red florescence to green fluorescence (Fig. 2Bc and g), indicating the excision of DsRed by *loxP*-site recombination. In contrast, the Rep250 cells that were treated with vehicle or PTD-β-Gal did not excise the DsRed sequence and continued to express DsRed (Fig. 2Bb and f). The number of EGFP-expressing cells in the Rep250 cell line increased with the addition of PTD-Cre and transfection of pCMV-Cre plasmids (Fig. 2Bd and h). This observation was confirmed by flow cytometry (Fig. 2C). Moreover, site-specific genomic recombination with PTD-Cre was verified by southern blot analysis, using probes specific to EGFP (Fig. 2D). Truncated DNA fragments from the reporter cell genome were detected in PTD-Cre-treated cells, but not in PTD-β-Gal-treated cells. These results suggest that the PTD-Cre recombinase was transduced and involved in *loxP*-specific genome recombination of the mammalian cells.

*Cell Cycle-dependent Recombination by His-PTD-Cre*—Despite successful transduction and nuclear localization (Fig. 1D) through the use of PTD-Cre protein, the efficiency of recombination by protein transduction was low compared to that of pCMV-Cre plasmids (Fig. 2Bh). There are many factors influencing recombination efficiency; however, this study aimed to explore the role of cell cycle stages in the induction of genomic recombination. Generally, DNA transfection using plasmid vectors is able to maintain protein expression throughout several phases of the cell cycle; however, PTD-Cre-mediated protein transduction was transient for only 2 h (Fig. 1D).

To synchronize the cell cycle of Rep250 cell culture, through the use of the thymidine-hydroxyurea method ($G_1$/S arrest) (*26*), it was crucial to determine the length of the cell cycle and S phase. The population doubling-time of proliferating Rep250 cells indicated that cell cycle length was 37.6 h, while the cell cycle length of HeLa parent cells was 25.6 h (Fig. 3A). Rep250 cells expressing DsRed displayed a prolonged cell cycle, particularly during $G_2$/M phase. S phase duration (12 h in Rep250 cells) was calculated by the percentage of cells that had an intermediate DNA content range (between diploid and tetraploid), determined by flow cytometry of PI-stained cells.

Following cell cycle synchronization of the Rep250 cells, >80% of Rep250 cells were adjusted to $G_1$/S transition phase, determined by flow cytometry of DNA content (Fig. 3B, right panel). The synchronized cell proliferation of $G_1$/S-arrested cells was initiated by the removal of HU. Synchronous DNA synthesis was observed for 12 h until the point of tetraploid formation, followed by $G_2$ and M phases (Fig. 3C). However, cell cycle of the synchronized cell culture became erratic by 30 h after HU removal, which could have been caused by aberrant DsRed expression.

To address the question of whether protein transduction mediated by PTD depends on a particular cell cycle phase, we used synchronized cell cultures expressing PTD-EGFP to determine the protein transduction efficiency through the use of flow cytometry (Fig. 3D). Consequently, every cell cycle stage in the synchronized, as well as asynchronized, cell culture resulted in the same level of transduction efficiency, suggesting that protein transduction is independent of cell cycle. Therefore, in the Rep250 cell line that was transduced with PTD-Cre protein, we analysed the cell cycle-dependency of genomic recombination. Synchronized cell cultures received purified PTD-Cre fusion protein (1.5 μM) at every stage of cell cycle for 2 h, followed by incubation in fresh growing medium for 5 days.

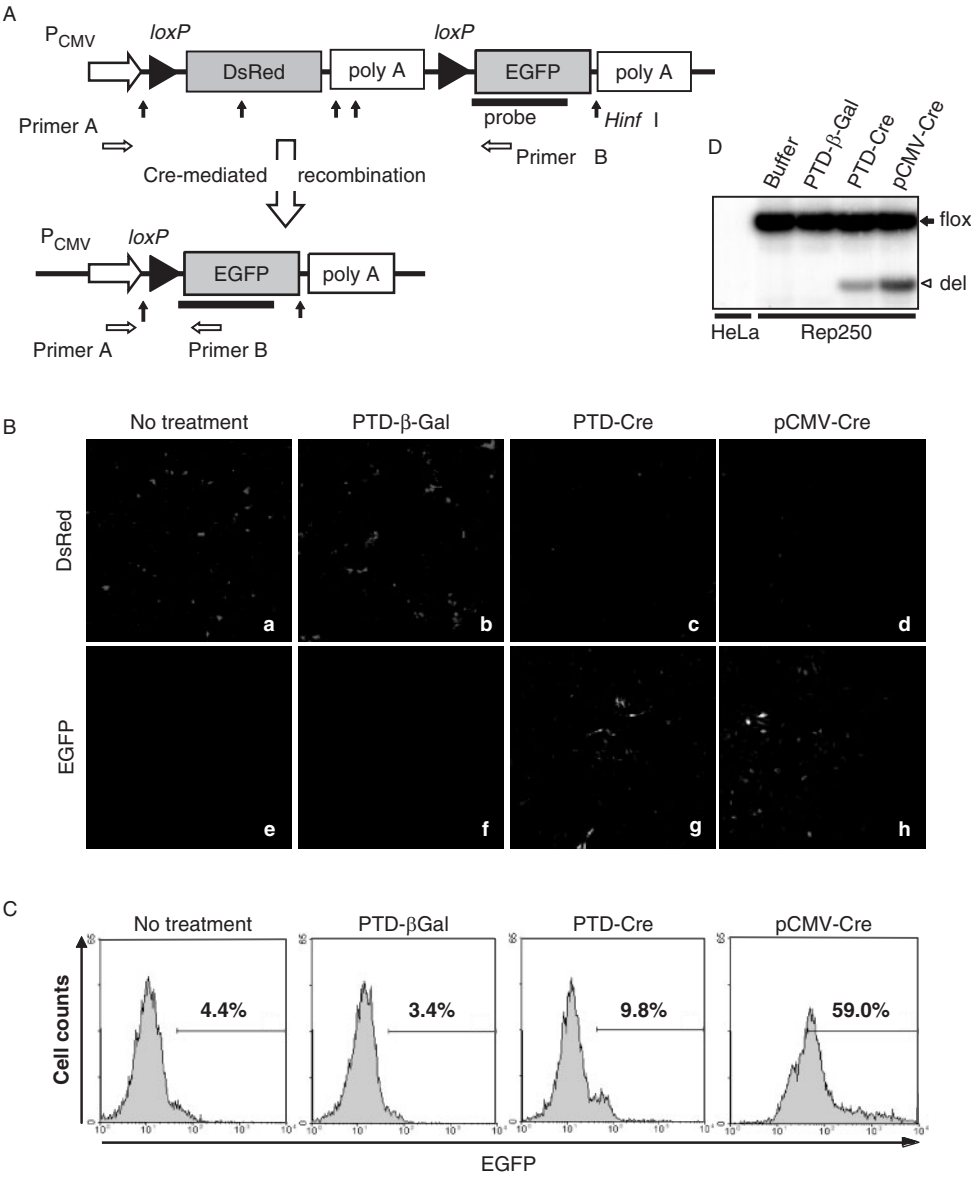


Fig. 2. **Generation of reporter cell line for Cre-mediated recombination.** (A) Schematic representation of the reporter gene construct in the reporter cell line Rep250. DsRed gene and polyA signal expression unit was flanked by *loxP* sites and followed by EGFP expression unit. Cre-mediated recombination resulted in removal of DsRed, thus permitting the expression of EGFP. The $^{P}$CMV indicates cytomegalovirus early promoter, and vertical arrows show the sites digested by *Hinf* I for Southern blot analysis. Bar (probe) indicates probe fragment used in Southern blot analysis, and open arrows show primer set used in genomic PCR (Fig. 4). (B) The reporter cell line (Rep250) was established by transformation with the reporter plasmid. *loxP* site-specific recombination was verified by confocal microscopy; DsRed (a-d) and EGFP (e-h) fluorescence. Rep250 cells were treated with 1.5 μM PTD-β-Gal (b and f), PTD-Cre (c and g) or no protein (a and e) for 2 h, followed by incubation in growth medium for 5 days. pCMV-Cre plasmid vector (Cre expression driven by $^{P}$CMV) was also used to transfect Rep250 cells, followed by incubation in growth medium for 2 days (d and h). (C) Flow cytometric analysis of Rep250 cells treated with PTD-Cre in the same condition as (B). Panels indicate EGFP expression levels. Horizontal lines show the intensity designated as EGFP-positive cell that are indicated in percentage against total cells on the lines (D) Southern blot analysis to detect genomic recombination. Rep250 cells were incubated with 1.5 μM PTD fusion proteins for 2 h, followed by further incubation in growth medium for 1 week. Genomic DNA was digested with *Hinf* I, applied to Southern blot hybridization, and probed by EGFP fragment. Upper band (arrow; flox) shows original pReporter construct, and lower band (arrowhead; del) indicates the truncated sequence resulting from *loxP* site-specific recombination.

Conversion of Rep250 cells from DsRed to EGFP expression was determined by flow cytometry, and the percentage of EGFP positive cells was determined (Fig. 3E and F). Interestingly, the conversion efficiency to EGFP was the highest (2-fold above average levels) during the mid-S phase of cell cycle, 6 h following HU removal. These results suggest that *loxP*-specific genomic recombination, mediated by transduced PTD-Cre

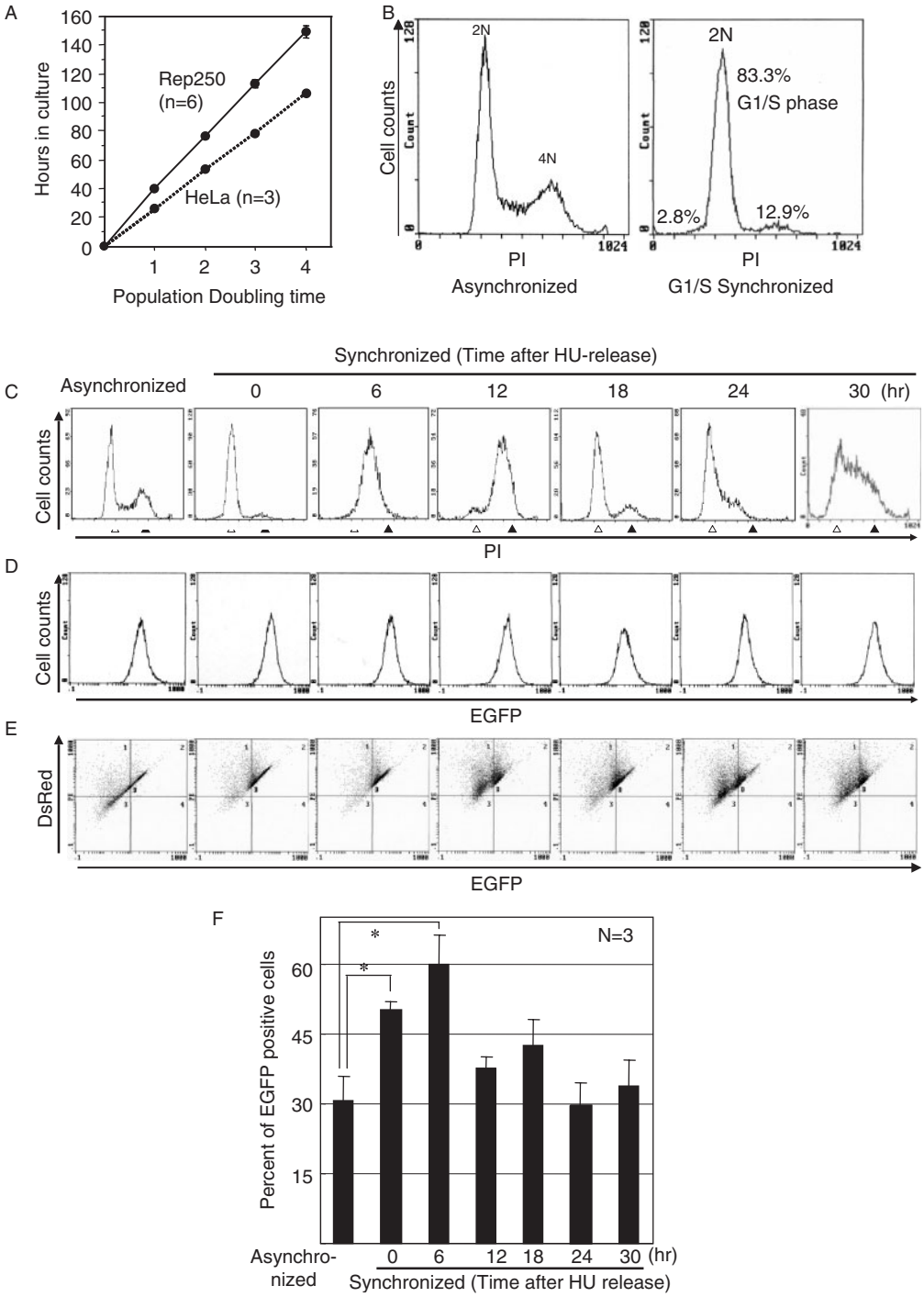


Fig. 3. **Cell cycle-dependent *loxP* site-specific recombination by PTD-Cre in synchronous Rep250 cell cultures.** (A) Population doubling time of HeLa and Rep250 cells. (B) Flow cytometry analysis of cell cycles in asynchronized cultures and synchronized Rep250 cells in $G_1$/S transition phase. Synchronized Rep250 cell cultures were induced by treatment of 2.5 mM thymidine for 36 h, followed by administration of 1 mM HU for 1 day. Cells were stained with PI and applied to flow cytometer. (C) Progression of cell cycle. The synchronous proliferation was re-initiated by HU removal, and time-dependently recovered cells were stained with PI. Open and closed arrowheads show $G_0/G_1$ to S phase and $G_2$/M phase, respectively. (D) Quantification of cell cycle-dependent uptake of PTD-EGFP. Synchronized Rep250 cell was treated with 1.5 μM PTD-EGFP for 2 h and analysed by flow cytometry. (E). Flow cytometry analysis of cell cycle-dependent Cre-recombination. A total of 1.5 μM PTD-Cre was administered to synchronized Rep250 cell cultures for 2 h, followed by further incubation for 5 days. Dot plots show flow cytometry analysis of EGFP-fluorescence (*x*-axis) versus DsRed-fluorescence (*y*-axis). (F) Cell cycle-dependent Cre-recombination. The numbers on each histogram indicate the percentage of EGFP-positive cells. Each column represents the mean ± SD of three independent experiments. Asterisks indicate significance level ($P$-value < 0.05) attained in comparison to the asynchronous group.

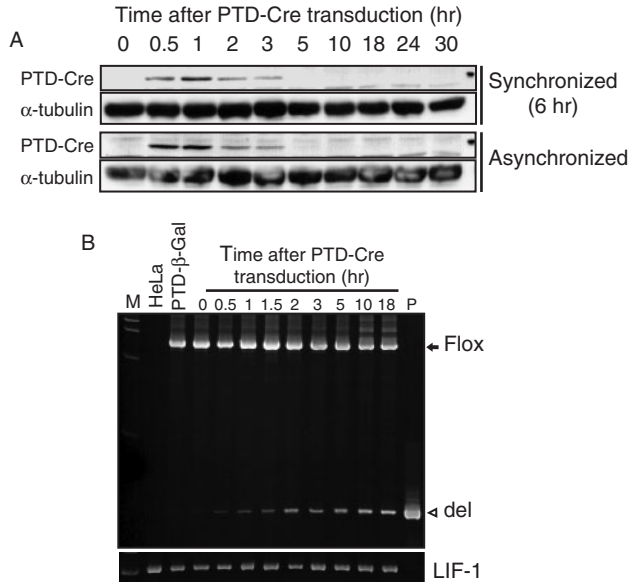


Fig. 4. **Characterization of PTD-Cre mediated genomic recombination.** (A) Time-course of transduction with PTD-Cre. 1.5 μM PTD-Cre was administered to asynchronous or synchronous cultures of Rep250 cells for 2 h at every stage of cell cycle. Incorporated protein in whole cell lysate was determined by western blot analysis using anti-His tag antibody. Anti-α-tubulin antibody was utilized for normalizing controls for protein loading onto electrophoresis. (B) Time-course of *loxP* site-specific genome recombination mediated by transduced PTD-Cre. Transduction of PTD-Cre was performed as described in (A). Genomic DNA (0–18 h) isolated from every phase of cell cycle and plasmid DNA (P) bearing the deletion were applied to PCR using the primer set indicated in Fig. 2A. Arrow and arrowhead indicate the original reporter gene construct (flox) and the truncated recombinant structure (del) generated by the *loxP* site-specific recombination.

recombinase protein, proceeded preferentially during the early stages (0–6 h) of S phase.

*Characterization of PTD-Cre-mediated Genomic Recombination*—In order to examine lifetime of the transduced PTD-Cre protein in Rep250 cells, we first determined PTD-Cre protein expression in reporter cells by western blot analysis using an anti-His tag antibody. Synchronized Rep250 cells that were in mid-S phase of cell cycle (6 h post-HU removal) were given PTD-Cre protein; cells were recovered at various times from 0 to 30 h post-administration, and protein was further detected by western blot. As shown in Fig. 4A, PTD-Cre protein was incorporated into the synchronized cells at 0.5 h post-administration, reached maximum incorporation at 1 h, and disappeared from the reporter cells after 3 h. This incorporation profile is also observed in the asynchronized reporter cells. The fate of transduced Cre recombinase in the Rep250 cells suggests that *loxP* site-specific genomic recombination occurred soon after Cre administration, and that the conversion of DsRed to EGFP expression (Fig. 2B), due to *loxP* site-recombination, took place shortly after Cre administration.

To confirm *loxP* site-specific recombination in Rep250 cells transduced with PTD-Cre recombinase, we examined the conversion of genomic structures by PCR, using reporter gene-specific primers as indicated in Fig. 2A. Genomic DNA was isolated from Rep250 cells that were synchronized in mid-S phase and received PTD-Cre protein. PCR products from the original reporter gene were truncated to a short fragment by *loxP* site-specific recombination. As shown in Fig. 4B, the truncated fragment (del) was detectable 1 h post-Cre administration, reached a maximum level by 2 h, and sustained the level up to 18 h. Taken together, these results suggest that PTD-Cre recombinase, transduced into synchronized Rep250 cells during mid-S phase, underwent *loxP* site-specific genomic recombination shortly after transduction.

## DISCUSSION

Protein delivery systems using PTD, which facilitates protein translocation across the plasma membrane, has seen rapid development in protein transduction therapy, in order to compensate for gene therapy and regenerative medicine. In this study, we produced physiologically active Cre recombinase protein, which contained PTD, to perform efficient chromosome rearrangement mediated by *loxP*-site recombination. In addition, we determined the phase of cell cycle during which Cre-recombination takes place. Although protein transduction is typically a transient effect, the use of PTD has allowed for intracellular transduction of target cells and tissues with specific proteins for a particular amount of time. In this report, we have shown that through the use of PTD, transduction of P1 Cre recombinase takes place preferentially during the early stages of S phase of cell cycle and is capable of initiating *loxP*-specific genomic recombination. Within 30 min following protein administration, His-PTD-Cre was detected; however, it was no longer measurable 3 h post-treatment. Cre/*loxP* recombination was observed at least 30 min after PTD-Cre transduction. Based on these observations, we believe that Cre-mediated recombination of genomic targets occurs predominantly during S phase of cell cycle, rather than $G_0/G_1$ and $G_2$/M phase.

Why did Cre-mediated recombination preferentially occur during S phase? It has been previously reported that histones at the replication origins are hyperacetylated during early S phase and that chromatin conformation is relaxed, coincidental with binding of the origin recognition complex (ORC) (*27*). The ORC is highly conserved among eukaryotes and regulates the initiation of replication through progression of bidirectional replication forks. Moreover, transcription factors and other DNA binding factors can more easily gain access to target regions in a relaxed chromosome structure (*28*). Therefore, the Cre/*loxP* recombination reaction also appears to be enhanced in a relaxed region of the chromosome.

The Cre recombinase of bacteriophage P1 belongs to the integrase family of site-specific recombinases. Recombination with this integrase family member is preceded by a common mechanism of catalysis that involves the formation of a cruciform DNA intermediate, known as 'Holliday junction' (*9*, *11*). Cre-mediated recombination is initiated on one pair of DNA strands to form a Holliday conservative intermediate,

which is then resolved by cleavage and exchange of the other pair of strands to yield recombinant products (*9–12*).

Resolvase, which recombines DNA strands at Holliday junctions, carries out the resolution of homologous recombination intermediates (*29*, *30*) and initiates replication once again at the replication forks (*31*). Resolvase activities have been identified in a variety of organisms, including bacteriophage, bacteria (*E. coli* RuvC and RusA) (*32*), yeast (Cce1, Ydc1, Ydc2) (*33*, *34*), archaea (Hje, Hjc) (*35*, *36*) and eukaryotic viruses (*37*); however, much less is known in mammalian cells. Recently, two distinct endonucleases that can resolve Holliday junctions have been detected in mammalian cell-free extracts. One of two enzymes was identified as a complex containing the Mus81 protein (*38*), a member of the XPF family of endonucleases that physically interacts with Rad54 and Cds1 in yeast (*39*, *40*). These factors are necessary for homologous recombination-mediated DNA repair and the rescue of stalled and collapsed replication forks induced by DNA damage during S phase (*31*). Moreover, previous reports demonstrated that sliding clamp proliferating cell nuclear antigen (PCNA) forms a physical interaction with Hjc and promotes Holliday junction cleavage activity of Hjc *in vitro* (*35*). Thus, our observation of incomplete recombination *in vitro* suggests that some endogenous factors are involved in the Cre recombinase reaction to resolve Holliday intermediates.

Protein transduction displays several characteristic qualities for the targeting of cells: (i) it is non-mutagenic for genomic DNA; (ii) the effect is transient (rapid incorporation and clearance) and (iii) there is a high transduction efficiency. Therefore, these techniques could overcome the hazardous limitations of conventional methods, such as transfection of plasmid DNA and infection of viral vectors. The induction of biological activities through protein transduction should be developed as a therapeutic technique for various diseases, as well as for gene therapy and regenerative medicine.

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