Full Paper

Measurements of transposition frequency of insertion sequence IS1 by GFP hop-on assay

Takashi Saito,¹ Taku Chibazakura,² Kiwamu Takahashi,^{2,†} Hirofumi Yoshikawa,² and Yasuhiko Sekine^{1,*}

¹ Department of Life Science, College of Science, Rikkyo (St. Paul's) University, Toshima-ku, Tokyo 171-8501, Japan

² Department of Bioscience, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156-8502, Japan

(Received November 18, 2009; Accepted November 30, 2009)

Transposons play a significant role in the evolution of bacterial genomes. Quantifying frequency of transpositional events caused by a transposon will facilitate understanding its role. Here, we report successful measurement of the frequency of IS1 transposition using “GFP hop-on assay” in which transposition-dependent GFP expression is monitored by FACS. This assay allows easy assessment of IS transposition into the chromosomal DNA on a single-cell scale; this is an advantage over other conventional methods to measure transposition frequency.

Key Words—*Escherichia coli*; fluorescence-activated cell sorting (FACS); green fluorescent protein (GFP); H-NS; insertion sequence; IS1; transposition

Introduction

Many assays have been developed to detect transposition events in bacterial cells. One common assay is to monitor the translocation of a transposon-encoded marker, for example an antibiotic resistance determinant, to a target plasmid. To detect transposition product, the transposon donor and target replicons are separated by transfer of a conjugative target plasmid to a new host in “mating-out assay” (for a review, see Craig, 1996), or by introduction of a target plasmid to a new host by use of electroporation in “intermolecular transposition assay” (Sekine et al., 1997). In these assays the target replicons are plasmids, whereas the chromosome is the target for transposition in

“papillation assay” (Huisman and Kleckner, 1987). The plasmid for papillation assay carries a transposon containing a truncated *lacZ* gene that lacks transcription and translation start signals; hence, β -galactosidase is not expressed at the original location in the plasmid. Transposition of the transposon into the chromosome of *Escherichia coli lacZ*[−] cells would lead to productive fusions in which the *lacZ* gene within the transposon is expressed from external chromosomal signals, resulting in the emergence of blue (LacZ⁺) papillae inside of white (LacZ[−]) colonies on agar plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). The length of time required to see blue papillae and the number of the papillae appearing in a white colony reflects the transposition frequency of the transposon within the colonies. Considering that transposition frequency and target site selection are influenced by various aspects related to chromosome dynamics, such as termination of chromosome replication (Peters and Craig, 2000), papillation assay to monitor transposition into chromosomal sites is a powerful tool for transpositional analysis. However, the assay can be applied only to cells growing on a solid media; this is one critical limitation of the assay.

* Address reprint requests to: Dr. Yasuhiko Sekine, Department of Life Science, College of Science, Rikkyo (St. Paul's) University, Nishi-Ikebukuro, Toshima-ku, Tokyo 171-8501, Japan.

† Present address: R&D Department, SBI ALApromo Co., Ltd., Roppongi, Minato-ku, Tokyo 106-6018, Japan.

Tel: +81-3-3985-3408 Fax: +81-3-3985-3408

E-mail: ysekine@rikkyo.ac.jp

In this paper, we report quantitative analysis of transpositional events into chromosomal sites in cells growing in a liquid media by use of “green fluorescent protein (GFP) hop-on assay,” which was originally developed for estimation of transposition in *Bacillus subtilis* cells (Takahashi et al., 2007). In this assay, fluorescent cells resulting from transpositional events can be detected by fluorescence-activated cell sorting (FACS). We choose IS1, an active transposon resident in the chromosome of *E. coli*, and successfully assessed IS1 transposition activity by this assay method. We characterized the IS1 transposition product to prove the adequacy of the assay system.

Materials and Methods

Bacterial strains and media. The strains used were *E. coli* K12 derivatives YS26 and YS27, which are derivatives with $\Delta(srl-recA)306::Tn10$ (Tet^r) from CSH26 ($\Delta(pro-lac) ara thi$) and CU211 (CSH26 $\Delta hns::Km^r$; obtained from C. Ueguchi), respectively. These strains were constructed by P1 transduction to tetracycline resistance using a P1 lysate grown on MV1184 (Vieira and Messing, 1987). L broth and L-rich broth (Yoshioka et al., 1987) were used. Antibiotics were used, when necessary, at a concentration of 100 μ g ampicillin (Wako)/ml and 30 μ g chloramphenicol (Wako)/ml.

Plasmid construction. Plasmid pSAT13 was constructed by assembly of three kinds of DNA fragments (DNA fragments 1–3) on pRS414 (Simons et al., 1987) as a backbone. DNA fragment 1 contains the GFP-hopper consisting of two copies of inverted repeat sequence (26 bp) in the right terminal region (IRR) of IS1 separated by the DNA segment of the *gfpmut2* gene encoding a FACS-optimized mutant of GFP, whose peak excitation wavelength is 488 nm. The *gfpmut2* gene was obtained from pKEN2-GFPmut2 (Cormack et al., 1996), and its promoter sequence and the initiation codon were eliminated. Note that there is no stop codon in the *gfpmut2* reading frame within the preceding IRR sequence. The *EcoRI-SalI* fragment containing the *lac* operon in pRS414 was replaced by DNA fragment 1. DNA fragment 2 contains the IS1 segment (nt 2–753; according to coordinates [1–768] given to the nucleotide sequence of IS1 [Ohtsubo and Ohtsubo, 1978]) covering the transposase gene and its promoter sequence. This fragment was obtained from a derivative of pSEK117 (Sekine et al., 1992) carrying IS1, in which the A₆C sequence was changed to

GA₂GA₃C, leading to production of transposase without translational frameshifting (Sekine and Ohtsubo, 1989) and no change of the amino acid sequence of transposase. DNA fragment 3 contains the sequence required for replication of plasmid p15A, and corresponds to nucleotide positions 583–1705 defined by coordinates given to pSTV28 (1–2999, TaKaRa). The *BspHI-SalI* fragment containing the sequence required for replication of plasmid pBR322 in pRS414 was replaced by DNA fragments 2 and 3. Plasmid pSAT13 Δ tnp, a derivative of pSAT13 without the transposase gene, was constructed by elimination of fragment 2 from pSAT13. Plasmid pBUN40, a derivative of pSAT13 without the GFP-hopper was constructed by replacement of the GFP-hopper in pSAT13 with the *lacZ*-hopper (consisting of IRR-*lacZ*-IRR) originally designed for the papillation assay. Details of procedures to construct these plasmids will be sent on request.

Detection of GFP-hopper transposition. YS26 and YS27 were transformed by pSAT13, pBUN40, or pSAT13 Δ tnp. The transformants were grown overnight at 37°C in L broth. The cultures were then diluted 1,000-fold with fresh medium and grown at 37°C to an optical density at 600 nm of 0.5. Cells were harvested by centrifugation and then suspended in phosphate-buffered saline (PSB) at an optical density at 600 nm of 1.0. Flow cytometry was performed on a FACSCalibur cytometer using CELLQUEST software (BD Bioscience). Cells were gated according to the forward scatter intensity (FSC-H) and side scatter intensity (SSC-H), compared with the PBS control as shown in Fig. 1. GFP fluorescence was detected with a FL1 detector; its intensity was expressed as an arbitrary logarithmic value (FLH-1) within 10⁰–10⁴.

Determination of the insertion site of the GFP-hopper. A portion of the culture of YS26 harboring pSAT13 used for FACS was plated on an L agar plate containing ampicillin and incubated at 37°C, and fluorescent colonies were selected. To segregate pSAT13 from the fluorescent cells, pACYC184, which is incompatible with pSAT13, was introduced, and chloramphenicol-resistance (Cm^r) and ampicillin-sensitive (Ap^s) transformants were obtained. Chromosome DNA was extracted by the CTAB method (Murray and Thompson, 1980) from the Cm^r Ap^s cells, and was digested with *Asel*, followed by treatment with T4 DNA ligase. (Note that there is no recognition site for *Asel* in the sequence of the GFP-hopper.) Polymerase chain reaction (PCR) was performed using the DNA preparation as template

Fig. 1. Structure of pSAT13.

pSAT13 carries the GFP-hopper, which consists of two copies of the IRR sequence (26 bp) (open box with filled triangle) of IS1 separated by the 0.7-kb DNA segment containing the *gfpmut2* gene lacking transcription and translation start signals. Four tandem copies of the strong transcriptional terminator, T1, of the *E. coli rrmB* operon is present upstream of the GFP-hopper to eliminate fortuitous readthrough transcription. pSAT13 carries the ampicillin-resistance gene (Ap^r) and the transposase (*tnp*) gene of IS1, in which the A_6C sequence, the site of translational frameshifting to produce transposase (Sekine and Ohtsubo, 1989), was changed to GA_2GA_3C , leading to production of transposase without frameshifting. Transcription of the transposase gene occurs from a promoter, P_{IS1} (filled triangle), present in the left-terminal region containing IRR. The origin of replication for p15A is shown by a filled circle.

and a pair of primers P1 (5'-GCATTGAAGACCATAC GCG-3') and P2 (5'-GGAAGCGTTCAACTAGCAG-3'). The DNA sequences of the PCR-amplified fragments were determined with primer P2 or P3 (5'-GTGCCCCA TTAACATCACCATC-3').

Results and Discussion

Development of the GFP hop-on assay

IS1 is an insertion element first identified in *Escherichia coli*. IS1 (768 bp) has imperfect terminal inverted repeat sequences (IRs), IRL and IRR of about 30 bp (Ohtsubo and Ohtsubo, 1978). IS1 transposes by the action of its transposase to a new site and generates a duplication of a target sequence of 9 bp (in most cases) or 8 bp (in some cases) (Galas et al., 1980; Grindley, 1978; Iida et al., 1985). Elements similar to IS1 in *E. coli* are widely distributed not only in eubacteria but also in Archaea (Ohta et al., 2002). To develop an assay that allows the quantitative assessment of IS1 transposition into chromosome, we constructed the GFP hop-on assay plasmid pSAT13 (Fig. 1), which

carries the IS1 transposase gene expressed from its own promoter sequence as well as an IS1 derivative containing a gene for GFP, namely the "GFP-hopper." It contains the *gfpmut2* gene, which lacks transcriptional and translational start signals and is located between two copies of IRR in IS1. The gene in the GFP-hopper is not expressed at its original location in pSAT13. Transposition of the GFP-hopper, which is provoked by transposase, into an expressed gene in the *E. coli* chromosome in the correct orientation and reading frame can lead to fusion of the expressed gene to the *gfpmut2* gene, resulting in the production of a green fluorescent fusion protein. Fluorescent cells resulting from such transpositional events can be detected by FACS.

Detection of transposants of the GFP-hopper as fluorescent cells

First, to distinguish between bacterial cells and the other particles originating from the PBS used, we performed flow cytometry using PBS only. The result was plotted on a graph where SSC-H and FSC-H of each event are indicated on X and Y axes, respectively (Fig. 2A, left panel). SSC-H represents the complexity of structure inside the particle, while FSC-H represents the size of the particle. We then set region R1, in which there were few plots (0.13% of total events). Next, we performed flow cytometry using the *E. coli* strain harboring pBUN40, which lacks the GFP-hopper as a negative control. Many events were detected in region R1 (Fig. 2B, left panel), showing that these events represented *E. coli* cells detected. We examined the GFP fluorescence intensity of the events within region R1, and found that almost all events showed a fluorescence intensity less than 3.3×10^0 FL1-H (Fig. 2B, middle panel). Therefore, we established the definition that fluorescent events under 3.3×10^0 FL1-H include background in this detection system.

We carried out the same assay using the *E. coli* strain harboring pSAT13. Among the events within region R1, significant events exceeded the background in fluorescence intensity. We set region R2 to collect events of which fluorescence intensity is more than 3.3×10^0 FL1-H, and such events were found to account for 0.3% of the total events within region R1 (Fig. 2C, middle panel). Deletion of the IS1 transposase gene from pSAT13 resulted in a remarkable decrease of events within region R2 (Fig. 2D, middle panel), demonstrating that the appearance of fluorescent cells de-

Fig. 2. Flow cytometric analysis of GFP-hopper transposition. *E. coli* cells suspended in PBS were analyzed by FACS. Samples used are PBS without cells (A), *E. coli* strain YS26 harboring pBUN40 (B), pSAT13 (C), pSAT13Δtnp (D), and *E. coli* strain YS27 harboring pSAT13 (E). Left panel: SSC-H and FSC-H of events are indicated on the X axis and the Y axis, respectively. Middle panel: Green fluorescence intensities (FL1-H) and FSC-H of events are indicated on the X axis and the Y axis, respectively. Only events which were gated by region R1 are shown, except for panel A where total events, which were not gated, are shown. Experiments were done in triplicate and representative results are shown. Numbers of events within region R1 and R2 are shown on the right side of the figure.

depends on the IS1 transposase. These results strongly suggest that the generation of fluorescent cells was the consequence of GFP-hopper transposition events. Thus, the transposition frequency of the GFP-hopper was estimated as the ratio of the number of events

within region R2 to that within region R1, and to be $2.78 \pm 0.23 \times 10^{-3}$ (Table 1). Fluorescence intensity detected from probable transposants generated from *E. coli* cells harboring pSAT13 varied from 3.3×10^0 to 2.0×10^2 FL1-H (Fig. 2C, middle panel). This was

Table 1. GFP-hopper transposition frequency.

Host plasmid	<i>hns</i> ⁺ pSAT13	<i>hns</i> ⁺ pSAT13Δtnp	<i>hns</i> ⁺ pBUN40	<i>hns</i> [−] pSAT13
Frequency ^a	2.8±0.24×10 ^{−3}	7.7±7.2×10 ^{−6}	1.8±1.2×10 ^{−5}	4.7±2.3×10 ^{−5}

^a The frequency of transposition of the GFP-hopper was calculated from triplicate experiments as the ratio of the number of events within region R2 to that within region R1.

probably caused by a variety of expression levels of the gene on which the GFP-hopper inserts.

A nucleoid-associated DNA binding protein H-NS is required for transposition of IS1 (Shiga et al., 2001). We examined the effect of *hns*-deficiency on IS1 transposition by the GFP hop-on assay. We introduced pSAT13 into *hns*-deficient strain YS27, and then performed the assay using this strain. The number of fluorescent cells was dramatically decreased (Fig. 2E, middle panel and Table 1) compared with that detected in the isogenic *hns*-proficient strain harboring pSAT13. This result confirms that the generation of fluorescent cells was the consequence of GFP-hopper transposition events.

Determination of the insertion site of the GFP-hopper

To verify the propriety of the GFP hop-on assay, we examined insertion sites of the GFP-hopper in several transposants appearing as fluorescent cells generated from YS26 harboring pSAT13. Analysis of a transposant by the inverse PCR method (see MATERIALS AND METHODS) revealed that the GFP-hopper inserted into the coding region of the *pgaB* gene that encodes poly-β-1,6-*N*-acetyl-D-glucosamine *N*-deacetylase involved in biofilm formation (Wang et al., 2004). This insertion caused in-frame fusion between the *pgaB* gene and the *gfpmut2* gene, resulting in N-terminal addition of 334 amino acids to GFP. A 9-bp sequence, 5'-GAT TACGTT-3', appeared on both sides of the inserted GFP-hopper. This shows that the GFP-hopper actually transposed by the action of IS1 transposase and duplicated the sequence.

Comparison with other methods

The fundamental principle of the GFP hop-on assay is similar to that of the papillilation assay in which, however, papillae inside of bacterial colonies on agar plates indicate transposition activity. This means that this assay can be applied only to cells grown on solid media. On the other hand, since the GFP hop-on assay facilitates the detection of transposition-dependent

GFP expression in a single cell, there is no restriction regarding forms of culture media. Cells grown in liquid media may be suitable for investigation of the influence of cell growth phase on transposition, for instance.

An advantage of the GFP hop-on assay over conventional methods, mating-out assay and intermolecular transposition assay, is that the target for transposition is chromosome DNA instead of plasmid DNA. This allows investigation of a host mutation which disturbs the stability or mobility of the plasmids. It was reported that change of the local and global structure of the chromosome induced by binding of nucleoid proteins or chromosomal DNA replication affects target site selection of transposons (Peters and Craig, 2000; Swingle et al., 2004). The GFP hop-on assay system represents a powerful tool for a better understanding of the roles of chromosome architecture on transpositional recombination.

Acknowledgments

We thank Shuhei Matsuyama and Yuki Okoji for their technical assistance. This work was supported by the Frontier Project "Adaptation and Evolution of Extremophiles" from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a Grant-in-Aid for Creative Scientific Research (17GS0314 to Y.S.) from the Japan Society for the Promotion of Science.

References

- Cormack, B. P., Valdivia, R. H., and Falkow, S. (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene*, **173**, 33–38.
- Craig, N. L. (1996) Transposition. In *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology, ed. by Neidhardt, F.C., Curtiss III, R., Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., Schaechter, M., and Umberger, H. E., American Society for Microbiology, Washington, DC, pp. 2339–2362.
- Galas, D. J., Calos, M. P., and Miller, J. H. (1980) Sequence analysis of Tn9 insertions in the *lacZ* gene. *J. Mol. Biol.*,

- 144, 19–41.
- Grindley, N. D. F. (1978) IS1 insertion generates duplication of a nine base pair sequence at its target site. *Cell*, **13**, 419–426.
- Huisman, O. and Kleckner, N. (1987) A new generalizable test for detection of mutations affecting Tn10 transposition. *Genetics*, **116**, 185–189.
- Iida, S., Hiestand-Nauer, R., and Arber, W. (1985) Transposable element IS1 intrinsically generates target duplications of variable length. *Proc. Natl. Acad. Sci. USA*, **82**, 839–843.
- Murray, M. G. and Thompson, W. F. (1980) Rapid isolation of high-molecular-weight plant DNA. *Nucleic Acids Res.*, **8**, 4321–4325.
- Ohta, S., Tsuchida, K., Choi, S., Sekine, Y., Shiga, Y., and Ohtsubo, E. (2002) Presence of a characteristic D-D-E motif in IS1 transposase. *J. Bacteriol.*, **184**, 6146–6154.
- Ohtsubo, H. and Ohtsubo, E. (1978) Nucleotide sequence of an insertion element, IS1. *Proc. Natl. Acad. Sci. USA*, **75**, 615–619.
- Peters, J. E. and Craig, N. L. (2000) Tn7 transposes proximal to DNA double-strand breaks and into regions where chromosomal DNA replication terminates. *Mol. Cell*, **6**, 573–582.
- Sekine, Y., Izumi, K., Mizuno, T., Mizuno, T., and Ohtsubo, E. (1997) Inhibition of transpositional recombination by OrfA and OrfB proteins encoded by insertion sequence IS3. *Genes Cells*, **2**, 547–557.
- Sekine, Y., Nagasawa, H., and Ohtsubo, E. (1992) Identification of the site of translational frameshifting required for production of the transposase encoded by insertion sequence IS1. *Mol. Gen. Genet.*, **253**, 317–324.
- Sekine, Y. and Ohtsubo, E. (1989) Frameshifting is required for production of the transposase encoded by insertion sequence 1. *Proc. Natl. Acad. Sci. USA*, **86**, 4609–4613.
- Shiga, Y., Sekine, Y., Kano, Y., and Ohtsubo, E. (2001) Involvement of H-NS in transpositional recombination mediated by IS1. *J. Bacteriol.*, **183**, 2476–2484.
- Simons, R. W., Houman, F., and Kleckner, K. (1987) Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene*, **53**, 85–96.
- Swingle, B., O'Carroll, M., Haniford, D., and Derbyshire, K. M. (2004) The effect of host-encoded nucleoid proteins on transposition: H-NS influences targeting of both IS903 and Tn10. *Mol. Microbiol.*, **52**, 1055–1067.
- Takahashi, K., Chibazakura, T., Sekine, Y., and Yoshikawa, H. (2007) Development of a new "GFP hop-on assay" system for insertion sequence transposition in *Bacillus subtilis* 168 using IS4Bsu1 from *B. subtilis* (natto). *Biochem. Biophys. Res. Commun.*, **355**, 426–430.
- Vieira, J. and Messing, J. (1987) Production of single stranded plasmid DNA. *Methods Enzymol.*, **153**, 3–11.
- Wang, X., Preston, J. F., III, and Romeo, T. (2004) The *pgaABCD* locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. *J. Bacteriol.*, **186**, 2724–2734.
- Yoshioka, Y., Ohtsubo, H., and Ohtsubo, E. (1987) Repressor gene *finO* in plasmid R100 and F: Constitutive transfer of F is caused by insertion of IS3 into *finO*. *J. Bacteriol.*, **169**, 619–623.