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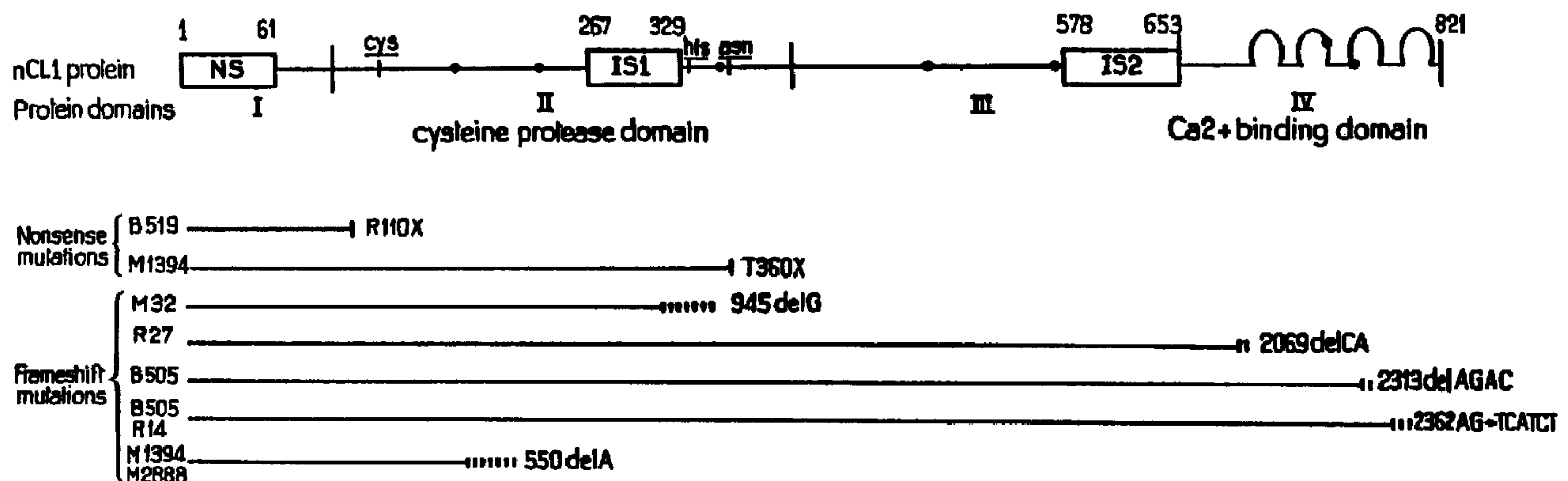
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(54) Title: LGMD GENE CODING FOR A CALCIUM DEPENDENT PROTEASE



(57) Abrégé/Abstract:

A nucleic acid sequence comprising: 1) the sequence represented in figure 8; or 2) the sequence represented in figure 2; or 3) a part of the sequence of figure 2 with the proviso that it is able to code for a protein having a calcium dependant protease activity involved in a LGMD2; or 4) a sequence derived from a sequence defined in 1), 2) or 3) by substitution, deletion or addition of one or more nucleotides with the proviso that said sequences still codes for said protease.

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(54) Title: LGMD GENE CODING FOR A CALCIUM DEPENDENT PROTEASE (57) Abstract A nucleic acid sequence comprising: 1) the sequence represented in figure 8; or 2) the sequence represented in figure 2; or 3) a part of the sequence of figure 2 with the proviso that it is able to code for a protein having a calcium dependant protease activity involved in a LGMD2; or 4) a sequence derived from a sequence defined in 1), 2) or 3) by substitution, deletion or addition of one or more nucleotides with the proviso that said sequences still codes for said protease.		

LGMD gene coding for a calcium dependent protease

The invention relates to the isolated gene coding for a calcium dependent protease belonging to the Calpain family which, when it is mutated, is a cause of a disease called Limb-Girdle Muscular Dystrophy (LGMD).

The term limb-girdle muscular dystrophy (LGMD) was first proposed by Walton and Nattrass (1954) as part of a classification of muscular dystrophies. LGMD is characterised by progressive symmetrical atrophy and weakness of the proximal limb muscles and by elevated serum creatine kinase. Muscle biopsies demonstrate dystrophic lesions and electromyograms show myopathic features. The symptoms usually begin during the first two decades of life and the disease gradually worsens, often resulting in loss of walking ability 10 or 20 years after onset (Bushby, 1994). Yet, the precise nosological definition of LGMD still remains unclear. Consequently, various neuromuscular diseases such as facioscapulohumeral, Becker muscular dystrophies and especially spinal muscular atrophies have been occasionally classified under this diagnosis. For example, a recent study (Arikawa et al., 1991) reported that 17% (out of 41) of LGMD patients showed a dystrophinopathy. These issues highlight the difficulty in undertaking an analysis of the molecular and genetic defect(s) involved in this pathology.

Attempts to identify the genetic basis of this disease go back over 35 years. Morton and Chung (1959) estimated that "the frequency of heterozygous carrier ... is 16 per thousand persons". The same authors also stated that "the segregation analysis gives no evidence on whether these genes in different families are allelic or at different loci". Both autosomal dominant and recessive transmission have been reported, the latter being more common with an estimated prevalence of 10^{-5} (Emery, 1991). The localisation of a gene for a recessive form on chromosome 15 (LGMD2A, MIM 253600; Beckmann et al., 1991) provided the definitive proof that LGMD is a specific genetic entity. Subsequent genetic analyses confirmed this chromosome 15 localisation (Young et al., 1992; Passos-Bueno et al., 1993), the latter group demonstrating genetic heterogeneity of this disease. Although a recent study localised a second mutant

gene to chromosome 2 (LGMD2B, MIM 253601; Bashir et al., 1994). there is evidence that at least one other locus can be involved.

Genetic analyses of the LGMD2 kindreds revealed unexpected findings. First genetic heterogeneity was demonstrated in the highly inbred Indiana Amish community. Second although the Isle of la Réunion families were thought to represent a genetic isolate, at least 6 different disease haplotypes were observed, providing evidence against the hypothesis of a single founder effect (Beckmann et al., 1991) in this inbred population.

The nonspecific nosological definition, the relatively low prevalence and genetic heterogeneity of this disorder limit the number of families which can be used to restrict the genetic boundaries of the LGMD2A interval. Cytogenetic abnormalities, which could have helped to focus on a particular region, have not been reported. Immunogenetic studies of dystrophin-associated proteins (Matsumura et al., 1993) and cytoskeletal or extracellular matrix proteins such as a merosin (Tomé et al., 1994) failed to demonstrate any deficiency. In addition, there is no known specific physiological feature or animal model that could help to identify a candidate gene. Thus, there is no alternative to a positional cloning strategy.

It is established that the LGMD2 chromosomal region is localized on chromosome 15 as 15q15.1 - 15q21.1 region (Fougerousse et al., 1994).

Construction and analysis of a 10-12 Mb YAC contig (Fougerousse et al., 1994) permitted the mapping of 33 polymorphic markers within this interval and to further narrow the LGMD2A region to between D15S514 and D15S222. Furthermore, extensive analysis of linkage disequilibrium suggested a likely position for the gene in the proximal part of the contig.

The invention results from the construction of a partial cosmid map and the screening by cDNA selection (Lovett et al., 1991 ; Tagle et al., 1993) for muscle-expressed sequences encoded by this interval led to the identification of a number of potential candidate genes. One of these, previously cloned by Sorimachi et al. (1989), encodes a muscle specific protein, nCL1 (novel Calpain Large subunit 1), which belongs to the calpain family (CANP, calcium-activated neutral protease; EC 3.4.22.17), and appeared to be a functional candidate gene for this disease.

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Calpains are non-lysosomal intracellular cysteine proteases which require calcium for their catalytic activities (for a review see Croall D.E. et al, 1991). The mammalian calpains include two ubiquitous proteins CANP1 and CANP2 as well as tissue-specific proteins. In addition to the muscle specific nCL1, stomach specific nCL2 and nCL2' proteins have also been described; these are derived from the same gene by alternative splicing. The ubiquitous enzymes consist of heterodimers with distinct large subunits associated with an common small subunit ; the association of tissue-specific large subunits with a small subunit has not yet been demonstrated. The large subunits of calpains can be subdivided into 4 protein domains. Domains I and III, whose functions remain unknown, show no homology with known proteins. Domain I, however, seems important for the regulation of the proteolytic activity. Domain II shows similarity with other cysteine proteases, sharing histidine, cysteine and asparagine residues at its active sites. Domain IV comprises four EF-hand structures which are potential calcium binding sites. In addition, three unique regions with no known homology are present in the muscle-specific nCL1 protein, namely NS, IS1 and IS2, the latter containing a nuclear translocation signal. These regions may be important for the muscle specific function of nCL1.

It is usually accepted that muscular dystrophies are associated with excess or deregulated calpains, and all the known approaches for curing these diseases are the use of antagonists of these proteases ; examples are disclosed in EP 359309 or EP 525420.

The invention results from the finding that, on the opposite to all these hypothesis, the LGMD2 disease is strongly correlated to the defect of a calpain which is expressed in healthy people.

The invention relates to the nucleic acid sequence such as represented in Figure 2 coding for a Ca^{++} dependent protease, or calpain, which is involved in LGMD2 disease, and more precisely LGMD2A. It also relates to a part of this sequence provided it is able to code for a protein having a calcium-dependent protease activity involved in LGMD2, or a sequence derived from one of the above sequences by substitution, deletion or addition of one or more nucleotides provided that said sequence is still coding for said protein, all the nucleic acids yielding a sequence complementary to a sequence as defined above.

The genomic organisation of the human nCL1 gene has been determined by the inventors, and consists of 24 exons and extends over 40 kb as represented in Figure 8, and is also a part of the invention. About 35 kb of this gene have been sequenced. A systematic screening of this gene in LGMD2A families led to the identification of 14 different mutations, establishing that a number of independent mutational events in nCL1 are responsible for LGMD2A. Furthermore, this is the first demonstration of a muscular dystrophy resulting from an enzymatic rather than a structural defect.

In the present specification, CANP3 means the protein which is a Ca^{++} dependent protease, or calpain, and coded by the nCL1 gene on chromosome 15.

The invention relates also to a protein, called CANP3, consisting in the amino acid sequence such as represented in figure 2 and which is involved, when mutated, in the LGMD2 disease.

The cDNA of the gene coding for CANP3, which is coding for the protein, is also represented in Figure 2, and is a part of the invention.

The protein coded by this DNA is CANP3, a calcium-dependent protease belonging to the Calpain family.

Are also included in the present invention the nucleic acid sequences derived from the cDNA of Figure 2 by one or more substitutions, deletions, insertions, or by mutations in 5' or 3' non coding regions or in splice sites, provided that the translated protein has the protease, calcium-dependent activity, and when mutated, induce LGMD2 disease.

The nucleic acid sequence encoding the protein might be DNA or RNA and be complementary to the nucleic acid sequence represented in Figure 2.

The invention also relates to a recombinant vector including a DNA sequence of the invention, under the control of a promoter allowing the expression of the calpain in an appropriate host cell.

A procaryotic or eucaryotic host cell transformed by or transfected with a DNA sequence comprising all or part of the sequence of Figure 2 is a part of the invention.

Such a host cell might be either :

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- a cell which is able to secrete the protein and, this recombinant protein might be used as a drug to treat the LGMD2, or

- a packaging cell line transfected by a viral or retroviral vector: the cell lines bearing recombinant vector might be used as a drug for gene therapy of LGMD2.

5 All the systems used today for gene therapy including adenoviruses and retroviruses and others are described for example in "l'ADN médicament", (John Libbey, Eurotext, 1993).

The examples hereunder and attached figures indicate how the structure of the gene was established, and how relationship between the gene and the LGMD was
10 established.

According to one aspect of the present invention, there is provided a nucleic acid selected among: a nucleic acid comprising the nucleotide sequence represented in Figure 8; a nucleic acid comprising the nucleotide sequence represented in Figure 2; a nucleic acid encoding the amino acid sequence represented in Figure 2;
15 and a nucleic acid obtained by substitution of one or more nucleotides of the nucleic acid defined above, with the proviso that said nucleic acid codes the Ca^{2+} dependent protease represented in Figure 2.

According to another aspect of the present invention, there is provided a nucleic acid that is complementary over its whole length to the nucleic acid as
20 described above.

According to still another aspect of the present invention, there is provided a nucleic acid comprising in its structure the nucleic acid as described above, under the control of regulatory elements, and involved in the expression

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5a

of a calpain enzyme active in a Limb Girdle Muscular Dystrophy type 2 (LGMD2) disease.

According to yet another aspect of the present invention, there is provided a polypeptide which is encoded by
5 the nucleic acid sequence as described above.

According to a further aspect of the present invention, there is provided a host cell which, when transformed or transfected with the nucleic acid sequence as described above, expresses a calpain enzyme activity.

10 According to yet a further aspect of the present invention, there is provided use of the nucleic acid as described above in the manufacturing of a drug for the prevention or the treatment of an LGMD2 disease.

According to still a further aspect of the present
15 invention, there is provided use of the polypeptide as described above in the manufacturing of a drug for the prevention or the treatment of an LGMD2 disease.

According to another aspect of the present invention, there is provided use of the polypeptide as
20 described above for the screening of the ligands of said amino acid sequence, said ligand being selected in a group consisting of substrate(s), co-factors and regulatory components.

According to yet another aspect of the present
25 invention, there is provided use of the nucleic acid as described above in a screening method for the determination of the components which may act on the regulation of gene expression of calpain.

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5b

According to yet another aspect of the present invention, there is provided use of the host cell as described above in a screening method for the determination of components active on the expression of the calpain.

5 According to yet another aspect of the present invention, there is provided a method for screening for the presence of nucleotide changes or LGMD2 causative mutations in a family or a human being, such method comprising the steps of: selecting one or more exons or their flanking sequences of
10 a nCL1 (novel Calpain Large subunit 1) gene, selecting primers specific for these exons, or their flanking sequences, or a hybrid thereof, amplifying the nucleic acid sequences with these primers, the substrate for this amplification being DNA of a human being; and comparing the amplified sequence to the
15 corresponding sequence of Figure 2 or Figure 8.

 According to yet another aspect of the present invention, there is provided a kit for the screening for the presence of nucleotide changes or LGMD2 causative mutations by
20 nucleic acid amplification, wherein said kit comprises primers as defined above.

 According to another aspect of the present invention, there is provided pharmaceutical composition for the treatment of an LGMD2 disease, wherein said pharmaceutical
25 composition contains a pharmaceutically acceptable carrier and a component selected from the group of: a) the nucleic acid as described above, and b) the polypeptide as described above.

Legend of the figures:

Figure 1:

30 A) Genomic organisation of the nCL1 gene

 The gene covers a 40 kb region of which 35 was sequenced (Accession number pending). Introns and exons

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are drawn to scale, the latter being indicated by numbered vertical bars. The first intron is the largest one and remains to be fully sequenced. Position of intragenic microsatellites are indicated by asterisks. Arrows indicate the orientation of Alu (closed) and of Mer2 (greyed) repeat sequences.

B) *Eco*R1 restriction map

An *Eco*R1 (E) restriction map of this region was established with the help of cosmids from this region. The location of nCL1 gene is indicated as a black bar. The size of the corresponding fragments are indicated and are underlined when determined by sequence analysis.

C) Cosmid map of the nCL1 gene region.

Cosmids were from a cosmid library constructed by a subcloning YAC 774G4 (Richard in preparation) and are presented as lines. Dots on lines indicate positive STSs (indicated in boxed rectangles). A minimum of three cosmids cover the entire gene. T3, T7

Figure 2: Sequence of the human nCL1 cDNA (B) , and the flanking 5' (A) and 3' (C) genomic regions.

A) and C) The polyadenylation signal and putative CAAT, TATAA sites are boxed. Putative Sp1 (position -477 to -472), MEF2 binding sites (-364 to -343) and CArG box (-685 to -672) are in bold. The Alu sequence present in the 5' region is underlined.

B) The corresponding amino acids are shown below the sequence. The coding sequence between the ATG initiation codon and the TGA stop codon is 2466 bp, encoding for a 821 amino acid protein. The adenine in the first methionine codon has been assigned position 1. Locations of introns within the nCL1 gene are indicated by arrowheads. Nucleotides which differ from the previously published ones are indicated by asterisks.

Figure.3: Alignments of amino acid sequences of the muscle-specific calpains.

The human nCL1 protein is shown on the first line. The 3 muscle-specific sequences (NS, IS1 and IS2) are underlined. The second line corresponds to the rat sequence (Accession no P). The third and fourth lines show the deduced amino acid sequences encoded by pig and bovine Expressed Sequences Tagged (GenBank accession no U05678 and no U07858, respectively). The amino acids residues which are conserved among all known members of the calpains are in reverse letters. A period indicates that the same amino acid is present in the sequence. Letters refer to the variant amino acid found in the homologous sequence. Position of missense mutations are given as numbers above the mutated amino acid.

Figure 4: Distribution of the mutations along nCL1 protein structure.

A) Positions of the 23 introns are indicated by vertical bars in relation to the corresponding amino acid coordinates.

B) The nCL1 protein is depicted showing the four domains (I, II, III, IV) and the muscle specific sequences (NS, IS1 and IS2). The position of missense mutations within nCL1 domain are indicated by black dots. The effect of nonsense and frameshift mutations are illustrated as truncated lines, representing the extent of protein synthesised. Name of the corresponding families are indicated on the left of the line. The out of frame ORF is given by hatched lines.

Figure 5: Northern blot hybridisation of a nCL1 clone

A mRNA blot (Clontech) containing 2 µg of poly(A)+ RNA from each of eight human tissues was hybridised with a nCL1 genomic clone spanning exons 20 and 21. The latter detects a 3.6 kb mRNA present only in a line corresponding to the skeletal muscle mRNA.

Figure 6: Representative mutations identified by heteroduplex analysis.

Examples of mutation screening by heteroduplex analysis. Pedigree B505 shows the segregation of two different mutations in exon 22.

Figure 7: Homozygous mutations in the nCL1 gene

Detection by sequencing of mutations in exons 2 (a), 8 (b), 13 (c) and 22 (d). Sequences from a healthy control are shown above each mutant sequence. Asterisks indicate the position of the mutated nucleotides. The consequences on codon and amino acid residues are indicated on the left of the figure together with the name of the family.

Figure 8 : Structure of nCL1 gene

Figure 8A represents the 5' part of the gene with exon 1.

Figure 8B represents the part of the gene including exons 2 to 8,

Figure 8C represents the part of the gene including exon 9,

Figure 8D represents the part of the gene including exons 10 to 24 including the 3' non transcribed region.

EXAMPLES**EXAMPLE 1****Localisation of the nCL1 within the LGMD2A interval**

Detailed genetic and physical maps of the LGMD2A region were constructed (Fougerousse et al., 1994), following the primary linkage assignment to 15q (Beckmann et al., 1991). The disease locus was bracketed between the D15S129 and D15S143 markers, defining the cytogenetic boundaries of the LGMD2A region as 15q15.1-15q21.1 (Fougerousse et al., 1994). Construction and analysis of a 10-12 Mb YAC contig (Fougerousse et al., 1994) permitted us to map 33 polymorphic markers within this interval and to further narrow the LGMD2A region to between D15S514 and D15S222.

The nCL1 gene had been localised to chromosome 15 by hybridisation with sorted chromosomes and by Southern hybridisation to DNA from human-mouse cell hybrids (Ohno et al., 1989). cDNA capture using YACs from the LGMD2A interval allowed the identification of thirteen positional candidate genes. nCL1 was one of the two transcripts identified that showed muscle-specific expression as evidenced by northern blot analysis. The localisation was further confirmed by STS (for Sequence Tagged Site) assays. Primers used for the localisation of the nCL1 gene are P94in2, P94in13 and pcr6a3, as shown in Figure 1 and their characteristics being defined in Table 1.

10 Table 1: PCR primers used for localisation of the nCL1 gene.

Primer name	Primer sequence (5'-3')	Position within the cDNA	Annealing temp (°C)	PCR product size on	
				cDNA	genomic DNA
P94in2	ATGGAGCCAACAGAACTGA C GTATGACTCGGAAAAGAAG GT	341-360 428-448	58	108	1758
P94in13	TAAGCAAAAGCAGTCCCCA C TTGCTGTTCTCACTTTCT G	1893-1912 1936-1956	58	64	1043
P94-6a3	GTTTCATCTGCTGCTTCGTT CTGGTTCAGGCATACATGG T	2342-2361 2452-2471	56	130	818
P94ex1ter	TTCTTTATGTGGACCCTGAG TT ACGAACTGGATGGGGAAC	218-239 275-293	55	76	76

These primers are designed from different parts of the published human cDNA sequence (Sorimachi et al., 1989), and were used for an STS content screening on DNA from three chromosome 15 somatic cell hybrids and YACs from the LGMD2A contig. The results positioned the gene in a region previously defined as 15q15.1-q21.1 and on 3 YACs (774G4, 926G10, 923G7) localised in this region. The relative positions of STSs along the LGMD2A contig allowed to localise the gene between D15S512 and D15S488, in a candidate region suggested by linkage disequilibrium studies.

The same primers as above were used to screen a cosmid library from YAC 774G4. A group of 5 cosmids was identified (Fig. 1). Experiments with another nCL1 primer pair (P94ex1ter; Table 1) established that these cosmids cover all nCL1 exons except number 1, and that a second group of 4 cosmids contain this

exon (Fig. 1). A minimal set of three overlapping cosmids (2G8-2B11-1F11) covers the entire gene (Figure 1). DNA from these cosmids was used to construct an *EcoRI* restriction map of this region (Figure 1B).

EXAMPLE 2

5 **Determination of the nCL1 gene sequence**

Most of the sequences were obtained through shotgun sequencing of partial digests of cosmid 1F11 subcloned in M13 and bluescript vectors, and by walking with internal primers. The sequence assembly was made using the XBAP software of the Staden package (Staden) and was in agreement with the
10 restriction map of the cosmids. Sequences of exon 1 and adjacent regions were obtained by sequencing cosmid DNA or PCR products from human genomic DNA. The first intron is still not fully sequenced, but there is evidence that it may be between 10 to 16 kb in length (based on hybridisation of restriction fragments; data not shown). The entire gene, including its 5' and 3' regions, is more than 40
15 kb long, and shown in Figure 8.

a) the cDNA sequence

The used technology allows the implementation of the published human cDNA sequence of nCL1 (Sorimachi 1989). It contains the missing 129 bases corresponding to the N-terminal 43 amino acids (Figure 2). It also differs from it
20 at 12 positions. Three of which occur at third base positions of codons and preserve the encoded amino acid sequence. The other 9 differences lead to changes in amino-acid composition (Figure 2). As these different exons were sequenced repeatedly on at least 10 distinct genomes, we are confident that the sequence of Fig. 2 represents an authentic sequence and does not contain
25 minor polymorphic variants. Furthermore, these modifications increase the local similarity with the rat nCL1 amino acid sequence (Sorimachi), although the overall similarity is still 94 %.

The ATG numbered 1 in Figure 2 is the translation initiation site based on homology with the rat nCL1, and is within a sequence with only 5 nucleotides out
30 of 8 in common with the Kosak consensus sequence (Kosak M, 1984). Putative CCAAT and TATA boxes were observed 590, 324, (CCAAT) and 544 or 33 bp (TATA) upstream of the initiating ATG codon, respectively (Bucher, 1990). A GC-box binding the Sp1 protein (Dyran et al., 1983) was identified at position -477.

Consensus sequences corresponding to potential muscle-specific regulatory elements were identified (Fig. 2). These include a myocyte-specific enhancer-binding factor 2 (MEF2) binding site (Cserjesi P. 1991), a CArG box (Minty A. 1986) and 6 E-boxes (binding sites for basic Helix-Loop-Helix proteins frequently found in members of MyoD family; Blackwell et Weintraub, 1990). The functional significance of these putative transcription factor binding sites in the regulation of nCL1 gene expression remains to be established.

Two potential AAUAAA polyadenylation signals, were identified 520 and 777 bp downstream of the TGA stop codon. The sequencing of a partial nCL1 cDNA containing a polyA tail, demonstrated that the first AAUAAA is the polyadenylation signal. The latter is embedded in a region well conserved with the rat nCL1 sequence and is followed after 4 bp by a G/T cluster, present in most genes 3' of the polyadenylation site (Birnstiel et al., 1985). The 3'-untranslated region of the nCL1 mRNA is 565 bp long. The predicted length of the cDNA should therefore be approximately 3550 or 3000 bp.

b) Comparison with calpain

The sequence of the human nCL1 gene was compared to those of other calpains thereof (Figure 3). The most telling comparisons are with the homologous rat (Accession no J05121), bovine (Accession no U07858) and porcine (Accession no U05678) sequences. The accession numbers refers to those or international genebanks, such as GeneBank (N.I.H.) or EMBL Database (EMBL, Heidelberg). High local similarities between the human and rat DNA sequences are even observed in the 5' (75%) or in different parts of the 3' untranslated regions (over 60%) (data not shown). The high extent of sequence homology manifested by the human and rat nCL1 gene in their untranslated regions is suggestive of evolutionary pressures on common putative regulatory sequences.

c) Genomic organisation of the nCL1 gene

A comparison of the published nCL1 human cDNA (Sorimachi et al., 1989) with the corresponding genomic sequence led to the identification of 24 exons ranging in length from 12 bp (exon 13) to 309 bp (exon 1), with a mean size of 100 bp (Figure 1). The size of introns ranges from 86 bp to about 10-16 kb for intron 1.

The intron-exon boundaries as shown in Table 2 exhibit close adherence to 5' and 3' splice site consensus sequences (Shapiro and Senapathy, 1987).

Table 2: Sequences at the intron-exon junctions. A score expressing adherence to the consensus was calculated for each site according to Shapiro and Senapathy (1987). Sequences of exons and introns are in upper and lower cases, respectively. Size of exons are given in parenthesis.

splice donor site	score (%)	Intron	score (%)	splice acceptor site	Exon
					Exon 1 (309 bp) ->
...CTCCGgtgagt...	88.5	<-Intron 1->	99.0	...ttttgtttcacagGAAAT...	Exon 2 (70 bp) ->
...GCTAGgtagga...	83.5	<-Intron 2->	90.0	...gtgtcgcctgcagGGGAC...	Exon 3 (119 bp) ->
...TCCAGgtgagg...	92	<-Intron 3->	81.5	...acgcttctgtgcagTTCTG...	Exon 4 (134 bp) ->
...GCTAAgtaagc...	82	<-Intron 4->	81.5	...atcctctctctaagGCTCC...	Exon 5 (169 bp) ->
...TTGATgtaagt...	87	<-Intron 5->	79.5	...ccatcgggcctcagGATGG...	Exon 6 (144 bp) ->
...CCCGGgtgtgt...	77.5	<-Intron 6->	91	...ttactgtcttacagACAAT...	Exon 7 (84 bp) ->
...ATGAGgtaagc...	94	<-Intron 7->	78.5	...tctgtgtgctaagGTCCC...	Exon 8 (86 bp) ->
...GATAGgtaggt...	89	<-Intron 8->	91.5	...cattttcccaccagATGGA...	Exon 9 (78 bp) ->
...TTCTGgtgagt...	88	<-Intron 9->	92	...ttccaacctctcagGATGT...	Exon 10 (161 bp) ->
...CCCAGgtggga...	80	<-Intron 10->	68.5	...ttctgggggtgcagATACT...	Exon 11 (170 bp) ->
...ACGAGgtgtgt...	85.5	<-Intron 11->	86	...tgtttcttctcaagGTTCC...	Exon 12 (12 bp) ->
...AAGAGgtatag...	70	<-Intron 12->	87	...tccccatctctcagATGCA...	Exon 13 (209 bp) ->
...TCTGAgtagt...	76.5	<-Intron 13->	97	...tgtattcctcacagGGAAG...	Exon 14 (37 bp) ->
...CAGTGgtgagt...	89	<-Intron 14->	93.5	...cttttcttaigcagAAAAA...	Exon 15 (18 bp) ->
...CCAAGgtaggt...	89	<-Intron 15->	87	...cctcctctctccagCCCAT...	Exon 16 (114 bp) ->
...CACAGgtgtct...	80	<-Intron 16->	88	...ttgtgcctccacagCCACA...	Exon 17 (78 bp) ->
...GAGATgtgagt...	84	<-Intron 17->	92.5	...cccttccctcctcagGACAT...	Exon 18 (58 bp) ->
...CAAACgtgagt...	83	<-Intron 18->	90	...ctccatccccccagACAAG...	Exon 19 (65 bp) ->
...TGGATgtatcc...	56	<-Intron 19->	88	...cctccctcctccagACAGA...	Exon 20 (69 bp) ->
...GGCAGgtggga...	80	<-Intron 20->	94	...tttctattgccagAAATA...	Exon 21 (79 bp) ->
...CGCAGgtgtgt...	66	<-Intron 21->	91	...ggccccctccacagGATTC...	Exon 22 (117 bp) ->

...GTTCAGtaagt... 79 <-Intron 22-> 93.5 ...gcattctttcacagGAGCT... Exon 23 (59 bp) ->
 ...TGGAGgtaaag... 81 <-Intron 23-> 79 ...gggacttctttcagTGGCT... Exon 24 (27 bp) ->

When the genomic sequence was submitted to GRAIL analysis (Uberbacher et al., 1991), 11 exons were correctly recognised, 4 were not identified, 6 were inadequately defined and 2 were too small to be recognised (data not shown).

5 As already noted, the nCL1 gene has three unique sequence blocks, NS (amino acid residues 1 to 61), IS1 (residues 267 to 329) and IS2 (residues 578 to 653). It is interesting to note that each of these sequences, as well as the nuclear translocation signal inside IS2, are essentially flanked by introns (Fig. 4). The exon-intron organisation of the human nCL1 is similar to that reported for
 10 the chicken CANP (the only other large subunit calpain gene whose genomic structure is known; (Emori et al., 1986).

Four microsatellite sequences were identified. Two of them are in the distal part of the first intron: an (AT)₁₄ and an previously identified mixed-pattern microsatellite, S774G4B8, which was demonstrated to be non polymorphic
 15 (Fougerousse et al., 1994). A (TA)₇(CA)₄(GA)₁₃ was identified in the second intron and genotyping of 64 CEPH unrelated individuals revealed two alleles (with frequencies of 0.10 and 0.90). The fourth microsatellite is a mixed (CA)_n(TA)_m repeat present in the 9th intron. The latter and the (AT)₁₄ repeat have not been investigated for polymorphism. Fourteen repetitive sequences of
 20 the Alu family and one Mer2 repeat were identified in the nCL1 gene (Fig. 1C), which has, thus, on the average one Alu element per 2.5 kb.

Southern blot experiments (Ohno et al., 1989) and STS screening (data not shown) suggest that there is but one copy per genome of this member of the calpain family.

25 EXAMPLE 3

Expression of the nCL1 gene

The pattern of tissue-specificity was investigated by northern blot hybridisation with a genomic subclone probe from cosmid 1F11 spanning exons 20 and 21. There is no evidence for the existence of an alternatively spliced form
 30 of nCL1, although this cannot be excluded. A transcript of about 3.4-3.6 kb was

detected in skeletal muscle mRNA (Figure 5). This size therefore favours that the position -544 is the functional TATA box.

Transcription studies suggested that it is an active gene rather than a pseudogene and its muscle-specific pattern of expression is consistent with the phenotype of this disorder (Sorimachi et al., 1989 and Figure 5).

EXAMPLE 4

Mutation screening

nCL1 fulfils both positional and functional criteria to be a candidate gene for LGMD2A. To evaluate its role in the etiology of this disorder, nCL1 was systematically screened in 38 LGMD2 families for the presence of nucleotide changes using a combination of heteroduplex (Keen et al., 1991) and direct sequence analyses.

PCR primers were designed to specifically amplify the exons and splice junctions and also the regions containing the putative CAT, TATA boxes and the polyadenylation signal of the gene as shown in Table 3.

Table 3: PCR primers used for the analysis of the nCL1 gene in LGMD patients.

amplified region	Primer sequences (5'-3')	Size (bp)	Annealing temp. (°C)
promotor	TTCAGTACCTCCCGTTCACC GATGCTTGAGCCAGGAAAAC	296	59
exon 1	CTTTCCTTGAAGGTAGCTGTAT GAGGTGCTGAGTGAGAGGAC	438	60
exon 2	ACTCCGTCTCAAAAAAATACCT ATTGTCCCTTTACCTCCTGG	239	57
exon 3	TGGAAGTAGGAGAGTGCGCA GGGTAGATGGGTGGGAAGTT	354	58
exon 4	GAGGAATGTGGAGGAAGGAC TTCCTGTGAGTGAGGTCTCG	292	59
exon 5	GGAACTCTGTGACCCCAAAT TCCTCAAACAAAACATTTCGC	325	56
exon 6	GTTCCCTACATTCTCCATCG GTTATTTCAACCCAGACCCTT	315	57
exon 7	AATGGGTTCTCTGGTTACTGC AGCACGAAAAGCAAAGATAAA	333	56
exon 8	GTAAGAGATTTGCCCCCAG TCTGCGGATCATTGGTTTGTG	321	58
exon 9	CCTTCCCTTCTTCCTGCTTC CTCTCTTCCCCACCCTTACC	173	56
exon 10	CCTCCTCACCTGCTCCCATA TTTTTCGGCTTAGACCCTCC	251	56
exon 11	TGTGGGGAATAGAAATAAATGG CCAGGAGCTCTGTGGGTCA	355	57
exon 12	GGCTCCTCATCCTCATTCACA GTGGAGGAGGGTGAGTGTGC	312	61
exon 13	TGTGGCAGGACAGGACGTTC	337	60

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		TTCAACCTCTGGAGTGGGCC		
exon 14		CACCAGAGCAAACCGTCCAC	230	61
		ACAGCCCAGACTCCCATTCC		
exon 15		TTCTCTTCTCCCTTCACCCT	225	57
		ACACACTTCATGCTCTCTACCC		
exon 16		CCGCCTATTCTTTCCTCTT	331	56
		GACAAACTCCTGGGAAGCCT		
exon 17		ACCTCTGACCCCTGTGAACC	270	61
		TGTGGATTTGTGTGCTACGC		
exon 18		CATAAATAGCACCGACAGGGA	258	59
		GGGATGGAGAAGAGTGAGGA		
exon 19		TCCTCACTCTTCTCCATCCC	159	57
		ACCCTGTATGTTGCCTTGG		
exons 20-21		GGGGATTTTGCTGTGTGCTG	333	61
		ATTCCTGCTCCCACCGTCTC		
exon 22		CACAGAGTGTCCGAGAGGCA	282	57
		GGAGATTATCAGGTGAGATGCC		
exons 22-23		CAGAGTGTCCGAGAGGCAGGG	608	61
		CGTTGACCCCTCCACCTTGA		
exon 24		GGGAAAACATGCACCTTCTT	375	58
		TAGGGGGTAAAATGGAGGAG		
polyadenylation signal		ACTAACTCAGTGGGAATAGGG	413	56
		GGAGCTAGGATAGCTCAAT		

PCR products made on DNA from blood of specific LGMD2A patients were then subjected either to heteroduplex analysis or to direct sequencing, depending on whether the mutation, based on haplotype analysis, was expected to be homozygous or heterozygous, respectively. It was occasionally necessary to clone the PCR products to precisely identify the mutations (i.e., for microdeletions or insertions and for some heterozygotes). Disease-associated mutations are summarised in Table 4 hereunder and their position along the protein is shown in Fig. 4.

Table 4: nCL1 mutations in LGMD2A families.

Codons and amino acid positions are numbered on the basis of the cDNA sequence starting from ATG.

Exon	Families	Nucleotide position	Nucleotide change	Amino acid position	Amino acid change	Restriction si
2	B519*	328	<u>C</u> GA-> <u>T</u> GA	110	Arg->stop	
4	M42	545	CT <u>G</u> -> C <u>A</u> G	182	Leu->Gln	
4	M1394; M2888	550	CAA -> CA	184	frameshift	
5	M35; M37	701	G <u>G</u> G -> G <u>A</u> G	234	Gly->Glu	

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6	M32	945	CGG -> CG	315	frameshift	-SmaI
8	M2407*	1061	G <u>T</u> G -> G <u>G</u> G	354	Val-> Gly	
8	M1394	1079	T <u>G</u> G -> T <u>A</u> G	360	Trp->stop	-BstNI, -Eco
11	M2888	1468	<u>C</u> GG -> <u>T</u> GG	490	Arg->Trp	
13	R12*	1715	C <u>G</u> G -> C <u>A</u> G	572	Arg->Gln	-MspI
19	R27	2069-2070	deletion AC	690	frameshift	
21	R14: R17	2230	<u>A</u> GC -> <u>G</u> GC	744	Ser->Gly	-AluI
22	A*: B501*: M32	2306	C <u>G</u> G -> C <u>A</u> G	769	Arg->Gln	
22	B505	2313-2316	deletion AGAC	771-772	frameshift	
22	R14: B505	2362-2363	AG -> TCATCT	788	frameshift	

The first letter of the family code refers to the origin of the population B= Brazil, M= metropolitan France, R = Isle of La Réunion, A= Amish.

Each mutation was confirmed by heteroduplex analysis, by sequencing of both strands in several members of the family or by enzymatic digestion when the mutation resulted in the modification of a restriction site. Segregation analyses of the mutations, performed on DNAs from all available members of the families, confirmed that these sequence variations are on the parental chromosome carrying the LGMD2A mutation. To exclude the possibility that the missense substitutions might be polymorphisms, their presence was systematically tested in a control population: none of these mutations was seen among 120 control chromosomes from the CEPH reference families.

EXAMPLE 5 :

Analysis of families genes, chromosome-15 ascertained families

The initial screening for causative mutations was performed on families, each containing a LGMD gene located on chromosome 15. These included families from the Island of La Réunion (Beckmann et al., 1991), from the Old Order Amish from northern Indiana (Young et al., 1992,) and 2 Brazilian families (Passos Bueno et al., 1993).

a) Reunion Island families

Genealogical studies and geographic isolation of the families from the Isle of La Réunion were suggestive of a single founder effect. Genetic analyses are,

however, inconsistent with this hypothesis as the families present haplotype heterogeneity. At least, six different carrier chromosomes are encountered, (with affected individuals in several families being compound heterozygotes). Distinct mutations corresponding to four of these six haplotypes have been identified
5 thus far.

In family R14, exons 13, 21 and 22 showed evidence for sequence variation upon heteroduplex analysis (Fig. 6). Sequencing of the associated PCR products revealed (i) a polymorphism in exon 13, (ii) a missense mutation (A->G) in exon 21 transforming the Ser⁷⁴⁴ residue to a glycine in the loop of the second EF-
10 hand in domain IV of the protein (Figure 4), and (iii) a frameshift mutation in exon 22. The exon 21 mutation and the polymorphism in exon 13 form an haplotype which is also encountered in family R17. Subcloning of the PCR products was necessary to identify the exon 22 mutation. Sequencing of several clones revealed a replacement of AG by TCATCT (data not shown). This frameshift
15 mutation causes premature termination at nucleotide 2400 where an in frame stop codon occurs (Figure. 4).

The affected individuals in family R12 are homozygous for all markers of the LGMD2A interval (Allamand, submitted). Sequencing of the PCR products of exon 13 revealed a G to A transition at base 1715 of the cDNA resulting in a
20 substitution of glutamine for Arg⁵⁷² (Figure. 7) within domain III, a residue which is highly conserved throughout all known calpains. This mutation, detectable by loss of *MspI* restriction site, is present only in this family and in no other examined LGMD2A families or unrelated controls.

In family R27, heteroduplex analysis followed by sequencing of the PCR
25 products of an affected child revealed a two base pair deletion in exon 19 (Figure. 6 and table 4). One AC out of three is missing at this position of the sequence, producing a stop codon at position 2069 of the cDNA sequence (Figure 4).

b) Amish families

30 As expected, due to multiple consanguineous links, the examined LGMD2A Northern Indiana Amish patients were homozygous for the haplotype on the chromosome bearing the mutant allele (Allamand, submitted). A (G->A) missense mutation was identified at nucleotide 2306 within exon 22 (Fig. 7). The

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resulting codon change is CGG to CAG, transforming Arg⁷⁶⁹ to glutamine. This residue, which is conserved throughout all members of the calpain family in all species, is located in domain IV of the protein within the 3rd EF-hand at the helix-loop junction. This mutation was encountered in a homozygous state in all patients from 12 chromosome 15-linked Amish families, in agreement with the haplotype analysis. We also screened six Southern Indiana Amish LGMD families, for which the chromosome 15 locus was excluded by linkage analyses (Allamand ESHG, submitted, ASHG 94). As expected, this nucleotide change was not present in any of the patients from these families, thus confirming the genetic heterogeneity of this disease in this genetically related isolate.

c) Brazilian families

As a result of consanguineous marriages, two Brazilian families (B501, B519) are homozygous for extended LGMD2A carrier haplotypes (data not shown). Sequencing PCR products from affected individuals of these families demonstrated that family B501 has the same exon 22 mutation found in northern Indiana Amish patients (Figure 7), but embedded in a completely different haplotype. In family B519, the patients carry a C to T transition in exon 2, replacing Arg³²⁸ with a TGA stop codon (Figure 7), thus leading, presumably, to a very truncated protein (Figure 4).

d) Analysis of other LGMD families

Having validated the role of the candidate gene in the chromosome 15 ascertained families, we next examined by heteroduplex analysis LGMD families for which linkage data were not informative. These included one Brazilian (B505) and 13 metropolitan French pedigrees.

Heteroduplex bands were revealed for exons 1, 3, 4, 5, 6, 8, 11, 22 of one or more patients (Figure 6). Of all sequence variants, 10 were identified as possible pathogenic mutations (5 missense, 1 nonsense and 4 frameshift mutations) and 3 as polymorphisms with no change of amino acid of the protein. All causative mutations identified are listed in Table 4 here-above. Identical mutations were uncovered in apparently unrelated families. The mutations shared by families M35 and M37, and M2888 and M1394, respectively, are likely to be the consequence of independent events since they are embedded in different marker haplotypes. In contrast, it is likely that the point mutation in exon

22 of the Amish and in the M32 kindreds corresponds to the same mutational event as both chromosomes share a common four marker haplotype (774G4A1-774G4A10-774G454D-774G4A2) around nCL1 (data not shown), possibly reflecting a common ancestor. The same holds true for the AG to TCATCT substitution mutation encountered in exon 22 in families B505 and R14. The exon 8 (T->G) transversion is present in the two carrier chromosomes of M2407, the only metropolitan family homozygous by haplotype, possibly reflecting an undocumented consanguinity. For some families, no disease-causing mutation has been detected thus far (M40 for example).

In addition to the polymorphism present in exon 13 in families R14 and R17 (position 668) and in the intragenic microsatellites, four additional neutral variations were detected: a (T->C) transition at position 96, abolishing a *Ddel* restriction site in exon 1 in M31; a (C->T) transition in exon 3 (position 495) in M40 and in M37 forming a haplotype with the exon 5 mutation (in the former family, this polymorphism does not cosegregate with the disease); a (T->C) transition in the paternally derived promotor in M42 at position -428, which was also evidenced in healthy controls; and a variable poly(G) in intron 22 close to the splice site in families R20, R11, R19, M35 and M37. The latter is also present in the members of the CEPH families, but is not useful as a genetic marker as the visualisation and interpretation of mononucleotide repeat alleles is difficult.

In total, sixteen independent mutational events representing fourteen different mutations were identified. All mutations cosegregate with the disease in LGMD2A families. The characterised morbid calpain alleles contain nucleotide changes which were not found in alleles from normal individual. The discovery of two nonsense and five frameshift mutations in nCL1 supports the hypothesis that a deficiency of this product causes LGMD2A. All seven mutations result in a premature in-frame stop codon, leading to the production of truncated and presumably inactive proteins (Figure 4). Evidences for the morbidity of the missense mutations come from (1) the relative high incidence of such mutations among LGMD2A patients ; although it is difficult in the absence of functional assays to differentiate between a polymorphism and a morbid mutation, the occurrence of different "missense" mutations in this gene cannot all be

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accounted for as rare private polymorphisms: (2) the failure to observe these mutations in control chromosomes; and (3) the occurrence of mutations in evolutionarily conserved residues and/or in regions of documented functional importance. Four of seven missense mutations change an amino acid which is conserved in all known members of the calpain family in all species (Figure 3). Two of the remaining mutations affect less conserved amino acid residues, but are located in important functional domains. The substitution V354G in exon 8 is 4 residues before the asparagine at the active site and S744G in exon 21 is within the loop of the second EF-hand and may impair the calcium-dependent regulation of calpain activity or the interaction with a small subunit (Figure 4). Several missense mutations change a hydrophobic residue to a polar one, or vice versa (Table 4) possibly disrupting higher order structures.

METHODS

Description of the patients

The LGMD2A families analysed were from 4 different geographic origins. They included 3 Brazilian families, 13 interrelated nuclear families from the Isle of la Réunion, 10 French metropolitan families and 12 US Amish families. The majority of these families were previously ascertained to belong to the chromosome 15 group by linkage analysis (Beckmann, 1991; Young, Passos-Bueno et al., 1993). However, some families from metropolitan France as well as one Brazilian family, B505, had non significant lodscores for chromosome 15. Genomic DNA was obtained from peripheral blood lymphocytes.

Sequencing of cosmid c774G4-1F11 and EcoRI restriction map of cosmids.

Cosmid 1F11 (Figure 1C) was subcloned following DNA preparation through Qiagen® procedure (Qiagen Inc., USA) and partial digestion with either *Sau3A*, *RsaI* or *AluI*. Size-selected restriction fragments were recovered from low-melting agarose and eventually ligated with M13 or Buescript® (Stratagene, USA) vectors. After electroporation in *E. coli*, recombinant colonies were picked in 100 µl of LB/ampicillin media. PCR reactions were performed on 1 µl of the culture in 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100®, 0.01 gelatine, 200 µM of each dNTP, 1 U of Taq Polymerase® (Amersham) with 100 ng of each vectors primers. Amplification was initiated by 5 min denaturation at 95°C, followed by 30 cycles of 40 sec denaturation at 92°C and 30 sec annealing

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at 50°C. PCR products were purified through Microcon devices (Amicon, USA) and sequenced using the dideoxy chain termination method on an ABI sequencer (Applied Biosystems, Foster City, USA). The sequences were analysed and alignments performed using the XBAP software of the Staden package, version 93.9 (Staden, 1982). Gaps between sequence contigs were filled by walking with internal primers. *EcoRI* restriction map of cosmids was performed essentially as described in Sambrook et al. (1989).

Northern Blot analysis

The probes were labelled by random priming with dCTP-($\alpha^{32}\text{P}$). Hybridisation was performed to human multiple tissue northern blots as recommended by the manufacturer (Clontech, USA).

Analysis of PCR products from LGMD2A families

One hundred ng of human DNA were used per PCR under the buffer and cycle conditions described in Fougere (1994) (annealing temperature shown in Table 3). Heteroduplex analysis (Keene et al., 1991) was performed by electrophoresis of ten μl of PCR products on a 1.5 mm-thick Hydrolink® MDE gels (Bioprobe) at 500-600 volt for 12-15 h depending of the fragment length. Migration profile was visualised under UV after ethidium bromide staining.

For sequence analysis, the PCR products were subjected to dye-dideoxy sequencing, after purification through microcon devices (Amicon, USA). When necessary, depending on the nature of the mutations (e.g., frameshift mutation or for some heterozygotes), the PCR products were cloned using the TA cloning kit from Invitrogen (UK). One μl of product was ligated to 25 ng of vector at 12°C overnight. After electroporation into XL1-blue bacteria, several independent clones were analysed by PCR and sequenced as described above.

The invention results from the finding that the nCL1 gene when it is mutated is involved in the etiology of LGMD2A. It is exactly the contrary to what is stated in the literature, e.g. that the disease is accompanied by the presence of a deregulated calpain. Identification of nCL1 as the defective gene in LGMD2A represents the first example of muscular dystrophy caused by mutation affecting a gene which is not a structural component of muscle tissue, in contrast with previously identified muscular dystrophies such as Duchenne and Becker (Bonilla et al., 1988), severe childhood autosomal recessive (Matsumura et al.,

1992), Fukuyama (Matsumara et al., 1993) and merosin-deficient congenital muscular dystrophies (Tomé et al., 1994).

The understanding of the LGMD2A phenotype needs to take into account the fact that there is no active nCL1 protein in several patients, a loss compatible
5 with the recessive manifestation of this disease. Simple models in which this protease would be involved in the degradation or destabilisation of structural components of the cytoskeleton, extracellular matrix or dystrophin complex can therefore be ruled out. Furthermore, there are no signs of such alterations by immunocytogetic studies on LGMD2 muscle biopsies (Matsumara et al., 1993;
10 Tomé et al., 1994). Likewise, since LGMD2A myofibers are apparently not different from other dystrophic ones, it seems unlikely that this calpain plays a role in myoblast fusion, as proposed for ubiquitous calpains (Wang et al., 1989).

All the data disclosed in these examples confirm that the nCL1 gene is a major gene involved in the disease when mutated.

15 The fact that morbidity results from the loss of an enzymatic activity raises hopes for novel pharmaco-therapeutic prospects. The availability of transgenic models will be an invaluable tool for these investigations.

The invention is also relative to the use of a nucleic acid or a sequence of nucleic acid of the invention, or to the use of a protein coded by the nucleic acid
20 for the manufacturing of a drug in the prevention or treatment of LGMD2.

The finding that a defective calpain underlies the pathogenesis of LGMD2A may prove useful for the identification of the other loci involved in the LGMDs. Other forms of LGMD may indeed be caused by mutations in genes whose products are the CANP substrates or in genes involved in the regulation of nCL1
25 expression. Techniques such as the two-hybrid selection system (Fields et al., 1989) could lend themselves to the isolation of the natural protein substrate(s) of this calpain, and thus potentially help to identify other LGMD loci.

The invention also relates to the use of all or a part of the peptidic sequence of the enzyme, or of the enzyme, product of nCL1 gene, for the screening of the
30 ligands of this enzyme, which might be also involved in the etiology and the morbidity of LGMD2

The ligands which might be involved are for example substrate(s), activators or inhibitors of the enzyme.

The nucleic acids of the invention might also be used in a screening method for the determination of the components which may act on the regulation of the gene expression.

A process of screening using either the enzyme or a host recombinant cell, containing the nCL1 gene and expressing the enzyme, is also a part of the invention.

The pharmacological methods, and the use of nucleic acid and peptidic sequences of the invention are very potent applications.

The methods used for such screenings of ligands or regulatory elements are those described for example for the screening of ligands using cloned receptors.

The identification of mutations in the nCL1 gene provides the means for direct prenatal or presymptomatic diagnosis and carrier detection in families in which both mutations have been identified. Gene-based accurate classification of LGMD2A families should prove useful for the differential diagnosis of this disorder.

The invention relates to a method of detection of a predisposition to LGMD2 in a family or a human being, such method comprising the steps of :

- selecting one or more exons or flanking sequences which are sensitive in said family;
- selecting the primers specific for the or these exons or their flanking sequences, a specific example being the PCR primers of Table 3, or an hybrid thereof,
- amplifying the nucleic acid sequence, the substrate for this amplification being the DNA of the human being to be checked for the predisposition, and
- comparing the amplified sequence to the corresponding sequence derived from Figure 2 or Figure 8.

Table 2 indicates the sequences of the introns-exons junctions, and primers comprising in their structure these junctions are also included in the invention.

All other primers suitable for such RNA or DNA amplification may be used in the method of the invention.

In the same way, any suitable amplification method : PCR (for Polymerase Chain Reaction ®) NASBA ® (for Nucleic acid Sequence Based Amplification), or others might be used.

The methods usually used in the detection of one site mutations, like ASO (Allele specific PCR), LCR, or ARMS (Amplification Refactory Mutation System) may be implemented with the specific primers of the invention.

The primers, such as described in Tables 1 and 3, or including junctions of Table 2, or more generally including the flanking sequences of one of the 24 exons are also a part of the invention.

The kit for the detection of a predisposition to LGMD2 by nucleic acid amplification is also in the scope of the invention, such a kit comprises a least PCR primers selected from the group of :

- 10 a) in those described in table 1
- b) in those described in table 3
- c) those including the introns-exons junctions of Table 2.
- d) derived from primers defined in a),b) or c).

The nucleic acid sequence of claim 1 to 3 might be inserted in a viral or a retroviral vector, said vector being able to transfect a packaging cell line.

The packaging transfected cell line, might be used as a drug for gene therapy of LGMD2.

The treatment of LGMD2 disease by gene therapy is implemented by a pharmaceutical composition containing a component selected from the group of :

- 20 a) a nucleic acid sequence according to claims 1 to 4,
- b) a cell line according to claim 24,
- c) an aminoacid sequence according to claims 5 to 9.

REFERENCES

- 5 Arikawa, E., Hoffman, E. P., Kaido, M., Nonaka, I., Sugita, H. and Arahata, K. (1991). The frequency of patients with dystrophin abnormalities in a limb-girdle patient population. *Neurology* 41, 1491-1496.
- 10 Bashir, R., Strachan, T., Keers, S., Stephenson, A., Mahjneh, I., Marconi, G., Nashef, L. and Bushby, K. M. D. (1994). A gene for autosomal recessive limb-girdle muscular dystrophy maps to chromosome 2p. *Hum. Mol. Genet.* 3, 455-457.
- 15 Beckmann, J. S., Richard, I., Hillaire, D., Broux, O., Antignac, C., Bois, E., Cann, H., Cottingham, R. W., Jr., Feingold, N., Feingold, J., Kalil, J., Lathrop, G. M., Marcadet, A., Masset, M., Mignard, C., Passos-Bueno, M. R., Pellerain, N., Zatz, M., Dausset, J., Fardeau, M. and Cohen, D. (1991). A gene for limb-girdle muscular dystrophy maps to chromosome 15 by linkage. *C. R. Acad. Sci. Paris. III* 312, 141-148.
- 20 Birnstiel, M. L., Busslinger, M. and Sturb, K. (1985). Transcription termination and 3' processing: The end is in site! *Cell* 41, 349-359.
- 25 Blackwell, T. K. and Weintraub, H. (1990). Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. *Science* 250, 1104-1110.
- Bonilla, E., Samitt, C. E., Miranda, A. F., Hays, A. P., Salviati, G., DiMauro, S., Kunkel, L. M., Hoffman, E. P. and Rowland, L. P. (1988). Duchenne muscular dystrophy: deficiency of dystrophin at the muscle cell surface. *Cell* 54, 447-452.
- 30 Bucher, P. (1990). Weight matrix descriptions of four eukaryotic RNA polymerase II promoter elements derived from 502 unrelated promoter sequences. *J. Mol. Biol.* 212, 563-578.

Bushby, K. M. D. (1994). Limb-girdle muscular dystrophy. In Diagnostic criteria for neuromuscular disorders, A. E. H. Emery, ed. (Baarn, The Netherlands: ENMC), pp 25-31.

- 5 Croall, D. E. and Demartino, G. N. (1991). Calcium-activated neutral protease (calpain) system: structure, function, and regulation. *Physiol. Rev.* 71, 813-847.

Dynan, W. S. and Tjian, R. (1983). The promoter-specific transcription factor Sp1 binds to upstream sequences in the SV40 early promoter. *Cell* 35, 79-87.

10

Emery, A. E. H. (1991). Population frequencies of inherited neuromuscular diseases - a world survey. *Neuromuscular Disorders* 1, 19-29.

- 15 Emori, Y., Ohno, S., Tobita, M. and Suzuki, K. (1986). Gene structure of calcium-dependent protease retains the ancestral organization of the calcium-binding protein gene. *FEBS lett.* 194, 249-252.

Fields, S. and Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature* 340, 245-246.

20

- Fougerousse, F., Broux, O., Richard, I., Allamand V., Pereira de Souza, A., Bourg N., Brenguier L., Devaud C., Pasturaud P., Roudaut C., Chiannilkulchai N., Hillaire D., Bui H., Chumakov I., Weissenbach J., Cherif D., Cohen D. and J. S. Beckmann (1994). Mapping of a chromosome 15 region involved in Limb-Girdle Muscular Dystrophy. *Hum. Mol. Genet.* 3, 285-293.

25

Goll, D. E., Thompson, V. F., Taylor, R. G. and Zalewska, T. (1992). Is Calpain activity regulated by membranes and autolysis or by calcium and calpastatin? *BioEssays* 14, 549-556.

30

Gosset, L. A., Kelvin, D. J., Sternberg, E. A. and Olson, E. (1989). A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. *Mol. Cell. Biol.* 9, 5022-5033.

Hirai, S., Kawasaki, H., Yaniv, M. and Suzuki, K. (1991). Degradation of transcription factors, c-Jun and c-Fos, by calpain. *FEBS lett.* 1, 57-61.

- 5 Imajoh, S., Kawasaki, H. and Suzuki, K. (1986). Limited autolysis of calcium-activated neutral protease (CANP): reduction of the Ca^{2+} requirement is due to the NH₂-terminal processing of the large subunit. *J. Biochem.* 100, 633-642.

- Jackson, C. E. and Carey, J. H. (1961). Progressive muscular dystrophy:
10 autosomal recessive type. *Pediatrics* 77-84.

Keen, J., Lester D., Inglehearn, C., Curtis, A. and Bhattacharya, S. (1991).
Rapid detection of single base mismatches as heteroduplexes on Hydrolink gels.
Trends Genet. 7, 5.

15

Kosak, M. (1984). Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res.* 12, 857-872.

- Lovett, M., Kere, J. and Hinton, L. M. (1991). Direct selection: a method for the
20 isolation of cDNAs encoded by large genomic regions. *Proc. Natl. Acad. Sci. USA* 88, 9628-9632.

- Matsumara, K., Tomé F. M. S., Collin H., Azibi K., Chaouch M., Kaplan J-K.,
Fardeau M. and Campbell K., P. (1992). Deficiency of the 50K dystrophin-
25 associated glycoprotein in severe childhood autosomal recessive muscular dystrophy. *Nature* 359, 320-322.

- Matsumura, K., Nonaka, I. and Campbell, K. P. (1993). Abnormal expression of dystrophin-associated proteins in Fukuyama-type congenital muscular dystrophy.
30 *Lancet* 341, 521-522.

- Minty, A. and Kedes, L. (1986). Upstream regions of the human cardiac actin gene that modulate its transcription in muscle cells: presence of an evolutionarily conserved repeated motif. *Mol. Cell. Biol.* 6, 2125-2136.
- 5 Miyamoto, S., Maki, M., Schmitt, M. J., Hatanaka, M. and Verma, I. M. (1994). TNF- α -induced phosphorylation of I κ B is a signal for its degradation but not dissociation from NF- κ B. *Proc. Natl. Acad. Sci. USA* *in press*.
- Morton, N. E. and Chung, C. S. (1959). Formal genetics of muscular dystrophy.
10 *Am. J. Hum. Genet.* 11, 360-379.
- Murachi, T. (1989). Intracellular regulatory system involving calpain and calpastatin. *Biochemistry Int.* 18, 263-294.
- 15 Ohno, S., Emori, Y., Imajoh, S., Kawasaki, H., Kisaragi, M. and Suzuki, K. (1984). Evolutionary origin of a calcium-dependent protease by fusion of genes for a thiol protease and a calcium-binding protein? *Nature* 312, 566-570.
- Ohno, S., Minoshima, S., Kudoh, J., Fukuyama, R., Shimizu, Y., Ohmi-Imajoh, S.,
20 Shimizu, N., Suzuki, K. (1989). Four genes for the calpain family locate on four different chromosomes. *Cytogen. Cell Genet.* 51, 1054.
- Passos-Bueno, M.-R., Richard, I., Vainzof, M., Fougèrouse, F., Weissenbach, J., Broux, O., Cohen, D., Akiyama, J., Marie, S. K. N., Carvalho, A. A.,
25 Guilherme, L., Kalil, J., Tsanaclis, A. M., Zatz, M. and Beckmann, J. S. (1993). Evidence of genetic heterogeneity in the autosomal recessive adult forms of limb-girdle muscular dystrophy following linkage analysis with 15q probes in Brazilian families. *J. Med. Genet.* 30, 385-387.
- 30 Richard, I., Broux, O., Chiannilkulchai, N., Fougèrouse, F., Allamand, V., Bourg, N., Brenguier, L., Devaud, C., Pasturaud, P., Roudaut, C., Lorenzo, F., Sebastiani-Kabatchis, C., Schultz, R. A., Polymeropoulos, M. H., Gyapay, G.,

Auffray, C. and Beckmann, J. (1994). Regional localization of human chromosome 15 loci. *Genomics* 23, 619-627.

5 Sambrook, J., Fritsh, E. F. and Maniatis, T. (1989). Molecular cloning: a laboratory manual. Cold spring Harbor Laboratory Press, Cold spring Harbor, USA.

Shapiro, M. and Senapathy, P. (1987). RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene
10 expression. *Nucleic Acids Res.* 15, 7155-7174.

Sorimachi, H., Imajoh-Ohmi, S., Emori, Y., Kawasaki, H., Ohno, S., Minami, Y. and Suzuki K. (1989). Molecular cloning of a novel mammalian calcium-dependant protease distinct from both m- and mu- type. Specific expression of
15 the mRNA in skeletal muscle. *J. Biol. Chem.* 264, 20106-20111.

Sorimachi, H., Ishiura, S. and Suzuki, K. (1993a). A novel tissue-specific calpain species expressed predominantly in the stomach comprises two alternative splicing products with and without Ca^{2+} -binding domain. *J. Biol. Chem.* 268,
20 19476-19482.

Sorimachi, H., Toyama-Sorimachi, N., Saïdo, T. C., Kawasaki, H., Sugita, H., Miyasaka, M., Arahata, K., Ishiura, S. and Suzuki, K. (1993b). Muscle-specific calpain, p94, is degraded by autolysis immediately after translation, resulting in
25 disappearance from muscle. *J. Biol. Chem.* 268, 10593-10605.

Staden, R. (1982). An interactive graphic program for comparing and aligning nucleic acid and amino acid sequences. *Nucleic Acids Res.* 10, 2951-2961.

30 Suzuki, K. and Ohno, S. (1990). Calcium activated neutral protease. Structure-function relationship and functional implications. *Cell Struct. Funct.* 15, 1-6.

Tagle, D. A., Swaroop, M., Lovett, M. and Collins, F. S. (1993). Magnetic bead capture of expressed sequences encoded within large genomic segments. *Nature* 361, 751-753.

- 5 Tomé, F. M. S., Evangelista T., Leclerc A., Sunada Y., Manole E., Estournet B., Barois A., Campbell K. P. and Fardeau M. (1994). Congenital muscular dystrophy with merosin deficiency. *C. R. Acad. Sci. Paris* 317, 351-357.

- 10 Uberbacher, E. C. and Mural, R. J. (1991). Locating protein-coding regions in human DNA sequences by a multiple sensor-neural network approach. *Proc. Natl. Acad. Sci. USA* 88, 11261-11265.

Walton, J. N. and Nattrass, F. J. (1954). On the classification, natural history and traitment of the myopathies. *Brain* 77, 169-231.

15

Wang, K. W., Villalobo, A. and Roufogalis, B. D. (1989). Calmodulin-binding proteins as calpain substrates. *Biochem. J.* 262, 693-706.

- 20 Young, K., Foroud, T., Williams, P., Jackson, C. E., Beckmann, J. S., Cohen, D., Conneally, P. M., Tischfield, J. and Hodes, M. E. (1992). Confirmation of linkage of limb-girdle muscular dystrophy, type-2, to chromosome 15. *Genomics* 13, 1370-1371.

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CLAIMS

1. A nucleic acid selected among:

- 1) a nucleic acid comprising the nucleotide sequence represented in Figure 8;
- 2) a nucleic acid comprising the nucleotide sequence represented in Figure 2;
- 5 3) a nucleic acid encoding the amino acid sequence represented in Figure 2;
and
- 4) a nucleic acid obtained by substitution of one or more nucleotides of the nucleic acid defined in 1) or 2), with the proviso that said nucleic acid codes for the Ca²⁺ dependent protease represented in Figure 2.

10 2. A nucleic acid that is complementary over its whole length to the nucleic acid according to claim 1.

3. A nucleic acid comprising in its structure the nucleic acid according to claim 1, under the control of regulatory elements, and involved in the expression of a calpain enzyme active in a Limb Girdle Muscular Dystrophy type 2 (LGMD2) disease.

15 4. The isolated nucleic acid according to claim 1, which is mutated in any of the following positions numbered according to Figure 2B and causes Limb Girdle Muscular Dystrophy type 2A (LGMD2A);

Nucleotide position	Nucleotide changes
328	CGA →TGA
545	CTG → CAG
550	Deletion A
701	GGG → GAG
945	CGG → CG
1061	GTG→ GGG
1079	TGG → TAG
1468	CGG → TGG
1715	CGG → CAG
2069-2070	Deletion AC
2230	AGC → GGC
2306	CGG → CAG
2313-2316	Deletion AGAC
2362-2363	AG → TCATCT

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5. A polypeptide which is encoded by the nucleic acid sequence according to claim 1 or 3.

6. The polypeptide according to claim 5, wherein said polypeptide contains the sequence as represented in Figure 2.

7. The polypeptide according to claim 5 or 6, wherein said polypeptide has a calpain enzyme activity involved in a LGMD2 disease.

8. A host cell which, when transformed or transfected with the nucleic acid sequence according to claim 1 or 3, expresses a calpain enzyme activity.

9. Use of the nucleic acid according to claim 1 or 3 in the manufacturing of a drug for the prevention or the treatment of an LGMD2 disease.

10. Use of the polypeptide according to any one of claims 5 to 7 in the manufacturing of a drug for the prevention or the treatment of an LGMD2 disease.

11. The use according to claim 9 or 10, wherein LGMD2 is LGMD2A.

12. Use of the polypeptide according to any one of claims 5 to 7 for the screening of the ligands of said amino acid sequence, said ligand being selected from the group consisting of substrate(s), co-factors and regulatory components.

13. Use of the nucleic acid according to claim 1, 3 or 4 in a screening method for the determination of the components which may act on the regulation of gene expression of calpain.

14. Use of the host cell according to claim 8 in a screening method for the determination of components active on the expression of the calpain.

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15. A method for screening for the presence of nucleotide changes or LGMD2 causative mutations in a family or a human being, such method comprising the steps of:

- 5 - selecting one or more exons or their flanking sequences of a nCL1 (novel Calpain Large subunit 1) gene,
 - selecting primers specific for these exons, or their flanking sequences, or a hybrid thereof,
 - amplifying the nucleic acid sequences with these primers, the substrate for
 10 this amplification being DNA of a human being; and
 - comparing the amplified sequence to the corresponding sequence of Figure 2 or Figure 8.

16. The method according to claim 15, wherein the primers are those selected
 15 from the group consisting of:

a) those described in Table 1;

	Primer name	Primer sequence (5'-3')	Position within the cDNA	Annealing temp (°C)	PCR product size on	
					cDNA	genomic DNA
20	P94in2	ATGGAGCCAACAGAACTGA C	341-360 428-448	58	108	1758
		GTATGACTCGGAAAAGAAG GT				
	P94in13	TAAGCAAAAGCAGTCCCCA C	1893-1912 1936-1956	58	64	1043
		TTGCTGTTCTCCTCACTTTCCT G				
25	P94-6a3	GTTTCATCTGCTGCTTCGTT CTGGTTCAGGCATACATGG	2342-2361 2452-2471	56	130	818
		T				
	P94ex1ter	TTCTTTATGTGGACCCTGAG TT	218-239 275-293	55	76	76
		ACGAACTGGATGGGGAACT				

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b) those described in Table 3;

	amplified region	Primer sequences (5'-3')	Size (bp)	Annealing temp. (°C)
5	promotor	TTCAGTACCTCCCGTTCACC GATGCTTGAGCCAGGAAAAC	296	59
	exon 1	CTTTCCTTGAAGGTAGCTGTAT GAGGTGCTGAGTGAGAGGAC	438	60
	exon 2	ACTCCGTCTCAAAAAAATACCT ATTGTCCCTTTACCTCCTGG	239	57
	exon 3	TGGAAGTAGGAGAGTGGGCA GGGTAGATGGGTGGGAAGTT	354	58
	exon 4	GAGGAATGTGGAGGAAGGAC TTCCTGTGAGTGAGGTCTCG	292	59
10	exon 5	GGAACCTCTGTGACCCCAAAT TCCTCAAACAACCATTCGC	325	56
	exon 6	GTTCCCTACATTCTCCATCG GTTATTTCAACCCAGACCCTT	315	57
	exon 7	AATGGGTTCTCTGGTTACTGC AGCACGAAAAGCAAAGATAAA	333	56
	exon 8	GTAAGAGATTTGCCCCCAG TCTGCGGATCATTGGTTTTC	321	58
	exon 9	CCTTCCCTTCTTCCTGCTTC CTCTCTTCCCCACCCTTACC	173	56
15	exon 10	CCTCCTCACCTGCTCCCATTA TTTTTTCGGCTTAGACCCTCC	251	56
	exon 11	TGTGGGGAATAGAAATAAATGG CCAGGAGCTCTGTGGGTCA	355	57
	exon 12	GGCTCCTCATCCTCATTACACA GTGGAGGAGGGTGAGTGTGC	312	61
	exon 13	TGTGGCAGGACAGGACGTTT TTCAACCTCTGGAGTGGGCC	337	60
	exon 14	CACCAGAGCAAACCGTCCAC ACAGCCCAGACTCCCATTCC	230	61
20	exon 15	TTCTCTTCTCCCTTCACCCT ACACACTTCATGCTCTCTACCC	225	57
	exon 16	CCGCCTATTCTTTCTCTCTT GACAAACTCCTGGGAAGCCT	331	56
	exon 17	ACCTCTGACCCCTGTGAACC TGTGGATTGTGTGCTACGC	270	61
	exon 18	CATAAATAGCACCGACAGGGA GGGATGGAGAAGAGTGAGGA	258	59
	exon 19	TCCTCACTCTTCTCCATCCC ACCCTGTATGTTGCCTTGG	159	57
25	exons 20-21	GGGGATTTTGCTGTGTGCTG ATTCTGCTCCCACCGTCTC	335	61
	exon 22	CACAGAGTGTCCGAGAGGCA GGAGATTATCAGGTGAGATGCC	282	57
	exons 22-23	CAGAGTGTCCGAGAGGCAGGG CGTTGACCCCTCCACCTTGA	608	61
	exon 24	GGGAAAACATGCACCTTCTT TAGGGGGTAAATGGAGGAG	375	58
	polyadenylation signal	ACTAACTCAGTGAATAGGG GGAGCTAGGATAGCTCAAT	413	56

and

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c) those including the introns-exons junctions of Table 2;

	splice donor site	score (%)	Intron	score (%)	splice acceptor site	Exon
5						Exon 1 (309 bp) ->
	...CTCCGgigagi...	88.5	<-Intron 1->	99.0	...mugttacacagGAAAT...	Exon 2 (70 bp) ->
	...GCTAGgtagga...	83.5	<-Intron 2->	90.0	...gtgtcgcacagGGGAC...	Exon 3 (119 bp) ->
	...TCCAGgigagg...	92	<-Intron 3->	81.5	...acgcttcgtgcagTTCTG...	Exon 4 (134 bp) ->
10	...GCTAAGtaagc...	82	<-Intron 4->	81.5	...atccctctctaaagGCTCC...	Exon 5 (169 bp) ->
	...TTGATgtaagi...	87	<-Intron 5->	79.5	...ccatcggggccacagGATGG...	Exon 6 (144 bp) ->
	...CCCGGgiggi...	77.5	<-Intron 6->	91	...taactgcctacagACAAT...	Exon 7 (84 bp) ->
	ATGAGgtaagc...	94	<-Intron 7->	78.5	...tctgtgtgctaaagGTCCC...	Exon 8 (86 bp) ->
15	...GATAGgtaggi...	89	<-Intron 8->	91.5	...catttccacacagATGGA...	Exon 9 (78 bp) ->
	...TTCTGgigagi...	88	<-Intron 9->	92	...tccaacccttcagGATGT...	Exon 10 (161 bp) ->
	...CCCAGgtagga...	80	<-Intron 10->	68.5	...tctgggggtgcagATACT...	Exon 11 (170 bp) ->
	...ACGAGgiggi...	85.5	<-Intron 11->	86	...tgttctctcaagGTTCC...	Exon 12 (12 bp) ->
	...AAGAGgtaga...	70	<-Intron 12->	87	...tcccatctctcagATGCA...	Exon 13 (209 bp) ->
20	...TCTGAgigagi...	76.5	<-Intron 13->	97	...tgtattctcacagGGAAG...	Exon 14 (37 bp) ->
	...CAGTGgigagi...	89	<-Intron 14->	93.5	...ctttcttaigcagAAAAA...	Exon 15 (18 bp) ->
	...CCAAGgtaggi...	89	<-Intron 15->	87	...cttctctctccagCCCAT...	Exon 16 (114 bp) ->
	...CACAGgigtct...	80	<-Intron 16->	88	...ttgtgcctccacagCCACA...	Exon 17 (78 bp) ->
25	...GAGATgigagi...	84	<-Intron 17->	92.5	...cccttctctccagGACAT...	Exon 18 (58 bp) ->
	...CAAACgigagi...	83	<-Intron 18->	90	...ctccatcccccagACAAG...	Exon 19 (65 bp) ->
	...TGGATgtaacc...	56	<-Intron 19->	88	...ccctctctctccagACAGA...	Exon 20 (69 bp) ->
	...GGCAGgtagga...	80	<-Intron 20->	94	...tttctattgccagAAATA...	Exon 21 (79 bp) ->
	...CCGAGgtagc...	66	<-Intron 21->	91	...ggctccctccacagGATTC...	Exon 22 (117 bp) ->
30	...GTTCAgtaagi...	79	<-Intron 22->	93.5	...gcattcttcacagGAGCT...	Exon 23 (59 bp) ->
	...TGGAGgtaagc...	81	<-Intron 23->	79	...gggaactcttcagTGGCT...	Exon 24 (27 bp) ->

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17. The method according to claim 15 or 16, wherein LGMD2 is LGMD2A.

18. A kit for the screening for the presence of nucleotide changes or LGMD2 causative mutations by nucleic acid amplification, wherein said kit comprises primers as defined
5 in claim 16.

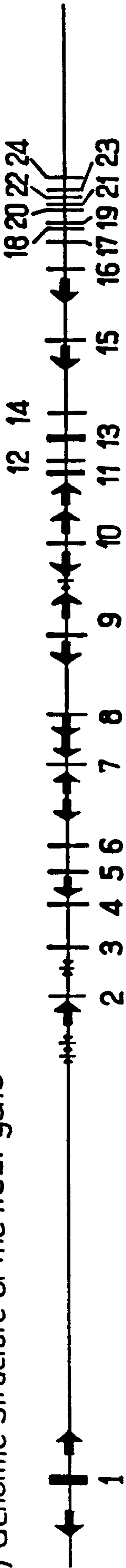
19. A pharmaceutical composition for the treatment of an LGMD2 disease, wherein said pharmaceutical composition contains a pharmaceutically acceptable carrier and a component selected from the group consisting of:

- 10 a) the nucleic acid according to claim 1 or 3, and
 b) the polypeptide according to any one of claims 5 to 7.

FETHERSTONHAUGH & CO.
PATENT AGENTS
MONTREAL, CANADA

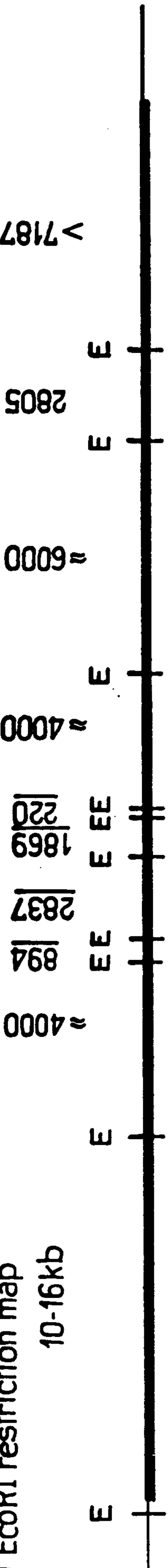
FIG. 1

A) Genomic structure of the nCL1 gene



B) EcoRI restriction map

10-16 kb



C) Cosmid map

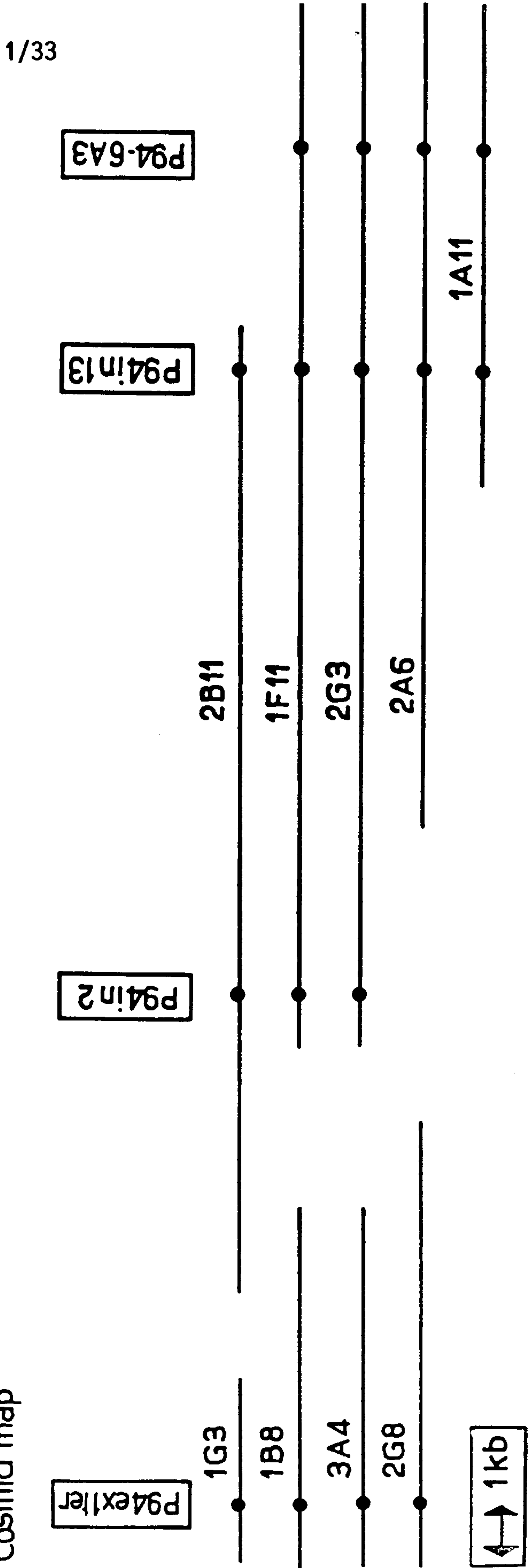


FIG. 2A

[illegible]

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1 10 30 50 70 90 110
ATGCCGACCGTCATTAGCGCATCTGTGGCTCCAAGGACAGCGGCTGAGCCCGGTCCTCACCAGGCAAGCCACTGAGGCTGGGGTGGAAACCCCAAGT
M P T V I S A S V A P R T A A E P R S P G P V P H P A Q S K A T E A G G G N P S

130 150 170 190 210 230
GGCATCTATTGAGCCATCATCAGCCGCAATTTCTCTATTATCGGAGTGAAGAGAGACATTGAGCAACTTCACAAGAAATGTCTAGAAAGAAAGTTCTTTATGTGGACCCCTGAGTTT
G I Y S A I I S R N F P I I G V K E K T F E Q L H K K C L E K K V L Y V D P E F

250 270 290 310 330 350
CCACCGGATGAGAGCCTCTCTCTTTTATAGCCAGAAAGTTCCCCATCCAGTTCTGTCTGGAAGAGAGACCTCCGGAATTTGCGAGAAATCCCCGATTTTATCATTTGATGGAGCCAAACGAACTGAC
P P D E T S L F Y S Q K F P I Q F V W K R P P E I C E N P R F I I D G A N R T D

370 390 410 430 450 470
ATCTGTCAAGGAGAGCTAGGGACTGCTGGTTCTCGCAGCCATTGCCCTGACCTGAACCAGCCTTCTTTCCGAGTCATACCCCATGATCAAGTTTCATCGAAAGCTACGCA
I C Q G E L G D C W F L A A I A C L T L N Q H L L F R V I P H D Q S F I E N Y A

490 510 530 550 570 590
GGGATCTTCCACTTCCAGTTCTGGCGCTATGGAGAGTGGTGACGTGTTATAGATGACTGCTGCCAAGCTACAACTCAAGTCAAGTTTCACCAAGTCCACCCGCAATGAGTTT
G I F H F Q F W R Y G E W V D V V I D D C L P T Y N N Q L V F T K S N H R N E F

610 630 650 670 690 710
TGGAGTCTCTGCTGGAGAAGGCTTATGCTAAGCTCCATGGTTCTCTACGAAGCTCTGAAGGTGGGAACACCAAGGCGCATGGAGGACTTCACAGGGGGTGGCAGAGTTTTCGAG
W S A L L E K A Y A K L H G S Y E A L K G G N T T E A M E D F T G G V A E F F E

730 750 770 790 810 830
ATCAGGGATGCTCCTAGTGACATGTACAGATCATGAAGAAGCCATCGAGAGAGGCTCCCTCATGGCTGCTCCATTGATGATGCCACCAACATGACCTATGGAACCTCTCCTCTGCT
I R D A P S D H Y K I M K K A I E R G S L H M G C S I D D G T N M T Y G T S P S G

850 870 890 910 930 950
CTGAACATGGGGAGTTGATTGCACGGATGTTAAGGAATATGGATAACTCAGTCTCAGGAGGCTCGACCTCGACCCAGAGGCTCAGATGAAGACCGACCGCAATCATTCGGGTT
L N M G E L I A R M V R N M D N S L L Q D S D L D P R G S D E R P T R T I I P V

970 990 1010 1030 1050 1070
CAGTATGAGACAAAGATGGCCTGCGGCTGCTCAGAGGTACGGCTACTCTGTACCGGGCTGGATGAGTCCGTTCAAGGTGAGAAAGTGAAGCTGGTGGGCTGCGGAATCCGTGG
Q Y E T R M A C G L V R G H A Y S V T G L D E V P F K G E K V K L V R L R N P W

1090 1110 1130 1150 1170 1190
GGCCAGGTGGAGTGAACGGTTCTTGGAGTGATAGATGAAGGAGTGGAGCTTTGTGGACAAGATGAGAAGCCCGTCTGCAGCACCAGGTCACTGAGGATGGAGAGTTCTGCAATGCC
G Q V E W N G S W S D R W K D W S F V D K D E K A R L Q H Q V T E D G E F W H S

1210 1230 1250 1270 1290 1310
TATGAGGATTTTACCATTTTCACAAAGTTGGAGATCTGCAACCTCAGGGCGGATGCTCTGCAGTCTGACAAAGCTTCAGACCTGGACAGTGTCTGTGAACGAGGGCGGCTGGGTACGG
Y E D F I Y H F T K L E I C N L T A D A L Q S D K L Q T W T V S V N E G R W V R

1330 1330 1350 1370 1390 1410 1430
GTTGCTGCGCGAGGCTGCCGCACTATCTTCTGGACCAACCCCTCAGTACCGTCTGGAAGCTCCTGGAGGAGGACCGATGACCCCTGATGACTCGGAGGTGATTTGCAGCTTC
G C S A G G C R N F P D T F W T N P Q Y R L K L L E E D D P D D S E V I C S F

1450 1470 1510 1530 1550
CTGGTGCCCTGATGCAGAAACCGCGGAGGACCGGAGGCTAGGGCGCAGTCTCTTCAACCATTTGGCTTCGCCATCTACGAGCTTCCCAAGACATGCGACGGGAACGACGACCTG
L V A L H Q K N R R K D R K L G A S L F T I G F A I Y E V P K E M H G N K Q H L

1570 1590 1610 1630 1650 1670
CAGAGGACTTCTCTGTACNAGCCTCCAGGCCAGGACCAACCTACATCAACATGCGGAGGTGTCAGCGCTTCGGCTGCCAGCGAGTACGTACGTCTCGTCCCTCCAGC
Q K D F F L Y N A S K A R S K T Y I N M R E V S Q R F R L P P S E Y V I V P S T

1690 1710 1730 1750 1770 1790
TAGGAGCCCAAGGAGGGGAATTCATCTCCGGTCTTCTCTGMAAGAGGAACCTCTCTGAGGAAGTTGMAATACCATCTCCGTGGATCGGCCAGTCAAAAGAAAAGCAAG
Y E P H Q E G E F I L R V F S E K R N L S E E V E N T I S V D R P V K K K T K

1810 1830 1850 1870 1890 1910
CCCATCATCTTCTGACAGAGCAACAGCAAGGAGCTGGGTGTGGACCAAGGAGTCAAGGAGGCAAGGCAAGCCCTGATAGCAAGGAGGAGTCCCAAGCAAGCAG
P I I F V S D R A N S N K E L G V D Q E S E G K T S P D K Q K Q S P Q P Q

1930 1950 1970 1990 2010 2030
CCTGGCAGCTCTGATCAGGAAGTGAGGAACAGCAATTCGGAACATTTTCAAGCAGATAGCAGGAGATGACATGGAGATCTGTGCAGATGAGCTCAAGAGGTCTTAAACACAGTC
P G S S D Q E S E Q Q Q F R N I F K Q I A G D D H E I C A D E L K K V L N T V

2050 2070 2090 2110 2130 2150
GTGMAACAACAAGGACCTGNAAGACACACGGGTTCACTGGAGTCTGCGCTCATGGATACAGATGGCTCTGGAAGCTCAAGCTGAGGAGTTCACACCTC
V N K H K D L K T H G F T L E S C R S H I A L M D T D G S G K L N L Q E F H H L

2170 2190 2210 2230 2250 2270
TGGMAAAGATTAGGCGCTGGCAAAATTTCAAACTATGACACAGACCAAGTCCGGCACCATCAACAGCTACGAGATGCGMAATGCAAGTCAAGCGGAGGATTCACCTCAAGCAAC
W N K I K A W Q K I F K H Y D T D Q S G T I N S Y E M R N A V N D A G F H L N N

2290 2310 2330 2350 2370 2390
CAGCTCTATGACATCATTAACCATCGGTCAGGACCAACACATCAACATCGACTTTGACAGTTTCATCTGCTTCTGTTAGCTGGAGGGCATGTTCAAGAGCTTTTCATGCTTTCATG
Q L Y D I I T M R Y A D K H M N I D F D S F I C C F V R L E G M F R A F H A F D

2410 2430 2450
AAGGATGGAGATGGTATCATCAAGCTCAACGTTCTGGAGTGGCTGCAGCTCAACCATGTATGCCTGA
K D G D G I I K L N V L E W L Q L T M Y A

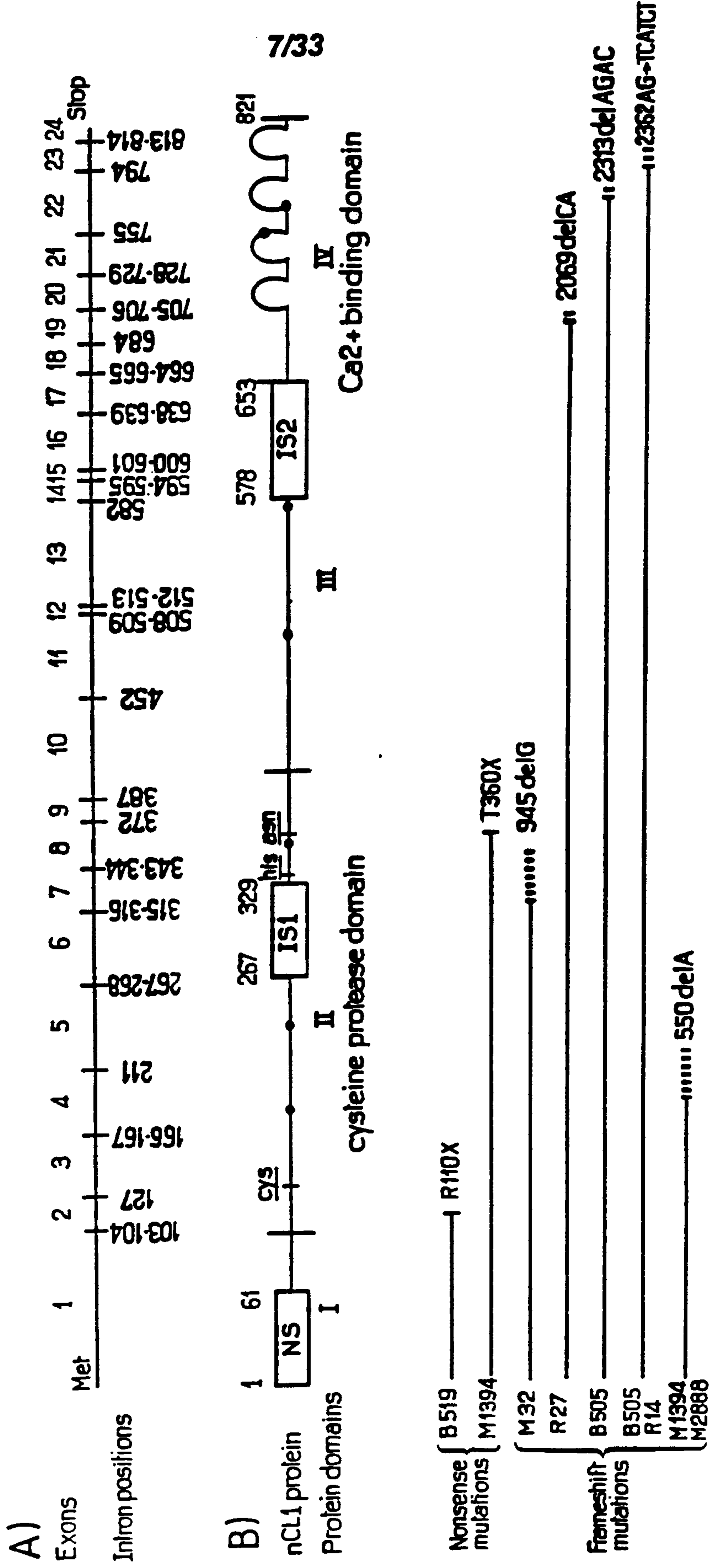
FIG. 2C

[illegible]

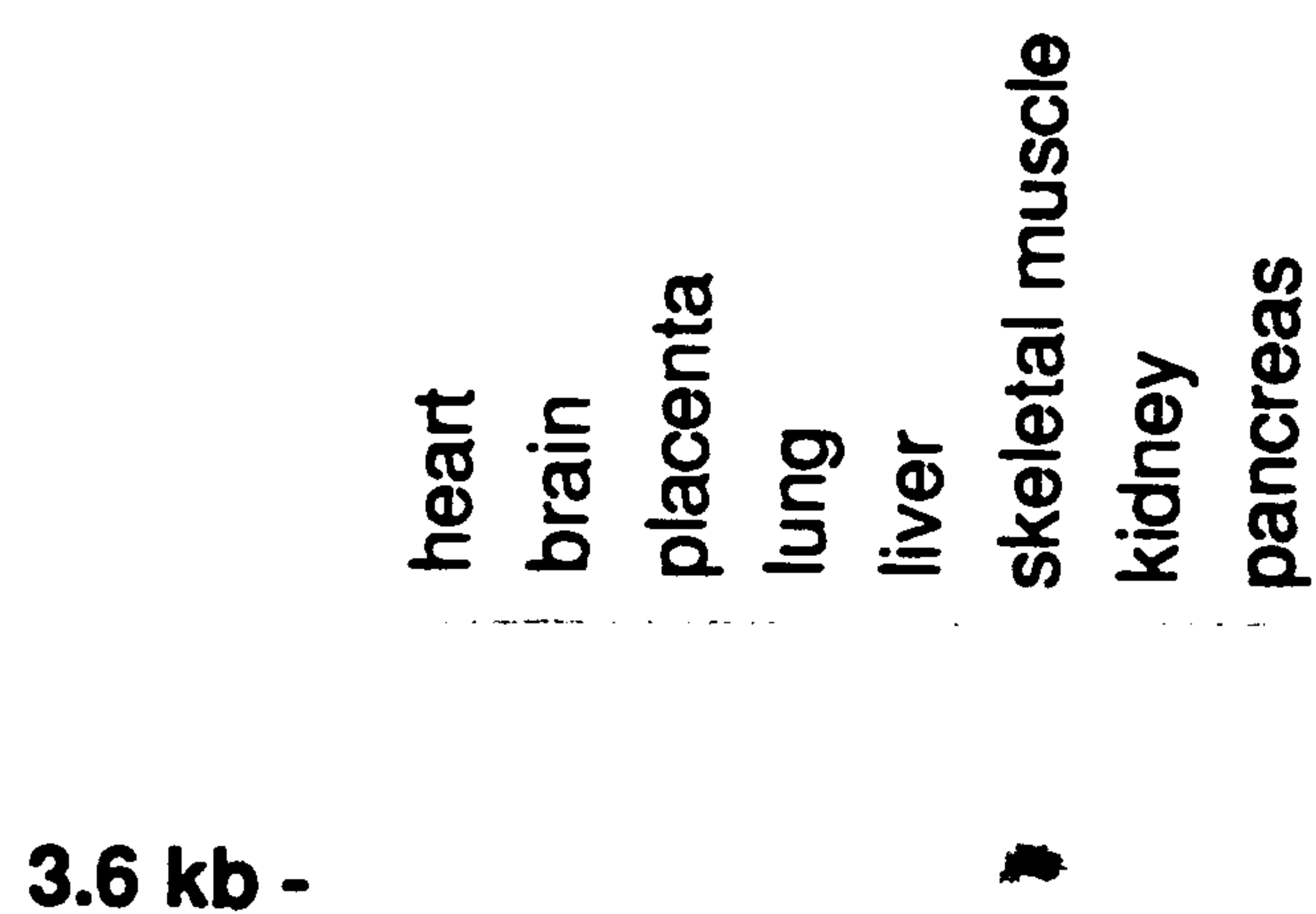
Figure 3:

Human	1	MPTVISASVAPRTAAERSRGPVPHPAOSKATEAGGNPSGLYSALISRNFLIGVKEKTFEQLHKKCLEKKVLYVDPEDPEPDETSIFYSQKFIQFVWKKRP	50	100
Rat	2	PT.....G.....G.T.....H.G.....		
Pig	3			
Cow	4			
	1	EICENPFFIIDGANKRTDICQGE [*] LGDCWFFLAIAIACLTNLQHLLFRVTPHDQSFNIENYAGIFHFQFWRRYGEVVDVVIDDCLEPTYNNOQLVETKSNHRNFEWSALLF	150	200
	2	G.....D.....L.....ER.....T.....D.....		
	1	KAYAKLHGSYEALKKGNTTEAMEDF ^② ICGVAEFFFEIRDA ^② PSDMYKIMKKAIERGSIMGCSIDDDGTNMTYGTSPSGINMGELIARMVNRMDNSLLQDSDLDPRGS	250	300
	2	T.....K.....R.....		
	3			
	4			
	1	DERPTRTLIPVOXETRMACGLVRGHAYSVTGLDEVFPFKGEKVKLVRLRNPQRQVEWNGS [*] SDRWKDSFVDKDEKARLQHQVTE ^④ DGEETMSYE ^④ FIYHFTKLE	350	400
	2	.D..S...V.....E.AL.....G.....D..V.....		
	3	.D.....V..F.....E.AL.....S.....D.....		
	4	.D.....M.V..F.....E.ALY.....S.....Y.....		
	1	IGNLTADA ^④ QSDKQLQWTVSVNEGFWRGCSAGGCRNFPD ^④ FTNPOYRLKLLLEE ^④ DDPDDSEVICSEFLVAMOKNRRKDRKIGASLFTIGFAIYEVEKEMHG	450	500
	2	E.....TG.....R.....N.....N.....		
	3	E.....TG.....R.....N.....N.....		
	1	NKQH ^⑤ LQKDFFLYNASKARSKTYINMREVSQRFRLPESEYVIVESTYEPHQEGE ^⑤ ILRVSEKRNLSEEVENTISVDRPVKKKTKPIIEVSDRANSNKELGVD	550	600
	2	R.....E.....M.....K.....R.....		
	3	QD.....EK..K.E.SNT.....		
	1	QEESEGKGTSPDKOKOSPQOPGSSDOESEEQQQFRNIFKQIAGDDME ^⑥ ICADE ^⑥ LKKV ^⑥ NTVVNKHKDLKTH ^⑥ GFTLESCRSMIALMDTDGSGKLNLC ^⑥ HH ^⑥ LW	650	700
	2	..A...D..G...GE...R..HT.....R.....Q.....		
	3	QD.....EK..K.E.SNT.....		
	1	NKIKAWQK ^⑦ FKHY ^⑦ DTDQSG ^⑦ TINSY ^⑦ EM ^⑦ AVNDAG ^⑦ FHLNNQLYDIITMR ^⑦ YADKHMNID ^⑦ SD ^⑦ IC ^⑦ FV ^⑦ REGME ^⑦ RAFHAFDKDGD ^⑦ GI ^⑦ IKLVLEW ^⑦ QLTMYA	750	800
	2	K.....H.....S.....		

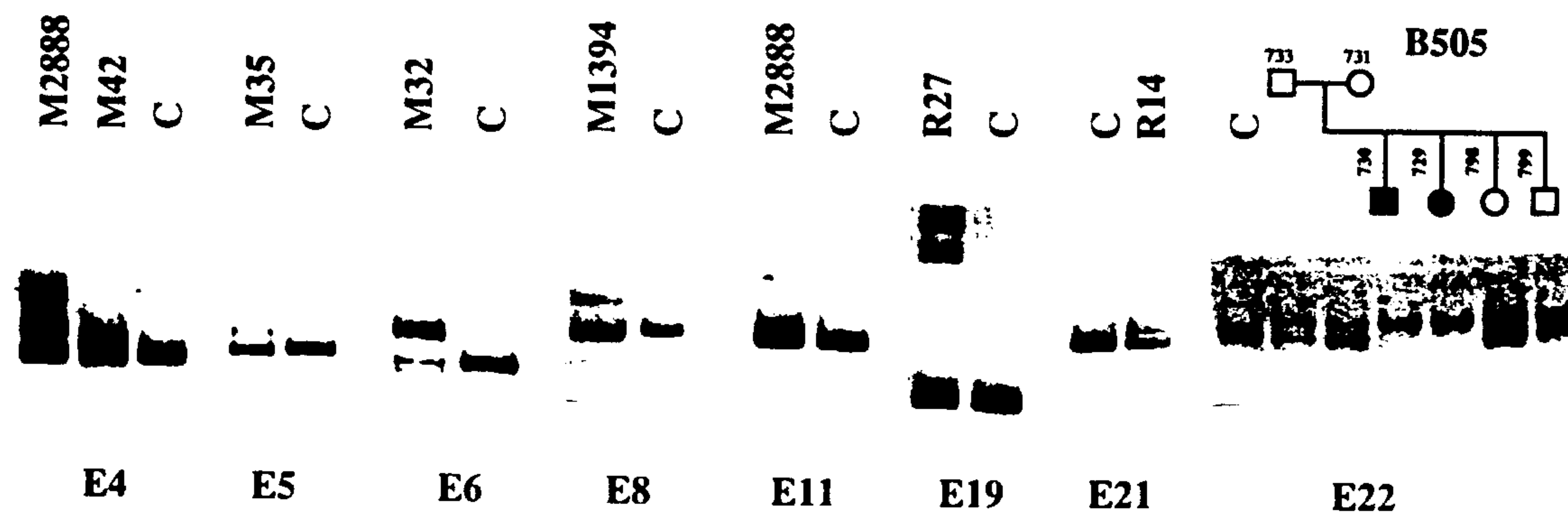
FIG. 4



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**FIG. 5**

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**FIG. 6**

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FIG. 7A) EXON 2Normal
sequence

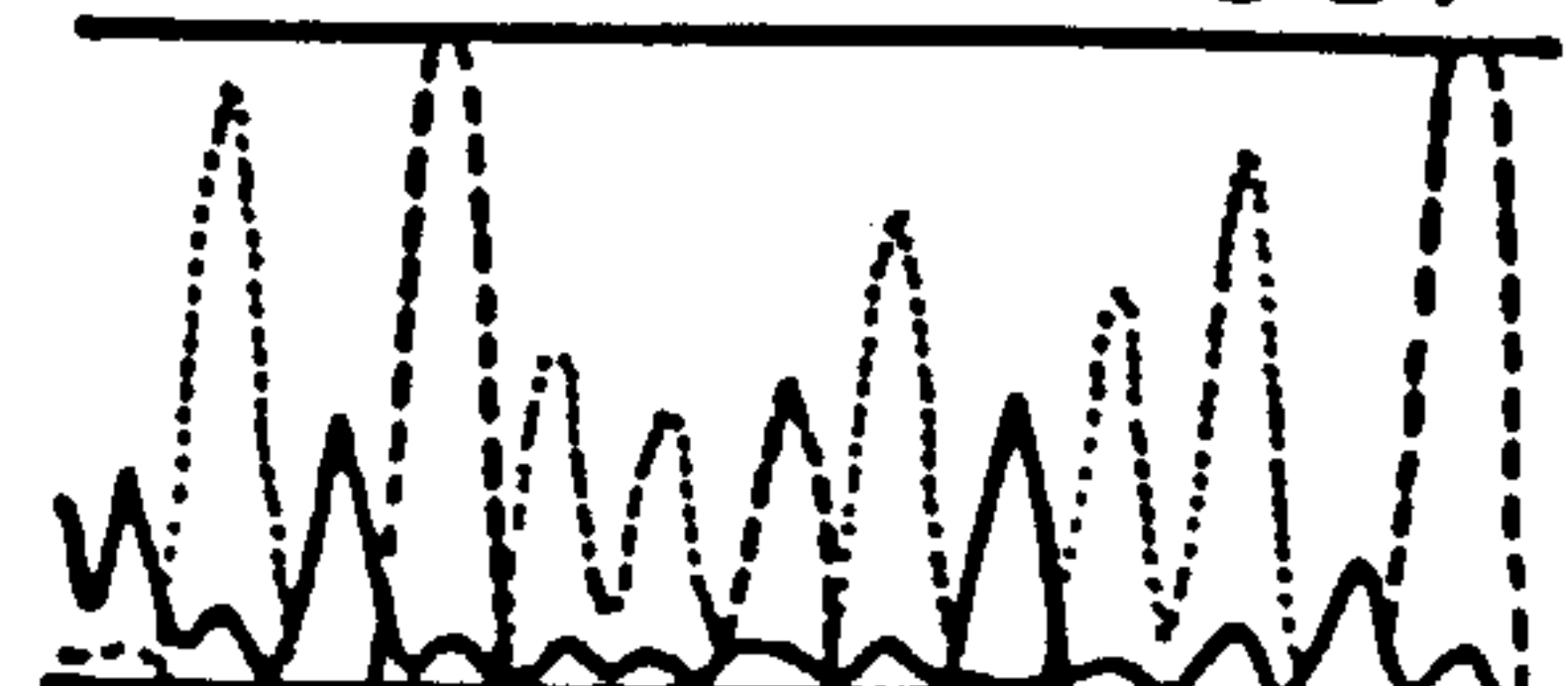
AATCCCCGATTTA

**B519**CGA → TGA
Arg110 Stop

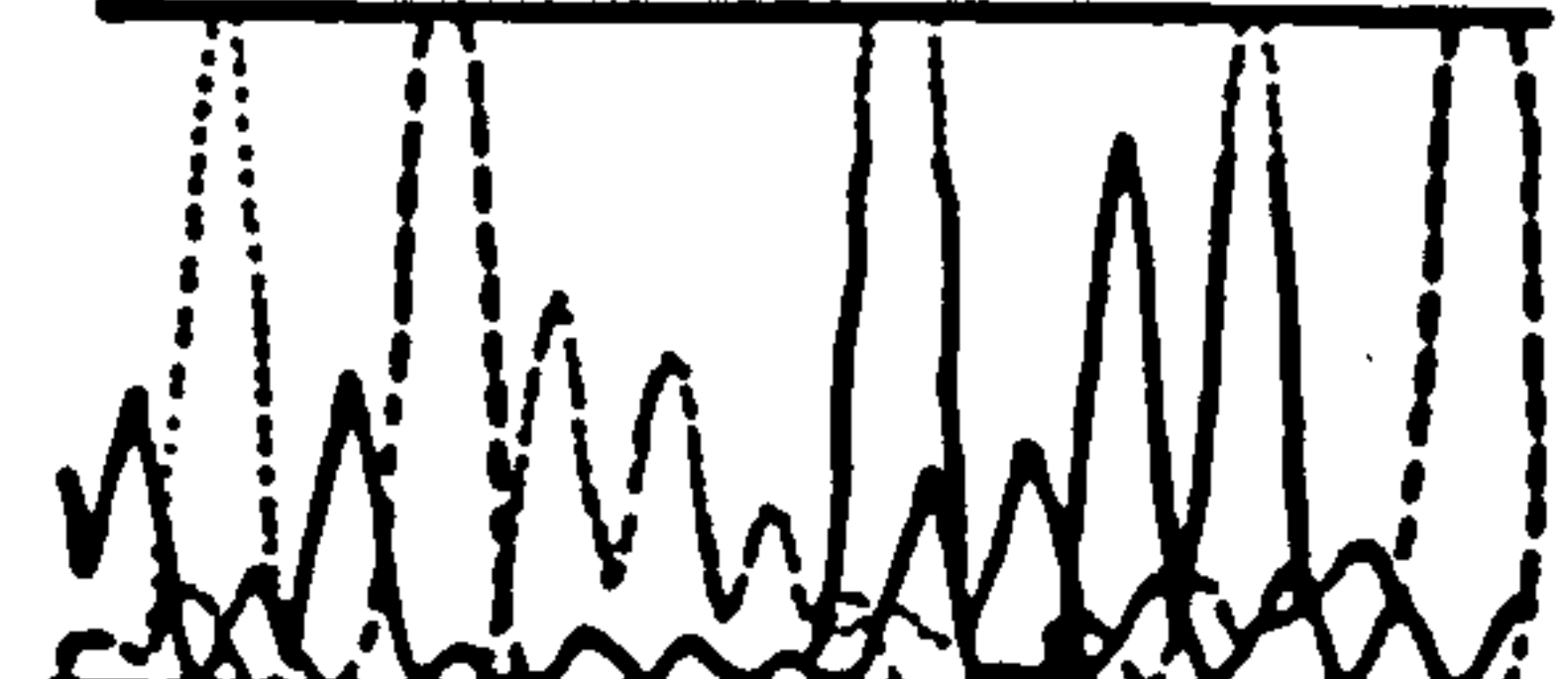
AATCCCT*GATTTA

B) EXON 8Normal
sequence

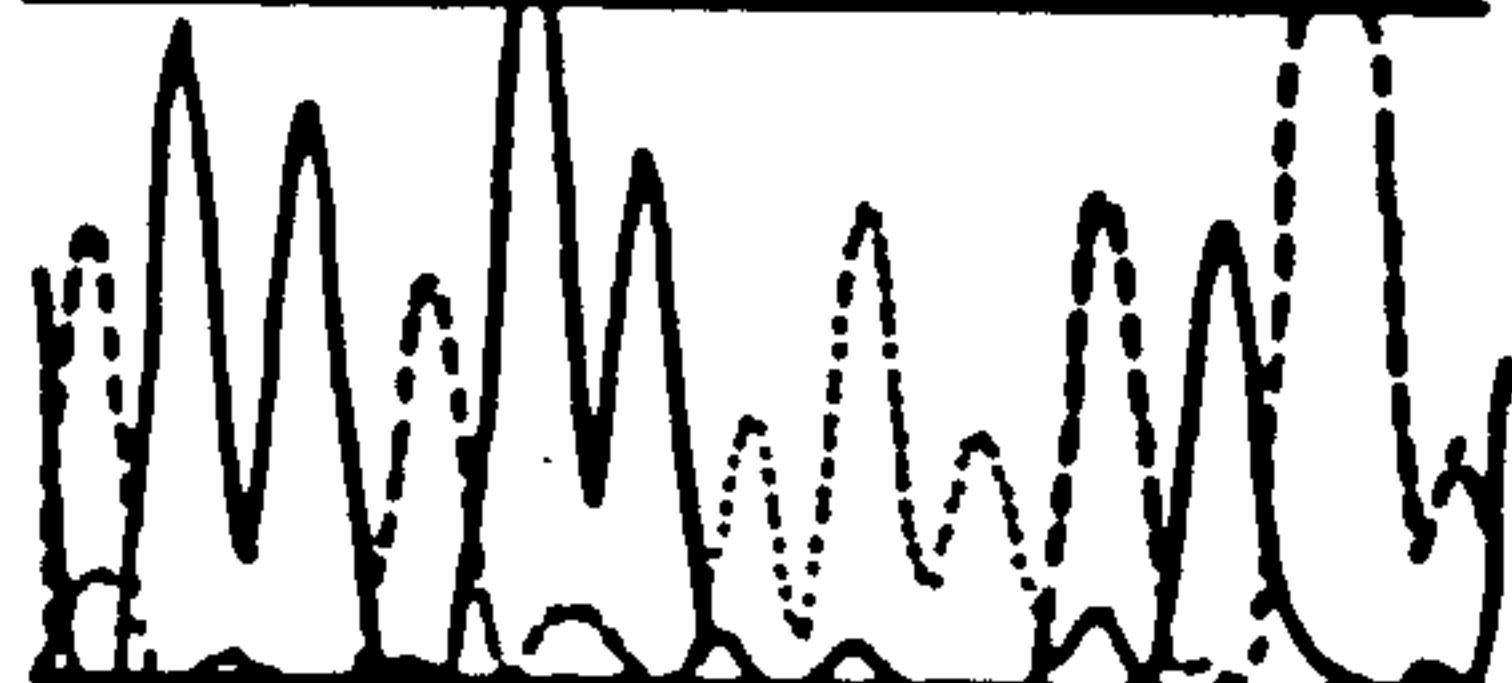
AGCTGGTGCGGCT

**M2407**GTG → GGG
Val354 Gly

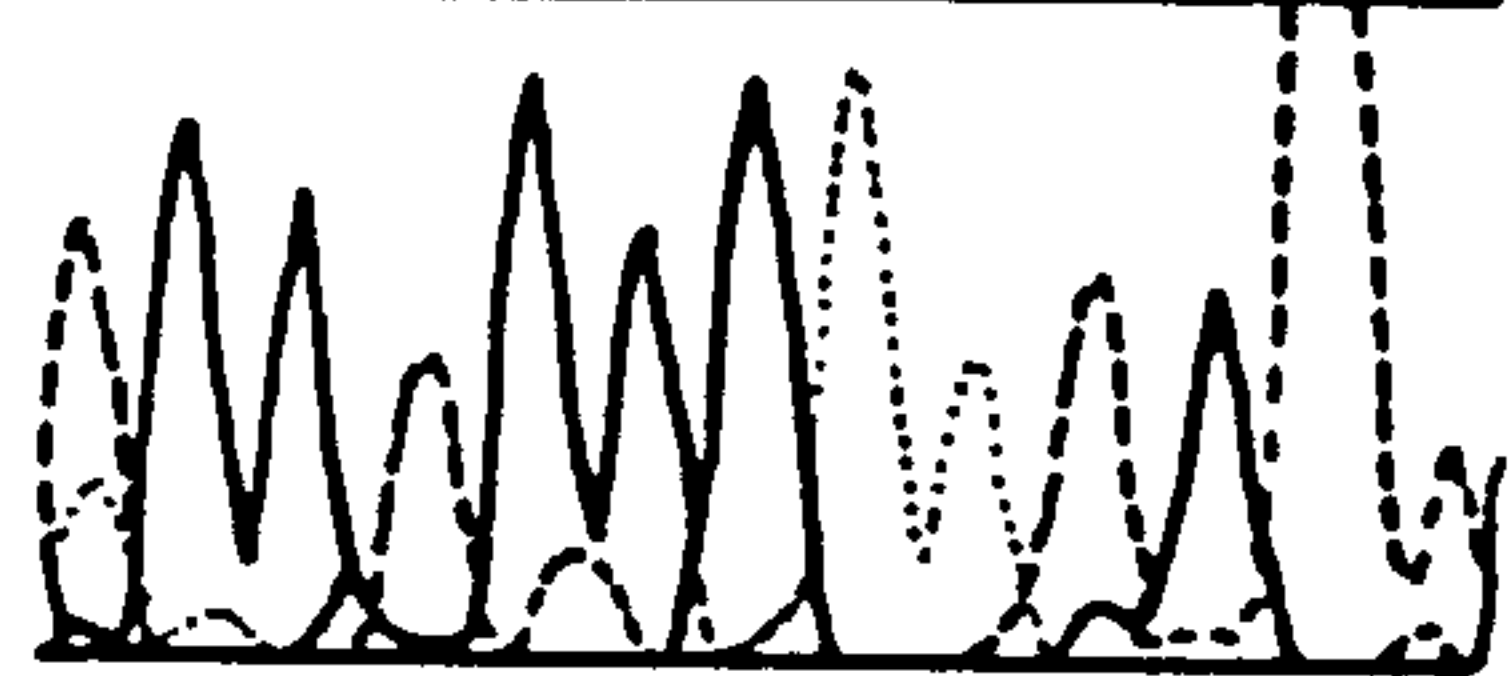
AGCTGGG*GCGGCT

C) EXON 13Normal
sequence

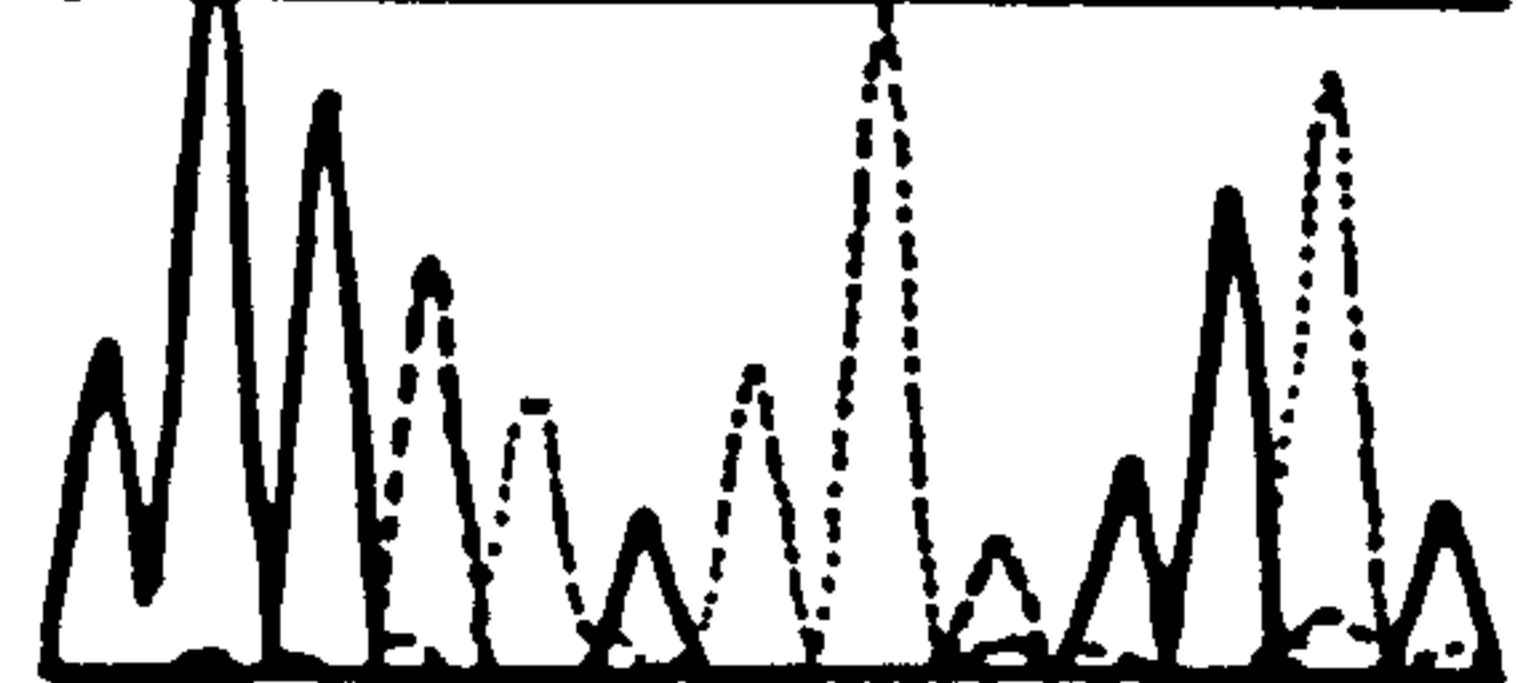
TCCTCCGGGTCTT

**R 12**CGG → CAG
Arg572 Gln

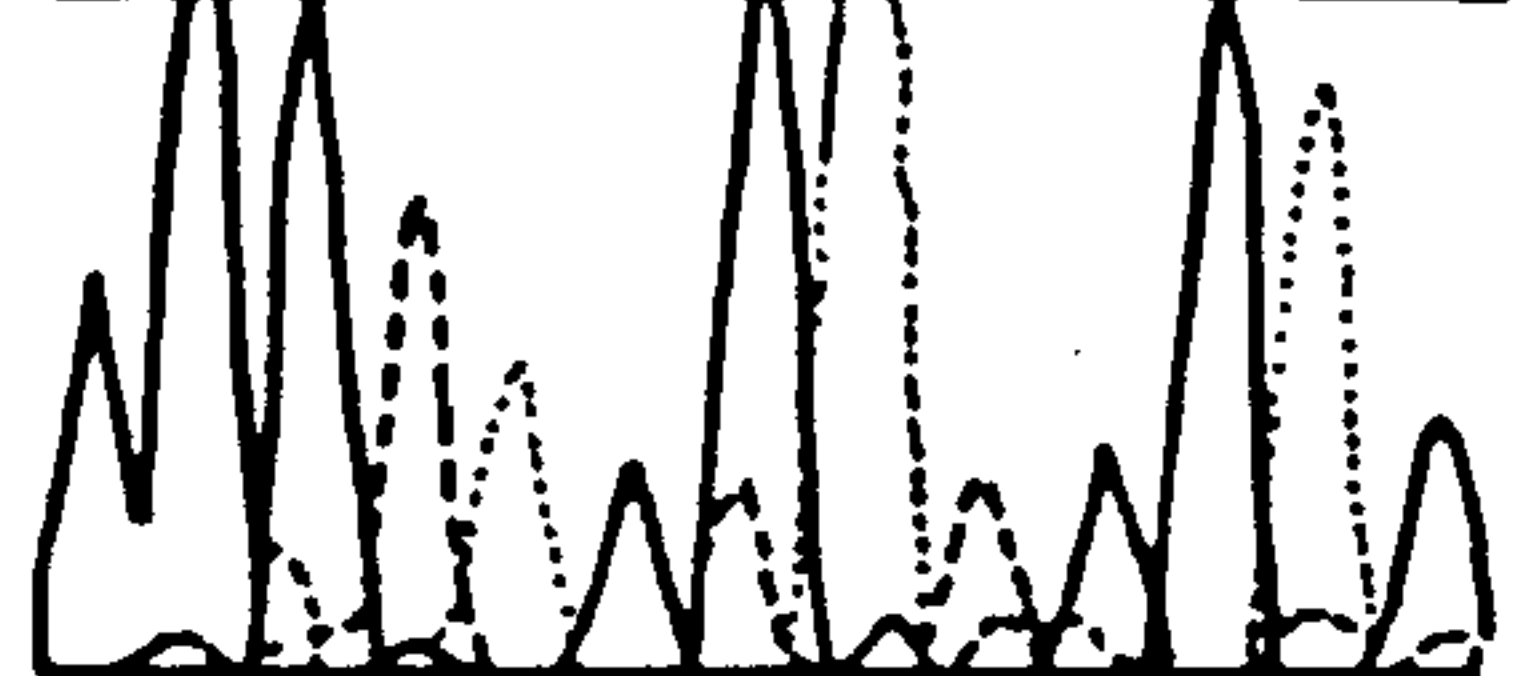
TCCTCC*AGGTCTT

D) EXON 22Normal
sequence

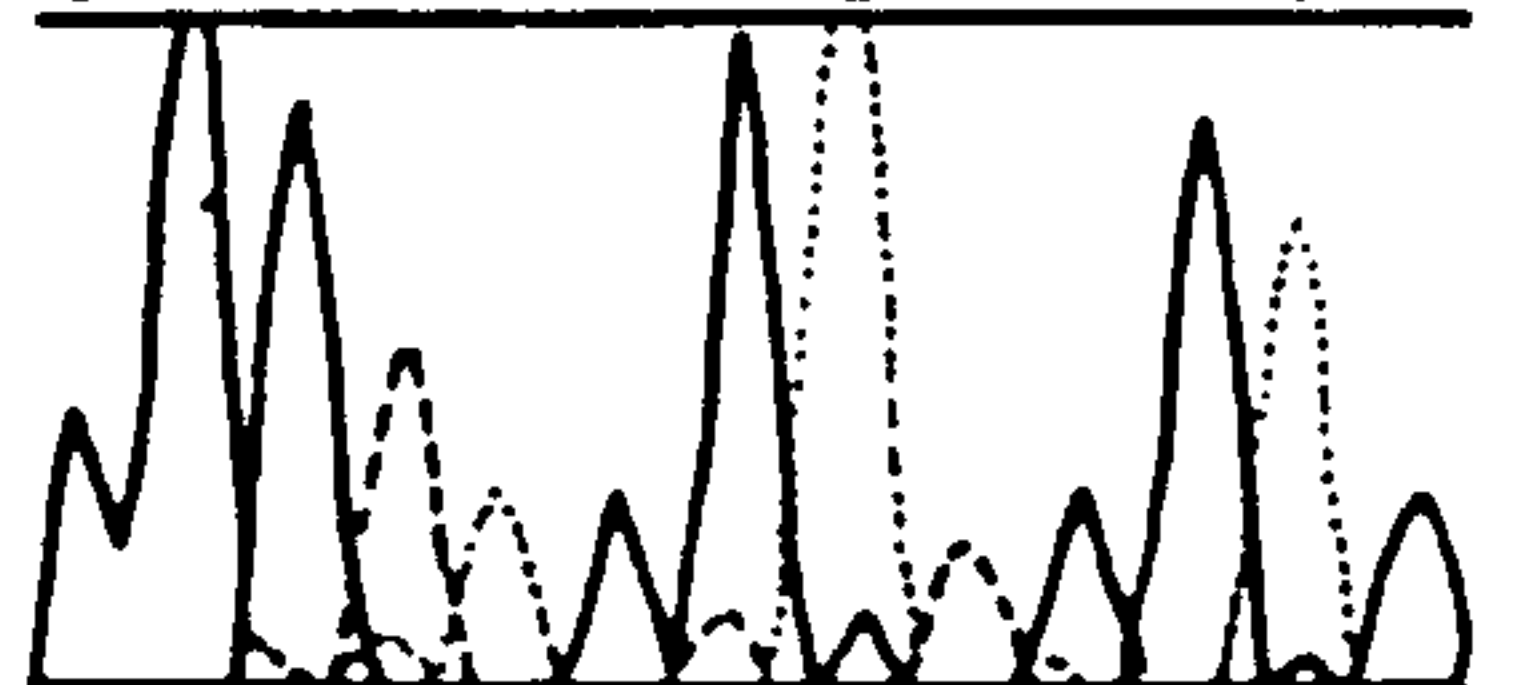
CCATGCGGTACGC

**Amish**CGG → CAG
Arg769 Gln

CCATGC*AGTACGC

**B501**CGG → CAG
Arg769 Gln

CCATGC*AGTACGC



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LISTE DE SEQUENCES

(1) INFORMATION GENERALE:

(i) DEPOSANT:

- (A) NOM: AFM
- (B) RUE: 13, place de Rungis
- (C) VILLE: PARIS
- (E) PAYS: FRANCE
- (F) CODE POSTAL: 75013
- (G) TELEPHONE: (1) 45 65 13 00

(ii) TITRE DE L' INVENTION: LGMD GENE

(iii) NOMBRE DE SEQUENCES: 4

(iv) FORME LISIBLE PAR ORDINATEUR:

- (A) TYPE DE SUPPORT: Floppy disk
- (B) ORDINATEUR: IBM PC compatible
- (C) SYSTEME D' EXPLOITATION: PC-DOS/MS-DOS
- (D) LOGICIEL: PatentIn Release #1.0, Version #1.25 (OEB)

(2) INFORMATION POUR LA SEQ ID NO: 1:

(i) CARACTERISTIQUES DE LA SEQUENCE:

- (A) LONGUEUR: 3018 paires de bases
- (B) TYPE: acide nucléique
- (C) NOMBRE DE BRINS: double
- (D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 1:

TGATAGGTGC TTGTAACTG TGCTTAACGA AAACATACCG TGTGCTGTAG GGA	60
CTTGTTTATA TCAGTTAGCC TGGTTTCGCT AACAGTACAT CATT	120
CTTACGAGAA CCTATCGATG ATGTAAAGTG AGGATTTTCT CTGCTCAGGT	180
TTTTTTTTTAA GACGGAGTCT CTTTCTGTCA CCTGGGCTGG AGTGCAGTGG	240
GTTCAACAACA ACCTCTGCCT CCTGGGTTCA AGCAATTCTT CTGTCTCAGC	300
GCTGGGATTA CAGGCACCCG CCGCCACACC CGGCTTATTT TTGTATTTT	360
GGGTTTCACT ATTGTTGACC ATGCTGGTCT CGAACTCGTG ACCTCATGTG	420
CTCGGCCTCC CAAAGTGCAG AGATTAGAGA CGTGAGCCAC ATGGCCCAGC	480

FIG 8A/1

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TTTAGCAGAT TCAGTCCCAG TGTTCATTTT GTGGATGGGG AGAGACAAGA GGTGCAAGGT	540
CAAGTGTGCA GGTAGAGACA GGGATTTTCT CAAATGAGGA CTCTGCTGAG TAGCATTTTC	600
CATGCAGACA TTTCCAATGA GCGCTGACCC AAGAACATTC TAAAAAGATA CCAAATCTAA	660
CATTGAATAA TGTTCGATA TCCTAAAATT TTAGGACTAA AAATCATGTT CTCTAAAATT	720
CACAGAATAT TTTTGTAGAA TTCAGTACCT CCCGTTACCC CTAAGTAGCT TTTTGTCAAT	780
ATTGTTTTCC ATTCATTGTA TGGGCAGTAG TTGGGTGGTC TGTATAACTG CCTACTCAAT	840
AACATGTCAG CAGTTCTCAG CTTCTTTCCA GTGTTACCT TACTCAGATA CTCCCTTTTC	900
ATTTTCTGTC AACACCAGCA CTTCATGTCA ACAGAAATGT CCCTAGCCAG GTTCTCTCTC	960
TACCATGCAG TCTCTCTTGC TCTCATACTC ACAGTGTTTC TTCACATCTA TTTTLAGTTT	1020
TCCTGGCTCA AGCATCTTCA GGCCACTGAA ACACAACCCT CACTCTCTTT CTCTCTCCCT	1080
CTGGCATGCA TGCTGCTGGT AGGAGACCCC CAAGTCAACA TTGCTTCAGA AATCCTTTAG	1140
CACTCATTTT TCAGGAGAAC TTATGGCTTC AGAATCACAG CTCGGTTTTT AAGATGGACA	1200
TAACCTGTCC GACCTTCTGA TGGGCTTTCA ACTTTGAACT GGATGTGGAC ACTTTTCTCT	1260
CAGATGACAG AATTACTCCA ACTTCCCCTT TGCAGTTGCT TCCTTTCCTT GAAGGTAGCT	1320
GTATCTTATT TTCTTTAAAA AGCTTTTTTCT TCCAAAGCCA CTTGCCATGC CGACCGTCAT	1380
TAGCGCATCT GTGGCTCCAA GGACAGCGGC TGAGCCCCGG TCCCAGGGC CAGTTCCTCA	1440
CCCGGCCAG AGCAAGGCCA CTGAGGCTGG GGGTGGAAC CCAAGTGGCA TCTATTCAGC	1500
CATCATCAGC CGCAATTTTC CTATTATCGG AGTGAAAGAG AAGACATTCG AGCAACTTCA	1560
CAAGAAATGT CTAGAAAAGA AAGTTCTTTA TGTGGACCCT GAGTTCCAC CGGATGAGAC	1620
CTCTCTCTTT TATAGCCAGA AGTTCCCCAT CCAGTTCGTC TGCAAGAGAC TCCGGTGAGT	1680
AGCTTCCTGC TTGCTGGCTG GGTTTCCCCC CCACGGAGGA GTCCTCTCAC TCAGCACCTC	1740
CGGCAGCTCA GCTGTGCACA TGGGCACTGG GGAAGGATC CTGGCAGCAG CTCTGCTGGG	1800
CTCTGTCTTT AAGTGTGAAG CAGGGAGGAG AGGAACAGGT CTCAGATATT TCACCAAATC	1860
TCAGCAAAAT CCAGAGGGAG AGCGCAGGAG GTGGGGTGAT TCTTATGCTC TGGCTCTTTC	1920
TCTCTGAAAA AAAAAAAAAA ATCTTGCTTT TTATAAAAGT GGGTGGAAC CAGTTTAATT	1980
CATCCTGTAA AAATAAATAT TCCTTTCTCA GAACAAATTC CAGACAGCCC AGATGTACCT	2040
GTTCGTTTTA ATATTATTCA TCTTGGTAAG ATTATTTTCA TTTCTCTGGC TAAAATCATG	2100

FIG 8A/2

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ATGTTATTCT TCTTTAATTT ACCAATGGCC ATTCTTTCTG AAACACAGAA ACCCTAGAAA	2160
GAGAAGAGTC ATAGGCAAGG AATTTTTTTC ATGCATAAAA TGTGGGGT AAAGAGAGAG	2220
AGACCTAGCA ATCGCTTTGG TCCACCTACC TCACCTCATA AGTGAGGAGT CAAGGCACAC	2280
TAGAGTGAAA TATATCTAGT GGCCACATGA CAGAGCCCGG ATTAAAACTT TGTTTTAGGA	2340
AACTCTCCCA GCCTCTGGGT TTCATTTACA GTGATCGCCA GGAGGGAAAT CACATTCCCC	2400
TGGCTCACCT CTCTGATCAT CCCTCCAGTG TGA CTCTTGT TCTTAATTCG AGAAATATTT	2460
ATTGAGCATC TACTAGTGCC AGCACTGGGC AAGCAACTGG GGGGACAGCA GTGAGTAAGA	2520
AAGACCAAAA TTCCAGCTGT CTTGGAACCT AGGGTCCTGA AGGGAAGATG GGCATTGAAC	2580
AAGAGTGACA TTGTCAGGAG ACGATGTTCT GGGTGCCACA GGATCATGTG GCAAGGAGAG	2640
CTAACCTGGT CCAGGGAGAC AAACCCTCTC TGAGGAAATG ATGACAAGCT GAGACCCAAT	2700
ACTATTGATT AGCCATGGTT TTCTTTAACC TAAGGTGGGC CAGGCATGGT GGCTCATGCC	2760
TATAAACCCA GCATTTTGGA AGGCCCAGGC TGGAGGATTG CTTGAGCCCA AGAGTTAGAG	2820
ACCAGCCTGG GCAACAGGGT GAAAACCTAT CTCTTTTGTA CTAAAAATTC AAAAAATTAT	2880
CCAGGCATGG TGGCACATGC CTGTGGTCCT AGCTACTCAG AGGCTGAGGT GGGAAGATCA	2940
CTTGA ACTCG GGGAGTTTGA GGCAGCAGTG AGCCGAGATC ATGCCACTGC ACTCCAGGCT	3000
GGGTGACAGG AGTGAGAC	3018

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(2) INFORMATION POUR LA SEQ ID NO: 2:

(i) CARACTERISTIQUES DE LA SEQUENCE:

- (A) LONGUEUR: 11451 paires de bases
- (B) TYPE: acide nucléique
- (C) NOMBRE DE BRINS: double
- (D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 2:

GATCCACCCG CCTTGGCCTC CCAAAGTGCT GAGATTACAG GTGTGAGCCA CCACGCCCAG	60
CCGACACTGC CCTAACTCTC AAGTTGCATC CTTACTCGAA TAGTATGACA GTGTGGGAAG	120
CAGCATGGGA CAATGTAAAA AGGAGGCATG TTTCTGGCTT CTGCTACTTA CTAGCTGTGT	180
GTCTTTGCAC GAGTTTCTTA ACCTCTCTGG GCCTCAGTTT CCTTATCTGA AAAATAACAA	240
TGATAGTATT CCCTTCACAG GGCCAAATGG AATACTATCA GGAACACTAC ATAATGGAAC	300
TCAATAAATA ATAGCTACTG CGGCCGGGCG CGGTGGCTCA CATCTGTAAT CCCAGCACTT	360
TGGGAGGCCG AGGCGGGTGG ATCACAAGGT CAAGAGATGG AGACCATCCT GGCCAACATG	420
GTGAAACCGT ATCTCTACTA AAGATACAAA AATTAGCTGG GCATGGTGGC GCATGCCTAT	480
AGTCCCAGCT ACTCGAGAGG CTGAGGCAGG AGAATCACTT GAACCCCGGA GGCAGAGGTT	540
TCAGTGAGCC AAGATTGCAC CAGTGCACTG CAGCCTGGCG ACAGAGTGAG ACTCCGTCTC	600
AAAAAAATAC CTATCTATCT ATCTGTCTAT CTACTGTTAT TCTTACCTGG TCATTTCCTT	660
TTTGTTTCAC AGGAAATTG CGAGAATCCC CGATTTATCA TTGATGGAGC CAACAGAACT	720
GACATCTGTC AAGGAGAGCT AGGTAGGAAA GTGCCTCAGG TCAGATCCTG CCAGATGATC	780
AAGGGGTGAT TACAAGGTGT GATCCCCTTC CAGGAGGTAA AGGGACAATC TGTGCTTGCT	840
TCCAGTAACT TTTTGGAAGA TTTTTTATAA CAGTTGCTTT ATGGTCGTTT ATCTACATGC	900
TGGCGATTGC TTCATTTCCT CCTACATGCC TCTTTAGCAC TCTGCCATGC ATCACAGGGG	960
GTATCTGCAT CCTGTGGCCT CCTCTCCAGT ATCTCAAGGA CACTTACATA CCCCACTCAG	1020
CATGACAAAA GCCCTGCTTT TCACTGTATC GTCTTTCTTG GAAGACAGCT CTGTGACTGT	1080
GCACCAAGCA TGCCCCTTGG GCATGGAGAT TCTAGATACA CACACAAAAG GCATCGCCAA	1140
GGAAAGCACT TGTAAGTGA ACCCTTGTT TAAATTGGCC CAGCATAGCT CCATCTTTAA	1200

FIG. 8/B1

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AAGAGTCTTT	CCACAAAGAT	GGCATCCGCC	ATGTGGATGA	GCATCCAATT	TTCTCTTTGA	1260
TTGGTTAGCT	TGACTGCTCC	ATCTGATCTT	CCTCTCTCTC	GACCTCTTGT	TCAGAAAGTA	1320
TTGTCTTTGG	TGTGGACTAT	AAGCAAGCTC	TGTGAAGTAA	AATTGGAGAG	AACACCAACA	1380
GAAACAATTT	AAATTTGAGG	AAAAGGGGGC	ACCTAAGACC	AAAGGAATTT	GGCTTATTTT	1440
ATTCCAGAAG	GGGAGGCTGA	GAATAAATCA	GATGAATATC	TGGGTTCTTG	CACCTGAGGG	1500
AAGGCTTCCT	GCAGAGCCCT	GGGCATAATA	ATCTGGGACC	TTCAAACCAA	TAACCTCTTT	1560
TCCAAGGAAA	GACTGGCTGC	TTCCAAGGAG	GGTAGGGGAG	AGTCGGGCTG	CAGGCAGCTC	1620
TCAAGTCTCC	CCTTGACAC	TCTCAGGTTG	GCATTTTCAC	TTTAACCCAT	CCTCCCTTAA	1680
GAAGGCAGTT	CTTTGTGACC	AGGGTACACC	CCCTATTATA	TATATATATA	CACACACAGA	1740
GAGAGAGAGA	GAGAGAGAGA	GAGAGAAAGA	GAGCAAAGTG	TTACCTCCAA	CTACATACAG	1800
TACTCTGTCA	GAAAAGAGGT	TCAGAGAATA	AGAAAACGTC	CCGAGCTCAT	TCCGTTGCCA	1860
GCAATGTCTT	ACTGCCCCCT	ATAGACGGGT	TCCAGGGCAG	CTGCCTACCT	GGCCTTCCTT	1920
CCAATACAAA	TCATCTTGGT	GGATGGTTCT	CTGAGGCTCA	GTCTTCGCTG	AAGTCAGAAG	1980
AGGAATTGGA	CTCACATTGC	AAAGGCACAG	GGCAGGGCAG	ATTCCTTACA	GGTGTTAGGA	2040
AGAACAACCC	AGTTATGATC	ACCTACTGCT	CTGTCTCCAT	TGAGGCCTAA	AAAGGAAGTG	2100
AGTTTATACT	GCAGTTGGAG	GAAGTGCCTG	CAGCCTTGAG	GAAAATGTCT	AGTCACAAGG	2160
GAGTAAGTTA	CCTGTTGATC	ATATTGTCAA	GGAATTCCTG	TCCAATTCTC	CTTCCCTGGG	2220
TTGACACCTC	TGTAAGGTCA	GATCTGGAAG	TAGGAGAGTG	GGCACCAAGG	GAGTCCCCGT	2280
TCAGGGAAGT	GGAGTGGCTG	GCTGGGATTG	GGGCTTTTTT	TTCCCAGGAG	GAGCAGGAGT	2340
GCTCACGATC	TGTGCCCTGT	GTCTGCCTGC	AGGGGACTGC	TGGTTTCTCG	CAGCCATTGC	2400
CTGCCTGACC	CTGAACCAGC	ACCTTCTTTT	CCGAGTCATA	CCCCATGATC	AAAGTTTCAT	2460
CGAAAACTAC	GCAGGGATCT	TCCAATTCCA	GGTGAGGTAA	TGAGAGTGTA	GTAAAGAGGG	2520
CCAGCGGCAG	GCCACCCACC	GCTGGTCTCC	TGGCCTTGAC	TTCCCAGAAG	CTGGAGGAAA	2580
CTTCCCACCC	ATCTACCCGC	AGCGGCAACA	GTCGGCATGG	ACCCCTTAA	GGCTTCAAGC	2640
CTGGGAGGAA	GCAGTTGCTT	ATCTCTGGCT	CCCTAATCCC	TCCCCACCA	CCTTCCACTA	2700
TGTCCCAGAA	AGACAGGAAG	ACATCCTGTT	TACTGTGGGT	CTATTTTGT	CTTTGCAGCT	2760
GTCTGGCTGC	TTTTATTGCC	TGCAGCCCTT	CTCAAGTAGG	TCCCTAAGAT	ATTAGCACTG	2820

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TGACACCACA	GGACCCTTCA	GGTTGTACAG	GAACCCCTGT	CCAGGGCTCC	TGTATACTTC	2880
TTCCTCTCTA	AGGCATGGCG	GTACCAAGGC	TATCACTCCT	CTCTTCCAAG	CCCTGGAAGA	2940
AGAGTCTGCT	TAACCTGGGG	ATCAGGCTTC	TTGTTTGCCC	TAGAACTGAA	TCTGATGGTT	3000
CTAGAATCCA	TCCAGCTACT	GGAAATTTTC	TGGGTCCCAG	TCACCTTGGC	ATAGAGCTGG	3060
TGCTAGAGCA	GAACCAAAC	GAATTCTACC	TGTGAGGGTC	TCGTAGCTTC	CGGGATGCTG	3120
GGGAGTCAGC	CTGTCTCCAG	CTTCAAAGGC	TCCCTCATGT	CCCAGGATGA	CCCACATTAT	3180
CAGTTCTTGC	TCCCCGGGTC	TTGCACCTCA	GCACGGAAGG	CCTCAGAAAA	GGTCTGTCTC	3240
CAGGCTCAGA	CTCCCCCTCC	TGCCGCCTTG	GGAACATGGC	ATATTTAAAG	GGTCTCAGAT	3300
CTAAAGGGCC	TTACATACAA	ATATCAGATA	GATTTCTGTT	CTCATTTCAA	TGAGGGAGAA	3360
AGTGCCATTG	AAAAGGAGAC	TAAACCACAT	TTGGCCCTTT	TCAGTTCAAA	CTGATTCATT	3420
CAAAAAGAG	CGACATCCAA	ACTTGAAATG	ATTGAACAAT	GTTCTGCTA	CAGCTAGAAT	3480
AGATTCTGGG	TCACTTTGTT	CCTCCGTTTC	AATCCTTGTT	CTTCAGTTTG	GCATCAAGAA	3540
ATACCTAAAT	CAGCACAGTG	CCTTCACTGC	ATAGTTCCCA	ATCCTGGCCA	CATTGAATCA	3600
GCTGGGGGCA	CCTGAGAGTG	CTGACACCCA	GGCCCTGCCC	CAGACCTGCT	GAGCAGGAGA	3660
ATGAAAATCT	TACATCCTAA	GACACTCATG	GAGCACCTAC	TCTACCCATT	ACTGGGCTGG	3720
ACTCTGTGGA	AGACATGAAG	TATATGTAAC	TCACTTCCAG	CTCTCAAAAA	GCACCCAGTC	3780
CAGTTAGAGA	CAGATTTACA	CACCCCAAAC	ACAAAATAGG	ATGAACAGGC	ACCCAGATGC	3840
AGAGTCCAGG	AAATGATGCT	GCTTTGGGAT	TCAAGAACCC	CCTGAGGAAT	GTGGAGGAAG	3900
GACACATTTT	CTAACAGTAA	TTTGAGTATG	TGACTCTGTG	CGTGACGCTT	CTGTGCAGTT	3960
CTGGCGCTAT	GGAGAGTGGG	TGGACGTGGT	TATAGATGAC	TGCCTGCCAA	CGTACAACAA	4020
TCAACTGGTT	TTCACCAAGT	CCAACCACCG	CAATGAGTTC	TGGAGTGCTC	TGCTGGAGAA	4080
GGCTTATGCT	AAGTAAGCAA	CACTTTAGAA	TGTGAGGTGG	GGCTAGAGGT	GAGAAAGTGG	4140
GTTGCAAAAT	CCAGCCGAGA	CCTCACTCAC	AGGAAGAGGC	ATGTGCCTCT	ATACGTGCAT	4200
ATGTGTGGGC	ATGCAAGTCC	AACTGTGACC	CAAAGTTAGA	GATCAGTTCC	AGGCAACAAC	4260
AGCTCTAACT	AAAAACATTA	AATTTAAGAG	TAGAAATGAA	GATTTGCATA	GAAGACCTTT	4320
AGCTTTAGCT	CACCATAGCG	AGTTCTTTCA	TTGCACCTCC	ATGGTGGCAT	TGCAAGTCTT	4380
GGGATCAGAG	CATTGTCCCA	GGGTCTCGAT	TGGCTCAACC	TCATGTGCTT	ATAGAAGATT	4440

FIG. 8B/3

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TATAAAGACA TGTGTCTCT CAACTTAAAA GCTCCACCCC AGATGATAAT AATGGATTTT	4500
CAAATTTTGG AACAAAGGTCA CTCTGTAATG CAGGCTGGAG TGCAGTGGTG CAGTCACGGA	4560
TCAGTGTAGA TTGACCTCCT GGGTTCAAGG TGCTCCTCCC ACCTCAGCCT CCCAAGTAGC	4620
TGGGACTACA TGCGGGCATC ACCATGGCCC TTTTATTTTT GTATTTTTTT GTAGAGCGGG	4680
GTTTTCCCAT GTTGACCCAG ACTGTTCTCG AACTCTTGGG CTCATACAAT CCACCAGCCT	4740
TGCCCTCCCG AAGCGCTGGG ATTGCCGGTG TGAGCCACCA CACCGGCAGC TGCTAATGGC	4800
TTAATGCAG CCCTTCCTCA ACGTTCAGGA TGAGTGGAA AGAGCTCTCA GGAAGTGGGG	4860
ATAGCTGGGT TTCAATCCCA GTGCTTCTGG CTCTCTGTGG TCTTGGGTGG GTCAGTTAGC	4920
CTCTTGAGCT CAGTTTCTTC ATTATGAAGA AAGGGAATCA TTGTTTCCAT CCCATGAGCT	4980
CATAGGGTTA ATGTGGAATT GATGAAAGAA CATCACAGCA TCCAAGAGGT AAAGTTCTGG	5040
TGGCAGTGGT ACCTGGGTTT TGTTCCCTGG AACTCTGTGA CCCCAAATTG GTCTTCATCC	5100
TCTCTCTAAG GCTCCATGGT TCCTACGAAG CTCTGAAAGG TGGGAACACC ACAGAGGCCA	5160
TGGAGGACTT CACAGGAGGG GTGGCAGAGT TTTTGTAGAT CAGGGATGCT CCTAGTGACA	5220
TGTACAAGAT CATGAAGAAA GCCATCGAGA GAGGCTCCCT CATGGGCTGC TCCATTGATG	5280
TAAGTCTGGG GTGTGGGGCA CAGGGTGGGG AGCTCCAAGT GTCAGGAAGC CTTTACCCA	5340
ATGAAGGGCA GCATAGAGCT TTTGTGTGGG ACAGAGCGAA TGTTTTGTTT GAGGAAGCAG	5400
GAAGTGGCTC TCAACTTTGA GGAAGTGGAA TTTCTCAAGG GAGAAGAGTT CTTCCGGATT	5460
TTCAATAAAG AACTGGTCA AGGACATTTC AAGCCCTGGA ATGTCAGTGG AAATCAGTCC	5520
AGAGGCCTGT GTCAGTGGAG GCCTCCCTTG CTGGTGCTCC TCAGTCTCAG CACGCTCCCA	5580
TTAAGCTGGC CACGTAATTG GCTGTGGACC TGAGCCCACC ATTTCCCTAA GAAAGCCTCC	5640
CAGTCACTGG GCTTTCACCA CACCTCCCCG CTTGAGACGT GGGCTTTGTG TTGTTACCTG	5700
GGAGAAGCTA AGCCTGCAGC ACCTTTCAGT GCAAAGAAAT GCTGTGAACT GAGACAGGAG	5760
CCAAGGGTAG GGAGATGGCC GCCCATGGCC AGGCCTCCTT CAGGGGGCAT GCCTTCCCTG	5820
AGGGCTGCTC AGTATATTGA TATGATAATC TTAGTGGTTT CCATTGGGGA GGATGGGGCT	5880
GAAGCTGAAT TCCTGCCCCCT TCTTCTCCCA ACACGCCCAA TGGACAGCTT GGAAGGTCAG	5940
TTAGCACACA ACACCATGGA TGAAGTTTTT TTCTGTATCA CTTTCTCCG TCTTTCCTCC	6000
ATTCGTGCTC TGTTGATCTC TCCTCTCTCC CTTTGTCTGT CCCATCTCTT TCTCCTCTCT	6060

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CCTTCCCTTT	CCACCCTTCT	GTGTTTGTTT	TCTCCCTCCC	CTGTGTTGTT	CCCTACATTC	6120
TCCATCGGGC	CTCAGGATGG	CACGAACATG	ACCTATGGAA	CCTCTCCTTC	TGGTCTGAAC	6180
ATGGGGGAGT	TGATTGCACG	GATGGTAAGG	AATATGGATA	ACTCACTGCT	CCAGGACTCA	6240
GACCTCGACC	CCAGAGGCTC	AGATGAAAGA	CCGACCCGGG	TGTGTACACC	TCCGATTATC	6300
AGAACTGACC	ATCCCTCCAA	CCCACATGAC	CCCGCCCTAT	TAGTGTGAGA	CTCCCCTCAG	6360
CAGCCAGGGC	CTTACCCACA	CACCCCCACC	TGGCACCTCC	CAAGGGTCTG	GGTTGAAATA	6420
ACTTGCTCAG	CCAAGGCTCC	TGAAGAGGGT	GCAAGAACCA	GGATTTTGGA	GGGAATCTCT	6480
GCTGGAGTTT	CTGCATATTC	CATGGTCCAG	GCAGTTCCTC	TCATAACGAA	CTATCAGACA	6540
GAAATACTTG	TAAAGATACT	TCATTTATTT	TGAAATATTT	TTCCTCTTCT	AATGTATTCA	6600
TTTATTCATT	CAACACTTAT	TTTTGAGCTC	CTACTATGTT	CCAGGCACTC	CTCTAGCAAA	6660
CAAAGCAAAT	TCTCTCCTCT	TTTTCAATAT	TTGTGGAAAA	AGCAAGGTCT	CCCTCTTGTA	6720
GAGTTTATAT	TCTAGTATTT	TCATAAGTTA	TACCTGCTCA	CTGGAGAATA	CTGAGCCATA	6780
CAGAAAAACA	CAGAGGAAAA	TTTCACTTAT	ATTTTTCCCC	ATGTAAAGAT	AACCACTCTT	6840
AACATCTAGT	ATATGTTCTT	CCAGGATTTT	TCTATGCACA	CACTGAATCT	GTATTTTTAT	6900
TTTTAAAATG	TTATCATATT	GTATGTACCT	CTTTGCAGCC	TGCTTTTTTC	AGTTAGTTTT	6960
TTTGGTTTTT	TGGTTTTTTT	TTTTTTTTTG	AAACCAAGTC	TTGCTCTATT	CCCTAGGCTG	7020
GAGCACAGTT	GTTGCCATCT	CGGCTCACTG	CAACCTCTGC	CTCCAAAGTT	AAACTAATTC	7080
TCCTGCCTCA	GCCTCCCGAC	ATAGCTGGGA	TTACAGGCAC	ACACCACCAC	ACATGGCTAA	7140
TTTTTGATTT	TTTGTAGTAG	GACGGGGTTT	CACCATGTTG	GCTGGAATGG	TCTTGAACTC	7200
CTGACCTCAA	GTGATCCACC	TGCCTCAGCC	TCCCAAAGTG	CTGGGATTAC	AAGTGTAAGC	7260
CACCACACCC	GGCCTAGTTT	GATATTCTTA	ATGTGCCCAA	AGTATTCTCC	TGTAACATTT	7320
TTTAATAGCT	ACACAATATT	CAAACACACA	GATATGTTAT	AATTTATTTA	CCCAATACCC	7380
TATTATTGGA	AAGTTGAGTT	CTTTTTTTTC	TTTGTTTTGT	TTTGTTTTGC	TACTATTCTA	7440
AAATGCTATA	ACGAACATCC	CAATAGATAC	ATCTTTGTAT	ACATCCATGG	TGACTTCCAT	7500
AGGACAGATT	CCCAGCAGTA	GAATTGCTGG	GTTGAATGAT	ATGCTTAGGG	TAATGACAGA	7560
AGAGTCATTT	CAAGCAGCTT	CCTAGGGTCT	TAGAACTTAA	GGATTAATGA	GTCTTCCCGC	7620
CCCCTCCCAG	TCTATTCAGC	ATGATCTGGA	TCATGAGGAC	TGAGATCTGG	AAGAGACTGA	7680

FIG. 8B/5

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GATCTGGGAG AGGCTGAGAT ACCAAAAGCC CTGGCTCCAC CCATACCCCT CGCCCTGAAA	7740
ACAGCTCTAG GAATTCCGCG GCCTAGCAAG GCTCCGGGAA GCTCCTTTTA AAGCTGTGAC	7800
GTTAGTAGGC ACATGGACCA TAGAGACCTA TCCAGGGCTC ATGGGACTTT AGTGATCCTG	7860
CCCTTCTCCC AAGGATCCCC CATGGCTGCA ACTTGGAAT TTCTGCAAAT GGAAGAGCTA	7920
CTCCTTAGGC ACGGTCATGT CTGAGCAGGG ATCTCCTCGG GCTTTCTTAG AATTCTCTCC	7980
CTGGGCACTG GGA CTCTTGA TTTCTTGAAT ATTATGTTCC AGGTGGGTGT GGAGGAGGTG	8040
AGGGGATGTA AAGAAGGCTA GACTTGGCCA GGCGCAGTGG CTCATGCCTG TAATCCCAGC	8100
ACTTTGGGAG GCTGAGGCGG GTGGATCACC TGAGGTCAGG AGTTCGAGAC CAGCCTGGCT	8160
AACATGGTGA AACCCCGTTT CTAATAAAAA TACAAAAAAT TAGCTGAGCA TGGTGGCAGC	8220
TGCCTGTAAT CCCAGCTACT CGGGAGGCTG AGGCAGGAGT ATCGCTGGAA CACGGGAGGC	8280
AGAGATTGCA GTGACCCGAG ATCGCGCCAC TGCACTCCAG CCTGGGCGAC ACAGCAAGAC	8340
TCTGTCTCAA AAAACAAAAA AGAAAGAAAA AAAGGAAAAG CTAAGACTTA CATGTGTCAC	8400
TTAACCCTT TTCTCAAACC TCTTTCTCTT CCAGGAATAG TCAACCCTG GATGGCTTCA	8460
GGGAAGGGG GATCCTGAAG CCCAGGGCAG CCTCCAATC TACCCCTTCC TCCTTTGAAG	8520
GATACTAAGG GGTCCAGAAA GGAGGGGCAG GACACTGTTA CCCACCCAC ATCCCAGCAT	8580
CCACATTGCT CTCTGATGGT CAGGACAGAG CCTTCTCAGG GAGACCAGCC TGTCTGGAGC	8640
TGTGTCTCTT GGC ACTCTTA AAGGGCCACT GAAGGTCCGT TCGTGGTCGT GAGGCACACT	8700
TTCAGGGAGC AGAGTGGTCT GTGTCTTCAC AGAGCCCGGA AAATGAACTA GTATGAACTT	8760
TGCCTCCAAG CAGCAGAACT TCTGTTCCCC CGCCCCTAAT GGGTTCTCTG GTTACTGCTC	8820
TACAGACAAT CATTCCGGTT CAGTATGAGA CAAGAATGGC CTGCGGGCTG GTCAGAGGTC	8880
ACGCCTACTC TGTCACGGGG CTGGATGAGG TAAGCCTGGT GGGGCTTGGT GGGGCAAGGG	8940
CACCCTCCTG GGTAAACCTC ATGAAGTCAG GACTTAGCTG TTGGGGCCCC TGCCCTGTCT	9000
GCAGAGCTTG CCTCCAATCA GGACATTCAG TTCAAGGTCC AAGCCACGCC TGGGAGCAGA	9060
GGGGCCTGTG AAAGTGGTAG AGGTGGATCC TGCCACAGTT GGTGCACAGT TTATCTTTGC	9120
TTTTCGTGCT AAAGATGGCA ATTTTCCAA CATTTCCAAT GAACAAATTG AAATATCACT	9180
TAACTTTGCT TTTACAAAGT TGGTTTCATG TGTTCTTGAG CTTCTGTTC TCTCGTGTTT	9240
AGATAGCTAC AGTTGTCTCT GGGTAGCCAC GGGGACTGGT TCCAGAAGCC CCAACAGTAA	9300

FIG. 8B/6

SUBSTITUTE SHEET (RULE 26)

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CAAAATCTGC AGATGCTCAA GTCCCTTCTG TAAAATGGAG TAGTATTTGC ATATAACCTA	9360
TGCACATCCT CCCATATACT TTAAGTCATC TCTGGATTAC TTACGATACC TAACACAATG	9420
GAAATGCTAT GTAAATAGTT ATTGCACTGC ATTGGGTTTT TTTGGTATTA TTTTCTGTTG	9480
TTGTATTATT ATTTTTTCTT TTTTGAATA TTTTGTATCC ACAATTGGTT ATATGCCAAA	9540
GCCATGGATA CGAGAGGCTG ACTGTTCTGT TTTGCTCCTT CTGGGACTTC TGGGTTTTCC	9600
TGGACCATGT CTGAGACAGG AACGTTGTAA GACCTGTTGC ACACAGTTGG GCAGGTTGTG	9660
CCCTGTACAG AGGGATGGGC TGAGAGGGGC AGTTGCCTGC ATCACCCATT GCAGCAGACT	9720
GGAGGGAGTC TGCTTGTTTG TAGTTCCTCA GTCAGCAGGG GCCTTTTGTC TTCCTTCCT	9780
TTCCTTTTTT TTTTTTTTG AGACGGAGTC TCACTCTGTT GCCCAGGCTG GAGTGTAGTG	9840
GCACAGTCTC GGCTCACTGC AATGTCCGCC TCCTGGATTC AAGCGATTTT CCTGCCTCAG	9900
CCTCCTGAGT AGCTGGGATT ACAGGCGCGT GTCACCATGC CCAGCTAATT TTTGTATTTT	9960
TAGTAGAGAT GGGGGTTTCT CCATGTTGAT CAGGCTGGTC TCGAACTCCT GACCTCGTGA	10020
TCCGCCACC TCGGCCTCTC AAAGTGCTGG GATTACAGGC GTGAGCCACC ACGCCTGGCC	10080
AGCAGGGGCC TTTTTCTAA TTTATATGAA GACACCTAAT TTATATGTGT TAGCAAAGCC	10140
CTCCTGTTTA TGCCTCACCT CCTCCCCGA AGCTCATACG GCAGGATGTT CCTGAGAAAA	10200
TTGCCTCTTA GAAGATAGAG AGGAGATGCC AAGCCTAAGT TAGGCAGACT CAGGAGGATA	10260
GGTCTGACCC ACCCCCTGCC ATTCCCCAGC ACACTTGTGA TTAATCTCCT TGGCCAGAGC	10320
CAGGCAGAAC ACCCTCGCGT AAGAGATTTG CCCCCAGCC CCGTCCCAGC CCTCAGCTAG	10380
ACAGAAGATT CCCTTTCCAG AGAGGCTGCA GAGCATGAGA GCTCTTTCTG TGTGCTTAAG	10440
GTCCCGTTCA AAGGTGAGAA AGTGAAGCTG GTGCGGCTGC GGAATCCGTG GGGCCAGGTG	10500
GAGTGGAACG GTTCTTGGAG TGATAGGTAG GTGAGGGGAC CCCACGGGAT TGGCGGTGGC	10560
GGGGAACAGG GTCCGGGACA AGGCTGTGTT GGGAAGTGA CCATGAGAGT ATTGAAGATG	10620
CTTGGTATAA AATCACCTC AAAACCAATG ATCCGCAGAG AAGAGGGGCA CAGGTGTTGG	10680
CTCCAGGGAA GGGCCAGGAG TGGAAGCGGG GTGCTGGGGA CCCAGAGAGG TTGCTGACAA	10740
CCATTGGCTG GAAAGGAAGG ATTCCAGAAA GCGTGGGGAA GGTCCAGGCA GGAAAAGCGT	10800
ATGAATGCAG GGTTCTGGGC TAGAGAAGTG ACTTCCCTTC TTGGGGTCTT GTGTTGCCTT	10860
TCCTGTGAAA TGGGAACAGT ATTATTAGCA CTTACCTTGT GGGCTGATAT TGAGGAGTAA	10920

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CTGGGACTTG	TTTTTGGGCA	AGTGCTGAGC	CATTGCTAAG	ATTCCCCTTA	CCCGTGCTTG	10980
TCCCTTGTAT	TAAGGCACAA	GGGCCCTTTG	AAAAGAATTT	TACCTGCTTT	ATCAATTGAA	11040
AGGGATTAAG	ACCTTGGGGG	CCAACCCAAA	ATAAACATGC	GAAGTTATTA	TTTATAGGCT	11100
CCATGCACAC	TTCGTAA AAC	CTCCATGGTC	CTACTGGTTC	CTGATTACCT	CCACTCAATG	11160
AGAGGCAATT	CATTACTGAA	TGAGCCATAA	GCGCCTCTTA	TTTCGAGAGG	GGGATGGCAG	11220
GA CTCAGTCG	AGGAGAAGGA	CCGCACCCAG	GCAGCCTGGG	CCCCTCGGCT	CCTGTACTTA	11280
TTTACTGCTG	GGTACTTCCT	AGCCCAGCAT	GTAATTACTG	GTTCG TTCAG	TCATTCGTTT	11340
AGTAAATGTT	TCTTGGGCAC	CTACTACATA	GGAGGCACAG	GTCAAGGCAC	TGGGGATATT	11400
CTTTCTACCC	ACCCCTCCC	TCCCTACACT	GTGATTAGGG	ACTGACCGAT	C	11451

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(2) INFORMATION POUR LA SEQ ID NO: 3:

(i) CARACTERISTIQUES DE LA SEQUENCE:

(A) LONGUEUR: 1834 paires de bases

(B) TYPE: acide nucléique

(C) NOMBRE DE BRINS: double

(D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 3:

ATTTTTTTTT TTTTTTTGA GACGGAGTCT CACTCTGCCA CCCAGGCTGG AGTGCAATGG	60
CGCGATCTTG GCTCACTGCA ACCTCCGCCT CCCGGGTTC AAGTGATTCTT CTGCCTTAGC	120
CTCCTGAGTA GCTGAGACTA TAGGTGCCCC CCACCACGCC CAGCTAATTT TTGTATTTTT	180
ATTAGGACGG GGTTCACCA TATTGGCCAG GCTGGTCTCG AAATCCTGAC CTTGTGATCC	240
GCCACCTCG GCCTCCCAA GTGCTGGGAT TACAGGTGTG AGCCATTGCG AGCAGCCCAG	300
AACTCAATTC TTAACCTTTA AAGTATGATG AGAAGAAGGA TCAAGCCCTC ACCAGCCCAT	360
TTAAGGAGTT TAGGCTCAGT CTTGAGGATG TGAGAAGTCA TTGCTATTGG GTTTCACACT	420
GAGGTAAACA GGTGAAGTCA GCATTTTGGT AGTTCACAGC AGCTGCAACT CTTTGTATTT	480
CTCTGATACC TCCTGTCCCA ACCTACATCA GGCCTTCCCT TCTTCCTGCT TCCTTAATTC	540
CTCCATTTTC CCACCAGATG GAAGGACTGG AGCTTTGTGG ACAAAGATGA GAAGGCCCGT	600
CTGCAGCACC AGGTCACTGA GGATGGAGAG TTCTGGTGAG TCCAGAACCC AGGAAGACCC	660
AGAAGGGTAA GGTGGGGAA GAGAGGGGAA ATCTCAGACC TCAGTCCCCA GCTAAGGTAA	720
TCAGATTCCA GCCCTTGGGA GATCTTGGCT GTGTTCTCCT CCAGCCCAAG GCCCAGCAAG	780
GATGAGGTTC TGAGAGGAGC CTTCCAGGCC ACAGGGACAA TGAGCCCAGG ACCAGGCCAA	840
CATGACATGG CTCTTGCCCT CTGTGTGCCC CTCCGCCACA CACTCTATTC CAGCCACAGG	900
CACCCTGGCC TTAGCACAAT TCTTTTCTGA GCCTAGGAAG CTCCACTTAC CCTGATCTTC	960
CAACGTCAAC CTCACCCTCT CTCAGGTTGT TTCTATTGAG GCTTCAAGTC TCAGCTTAAG	1020
GAGAATTTTC AAGTCTCAGC TTAAGGAGAG CCCCCTAAGT TCCCCGAGGA CTGGGATTAA	1080
TTTATGATGC TCATCACCTT TAAATTGTT TGCTTAAGCC GGGCGCGGTG GCTCACGCCT	1140
GTAATCCCAG CACTTTGGGA GGCCGAGGTG AACGGATCAC GAGGTCAGGA GATCGAGAAC	1200

FIG. 8C / 1

SUBSTITUTE SHEET (RULE 26)

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ATCTTGGCTA ACACGGTGAA ACCCTGTCTG TACTAAAAAT ACACAAAAAA AGTAGCCGGG	1260
CGTGGCAGCG TCGCCTGTA GTCCTAGCTG CTGGGGAGGC TGAGGCAGGA GAATCACTTG	1320
AACCTGGGAG GCAGAGGTTA CAGTGAGCCC AGATTGCGCC ACTGCACTCC AGCCTGGGCG	1380
ACAAGAGAGA CTCTGTCTTG GAAAAAAAAA AAAAAATGTG GTCTTAGTTT AATGTCAAGG	1440
GAAAGGTTTT GGGTGTTTTT ATTACTTTAT TTTTATTTA AAAACTATAA TAGAGACGGG	1500
CCTCGCTATA TTTCTCGGGC TGGTCTCAA CTCCTGGGCT CAAGCGGTCC TCCCACCTTG	1560
GCCTCCCAA ATGCTGGCAT GTGGGCCTGG TCAACATATG GGACCCCAAC TCTACAAAAA	1620
ATTTTAAAT TAGCCAGATG TGGTGGCGTG TGCCTGTAGT CCCAGCTACT TGGGAGGCTG	1680
AAGCAGGGGG TCACTTGAGC CCAGGAGGTT GAGGCTGCAG TGAACATGA TTGTCGTTCA	1740
CTTTTCTTCT GAACGTGAGA TTAAGTGTAG TCAGCAATTT GGCTTAGGAT TATTTATTCA	1800
GAATTTTAA CCGTCACGTT GCGGCAAACC AGGT	1834

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(2) INFORMATION POUR LA SEQ ID NO: 4:

(i) CARACTERISTIQUES DE LA SEQUENCE:

- (A) LONGUEUR: 14664 paires de bases
- (B) TYPE: acide nucléique
- (C) NOMBRE DE BRINS: double
- (D) CONFIGURATION: linéaire

(ii) TYPE DE MOLECULE: ADN (génomique)

(xi) DESCRIPTION DE LA SEQUENCE: SEQ ID NO: 4:

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AGGAGGTGGA GGTGTCAGTG AGCCAAGATC ATGCCACTGC ACTCTAGCCT GGGCAACAGA      60
GCGAGACTCT GTCTCAAAAA ATACACACAC ACACACACAC ACACACACAC ACACACACAC      120
ACACACATAT ATATACACAC ATATATATAC ACACACATAT ACACACACAC ACGTCTGTAT      180
ATATATGTGT GTGTGTATAT ATACACACAC ACACTATTCT ATATATTCTT GTAGAGCTAT      240
GTGTGTCTCC TGTGCTATTG AGCATGAGCC CTTTTTTTTT TTTTTTTTTT TTGAGACAGA      300
GTCTCACTTT GTCGCCCAGG CTGGCATACA ATGGCGCAAT ATCGGCTCAC TGCAACCTCC      360
GCCTCCTGGG TTCAAGTGAT TCTCCTGCCT CAGCCTCCCA AGTAACTAGG ATTACAAGTG      420
CCCGCCATAA TGCTCAGCTA ATTTTGTAT TTTCAGTAGA GATGGGGTTT CACCATGTTG      480
GCCAAGCTGG TCTCAAATC CTAGCCTCAG GTGATCCACC TGCCTCAGCC TCCCAAAGTG      540
CTGGGATTAC AGGCATGAGC CACAGCACCC TGGTGAGCAC TAGAGCTTAT TTCTTCTATC      600
TAACTGTATT TTTGTATCCA TTAGCCACCC TCTTTTCATC CTCCCCTCTC CTTCCCTTCC      660
CAGCCTCTGG TAACCACTGT CTGCTCTCTA CTTCCATGAC ATATGCTTTG TTTTAGCTCT      720
CACATATGAG TGAGAGCATG CGACATTTAT CTTTCTGGCC CTGGCACATT TTTGAATCAT      780
TGTTAGAAAA GATGATGGTT TGGAGTAGAT ACATCAGAAG TGACAGCGTT TGCCCTAAAA      840
AGGAAAGACA GGCTCCTCTG GGACCCTGAC CAAGTTCCTG TGAAC TATTATTGTG      900
CTGTGTTAGT CCTGGGGTCT TCCGTTCCCA GCCCTCCTCA CCTGCTCCCA TATGGCTCTC      960
TCTCTTCTTC CAACCTCTCA GGATGTCCTA TGAGGATTTT ATCTACCATT TCACAAAGTT     1020
GGAGATCTGC AACCTCACGG CCGATGCTCT GCAGTCTGAC AAGCTTCAGA CCTGGACAGT     1080
GTCTGTGAAC GAGGGCCGCT GGGTACGGGG TTGCTCTGCC GGAGGCTGCC GCAACTTCCC     1140
AGGTGGGAGA TGCTCTTGAT GGGGGGAGGG TCTAAGCCGA AAAAGTTCCA GGCAGAAGAA     1200

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FIG. 8D/1

SUBSTITUTE SHEET (RULE 26)

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GCCTAACTAG TGCTTATTAA GTCTCTCTGT TCCAGACGTC CACTATCTTA TTAAACCTTC	1260
CCTGTTTTAC TGAGAAGGAA ACCACCATGC TGAGAAGTTT GCAATAGGGA GCTGGGTAGC	1320
AACTTTGGAA GCAGGAACTT GTGGGAACAA TGCAGATGCT GCTTGGACTT ACGATGAGGT	1380
TATGTCCAGA TAAGCCCATC CATCTTTTGA AAATACCCTA AGTGAAAAGT GCATCCAATA	1440
TGCCTAACCC CCCAAACCTC ATAGCTTACC CTGGCCTACC CTCAAACATT GCTCGGAACC	1500
CTTGACCTTA AGCCTAAAGT TGGGCCAAAT CATCTAACTC CAAAGCCTAT TTTACAAAGA	1560
AAGTTGTTGT AATATCTCCA TGTAACCTAG TTAATACTTG TACCTAAAAA GTGAAAAACA	1620
AGAATGGTTG TACGGGTACT CGAAATCCAG TTTCTACTGA ATGTGCATCT CTTTCACATT	1680
GTAAAGTTAA AAAATTGTAG CCGAACCATC CTAAGTCAGG GACTGTGAGT ACTGTGTCAG	1740
TAACAGTAAG GGCCTATTG GAGAACCAAG TTAGCAGCTG CTGCAATAGT TCAAGTCAGA	1800
GATGATGAAA ACCTAGACCA AGTCAGTAGC AGCAGAGATG GAGGGGAGAC AGCAGATTTA	1860
GGGAGAGCAT ATTGGGTGAT GTAGGGAAGG AAGAAGAATG ATGTCAAGAT TCCCAGTTGG	1920
GGACCTGACA ACATTGCAAC ATAAGACACA CAAGAAGATC GGGTGGGTGG CTCATGCCTA	1980
TAATCCCAGC ACTTTGGGAG GCAGAGCCAG GAGGATCACT TGAGCCCAGG AGTTCAAGAC	2040
CAGCACAGGC AACATAGTGA CACCTCATCG TTACCCAAAA TAAAAAATAA AATGAGGTGG	2100
GAGGATTGCT TGAGCTCGGG AGGTTGAGGC TACAATAAAC TGTGATCATG CCACTGCACT	2160
CCTGCCTGGG TGACAGAGTG AGACCCTGCC TCAAAAAAAA AAGACACACA AGAGAAAAAT	2220
ATCAGCGTGT TGTTTGTTTT TGGTGGAGTT AATTGTGGGG TTCTAGGGAA AGGAATTTAG	2280
CTTGGGACAT GGAAAGTTTG AGGTTCTGT AGAGTGTCCT AGTGAAGATT TGTAATAGAG	2340
CATCGGATGC GCATATTAGA TGGCACTTGG TGATATGATA AGAACTCAAA AAATATTTGA	2400
GGAATAAAGG AAAGAAGAGG CCAGACGTGG TGGCTTATGC CTGTAATCCC AGCACTTTGG	2460
GAGGCTGAGG CAGGCGGATC ACTTGTGGTC AGGAGTTCGA GACCAGCTTG GCTAACATGG	2520
TGAAAACCCA TCTCTACTAA AGATACAAAA ATTAACCGGG GATGATGGTG GGTGCCTGTA	2580
ATCCCAGCTA CTTGGGAGGC TCAGTCAGAA GAATCGCTTG AACCAGGAG GCGGAGGCTG	2640
CAGTGAGCCG AGATCGCGCC ACTGCACTCT AGCCTGGGCA ACAGAGCCAG ACTCCGTCTC	2700
AAAAAAAAAA AAGTGAGAGA GATTGAGGCT GGGATATATG GCTCAGGCAT CATGCGCGTG	2760
TAGGGGGCAG TTAATAAGCA GAAGTAAGAA AGATTGCCTA GGGAGGCAGG AAGGGTGAGG	2820

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TGAGAGGAGA AGAGGCCAG GACCAGATTC TAGTCACCAA CAGCGTTTAA GGGGCAGGTA	2880
AGGAAAACAA AACCATCAGC AAAGACTGAG AATGAAAGCC CAGAGAGGAA GGAAAAGCCA	2940
CACATACAAT CAGTACAGCT CCATCTGAAT AAAGGTAGCG CCCCCCCCCC CCCAAATCAT	3000
TAGAGAAATG CCTGATTTCG TTTTCTGTGG ATTTTTCCTA AGAACCTAGA TGTGGGGAAT	3060
AGAAATAAAT GGTTCCTCT GTCTCATCCC CTCCTGCCC TCTGAGAGGA AGCTGTGATT	3120
GCGTGCTCCC TTTCTGGGGG TGCAGATACT TTCTGGACCA ACCCTCAGTA CCGTCCGAAG	3180
CTCCTGGAGG AGGACGATGA CCCTGATGAC TCGGAGGTGA TTTGCAGCTT CCTGGTGGCC	3240
CTGATGCAGA AGAACCGGCG GAAGGACCGG AAGCTAGGGG CCAGTCTCTT CACCATTGCC	3300
TTCGCCATCT ACGAGGTGTG TAGTCCTGAT TGGCTCCAGC CCAGGAAACA TACTTTCCCA	3360
GAGAGGACGC TTCCAGGGGC TTCTAGAGGG GCCCTCTGCT TCCTCAATAC CAGTGACCCA	3420
CAGAGCTCCT GGTATCAGGA CCACTTGTGT TTGTAACAAG CAAAAAATAC CAGGGGGGGC	3480
ATTAGAGAGG CAGTGGAGCG GGCCTGGCAG AACAGGTGCC TGGGGGTCAG GCTTCCGCAT	3540
GCGGGCTGCA GTTGCTGGCA TTGCCTTCCG CAGGCTCCTC ATCCTCATTC ACATCTGAAG	3600
CATCTTCCTT TCTGTTTCTT CTCAAGGTTT CCAAAGAGGT ATAGCAGCAG CAGCGGCCAG	3660
CAGTTGTGTG CAGCACTACC CAGGGGGGCC CGAGTCTGTC TGTGGCTCGT CGAGAAGCTT	3720
CCTGGTGGGG TTTGTGGGCA GGAATTGTGA TAGGAGAGGG CCTTGCCTGT TGTATTTC	3780
CACTTGCAGA GCAGGTTGCC TCAGGGCATT GCATGACCCA TGA CTACCAC CCCAGGATG	3840
TGCACTTTCT CCCTCGCACC AGACACTGCA CGTCACACAC ATGCCTTTGC AACTCACCC	3900
TCCTCCACGC TTACAGCCAC ACACACAGTC ACACAGACGC GTTCTGAGGG TGGCTGCCCC	3960
CTTGGGATGG AGGAATCACT TCCCTCAGAA CCCAGCCAAG TCCTCTAGGC CTCCTTGGGG	4020
GTCCTTCCAG CCTGAGGGGC TTCGGAGCTG AGGACAGCTG TTCTGGTAAG TGTCCCTGAG	4080
TGTGGGGATG ACACATTTCC ATTCACTCTG AATCACAACA GAAAAGGGAA GAGGAATTGA	4140
GGTAGGGAGC CTATTTAACC CTTGGGAGTC GGAAGTAGG GAGGTTGAAA CTGTGACATG	4200
GGTGACCAGG GAGTTGGGAA GGGACCCTTG GAGGTGGCTG TGGCAGGACA GGACGTTCT	4260
CCCGAGGGGC TCATGTGCCC TGGGCTCTCC CCATCTCTCA GATGCACGGG AACAAGCAGC	4320
ACCTGCAGAA GGA CTCTTC CTGTACAACG CCTCCAAGGC CAGGAGCAAA ACCTACATCA	4380
ACATGCGGGA GGTGTCCAG CGCTTCCGCC TGCCTCCAG CGAGTACGTC ATCGTGCCCT	4440

FIG. 8D/3

SUBSTITUTE SHEET (RULE 26)

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PCT/EP95/04575

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CCACCTACGA	GCCCCACCAG	GAGGGGGAAT	TCATCCTCCG	GGTCTTCTCT	GAAAAGAGGA	4500
ACCTCTCTGA	GTGAGTGCTG	GCCCAGCTTT	CCCACGTGTT	TCTAAAAGCT	CACATGGCCC	4560
ACTCCAGAGG	TTGAAGGCAT	GAGGCAGCTA	GACACGTCTC	CTCCAGGGTC	CTTCTGCTGC	4620
TCCTGAGCCA	CTGGCCACAT	TACCCCCATT	CATTCAATCA	TCCATTCTGT	GATATTTATT	4680
GAGCACCTAC	TATGTTCCAG	GCACTGTCCT	AGGCACTAAG	GATAGAGTAG	TGAAGTAAAC	4740
AGAAAGAAAT	CCCTGCCTTC	ATGGAGCTTA	ATATTCTAAC	ATGAGACAAT	AATGGATAGG	4800
AAAAACATAT	GTAGCATGTT	AGATTTGGAG	AGGTGATATG	GAGCAAAAAT	AAAGTAGGGA	4860
AGAGGGATAG	GAGGTGTTGG	GGATGCTTGA	AATTTTAGGT	TAGCATGGCC	AGGAAAGCCA	4920
CATCCTGTCC	CTGGCCACCA	CAGATGAGCT	CATAGCCCCT	GCCACTCTGA	TCTCTGTCCT	4980
TGGAAGATGC	ACCAGGTCCA	TGGGTAGGTG	GCTGGGTCAT	GCCTTTGGGG	GGCTCTGAGC	5040
AATACTAACA	AGAACCTGCG	TGCCTGGGCT	TGGCTGTGGG	GGATGGTGCT	GACATGGGGC	5100
TGGTTCCTGG	GGTTGGGGTG	TTCCAGGGGT	TCTCTAGAGG	CTGGTTCTGG	CTTGGCTGCC	5160
AGGAAGCCGT	GCACCAGAGC	AAACCGTCCA	CGGGCCTCCT	GCTTGCTTCT	GGTGACACTG	5220
AGACCCACAC	TGTCTGTATT	CCTCACAGGG	AAGTTGAAAA	TACCATCTCC	GTGGATCGGC	5280
CAGTGGTGAG	TGGTTTAGAT	CTTCTGTGCG	AAAAGTCCAG	AGGGTCCCCT	TCCCTGACCA	5340
TGCAGGGGAC	AGATGGTGCA	GGGGAGAATG	GGCACTGGCA	GAGGGAATGG	GAGTCTGGGC	5400
TGTGCTGAGC	AGTCCCTCCT	TGGCACTGCA	AATCCTACTT	TGGCATGGCC	AGAAGTAATC	5460
GGCCTTAAGC	ACCGGGGGCC	ATTGAGGCAG	TTCAGGGGCT	GGGAAATATG	GAAGAGGGTC	5520
CTGGAAAGGA	GAAGCAATTT	GAACAATCGG	AGGGAACAAG	GCCACAGGAA	GGGATGACAA	5580
GAGCCGCAGC	GAACACTGGA	TTCTGAGACT	GGATAACATT	GGATTTACAC	CATAGAGAAA	5640
AGAAAGTAAG	CTGGTGCCGG	ACCTGGTGTT	GACACTTGGA	TCCTCCACTT	ACCAGCGGGG	5700
TGACCTGGAC	AATTTCTGTA	ATCCCTCTCA	CTCAGTTTCC	TACTCAGTAA	AACGGGGATG	5760
ATAATGTGCC	TTGCAAGGCT	TTTGTGAGGC	TTCATCAATG	AGGTGATGTA	TGTGAAGTGT	5820
CTGGCACAGC	ATGGGCACTC	AAACAGAGGT	GCTTTTTCAC	ACTTTACACC	TTACAAGGTA	5880
CTTTTCACAT	GTGTCATCGC	GATACTTGCA	AGGTTGCTGA	GAGGTAGATG	GGGTTATAAT	5940
CCCTGGTGTT	CAAGAAAGGA	AGCAGAGGCT	CAATGGGGTT	GAATGACTTC	TCTGAGTTCA	6000
CAGAGCTCAG	TAAGTGGCAG	GGTTTGGAAC	TCACATTCAG	ACTCTCTGAC	TCCAGACTTA	6060

FIG. 8D/4

SUBSTITUTE SHEET (RULE 26)

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GGTTTTTCCG CACCTCCACG CTGAGGCCAG CCCCAGGCAG TGAGAAGCCC AAAGTCCGAA	6120
GCACAGAGTG CTGTGTGTTG GGCTCTGTGT GTTGAGGAGT CTTGTGACTG CCTTGGGGCT	6180
TTGGGCTGTA GTCAGCTGAC AGTCCTTTGT GCTCTGTGGG GATGACGTAG GCCAATGGGA	6240
GGACAAATGC CCCTCTGAAC TGTCTTCTGG GCAGTGACAG TCATGGTCAT AATCCTGACC	6300
CTGAGCCAGT GCCAGGTCTC CAAGTGCCTT CTGAATGACC ACAGGCGATT GGTTTTAGTG	6360
GTAGGTGCGT GGGGATCTGT TCTGGTCATC TGGATGCTGG TCATCGGGTG CAGTATTGAT	6420
CAGGACCTGC AAACCCAAAA GCTTATGGGA GCTGGCACGT CACGTGAGTA GAGCAGGCAG	6480
GTGCAGGGTT TTTGATGTCC CTGCACTGAC ACAGTTGTCT GCAGTTCTCC AATTTGACAT	6540
TTGGGCTCCA GTGTCGAGGG TCAAACAAGG AATTTTGGGG CGTGGGCCAA ATCTGGGAAG	6600
ACACAGGGAG CAGGGCCCTT TGGCTCAAGC TGATAGTTGC CGCAGGGATT ACCAGGCCCA	6660
GGGCAGCCTG CCACAAGCTG GGGCTTTTAC CAAAGAAAAT CTCCCTATGT TAAATGCTTG	6720
CTCAAAAATT TTTAAAAAAT ATTCTGTAAG TCAAAATCCA TTGTTAGGTC AGTTTGAGAG	6780
AGCCATGTTT TTGGTGTTTT AGTAACCAAT TTCATTTTTT TATTATTTAT TTATTTGTTT	6840
ATTTTTGAGA CGGAGTTTCA CTCTTGTCAC CCAGGCTGGA GTGCAATGGC ATGATCTCAG	6900
CTCACTGCAA CCTCCGCCTC CCGGGTTCAA GCAATTCTCC TGCCTCAGCC TCCTGAGTAG	6960
CTGAGATTAC AGGTGCCCAC CATCACGCCT GGATAATTTT TGTATTTTTT AGTCGAGATG	7020
GGGTTTCACC ATGTTGGCCA GGATAGTCCT GAACTACTGA CCTCAGATAA TCCGCCCACC	7080
TCAGCCTCCC AAAGTGCTGG GATTACAGGC ATGAGCCAGC ACGCCCGGCC ACCAATTTCA	7140
TTTTTTAAAA AAGGAAGAAA GAAAACCTTA GCCAGAAGAT CTTTTTCCTT GCCATATGCA	7200
GTAAGAGTAG ATTATAAAAA CAAAGTCAGA GCAGTCACTG GTGTCTGGGC ATGGAGGAGA	7260
AAGAAGAATT CTCTTCTCCC TTCACCCTCC ATGCCCCTTT TTGGCTCCAT GTGATTCAGA	7320
TTTCTGGACC CTGGAGCCCC ACCCCAAGCT AAAGACCAGG ATACAGGGAA GCCACAACCA	7380
CTGGCGGTTT TGAGAACTTA CTTTTCACTT ATTCTGCATT TACTGTTTCC TTTTCTTATG	7440
CAGAAAAAGA AAAAAACCAA GGTAGGTGTG TGGGTAGAGA GCATGAAGTG TGTGTACTCA	7500
TGCATATGTA TGTGCATGCA TGTGAAGTGT GCATGTGTGA GCTCATATGC ATCCATGCAC	7560
CAGACTTGCC TCTTCCTCCC CCTCCTTCCT GAGCTTCTGC TGGGGCCGAG CGTGCAGTAA	7620
TGACAACTAC GATTGCTGG GGGAAGGCTA CGTGCCAAGC ACTCTTTTAG GTGCTTTCCA	7680

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TGATTAATTC	CTTCCTCACA	ACAGCCCTAT	GAGATTAGTA	CTATAACTAT	CCCCATTTTC	7740
AGAGGGAGAA	AAGGTACAGA	CTTGACTAAC	TTGCCCAAGG	CCACACAGCC	AGAGAGGGGC	7800
AGAGCCAGTA	CTTAGAGCCA	GGCAGTCTGG	GTCCAGAGTC	CGTGTCTGA	ACCACAAGAG	7860
GCCATCATAC	GCCATCAGAT	TTGGTGCTAG	CATTTCTGGT	GGTGCCTGGT	GGTGATGGAT	7920
CCATCACAGG	GGTCCTCCAG	GTACTGGTGC	TGCCCCAGAC	CAGAGCTGAC	ACTCCTCAGG	7980
CACTACCACA	TTCCAGGCAC	TGTGCTTGGG	GTCAGTCCCT	CTCTTTTTTT	TCCCCCCCCA	8040
TTATAACAGT	ATCTACAAAG	TAGGTGCTGT	TATTTTCCC	CTTTCACAGG	TGAGATAGAC	8100
TCAAAGAAGT	GAACCTGCCC	AAGGAACAGA	ACTAATGAGT	GGGAAAATG	GAACCTGGAAA	8160
CCATGTCTGT	TTACTCCAAA	ACCTGTGTTT	CTTGCCCTCT	TTCTCTGATG	CCAGCCCCCT	8220
ACACTTCAAG	GCCTGTGTTG	TCCAGACCCA	CACTCGGGCC	TGCCAGTGTG	TGCCTGGCAG	8280
GGATGCTCCA	TGGCCACACC	ATATCCATCC	TACACATCCC	CCCTCAGACT	GTGACCTCCA	8340
TTTGCTCTGG	GATCCCCACA	AGCTTCAGCT	GCTTGAGCAA	GACACTGCTT	AGAAGGCAGA	8400
GCAAGCCAAG	GCCTCTGGGG	CCTGCTGGGA	GCCAAAGCTG	GGGAGCCGTT	TCCACGGGTC	8460
TATCTGCTTG	AGCTGTCCTA	GATGAGCAGC	ATGGAAGGGC	AGTGGTGCAT	GAGTCCAGGC	8520
GGGCTGCTTT	TCTGCTCCGA	GAGGCTCTGC	CTGCCAGTT	GTTCTCTGCA	TTGCAGCCTC	8580
AATCCCCACA	GCCTTGCCCTT	CCCCGGCTT	TCCCTACAGG	TGCACCGCAT	CCACAGTGTT	8640
GGCACCATGC	AGCAGCCGCT	CTCCGTCCTT	TTCATATCCT	TGTCACTTGC	ACGAGCATGT	8700
CTTGAAAATA	TCCCTTGTTT	GTGTAGCATC	TTAAATGTTT	TTGCAGTATG	ATTTTGCATT	8760
CAGTATCTCA	TTTGATCCCC	ACAAGAGCCC	TATGAGGAGG	GAAAGCAGAT	TTTACCATTA	8820
AAGGATGAGT	AAACTGAGGC	CAGAGAGGAT	ATTTTGGTT	TTTTTTGAGA	CAGTCTCACT	8880
CTGTCACCCA	GCCTGGAGTG	CAGTGGCTTG	ATCTTGGCTC	ACTGCAAGCT	CCACCTCCCA	8940
TGTTACACAC	ATTTTCCTGC	CTCAGCCTCC	CAAGTAGCTG	GGACTACAGG	CACCCACCAC	9000
CACACCCAGC	TAATTTTTTT	GTATCTTTAG	TAGAGATGGG	GTTTCACCCA	GTTAGCCAGG	9060
ATGGTCTTGA	TCTCCTGACC	TTGTGATCTG	CCTGCTTCGG	CCTCCTAAAG	TGCTGGGATT	9120
ACAGGCGTGA	ACCCCCCTGC	CCGGCCAGAG	AGGATATTTT	TTAATGAGGG	GCAGGGCTGG	9180
GATTCCAGCC	CAGTGTTCTG	ATGGCTCACC	CACTGACCAT	TCCACTAATC	CGTGTCTTTT	9240
TTCAATCTAA	ACTTTCAGGG	TTGTAGAGGT	TCCTTTGAGG	TGCCTCAGTA	CTTCCATGGT	9300

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GATGTGGGGT	CTGAGGGCCA	AGAGCTCTGT	TCTCATTAAT	CAGAGAAGCT	TGTGTTTTTA	9360
AAAACACCAT	GTTTACTGCA	GGAAATTTAA	TTGGACAGTG	TTTCCATCTG	GAAAAAAAAA	9420
AGTCTACAAA	ATACTTGACA	ATCACTGCAC	TAGATCATGC	TGCTTTTAGC	ATTCTTAGCA	9480
TTTCACGTGC	TGAGCTCTCA	ATACTCTACC	ATGAGGAGGG	ATGGAGTGGG	TATGAAAAGA	9540
TAAAGAACTG	AAGTCACACG	GCTTGTCAGT	GGCAGAGATA	GAGCTTGAAC	CGAGGTTGAA	9600
GAGCTCCCGC	CTATTCCTTT	CCTCTTCTCA	CTGGATAAAG	CTGCTCCAAG	AGAGGTGCTG	9660
CCTCAGTGTG	CCTGTTCAGA	CTGTAATCCT	CCCTTCCTTC	CTGCCTCCTC	CCTCCTCTCT	9720
CCAGCCCATC	ATCTTCGTTT	CGGACAGAGC	AAACAGCAAC	AAGGAGCTGG	GTGTGGACCA	9780
GGAGTCAGAG	GAGGGCAAAG	GCAAAACAAG	CCCTGATAAG	CAAAAGCAGT	CCCCACAGGT	9840
GTCTGGGCAT	GTGGCATGGG	TGGGGTGGCC	AGCACGCTAC	AGGGGCTTCC	TATGCGCTTG	9900
GGATACACAG	GGGCTGGAGG	CTTCCCAGGA	GTTTGTCTTG	AACATCTGGA	GGTTTGAATT	9960
TGTCCCACTG	ACCTTTTCTT	TCAGCAAGTT	CCCCTGAAAT	TTGGGCTGCT	GCTTGGGTGA	10020
ATATCCCAGG	ATGGGGGTTC	CATTCTAGGA	GTGGACTGGC	AGGCTGAGCC	TCCCATGGAG	10080
CTGATCCAGC	CAGGATACAG	AGAAGGGGAG	GCAAAGGCTG	AGACAGAACC	AGCTTGAGAG	10140
CGGAGGCGCA	ACTCTTGTCT	CCTGGTGGCC	TTGAGCATTT	CACAATAGGG	GGATAAAGGA	10200
TAGGAGCAGA	AAAGTGGGGC	TGACTTCAGA	AATGGGGTCC	TCTAGAGCTC	ACGGGAGGGT	10260
GTTAGATTGG	AGTGGGAGCT	TAGTGGAGGT	GAGCCTTAGA	GGCAAAGTC	TCCAGACCAA	10320
TCCAGGCCCC	CTCTTCTATC	CGGGGGCCCC	TCTTCTATCC	AGGGCCCCTC	TTCTGTCTGG	10380
GAGCCCCTCT	TCTATCTGGG	GCCTCATGCA	GTGGGGCCTA	GGGAGGTTC	TCTGAGGACT	10440
TGGCCTTGAT	GACAGGGTGG	CTGGAGGAAT	CAGAACGGTC	AGACCTTCTT	TGACCTGCGG	10500
GCACCTTTAG	TTGGAATGCT	CAGGCCTGGG	ATGGTGGAGG	GGGCTCTTGC	AGGTGGGGAC	10560
TGGGGTGGCG	GGGAGGAGGC	TGTATGGCCG	CCATATCTCC	TTTGGCTGGG	GGCGTCAGGG	10620
CTGGAGAGGT	GTGAAGAGTC	CCTGAGGCCT	CGATGCATCT	CACTCCAGCT	CACCAGGTCT	10680
GCATTTGCCC	GTCCCCAGCT	CCTGCTGCCA	CCCCGGGCCG	TTTtagGCAC	TTGGCTCCCT	10740
TGGCCCAGAG	GAGCTTGCCT	CACAGGCCTG	TGCACCTCTG	ACCCCTGTGA	ACCAGTTTTC	10800
CTTTGTGCCT	CCACAGCCAC	AGCCTGGCAA	CTCTGATCAG	GAAAGTGAGG	AACAGCAACA	10860
ATTCCGGAAC	ATTTTCAAGC	AGATAGCAGG	AGATGTGAGT	ACCTCCAAGC	CCAGGACGCC	10920

FIG. 8D/7

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CACAGGTGCT	TCCTTCTCTC	CTGGATTAAAC	TGCTCAGATT	ACCAATTATT	TCATTATTGT	10980
TTGGTAGAGG	TCACTTTGGA	CTTCGGTGGA	GCCAGGGGAT	GTGTGCGTAG	CACACAAATC	11040
CACAAGCCCT	TGAGTTTTTG	ACTGCCACGT	CTGCTGGGGG	GCTCAGAGGC	CTTTTTGCTC	11100
TGAGCTGCCC	ACGGTGGTCC	TGATAGCTGA	GGTGCAGTAT	CTGGCCCCCT	GTCTTCCTCA	11160
GAAAAGCCCC	AGCTTCCCAT	GACATAATAG	CACCGACAGG	GATTTTACAA	ACACAGCCAG	11220
GTGGAATTTG	TTTTGCAAAG	TGTCCGCGCC	AGGAGCTGCT	GTACTCCTGA	ACCATGACCC	11280
TCCTCTCCCT	TCCTCCTCAG	GACATGGAGA	TCTGTGCAGA	TGAGCTCAAG	AAGGTCCTTA	11340
ACACAGTCGT	GAACAAACGT	GAGTTGCTCA	AACCAAATGG	GGGTGGGGTG	GGTGGGGAGT	11400
CCCGTTGTCT	CAAAGCAGCT	CCTCACTCTT	CTCCATCCCC	CCAGACAAGG	ACCTGAAGAC	11460
ACACGGGTTC	AACTGGAGT	CCTGCCGTAG	CATGATTGCG	CTCATGGATG	TATCCTTCCT	11520
GCCGCCCTT	CCCGACCCTC	TGTCATCAGC	CCACGGGGGC	CAAGGCAACA	TACAGGGTGC	11580
CCAGTCAGGC	AAAGGGCCCT	AATTTGTGCC	CAGGGAAACT	TAAGGAGACC	CTGATTCAGA	11640
ACATCTTGGA	TACTCGTCTG	AAAGGGGTTG	TTAGAGGCGG	AAGGGGAGGA	TGTTGGGTTG	11700
TAACTGCCCT	AACCCCTGTG	CTTCTCTCAG	GCCTGGGATC	CTGCCCAAGC	AAAAGTGGTC	11760
CTTAGGAGAG	CGGCTCCTGG	GTTACAGAGT	AGGCGCAATC	TCTGACTGGT	GGTGGAGTGG	11820
AGGGGAGGGT	TAAATAGTAC	AACAGGGCAG	TGGGTAGGAC	AGCCCCGAGT	CTCCTAGACC	11880
CTCCCTCCAA	ATCCAGGGGG	ATTTTGCTGT	GTGCTGTGTA	GCCCTGACCT	CCCTCCTCCA	11940
GACAGATGGC	TCTGGAAAGC	TCAACCTGCA	GGAGTTCCAC	CACCTCTGGA	ACAAGATTAA	12000
GGCCTGGCAG	GTGGGAAGAG	AAAATGAAGC	GTGGGAGTCA	AGAATGGGGT	TGATTTGGAG	12060
ATTCAGTGTG	TGACCTCCAT	CCTCAAATTT	TCTATTGCCA	GAAAATTTTC	AAACACTATG	12120
ACACAGACCA	GTCCGGCACC	ATCAACAGCT	ACGAGATGCG	AAATGCAGTC	AACGACGCAG	12180
GTGCTGAGAA	GGAAGGGGTG	TCAGGGATGT	GGACCCGAGA	CGGTGGGAGC	AGGAATGGGA	12240
GGGGACTAGC	TACTAGGGCC	CCACTAGAGA	AGGAGAGGGA	AAGGGCTTCT	CACTTTCCTT	12300
TCCCAGGTCA	CAGAGTGTCC	GAGAGGCAGG	GAAAATAGAA	GACAGGCCCA	AGGCCTCCAG	12360
CTCCACGTCC	ACCTCTAACA	TGGTCCCCTC	CACAGGATTC	CACCTCAACA	ACCAGCTCTA	12420
TGACATCATT	ACCATGCGGT	ACGCAGACAA	ACACATGAAC	ATCGACTTTG	ACAGTTTCAT	12480
CTGCTGCTTC	GTTAGGCTGG	AGGGCATGTT	CAGTAAGTGG	GAGAGGGGGG	CTGCCCTCTG	12540

FIG. 8D/8

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CTCTCTTGCA	GGGGCAGTTG	TGGCAACAGG	CATCTCACCT	GATAATCTCC	AGTCTGCTCC	12600
ATCCAGGCTG	AACAAGGGCC	AATGACCTCT	TTAGGCCCCAG	AATGGGATGG	CAAAGGGAGG	12660
GTTACTGGTG	ATTCTCTGCC	TGCACATCTT	TGTGCTGATG	AGGGACAGCA	CTGGGCACAC	12720
GGTCCTCTGA	GGGGAAGTTA	CAGTAGTAGA	GGCGGAGTGC	GCCTGTAACT	GGCCTCTGGC	12780
CTGTGCATTC	TTTCACAGGA	GCTTCTCATG	CATTTGACAA	GGATGGAGAT	GGTATCATCA	12840
AGCTCAACGT	TCTGGAGGTA	AAGCATAGGC	ACAGCACATT	CCCCCTACAC	ATTAAAACTC	12900
AAGGTGGAGG	GGTCAACGGG	GCGGACTGGA	CCCAGGGTGT	GCTCCTCATT	TCCACACAGT	12960
GGTGGAGGGA	AGGGATAGGA	ACAGAACATG	GAGGGAGGCT	CAGCAGGCTC	CCAGGACACA	13020
TGCACTTGAG	GCCCAAAGG	ACCTCTGCTC	CCCCAGTCAC	TTGATGCGGG	AAAACATGCA	13080
CCTTCTTAGG	GAAGATCTAG	GAGAAAGGAA	ACAGTAAGCC	ACTGCTTCTT	GGAAAATCTT	13140
CTGGGGGTCT	GACCTGCTGG	GACTGTTCCC	TTTCCTCTTG	CCCCGTAAGA	TTCTAGGGC	13200
GGGGGGGGGG	GGGGGTCACT	CTTTTCTGAT	CTACATTCTG	ATCTTGGGAC	TTCTTTCAGT	13260
GGCTGCAGCT	CACCATGTAT	GCCTGAACCA	GGCTGGCCTC	ATCCAAAGCC	ATGCAGGATC	13320
ACTCAGGATT	TCAGTTTCAC	CCTCTATTTC	CAAAGCCATT	TACCTCAAAG	GACCCAGCAG	13380
CTACACCCCT	ACAGGCTTCC	AGGCACCTCA	TCAGTCATGT	TCCTCCTCCA	TTTTACCCCC	13440
TACCCATCCT	TGATCGGTCA	TGCCTAGCCT	GACCCTTTAG	TAAAGCAATG	AGGTAGGAAG	13500
AACAAACCCT	TGTCCCTTTG	CCATGTGGAG	GAAAGTGCCT	GCCTCTGGTC	CGAGCCGCCT	13560
CGGTTCTGAA	GCGAGTGCTC	CTGCTTACCT	TGCTCTAGGC	TGTCTGCAGA	AGCACCTGCC	13620
GGTGGCACTC	AGCACCTCCT	TGTGCTAGAG	CCCTCCATCA	CCTTCACGCT	GTCCCACCAT	13680
GGGCCAGGAA	CCAAACCAGC	ACTGGGTTCT	ACTGCTGTGG	GGTAAACTAA	CTCAGTGGAA	13740
TAGGGCTGGT	TACTTTGGGC	TGTCCAACCT	ATAAGTTTGG	CTGCATTTTG	AAAAAAGCTG	13800
ATCTAAATAA	AGGCATGTGT	ATGGCTGGTC	CCCTTGTGTT	TTGTTGTCTC	ACATTTAGAT	13860
ATCAGCCATG	CATGACTGAA	TGGCTTCCAA	TCATATACTC	ACCTATCACC	TACAAGAGAA	13920
CAATGAAAAA	CACACACAAA	AACAAAATCT	TGAATTTTGT	AATCATGCCT	ATTGCTATTT	13980
CTTGAGCATA	AGAATGGCTC	AGATACTTTC	CAAGACATAA	AAGGAAGGCA	GAGGAATAGT	14040
TGTTGCTGTA	AAAGACATCA	AGAATAAATG	GGGTCATGTA	CAACGGGAGG	GGCCGGTTAC	14100
CTGAATAATG	GAGTGGAGAT	TGAGCTATCC	TAGCTCCTCT	GCTCACTAAC	TGACCTGTCC	14160

FIG. 8D/9

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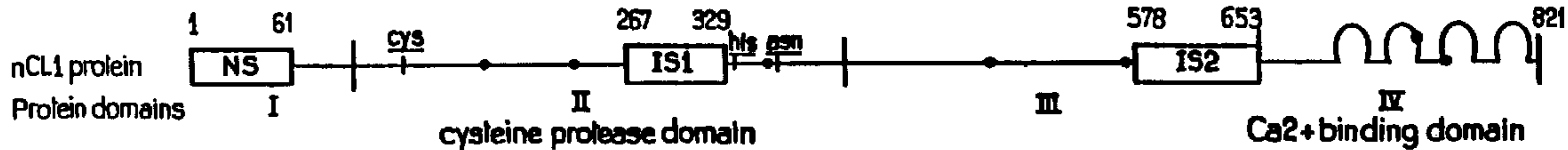
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CATGACCGTG GACAAAACCC TGAACGCAGC TGTTTGTTTG CTAAACTTCT CTGGACCATG	14220
GCCTGCGGCA TATCTATAGG CATCCTGTGT TTTCCACCCA GTTTCCTTCT TCCTCGCTAA	14280
GCCAACGTGG AAAGGGCTGG CCGTGAATAT GCAGACAAGG TAACGAAAGT AAACCGTCAA	14340
TTAGTAAAAG TACTTCATTT TCCTCTTGTA TTTGCTTCAT TCTTGCTTCA CAAAGTTACG	14400
AAGTCCACAG CTTTATACCA AAATGTAAGA AGGCTATTTG CTTATAAACA TTTTGAGTCA	14460
GGTGTCATCT GATTTCATTC TTCTAATCCA TATTCAATAT TAAAAAATCA GAAACCAAGG	14520
GTGCTGGAGC AGCTCTAGGG CATATATTTT TCTTAAATAG GAGAAAGATT TTCAACAGCT	14580
TTTCCTCCTT GACCCCCTCC TTTCCCAATT TATTTGGGTC ACTACCTTGA ATTTAGAGTG	14640
AATCTGGGAA ATGTAGTCAC CAGG	14664

FIG. 8D/10

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Nonsense mutations { B 519 —————> R110X

M1394 —————> T360X

M32 —————> 945delG

R27 —————> 2069delCA

B 505 —————> 2313delAGAC

B 505 —————> 2362AG→TCATCT

R14

M1394 —————> 550delA

M2888

Frameshift mutations {