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An *in Vivo* Approach to Identifying Sequence Context of 8-Oxoguanine Mutagenesis

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Base substitution mutations are not distributed randomly in that most are located at a few specific hotspots sites. We have been studying 7,8-dihydro-8-oxoguanine mutagenesis in *Escherichia coli* in the *supF* gene carried in a plasmid. Among hotspots, guanine within the 5′-AGA-3′ located in the anticodon site was susceptible to the induction of G:C→T:A transversion. In this study, we constructed variants of the *supF* gene in which the hotspot 5′-AGA-3′ was modified to 5′-AGT-3′, 5′-AGG-3′ and 5′-AGC-3′ to determine the influence of 3′ neighboring base on G:C→T:A mutational activity. Using these variant *supF* genes propagated in a 7,8-dihydro-8-oxoguanine repair-deficient host, we found that guanine within 5′-AGA-3′ and 5′-AGG-3′ produce G:C→T:A, but guanine within 5′-AGT-3′ and 5′-AGC-3′ reduce the formation of G:C→T:A. These changes were thus due to the effect of sequence context on the efficiency of mutation formation at the sites of 7,8-dihydro-8-oxoguanine. We also observed a longer range base-pair effect on hotspot formation.

Key Words: **mutation hotspot; 7,8-dihydro-8-oxoguanine; *supF* gene; *mutM; mutY*.**

Spontaneous mutations are not uniformly distributed throughout the genome. Benzer's classic study (1) of the *rII* system in phage T_4 first demonstrated the existence of hotspots, sites that are more mutable than other sites. Detailed analysis of spontaneous mutation in the *lacI* gene in a mismatch repair-defective strain of *Escherichia coli* indicated that the base substitutions are exclusively transitions, among which favored sites can be detected (2). The distribution of base substitutions in DNA of mammalian cells also appears to be highly uneven; clustering of base substitutions at selected sites was observed in genes such as *aprt* (3) and *p53* (4).

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Mutational hotspots induced by DNA damaging agents have also been observed. Skopek and Hutchinson (5) showed that bromouracil, which is specific for A:T→G:C transitions in the λCI gene, has sequence-dependent site specificity. A:T→G:C transitions at the sequence 5′-ACGC-3′ are at least an order of magnitude more mutable than any other site. They suggested that the 5′-CGC-3′ sequence following the adenine could affect the editing function of the DNA polymerase. Levy *et al.* (6) characterized UV-induced mutagenesis and found two UV-induced hotspots of C:G→T:A transitions in the *supF* tRNA gene propagated in repair-deficient xeroderma pigmentosum cells at the 5′-TC sites in the eight-base palindrome 5′-CTTCGAAG-3′.

We have been studying spontaneous as well as reactive oxygen species (ROS)-induced mutagenesis in *E. coli* cells using a plasmid carrying the *supF* gene (7–10). ROS-induced base substitutions, especially G:C→T:A and G:C→C:G transversions, which are induced by 8-oxoguanine (8-oxoG) (11, 12), occurred at four hotspot sites in the *supF* gene. These hotspots for mutagenesis by ROS were essentially the same as those observed in a collection of spontaneous mutations. Investigation of the influence of the local DNA sequence on the transversions due to 8-oxoG revealed that G at the center of the triplet 5′-PuGA-3′ context was more mutable than that at the center of 5′-PyGN-3′. Finally, we collected spontaneous G:C→T:A transversions in the *supF* gene in a plasmid replicated in an 8-oxoG repair-deficient *mutM mutY* double mutant strain (13). Among the hotspots, the guanine within the 5′-AGA-3′ sequence context located in the anticodon site in the *supF* gene was the strongest site. These observations suggested that as the average reactivity of ROS to guanine within the AGA may not be equal to the average reactivity to any other guanine in the DNA, preferential repair of 8-oxoG can occur at sites other than the AGA, and AGA sequence can affect the configuration at the replication fork so as to favor base mispairing.

Systematic study of the influence of sequence context on mutation activity at a specific site would require the

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methodical manipulation of sequences around the site, and determination of the impact on mutational activity at the site. This approach, however, has not been applied to base substitution mutagenesis. This is largely because of the difficulty of devising assay systems that are tolerant of the manipulation of sequence, and sensitive to base substitution mutations. As a result, most discussions of the topics have been limited to cataloguing sequences surrounding hotspots in a marker gene (8).

In the present study, using site-specific mutagenesis *in vitro*, we constructed *supF* variants in which the 3′ nucleotide of the 5′-AGA-3′ hotspot motif, located in the anticodon of the *supF* gene, was modified to all possible nucleotides; i.e., AGT, AGG, and AGC. The amber suppressor function of the variant tRNA was preserved. Consequently, the influence of different 3′ nucleotides on 8-oxoG mutational activity at the 5′-AGA-3′ hotspot was determined.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Escherichia coli* KS40 (*lacZ*am *rpsL gyrA*), SY1033 (*sup*^{0}/F′*pro81*:: Tn*10 lacZ*am), SY5 (wild-type), and SY302 (*mutM*::Tn*10 mutY*::Km^R) were described previously (14).

The plasmid pOF105 carries p15A *ori* of pACYC184, a *gyrA*am gene and *rpsL*am gene and a chloramphenicol-resistant (Cm^R) gene (14). pTW-A, a derivative of pTW-F (13), carries the *supF* gene, an ampicillin-resistance (Ap^R) gene and f1 *ori* (Fig. 1).

Media and reagents. L-broth, L-plates and phosphate buffer were described previously (15). Kanamycin (Km), Ap, and Cm were included, if necessary, in L-broth and L-plates at concentrations of 50, 50, and 30 μg/ml, respectively. Minimal agar medium, used for *supF* mutant selection, was composed of M56 salt plus 0.4% glucose, 0.4% casamino acids, and 1.5% agar supplemented with Ap (150 μg/ml), Cm (30 μg/ml), nalidixic acid (Nal; 50 μg/ml), and streptomycin (Sm; 100 μg/ml). The enzyme and reagents used for DNA manipulation and DNA sequencing were purchased from TaKaRa Biomedicals (Kyoto, Japan) and Applied Biosystems (Foster City, CA). Oligonucleotides used for site-directed mutagenesis, DNA amplification, and DNA sequencing were purchased from Sawady Technology Co., Ltd. (Tokyo, Japan).

Construction of variant supF genes. Site-directed mutagenesis was performed according to the TaKaRa Mutan-K system. Three oligonucleotides were designed that replaced the AGA motif in the pTW-A plasmid in the anticodon (AGA corresponding to positions 134, 133, and 132; see Fig. 1) by AGT, AGG, and AGC. Mutagenized *supF* genes were sequenced to ensure no other mutations had been introduced. Plasmids, pTW-T, pTW-G, and pTW-C thus obtained carried AGT, AGG, and AGC, respectively, instead of the AGA motif of the pTW-A plasmid.

β-Galactosidase assay. Exponentially growing cultures of strain KS40 (*lacZam*) containing pBR322, pTW-A, pTW-T, pTW-G, or pTW-C were used to measure β-galactosidase activity by the method described by Miller (16).

Mutant selection and DNA sequencing. Individual cultures of *E. coli* strains SY302 (*mutM mutY*) containing pTW-T, pTW-G, and pTW-C in 5-ml of L-broth with Ap were grown at 37°C overnight. The plasmids were extracted and introduced into competent KS40 harboring the plasmid pOF105 (designated as KS40/pOF105). To select transformants with *supF* mutant plasmids, the transformed cells were plated on M56 plates containing Nal, Sm, Cm, and Ap at 37°C for 2 days. Strain KS40/pOF105 was resistant to Nal and Sm if it contained a mutant *supF*, whereas cells carrying active *supF* suppressor tRNA produced no colonies on such plates. The total number of transformants was counted by plating aliquots of cells on L-plates containing Ap and Cm. The plates were incubated at 37°C overnight before counting the number of colonies. To collect *supF* mutant plasmids generated in the *mutM mutY* strains, plasmids were extracted from Nal^R and Sm^R KS40/pOF105 transformants and introduced into SY1033 cells to select for Ap^R for further analysis. The DNA was extracted and sequenced by the dideoxy method (17) with an appropriate sequencing primer using an ABI automated sequencer model 373A.

Calculation of mutation frequency and statistical analysis. The frequency in SY302 (*mutM mutY*) with which a transformant produced at least one *supF* mutant was estimated from the fluctuation test data by assuming that all the *supF* mutations occurred randomly following the Poisson distribution. The mean number of transformations producing at least one *supF* mutant per tube, mN, is given by the equation $P(0) = e^{-mN}$, where P(0) is the fraction of the tubes containing no *supF* mutants, m is the mutation frequency per replication and N is the average number of transformants.

To determine whether there were any differences in the mutation strength at position 133 in four variant plasmids, the χ^2 test was used. $P < 0.05$ was regarded as significant.

RESULTS

In our previous study, we determined the spontaneous as well as ROS-induced mutation pattern of the *supF* gene in the plasmid pZ189 and derivatives propagated in wild-type and *mutM mutY* strains (7, 8, 13). Assuming that G:C→T:A transversions observed originated from 8-oxoG, four hotspots in the spectra were at positions 133, 159, 168, and 169 (see Fig. 3; the numbering is in accordance with Seidman *et al.* (18). These were at the G within 5′-AGA-3′. 5′-GGA-3′ and 5′-AGG-3′ sequence context.

Construction of the variant supF genes. We were interested in studying the influence of nearby sequence changes on 8-oxoG mutational activity at the hotspot sites. Among hotspots in pTW-A *supF* gene (for comparison, we described here pTW-F as pTW-A), 3′ nearby nucleotide adenine (at position 132) in the position 133 hotspot located in the anticodon was found to be variable. Thus, we developed the variant *supF* gene to permit these experiments. The plasmids pTW-T, pTW-G, and pTW-C carried T, G, and C, respectively, at position 132 instead of A (Fig. 1). The tRNAs encoded by the genes were functional (Fig. 2), and so it was possible to determine the influence of 3′ nearby sequence for G:C→T:A transversion using these new plasmids.

Characterization of supF mutations in mutM mutY strain. pTW-T, pTW-G, and pTW-C were extracted from 92, 84, and 96 independent cultures of SY302, respectively, and used to transform KS40/pOF105. We identified at least one Nal^R and Sm^R transformant from each of 76, 70, and 80 cultures, respectively. Mutation frequencies were 1.7×10^{-6}, 2.7×10^{-6}, and 1.0×10^{-6} for pTW-T, pTW-G, and pTW-C, respectively. The val-

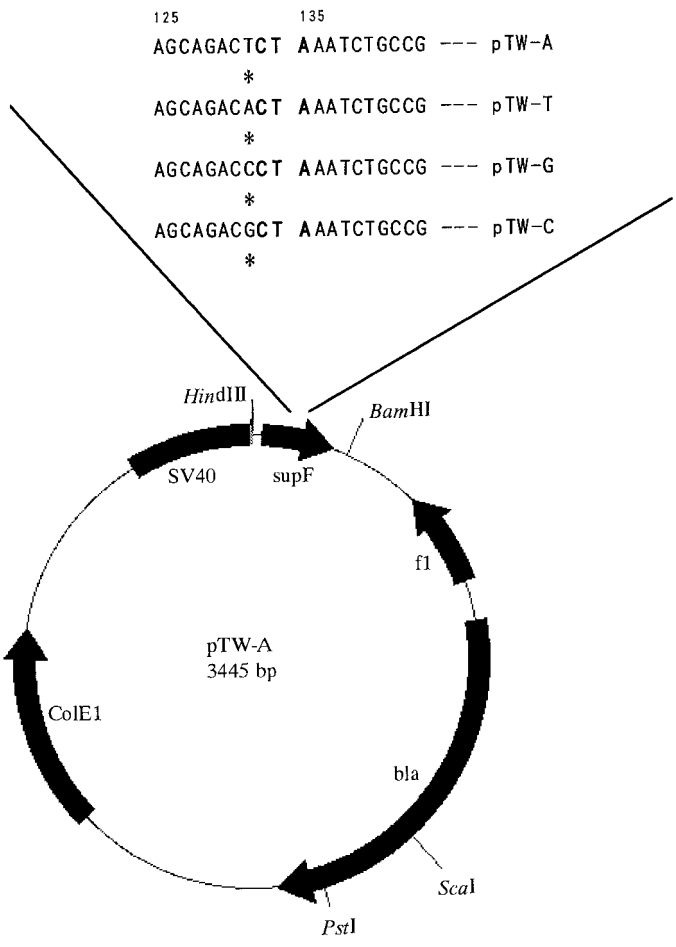

FIG. 1. Map of pTW-A, 3445 bp in length and details of the *supF* gene from position 125 to 144. Bases with asterisks at position 132 are the sites where base pair changes were made. Numbering is in accordance with Seidman *et al.* (18). The boxes with arrows represent the direction of transcription of genes or DNA replication.

ues were essentially the same as the *supF* mutation frequency of pTW-A extracted from SY302; i.e., 1.2×10^{-6} (13).

In the four plasmids, mutations were predominantly single base substitutions (Table 1), among which G:C→T:A transversions were the most common (Table 2). In the pTW-T, pTW-G, and pTW-C, relative to pTW-A, A:T→G:C transitions increased, while G:C→C:G transversions decreased (Table 2).

Characterization of mutational specificity and the effects of the 3′-flanking base on G:C→T:A transversion in mutM mutY cells. The distribution and type of base substitution mutations of pTW-A, pTW-T, pTW-G, and pTW-C are shown in Fig. 3. Due to the absence of 8-oxoG repair in SY302, mutations of G:C→T:A in these strains directly reflect replication miscoding (11). Among 35 G:C→T:A transversions in pTW-A, 11 were at the position 133 hotspot, 35 G:C→T:A transversions in pTW-T, 0 was at the position 133 hotspot, 26 G:C→T:A transversions in pTW-G, 10 were at the position 133 hotspot and 37 G:C→T:A transversions in pTW-C, 5 were at the position 133 hotspot (Table 2 and Fig. 3). Statistical analysis indicated that the frequencies of occurrence of G:C→T:A transversion at position 133 were significantly different among the four plasmids ($\chi^2 = 18.416$, degree of freedom = 3, $P < 0.0004$). In a simultaneous test procedure (19), the frequencies of occurrence of G:C→T:A transversion at position 133 were not significantly different among pTW-A, pTW-G, and pTW-C ($\chi^2 = 5.546$, degree of freedom = 3, $P > 0.05$), between pTW-A and pTW-G ($\chi^2 = 0.327$, degree of freedom = 3, $P > 0.05$) or between pTW-C and pTW-T ($\chi^2 = 5.083$, degree of freedom = 3, $P > 0.05$). These observations indicated that the frequency of occurrence of G:C→T:A transversion at position 133 for pTW-T was significantly lower than that for pTW-A and pTW-G. The frequency of occurrence of G:C→T:A transversion at position 133 for pTW-C was lower than that for pTW-A and pTW-G and was not different from that for pTW-T, but the difference of the frequency of occurrence of G:C→T:A transversion at position 133 was not significant among pTW-A, pTW-G, and pTW-C.

The results of these experiments indicated that the nature of the base at position 132 had a clear influence

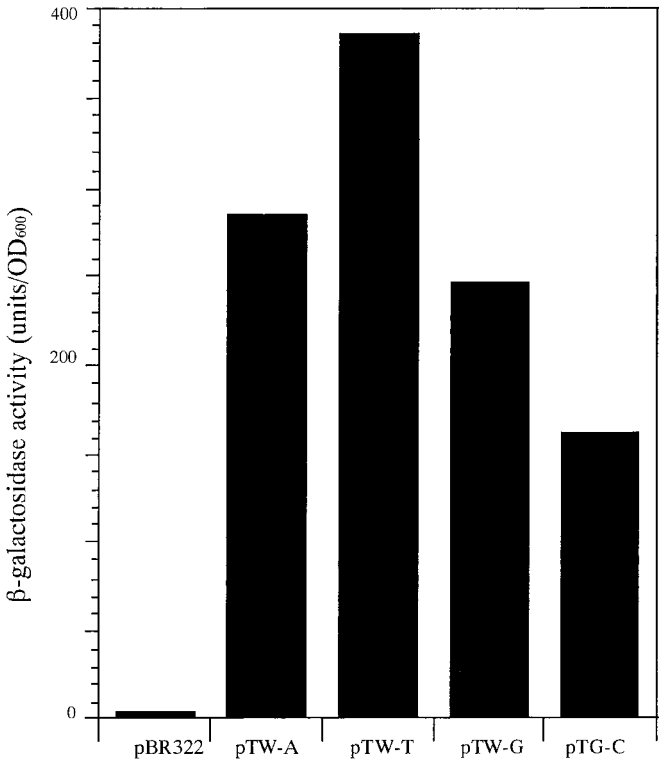

FIG. 2. Suppressor activity of variant *supF* genes in KS40 (*lac-Z*am) hosts. β-Galactosidase activities of exponentially growing KS40 with various plasmids were measured. The result is the average of two independent experiments.

TABLE 1

Number (%) of Spontaneous Mutations in the Variant *supF* Genes in *mutM mutY* Cells

	SY30 (wild-type)[a]	SY302 (*mutM*::Tn*10* *mutY*::Km)			
Class of changes	pTW-A	pTW-A[a]	pTW-T	pTW-G	pTW-C
Base substitution	17 (35)	54 (64)	50 (66)	51 (73)	57 (71)
Frameshift	3 (6)	9 (11)	6 (8)	9 (13)	6 (8)
Deletion/IS sequence	28 (59)	21 (25)	20 (27)	10 (14)	17 (21)
Total	48	84	76	70	80
Mutation frequency	2.8×10^{-7}	1.2×10^{-6}	1.7×10^{-6}	2.7×10^{-6}	1.0×10^{-6}

[a] Data from Watanabe *et al.* (13); in this case, the plasmid used was pTW-F. All constituents of *supF* in pTW-F and pTW-A were the same. Thus, for convenience, we described here pTW-A, but not pTW-F.

of G:C→T:A transversion at position 133. Hotspot 133 constitutes a 5′-AGA-3′ motif. Changing AGA (pTW-A) to AGG (pTW-G) did not affect 8-oxoG mutational activity. However, changing to AGT(pTW-T) or AGC (pTW-C) did reduce 8-oxoG mutational activity.

The spectra of pTW-T, pTW-G, and pTW-C showed significant differences to the pattern of pTW-A such that a new A:T→G:C transition hotspot at position 112 appeared. In pTW-T, a G:C→T:A transversion hotspot at 160 appeared. In pTW-G, G:C→T:A transversion hotspots at 159, 168, and 169 disappeared. These results indicated that the nature of the base at 132 affected mutagenesis on not only nearby bases but also those at a distance of more than 20 bases.

DISCUSSION

8-oxoG is one of the most prominent base oxidation products (20) and has been implicated in mutagenesis, carcinogenesis, and aging (21). It has been shown to cause G:C→T:A transversion (11, 22). In our previous 8-oxoG mutagenesis experiments, we demonstrated that guanine in the 5′-AGA-3′ sequence context in the anticodon site at position 133 in the *supF* gene is a strong hotspot (8, 13). The goal of our study was to understand the nearby base effects of 8-oxoG mutational hotspots. We developed a vector system that allows 133 *supF* hotspot sequence 5′-AGA-3′ manipulation to 5′-AGT-3′, 5′-AGG-3′, and 5′-AGC-3′. The modified *supF* genes were active. The plasmids were transfected into 8-oxoG repair-deficient *E. coli.*

We found that the nature of the base at 132 strongly affected the frequency of G:C→T:A transversion at position 133 (Fig. 3). When the 3′ base was adenine or guanine, G at position 133 was a hotspot for G:C→T:A transversion, whereas with thymine or cytosine at this position, transversion was not observed or was reduced. Thus, we can easily modify the 8-oxoG position 133 hotspot site by exchanging 3′ nearby base from a purine to pyrimidine. Previously, Akasaka and Yamamoto (8) reported that guanine at the center of 5′-PuGA-3′ was a putative hotspot for G:C→T:A transversion. The present results are consistent with their arguments about the influence of nearby bases on 8-oxoG mutagenesis.

Our finding that hotspots for 8-oxoG mutagenesis occurred at the sequence of 5′-PuGA-3′, agreed with those of Hatahet *et al.* (23). They studied sequence contexts that significantly enhanced the mutagenic potential of 8-oxoG *in vitro.* To do so, they isolated se-

TABLE 2

Number (%) of Spontaneous Base Substitution Mutations in the Variant *supF* Genes in *mutM mutY* Cells

	SY30 (wild-type)[a]	SY302 (*mutM*::Tn*10* *mutY*::Km)			
Type of mutation	pTW-A	pTW-A	pTW-T	pTW-G	pTW-C
Transition					
G:C→A:T	7 (41)	4 (7)	2 (4)	2 (4)	3 (5)
A:T→G:C	0 (–)	5 (9)	10 (20)	21 (41)	16 (28)
Transversion					
G:C→T:A	0 (–)	35 (65)	35 (70)	26 (51)	37 (65)
G:C→C:G	5 (29)	6 (11)	1 (2)	0 (–)	0 (–)
A:T→T:A	5 (29)	2 (4)	0 (–)	2 (4)	0 (0)
A:T→C:G	0 (–)	2 (4)	2 (4)	0 (–)	1 (2)
Total	17	54	50	51	57

[a] Data from Watanabe *et al.* (13); also see the legend of Table 1.

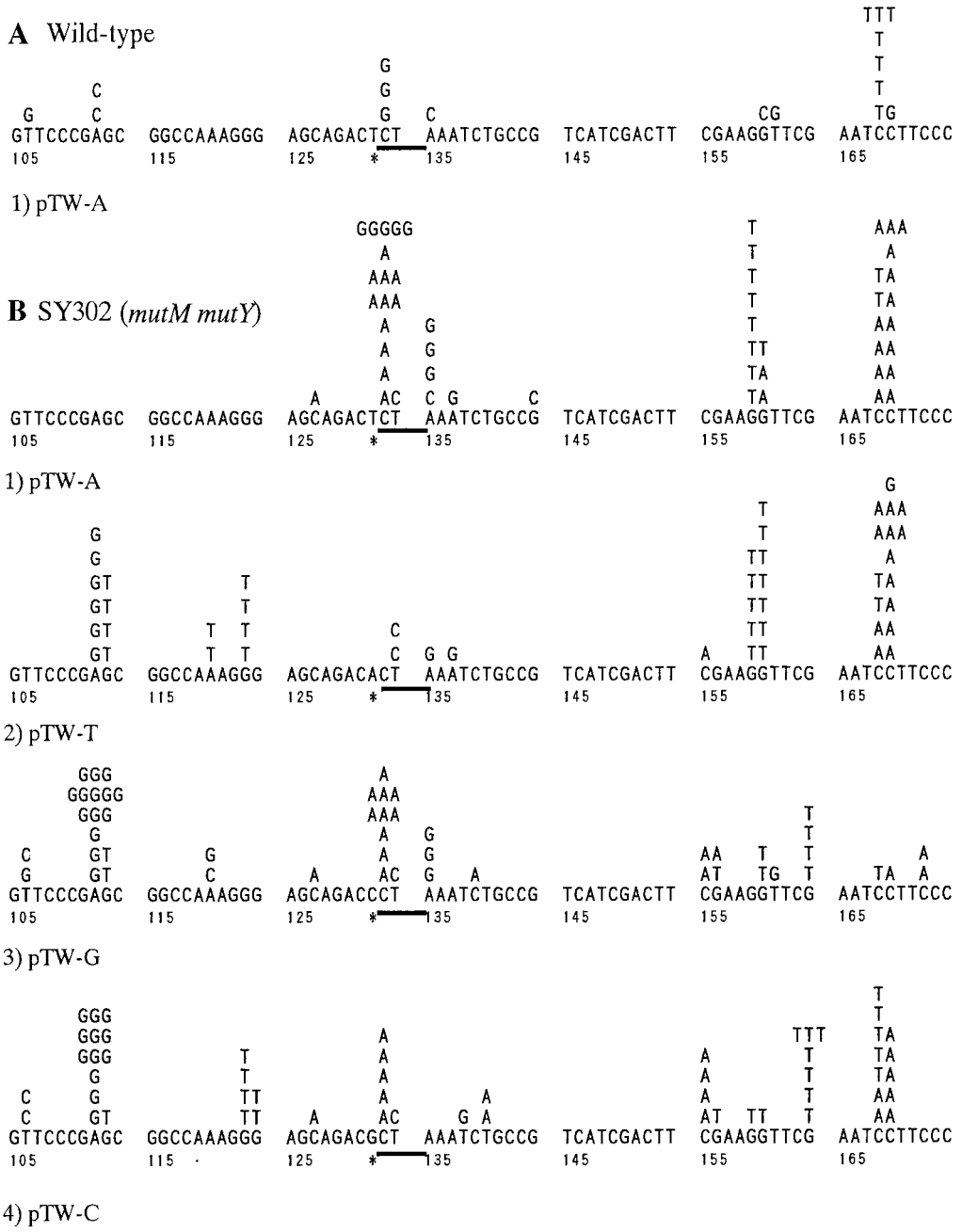

FIG. 3. Location of base substitution mutants of the *supF* genes. (A) Wild-type *supF* gene in the wild-type host (13). (B) Variant *supF* genes (pTW-A, pTW-T, pTW-G, and pTW-C) in the SY302 (*mutM mutY*) host. For each distribution, only the region of the nontranscribed strand mutations is shown. Numbering is in accordance with Seidman *et al.* (18). The anticodon is underlined. Altered bases at position 132 are marked with asterisks.

quence contexts that simultaneously reduced the efficiency of 8-oxoG cleavage by the formamidopyrimidine DNA N-glycosylase (Fpg) and increased the efficiency of miscoding A opposite 8-oxoG by DNA polymerase from a pool of 4^8 random octanucleotide sequences *in vitro.* From these experiments, they concluded that the 5′-G(8-oxoG)A-3′ context is a putative hotspot. Based on a database search, the authors further reported that G:C→T:A transversions are significantly over-represented in the 5′-GGA-3′ hotspot context in the *E. coli lacI* gene, human *p53* gene, and human factor IX gene (23 and citations therein). These *in vitro* and *in vivo* data agree well with our results indicating that 5′-PuGPu-3′ is the 8-oxoG hotspot context.

The 5′-G(8-oxoG)A-3′ sequence context was shown to support higher efficiency of misincorporation A opposite the lesion by T_4 DNA polymerase *in vitro* relative to other contexts (23). Fpg, which acts on 8-oxoG paired

with C, reduced the efficiency of removing 8-oxoG within the putative hotspot 5′-G(8-oxoG)A-3′ paired with C (23). Thus, at least two mechanisms can operate to make G within 5′-PuGPu-3′ G:C→T:A transversion mutable.

In a previous study, Koch (24) reported that a base pair change one or two sites away can alter mutation rates, but the magnitudes of the effects decreased with distance. However, Sugino and Drake (25) demonstrated a nonneighbor base pair effect on site specific mutabilities; a single base pair change altered mutation rates at sites more than 10 base pairs away. Thus, the nonneighbor base pair effect appeared to be highly site-specific and was not simply related to distance. In this study, we observed that changing of A at position 132 to T, G, or C altered mutability at not only the nearby site (position 133) but also at a position 20 bases away (position 112), where G:C→A:T transition was increased (Fig. 3). In pTW-T as compared to pTW-A, G:C→T:A transversion at position 160 was increased, and in pTW-G, G:C→T:A transversion at position 169 was decreased (Fig. 3). These results strongly suggested the existence of long range effects on hotspots. Although our results did not suggest any possible mechanisms for the long range effects, Sugino and Drake (25) favored explanations based on DNA-protein interaction during DNA synthesis.

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