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Reevaluation of the role of DNA polymerase θ in somatic hypermutation of immunoglobulin genes

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Abstract

DNA polymerase θ has been implicated in the process of somatic hypermutation in immunoglobulin variable genes based on several reports of alterations in the frequency and spectra of mutations from *Polq*$^{-/-}$ mice. However, these studies have contrasting results on mutation frequencies and the types of nucleotide substitutions, which question the role of polymerase θ in hypermutation. DNA polymerase η has a dominant effect on mutation and may substitute in the absence of polymerase θ to affect the pattern. Therefore, we have examined mutation in mice deficient for both polymerases θ and η. The mutation frequencies in rearranged variable genes from Peyer's patches were similar in wild type, *Polq*$^{-/-}$, *Polh*$^{-/-}$, and *Polq*$^{-/-}$*Polh*$^{-/-}$ mice. The types of substitutions were also similar between wild type and *Polq*$^{-/-}$ clones, and between *Polh*$^{-/-}$ and *Polq*$^{-/-}$*Polh*$^{-/-}$ clones. Furthermore, there was no difference in heavy chain class switching in splenic B cells from the four groups of mice. These results indicate that polymerase θ does not play a significant role in the generation of somatic mutation in immunoglobulin genes.

Keywords

Somatic hypermutation; Class switch recombination; Immunoglobulin gene; DNA polymerase θ; DNA polymerase η

1. Introduction

Somatic hypermutation of immunoglobulin variable and switch regions is initiated by the activation-induced cytosine deaminase protein which deaminates cytosine to uracil in DNA [1,2]. Uracil can either be faithfully repaired in the base excision repair pathway [3], or become mutagenic in the hypermutation pathway, when it is removed by uracil DNA glycosylase (UNG) [4], or recognized as a U:G mismatch by MSH2–MSH6 mismatch repair proteins [5, 6]. Mutations can be made when low-fidelity DNA polymerases copy UNG-generated abasic sites or synthesize repair patches made by MSH2–MSH6 and exonuclease 1 [7]. Eight low-fidelity polymerases have been studied for their role in this process, using mice deficient for the polymerases. Polymerases ι [8], κ [9], λ [10], and μ [10] are not involved, and polymerase η (pol η, encoded by the *Polh* gene) [11,12] and Rev1 [13] are involved. The role of polymerase

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ζ is less clear [14], due to the nonviability of gene-deficient mice, and, as discussed below, the role of polymerase θ (pol θ, encoded by the *Polq* gene) is controversial.

Concerning pol θ, a report by Casali and coworkers [15] stated that the polymerase has a dominant role in hypermutation since mice deficient for the protein (pol θ-null) had a dramatic decrease in the frequency of mutations and an increase in transitions of G:C base pairs (bp). These mice contained a modification of exon 1 and a deletion of exons 2–5 encoding pol θ [16]. In marked contrast, a study by O-Wang and colleagues [17] reported that their pol θ-null mice, with a deletion of exons 1–3, had only a mild reduction in the number of mutations and an increase in G to C transversions. The O-Wang group [18] also studied mutation in mice missing exons 25 and 26 in the polymerase domain of pol θ (pol θ-inactive), and found an actual decrease in mutations of G:C bp. Finally, a recent paper [19] by their lab looked at hypermutation in mice doubly deficient for pol θ and pol η to see if the absence of two polymerases compounded the phenotype. The results demonstrated that the absence of pol θ did not change the frequency or pattern of mutation caused by the lack of pol η, which was a substantial decrease in mutations of A:T pairs. Although pol θ did not change the mutations induced by pol η, the authors concluded that pol θ functions in the same genetic pathway as pol η, raising the question about what pol θ does.

Because of the above differences in the literature, we have reexamined the role of pol θ in $Polq^{-/-}$ mice and $Polq^{-/-}Polh^{-/-}$ double knockouts. After measuring the frequency of mutation, spectra of mutations, and frequency of heavy chain class switching, we conclude that pol θ has no major involvement in somatic hypermutation.

2. Materials and methods

2.1. Mice

All mice were on a C57BL/6 background, and bred to homozygousity in our animal facilities. $Polq^{+/-}$ mice were originally obtained from Schimenti [16]; $Polh^{+/-}$ mice were from Hanaoka [20]; and $Polq^{-/-}Polh^{-/-}$ mice were produced by crossing the respective strains. Genotypes were confirmed by PCR of tail DNA using the following primers. For pol θ, primer sequences were provided by Schimenti. The wild type *Polq* gene was detected using Polqe3L, 5′ TGCAGTGTACAGATGTTACTTTT, and Polq21R, 5′TGGAGGTAGCATTTCTTCTC, which generated a 150-bp band containing exon 3. For the disrupted gene lacking exons 2–5, generic neomycin primers were IMR13, 5′CTTGGGTGGAGAGGCTATTC, and IMR14, 5′ AGGTGAGATGACAGGAGATC, which generated a 280-bp band containing the neomycin resistance gene. For pol η, the wild type *Polh* gene was detected using primer 1, 5′ GTAGTCTGGGGGGTTGAATC, and primer 2, 5′TTTCGATCTTTGGTTAGCCTCTCC, which generated a 1.95-kb band containing exons 8 ands 9. For the disrupted gene lacking exon 8, primer 1 was used with primer 3, 5′GTCTGTTGTGCCCAGTCATAGC, which generated a 1.4-kb band containing part of the *Polh* intron and the neomycin resistance gene. All animal procedures were reviewed and approved by the NIA Animal Care and Use Committee.

2.2. Hypermutation

Cells from the Peyer's patches of 2–8 mice, age 4–8 months, from each genotype were stained with phycoerythrin-labeled antibody to B220 (eBioscience, CA) and fluorescein-labeled peanut agglutinin (EY Laboratories, CA). Cells binding both anti-B220 and peanut agglutinin were isolated by flow cytometry, and DNA was prepared. The 492-bp intron region downstream of J_H4 from rearranged V_HJ558 genes was amplified using previously described forward and reverse primers [21]. The amplified DNA was then TA-cloned into pGEM-T Easy vector (Promega, WI) and sequenced.

2.3. Class switch recombination

Splenic B cells from two to five mice, age 3–4 months, of each genotype were isolated using negative selection with anti-CD43 and anti-CD11b magnetic beads (Miltenyi Biotech, CA), following the manufacturer's directions. Isolated B cells were plated at a density of 0.2–0.5 million cells per ml. The cells were stimulated with 5 μg/ml *Escherichia coli* lipopolysaccharide (LPS) serotype 0111:B4 (Sigma–Aldrich, MO) alone to induce switching to IgG3; LPS plus 5 ng/ml mouse IL-4 (BD Biosciences, NJ) to induce switching to IgG1; LPS plus 25 ng/ml IFNγ (R&D Systems, MN) for switching to IgG2a; or LPS plus 2 ng/ml TGF-β (R&D Systems, MN) for switching to IgG2b. After 3–4 days, the cells were stained with fluorescein-conjugated antibody to B220 (Southern Biotech, AL) and phycoerythrin-conjugated antibodies to mouse IgG1, IgG2a, IgG2b or IgG3 (Southern Biotech, AL) for analysis by flow cytometry.

3. Results

3. 1. Similar mutation frequencies in J_H4 introns from Peyer's patches of mice deficient for pol θ and pol η

The J_H4 intronic region from Peyer's patch B cells was analyzed for three reasons: (1) DNA was amplified from primers recognizing the middle of several V_HJ558 genes and the intronic region downstream of these rearranged genes, so the clones derive from a heterogeneous population, (2) the intron contains unselected mutations, and (3) Peyer's patch cells contain a high frequency of mutations due to continual stimulation from gut bacteria. For each of the four genotypes, over 100 clones were examined, and around 300 substitutions were recorded (Table 1). About half of the clones contained mutations, as shown in Fig. 1A. The averages of the mutation frequencies are plotted in Fig. 1B, and are around 5×10^{-3} mutations per bp sequenced. There was no statistically significant difference in frequency between wild type and *Polq*$^{-/-}$ clones ($p > 0.2$, χ^2 test). The range of frequencies is somewhat broad, as it reflects the arbitrary exposure of Peyer's patch B cells to environmental antigens.

3.2. The absence of pol θ does not have a major effect on the spectrum of substitutions

The types of nucleotide changes were recorded from the nontranscribed DNA strand, and are shown in Fig. 2A. The spectra of substitutions were similar between wild type clones and *Polq*$^{-/-}$ clones, with an increase in mutations of A compared to the complementary T, and an equal number of mutations of G and C bases. Zan et al. [15] reported that *Polq*$^{-/-}$ clones had an increase in transitions of G:C and A:T, and specifically an increase in G to A transitions, which was not apparent in this study (Table 1 and Fig. 2B). Masuda et al. [17] found an increase in G to C transversions in the absence of pol θ, which again was not seen here (Fig. 2B). The absolute frequencies of mutations of A:T bp compared to mutations of G:C bp is recorded in Table 1. Wild type clones had a frequency of mutation of A:T bp of 0.31%, and mutation of G:C bp of 0.23%. For *Polq*$^{-/-}$ clones, the frequencies were moderately lower for both A:T and G:C bp (0.23% and 0.18%, respectively). As previously reported [20], *Polh*$^{-/-}$ clones have a decreased frequency of mutations of A:T bp (0.087%), and subsequent rise in mutations of G:C bp (0.40%), and mice doubly deficient for both pol 3 and pol η had the same mutational spectrum as *Polh*$^{-/-}$mice.

3.3. Class switch recombination is normal in mice deficient for pols θ and η

Splenic B cells were stimulated in vitro with LPS and several cytokines to induce switch recombination to various heavy chain classes. After culture for 3–4 days, B cells were stained with fluorescent antibodies to the individual constant regions, and the percentage of cells that switched was measured by flow cytometry (Fig. 3A). Switching was compared among wild type mice, mice heterozygous for pols θ and η, and mice singly and doubly deficient for those

polymerases. Five experiments were performed, and the average frequency of switching was normalized to the percent switching in wild type mice. As seen in Fig. 3B, there was no significant difference between mice with and without pols θ and η.

4. Discussion

DNA polymerases are responsible for generating base substitutions in immunoglobulin genes during the process of somatic hypermutation. The types of mutation can be grouped into three categories. (1) Transitions of G:C can occur by replication past dU with high fidelity polymerases such as polymerase δ or polymerase *ε*. (2) Transversions of G:C may be due to replication past an abasic site caused by removal of U by UNG. Rev1, a cytidyl transferase, has been shown to play in role in causing G:C to C:G transversions in mice [13] and the chicken DT40 cell line [22]. However, another low fidelity polymerase must generate the G:C to T:A transversions. (3) Transitions and transversions of A:T are likely caused by pol η. Humans and mice deficient for pol η have a 80% reduction in A:T substitutions [11,12,21]. Another polymerase could generate the residual A:T mutations seen in the absence of pol η. Thus, it is logical to examine mice deficient for different polymerases to identify the ones that produce G:C to T:A transversions and the residual A:T mutations found in the absence of pol η.

The low fidelity polymerase pol θ is a candidate for hypermutation, since it is highly expressed in lymphoid tissues, including germinal centers [23]. Pol θ has a distinguishing characteristic of bypassing an abasic site by inserting an A, which would produce G:C to A:T transitions at the UNG-generated abasic sites, and it can efficiently extend the A to continue DNA synthesis [24]. Pol θ also has the ability to extend from mismatched base pairs generated by its own low-fidelity synthesis or generated by other DNA polymerases such as pol η [25]. If pol θ was involved in hypermutation, one would expect to see a decrease in G:C transitions in its absence, and a decrease in the overall mutation frequency, since DNA synthesis would stop at the multiple abasic sites caused by UNG, and mismatches would not be efficiently extended.

Both these effects were reported by two research groups, but to varying degrees. Casali and colleagues [15] used pol θ-null mice from the Schimenti lab which had a stop codon in exon 1 and deletion of exons 2–5 [16], so it is unlikely that any protein was produced in these mice. They reported a 60–80% decrease in the mutation frequencies of rearranged V_H genes from spleen and in the J_H4 intron from Peyer's patch cells. They also found an increase in transitions of G:C and A:T pairs. However, we have studied the same Schimenti mice and found no significant decrease in mutation in the J_H4 intron from Peyer's patch cells. There was no difference in the percentage of transition mutations compared to those from wild type clones. As the frequency of mutation in wild type mice was similar in both the Zan et al. [15] report and this study, it is unclear why the mutation frequencies in the pol θ-deficient mice are so disparate.

O-Wang and colleagues have studied two genetic models of pol θ: a mouse with an inactive form of the enzyme [18], and a mouse deficient for the enzyme [17]. The pol θ-inactive mice produced a protein, but it lacked catalytic activity because exons 25 and 26 were deleted. The authors reported a 20% decrease in mutations in V_H genes from spleens of immunized mice. There was a moderate decrease in mutations of G:C compared to those of A:T, suggesting that pol θ participates in synthesizing mutation opposite an abasic site caused by removal of U by UNG. The pol θ-null mice had a deletion of exons 1–3 and produced no RNA transcripts, so there would be no protein in these mice. The authors again found a 20% reduction in total mutations, but now both G:C and A:T mutations were affected. A significant increase in G to C transversions was reported, although this was not observed in the *Polq*$^{-/-}$clones in this study or elsewhere [15,19].

To see if pol θ had a more prominent role in hypermutation when the dominant pol η polymerase was absent, we studied mice deficient for both enzymes. Our data showed no difference in the frequency and spectra of mutations in the J_H4 intronic regions from *Polq*$^{-/-}$*Polh*$^{-/-}$ mice compared to those from *Polh*$^{-/-}$ mice. These results, which are similar to those reported by Masuda et al. [19], support the commanding role of pol η in hypermutation, and suggest that pol θ is a minor player at best, even in the absence of pol η. The lack of pol θ and pol η did not affect heavy chain class switch recombination, as measured by an in vitro assay of stimulating spleen cells in the presence of mitogen and cytokines. Switching to IgG1, IgG2a, IgG2b, and IgG3 was comparable to switching in wild type cells and cells heterozygous for the polymerases. Thus, the enzymatic activities of these two polymerases likely do not generate or resolve recombinational substrates in the immunoglobulin switch regions.

O-Wang and colleagues have suggested that pol θ functions in the same pathway as pol η by extending mismatches formed by pol η [19]. The basis of this hypothesis rests on the 20% decrease in mutation frequency in *Polq*$^{-/-}$ clones compared to wild type clones. In our study, the range in frequencies found in pol θ-null mice overlapped with the range seen in experiments from wild type mice, and the difference was not significant. There is still a possibility that pol θ occasionally extends mismatches introduced by pol η. However, pol η has been shown to bypass abasic sites [26,27] and extend mismatches [28] by itself, and may not require a second polymerase for this function in vivo. Thus, the data do not support a compelling role for pol θ in the hypermutation pathway.

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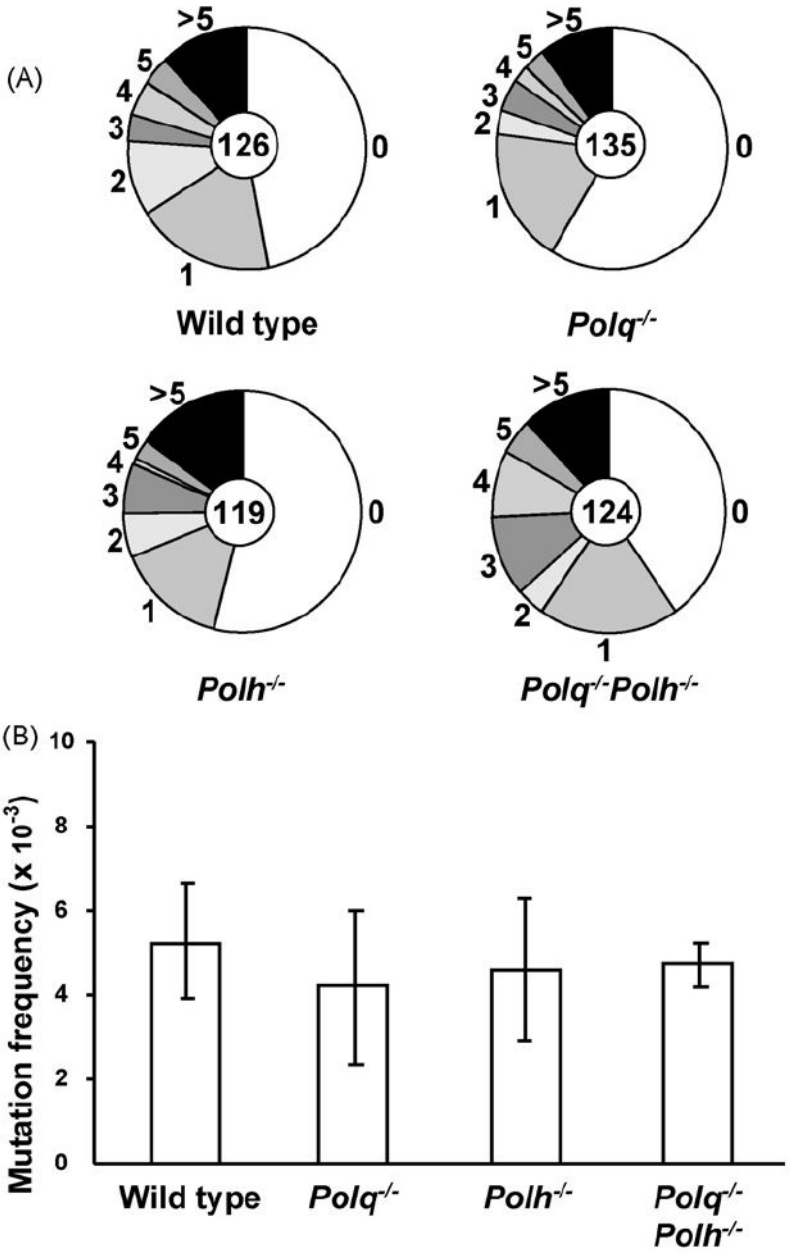

Fig. 1. Frequencies of hypermutation in Peyer's patches from mice deficient for pol θ and pol η

(A) The total number of clones analyzed is shown in the center of each circle. Segments represent the proportion of clones that contain the indicated number of mutations. (B) The mean frequency with error bars from two to three experiments is depicted as mutations per bp $\times 10^{-3}$. Frequencies are as follows: wild type mice, three experiments, 6.7×10^{-3} (3 mice), 5×10^{-3} (three mice), and 3.9×10^{-3} (two mice); *Polq*$^{-/-}$ mice, two experiments, 2.5×10^{-3} (two mice), and 6×10^{-3} (one mouse); *Polh*$^{-/-}$ mice, three experiments, 6.4×10^{-3} (two mice), 4.1×10^{-3} (three mice), and 3.0×10^{-3} (one mouse); and *Polq*$^{-/-}$*Polh*$^{-/-}$ mice, two experiments, 5.3×10^{-3} (one mouse), and 4.2×10^{-3} (one mouse).

(A) **Wild type (301 mutations)**

From \ To	A	T	G	C	Total
A	-	11	22	4	37
T	4	-	3	14	21
G	15	2	-	3	20
C	6	13	3	-	22

***Polq*$^{-/-}$ (272 mutations)**

From \ To	A	T	G	C	Total
A	-	15	14	9	38
T	4	-	4	11	19
G	14	5	-	4	23
C	3	15	2	-	20

***Polh*$^{-/-}$ (285 mutations)**

From \ To	A	T	G	C	Total
A	-	4	1	5	10
T	1	-	3	3	7
G	21	5	-	11	37
C	8	30	8	-	46

***Polq*$^{-/-}$*Polh*$^{-/-}$ (294 mutations)**

From \ To	A	T	G	C	Total
A	-	3	4	6	13
T	2	-	1	1	4
G	25	4	-	11	40
C	7	29	7	-	43

(B)

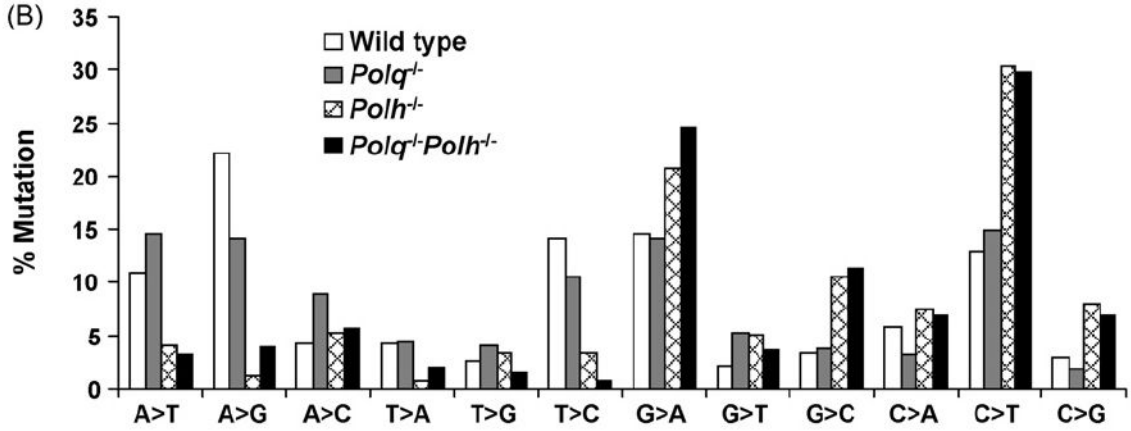

Fig. 2. Types of substitutions in *Polq*$^{-/-}$ and *Polh*$^{-/-}$ clones

(A) Mutations were recorded from the nontranscribed strand and have been corrected for base composition of the nucleotide sequence. Data are expressed as percent of the number of mutations. (B) Each category is plotted in groups to facilitate comparison between genotypes.

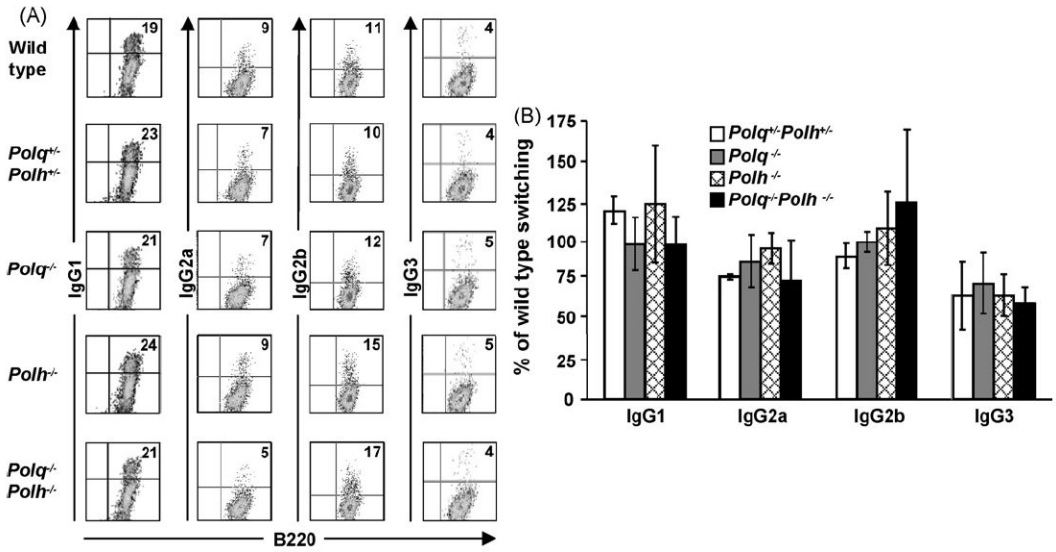

Fig. 3. Heavy chain class switching after in vitro stimulation

(A) Spleen cells were stimulated with LPS and a variety of cytokines for 4 days. Cells were stained with antibodies to several isotypes and analyzed by flow cytometry. The percentage of cells that switched is shown in the upper right quadrant. (B) Data from five experiments of cells stimulated for 3–4 days were normalized to the percent switching in wild type mice, which is expressed as 100%.

Table 1

Comparisons of mutation in the J_H4 intron in Peyer's patch B cells from *Polq*$^{-/-}$ and *Polh*$^{-/-}$ mice

	Wild type	*Polq*$^{-/-}$	*Polh*$^{-/-}$	*Polq*$^{-/-}$*Polh*$^{-/-}$
Number of sequences	130	135	119	124
Mutated sequences (%)	53	41	46	60
Total mutations	301	272	285	294
Total bp	55,820	66,420	58,548	61,008
Mutations/bp	5.4×10^{-3}	4.1×10^{-3}	4.9×10^{-3}	4.8×10^{-3}
Mutations at A:T (%)[a]	58 (0.31)[b]	57 (0.23)	18 (0.087)	17 (0.082)
Mutations at G:C (%)	42 (0.23)	43 (0.18)	82 (0.40)	83 (0.40)
Transitions (%)	53	54	56	59
Transversions (%)	47	46	44	41
Transitions:Transversions at A:T (%)	43:57	44:56	25:75	27:73
Transitions:Transversions at G:C (%)	67:33	67:33	62:38	65:35

[a] Data are recorded from the nontranscribed strand and are corrected to represent a sequence with equal amounts of the four nucleotides.

[b] Parentheses contain the absolute frequency of substitutions, determined by dividing the number of mutations at A and T, or G and C, by the total bp sequenced. Values are expressed as %.