Review

The Achievement of Shuttle Vector Techniques in Mammalian Cell Mutation Research

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Received March 16, 2013; Revised April 20, 2013; Accepted April 22, 2013
J-STAGE Advance published date: July 1, 2013

Shuttle vector plasmids have made major contributions to the advancement of mutation research for the last two decades. I have been involved in the development of shuttle vector plasmids and their experimental protocols, and herein provide a chronicle of shuttle vector studies. In the late 1960s, *in vitro* mammalian cell culture became a common technique in cell biology. Geneticists established a method to detect gene mutation as 8-azaguanine or 6-thioguanine resistance after cells had been exposed to radiation or chemicals. The resistance of the cells was found to be due to a mutation of the hypoxanthine-guanine phosphoribosyl transferase gene; however, which base was changed in the gene remained difficult to determine until DNA sequencing techniques were developed. After the Sanger method of DNA sequencing became available to geneticists, the next issue was how to identify numerous base changes in mutants easily and rapidly. A breakthrough on this issue was the use of the shuttle vector plasmid pZ189, which was developed for practical applications by Seidman and colleagues. Along with the development of new molecular biology techniques such as polymerase chain reaction and automatic DNA sequencing, pZ189 has been modified to several forms as adaptations to the new techniques. These shuttle vector plasmids remain useful tools to reveal what types of mutations are induced by newly identified mutagens at the DNA sequence level. This article describes some of the contents of my JEMS award lecture in 2011.

Key words: mutation, shuttle vector plasmid, *supF*, pZ189, pMY189, pNY200

Introduction: A Chronicle of Methods in Mutation Research

In the early 1960s, *in vitro* culture of mammalian cells was established, although by then bacterial genetics had already been prospering for a long time. Scientists attempted to study genetics using the cultures of mammalian cells, as with bacteria, and mutation induction was first demonstrated as 8-azaguanine resistance by alkylating agents in Chinese hamster V79 cells (1). Sato,

Slesinski and Littlefield (2) measured the frequencies of 6-thioguanine resistant mutations induced by alkylating agents in cultured human lymphoblastoid cells. Subsequently, studies on 8-azaguanine- and 6-thioguanine-resistant mutations induced by various chemicals and types of radiations became common among geneticists who soon found that these mutations were induced in the hypoxanthine-guanine phosphoribosyl transferase gene (*hprt*). The development of the Sanger method of DNA base sequencing allowed the identification of mutated bases in the late 1970s (3,4); however, the method was very complex and laborious in terms of identifying the base changes of many mutants.

For the rapid identification of many mutations at the base sequence level, geneticists developed shuttle vector plasmids that carry mutation and selection marker genes and can replicate in both mammalian and *Escherichia coli* cells. One of the reliable and practical plasmids is pZ189, which was developed by Seidman *et al.* (5). The plasmid pZ189 has the bacterial *supF* gene as a mutation target, and the gene is about 160 bp, which is short enough to read all base sequences with a ³²P-label by one run on a polyacrylamide gel sequencing plate. *E. coli* with the *supF* gene mutated in human cells is easily selected as a colony on an agar plate. In this way, pZ189 constituted major progress in revealing the mutational spectra of various mutagens. The details of this are described in the next section.

The invention of the polymerase chain reaction (PCR) was a breakthrough in mutation research (6,7). Mutations of *hprt* cDNA of ethyl nitrosourea-induced 6-thioguanine-resistant cells were identified at the base sequence level with reverse transcriptase PCR (RT-PCR) and ³²P-labeling DNA sequencing methods (8). Yu *et al.* (9) identified base changes of the *hprt* gene by

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doi: org/10.3123/jemsge.2013.001

the multiplex PCR method. In the 1990s, automatic DNA sequencing was developed and adapted in genetic research (10,11). The combination of PCR and automatic DNA sequencing has promoted the molecular study of chemical and radiation mutagenesis up until the present. The shuttle vector plasmid pZ189 has also been improved with the progress of these new techniques, as described below.

In the 1990s, *in vivo* mutation assays using shuttle vector transgenic mice were established. The transgenic mice called Mutamouse and Big Blue mouse, possessing bacterial *lacZ* and *lacI* genes, respectively, can be treated with chemicals or radiation, and the mutation target transgenes can then be recovered from the mouse cells into plaques of bacteriophage λ (12,13). Nohmi *et al.* (14) also established a new transgenic mouse carrying the bacterial *gpt* Δ gene that can be recovered into bacteriophage λ . This *gpt* Δ assay system was adopted as the *in vivo* mutation assay in the OECD guidelines (15).

Development of the First Practical Shuttle Vector Plasmid pZ189

The shuttle vector plasmid pZ189 developed by Sedman *et al.* (5) was extensively characterized by his colleagues, namely, Razzaque A, Dixon K, Bredberg A, Seetharam S and Kraemer KH, who belonged to the laboratory of Molecular Carcinogenesis, National Cancer Institute, NIH. I was a member of the laboratory from 1982 to 1984. The plasmid pZ189 contains the following elements: the T antigen coding region and origin of replication from the SV40 virus; the ampicillin-resistance gene (Ap^R , *Amp*), the pBR327 origin of replication (pBR327 ori); the suppressor tRNA (*supF*) gene that serves as the mutagenesis marker; and the IG region from the bacteriophage M13 DNA to permit the production of single-stranded plasmid DNA in M13-infected cells. The *supF* tRNA gene with regulatory regions consists of about 160 bp and lies between two essential components of the plasmid, the *Amp* gene and the ori of the plasmid.

The plasmid treated with a mutagen was propagated in human cells for 48–72 h. The plasmid was extracted from the cells, and then digested by the restriction endonuclease *DpnI* to eliminate non-replicated input plasmids with the bacterial methylation pattern. *E. coli* MBM7070, which has an amber mutation in its *lacZ* gene [*lacZ(am)CA7020*], was transformed by the plasmid and spread onto LB agar plates containing ampicillin, 5-bromo-4-chloro-3-indoyl- β -D-galactoside (X-gal) and isopropyl- β -D-thiogalactoside (IPTG). After 24 h incubation at 37°C, blue colonies having normal *supF* and white or light-blue colonies having mutated *supF* appeared on the plate. The total number of colonies was counted as an indication of plasmid survival, and the proportion of white or light-blue colonies was deter-

mined as a measure of mutant frequency. The *supF* gene of the white or light-blue colonies was sequenced to reveal which base had been mutated by the mutagen.

Modification of the Shuttle Vector Plasmid

The first automatic DNA sequencer with a reliable DNA sequencing reagent kit was distributed by Applied Biosystems Inc. (ABI) (16). The –M13 primer, whose 5'-end had been labeled with fluorescent dye, became the standard primer (Dye-primer) for sequencing with ABI Model 373. Matsuda *et al.* (17) synthesized a 13-mer oligonucleotide sequence that can hybridize with the fluorescent –M13 primer, and inserted it into the *EcoRI* site of pZ189. The plasmid was named pMY189 (Fig. 1A). To propagate pMY189 in rodent cells, a part of the pMY189 sequence derived from SV40 was replaced by the whole genome of polyoma virus, and the plasmid was called pYZ289 (18). An unnecessary part of the polyoma virus sequence of pYZ289 for plasmid replication was removed by Nakayama *et al.* (19), and finally the plasmid pNY200 for rodent cells was established as a counterpart of pMY189 for human cells (Fig. 1B). Both shuttle vector plasmids were applied for the ABI Dye-primer sequencing and later for Dye-terminator sequencing, according to technical innovations of automatic DNA sequencing.

In the shuttle vector plasmid assay, there has been a fundamental issue that mutants arising from an independent event cannot be distinguished from siblings of an initial event when an identical base change appears from a plasmid transfection plate. Parris and Seidman (20) resolved this issue by developing a population system, pSP189 plasmids, each of which contains an 8-bp 'signature sequence'. This sequence confers a unique identification tag to each plasmid and allows individual members to be identified by a distinctive signature. This system is particularly advantageous when low mutant frequencies are obtained by weak mutagen treatment.

As indicator bacteria for *supF* mutation, *E. coli* KS40/pKY241 was established (21) instead of the MBM7070 strain. The *E. coli* KS40 has a MBM7070

Fig. 1. Shuttle vector plasmids, pMY189 (A) and pNY200 (B), that can replicate in human and mouse cells, respectively. These plasmids can also replicate and undergo selection of their *supF* mutations in *E. coli*.

derivative that has a DNA gyrase A (*gyrA*) gene with an amber mutation, and the plasmid pKY241 has a wild-type *gyrA* gene and a chloramphenicol-resistant marker gene. The *E. coli* carrying pKY241 is therefore resistant to nalidixic acid and chloramphenicol. The bacteria carrying pKY241 and pMY189 with wild-type *supF* cannot make colonies on an LB agar plate containing nalidixic acid, ampicillin and chloramphenicol, whereas those carrying pKY241 and pMY189 with mutated *supF* can. The addition of IPTG and X-gal in the plate can confirm the selection of the mutated *supF* gene by the color of the colonies, like the MBM7070 strain. The *E. coli* KS40/pKY241 strain increased accuracy of measurement of *supF* mutant frequencies.

Application of the Shuttle Vector Plasmids to UV Mutagenesis

As soon as the plasmid pZ189 became available (5), a series of studies to elucidate the mutation spectrum of ultraviolet light (UV) in human cells were carried out by Kraemer and colleagues (22–25). UV was the first subject of the studies because UV-induced DNA damage, cyclobutane pyrimidine dimers (CPDs) and 6–4 photoproducts (6–4 PPs), had been well characterized and the DNA repair-deficient cells derived from xeroderma pigmentosum (XP) patients with 7 genetic complementation groups were by then available. The lesions induced in pZ189 by UV irradiation *in vitro* are not removed in XP cells, and therefore, all lesions become triggers of mutations upon plasmid replication in XP cells.

As a result of various studies on UV-induced mutations in the shuttle vector plasmids introduced into normal human and XP cells (22–27), some key findings that are now common knowledge were obtained. The G:C to A:T transition is the major type of UV-induced mutation, and its frequency is higher in DNA repair-deficient XP cells than in normal human cells. The C to T transitions occur mostly at the 3'-C of 5'-TC-3' and 5'-CC-3' sequences. Tandem CC to TT transitions at 5'-CC-3' sequences are also a feature of UV-induced mutation. The most abundant DNA lesion, cyclobutane thymine dimer, which is expected to cause base substitution at 5'-TT-3' sequences, is not a premutagenic lesion (28,29). More precise analysis exploiting photoreactivating enzymes, CPDs and 6–4 PPs photolyases, following UV irradiation to the shuttle vector plasmid was carried out by Otsushi *et al.* (30). They revealed that both cytosine-containing CPDs and 6–4 PPs are premutagenic lesions for G:C to A:T transitions, while the premutagenic lesions for A:T to G:C transitions are 6–4 PPs.

A tumor suppressor gene, *p53*, has been studied extensively; these studies revealed that more than 50% of human tumors including skin tumors have mutations in the *p53* gene (31,32). XP patients are predisposed to

skin tumors because they are deficient in the ability to repair UV-induced DNA lesions. Identification of base changes in the *p53* gene of XP skin tumors revealed that predominant base changes are C to T transitions at the 3'-C of 5'-TC-3' and 5'-CC-3' sequences, and tandem CC to TT transitions at 5'-CC-3' sequences have also been characterized (33–35). Similar types of *p53* base changes are also found in the skin tumors of sun-exposed body areas, but not in skin tumors of non-exposed areas (36). These mutation characteristics are similar to those found in the UV-induced mutations of shuttle vectors, indicating that *p53* gene mutation is a cause of skin tumors induced by sunlight.

Application of the Shuttle Vector Plasmids to Chemical Mutagenesis

The shuttle vector plasmids have been applied to reveal the types of mutations induced by various chemical mutagens. *N*-methyl nitrosourea (MNU)-induced mutations in the plasmids were more frequent in *O*⁶-methylguanine-DNA methyltransferase (MGMT)-deficient cells than in proficient cells (37). The G:C to A:T transition is dominant, particularly in MGMT-deficient cells, which agrees with the findings that unrepaired *O*⁶-methylguanine can form a mispair with thymine at DNA replication (38,39).

Carcinogenic polyaromatic hydrocarbons (PAHs) are emitted mainly by industry and automobiles, but most of them do not react with DNA directly. These PAHs are biotransformed to reactive intermediates by cellular metabolic enzymes. The reactive metabolites of the PAHs, 1-nitropyrene, benzo[*a*]pyrene (40), aminofluorene (41) and 3-nitrobenzanthrone (42,43), make bulky DNA adducts in DNA. The bulky adducts formed in the shuttle vector plasmids yield mainly G:C to T:A transversions in human cells. Recent progress of translesion DNA polymerases (Pol η , ι , κ and ζ) shows that Pol η , the major polymerase for translesion DNA synthesis (TLS), predominantly inserts adenine opposite a guanine carrying such a bulky adduct (44,45), which is consistent with the results from shuttle vector mutagenesis. This is also consistent with the A-rule of base misincorporation, which has been established in molecular genetics (46).

Various aldehydes such as acetaldehyde, 2-chloroacetaldehyde, crotonaldehyde, acrolein, glyoxal and methylglyoxal are formed in cells through the intake of food, drink and air. These aldehydes react with DNA and form exocyclic guanines, ethenoguanine and propanoguanine (47,48). These DNA lesions generates mutations mainly at G:C sites, but the types of base change are different among these aldehydes in the shuttle vector assays (49–54). It is noteworthy that acetaldehyde induced tandem base changes at 5'-GG-3' sites, suggesting that it forms intra-strand crosslinks between

adjacent guanines through propanodeoxyguanine formation (51).

Benzene is biotransformed to several products, muconic acid, *p*-benzoquinone, catechol and hydroquinone, in the body (55). Hydroquinone produces reactive oxygen species in cells. In the shuttle vector assay, hydroquinone induced deletion mutations frequently in the presence of NADH and Cu (II), which is consistent with the reaction of reactive oxygen species to DNA (56). Other benzene biotransformants were less mutagenic than hydroquinone (19,56,57).

Prospects for New Shuttle Vector Development to Study TLS

TLS is a tolerance pathway of replication block caused by DNA lesions (58,59). To measure the efficiency and fidelity of TLS in human cells, a new shuttle vector assay system was established recently (60). This assay consists of transfection of DNA repair-deficient human cells with a plasmid possessing a single DNA adduct, and transformation of indicator bacteria with plasmids extracted from the cells. The plasmid consists of SV40 replication origin, T antigen gene, bacterial *Amp* gene and *lacZ* gene, where a single site-specific DNA adduct is produced. The plasmid DNA strand with an adduct replicates by bypassing the adduct in human cells and forms a blue bacterial colony, whereas the other DNA strand that has a 2-base frameshift without the adduct replicates in human cells and forms a white bacterial colony. Replication of the plasmid possessing a single aminobiphenyl-dG adduct was suppressed to 1/9 of that of the normal plasmid, and the mutant frequency of the TLS-operated plasmids was 0.31, of which the major mutation (78%) was a G to T transversion (60). Using this assay system, we showed that a new inhibitor of proliferating cell nuclear antigen (PCNA) suppressed TLS of cisplatin-guanine adducts (61). These results demonstrate that this assay is applicable for investigating TLS in human cells. This cellular TLS assay is superior to the cell free TLS assay in a test tube using adducted oligonucleotide, extension primer and purified TLS polymerases because the *in vitro* test tube assay is less reflective of the cellular process. A project to measure TLS efficiencies and mutation frequencies of various DNA adducts formed by 3-nitrobenzanthrone, a carcinogenic nitro-PAH from diesel exhaust, is in progress (62).

In conclusion, the shuttle vector assays that were improved by many investigators have been disclosing the mutation spectra of various chemical and physical mutagens in mammalian cells. These assays will further contribute to elucidate the TLS mechanism of various DNA adducts in human cells. The remaining issues are the elucidation of which TLS polymerase operates to bypass various kinds of DNA lesions, and what type of

mutation is induced by each TLS polymerase in the cells. The clarification of mutation mechanisms at the molecular level would be important for studies on human cancer prevention.

Acknowledgments: The author thanks all instructors, collaborators and students who have provided support via their participation in the field of mutation research. The author would not have received the award and written this review without this earnest support. This research was supported by Grants in Aid for Scientific Research (B) No. 24310047 and for Challenging Exploratory Research No. 24651063 from the Japan Society for the Promotion of Science.

Conflicts of interest statement: The author declares that there are no conflicts of interest.

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