

Identification of a unique splice site variant in *SLC39A4* in bovine hereditary zinc deficiency, lethal trait A46: An animal model of acrodermatitis enteropathica[☆]

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Abstract

Lethal trait A46, also known as bovine hereditary zinc deficiency, Adema disease, and hereditary parakeratosis, is an autosomal recessive disorder first described in 1964, with a clinical presentation similar to that of acrodermatitis enteropathica (AE) in humans. The molecular basis of the defect has not been previously identified. Recently, the basic defect in AE was found to lie in *SLC39A4*. We report the characterization of the bovine ortholog of *SLC39A4* and identification of a unique splice site variant within this gene in affected animals. The mutation leads to exon skipping, leaving the coding region in frame. The gene product is predicted to lack two critical motifs, which lie in adjacent transmembrane domains implicated in the formation of a pore responsible for the transport of zinc. While further functional studies are warranted, this unique variant is likely to be responsible for the impaired zinc absorption in this disease.

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Zinc is an essential nutrient and plays a critical role in the function of many biological processes as a cofactor of many enzymes, transcription factors, and other critical molecules in both the animal and the plant kingdoms [1]. Symptoms of zinc deficiency in animals are similar across various species and include diarrhea, skin lesions, immunodeficiency, and growth retardation. Studies of zinc transport in plants, bacteria, yeast, and other eukaryotes have culminated in the recent identification of two major solute-linked carrier (SLC) gene families, the *ZnT* (*SLC30*) and *Zip* (*SLC39*) families, with at least 9 *ZnT* and 15 *Zip* transporters being identified in human cells [1].

Three mammalian genetic disorders have been known to involve zinc transport, lethal milk syndrome (*lm*) in mice, acrodermatitis enteropathica (AE) in humans (OMIM No: 201100), and bovine hereditary zinc deficiency (BHZD), also known as lethal trait A46 (OMIA 000593), in cattle [2–4]. All of these disorders display an autosomal recessive inheritance. Of these, lethal milk syndrome, so named because pups of any genotype suckled on *lm/lm* dams die before weaning, is caused by a mutation in the *SLC30A4* (*ZnT4*) gene [2], which appears to be critical in the transport of zinc into the milk. Supplementation of pups with zinc or nursing on foster dams alleviates the symptoms, but adult mutant mice display abnormalities including difficulty in righting, tail-spinning, and instability when swimming, possibly due to the role of *SLC30A4* in zinc transport in the brain. Dramatic effects of zinc deficiency are also observed in AE, a rare human disorder, in which affected infants usually present with skin lesions and diarrhea, infections, and, if diagnosis and intervention are delayed, growth retardation [5–7]. Investigations by two groups, led by Gitschier at the

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University of San Francisco (CA, USA) [8] and by Kury at Laboratoire d'Etude de l'AND, in Nantes, France [9], concurrently identified mutations in *SLC39A4* as being responsible for the disorder. Supplementation of AE infants with zinc in very large doses (2- to 10-fold the required daily intake) has been extremely effective in treatment of this disorder [10]. Interestingly, the identification of the central role of zinc in AE and its subsequent treatment with zinc were furthered by the observation of the similarity of the manifestations of AE with those of both acquired [7] and hereditary zinc deficiency in cattle [11,12]. Of note, successful treatment of parakeratosis in cattle with zinc was reported in 1960 [13], 4 years before the hereditary defect was described.

Bovine hereditary zinc deficiency, also known as lethal trait A46, Adema disease, hereditary parakeratosis, and hereditary thymus hypoplasia, was first described in 1964 [14], in Black Pied Friesian cattle. Black Pied Friesian cattle descended from two breeds, Black Pied Jutlands from Denmark and Friesians from Holland [15], which serve as the ancestors to the Holstein breed, the major breed involved in dairy production today. A similar disorder has been more recently described in Shorthorn cattle in the United States [16] and in Angus cattle in Australia [17]. In their report of the mutation in AE, Wang et al. [8] mention personal communications with Australian colleagues indicating a defect in the orthologous gene in Angus cattle. Detailed description of the findings in Angus cattle has not been reported except for the claim of an undisclosed missense mutation in this gene in Angus cattle, which was not present in Friesian cattle, in an abstract [18].

The presenting symptoms of BHZD are diarrhea and skin lesions, followed by secondary infections and death if left untreated. However, prompt treatment of calves with oral zinc alleviates all signs and leads to normal development. In our earlier studies, detailed characterization of the disorder and the establishment of a family for genetic studies were reported [19]. The pedigree established by embryo transfers is shown in Fig. 1. The two affected bulls thus obtained (VYG-1 and VYG-3) were raised to 2.5 years of age and semen was banked from them for future studies. Our treatment protocol from birth to 2 years of age was described [19]. The adult animals have been maintained on a regimen of oral boluses of 45 g of elemental zinc administered as

zinc acetate in gelatin capsules twice a week. With this treatment, the animals have all been free of clinical signs. When treatment lapses of a few weeks have occurred unintentionally, i.e., due to unavailability of the gelatin capsules, skin lesions appeared in the perianal area and on the lower extremities and resolved completely within 2–3 weeks of reinstating treatment. At the moment, the oldest affected animal is a healthy 11-year-old heifer, VYG-6.

In recent studies, we provided evidence that the locus mapped to a region orthologous to that of AE in the human [20]. In the current study, we report the identification of a single base change that results in a unique splice site mutation in the bovine ortholog of *SLC39A4* as an excellent candidate to be the causative mutation in lethal trait A46, the classical BHZD.

Results

Primers that successfully amplified bovine cDNA and genomic samples are shown in Table 1, along with the optimal annealing temperatures. Reverse transcription-polymerase chain reaction (RT-PCR) of the samples from affected animals with primer set 3 (VYG1180/VYG1170) produced a band about 150 bp shorter than those from unaffected animals. Sequencing of the amplified products revealed the affected sample to be missing exactly 153 bp, corresponding to exon 10 of the human *SLC39A4* gene, with the resulting sequence being in frame. Amplification of genomic DNA fragments containing the splice junctions on both ends of this exon was carried out using primers VYG1277/VYG1278 and VYG1298/VYG1299 as shown in Table 1. The amplified fragments from normal and affected samples were sequenced. The sequences were deposited in GenBank (intron 9, Accession No. DQ336348, and intron 10, Accession No. DQ226349). The corresponding sequences between the affected and the normal were all identical except for a single nucleotide change from G to A identified in the splice donor position of intron 10 in the affected animals as seen in Fig. 2. Inspection of the intron sequences of the normal genotype indicated conformity to the GT/AG rule [21] and the presence of a C-rich pyrimidine tract near the splice acceptor site.

The G to A mutation was predicted to result in the generation of a *FokI* restriction enzyme recognition site. To screen samples rapidly and reliably for this mutation using PCR, an artificial introduction of restriction sites test [22] was designed, incorporating an engineered cut site for *FokI* in one of the primers to serve as an internal control for digestion (primers VYG1336/VYG1337). As indicated in Table 2 and shown in Fig. 3, this results in the generation of a diagnostic band of 143 bp for the G allele and of 98 bp for the A allele, with the obligate heterozygous animals showing both alleles. Our archived samples from the two affected bulls (VYG-1 and VYG-3) and three affected heifers and three obligate heterozygotes conform to the expected band pattern. All of the 104 clinically normal Holsteins screened were found to be homozygous for the G allele.

The remaining coding region of bovine *SLC39A4* was amplified using RT-PCR and sequenced from both normal and affected animals. No further differences were identified and the

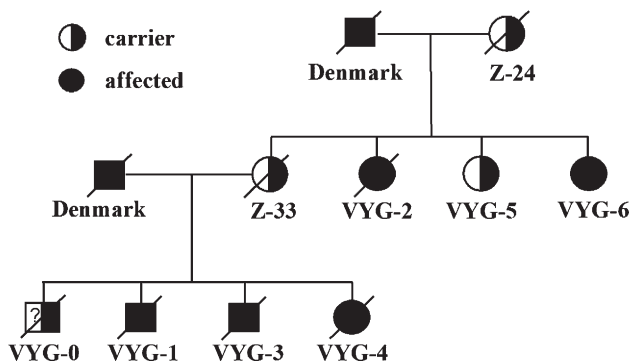


Fig. 1. The pedigree of BHZD cattle established at Michigan State University. VYG-0 died within 24 h of birth from pneumonia and could not be phenotyped with regard to BHZD status.

Table 1
Primers used in polymerase chain reaction and sequencing

Template	Primer set	Primer name	Sequence (from 5')	Primer position	Product size (bp)	Annealing temperature (°C)	Other PCR conditions
cDNA	1	VYG1313F ^a	CAGGCAAGCTCGGCCTAC	5' UTR	803	58	10% DMSO
		VYG1314R ^b	CTGTCGTCACTGTGGCTGTC	Exon 4			
cDNA	2	VYG1184F	GTACTTCGTGGACTTTGTGTT	Exon 3	479	56	—
		VYG1187R	ATGTAGTGGGAGGTGGTGCT	Exon 6			
cDNA	3	VYG1180F	CTGAGAGGTACCTGTATGGC	Exon 5–exon 6	980 ^d (827)	58	10% DMSO
		VYG1170R ^c	CAGAAGCTCTCAGAAGGCCGA	Exon 12–3' UTR			
Genomic DNA	4	VYG1277F	GAGCCCGGAGCCCGGAGAC	Exon 9	200	65	10% DMSO
		VYG1278R	CACGGCGTCGCCCAAAGTGAT	Exon 10			
Genomic DNA	5	VYG1298F	GGAACCGGGCCGGGCAAATACG	Intron 9	308	67	10% DMSO
		VYG1299R	GCCGCGCCCACTCCCCGATAGT	Intron 10			
Genomic DNA	6	VYG1214F	AGCCCAAGCAGCTCCATGA	Exon 8	1005	58	5% DMSO
		VYG1189R	AACGTAGAGGCCGATGAAGG	Exon 11			

^a Primer designed based on sequence reported in GenBank trace sequence entry 410393809.

^b Primer designed based on sequence reported in GenBank trace sequence entry 641547542.

^c Primer designed based on sequence reported in GenBank entry AW477707.

^d Product length from normal mRNA. In affected mRNA (length in parentheses) the product is lacking 153 nucleotides.

full-length coding region was deposited in GenBank (Accession No. DQ336345) along with that of the mutant (Accession No. DQ336345). The aberrant message is in frame. The normal bovine coding region shows about 80% identity with the human at the nucleotide level. Alignment of the predicted bovine protein with the human (GenBank Accession No. GI 19115957) as optimized by ClustalW [23] using the DNA Star package from Lasergene is shown in Fig. 4. The last four amino acids of the bovine gene product are predicted from the primer, based on a partial bovine cDNA, GenBank Accession No. AW47707, and have not been independently verified. With that said, the human and the bovine predicted proteins are 74% identical and 78% similar. The normal bovine product is predicted to have 653 amino acid residues compared to the normal human product of 647 residues. The mutant transcript would code for a protein lacking amino acids 498–548 as indicated in Fig. 4. This deleted region is highly conserved, showing 92% identity between the human and the bovine, and covers large parts of two critical domains. All of transmembrane domain 4, which contains the Zip motif (GDAVHNF), is deleted, as well as most of transmembrane domain 5, including all but the last two

amino acids of the HEXPHEXGD motif, identified as the defining motif of the LZT family, LIV-1 subfamily of zinc transporters [24]. These two domains appear to be critical in the formation of a pore structure, which is proposed to be potentially responsible for the transport of zinc ions [24].

Discussion

Our findings show that a single nucleotide mutation of the splice donor site in intron 10 of the bovine *SLC39A4* leads to a unique transcript in the animals harboring the defective gene. This mutation is predicted to have severe consequences for the protein known to be involved in AE. The absence of this allele in 208 chromosomes from 104 Holsteins studied indicates that this is not a common variant in Holsteins. In our search for this or a similar variant, we did not find any transcripts from unaffected individuals of any species that were missing exon 10. (Databases searched include ESTs, nonredundant sequences in GenBank, and other resources from the GeneCards interface.) Interestingly, we identified three entries (BF685329, BQ432515, BM826539) representing partial cDNAs containing a fragment matching the

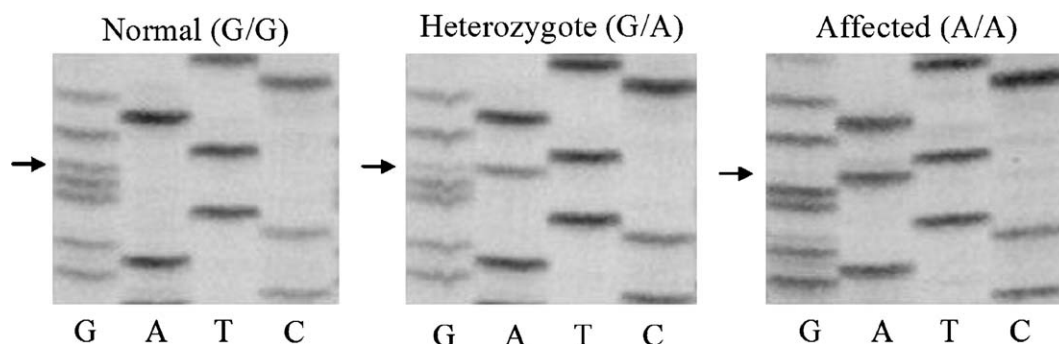


Fig. 2. Radiolabeled dideoxynucleotide sequencing reaction products separated in a 6% acrylamide sequencing gel and then exposed to X-ray film. A single nucleotide substitution (G to A) was discovered in the first position of intron 10 in affected animals, indicated by the arrow. This destroys the conserved donor splice site of the intron and explains the exon 10 skipping observed in cDNA from affected animals.

Table 2
Amplified fragment length polymorphism assay

Primer set ^a	Annealing temperature (°C)	PCR product length (bp)	Restriction enzyme ^b	Band sizes (bp) after restriction digest ^c		
				G	G/A	A
Forward: VYG1336, 5'-GTGTTCTGCCACGAGGTGCC (located in exon 10 of bovine ZIP4)	61	164	<i>FokI</i>	143	143	—
Reverse: VYG1337, 5'-GGCGGGGATGGGGTCAGTCAAGG (located in intron 10 of bovine ZIP4)				—	98	98
				—	45	45
				21	21	21

^a Mutagenesis nucleotide is in bold and underlined (A replaces C). 10% DMSO is included in PCR.

^b Prior to digestion, MgCl₂ is added to the PCR mix to a final concentration of 10 mM.

^c Diagnostic bands are in bold.

exon 9–exon 11 junction—which most likely represent mutation events in these tumors. These sequences represent partial human cDNA entries (498–879 bp long) from tumors (two from leiomyosarcoma cell lines from ATCC—possibly the same source—and one described as being from a “lymphoblast-like cell line” from ascites fluid from a gastric cancer patient). The absence of transcripts lacking exon 10 in normal tissues of any species studied to date, the high degree of conservation of this exon, and the importance of the domains it encodes strongly supports that this uncommon variant in Holsteins is likely to be the causative mutation rather than a linked marker, although functional studies will provide the conclusive proof.

This finding brings to full circle the utility of comparative observations made in cattle that provided a simple, yet life-saving, treatment for humans affected with this disorder. Producers now can choose to eliminate animals carrying this variant from their breeding stocks. While the frequency of carriers of this gene is likely to be low in the current cattle gene pool, the need for vigilant screening of bulls is accentuated by the large contribution to the gene pool of popular bulls through the use of artificial insemination. The experience with another autosomal recessive disorder in Holstein cattle, leukocyte

adhesion deficiency, provides a good example [25]. The mutation is in the gene coding for CD18, the common β subunit of the β 2 integrins in both humans and cattle [26]. In this disorder, deficiencies in leukocyte function lead to death of calves due to infections. With the identification of the mutation, population studies were able to be carried out in 1992, revealing the carrier frequency of the mutation in bulls and cows to be 15 and 9%, respectively, and showing all the cattle with the mutant allele to be related to the same bull [27]. While subsequent studies revealed the mutant allele to have spread throughout the world, as recently reviewed [28], there has been a dramatic decline in the frequency of this disorder with the use of genetic testing by the Holstein breeders and the dairy industry. It is possible that some of the remaining calf morbidity and mortality in Holsteins is attributable to BHZD and may be controlled by genetic testing.

The splice donor site mutation identified in this study has not been reported in any of the human cases studied to date. Over 25 mutations have been described in *SLC39A4* in humans. Of these, 3 involve splicing abnormalities. One mutation is that of c.192 + 19G → A [9], which creates a false splice site. Two other mutations involve abolition of a splice site. One of these, c.475–2A → G, leads to skipping of exon 2 and is predicted to result in a frameshift [29] and a premature stop codon. The other (c.1150–2A → G), which has been described [8,29], is a splice-acceptor mutation that changes the AG preceding exon 7 to GG. It is possible that future studies will identify other splicing defects. The present finding adds to the importance of the GT/AG splice donor–splice acceptor consensus sequences in introns in bovine mRNA processing, as is the case in humans.

We propose that BHZD will be useful not only for the study of the inherited disorder of zinc deficiency, but also of zinc deficiency in general, including the more subtle effects of marginal zinc deficiency on the immune system. The availability of a growing list of genomic resources for the bovine will make possible highly sophisticated studies. As many transcription factors rely on zinc for their activity, we hypothesize that changes in gene expression are a major target for complications of marginal zinc deficiency, which may be prevalent in both underdeveloped and developing countries as well as in segments of the population such as the elderly in the developed world [30]. The availability of a growing number of genomic resources, including microarrays, will allow us to use this model

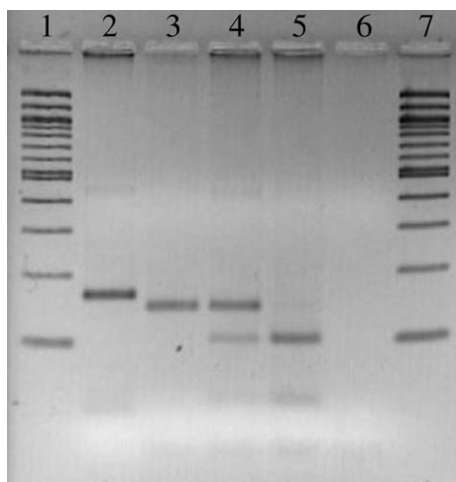


Fig. 3. Agarose gel with PCR products and *FokI*-digested PCR products. Lanes: 1 and 7, 100 bp DNA ladder (New England Biolabs); 2, undigested PCR product; 3, normal (G/G) *FokI* cut; 4, heterozygote (G/A) *FokI* cut; 5, affected (A/A) *FokI* cut; 6, negative control (water blank) for PCR and digest. Table 2 lists band sizes for each genotype.

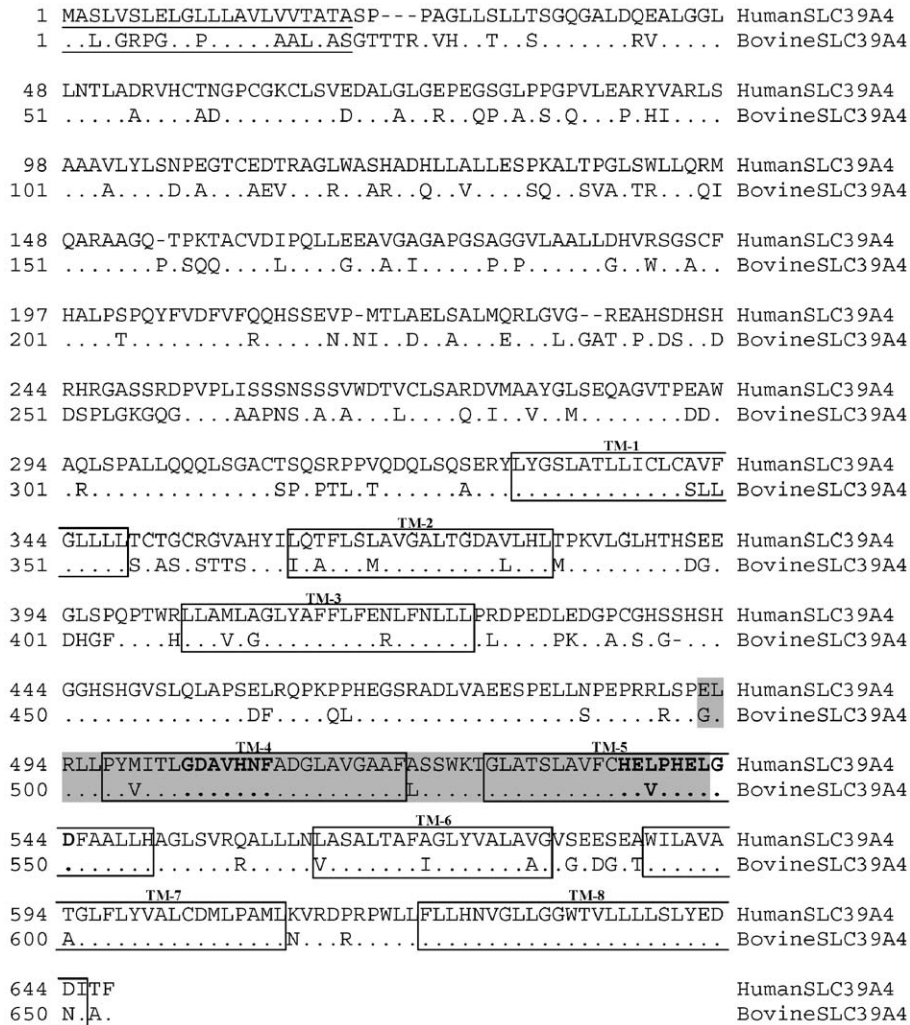


Fig. 4. ClustalW alignment of the predicted bovine and human *SLC39A4* gene product. The underlined residues are predicted to be the signal peptide. The transmembrane domains (TM-1 to TM-8) are indicated by the boxed sequences. The core residues that make up the Zip and HEXPHXGD motifs, in TM-4 and TM-5, respectively, are indicated in bold. The shaded residues correspond to exon 10, the region deleted in the mutant transcripts. The last four amino acids of the bovine sequence are predicted from a primer based on GenBank entry AW477707 and have not been independently sequenced by us.

system to look into the effects of different degrees of zinc deficiency on the transcription of many genes across different organ and cellular systems and to make further contributions to biology and human health.

Cases, material, and methods

Normal and affected animals

Animals affected with or heterozygous for BHZD were from a pedigree established at Michigan State University. Semen from an affected bull, “Denmark,” was used for artificial insemination to produce obligate heterozygotes, which were then used in artificial insemination and embryo transfer studies to establish a family segregating for BHZD as described [19]. Clinically normal Holsteins (98 females and 6 males) from a Michigan dairy farm were included for DNA studies using leftover clotted blood from samples taken for unrelated studies.

Isolation of DNA samples was carried out by use of a Trizol reagent (Invitrogen) protocol on about 100 mg of tissue according to the manufacturer’s instructions or by phenol–chloroform extraction after digestion of a clotted blood sample in proteinase K based on a slight modification of the protocol described [31]. The modification included the use of 200 µl of 5% SDS per 100–200 mg of clotted blood sample to assist the lysis. Isolation of total RNA was

carried out using the Trizol reagent or the PureLink RNA Purification System (both from Invitrogen). Intestinal tissues (ileum and jejunum) were used for the RNA studies.

Sequence alignments

Sequence alignments were constructed using the Lasergene software package of DNASTAR, Inc., as well as the BLAST algorithms of NCBI. Primers were designed based on either trace sequences from the bovine genome project (as in primer VYG1313F) or existing GenBank partial cDNA entry AW47707 (as in primer VYG1170R) or from consensus primers based on human (GI: 19115957) or mouse (GI: 27754010) sequences.

RT-PCR analysis and sequencing

Total RNA samples isolated from intestinal tissues of normal, heterozygous, and affected bovine cases were reverse transcribed using either the M-MLV or the Superscript III reverse transcriptase (Invitrogen). Consensus primers derived either from alignment of human and mouse *SLC39A4* coding regions or from available bovine trace sequences were used to amplify the target regions from normal, heterozygous, and affected bovine cDNA samples. The resulting fragments were resolved on agarose gels and visualized under UV light after ethidium bromide staining. Typical amplification reactions were prepared in a

25- μ l total reaction volume, with 5 μ l (50–200 ng) cDNA template, 5 pmol of each primer, 0.5 units of *Taq* polymerase (Invitrogen, Carlsbad, CA, USA), and final concentrations of 80 μ M dNTPs, 2 mM MgCl₂, 20 mM Tris–HCl, and 50 mM KCl. When necessary, the PCR was adjusted to contain 5–10% DMSO as indicated in Table 1. Cycling conditions were as follows: 94°C for 4 min; 35–40 cycles at 94°C for 1 min, optimal annealing temperature (as indicated in Table 1) for 1 min, 72°C for 1 min; followed by 72°C for 5 min. PCR products were analyzed on agarose gels stained with ethidium bromide, then amplified fragments were cut and purified using the QiaexII Gel Extraction kit (Qiagen). The fragments were then sequenced either with automated sequencing or manually with the amplification primers using the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing kit (USB Corp., Cleveland, OH, USA) and ³³P-labeled dideoxy-nucleotide triphosphates according to the manufacturer's protocol. Sequencing products were analyzed on 6% acrylamide denaturing sequencing gels, dried, and exposed to Biomax MR Scientific Imaging Film (Kodak, Rochester, NY, USA) for 48–72 h and developed.

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