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Random insertion of injected DNA molecules into the macronuclear chromosome of *Paramecium caudatum*

Yasuhiro Takenaka[1,2], Akira Yanagi[3], Hiromi Masuda[4], Youji Mitsui[5], Hiroshi Mizuno[1] and Nobuyuki Haga[3,*]

[1]Department of Biochemistry, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan, [2]Health Technology Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8569, Japan, [3]Department of Biotechnology, Ishinomaki Senshu University, Ishinomaki, Miyagi 986-8580, Japan, [4]VALWAY Technology Center, NEC Soft, Ltd., 1-18-7, Koto-ku, Tokyo 136-8627, Japan, [5]Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences at Kagawa Campus, Tokushima Bunri University, Kagawa 769-2193, Japan

Key words: genome integration, transformation, microinjection, plasmid rescue, telomere

SUMMARY

Linear DNA molecules injected into the macronucleus of *Paramecium caudatum* became integrated into genomic DNA. The plasmid harboring fusion gene of *P. caudatum* histone H2B and a yellow fluorescent protein (YFP) named PcVenus was linearized and microinjected into the macronucleus of *P. caudatum*. Southern blots of total cellular DNA from three fluorescence-positive transformant clones probed with the PcVenus sequence revealed that the injected DNA had become randomly inserted into the chromosome. Free linear monomers or multimers of injected DNA molecules as seen in *P. primaurelia* and *P. tetraurelia* were not evident even though the plasmid possessed extant telomeric repeats at its both extremities. We also cloned fragments containing the integration site of genomic DNA from transformant cells by plasmid rescue. Sequence analysis of the flanking DNA confirmed random insertion of the linear plasmid into the chromosomal DNA with extant telomere repeats at the fusion junction. Therefore, *P. caudatum* maintains introduced DNA in a unique manner by non-homologous or illegitimate, rather than homologous recombination.

*Corresponding author
Tel: +81-225-22-7716
Fax: +81-225-22-7746
E-mail: haga@isenshu-u.ac.jp
Received: 1 May 2008; Accepted: 31 July 2008.
Note: The sequences described herein have been deposited in the DDBJ/EMBL/GenBank databases (accession numbers AB195237 and AB195239).

INTRODUCTION

The ciliated protozoan, *Paramecium caudatum*, possesses both a micronucleus and a macronucleus. The micronucleus is considered to have a germinal function because it divides mitotically and undergoes meiosis during conjugation. On the other hand, the macronucleus divides amitotically and is transcriptionally active, implying a somatic function. The macronucleus is highly polygenomic (n = 3,400) (Soldo et al., 1981) and is formed *de novo* after meiotic events by excising internal eliminated sequences (IESs) from micronuclear DNA and ligating fragmented DNA. Exogenous DNA molecules microinjected into the macronucleus of *Paramecium* replicated and were maintained in the macronucleus. Both the integration of injected DNA into host chromosomal DNA and independent replication of injected DNA as an episome in *Paramecium* have been reported. Injected DNA molecules are randomly inserted into the chromosomal DNA of *P. primaurelia* and *P. tetraurelia* (Katinka and Bourgain, 1992; Haynes et al., 1995), both of which can also harbor episomal forms of microinjected plasmid DNAs that autonomously replicate in the macronucleus (Godiska et al., 1987; Gilley et al., 1988; Bourgain and Katinka, 1991). However, the fate of DNA molecules microinjected into *P. caudatum* remained to be determined.

We constructed the expression vector, pTubtel3 (pTT3), that constitutively expresses a target protein in *P. caudatum* (Takenaka et al., 2002). We then created a codon-optimized and a superenhanced yellow fluorescent protein, PcVenus, by altering 8 amino acid residues of codon-optimized green fluorescent protein (GFP). PcVenus was expressed as a fusion protein with *P. caudatum* histone H2B to localize it in macro- and micronuclei (Takenaka et al., 2007). The stable expression of the histone H2B-PcVenus in *P. caudatum* was monitored for more than 300 successive divisions of each transformant clone.

We describe herein how the injected expression vector, pTT3 H2B-PcVenus, was maintained in the macronucleus of *P. caudatum*. Genomic Southern blots of 3 transformant clones that underwent 50-100 fissions after injection revealed that the injected DNA had randomly inserted into the chromosome, and free linear monomers or multimers of the injected DNA molecules were undetectable. We also confirmed the non-homologous and random insertion of injected DNA by sequencing insertion boundaries.

MATERIALS AND METHODS

Cell culture and microinjection

Strain KoscA4 [the trichocyst non-discharge, syngen 3 (O-type) mutant (*tnd2*/*tnd2*)] was produced by cross breeding (A. Yanagi, unpublished data). The culture medium and conditions were as described (Takenaka et al., 2002). The construction and microinjection of the *P. caudatum* histone H2B -PcVenus expression vector, pTT3 H2B-PcVenus, have been described elsewhere (Takenaka et al., 2007). Plasmid DNA (100 μg) was linearized by *Bam* HI digestion, purified by phenol/chloroform extraction, precipitated with isopropanol, and resuspended in sterile distilled water at a final concentration of 1 μg/μl. Approximately 1.5×10^5 copies in 10 pl of linearized plasmid DNA were microinjected into the macronucleus of KoscA4 cells at the stationary phase. Cells that remained viable after microinjection (about 30%) were isolated into 100 μl of culture medium in depression slides.

Southern blotting

Genomic DNA (0.8-1.4 μg) was prepared using a DNeasy Tissue Kit (QIAGEN) from transformed cells that underwent 50-100 fissions after injection. Nuclear DNA was digested with one or two restriction enzymes, resolved by electrophoresis on 0.7% TAE-agarose gels and transferred to Hybond N+ nylon membranes (GE Healthcare).

The membrane was hybridized with a randomly labeled full-length PcVenus probe in 5x SSPE, 5x Denhardt's solution, 0.5% SDS, 20 μg/ml salmon sperm DNA overnight at 42°C. The membranes were washed twice with 2x SSC, 0.1% SDS for 15-30 min at 65°C and exposed to BioMax MS film (Kodak).

Cloning of the integrated site on *P. caudatum* genome

Genomic DNA (5 μg) from the transformant PcV1 expressing histone H2B-PcVenus was digested with *Afl* II, extracted with phenol and precipitated with ethanol. Genomic DNA (~ 0.5 μg) was self-ligated with Ligation High (Toyobo) at a low DNA concentration to foster intramolecular circularization and transformed into TOP10 (Invitrogen) using an E. coli Pulser (Bio-Rad). Ampicillin-resistant positive clones were selected on LB plates containing carbenicillin (50 μg/ml). Twenty-six colonies were screened by colony PCR with KOD Dash® DNA polymerase (Toyobo) using the GFP-specific primers GFP RT-UP1 (5'-GCTGAAGTCAAGTTTGAAGGTGATA-3') and GFP RT-LP1 (5'-AGCTGTTACAAACTCAAGA AGGAC-3'). The plasmid was purified from 8 positive colonies using QIAprep Spin Miniprep Kit (Qiagen) and then sequenced using the dGTP BigDye Terminator Cycle Sequencing Kit and an ABI PRISM 3100 Gene Analyzer (Applied Biosystems).

RESULTS AND DISCUSSION

Southern blots of transformant clones

The expression vector pTT3 H2B-PcVenus (Takenaka et al., 2007) contained 350-bp subtelomeric sequences plus 41 (ampicillin resistance gene side) or 5 (PcVenus side) tandem TTGGGG repeats that originated from the *Tetrahymena* telomere fragment (Fig. 1A). The vector was digested with *Bam* HI to expose telomeric repeats at both ends and microinjected into the macronucleus of *P. caudatum*. Transformation was checked by microscopic observation of the codon-optimized yellow fluorescent protein (PcVenus) fused at C-terminus of *P. caudatum* histone H2B. The fusion protein was specifically localized in the macro- and micronucleus of transformant cells (Fig. 1B and C). To clarify how injected DNA is maintained in the macronuclear genome of *P. caudatum*, we performed Southern blot analyses of genomic DNA isolated from transformant cells that underwent 50 - 100 fissions after microinjection. Undigested or *Bam* HI-digested total cellular DNA from 4 transformant clones (PcV1-PcV4) and 1 untransformed clone was Southern blotted with a PcVenus specific probe (PcV probe) comprising a 726-bp PCR fragment spanning the entire sequence of the PcVenus gene (Fig. 1A, bar). Figure 1D (PcV1, lane Un) and E (PcV2 and PcV3, lanes Un) shows no predicted 5.2-kb band of linearized pTT3 H2B-PcVenus, indicating that the injected DNA was not maintained as an episome in the macronucleus. Genomic DNA cut with *Bam* HI and hybridized with the PcV probe resulted in a large, homogeneous, high molecular weight smear without any discrete band (Fig. 1D: PcV1, lane Bm; Fig. 1E: lanes Bm). Therefore, injected linear expression vectors did not form concatemers by ligating their *Bam* HI ends in the nucleus, but were randomly integrated into chromosome DNA. Hybridization signals from PcV2 and PcV3 were weak, but similar to those of PcV1 upon longer exposure (Fig. 1E), whereas PcVenus was undetectable in untransformed cells and in PcV4 (Fig. 1D and E). The copy numbers of the retained expression vector in PcV1 calculated using the signal intensity of digested pTT3 H2B-PcVenus vector as a standard ranged from 30,000 - 70,000 per cell. The copy numbers estimated in our experiment were similar to those of *P. tetraurelia* (Gilley et al., 1988) and of *P. primaurelia* (Bourgain and Katinka, 1991).

We next clarified whether an end-to-end fusion occurred between injected DNA and the chromosome. If the integrated vector was located close to

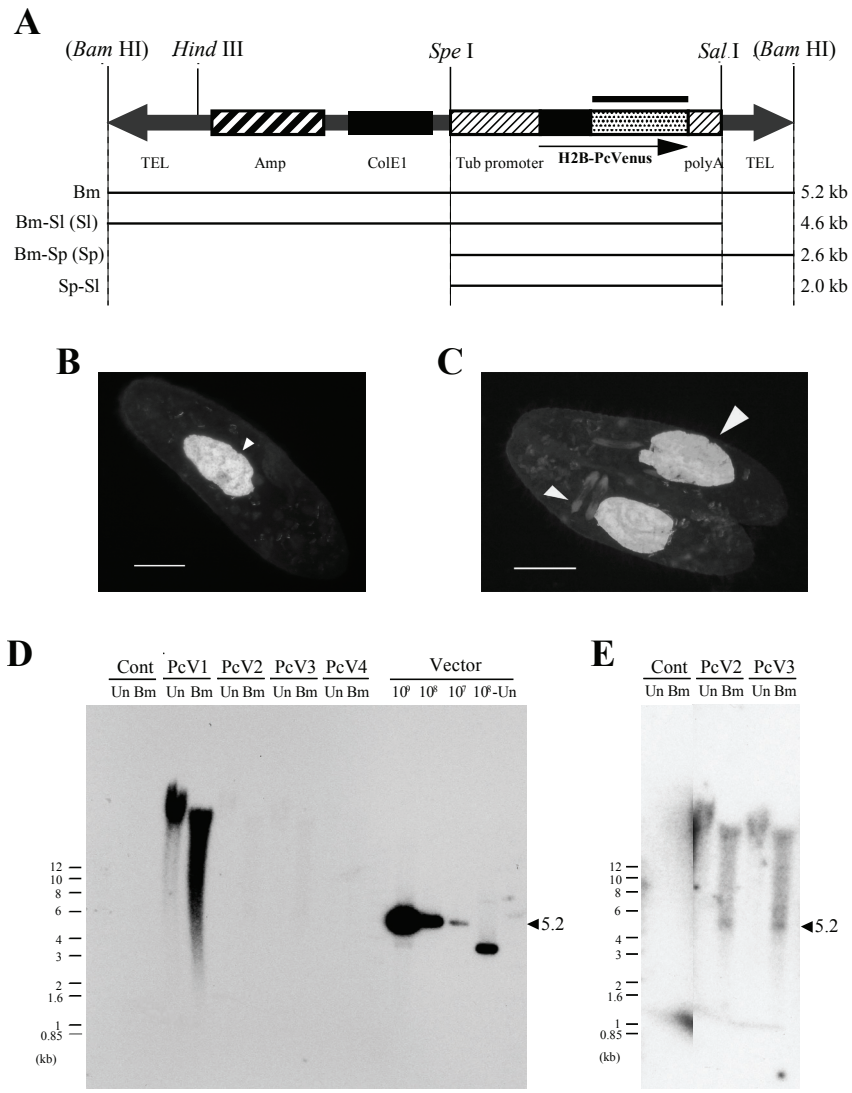

Fig. 1. Transformation of *P. caudatum* with pTT3 H2B-PcVenus vector and Southern blots of transformants. (A) Map of pTT3 H2B-PcVenus digested with *Bam* HI before microinjection to expose telomere sequences (TEL) at both ends. Solid bar indicates position of PcV probe. *Bam* HI (Bm), *Bam* HI-*Sal* I (Bm-Sl), *Bam* HI-*Spe* I (Bm-Sp) and *Spe* I-*Sal* I (Sp-Sl) restriction fragments of 5.2-, 4.6-, 2.6-, and 2.0-kb, respectively, are shown. (B) Fluorescent microscope photographs of cells expressing histone H2B-PcVenus. Arrowhead, the macronucleus. Bar, 50 µm. (C) Selfing conjugation of cells expressing histone H2B-PcVenus at about 12 hours after initiation of mating reaction. Large arrowhead, macronucleus; small arrowhead, micronucleus undergoing second meiotic division. Bar, 50 µm. These cells underwent about 100 fissions after microinjection of the pTT3 H2B-PcVenus expression vector. Photographs were taken under a fluorescent microscope (ZEISS AX10) with a digital camera (OLYMPUS E-330). (D) Restriction enzyme analysis of DNA from transformant clones (PcV1-PcV4) derived from cells injected with linearized pTT3 H2B-PcVenus vector and DNA derived from untransformed cells (Cont). Total cellular DNA was digested (Bm) or not (Un), blotted onto Hybond N+ membrane (Amersham Bioscience) and hybridized with PcV probe. Total DNA (1.4 µg) from 4 independently transformed clones is shown as PcV1-PcV4. Copy numbers in transformant clones were calculated based on signal strength of 10^9, 10^8, 10^7 *Bam* HI-digested, and 10^8 undigested (10^8-Un) pTT3 H2B-PcVenus vectors. Membranes were exposed to BioMax MS film (Kodak) for 3 h. (E) Same membrane as in (D) after exposure for 20 h. Signals from PcV2 and PcV3 became visible after longer exposure and were similar to those from PcV1. Molecular size markers are noted at left.

the chromosomal end, Southern blots of PcV1 genomic DNA digested by *Spe* I or *Sal* I, of which a recognition site exists between the PcVenus gene and chromosomal DNA, would result in the detection of a specific band upon hybridization with PcV probe (Fig. 2A). The results showed a smeared signal at a high molecular mass location (Fig. 2B, lanes Sp and Sl) with a very weak band around 2.6 (Sp) and 4.6 kb (Sl)(Asterisks in Fig. 2B), indicating that most of the vectors were not located close to the chromosomal end. Double digestion of genomic DNA by *Spe* I and *Bam* HI, or *Sal* I and *Bam* HI resulted in increased heterogeneous hybridization signals that were probably derived from DNA molecules at a higher molecular mass as shown in lane Sp or Sl, with a minor band (Fig. 2B, lanes Bm -Sp and Bm-Sl).

Digesting genomic DNA and the pTT3 H2B-PcVenus vector with *Spe* I and *Sal* I (Sp-Sl) or *Hin*d III and *Sal* I (Hd-Sl) resulted in discrete bands that co-migrated at the same location (2.0 or 4.0 kb, respectively; Fig. 2B). Digestion with these restriction enzymes generates a fragment that contains the α-tubulin expression cassette and histone H2B-PcVenus but lacks both telomere sequences. Therefore, integration occurred mainly in or close to the telomeric repeats of the injected vectors.

Direct cloning of the integration site

Although the Southern blots revealed that the injected DNA had not been concatenated by ligation at the *Bam* HI sites of the linearized vector (Fig. 1D), other types of concatenation cannot be ruled out. For example, if *Bam* HI-ends of the vector were trimmed by enzymes in the macronucleus after injection, and each molecule was ligated, such concatenation would still generate the same results. Therefore, we sequenced the DNA flanking the vector isolated from genomic DNA of PcV1. Southern blots of genomic DNA digested with *Hind* III and *Sal* I generated discrete signals at ~ 4.0 kb (Fig. 2B), suggesting that the ampicillin resistance

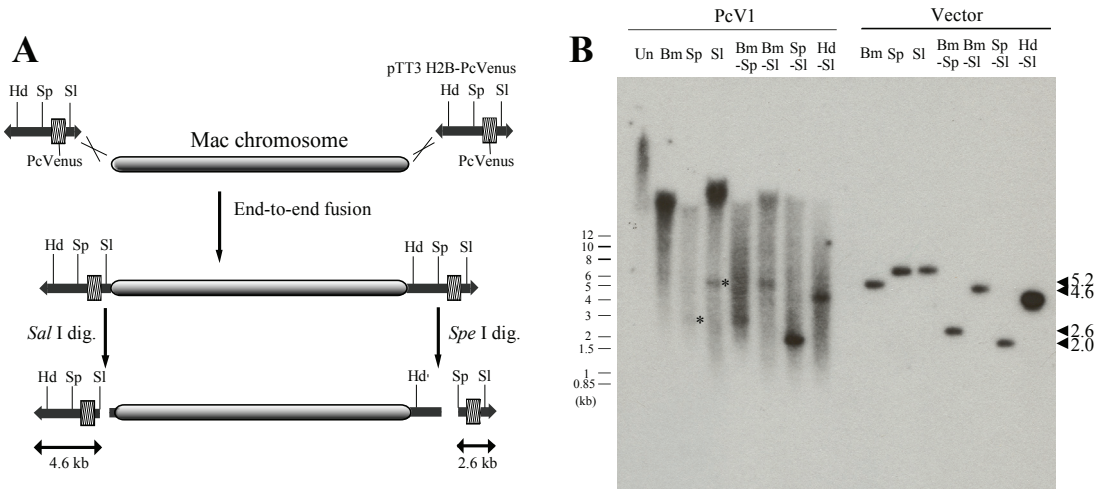

Fig. 2. (A) Speculative diagram of end-to-end fusion between injected pTT3 H2B-PcVenus and macronuclear chromosome. *Spe* I, *Sal* I, and *Hin*d III recognition sites are indicated by Sp, Sl, and Hd, respectively. Predicted length of fragments containing PcVenus after *Spe* I or *Sal* I digestion are 2.6 or 4.6 kb, respectively. (B) Additional Southern blots of genomic DNA (0.8 μg) from PcV1 and pTT3 H2B2-PcVenus plasmid (2 x 10^7) digested with *Bam* HI (Bm), *Spe* I (Sp), *Sal* I (Sl), *Bam* HI and *Spe* I (Bm-Sp), *Bam* HI and *Sal* I (Bm-Sl), *Spe* I and *Sal* I (Sp-Sl), *Hin*d III and *Sal* I (Hd-Sl). DNA was then resolved by electrophoresis, blotted and hybridized with PcV probe. Asterisks indicate minor 4.6-kbp *Sal* I and 2.6-kbp *Spe* I fragments that were probably generated by end-to-end fusion between macronuclear chromosomes and injected DNA molecules.

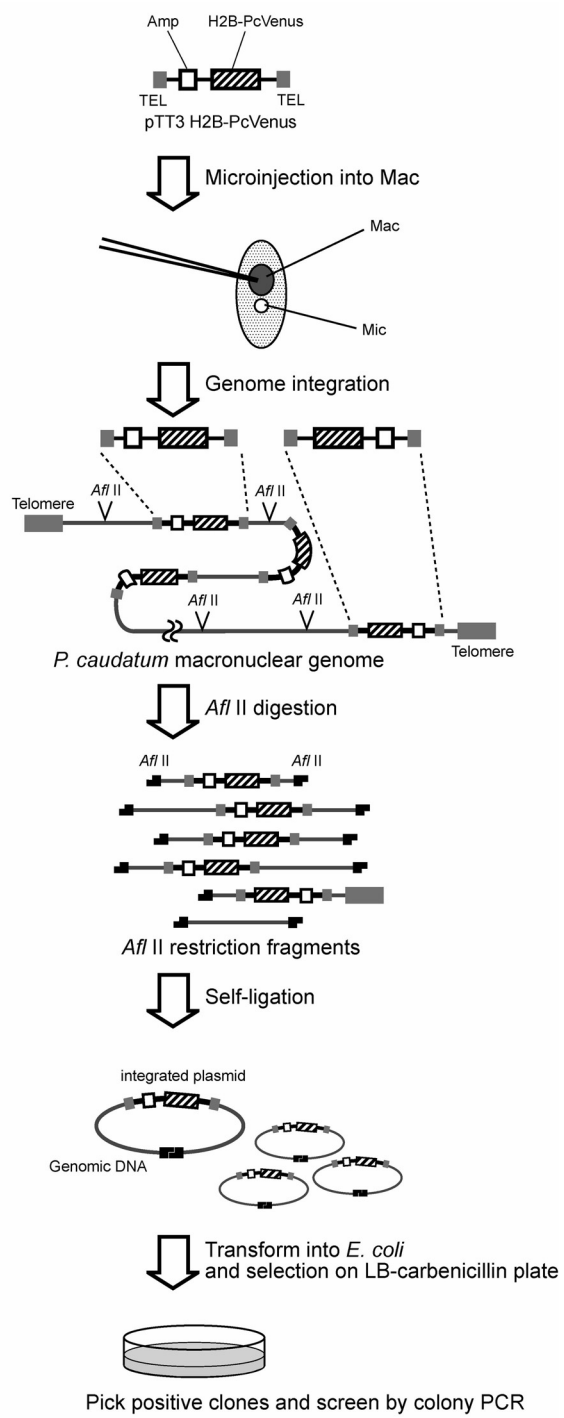

Fig. 3. Plasmid rescue procedure to isolate integration site of injected DNA and its flanking regions in transformant genomic DNA. Mic, micronucleus; Mac, macronucleus. Details are described in Materials and methods.

gene (Fig. 1A, Amp) and the replication origin (ColE1) on the pTT3 H2B-PcVenus vector were largely intact in the chromosome DNA of PcV1. Therefore, we isolated integrated plasmids with adjacent chromosomal DNA using a plasmid rescue method (Fig. 3). Genomic DNA of PcV1 was initially digested with *AfI* II which does not cut the pTT3 H2B-PcVenus sequence. The genomic DNA fragments were self-ligated and transformed into *E. coli* by electroporation. Transformants were then selected on LB-carbenicillin plates. We sequenced 8 *E. coli* clones, and confirmed that the DNA flanking the vector did not contain any sequences derived from the vector. A BLAST search of the flanking DNA resulted in no identity with any known DNA sequences. The vector retained the full or truncated telomeric repeats at the fusion junction in many *E. coli* clones (Fig. 4, left ends of clones 1 - 4, 7 and right ends of 3 - 7). Fusion points were found at subtelomeric or more inner regions of the vector in 4 *E. coli* clones (Fig. 4, left of clones 5 and 6; right of clones 1 and 2). Considering the results from the Southern blots and direct cloning of the fusion site, most of the injected vectors were non-specifically inserted into chromosomal DNA without any type of concatenation.

The telomeric repeats in *P. caudatum* consist of hundreds of base pairs and are elongated slowly during vegetative propagation (Takenaka et al., 2001). Although the injected linear DNA molecules contained telomeric repeats at both their extremities that telomerases could recognize as elongatable DNA ends, none of the *E. coli* clones contained telomeric repeats longer than those of the pTT3 H2B-PcVenus vector at the fusion junction (Fig. 4). This may be due to insertion of the introduced DNA molecules into the macronuclear chromosomes before telomere elongation by telomerases. Another explanation might be the lack of telomere binding proteins at the ends of the injected DNA molecules. Telomeres in ciliate, yeast, and mammalian comprise DNA-protein complexes, the core proteins of

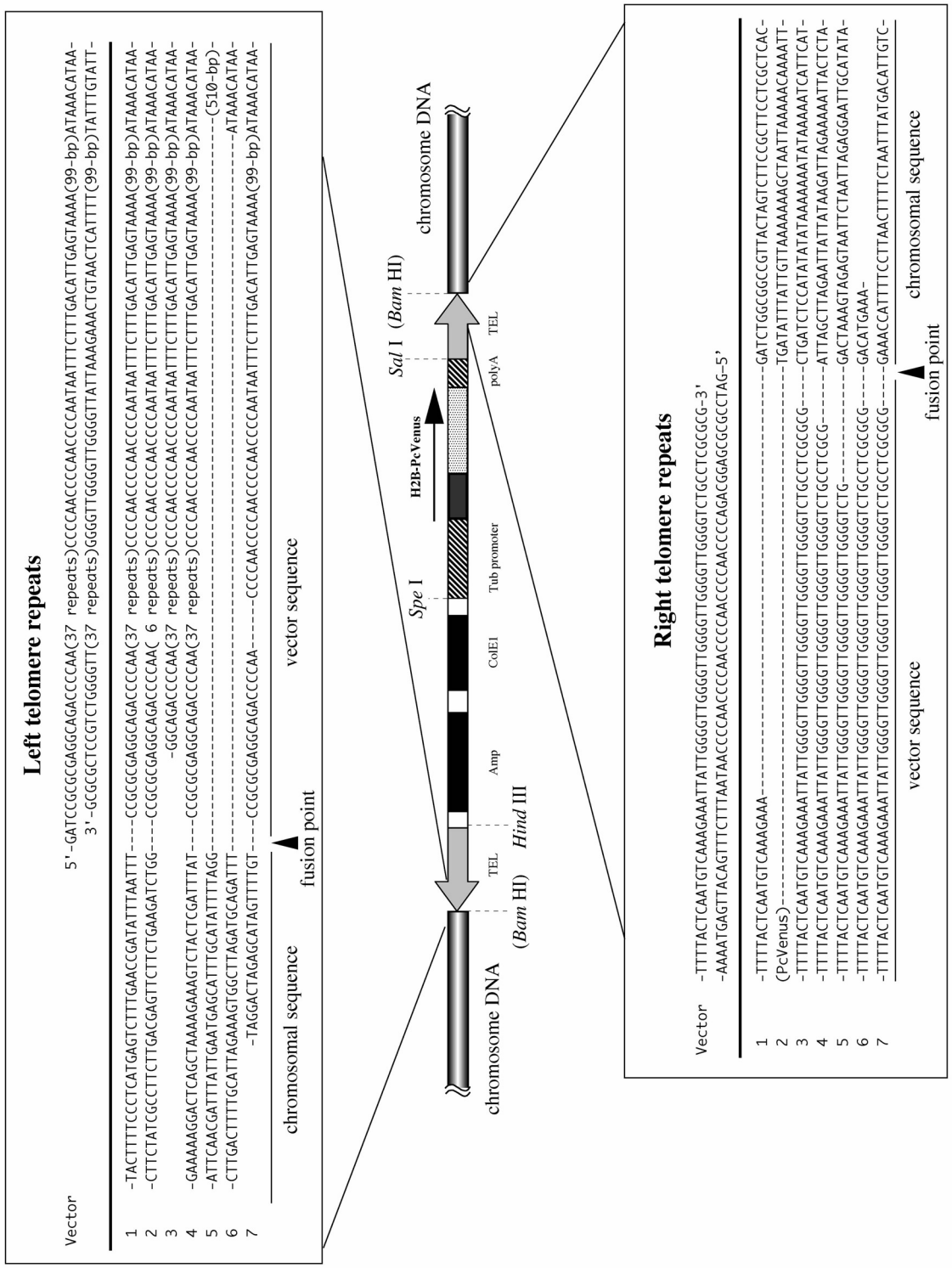

Fig. 4. Sequence alignment of genome integration site from total cellular DNA of PcV1. Numbers of *E. coli* clones are listed left of sequences. Boundary sequences obtained from 2 of 8 clones were identical. The hyphen (-) represents a gap in the alignment.

which directly bind to telomeric DNA to protect the chromosome ends. In yeast, the single-strand telomere binding protein, cdc13, and the telomerase accessory protein, Est1, function as co-mediators to tether telomerase to the telomere (Evans and Lundblad, 1999). Human POT1 and TPP1 that are structural homologues of TEBPα and TEBPβ (telomere end-binding protein) respectively, from the ciliate *Oxytricha nova*, are involved in telomerase processivity to the telomere (Wang et al., 2007). The *P. caudatum* macronucleus contains more than 100 chromosomes at a copy number of 3,400 (> 6.8 x 10^5 DNA ends). Thus, microinjection of ~ 1.5x10^5 copies of linearized pTT3 H2B-PcVenus vectors (~ 3.0 x 10^5 DNA ends) resulted in the abrupt emergence of exogenous DNA ends and the depletion of pre-existing telomere-associated proteins that are involved in the recruitment of telomerase to the telomere. If this were so, the injected vector would have been little, if any at all, telomerized due to poor interaction between telomerase and the naked telomere of the vector.

In *Tetrahymena*, DNA-mediated transformation has been achieved by autonomous replication of the ribosomal RNA (rDNA) vectors (Tondravi and Yao, 1986; Kahn et al., 1993; Cassidy-Hanley et al., 1997). The rDNA vectors contain replication origins and telomeres and exist as free linear palindromic dimers in the macronucleus (Bruns et al., 1985; Yu et al., 1988; Yu and Blackburn, 1989; Yu and Blackburn, 1990). When a circular or linear plasmid containing rDNA is microinjected (Tondravi and Yao, 1986), electroporated (Gaertig and Gorovsky, 1992), or bombarded with DNA-coated particles (Cassidy-Hanley et al., 1997) into *Tetrahymena*, they do replace the host rDNA due to a replication advantage. The results of our Southern and plasmid rescue experiments showed that no autonomous replication of free linear plasmid was evident.

Molecules of DNA without a *Paramecium* gene sequence or telomeric repeats microinjected into the macronucleus of *P. primaurelia* are mostly inserted into the chromosome by illegitimate recombination with a small proportion of free multimers (Katinka and Bourgain, 1992). Genome integration of injected DNA in *P. caudatum* resembles that observed in *P. primaurelia*, which evokes evolutionary insights into the conserved mechanism of recombination in *Paramecium* species. In *P. primaurelia*, however, the insertion sites were not completely random, but about 30% of the molecules were integrated next to or within interstitial telomere repeats, which were not identified by our sequencing of the insertion boundaries. Furthermore, we did not find any free multimers of injected DNA molecules in *P. caudatum*. The data presented here indicate that *P. caudatum* maintains introduced DNA in a unique manner because of (i) a tendency to randomly insert injected DNA into the macronuclear chromosomes by non-homologous or illegitimate recombination rather than by homologous recombination between *P. caudatum* genes of the injected DNA, (ii) the absence of free linear monomers or multimers of injected DNA fragments as found in other close species (Godiska et al., 1987; Gilley et al., 1988; Katinka and Bourgain, 1992) even when they possess extant telomeric repeats at both extremities, and (iii) the absence of insertion hot spots such as internal or interstitial telomeric repeats. The insertion mechanism of injected DNA molecules into *P. caudatum* genome requires further investigation to improve transformation efficiency.

ACKNOWLEDGEMENTS

This study was supported in part by Special Coordination Funds for the Promoting Science and Technology of the Ministry of Education, Culture, Sports, Science and Technology of Japan (H. Mizuno), matching research funds from NIAS & NEC Soft, Ltd. (H. Masuda and H. Mizuno), and funds

from the University of Tsukuba (Y. Takenaka and Y. Mitsui).

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