Induction of Various Mutations during PCRs with Manganese and 8-Hydroxy-dGTP

Hiroyuki KAMIYA,* Mana ITO, and Hideyoshi HARASHIMA

Faculty of Pharmaceutical Sciences, Hokkaido University; Kita–12, Nishi–6, Kita-ku, Sapporo 060–0812, Japan.
Received November 8, 2006; accepted January 5, 2007

To induce various mutations randomly, PCRs with Mn^{2+} and with a mutagenic deoxyribonucleotide, 8-hydroxy-dGTP (8-OH-dGTP), were performed. Mutations were induced by deoxyribonucleotide imbalance plus 500 μM Mn^{2+} in the Mn^{2+}-PCR, and the amplified DNA was inserted into a plasmid. The plasmid library obtained from the transformed bacterial cells was then used as the template in the next PCR, which was done with 50 or 100 μM 8-OH-dGTP. Four kinds of mutations, A:T→G:C and G:C→A:T transitions and A:T→T:A and A:T→C:G transversions, occurred with similar frequencies. These results suggest that this strategy will be useful in random PCR mutagenesis for the *in vitro* evolution of nucleic acids and proteins, and for analyses of residues in these biomolecules.

Key words mutagenesis PCR; manganese; 8-hydroxy-dGTP; transition; transversion

Random mutagenesis is a useful tool to analyze the roles of many residues in proteins or functional nucleic acids, such as RNA enzymes. This mutagenesis method has also been used for the *in vitro* evolution of bioactive molecules, in conjunction with an appropriate screening system. Modified deoxyribonucleotides with ambiguous base-pairing properties are used in random mutagenesis PCR. The use of 6-(2-deoxy-β-D-ribofuranosyl)-3,4-dihydro-8*H*-pyrimido-[4,5-*c*][1,2]oxazin-7-one 5′-triphosphate and 8-hydroxy-2′-deoxyguanosine 5′-triphosphate (8-OH-dGTP) induced A:T→G:C and G:C→A:T transitions, and an A:T→C:G transversion, respectively.[1] Recently, we reported that 2-hydroxy-2′-deoxyadenosine 5′-triphosphate induces both transition and transversion mutations, with A:T→G:C, A:T→C:G, and G:C→T:A substitutions.[2]

Decrease in fidelity of DNA polymerases is also used for random mutagenesis PCR. The random mutagenesis has been achieved with deoxyribonucleotide imbalance, the addition of Mn^{2+}, and error-prone DNA polymerases in the PCR. Previously, a combination of the deoxyribonucleotide imbalance and Mn^{2+} was used in the random mutagenesis PCR, and A:T→T:A, A:T→G:C, and G:C→A:T substitutions were effectively induced.[3,4] We noticed that four kinds of mutations could be induced when an A:T→C:G transversion was added to the three types of substitutions by the Mn^{2+} reactions. In this study, we found that PCR with 8-OH-dGTP, after error-prone PCR with Mn^{2+}, induced A:T→G:C and G:C→A:T transitions and A:T→T:A and A:T→C:G transversions with similar frequencies. These results indicate that the combination of the Mn^{2+}-PCR and 8-OH-dGTP-PCR may be useful to generate random mutant libraries of proteins or functional nucleic acids.

MATERIALS AND METHODS

Materials 8-OH-dGTP was prepared by the oxidation of dGTP, and was purified by HPLC as described.[5] Purified oligodeoxyribonucleotides were from Hokkaido System Science (Sapporo, Japan) and Sigma Genosys Japan (Ishikari, Japan). The plasmid pGST-dNK, containing a glutathione-*S*-transferase-deoxynucleoside kinase fusion gene, was derived from pGEX-6P-3 (Amersham Biosciences, Piscataway, New Jersey, U.S.A.). The construction of pGST-dNK will be reported elsewhere. $MnCl_2 \cdot 4H_2O$ (reagent grade, >99% purity) was from Wako Pure Chemical Industries (Osaka, Japan).

PCR and Mutation Frequency Determination Mutagenic PCR in the presence of Mn^{2+} was carried out as described.[3] The deoxynucleoside kinase gene in the pGST-dNK plasmid (50 pg) was amplified with primers (Upper: 5′-dCTGGAAGTTCTGTTCCAGG-3′ and Lower: 5′-dTCAGTCAGTCACGATGCGG-3′, 50 pmol each) and 4 units of *Taq* DNA polymerase, in a buffer (total 100 μl) containing 10 mM Tris–HCl (pH 8.3), 7 mM $MgCl_2$, 500 μM $MnCl_2$, 50 mM KCl, 0.01% gelatin, 0.01% Triton-X100, 1 mM dCTP, 1 mM dTTP, 200 μM dATP, and 100 μM dGTP. The PCR program used was 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 3 min, 45 cycles. The amplified DNA was digested by *Bam*H I and *Sal* I, which have recognition sites near the termini of the amplified DNA fragments. The DNA was then ligated into the pGEX-6P-3 plasmid, which had been pretreated with the aforementioned restriction enzymes, with a Ligation high kit (Toyobo, Osaka, Japan). The ligation mixture was transfected into *Escherichia coli* DH5α cells by the $CaCl_2$ method.[6] Plasmid DNA (Mn^{2+}-mutant library) was recovered collectively from an *E. coli* culture that was derived from the original 8×10^4 transformed *E. coli* cells.

The kinase gene of the plasmid in the Mn^{2+}-mutant library (20 pg) was amplified with primers (Upper: 5′-dCTGGAAGTTCTGTTCCAGG-3′ and Lower: 5′-ACAATTGTCGACTAATGGGATAATGGT-3′, 10 pmol each) and 0.8 units of *Taq* DNA polymerase, in a buffer (total 20 μl) containing 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM each of dATP, dGTP, dCTP and dTTP, and with 50 or 100 μM 8-OH-dGTP. The PCR program used was 95 °C for 30 s, 53 °C for 1 min, and 72 °C for 3 min, 30 cycles. One microliter of the 8-OH-dGTP-PCR mixture was transferred into a fresh tube as a template, and the same primer set (10 pmol each), the same buffer, and 0.8 units of *Taq* DNA polymerase were used in the subsequent PCR (total 20 μl) without 8-OH-dGTP. The same program, except for the cycle number (10 cycles), was employed in the PCR. This PCR product was

* To whom correspondence should be addressed. e-mail: hirokam@pharm.hokudai.ac.jp

Table 1. Mutations Induced by Mn^{2+}- and 8-OH-dGTP-PCRs

Mutation inducer added	Mutation frequency (%/bp)	No. of mutations found							
		Substitutions						Others	Total
		A:T→G:C	G:C→A:T	A:T→T:A	A:T→C:G	G:C→T:A	G:C→C:G		
500 μM $MnCl_2$	1.2	8	3	8	1	4	0	2	26
500 μM $MnCl_2$ + 50 μM 8-OH-dGTP	2.0	16	14	28	19	4	1	3	85
500 μM $MnCl_2$ + 100 μM 8-OH-dGTP	2.8	10	12	20	26	8	0	2	78

then inserted into pGEX-6P-3, as described above. Determination of the mutation frequency by sequencing was carried out essentially as described previously.[2)]

RESULTS AND DISCUSSION

Mutation Frequencies in Mutagenesis PCR Previously, Kawate *et al*. reported error-prone PCR using Mn^{2+} and unequal concentrations of dNTPs.[3)] It was found that A:T→T:A, A:T→G:C, and G:C→A:T substitutions effectively occurred. In the first PCR, we employed the same conditions as those in the previous report (see Materials and Methods). To induce an A:T→C:G transversion mutation during PCR, 8-OH-dGTP was included during the next PCR. Since the direct use of the products amplified in the Mn^{2+}-PCR inhibited the 8-OH-dGTP-PCR, we inserted the Mn^{2+}-PCR product into a plasmid, to construct a library of mutants generated in the Mn^{2+}-PCR, and the plasmids in the mutant library were then used as the templates in the 8-OH-dGTP-PCR. 8-OH-dGTP was added to a final concentration of 50 or 100 μM.

Table 1 shows the mutations detected in the deoxynucleoside kinase gene after the Mn^{2+}- and 8-OH-dGTP-PCRs. We detected mutations in the 694-bp region (positions 35—728 in the gene, where the first A of the start codon is denoted as 1). The average mutation frequency per bp was 2.0% in the case of 50 μM 8-OH-dGTP. This value was *ca*. 30-fold higher than that of the usual PCR that we reported previously (0.07%).[2)] The mutation frequency was further enhanced and reached 2.8% per bp by the use of 100 μM 8-OH-dGTP. This value was *ca*. 40-fold higher than that of the usual PCR. When 8-OH-dGTP was not included in the second PCR (amplification of the kinase gene on the plasmid in the Mn^{2+}-mutant library), the mutation frequency was 1.2% (Table 1).

Mutation Spectrum Eighty-two of the 85 mutations detected in the Mn^{2+} and 8-OH-dGTP (50 μM) mutagenesis were base substitutions (Table 1). As expected, the four kinds of substitution mutations, A:T→G:C and G:C→A:T transitions and A:T→T:A and A:T→C:G transversions, were observed. The A:T→T:A transversion was most abundant, and the A:T→C:G transversion induced by 8-OH-dGTP seemed to be less frequently induced than the A:T→T:A transversion.

Seventy-six of the 78 mutations detected in the Mn^{2+} and 8-OH-dGTP (100 μM) mutagenesis were base substitutions (Table 1). The ratio of the A:T→C:G transversion was increased, and this mutation was detected more frequently than the A:T→T:A transversion. This result was consistent with the increase in the 8-OH-dGTP concentration. The use of 50 μM 8-OH-dGTP was better, considering the nearly equal induction frequencies of the four kinds of mutations.

When compared with the result of no 8-OH-dGTP, it is clear that the nucleotide effectively induced A:T→C:G mutations (Table 1). The G:C→T:A and G:C→C:G transversions were minor mutations (Table 1). The ratio of the G:C→T:A mutation appeared to be increased when 100 μM 8-OH-dGTP was used. This type of mutation might be induced by 8-OH-dGTP, since 8-hydroxyguanine in DNA causes the G:C→T:A

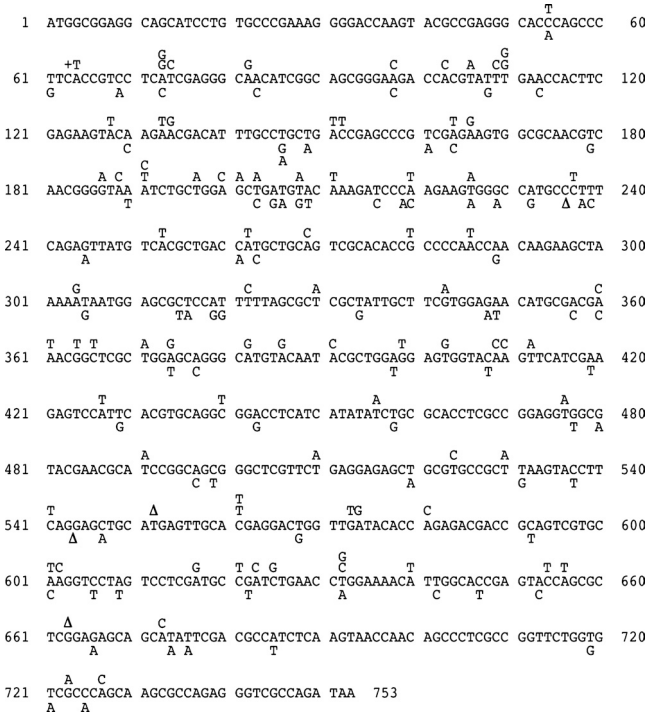

Fig. 1. Overall Distribution of the Mutations Detected in the Deoxynucleoside Kinase Gene

The sequence of the sense strand of the gene is shown. The mutations obtained with $MnCl_2$ and 50 μM 8-OH-dGTP, and those induced by $MnCl_2$ and 100 μM 8-OH-dGTP are shown above and below the sequence, respectively. The symbol Δ represents a deletion. The symbol +T above positions 63 and 64 represents the addition of T between positions 63 and 64 (CA→CTA). The symbol Δ below position 236 represents the deletion of either of the C residues located at positions 235—237. The symbol Δ below the midpoint of positions 543—544 represents the deletion of either of the G residues located at positions 543—544. The symbol Δ above position 663 represents the deletion of CG from CGG at positions 662—664 (CGG→G).

transversion.[7,8] However, no G:C→T:A mutation was induced by 100 μM 8-OH-dGTP in the PCR in which the pGST-dNK plasmid was used as the template (data not shown).

Distribution of Mutations The overall distribution of the mutations detected in the Mn^{2+}- and 8-OH-dGTP-PCRs is shown in Fig. 1. The mutations seemed to be uniformly distributed, and did not form a hotspot. This characteristic of this PCR mutagenesis method makes it attractive for random mutagenesis applications.

Possible Applications We found that the four kinds of substitution mutations, A:T→G:C and G:C→A:T transitions and A:T→T:A and A:T→C:G transversions, were induced by the PCR mutagenesis described in this study. Under our conditions, 2—2.8 mutations occurred per 100 bp, which could be an adequate mutation rate for random mutagenesis. This property will be useful for analyses of biomolecules and their *in vitro* evolution.

Acknowledgments We thank Dr. Hisaya Kawate of Kyushu University for valuable advice. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1) Zaccolo M., Williams D. M., Brown D. M., Gherardi E., *J. Mol. Biol.*, **255**, 589—603 (1996).
2) Kamiya H., Ito M., Harashima H., *Biol. Pharm. Bull.*, **27**, 621—623 (2004).
3) Kawate H., Landis D. M., Loeb L. A., *J. Biol. Chem.*, **277**, 36304—36311 (2002).
4) Cadwell R. C., Joyce G. F., *PCR Methods and Applications*, **2**, 28—33, (1992).
5) Cheng K. C., Cahill D. S., Kasai H., Nishimura S., Loeb L. A., *J. Biol. Chem.*, **267**, 166—172 (1992).
6) Sambrook J., Fritsch E. F., Maniatis T., "Molecular Cloning: A Laboratory Manual," 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989.
7) Kamiya H., *Biol. Pharm. Bull.*, **27**, 475—479 (2004).
8) Kamiya H., Sakaguchi T., Murata N., Fujimuro M., Miura H., Ishikawa H., Shimizu M., Inoue H., Nishimura S., Matsukage A., Masutani C., Hanaoka F., Ohtsuka E., *Chem. Pharm. Bull.*, **40**, 2792—2795 (1992).