A *VpreB3* homologue from a marsupial, the gray short-tailed opossum, *Monodelphis domestica*

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Abstract

A VpreB surrogate light (SL) chain was identified for the first time in a marsupial, the opossum *Monodelphis domestica.* Comparing the opossum *VpreB* to homologues from eutherian (placental mammals) and avian species supported the marsupial gene being *VpreB3*. VpreB3 is a protein that is not known to traffic to the cell surface as part of the pre-B cell receptor. Rather, VpreB3 associates with nascent immunoglobulin (Ig) chains in the endoplasmic reticulum. Homologues of other known SL chains *VpreB1, VpreB2*, and *λ5*, which are found in eutherian mammals, were not found in the opossum genome, nor have they been identified in the genomes of non-mammals. *VpreB3* likely evolved from earlier gene duplication, independent of that which generated VpreB1 and VpreB2 in eutherians. The apparent absence of VpreB1, VpreB2, and λ5 in marsupials suggests that an extra-cellular pre-B cell receptor containing SL chains, as it has been defined in humans and mice, may be unique to eutherian mammals. In contrast, the conservation of VpreB3 in marsupials and its presence in non-mammals is consistent with previous hypotheses that it is playing a more primordial role in B cell development.

Keywords

VpreB; Evolution; Marsupial; B cell onteny

B-lymphocytes progress through distinct stages of gene expression and gene rearrangement during their ontogeny (Hardy et al. 1991; Li et al. 1993). Two defining steps are the sequential rearrangement of Ig heavy (H) and light (L) chain genes through the process of RAG dependent V(D)J recombination (Schatz 2004). Associated with the rearrangement of the Ig H genes and expression of H chain proteins is the expression of the SL chains VpreB and λ5, which precedes the rearrangement and expression of Ig L chain genes. These SL chains associate with the IgM H (μH) chain, which along with the CD79a and CD79b (Igα and Igβ) signaling molecules form the extra-cellular pre-B cell receptor (BCR) (Kudo and Melchers 1987; Karasuyama et al. 1990; Tsubata and Reth 1990; Shirasawa et al. 1993; Mårtensson et al. 2007). Successful expression of the pre-BCR is critical for signaling developmental progression in B cells.

Three *VpreB* gene lineages, *VpreB1, VpreB2* and *VpreB3,* have been identified. Mice have all three genes, whereas humans only have *VpreB1* and *VpreB3*. VpreB1 and VpreB2 (VpreB1/2) are closely related and used on the cell-surface as part of the pre-BCR in early developing B cells and, although both genes are present, in mice VpreB1 appears to play a dominant role (Tsubata and Reth 1990; Ohnishi and Takemori 1994; Mundt et al. 2005;

Mårtensson et al. 2007). VpreB3 is a more distantly related gene whose function is less well studied. VpreB3 has been shown to associate with nascent μH chains and λ5 in the endoplasmic reticulum (ER) in mice, but not traffic to the cell surface (Ohnishi and Takemori 1994). VpreB3 has also been found in a non-mammalian vertebrate, the chicken, where it appears to participate in the retention of free Ig L chains in the ER to prevent their secretion independent of H chains (Rosnet et al. 2004). The presence of VpreB3 in birds as well as mammals have lead some investigators to speculate that it is the more ancient of the SL chains, whose primordial function may be to associate with Ig chains intra-cellularly (Vettermann and Jäck 2010).

Here we describe a VpreB homologue in a marsupial, the gray short-tailed opossum *Monodelphis domestica.* Marsupials are a mammalian lineage that diverged from eutherians at least 147 million years ago, and are noteworthy for giving birth to highly altricial young (reviewed in Old and Deane 2000; Bininda-Emonds et al. 2007). In general, newborn marsupials are unable to initiate B and T cell dependent immune responses until they are greater than a week old, which correlates well with the appearance of lymphocyte specific markers and gene expression in many cases (Kalmutz 1962; LaPlante et al. 1969; Rowlands et al. 1972; Parra et al. 2009; Duncan et al. 2010). As part of an ongoing study of postnatal ontogeny of the opossum immune system, we wished to identify markers of B cell development including the surrogate light chains; however, surrogate light chains have not been described previously for any marsupial species. Using the available opossum whole genome sequence (GenBank accession no. AAFR03000000; Mikkelsen et al. 2006) the identification of genes homologous to *VpreB* and *λ5* was attempted using *in silico* screening methods (Altschul et al. 1990). Using mouse *VpreB1, VpreB2*, and *λ5* was unsuccessful, although these sequences did identify previously annotated opossum Vλ and Cλ genes known to be used in the conventional Igλ repertoire (Wang et al. 2009). Mouse *VpreB3,* however, matched a partial gene among the unassembled opossum genome sequences (Scaffold Un.55000001-60000000). The partial *VpreB* gene contained a 329 bp gap at the 5' end of the gene, which was filled by sequencing a PCR fragment spanning the gap that was amplified directly from genomic *M. domestica* DNA (Fig. 1; The complete gene sequence was deposited in GenBank as accession number JN863116).

The *VpreB* gene in eutherian mammals consists of two exons that encode the leader peptide and extracellular V domain, respectively. Based on sequence similarity between the opossum gene and mouse and human VpreB, the presumptive opossum *VpreB* exons were identified along with the predicted mRNA splice sites (not shown). PCR primers located within the two exons were used to amplify a cDNA clone from splenic mRNA from an eight-week-old opossum. When compared to the genomic sequence, the cDNA sequence confirmed the opossum *VpreB* gene structure predicted from the alignments. When compared to *VpreB* genes from eutherians and birds the opossum gene clustered with other *VpreB3* genes in a phylogenetic analysis, consistent with the opossum gene being a *VpreB3* homologue (Figs. 1 and 2).

The translated sequence was aligned to VpreB protein sequence from other species and many key residues are conserved including cysteines (Cys) in predicted β-strands B and F, and the canonical WF/YQQ in the region corresponding to framework region (FR)-2 (Fig. 1; Williams and Barclay 1988). Also present is a conserved HXAC motif was evident in all VpreB3, providing an unusual unpaired Cys (Cys-99), which is absent from VpreB1/2 (Fig. 1). Chicken VpreB3 has been shown to form covalent heterodimers with Ig L chains, however these are unaffected by mutation of Cys-99 (Rosnet et al. 2004). Which of the three Cys in VpreB3 is forming the covalent bond with L chain in chicken has not been determined, nor has been the role of Cys-99 in any species. Such covalent binding has not

been reported for mammalian VpreB3 where the interactions with μH chain and λ5 may be transient early in B cell ontogeny (Ohnishi and Takemori 1994).

The predicted opossum VpreB3 protein, like VpreB3 from other species, lacked the extended carboxy-end tail (non-immunoglobulin domain) sometimes referred to as the unique region (UR) found in VpreB1/2 (Fig. 1). In VpreB1/2 the UR does not form β-strand G, as would the region corresponding to FR4 in conventional V domains (Bankovich et al. 2007; Williams and Barclay 1988). Rather an extended UR at the amino terminus of λ5 replaces the G-strand in VpreB1/2 in the complete pre-BCR (Bankovich et al. 2007). VpreB3 in mouse also interacts with μH and of λ5 presumably in a similar interaction (Ohnishi and Takemori 1994). Although a VpreB3 crystal structure is unavailable based on the sequence it appears that this molecule would also lack β-strand G, as the protein only extends four residues past the last Cys (Fig. 1). However opossum, like chicken, appears to lack λ5; therefore it is not clear what structural interaction the VpreB3 has with other pre-BCR components.

The UR of VpreB1/2 extends beyond the Ig domain and interacts with the complementarity determining region (CDR)-3 of the μH chain V domain (Bankovich et al. 2007). Intact Pre-BCR has been shown to form dimers and this cross-linking is dependent on the UR of VpreB1/2 and λ5 (Bankovich et al. 2007). The absence of an extended UR in VpreB3 suggests it would be incapable of performing this role on the cell surface, consistent with an intracellular role, early in the trafficking of the pre-BCR (Ohnishi and Takemori 1994; Rosnet et al. 2004). This looks to be as true for opossum VpreB3 as for chicken and mouse. One of the roles of the Ig domain part of SL, independent of an extended UR, may be to interact with nascent μH chain to evaluate its compatibility with L chains (Smith and Roman 2010). It is possible that this is a primary role for VpreB3 as well.

Phylogenetic analyses of representative *VpreB* genes from a variety of mammalian and non-mammalian species, that included Vλ and Vκ genes, was performed (Fig. 2). Also included was VpreB3 from turkey and zebra finch, which were identified in the available whole genome assemblies for these species (Dalloul et al. 2010; Warren et al. 2010). The results of these analyses support the origin of VpreB3 independently of VpreB1/VpreB2 from different ancestral Vλ genes (Fig. 2). An early duplication gave rise to the more ancient VpreB3, which is present in birds, opossum, and eutherians. A second duplication appears to be specific to eutherians and gave rise to the VpreB1 and VpreB2 lineages. In other words, the extant VpreB genes are not monophyletic. VpreB1 and VpreB2 share 97% amino acid identity, while VpreB3 shares only 37% amino acid identity with VpreB1 and 2 (Mårtensson et al. 2007). VpreB1 and VpreB2 appear to be recent duplications occurring independently in different species (Fig. 2). Alternatively gene conversion (concerted evolution) may be homogenizing VpreB1 and VpreB2 in a species-specific manner in different eutherian lineages, creating the pattern of clades found in mice, rats and rabbits in Fig. 2. A VpreB-like gene reported from an agnathan, the sea lamprey, was also included in the initial analyses (Cannon et al. 2005). However, in phylogenetic analyses this gene fell outside the clade containing VpreB, Vλ and Vκ, and is likely not directly ancestral to the *VpreB* genes found in amniotes (not shown).

The *VpreB* genes appear to have evolved from Vλ genes and the mouse *VpreB1* and *VpreB2* and human *VpreB1* genes remain linked to the *Igλ* locus (Kudo and Melchers 1987). Chicken and human *VpreB3* genes are also linked to the *Igλ* locus, however the mouse *VpreB3* gene is on chromosome 10, non-syntenic to *Igλ* (Rosnet et al. 1999; Rosnet et al. 2004). A search of the region on opossum chromosome 3, where *Igλ* is located (Deakin et al. 2006), did not uncover any genes resembling SL chain homologues (not shown). Unfortunately the *VpreB3* gene was found amongst the unassembled sequences in

the opossum genome database and, therefore, it is not currently known where *VpreB3* is physically located the opossum, although it is clear from the mouse genome that its linkage to *Igλ* is not required for its function. As with opossum and chicken there was no evidence of *VpreB1* or *VpreB2* in the turkey and zebra finch genomes (not shown).

To investigate transcription of the *VpreB3* gene in postnatal opossums, RT-PCR was performed using RNA isolated from neonatal animals. The earliest age which VpreB3 transcripts could be detected was postnatal day 6 (Fig. 3). This is following the detection of CD79a, CD79b, and IgH transcription in newborn opossums, but preceding Ig L chain gene rearrangement and transcription (not shown; XW and RDM manuscript in review). These results are consistent with marsupials not having mature B cells capable of generating antibody responses until greater than one week of age (Kalmutz 1962; Rowlands et al. 1972; Old and Deane 2000; Duncan et al. 2010). Furthermore, the expression of VpreB3 just prior to Ig light chain gene rearrangement and transcription in the opossum would be consistent with its apparent function in chickens, where it associates with nascent light chains in the ER.

Homologues of the *VpreB1, VpreB2,* and *λ5* genes were not identified in the opossum genome and, to our knowledge, have only been found in eutherian mammals. VpreB1 and/or VpreB2, and λ5, therefore, may be specific to eutherians; marsupials, birds, and other lineages may use an alternative pre-BCR form that lacks these surrogate light chains on the cell surface. If this is the case then the cell-surface pre-BCR, as it has been defined in eutherian mammals such as mice and humans, is both a unique and significant adaptation given its requirement for normal B cell development. In mice pre-BCR containing H chain and the CD79a and CD79b signaling molecules, but lacking one or both SL or conventional L chains can be expressed, signal phosphorylation and Ca^{2+} release, and mediate allelic exclusion (Shimizu et al. 2002; Schuh et al. 2003; Su et al. 2003). There is, however, severe impairment of B cell development in SL knockout mice, demonstrating the clear important role these chains play in eutherian B cell development (Kitamura et al. 1992; Ehlich et al. 1993; Pelanda et al.1996).

One hypothesis is that a functional cell-surface pre-BCR lacking SL or conventional L chains is present in birds and non-eutherian mammals and may be an ancestral form of the pre-BCR. However, there is the possibility of alternatives to the conventional SL. Rearrangement of the Igκ L chain genes prior to IgH rearrangement and expression has been proposed as an explanation for the development of some B cells in SL knockout mice (Ehlich et al. 1993; Pelanda et al 1996). Germline Vk and JCk transcripts encoding proteins that functionally substitute for VpreB1/2 and λ5 have been described in mouse and may be a partial explanation for why B cell development is not completely blocked in VpreB1/2 and λ5 deficient mice (Francés et al. 1994; Rangel et al. 2005). Indeed, an Igκ transgene, under some circumstances, can rescue impaired B cell development in SL knockout mice (Pelanda et al. 1996). Opossum does have the Igκ L chain, however the presence of germline Igκ transcripts has yet to be explored in this species (Miller et al. 1999).

In conclusion, *VpreB3* is an ancient gene whose function in B cell development has been retained at least among the amniotes (Rosnet et al. 2004; Vettermann and Jäck 2010). VpreB3 is the only of the Igλ derived SL that may be universally present in the amniotes. Further structural analyses of VpreB3 interactions with other chains in the BCR complex are needed.
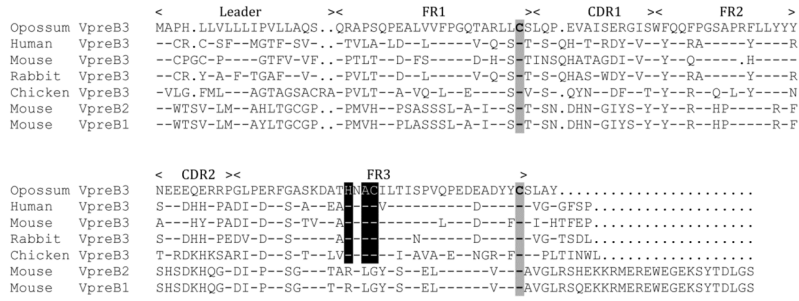
Acknowledgments

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               <      Leader        ><        FR1          ><    CDR1    ><     FR2       >
Opossum VpreB3 MAPH.LLVLLLIPVLLAQS..QRAPSQPEALVVFPGQTARLLCSLQP.EVAISERGISWFQQFPGSAPRFLLYYY
Human   VpreB3 --CR.C-SF--MGTF-SV-..-TVLA-LD--L-----V-Q-S-T-S-QH-T-RDY-V--Y--RA-----Y----R
Mouse   VpreB3 --CPGC-P----GTFV-VF..-PTLT--D-FS-----D-H-S-TINSQHATAGDI-V--Y--Q-----H-----
Rabbit  VpreB3 --CR.Y-A-F-TGAF--V-..-PVLT--D-L-----V-Q-S-T-S-QHAS-WDY-V--Y--RA-----Y----R
Chicken VpreB3 -VLG.FML---AGTAGSACRA-PVLT--A-VQ-L--E----S-V-S-.QYN--DF--T-Y--R--Q-L-Y----N
Mouse   VpreB2 --WTSV-LM--AHLTGCGP..-PMVH--PSASSSL-A-I--S-T-SN.DHN-GIYS-Y-Y--R--HP-----R-F
Mouse   VpreB1 --WTSV-LM--AYLTGCGP..-PMVH--PLASSSL-A-I--S-T-SN.DHN-GIYS-Y-Y--R--HP-----R-F


               <   CDR2  ><              FR3                   >
Opossum VpreB3 NEEEQERRPGLPERFGASKDATHNACILTISPVQPEDEADYYCSLAY......................
Human   VpreB3 S--DHH-PADI-D--S-A--EA--V---------D----VG-GFSP..................
Mouse   VpreB3 A---HY-PADI-D--S-TV--A--------L---D--F--I-HTFEP..................
Rabbit  VpreB3 S--DHH-PEDV-D--S-----A-----N------D----VG-TSDL..................
Chicken VpreB3 T-RDKHKSARI-D--S-T--LV---I-AVA-E--NGR-F---PLTINWL..................
Mouse   VpreB2 SHSDKHQG-DI-P--SG---TAR-LGY-S--EL------V--AVGLRSHEKKRMEREWEGEKSYTDLGS
Mouse   VpreB1 SHSDKHQG-DI-P--SG---T-R-LGY-S--EL------V--AVGLRSQEKKRMEREWEGEKSYTDLGS
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Figure 1.

Alignment of deduced amino acid sequences of opossum VpreB3 with mouse VpreB1, 2, and 3, human VpreB1 and 2, rabbit VpreB3, and chicken VpreB3. Leader peptide, and the regions that correspond to FR1 through 3 and CDR1 and 2 in Ig V domains are indicated above the alignment. Residues identical to the opossum sequence are indicated by dashes; dots indicate insertions necessary for generating the alignment. Conserved cysteines are shaded while the conserved HXAC motif is highlighted in black. The mouse *VpreB1, VpreB2,* and *VpreB3* sequences that were used to perform an *in silico* search of the opossum whole genome using the BLAST algorithm were Genbank accession nos. NM_016982, BC141459, and NM_009514). To complete the partial opossum *VpreB* gene identified, primers were designed to flank a 329 bp gap at the 5' end of the gene (5'-AGGAGGGCCTTCTCAGGA and 5'-GCTCCTGCTCCTCTTCATTG) and a product that covered the gap was cloned and sequenced.
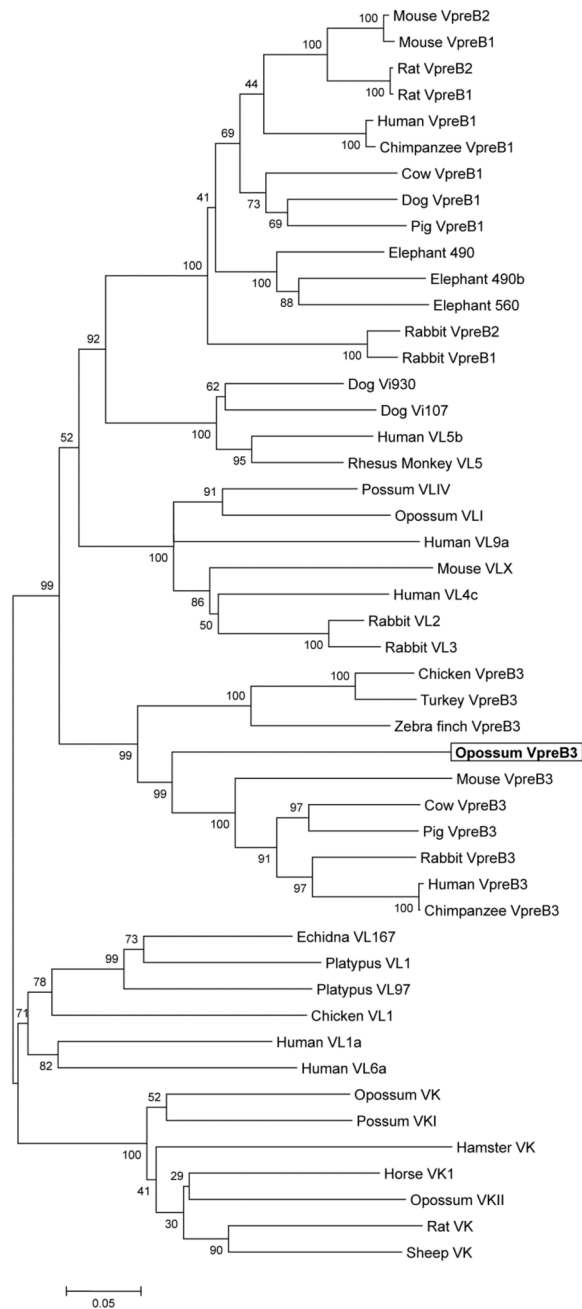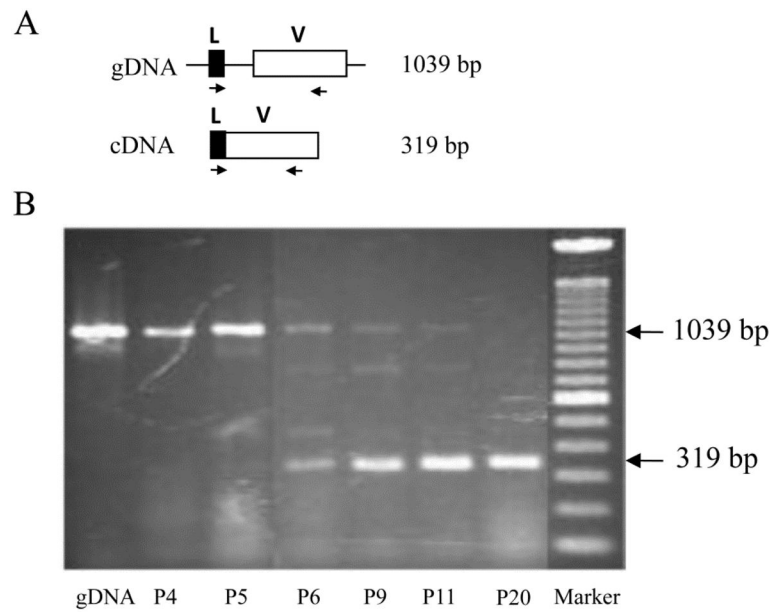
Figure 2.
Phylogenetic tree based on nucleotide alignments of *VpreB1, 2,* and *3* along with *Vλ* and
Vκ genes from the species indicated. The opossum VpreB3 is bolded and boxed. Analyses
were performed on nucleotide alignments using the neighbor-joining and minimal evolution
methods in MEGA4 with similar results; the minimal evolution tree is shown (Tamura et al.
2007). Amino acid translations were first aligned to establish gap position and then
converted back to nucleotide using the BioEdit program (Hall 1994). The GenBank
accession numbers of the sequences used in the phylogenetic analysis were: Opossum
VpreB3, JN863116; Human VpreB1, CR456609; Human VpreB3, NM_013378; Human
VL5b, BAA20017; Human VL4c, CAA80218; Human VL9a, CAP74528; Human VL1a,

BAA20004; Human VL6a, AAB33217; Chimpanzee VpreB1, NW_003458635; Chimpanzee VpreB3, NW_003458643; Rhesus Monkey VL5, AM056012; Mouse VpreB2, BC141459; Mouse VpreB1, NM_016982; Mouse VpreB3, NM_009514; Mouse VLX, AAA39169; Rat VpreB2, NM_001134788; Rat VpreB1, NM_001108845; Rat VK, EDL82784; Hamster VK, AAA82732; Rabbit VpreB2, AY351268; Rabbit VpreB3, XM_002724010; Rabbit VpreB1, AY351269; Rabbit VL2, PS0055; Rabbit VL3, PS0056; Sheep VK, S33161; Cow VpreB3, NW_003104461; Cow VpreB1, NW_003104461; Horse VK1, CAA53283; Pig VpreB3, NW_003611976; Pig VpreB1, NW_003611976; Elephant 490, NW_003573490 (1148141-1148468); Elephant 490b, NW_003573490 (3338560-3338236); Elephant 560, NW_003573560; Dog VpreB1, NC_0066083; Dog Vi930, XM_003639930; Dog Vi107, NW_003726107; Opossum VK, XP_003339882; Opossum VKII, XP_003339837; Possum VKI, AY074425; Possum VLIV, AAM09967; Opossum VLI, AF049774; Echidna VL167, AAM76525; Platypus VL1, AAO16067; Platypus VL97, AAO16074; Chicken VpreB3, XM_415223; Zebra finch VpreB3, NW_002197395; Turkey VpreB3, NW_003436164; Chicken VL1, I51216.

Figure 3.
A, Diagram of the VpreB3 gene (top) and cDNA (bottom). Leader region and V domain are represented by solid and open boxes, respectively. Location of the primers used for RT-PCR, complementary to leader exon and V exon are indicated as arrows. **B,** Detection of VpreB3 transcripts by RT-PCR in postnatal opossums. To investigate the timing of transcription of the opossum *VpreB3* gene RT-PCR was performed on total RNA isolated from newborn opossums at postnatal (P) days 4, 5, 6, 9, 11, and 20. gDNA is a control for the 1039 bp product produced when contaminating genomic DNA is present. Primers used were 5'-AGGAGGGCCTTCTCAGGA and 5-GCTCCTGCTCCTCTTCATTG.