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Influence of the Duplication of *CFTR* Exon 9 and Its Flanking Sequences on Diagnosis of Cystic Fibrosis Mutations

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The DNA sequences of seven regions in the human genome were examined for sequence identity with exon 9 of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which is mutated in cystic fibrosis, and its intronic boundaries. These sequences were 95% to 96% homologous. Based on this nucleotide sequence similarity, PCR primers for *CFTR* exon 9 can potentially anneal with other homologous sequences in the human genome. Sequence alignment analysis of the *CFTR* exon 9 homologous sequences revealed that five registered mutations in the Cystic Fibrosis Mutation Database may be due to the undesired annealing of primers to a homologous sequence, resulting in inappropriate PCR amplification. For this reason, we propose that certain pseudomutations may result from the similarity between *CFTR* exon 9 (and its flanking introns) and related sequences in the human genome. Here we show that two mutations previously described in the CFTR database (c.1392 + 6insC; c.1392 + 12G>A) were inappropriately attributed to two individuals who sought carrier testing. A more detailed study by either direct sequencing or subcloning and sequencing of PCR products using specially designed primers revealed that these apparent mutations were not, in fact, present in *CFTR*. In addition, we present new PCR conditions that permit specific amplification of *CFTR* exon 9 and its flanking regions. *(J Mol Diagn 2009, 11:488–493; DOI: 10.2353/jmoldx.2009.090005)*

The cystic fibrosis (CF) gene, encoding the cystic fibrosis transmembrane conductance regulator (CFTR), is located on the long arm of chromosome 7, at position 7p31. CFTR is involved in the active transport of ions through the apical membrane of epithelial cells.[1] The 250-kb gene, containing 27 exons, appears highly susceptible to mutations due to its large size.[2] More than 1500 genetic alterations have been described to date. Most are disease-causing mutations; about half lead to amino acid substitutions (missense mutations), 20% lead to splicing errors, and 30% appear to be nonsense and frame shift (including small deletions and insertions) or promoter mutations (*http://www.genet.sickkids.on.ca/cftr/*, last accessed November 29, 2006). Moreover, the type and distribution of mutations vary substantially between populations.[3,4]

Previous studies on the *CFTR* gene have reported that exon 9 and its flanking introns are present in multiple copies in the human genome. Indeed, this region is part of the large duplicated sequence unit LCR7-20 (low-copy repeats 7 to 20), which is dispersed on different chromosomes in human genome.[5,6]

Screening for *CFTR* exon 9 mutations is difficult due to the polymorphism of the (TG)*m* (T)*n* repeats located at the end of intron 8. This variation eludes common PCR-based techniques for mutation detection in this region, including direct sequencing, as well as denaturing high-performance liquid chromatography. Thus, if denaturing high-performance liquid chromatography analysis is used, it is necessary to use primers that have been documented[7] to prevent the variability that T/TG repeats can cause. Using this method, however, only the beginning of exon 9 is amplified. Because this region of *CFTR* has been duplicated in several regions of the genome, we suggest that using these classical primers could lead to misidentification of a *CFTR* pseudogene mutation as a CF-causative mutation.

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Here, we analyzed two cases in which there were molecular diagnosis difficulties in the exon 9 region of *CFTR*. We demonstrate that technical anomalies leading to incorrect molecular diagnosis are due to the primers used in the PCR protocol. The consequence of these primer mismatches could be that multiple mutations already registered in the Cystic Fibrosis Mutation Database (*http://www.genet.sickkids.on.ca/cftr/*; last accessed December 24, 2008; Hospital for Sick Children, Toronto, Canada) could be irrelevant.

To our knowledge, there has been no previous report demonstrating the effect of sequence similarity between *CFTR* exon 9 (encoding a part of the first nucleotide-binding domain[8]) and related sequences on *CFTR* mutation screening. Therefore, our primary aim in this paper is to provide evidence of mutations in the database that are in fact pseudomutations in duplicated regions of the genome, with normal sequences in exon 9 of the *CFTR* gene. Since traditional methods for amplification of exon 9 and its flanking sequences will amplify several ectopic regions on chromosomes other than chromosome 7, we define here conditions that can be used to study this region exclusively. We suggest that each patient who presents such mutations should be re-examined by our proposed method.

Cases

The first case was a pregnant woman (UNP 10186, French origin) tested at Poitiers Hospital, because her husband was heterozygous for a mutation in *CFTR*. The c.1392 + 6insC and c.1392 + 12G>A mutations were identified in intron 9 (T. Bienvenu, C. Cazaneuve, J. C. Kaplan, B. Neldjord, personal communication). Following this result, the parents of case 1 were tested at Bordeaux Hospital. Surprisingly, only one mutation was identified in her father (p.Asp443Tyr in exon 9), and no mutation was found in her mother. The same result (p.Asp443Tyr mutation) was obtained in Bordeaux with case 1. Using microsatellites analysis, the Bordeaux laboratory confirmed that no error of sampling (blood or DNA) was made between case 1 and her father in Poitiers or Bordeaux. For these reasons, Poitiers Hospital analyzed this case again using the original blood sample and a freshly drawn blood sample. The new analysis for this family confirmed the previous results: case 1 carries the c.1392 + 6insC and c.1392 + 12G>A mutations in intron 9, but not the Asp443Tyr mutation in exon 9. On analysis of fresh parental samples at Bordeaux Hospital, the previous parental results were also confirmed, showing that her father must be carrier for Asp443Tyr mutation in exon 9.

The second case was a pregnant woman (UNP 10481, French origin) tested in Poitiers Hospital for genetic counseling of cystic fibrosis. Her family history revealed that her cousin had died of this disease, but the underlying *CFTR* mutations had not been identified. The presence of mutations in the 27 exons and neighboring intronic regions of the *CFTR* gene was assessed by denaturing gradient gel electrophoresis (for exons 3, 4, 6b, 10, 11, 12, 14a, 20, 21) and by denaturing high-performance liquid chromatography (all others). The c.1392 + 6insC and c.1392 + 12G>A mutations were detected by sequencing. Moreover, no other mutations were identified.

Control groups used in these studies consist of: (1) six individuals with no history of CF, as a negative control, and (2) 30 patients with at least one clearly identified pathogenic *CFTR* mutation, as a positive control. The control samples were screened using the same methods used to detect the c.1392 + 6insC and c.1392 + 12G>A mutations in cases1 and 2.

Materials and Methods

DNA Extraction and Sequencing

Blood samples (5 ml) were collected in tubes containing EDTA. All genomic DNA samples were extracted from peripheral blood cells using the QIAamp DNA Blood Mini kit (Qiagen) according to the supplier's protocol. The concentration of DNA in the samples was determined by spectrophotometry to obtain the desired final concentration (5 mg/L for patients and controls [calibrator], and 10 mg/L for control samples used for standard curve). DNA samples were screened for mutations within the CFTR gene by direct sequencing using the PCR primers as described in Table 1. PCR products were purified on Dye Ex 2.0 Spin kit columns (Qiagen) and then subjected to automated sequence analysis, on an ABI Prism 310 Genetic Analyzer using the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems) with targeted primers.

Primer Design

CFTR gene exon 9 primers were tested for their ability to amplify CFTR exon 9 sequences from genomic DNA without interference from other sequences in the human genome. A map of primers in intron 8, exon 9, and intron 9 is shown in Figure 1. Primer sequences, annealing temperatures, and sizes and localization of the PCR products are shown in Table 1.

PCR Amplification

Genomic DNA used for mutation screening was amplified in a total volume of 50 μl containing 100 ng DNA, 1.5 mmol/L $MgCl_2$, 200 mmol/L dNTP, 1 U *Taq* polymerase (Amersham), and 20 pmol of each primer. The conditions used for amplification were as follows: 5 minutes at 94°C; 30 cycles of 30 seconds at 94°C followed by 30 seconds at annealing temperature (see Table 1), and 30 seconds at 72°C; and a final extension step for 5 minutes at 72°C.

Subcloning and DNA Sequencing

PCR products were subcloned into pGEM-T vector (Promega) to provide templates. Plasmid DNA was extracted by alkaline lysis using a plasmid Mega purification kit

Table 1. Primers Used in PCR Amplification and Sequencing of *CFTR* Exon 9 and its Intronic Boundaries

PCR Product	Primers names	Primer sequence (forward primer first)	Amplicon size (bp)	Annealing temp. (°C)
A	CF9.6	5′-TGGGGAATTATTTGAGAAAGC-3′	285	50
	CF9 + 58	5′-CCTTCCAGCACTACAAACTAGAAA-3′		
B	CF9-130	5′-ACAGTGTAATGGATCATGGGC-3′	395	55
	CF9 + 58	5′-CCTTCCAGCACTACAAACTAGAAA-3′		
C*†	CF9-268	5′-TGTATACATGTAGTAATTCAGT-3′	572	55
	CF9 + 121	5′-ACATTCTCCTAATGCTCATG-3′		
D*†	CF9-70	5′-GTACATAAAACAAGCATCTAT-3′	394	56
	CF9 + 121	5′-TGTATACATGTAGTAATTCAGT-3′		
E*‡	CF9-70	5′-GTACATAAAACAAGCATCTAT-3′	710	54
	CF9 + 437	5′-TCTAAATCTATTGAAAATTG-3′		
F	CF9-184	5′-CCTCTAGAAACCGTATGC-3′	364	54
	CF9.181	5′-TGCCTGCTCCAGTGGAT-3′		

*Indicates specific PCR amplification for *CFTR* exon 9 and its flanking introns.
†Two sets of primers were used in which the forward primers are outside the region of similarity, but the reverse primers are inside this region.
‡Another set of primers was used in which both the forward and reverse primers are outside the region of similarity. This primer design allows the selective amplification of the target region, avoiding amplification of any similar sequences.

(Qiagen). The inserted DNA was subjected to automated DNA sequencing (as described above). Sequences were deduced from data obtained for both strands.

Nucleotide Sequence Analysis

Sequence homology searches against *CFTR* exon 9 and flanking intronic regions were executed by means of BLAST (Basic Local Alignment Search Tool) computer-based analyses, made available through the National Center for Biotechnology Information.[9,10] Surprisingly, these sequences had high identity with sequences on other chromosomes. Another similarity was observed in the intronic boundaries of *CFTR* exon 9. These sequences were aligned/compared with human sequences in the GenBank database using the CLUSTALW program (*www.ebi.ac.uk/Tools/clustalw2/index.html,* last accessed 24/12/2008), which inserts gaps into one or the other sequence in a pair wise comparison with maximize similarities between the two sequences.

Data Bank Accession Numbers

Sequences homologous to *CFTR* exon 9 and its flanking introns analyzed in this study were obtained from GenBank accession numbers: NW_001839181.1 Hs9_WGA574_36 chromosome 9 genomic contig; NW_925362.1 HsCraAADB02_461 chromosome 12 genomic contig (Chr 12-a); NW_925284.1 HsCraAADB02_454 chromosome 12 genomic contig (Chr 12-b); NT_011387.8 Hs20_11544 chromosome 20 genomic contig (Chr 20-a); NT_025215.4 Hs20_25371 chromosome 20 genomic contig (Chr 20-b); NW_001840455.1 HsUn_WGA2630_36 genomic contig (Cx); NW_001841138.1 HsUn_WGA3313_36 genomic contig (Cy).

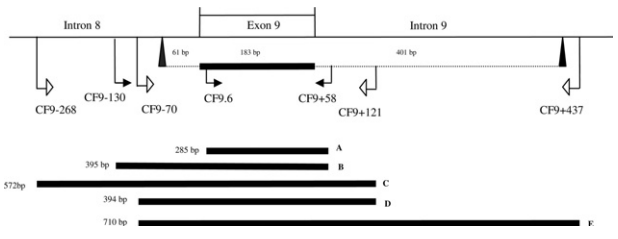

Figure 1. Schematic illustration of primers spanning exon 9 of the human CFTR gene and its intronic boundaries. Black bars, *CFTR* exon 9 coding region; dotted bars, intronic boundary region; **solid arrowheads**, beginning and end of similarity with other sequences in the human genome. DNA fragments were obtained using primers from the laboratories of Poitiers Hospital (**A**) and Bordeaux Hospital (**B**). **Open arrowheads** indicate positions of primers developed in this study for specific PCR amplification of the desired region: (**C** and **D**) mono specific primers and (**E**) double specific primers.

Results

Case 1

Sequence variations were identified for each PCR fragment that had an abnormal DHLPC pattern. The classical primers[7] used in Poitiers Hospital allow amplification of exon 9 and the beginning of intron 9 (Figure 1, PCR product A). Sequencing of the PCR fragments using CF9.6 and CF9 + 58 primers revealed two heterozygous mutations, c.1392 + 6insC and c.1392 + 12G>A. However, PCR using the CF9-130 and CF9 + 58 primers (PCR product B, Bordeaux primers), revealed only the G>T variation at position 1327, corresponding to the p.Asp443Tyr mutation, and not the expected c.1392 + 6insC and c.1392 + 12G>A mutations.

To rule out the possibility that samples had been mishandled, the samples (and controls) were reassessed by PCR using the same primers and conditions; this reanalysis revealed the same mutations identified in the previous analysis. We then tested whether the choice of the primers could have affected the results. Surprisingly, only the p.Asp443Tyr mutation was detected using another primers pair that spans from intron 8 to intron 9, (CF9-268 and CF9 + 121, PCR C, from intron 8 to intron 9 as for Bordeaux primers).

To explain the results obtained, additional PCR amplifications followed by direct sequencing were performed using intronic or exonic primers (Table 1). The results revealed that PCR product F covers only the Asp443Tyr mutation, whereas PCR products obtained using exonic

Table 2. Summary of Clones with Different Genotypes Used for PCR Amplification and Sequencing

Case	Clone genotype	N/N N/N	c.1327G>T(p.Asp443Tyr) N/N	N/N c.1392 + 6insC; c.1392 + 12G> A	c.1327G>T(p.Asp443Tyr) c.1392 + 6insC; c.1392 + 12G> A	Total number of clones tested
Case 1	% clones of PCR A	19	54	27	0	26
	% clones of PCR C	60	40	0	0	15
Case 2	% clones of PCR A	4	26	35	35	15
	% clones of PCR C	67	33	0	0	6

N/N = wild-type genotypes.

forward primers and intronic reverse primers cover the heterozygous c.1392 + 6insC and c.1392 + 12G>A mutations (data not shown).

To investigate the number of different alleles and confirm the unusual result from direct sequencing, PCR products of the region of interest have been cloned. Sequencing analysis of cloned PCR product C showed two alleles: one mutated (p.Asp443Tyr allele) and one wild-type. In contrast, by using cloned PCR (A) product, a third allele is detected containing the c.1392 + 6insC and c.1392 + 12G>A mutations. In addition, we found these apparent mutations in homozygous state, which clearly allows identification of the insertion. Table 2 shows the percentage of each genotype obtained by direct amplification and sequencing, confirming that different experimental protocol yielded o different numbers of alleles.

Case 2

Similar data have been obtained for case 2 either by direct sequencing or by subcloning of PCR products A and C. This individual carries a G>A polymorphism at position 1395 in exon 9. In fact, four alleles could be detected: two with G>A at position 1395 and two with G>T at position 1395 and c.1392 + 6insC; c.1392 + 12G>A. The c.1392 + 6insC and c.1392 + 12G>A mutations were never found without the G>T polymorphism at position 1395.

Most surprisingly, these results show that a different sequence, an apparent copy of *CFTR*, *was* amplified specifically in each case. Indeed, the amplification procedure revealed a G>T polymorphism at position 1395 in case 2 that was never observed in case 1.

DNA Sequence Comparisons

A BLAST search for regions of similarity to *CFTR* exon 9 and its flanking introns revealed an identical sequence in five regions in the human genome, named 20-a, 20-b, 12-a, 12-b, 9, and two genomic contigs named Cx and Cy. An alignment of these sequences was preformed to determine the common region of similarity. The beginning of similarity is located 61 bp upstream and continued 401 bp downstream of the exon 9 (Figure 1).

Comparison of the CFTR Exon 9 Coding Sequence

Exon 9 shares 95% identity with other homologous sequences. Several variations have been identified in exon 9, including four possible mutations shown in Figure 2A: p.Lys464Asn, c.1328_1329delAT, c.1235delC, and p.Asn416ser (*http://www.genet.sickkids.on.ca/cftr/*; last accessed 24/12/2008) [Personal communications: p.Lys464Asn (E. Bleth, V. Gaston, P. Gautry), c.1328_1329delAT (T. Bienvenu, L. Tchertkoff, C. Cazeneuve, C Beldjord), c.1235delC (C. Férec), and p.Asn416Ser (L. Picci, M. Cameran, O. Marangon, D. Marzenta, M. Scarpa)]. Table 3 summarizes the mutations that may be due to these homologous sequences.

Comparison of Intronic Sequences

We also studied the relationship of the intronic boundaries of *CFTR* exon 9 with homologous sequences. The c.1392 + 6insC and c.1392 + 12G>A mutations, previously thought to lie at the beginning of intron 9, were found in all homologous sequences (Figure 2B). Furthermore, an analysis of the number of thymines in a thymine repeat in intron 8 (IVS8) indicated that the duplicated sequences do not affect the T5/T7/T9 polythymidine tract situated in intron 8 near the acceptor splice site for exon 9. Moreover some other PCR products revealed addi-

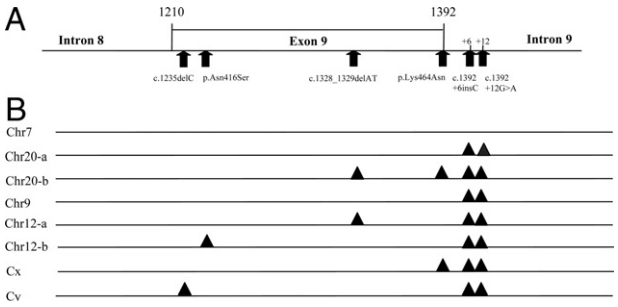

Figure 2. Mutations in human *CFTR* exon 9, its intronic boundaries, and homologous sequences in the human genome. **A:** Name and localization of potentially incorrect mutations (pseudomutations). These variants could be a consequence of local sequence identity with similar regions in the human genome that result in a severely increased frequency of priming artifacts. **B:** Horizontal lines show human chromosomal regions with homology to *CFTR* exon 9 and its intronic boundaries, and **arrowheads** show positions of mutations found within these homologous sequences (based on CLUSTALW multiple alignment analysis).

Table 3. Mutations in *CFTR* Exon 9 and its Intronic Boundaries that Have Homologous Sequences in Other Chromosomes

CFTR mutation	Common nomenclature	Nucleotide change	Site of mutation*	Consequences
p.Lys464Asn	K464N	G to T at 1392	Exon 9 (no. 10)	Lys to Asn at 464; mRNA splicing defect?
c.1328_1329delAT	1460delAT	Deletion of AT from 1328	Exon 9 (no. 10)	Frameshift
c.1235delC	1367delC	Deletion of C at 1235	Exon 9 (no. 10)	Frameshift
p.Asn416Ser	N416S	A to G at 1247	Exon 9 (no. 10)	Asn to Ser at 416
c.1392 + 6insC; c.1392 + 12G>A	1524 + 6insC 1524 + 12G>A	Insertion of C after 1392 + 6, G to A at 1392 + 12	Intron 9 (no. 10)	mRNA splicing defect?

These mutations were previously reported as personal communication to the CF Genetic Analysis Consortium (*http://www.genet.sickkids.on.ca/cftr*, last accessed December 24, 2008).

*Conventional *CFTR* exon/intron numbering includes exons 6a and 6b, exons 14a and 14b, and exons 17a and 17b; for exon/intron numbers in parentheses, these exon pairs are numbered sequentially, without modifiers such as "6a" and "6b."

tional substitutions, indicating that other sequences were co-amplified (data not shown).

PCR analyses were performed using specific primers directed to the ends of or inside the homologous sequences to confirm the presence or absence of the c.1392 + 6insC and c.1392 + 12G>A mutations (Figure 1). Sequencing of PCR products C, D, and E revealed the wild-type sequence of exon 9 on chromosome 7. Moreover, when the annealing temperature of PCR A was increased from 50°C to 60°C, the PCR product A presented only the p.Asp443Tyr mutation (G>T at 1327) in exon 9, and not the c.1392 + 6insC; c.1392 + 12G>A mutations.

Furthermore, the c.1392 + 6insC and c.1392 + 12G>A mutations were detected in DNA samples from each of the six negative control subjects and in patients with other *CFTR* mutations, when the classical primers were used, but were not detected in these same subjects when the specifically designed primers or the high annealing temperature was used. Taken together, our data confirm that the c.1392 + 6insC and c.1392 + 12G>A mutations are not actually present in the *CFTR* gene, but are in fact the consequence of multiple copies of this region throughout the human genome.

Discussion

The Cystic Fibrosis Mutation Database is useful for screening different populations, especially for laboratories involved in the diagnosis of *CFTR* mutations. Furthermore, to determine *CFTR* abnormalities in mutation carriers, these resources have important implications for continuing research on the molecular basis of CF. The identification and confirmation of newly discovered mutations is a challenge, particularly to CF clinics, for improving methods of detection.

The availability of complete genome sequences for many organisms in accessible databases has facilitated the assessment of DNA sequence similarities allowing a better understanding of both inter- and intragenomic homology, and thus of global genome structure.[11] The final and conclusive characterization of a genomic rearrangement usually relies on the identification of sequences that harbor the rearrangement/mutation(s).[12] Ultimately, the functional consequences of disease-associated genomic rearrangements/mutations can be assessed only by accurately determining the exact DNA sequences(s) responsible for the particular disease phenotype. BLAST analysis of *CFTR* exon 9 and its flanking intronic regions revealed several exon 9 repeat sequences in the human genome. Surprisingly, the c.1392 + 6insC and c.1392 + 12G>A mutations were found in all of these pseudogene sequences. Moreover, the T at position 1395 was also detected in one similar sequence. Therefore, sequence similarities and structure alignment indicate that these mutations could be pseudomutations. It is noteworthy that we identified four other mutations in the *CFTR* database (*http://www.genet.sickkids.on.ca/cftr/*, last accessed December 24, 2008) that are present in homologous sequences within *CFTR* exon 9. The above data are consistent with a report describing the amplification of this segment to multiple copies in human genome by cloning, sequence analysis, and chromosome localizations.[5] A detailed analysis of this segment has previously shown that this region is part of a large duplicated sequence unit (LCR7-20), and that duplicated units are localized on different human chromosomes. Furthermore, restriction fragment analysis and limited sequencing data have shown that the human genome contains approximately 30 copies of LCR7-20-like sequences.[6] Our present results, however, do not agree with this high copy number. Moreover, an insertion containing a 647-bp sequence with strong sequence similarity to *CFTR* exon 9 has been previously described,[6] and the authors suggested that these regions were copied and co-integrated with an L1 retrotransposon during its transposition to new locations in the genome.

Based on our results and those from other studies, we suggest that technical anomalies can occur when screening for *CFTR* mutations due to the presence of several genomic regions that are homologous to this gene. Therefore, we have studied human *CFTR* exon 9 and its intronic boundaries by investigating a wide range of highly similar/homologous sequences to design primers that precisely amplify only *CFTR* exon 9 and its flanking introns, and that can be used to identify true mutations in this gene. We used an upstream fragment in intron 8 near exon 9 to avoid amplifying the polymorphic tract upstream of exon 9; other amplified fragments were located in exon 9 or intron 8. This strategy successfully

excluded amplification of pseudogene sequences. By direct sequencing with non-specific primers, the findings revealed different sequences that were not located in the *CFTR* exon 9 regions. Subsequently, we used PCR cloning with specific and non-specific primers to confirm the pseudogene variants. We have described here three specific primer pairs for *CFTR* exon 9 that have no similarity to other pseudogene sequences. In one primers set (CF-70, CF + 437), both the forward and reverse primers are outside the region of homology. In the two other primer sets, forward primers (CF-70, CF-268) that are outside the region of homology, and a common reverse primer (CF + 121) is inside the region of homology. These designed primer sets, along with suitable melting temperatures, preclude co-amplification of pseudogene sequences.

Our results also suggest that several mutations that have been described in *CFTR* exon 9 and its flanking regions could, in fact, be ectopic variants in pseudogenes. These include p.Lys464Asn, c.1328_1329delAT, c.1235delC, and p.Asn416Ser in exon 9, and c.1392 + 6insC; c.1392 + 12G>A in intron 9. In conclusion, all patients carrying such mutations should be re-examined using the primers and protocol described here.

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