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A polymerization-based method to construct a plasmid containing clustered DNA damage and a mismatch

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ABSTRACT

Exposure of biological materials to ionizing radiation often induces clustered DNA damage. The mutagenicity of clustered DNA damage can be analyzed with plasmids carrying a clustered DNA damage site, in which the strand bias of a replicating plasmid (i.e., the degree to which each of the two strands of the plasmid are used as the template for replication of the plasmid) can help to clarify how clustered DNA damage enhances the mutagenic potential of comprising lesions. Placement of a mismatch near a clustered DNA damage site can help to determine the strand bias, but present plasmid-based methods do not allow insertion of a mismatch at a given site in the plasmid. Here, we describe a polymerization-based method for constructing a plasmid containing clustered DNA lesions and a mismatch. The presence of a DNA lesion and a mismatch in the plasmid was verified by enzymatic treatment and by determining the relative abundance of the progeny plasmids derived from each of the two strands of the plasmid.

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1. Introduction

Ionizing radiation, when directed at biological materials, delivers energy to a local area, generating clustered DNA damage or multiply damaged sites in DNA [1]. Clustered DNA damage is defined as two or more lesions formed within 1–2 helical turns of DNA by a single radiation track [2,3]. The lesions in such clusters include single strand breaks, oxidized bases, and apurinic/apyrimidinic sites (AP sites). Exposing cells to low linear energy transfer (LET) radiation such as X-rays or gamma-rays induces clustered DNA damage at least 4–8 times more frequently than it induces double strand breaks (DSBs) [4–6]. Furthermore, the complexity of the cluster is strongly dependent on the LET of the radiation [7–9].

Most of the details of repair and processing of clustered DNA damage have been obtained by using synthetic models of clustered DNA damage comprised of base lesions, AP sites, and/or strand breaks. *In vitro* studies have revealed that the efficiency of base excision repair (BER) of lesions such as mutagenic 8-oxo-7,8-dihydroguanine (8-oxoG) in a cluster depends on its configuration. Plasmid-based assays for *in vivo* investigations have complemented the *in vitro* studies by showing that clustered DNA damage sites can significantly increase the mutation frequency (the frequency with which an unrepaired mutagenic lesion results in a mutation) (for reviews, see Refs. [10–13]).

Fpg and MutY proteins are two of the main repair enzymes for reducing the mutagenic potential of 8-oxoG in *Escherichia coli* [14]. When plasmids with a single 8-oxoG were transfected into an *fpgmutY* mutant of *E. coli*, the mutation frequency was about 20%, but when plasmids with an 8-oxoG on one strand and another lesion at 1-bp separation on the complimentary strand were transfected, the mutation frequencies were around 30–45% [15–20]. The majority of mutations were found to originate from 8-oxoG. The results indicated that the repair efficiency of 8-oxoG alone cannot explain the increase of the mutagenic potential of 8-oxoG in a cluster. In addition, we found that the lack of DNA polymerase I enhances the mutagenic potential of 8-oxoG within a bistranded cluster in *fpgmutY* [21]. The mutation frequency of hydroxyl uracil (hU) within a three-lesion cluster is over 90%, which indicates that the strand opposite hU are rarely used as a template for replication [22]. These results led us and others to propose that the mutagenic potential of a lesion within a clustered DNA damage site is increased not only by a delay in repairing the lesion, but also by preferentially using one of the two templates as a result of some

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process, such as a collapse of replication fork or a post-replicational recombination.

A plasmid with a mismatch is useful for examining the extent of each strand used as a replication template, as the fraction of two progeny plasmids derived from each of the two strands of the transfected plasmid can be determined by DNA sequence. Pandya et al. (2000) constructed plasmids with a single DNA lesion and a mismatch by annealing a damage-containing oligonucleotide to a circular single-stranded (ss) DNA template, which was generated from a phagemid [23]. However, the method cannot be used to directly construct a plasmid with a bi-stranded clustered DNA damage site due to its inability to incorporate a DNA lesion in the circular ssDNA template. In the plasmid-based assay of clusters, plasmids were constructed by simply ligating the oligonucleotides that harbored the damage to the linearized plasmid vector. Although the conventional ligation method for examining the effect of clustered DNA damage is simple and straightforward, this method does not allow insertion of a mismatch at a given site in the plasmid. Further, in the ligation method, the transfected DNA is a mixture of circular and linear forms of plasmids ligated or unligated to oligonucleotides. It is difficult to know whether these byproducts have any influence on the transformation efficiency or the mutation efficiency of a cluster [24].

Here, we describe a method to construct a plasmid by sequential polymerization of each strand, which enables insertion of a mismatch and a clustered DNA damage site without contaminating the transfected DNA with undesired byproducts, such as linear and multimer molecules. With the method, we were able to insert both a clustered DNA damage site and a mismatch into a single plasmid. Our method could be a useful tool for analyzing the processing of clustered DNA damage *in vivo*.

2. Materials and methods

2.1. Bacterial strains

Strains CC104*mutY::TetR* [21] and CC104*mutY::TetR mutS::KanR* were used for transfecting the synthesized plasmids. CC104*mutY::TetR mutS::KanR* was constructed by transferring *mutS::KanR* from JW2703-2 to CC104*mutY::TetR* by P1 transduction. The strains CJ236 and JW2703-2 were provided by Coli Genetic Stock Center (CGSC) at Yale University.

2.2. Plasmid and oligonucleotides

Plasmid pGEM3Zf(−) were purchased from Promega K. K. (Tokyo, Japan) and helper phage VCSM13 were from Agilent Technologies Japan, Ltd. (Tokyo, Japan). Five primers were used (Fig. 1), including two undamaged primers (NAEP and BNG), a primer with uracil (U-1), a primer with 8-oxoG (8G) and a primer with a 6-base insert to form a mismatch (X1800). All were synthesized by Tsukuba Oligo Service Co., Ltd. (Ibaraki, Japan). NAEP and U-1 are identical except for the presence of uracil in U-1, and BNG and 8G are identical except for the 8-oxoG in 8G. BNG is complimentary to NAEP and uracil in U-1 and 8-oxoG in 8G are both placed within the Alw26I recognition sequence. When U-1 is annealed with 8-oxoG-carrying plasmid to generate uracil +8G cluster, uracil is located 1 bp 5′ from 8-oxoG on the complimentary strand. The 6-base insert (5′CTCGAG3′), which is the XhoI recognition sequence, is placed in X1800 so as to form a mismatch at the ScaI recognition sequence in pGEM3Zf(−).

2.3. Preparation of uracil-containing circular ssDNA

Uracil-containing circular ssDNA was prepared based on the protocol of Kunkel [25], with slight modifications. In brief, NAEP + BNG double-stranded (ds) oligonucleotides were inserted between EcoRI and PstI sites of pGEM3Zf(−) to construct pGEM3Zf(−)NAEPBNG, and a fresh colony of CJ236 harboring pGEM3Zf(−)NAEPBNG was inoculated into 4 ml LB broth with 100 μg/ml ampicillin and 15 μg/ml chloramphenicol, and incubated at 37 °C. After OD_{600} of the culture reached around 0.05, 1 ml of the culture was inoculated to 100 ml LB broth with 100 μg/ml ampicillin and 0.75 μg/ml uridine. After 3-h incubation with vigorous shaking, $>1 \times 10^{10}$ pfu of VCM13 helper phage and $MgCl_2$ to a final concentration of 5 mM were added. The culture was incubated for 0.5 h, prior to the addition of kanamycin (final concentration 50 μg/ml), and incubated for another 20 h at 37 °C. Subsequently, the culture was centrifuged and the supernatant containing phage particles was passed through a 0.22 μm filter to eliminate any debris. Polyethylene glycol (PEG, average MW 6000) and NaCl were added at a final concentration of 5% and 0.5 M, respectively, to the phage solution, and after incubation at 4 °C overnight, phage particles were precipitated by centrifugation at 9000g for 20 min. The phage pellet was suspended in TE buffer (pH 7.6) and centrifuged to remove any debris. The purified phage particle was again precipitated, and then suspended in 4 ml of TE. Uracil-containing circular ssDNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1), and further purified by ethanol precipitation (Fig. 2B, lane 1).

2.4. Strand synthesis and enzyme treatment of plasmid DNA

The first and second strands were synthesized as reported previously [26], with slight modifications. In brief, 30 pmol of the phosphorylated oligonucleotides were annealed with 20 pmol of

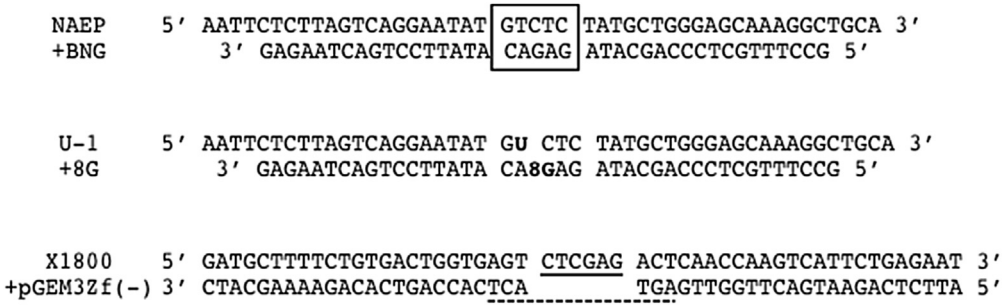

Fig. 1. Sequences of annealed primers. NAEP primer is annealed to BNG primer, U-1 primer to 8G primer, and X1800 primer to pGEM3Zf(−). Alw26I recognition sequence in NAEP + BNG is boxed. U (bold) denotes uracil and 8G (bold) denotes 8-oxoG. A 6-base mismatch in X1800 primer, which forms the XhoI recognition sequence, is underlined by a solid line. The ScaI recognition sequence is underlined by a dotted line.

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