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Universal primers suitable to assess population dynamics reveal apparent mutually exclusive transcription of the *Babesia bovis* *ves1* α gene

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Abstract

Babesia bovis is an intraerythrocytic hemoparasite of widespread distribution, which adversely affects livestock production in many regions of the world. This parasite establishes persistent infections of long duration, at least in part through rapid antigenic variation of the VESA1 protein on the infected-erythrocyte surface. To understand the dynamics of *in vivo* antigenic variation among the parasite population it is necessary to have sensitive and broadly applicable tools enabling monitoring of variation events in parasite antigen genes. To address this need for *B. bovis*, “universal” primers for the polymerase chain reaction have been designed for the *ves1* α gene, spanning from exon 2 to near the 3' end of cysteine-lysine-rich domain (CKRD) sequences in exon 3. These primers robustly amplified this segment, with minimal bias, from essentially the entire repertoire of full-length *ves1* α sequences in the *B. bovis* Mexico isolate genome, and are equivalently present in other isolates. On purified genomic DNA, this primer set can achieve a sensitivity of 10 genome equivalents or less. When applied to the amplification of cDNA derived from the *B. bovis* C9.1 clonal line evidence consistent with mutually exclusive transcription of the *ves1* α gene was obtained, concomitant with detection of numerous mutational events among members of the parasite population. These characteristics of the primers will facilitate the application of polymerase chain reaction-based methodologies to the study of *B. bovis* population and antigenic switching dynamics.

Keywords

Babesia bovis; antigenic variation; babesiosis; universal primers; *ves* multigene family

Introduction

The bovine hemoparasite, *Babesia bovis*, uses a combination of cytoadhesion and antigenic variation to persistently infect cattle, a combined mode of survival that is also used by the

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human malarial parasite, *Plasmodium falciparum* [1]. In *B. bovis* both processes rely upon the same parasite-derived protein, the variant erythrocyte surface antigen-1 (VESA1), expressed on the infected-erythrocyte (IE) surface [2-5]. VESA1 is a heterodimeric protein comprised of two structurally similar but distinct subunits, referred to as VESA1a and VESA1b [2,6-8]. VESA1a is encoded by members of the 1 α branch of the *ves* multigene family [7], whereas VESA1b is thought to be encoded by members of the 1 β branch ([8]; additional unpublished data). When recognized by antibody cytoadhesion of parasitized erythrocytes is prevented [5,9]. However, antigenic variation renders existing immune responses ineffective at recognizing parasitized erythrocytes *in vitro* [2] and, it is thought, *in vivo* [10]. Clearly, the kinetics of the immune response and antigenic variation will significantly impact upon the dynamics of parasite population growth and persistence. Although the capacity for rapid antigenic variation in the VESA1 protein is well established, essentially nothing is known regarding the dynamics with which the structure of each subunit is altered during infection *in vivo*, or even during *in vitro* growth. Knowledge of variation dynamics will improve our understanding of parasite persistence, and impact upon the development of any strategies targeting antigenically variant components for immunoprotection.

One robust approach to understanding better the dynamics of *in vivo* antigenic variation would be to directly monitor changes in VESA1 structure over time during an infection. This could be achieved by sampling the VESA1 isoforms transcribed at different phases of infection, establishing both the apparent rate of *in vivo* variation in the parasite population within a single host, and the complexity of isoforms produced under different circumstances. Given that a primary mechanism of antigenic variation in *B. bovis* is segmental gene conversion [7,8], there is considerable potential for the creation of expressed, unique, mosaic copies of the gene not present in the genome prior to the time of their formation.

While it has been possible to characterize changes that occur to expressed *ves* genes, it has not been feasible to follow the dynamics of their change in real time because the tools to follow *in vivo* behavior do not currently exist. We therefore initiated the development of such tools. Polymerase chain reaction (PCR) primers suitable for use either in PCR or reverse transcriptase-PCR (RT-PCR) reactions were developed to facilitate the acquisition of this information for the *ves1 α* gene. We describe here the development and characterization of a pair of “universal” primers encompassing a part of exon 2 and most of the CKRD domain-encoding region of exon 3 of the *ves1 α* gene. Within the VESA1a polypeptide this region is extremely rich in cysteine and lysine residues. Although full-length CKRD domains contain a long, highly conserved, complex motif [7]; additional unpublished results), it is overall highly polymorphic in sequence, and the CKRD region of each of the approximately 72 different *ves1 α* genes within the *B. bovis* genome appears to be unique [11]. Modifications by segmental gene conversion result in further expansion of the repertoire of unique expressed variants [8]. Thus, the CKRD domain-encoding region of the *ves1 α* gene should provide a sensitive estimation of structural, and presumably antigenic, changes occurring during the course of an infection. The ability to monitor changes in the CKRD region of the transcribed *ves1 α* gene would facilitate study of the dynamics of antigenic variation *in vivo*, both within the individual host and the parasite population as a whole.

Materials and Methods

Parasites

B. bovis parasites of the C9.1 clonal line were maintained in *in vitro* culture as described previously [6]. The derivation of the C9.1 line from the Mexico isolate is described elsewhere [2].

Nucleic acids isolation

Genomic DNA (gDNA) was isolated from in vitro-cultured *B. bovis* parasites by the use of sodium dodecylsulfate, Proteinase K, and phenol-chloroform extraction as described [7]. Prior to extraction, erythrocytes were treated on ice with 0.05% (w/v) saponin in VYM buffer [12] to permeabilize the erythrocyte membranes and release hemoglobin. Prior to isolation of RNA, the *B. bovis* C9.1 line was enriched once for parasites reactive with monoclonal antibody 4D9.1G1, as described [13]. When assayed by live-cell immunofluorescence [14] with 4D9.1G1 the selected parasitized erythrocytes were at least 70% reactive. Rather than being antigenically diverse at the 4D9.1G1 epitope, this primarily reflects disparate levels of VESA1 expression, with some cells being below the level of assay sensitivity ([13]; additional unpublished observations), and varies somewhat from day to day. Total RNA was isolated from approximately 1×10^6 *B. bovis* C9.1 parasitized erythrocytes, by using the ToTALLY RNA kit (Ambion, Inc.; Austin, TX). The RNA was digested with Turbo DNase (Ambion) for 30 min at 37°C, an additional 1 U DNase was added, and incubation continued for 30 min. The enzyme was then inactivated with Inactivation Reagent, according to manufacturer's protocol. The RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen; La Jolla, CA), using 1 µg of random hexamer primer.

Generation of universal primers

To design “universal” primers targeting the *ves1α* gene, optimized alignments were prepared of all *ves1α* gene sequences available at the time this work was first initiated [7,8]. Focusing on exons 1 and 2, and the 5' end of exon 3, it was determined that exon 2 is very highly conserved among *ves1α* genes. Four forward and three reverse primers were designed, based upon highly-conserved sequences of exon 2 and near the 3' end of sequences encoding the CKRD domain within exon 3. Based upon initial experiments we determined that the optimal combination was comprised of primers PD2Fa (AATGYATATGTGGCCTGG) and PD2Fb (AATGYATATGTGGCCTTG) as forward primers, mixed together in a 4:1 molar ratio (hereafter referred to as “PD2Fa,b”), coupled with primer PD1R (TACAANAACACTTGACAGCA) as the reverse primer. Primers were synthesized by Sigma-Genosys (Woodlawn, TX). As an internal control for reverse transcription and amplification, the β-tubulin gene was amplified, using forward primer AZT01 (TGCCAAATTCTGGGAAGTC) and reverse primer XW10 (CATACCACCAAAGGGACTCAA).

Amplification and cloning of *ves1α* sequences

Sequences encompassing the 3' end of exon 2, intron 2, and most of the CKRD-encoding sequences from exon 3 (Figure 1) were amplified by the polymerase chain reaction (PCR). PCR was carried out using either iProof Polymerase (BioRad; Hercules, CA) or Phusion Polymerase (New England Biolabs; Beverly, MA). In each case, the manufacturer's “high fidelity” buffer was used, employing 1.75 mM Mg²⁺, along with 0.2 mM deoxyribonucleotide-triphosphates, and 250 nM primers. Both polymerases gave comparable results in most respects. PCR reactions were conducted in an MJ Research PTC-200 thermal cycler as 50 µl reactions. Amplification involved an initial 150 sec. denaturation at 98°C, followed by 35 cycles of 30 sec. at 98°C, 30 sec. at 58.5°C, and 1 minute at 72°C. This was followed by a 7 minute incubation at 72°C to enhance completeness of products prior to cloning. Amplification of the *B. bovis* β-tubulin gene (accession no. L00978) was included as an internal positive control, using primers AZT01 and XW10. Amplified products were cloned either by using A overhangs, vector pCR4, and the Topo-TA cloning kit (Invitrogen) for iProof polymerase, or by topoisomerase-mediated blunt-end cloning into vector pCR2.1, using the Topo Zero-Blunt cloning kit (Invitrogen) for Phusion polymerase.

Analysis of PCR-amplified *ves1α* fragments

Individual cloned PCR products were cycle-sequenced using dye terminator technology and BigDye v1.1 reagents (Applied Biosystems; Foster City, CA), with analysis on an Applied Biosystems 3130 Sequencer, as described elsewhere [7]. Alternatively, high-throughput sequencing was performed by robotic picking of primary recombinants, rolling-circle amplification of plasmid DNAs with Phi29 polymerase, and dye terminator sequencing of amplified products with analysis on an Applied Biosystems 3730XL Sequence Analyzer. Analysis of sequences and high-throughput sequencing were performed by the DNA sequencing facilities of the University of Florida Interdisciplinary Center for Biotechnology Research. Sequences were trimmed, aligned, and relational trees drawn using CLC Sequence Viewer, v5.0.1 (CLC Bio; Aarhus, Denmark), with manual optimization of alignments. Relational trees were drawn employing the neighbor-joining method [15], using 200 bootstrap iterations. The nearest-neighbor method makes no assumptions regarding the rate of evolution of each sequence, and no evolutionary relationships are implied from our analyses. Alignments of cDNAs were performed including the already published p9.6.2 cDNA *ves1α* sequence (accession number [DQ267461](#)) obtained from the C9.1 clonal line [7], as “type sequence” for comparison. Genomic data for the unrelated *B. bovis* T2Bo isolate were downloaded from Genbank (National Center for Biotechnology Information), using genomic coordinates provided by Brayton et al. [11]. For comparison of cDNA and gDNA sequences, intron 2 was identified by its position relative to the p9.6.2 cDNA, and removed from all gDNA sequences prior to alignment. Incomplete sequences were removed from the alignment. Sequence recovery data were plotted and analyzed by non-linear regression analysis, using SigmaPlot v11 (Systat Software, Inc.; Point Richmond, CA).

Sequences

All sequences presented herein are deposited in GenBank, with accession numbers [FJ536943](#) through [FJ536983](#) (gDNAs) and [FJ536984](#) through [FJ537067](#) (cDNAs).

Results and Discussion

Expressed, full-length copies of the *ves1α* gene have a consistent 3-exon 2-intron structure ([7,8,11]; additional unpublished data). Primers were designed, based upon highly conserved sequences present in available *ves1α* genes, which spanned across intron 2 to enable the use of these primers for analysis of both gDNA and transcripts. A schematic diagram illustrating the overall structure of *ves1α* genes and the locations of the primers used in this study is given in Figure 1. Preliminary experiments revealed that a (4:1) molar ratio of PD2Fa and PD2Fb (PD2Fa,b) as forward primer, coupled with PD1R as reverse primer, consistently provided very sensitive and reproducible amplification of *ves1α* sequences from genomic DNA. We therefore focused in subsequent experiments upon optimizing the PCR reaction for annealing temperature, Mg²⁺ concentration, and primer concentration, using this mixture of primers and gDNA as template.

The amplicons recovered from gDNA were a diverse mixture of major products ranging in size from approximately 550-700 bp, with a lesser smearing of higher mass products (Figure 2). Reproducible and robust amplification could be achieved with as little as 100 fg of genomic DNA, although amplification could often be achieved with as little as 10 fg, or roughly one genome equivalent of total DNA. This level of sensitivity is likely possible only because the *B. bovis* genome contains approximately 100 *ves1α* genes, scattered over the four chromosomes [8,11,16]. However, amplification from such a small quantity would seriously skew representational aspects of the results. The high mass smears may be a result of crossover PCR amplification products resulting from the presence of conserved sequences within these variant genes, including the segment under study, and/or the predominant organization of this gene

family as clusters of divergently-oriented gene pairs [7,8,11]. These characteristics of the *ves* gene family could result in formation of some unanticipated products. Despite this, no such products were cloned, nor do any identifiable genomic copies of *ves1α* fall into that size range [11].

To determine how broadly this primer set would function for the recovery of any *ves1α* sequence present, *ves1α* sequences were amplified from 10 ng of genomic DNA, representing approximately 1,000 genome equivalents, and the amplicons were cloned and sequenced. Of 192 colonies picked for sequencing 176 provided useable, high quality sequence. The sequences recovered represented 58 unique CKRD sequences, of which 41 represented full-length sequences from the CKRD domain (Figure 3A and Supplementary Figure S1). The other 17 were incomplete sequences, presumably due to deletion of end sequences during cloning. This compares favorably with the 47 full-length CKRD domain sequences present among the 72 *ves1α* genes identified in the *B. bovis* genome [11] (Figure 3B). Sequences were considered to be unique if they had three or more nucleotide differences from other sequences. Of the 41 full-length CKRD sequences observed, 28 were recovered once only. All others were recovered from two to 33 times, with the distribution shown in Figure 4. The frequency distribution of individual sequence recoveries was fit well ($R^2 = 0.9506$) by a non-linear regression curve describing a dual parameter-exponent logarithmic distribution. Given this excellence of fit, we interpret this to indicate that the limiting factors in sequence detection are the points during the PCR process at which individual sequences begin to be amplified logarithmically, and whatever amplification bias exists for each gene copy once logarithmic amplification is initiated. If this distribution were due solely to initiation of logarithmic amplification, the recovery range from 1 to 33 copies would imply a difference of approximately 5.04 cycles of amplification between the latest and earliest to amplify sequences. Alternatively, if this were due solely to primer bias for specific loci, this would maximally represent a bias of approximately 10.5% per cycle for the most abundant products. It is likely that both factors come into play, particularly as the capacity for some quasi-palindromic *ves* loci to self-anneal when denatured (unpublished observations) is likely to be greater than others. This feature of these loci undoubtedly affects the ability of primers to anneal with one or the other of the sites, affecting the point at which logarithmic amplification predominates over linear amplification. Regardless, this primer set clearly amplifies a very wide variety of the *ves1α* repertoire efficiently. When applied to the study of antigenic variation dynamics at the transcript level, where the apposing *ves* sequence is not physically contiguous and continuous, it is apparent that it should be possible to capture most, if not all, full-length variant transcripts.

An alternative indication of the universality of the primers is provided by comparison of the relational trees made for non-redundant full-length sequences recovered from *B. bovis* C9.1 line and unrelated T2Bo isolate gDNAs (Figure 3). It is unfortunately not possible to directly compare the sequences as the *B. bovis* genome was sequenced from the T2Bo isolate. The T2Bo isolate was obtained from Texas in 1978 [17], whereas this work was done using the C9.1 clonal line, a derivative of the Mexico isolate [18], and the two original parasite populations were segregated both geographically and temporally. Being highly variant genes overall there is no direct *ves1α* gene-to-gene correspondence between isolates. Indeed, construction of a relational tree from an alignment containing C9.1 and T2Bo sequences together results almost exclusively in isolate-specific segregation and clustering of sequences (data not shown). However, it is apparent that the *ves1α* sequences in each isolate similarly clustered into four major groups (Figure 3, dashed boxes) with numerically similar distributions of members. This finding supports the probability that there is not a significant bias toward recovery of the members of one grouping relative to another, an important characteristic of primers intended for this usage. As no specific sequence motifs appear responsible for these groupings, it is assumed they simply reflect overall similarities among members. The cause of this similarity is unclear, but in the T2Bo isolate it is unrelated to their chromosomal origins

(not shown). It is possible that sequences within groups are favored interaction partners for other sequences within the same groups during gene conversion events, resulting in overall partial homogenization of sequences. This possibility remains to be explored.

The primers were tested for their intended application in a pilot experiment involving RT-PCR amplification of cDNA made from total *B. bovis* C9.1 line RNA. Prior to isolation of RNA the parasite population was selected once for binding of mAb 4D9.1G1 to provide as antigenically homogeneous a population as possible. This enabled us to ask whether transcripts appeared to arise from a single *ves1α* locus or from multiple loci. Of the 96 sequencing reactions performed, 84 gave useable results. Alignment of these sequences revealed that 67 of the sequences were identical or sufficiently similar to one another (tree distance ≤ 0.001), to clearly reflect transcription from a single *ves1α* locus that had undergone little mutation within the population (Figure S2). When aligned with the amplified gDNA sequences, all but seven of the 17 remaining cDNA sequences were more closely related to the major cDNA form, to one another, and to the p9.6.2 cDNA form of the *ves1α* gene than to any other genomic copy, strongly suggesting these, too, were derivatives of the same locus (Figure 5). The p9.6.2 form was that present in the *B. bovis* C9.1 line LAT at the time when this gene family was first described; it has since undergone some modification and continues to do so due to ongoing stochastic variation. Further evidence for a common locus origin of these transcripts is provided by the extreme conservation of sequences in the region from position 244-333 of the cDNA alignment (Figure S2), in contrast with the extreme diversity present in the corresponding region among gDNA sequences (Figure S1, positions 317-453 of alignment). This result virtually precludes a multi-locus origin for these transcripts. Six of the remaining seven cDNAs more closely resembled but were significantly different from other genomic copies of the *ves1α* gene, indicating either that they represent unique genes not captured during gDNA amplification, or that they have been so extensively modified as to no longer resemble any specific genomic locus. On the other hand, one cDNA, SSII-G09, perfectly matched another genomic locus represented by sequence DS2-B08. This cDNA was either transcribed from that locus, perhaps as “leaky transcription”, or sequences from that locus completely replaced the amplified region within the known LAT. Alternatively, this cDNA may reflect an *in situ* transcriptional switch from the known LAT to the DS2-B08 locus in a small fraction of the population. These data do not discriminate among these possibilities. Differences among the various p9.6.2-like cDNAs recovered were due to scattered point mutations or replacement of short sequence patches, consistent with the previously demonstrated pattern of sequence changes observed during segmental gene conversion [8]. Since the parasites used in this experiment had been growing in culture for several months without selection for a particular CKRD sequence, it would be expected for mutations to have accumulated, as modification of the *ves1α* gene occurs over time during *in vitro* culture [7]. This is true despite the selection of these parasites for immunoreactivity with monoclonal antibody 4D9.1G1 prior to RNA isolation, since the epitope recognized by this antibody maps to the VDCS domain encoded by sequences approximately 2000 nucleotides downstream [7]. Significantly, these results are consistent with and provide experimental support for mutually exclusive transcription of the *ves1α* gene. However, they do not imply that a single *ves* locus is competent for transcription, only that a single *ves* locus appears to be transcriptionally active at one time. It should be noted, too, that RT-PCR recovers RNA species roughly in proportion to their relative steady-state abundance, and short-lived RNAs that do not accumulate to significant levels may not be detected in a small scale analysis.

It is not unexpected that transcription among *ves1α* genes would be mutually exclusive. The success of antigenic variation depends upon the infectious agent not revealing more of its antigen repertoire than necessary at any given time [19]. Otherwise, a broadly reactive immune response could ensue that might eliminate the parasite before it could respond. Mutually exclusive transcription of variant antigen genes from within a multigene family has been demonstrated in other infectious agents, including among the 1000 or so variant surface

glycoprotein (VSG) genes of the African trypanosomes [20] and the 60 *var* genes of *P. falciparum* [21,22]. *B. bovis* shares with these two parasitic protozoa genetic mechanisms of antigenic variation and lifestyle within the host, respectively, as well as the ability to establish persistent infections.

A key characteristic of the primer set described herein is that it was designed to span intron 2 of the *ves1α* gene. This facilitates assessment of the nucleic acid source being amplified during RT-PCR experiments, a fundamental characteristic for interpreting the results of such studies. During numerous attempts to perform single-cell RT-PCR on *B. bovis* IRBCs this proved crucial in establishing that, with rare exception, amplification of transcripts was not being detected. Rather, an apparent “reassembly” of gDNA fragments during the process of reverse transcription seems to have occurred, despite consistent failure to amplify products in control samples lacking reverse transcriptase (unpublished results). A similar observation has been reported in the amplification of mammalian transcripts [23]. Unfortunately, similar studies performed on *P. falciparum* in the study of *var* gene transcription dynamics employed primer sets which could not provide such information due to the structure of *var* genes and the necessary location of primer binding within the gene [22,24]. Interestingly, *ves1α* intron 2 was observed to exist in two discrete size ranges among the *ves1α* sequences, 38-39 bp and 72-74 bp, yet to share essentially invariant sequences at the 5' (GTAAGTA) and 3' (TAG) ends and some internal sequences (Figure S3). The significance of this dichotomy is not clear.

Our results demonstrate that the PD2Fa,b combination of forward primers with the PD1r reverse primer provides for extremely sensitive amplification of the *ves1α* gene repertoire of *B. bovis*, with little bias. These primers also allow discrimination of transcript versus gDNA amplification, although unspliced precursor transcripts may be misinterpreted. When applied to RNA obtained from “bulk culture” of a clonal parasite line the results strongly suggest that transcription among the *ves1α* genes occurs in a mutually exclusive manner. Because of the characteristics and performance demonstrated by this primer set, we propose that it may provide a suitable tool to follow variation within this region of the *ves1α* gene during *in vivo* infection with *B. bovis*, and may provide a basis for the development of sensitive PCR-based diagnostics. Given their broad specificity, it is unlikely that such diagnostics would be adversely affected by the identity of the isolate under study or by antigenic variation within that isolate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

cDNA	complementary DNA
gDNA	genomic DNA
PCR	polymerase chain reaction
RT-PCR	

reverse transcriptase- polymerase chain reaction

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Figure 1. Location of the universal primers relative to *ves1α* gene structure

The general structure of the *ves1α* gene is shown. The *ves1α* gene is comprised of three exons, with two introns near the 5' end of the gene. Primers PD2Fa,b and PD1R anneal at the sites marked with arrowheads, spanning intron 2. CKRD, “cysteine-lysine-rich domain” region; VDCS, “variant domain conserved sequences” region; T, predicted transmembrane domain region; 1, intron 1; 2, intron 2; 5', 5' end of gene and 5'-untranslated region; 3', 3'-untranslated region.

Figure 2. Amplification of *ves1α* CKRD sequences with the *ves1α* “universal” primer pair
 Agarose gel electrophoresis of products amplified from *B. bovis* C9.1 line (a) and MO7 line (b) gDNAs over a range of template concentrations, using the *ves1α* universal primers. Lanes: 1a,b- 100 pg template; 2a,b- 10 pg template; 3a,b- 1 pg template; 4a,b- 0.1 pg template; 5a,b- 0.01 pg template; 6 and 7, water controls. In our experience, the products observed in the water controls are persistent artifacts of iProof polymerase in the absence of template and not contamination with babesial DNA. They fail to appear with Phusion polymerase.

Figure 3A

Figure 3B

Figure 3. Relational trees for *B. bovis* C9.1 and T2Bo gDNA-derived CKRD sequences
Alignments were made of all non-redundant, full-length CKRD sequences amplified from (A) the C9.1 line and (B) all T2Bo strain *ves1a* sequences containing a full-length CKRD domain (acquired from GenBank). Sequences from both parasite populations cluster into four similarity groups, indicated by dashed boxes. Relational trees were constructed using nearest-neighbor analysis, with 200 bootstrap iterations. Relational distances are provided for these and all subsequent relational trees.

Figure 4. Distribution of gDNA sequence recoveries

Shown is the distribution of frequencies with which all 58 unique sequences of the 186 total were recovered. The distribution is very well described by a dual-parameter exponent logarithmic distribution ($R^2 = 0.9506$).

Figure 5. Relational tree comparing PCR-recovered cDNA and gDNA sequences

All full-length cDNA (SSII series) and comparable non-redundant gDNA sequences (DS and DS2 series) were aligned, subsequent to removal of intron 2 sequences from the gDNA sequences, and a relational tree was constructed. The p9.6.2 cDNA sequence (boxed) was included for comparative purposes. It is clear that the large majority of the cDNA sequences are either identical or extremely similar and cluster together, whereas gDNA sequences are all unique. Only seven of the cDNA sequences cluster more closely with gDNA than with other cDNA sequences (boxed sequences), and only one is a perfect match.