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(54) Titre : MUTANTS DE LA LIPOPROTEINE-LIPASE
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(57) Abrégé/Abstract:
This invention provides DNA from exon 5 and 6 of the human lipoprotein lipase (LPL) gene each containing missense mutations resulting in the substitution of leucine at residue 207 and asparagine at residue 250, respectively, in LPL. Oligonucleotide probes for the two mutations are provided. Methods and kits are provided for screening patients for the mutations. The methods are useful for screening for LPL deficiency and for carriers, particularly in the French Canadian population.

ABSTRACT

This invention provides DNA from exon 5 and 6 of the human lipoprotein lipase (LPL) gene each containing missense mutations resulting in the substitution of leucine at residue 207 and asparagine at residue 250, respectively, in LPL. Oligonucleotide probes for the two mutations are provided. Methods and kits are provided for screening patients for the mutations. The methods are useful for screening for LPL deficiency and for carriers, particularly in the French Canadian population.

This invention relates to lipoprotein lipase (LPL) and missense mutations in the human LPL gene. LPL hydrolyses the triglyceride core of chylomicrons and very-low density lipoproteins (VLDL) and has a major modulating effect on the levels and lipid composition of high-density lipoproteins (HDL).

There is an inverse correlation between HDL levels and coronary artery disease. The mechanisms of variation of HDL levels in the general population are not well understood. However, levels of HDL show positive correlation with LPL. Individuals who are heterozygous for LPL deficiency have significantly reduced HDL cholesterol levels and it is possible that there may be an association between the heterozygous state for LPL deficiency and the present of premature coronary atherosclerosis.

Patients with type I hyperlipoproteinemia usually have triglyceride levels above 1500 mg/dl and HDL levels of less than 20 mg/dl in fasting plasma (1). They usually present in childhood with abdominal pain, recurrent attacks of pancreatitis, and eruptive xanthomas. In most cases, this disorder is inherited as an autosomal recessive trait, and is due to catalytically defective LPL.

The frequency of carriers for LPL deficiency is estimated at 1 in 500 in the general population (1). The highest reported worldwide frequency of LPL deficiency occurs in certain areas in the province of Quebec in Canada where the carrier rate is approximately 1 in 40 (2).

Human LPL is a glycoprotein with an apparent monomeric molecular weight of approximately 60kD (3). Human LPL cDNA has

been cloned and its sequence is known (4). The structure and polymorphic map of the human LPL gene has been reported (6,21) and the gene has been cloned (4,6,22). The coding sequence is 1425 base pairs (bp) in length and translates into a mature protein of 448 residues preceded by a signal peptide of 27 residues. The human LPL gene is located on the short arm of chromosome 8 (5) and consists of 10 exons (6,22) spanning approximately 30 kilobases (kb). Numerous DNA sequence alternations have been reported to cause LPL deficiency. These include large DNA rearrangements (7,8), but the majority comprise missense and nonsense single base substitutions in exons 4, 5, and 6 (9-15). In most instances, the mutations in the LPL gene are different in unrelated families and different population groups.

One of the previously reported mutations (11,12) is a missense mutation observed in exon 5 of the LPL gene resulting in an amino acid substitution of glutamic acid for glycine at residue 188. This Glu^{188} mutation was reported to be responsible for LPL deficiency in 21 of 88 LPL alleles and occurred on the same haplotype in unrelated affected persons. The Glu^{188} mutation lies in one of the longest conserved regions of the LPL gene. The region shows complete residue identity between man, cow, mouse and guinea pig, and spans residues 178 to 210 of human LPL.

The Glu^{188} mutation alters an AvaII restriction site in exon 5 and can thus be detected by amplification by the polymerase chain reaction (PCR) followed by digestion by AvaII and analysis of restriction fragment length polymorphism (RFLP); or, alternatively by allele specific oligonucleotide hybridization (11,12). Screening by RFLP analysis for Glu^{188} provides a rapid

test for unrelated patients having LPL deficiency (12). However, it is desirable to identify other mutations which will facilitate screening. It would be most desirable to be able to screen for a mutation that is most common in a population having LPL deficiency.

The discovery of a mutation that is most common in a population (e.g. French Canadian) would provide a unique opportunity for identification of large numbers of carriers in the population. This will permit an examination of the association between the heterozygous state for LPL deficiency and the presence of premature coronary atherosclerosis and, the relationship between heterozygosity for LPL deficiency and the present of lipid abnormalities. As well, the discovery would provide a further basis for genetic counselling for patients having LPL deficiency.

The inventors have identified a missense mutation for residue 207 which accounts for the majority of mutations underlying LPL deficiency in French Canadians. Sequence analysis reveals it to be a missense substitution of T for C on the coding strand in exon 5 resulting in a substitution of leucine (CTG) for proline (CCG) at amino acid residue 207 (hereinafter referred to as Leu^{207}). The mutation accounts for 54/74 (73%) of all mutant alleles in 27 apparently unrelated French Canadian patients with LPL deficiency. In vitro mutagenesis studies have confirmed that this mutation generates a catalytically inactive LPL and is the cause of LPL deficiency in these patients. The mutation can be easily detected by DNA amplification using PCR followed by dot-blot hybridization analysis using labelled allele specific oligonucleotide probes. This discovery provides a unique

opportunity for definitive DNA diagnosis of LPL deficiency; identification of heterozygous carriers and for studies of phenotypic expression.

The inventors have also identified a less common missense mutation in exon 6 of the LPL gene. This mutation (hereinafter referred to as Asn^{250}) is a substitution of A for G in the coding strand resulting in a substitution of the asparagine codon (AAC) for the aspartic acid codon (GAC) at amino acid residue 250. The mutation may also be detected by dot-blot analysis using PCR amplification followed by allele specific hybridization with an oligonucleotide probe. However, the mutant is more easily detected following PCR amplification by RFLP analysis since the mutation interrupts a TaqI endonuclease recognition site. Screening for this mutation provides an additional factor which will facilitate detection and study of LPL deficiency and heterozygous carriers.

This invention provides DNA in a substantially purified form which includes one or more from the group consisting of:

(a) all or a part of exon 5 of the human LPL gene comprising the codon for residue 207 of LPL or its complement in which said codon is a leucine codon, and said complement is a sequence complementary to a leucine codon; and

(b) all or a part of exon 6 of the human LPL gene comprising the codon for residue 250 of LPL or its complement in which said codon is an asparagine codon, and said complement is a sequence complementary to an asparagine codon.

This invention also provides an oligonucleotide probe

suitable for allele specific hybridization to the coding or the non-coding strand of the part of exon 5 of the human LPL gene which includes the codon for residue 207 of LPL wherein, the specific allele is a T for C substitution in the codon.

This invention also provides an oligonucleotide probe suitable for allele specific hybridization to the coding or the non-coding strand of the part of exon 6 of the human LPL gene which includes the codon for residue 250 of LPL wherein, the specific allele is a A for G substitution in the codon.

This invention also provides methods for screening for the Leu ^{207} and Asn^{250} mutations that include the common step of amplifying DNA by PCR followed by either allele specific hybridization of a suitable Leu^{207} or Asn^{250} probe, or digestion with TaqI endonuclease and size analysis of the resulting fragments.

This invention also provides kits for practising the above described methods which include PCR primers, and labelled probes or TaqI nuclease, as required.

In drawings which illustrate embodiments of the invention:

Figure 1 shows DNA sequences of portions of the strands from exon 5 of a French Canadian patient with LPL deficiency.

Figure 2 shows an autoradiograph of a nylon filter containing PCR amplified exon 5 DNA from 9 patients with LPL deficiency. The DNA was dot-blotted into the filter and hybridized with radio-labelled oligonucleotide probes complementary to the normal and mutant sequences. LPL gene haplotype nomenclature is according to Table 2.

Figure 3 shows a map of a pUC18 plasmid wherein the coding strand of exon 5 DNA containing the Leu^{207} mutation was cloned, together with PCR primer sequences.

Figure 4 shows a map of a pUC18 plasmid wherein the coding strand of exon 6 DNA containing the Asn^{250} mutation was cloned, together with PCR primer sequences.

A total of 37 French-Canadian probands with LPL deficiency were examined. Twenty nine probands, comprising 16 males and 13 females, were from eastern Quebec while 8 subjects (5 females and 3 males) were from the Montreal area. No genetic relationship was found between any of the affected probands over a minimum of three generations, and in all instances as far back as could be ascertained. None of the patients had a history of diabetes, renal disease or chronic alcoholism. Also included for comparison were an additional 34 unrelated probands of different ancestries with LPL deficiency.

Blood samples were also collected from 70 unrelated consecutively selected French Canadian clinic patients at the Clinical Research Institute of Montreal or the University Hospital in Vancouver. Also included for comparative purposes were 60 French Canadian normolipidemic control subjects attending genetic clinics in Montreal and Vancouver for other reasons.

Blood samples were collected from all patients after an overnight fast. Total cholesterol, triglycerides, LDL- and HDL-cholesterol levels were determined according to protocols previously described (2,16). Post heparin plasma was obtained from probands 10 minutes after an intravenous injection of heparin

given at 60U/kg body mass. Samples were immediately frozen and stored at -70°C.

LPL mass in pre and post heparin plasma an ELISA method (17) and LPL activity was determined using a radiolabelled substrate emulsion as previously described (16).

Genomic DNA was isolated from white blood cells of all LPL deficient probands and some of their family members by a known procedure (18). DNA haplotypes of mutant LPL alleles were constructed using the HindIII, BamHI, and PvuII restriction fragment length polymorphisms (RFLP) at the LPL locus by known procedures (19,20). For some patients, it was necessary to analyze DNA from other family members in order to determine their LPL gene haplotypes.

DNA haplotypes for 44 mutant alleles from 22 of the patients with LPL deficiency were constructed using HindIII, BamHI, and PvuII RFLPs. Only two haplotypes, of the possible eight reported (12), were found in this group of patients (see: Table 1). The majority of these patients proved to be homozygous for haplotype H2 (HindIII(+); BamHI(-); PvuII(+)), for which no mutation had previously been detected. One of the subjects homozygous for H2 was selected for sequence analysis.

Each of the 10 exons encoding the amino acid sequence of LPL, in the selected subject, were individually amplified from 0.5 - 1 ug of genomic DNA using (PCR) as previously described (12). The amplified exons were then purified and either sequenced directly or after cloning into a pUC18 vector using commonly known procedures.

The sequence analysis revealed homozygosity for a C to T

substitution at nucleotide 875 of the published LPL cDNA sequence (as shown in Figure 1). This mutation results in an amino acid substitution of leucine (CTG) for proline (CCG) at residue 207. Both the coding and non-coding strand of exon 5 DNA from several independent PCR reactions were sequenced to confirm the presence of the Leu^{207} mutation. No other DNA alteration was detected in exon 5, and the DNA sequence of the 9 remaining exons and the exon-intron junctions of the LPL gene were found to be normal. Since this single base mutation does not alter a restriction site, it would best be detected by using allele specific oligonucleotide hybridization analysis.

Haplotype H1 has previously been shown to be associated with the Glu^{188} mutation. In this study, Glu^{188} was found in 13 of the 14 alleles with haplotype H1 (Table 1). The remaining four alleles from the French Canadian patients did not carry either the Glu^{188} or the Leu^{207} mutation. Three of those were associated with haplotype H2 and one with H1. This suggests that there are more rare undescribed mutations underlying LPL deficiency in this group of French Canadian patients.

Exon 5 of the LPL gene from all available patients and controls was amplified by PCR and approximately 50 nanograms(ng) of the DNA was denatured and transferred in duplicate onto nylon membranes (Hybond™ N-Plus, Amersham). Oligonucleotides homologous to the normal and mutant sequences spanning the Leu^{207} substitution were synthesized on an automated synthesizer (Applied Biosystems Model 380A) and end-labelled with [γ^{3}P]ATP. The nylon membranes were prehybridized in 7 ml of buffer containing 1% BSA, 0.5 M sodium phosphate, 1 mM EDTA, and 7% SDS for 1 hour at 48°C.

A total of 1 x 10^6 cpm/ml of each oligonucleotide was added and the hybridization performed for 2 hours at 48°C. Filters were washed in several buffers under the following conditions: Buffer 1 (2X SSPE, 0.5% SDS) for 5 minutes at room temperature (RT); Buffer 2 (1X SSPE, 0.25% SDS) for 15 minutes at RT; Buffer 3 (0.1X SSPE, 0.1% SDS) for 30 minutes at 42°C for the mutant oligonucleotide and at 43°C for the wildtype probe. Filters were exposed to X-ray films for 2-16 hours at -70°C with an intensifying screen.

The sequences of the oligonucleotide probes used were 5'-CATTTACCCGAATGGAG- 3' for the coding strand of the normal allele and 5'-CATTTACCTGAATGGAG- 3' for the coding strand of the mutant allele. Alternatively, a probe for the non-coding strand of the mutant allele may be used (e.g. 5'-CTCCATTCAGGTAAATG- 3').

The autoradiograph of a filter containing amplified DNA hybridized with the normal and mutant oligonucleotides (Fig 2) clearly identifies individuals who are homozygous or heterozygous for the Leu^{207} substitution. The results of analysis of 71 probands are summarized in Table 2. Among the 37 French Canadian patients (74 alleles), Leu^{207} mutation occurs on 54 alleles (73.0%) whilst the Glu^{188} mutation accounts for 16 alleles (21.6%). In total, we have identified 70/74 (94.6%) mutant LPL alleles in this group of French Canadian patients.

The same experiments were also performed on 34 unrelated DNA samples for LPL deficiency patients of 21 different ancestries excluding French Canadian. Only one allele in a patient living in Germany was found to have the Leu^{207} mutation. The detailed ancestry of this patient could not be established.

Dot-blot hybridizations and restriction fragment analyses were also performed to determine the frequencies of the Leu^{207} and Glu^{188} mutations in French Canadian hyperlipidemic and normolipidemic control subjects. Four of 70 hyperlipidemic patients were found to be heterozygotes for the Leu^{207} mutation. This mutation was not found in the 60 normolipidemic French Canadian controls. None of these individuals carried the Glu^{188} mutation. This suggests that the Leu^{207} substitution might be overrepresented in French Canadian hyperlipidemic patients and may indicate that decreased LPL levels in carriers contributes to the development of hyperlipidemia in the lipid clinic patients.

Patients homozygous for the Leu^{207} mutation have a postheparin incremental LPL mass that is lower than seen in normal individuals. In Vitro mutagenesis studies by the inventors shows that Leu^{207} mutant and wildtype LPL mRNA levels within transfected COS-1 cells are similar and so are the levels of cell associated LPL mass. This suggests that the Leu^{207} substitution has no major effect on LPL gene expression, or the processing, and secretion of the mutant enzyme. There is a substantial decrease in mutant enzyme mass levels in the cell culture medium that suggests that the mutant enzyme is less stable and undergoese rapid degradation.

With the knowledge of the DNA sequence having the Leu^{207} mutation disclosed herein, suitable oligonucleotide probes for hybridization to the Leu^{207} mutant will be readily apparent to the person skilled in the art. Generally, the probes will have from 15 to 23 nucleotides complimentary to the DNA sequence surrounding the mutation. Preferably, the probes will have approximately 8 nucleotides upstream and downstream from the substitution site.

In some instances, it may be preferable to clone the amplified DNA from a patient with LPL deficiency into a vector. Procedures and suitable vectors are well known in the art. The inventors have cloned all of the exon 5 coding strand and associated PCR primer sequences used for amplification (total 256 nucleotides encompassing the Leu^{207} mutation) into the HincII site of the pUC 18 plasmid (see Figure 3).

Using the procedures described above, the inventors have also identified Asn^{250} on exon 6. The normal coding sequence for this region is:

5'-TCTTCATCGACTCTCTG-3'

The mutant sequences spanning the Asn^{250} mutation are as follows:

5'-TCTTCATCAACTCTCTG-3' (coding strand)

5'-CAGAGAGTTGATGAAGA-3' (non-coding strand)

The mutation results in an asparagine codon (AAC) being substituted for the aspartic acid codon (GAC) that codes for amino acid residue 250 in LPL.

The two mutant sequences described above would be suitable probes (for Asn^{250}) allele specific hybridization. Other probes may be constructed (as described above for Leu^{207}). However, the presence of the mutation may more easily be detected by RFLP analysis using TaqI endonuclease digestion after PCR amplification of exon 6 DNA. In a normal patient, digestion of exon 6 DNA by the nuclease will produce 98 bp and 234 bp fragments. Since there is no cleavage of the mutant DNA, at this site, a 332 bp fragment is observed.

The inventors have cloned exon 6 DNA having the Asn^{250} mutation, with PCR primers, into the HincII site of the pUC18

plasmid (as shown in Figure 4).

The Asn^{250} mutation appears to be more rare in the sample population than Leu^{207} or Glu^{188}. Asn^{250} was identified in 4 of 142 alleles (2.8%) from 71 patients of different ancestries having LPL deficiency. However, screening for this mutation will be a useful supplement to other LPL deficiency screening tests and provides another opportunity to examine the causes and origins of LPL deficiency.

As is shown in Table 2, a combination of screening tests for Leu^{207} and Glu^{188} would be particularly effective for screening of the French Canadian population for carriers. The combination, either alone or further combined with other screens (e.g. for Asn^{250}) would also be useful in respect of other ancestries.

Kits may be provided to facilitate testing. For example, a kit may be provided with exon 5 PCR primers, labelled Leu^{207} oligonucleotide probe, and the necessary reagents, buffers and enzymes (which are known) to carry out PCR amplification, allele specific hybridization and autoradiography for the detection of the Leu^{207} mutant. This kit may also include AvaII restriction nuclease and the additional known materials required to test the amplified exon 5 DNA for Glu^{188} by RFLP analysis. A Asn^{250} kit may include PCR primers and materials for amplification of exon 6 DNA and either a labelled Asn^{250} probe or, TaqI enzyme (plus necessary materials) for screening of Asn^{250} by either allele specific hybridization or RFLP analysis.

As will be appreciated by those skilled in this art, various modifications of detail may be made to the embodiments

described herein without departing from the scope of the present invention.

TABLE 1

LPL gene haplotypes associated with DNA mutations in 22 French Canadian probands with LPL deficiency

Haplotype	HindIII	BamHI	PvuII	Alleles	Mut188+	Mut207+	MutND
H1	+	-	-	14	13	0	1
H2	+	-	+	30	0	27	3

\+ presence and - absence of the restriction site

Mut188+ = presence of the Glu^{188} mutation

Mut 207+ = presence of the Leu^{207} mutation

MutND = mutation not detected

TABLE 2 Frequency of mutation Glu^{188} and Leu^{207} in LPL deficiency probands of different origins

Origin	No. of probands	No. of alleles	Mut 188 alleles	Mut 207 alleles	Total
French Canadian	37	74	16(21.6%)	54(73.0%)	94.6%
Other Ancestries Excluding French Canadian	34	68	9(13.2%)	1(1.5%)	14.7%
Total:	71	142	25(17.6%)	55(38.7%)	56.3%

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. DNA in a substantially purified form which includes one or more from the group consisting of:

(a) all or a part of exon 5 of the human LPL gene comprising the codon for residue 207 of LPL or its complement, in which said codon is a leucine codon and said complement is a sequence complementary to a leucine codon; and

(b) all or a part of exon 6 of the human LPL gene comprising the codon for residue 250 of LPL or its complement, in which said codon is an asparagine codon and said complement is a sequence complementary to an asparagine codon.

2. DNA of claim 1 wherein the DNA is all or part of exon 5.

3. DNA of claim 2 wherein the DNA is the LPL coding strand having a leucine codon for residue 207 of LPL.

4. DNA of claim 2 wherein the DNA is the LPL non-coding strand having a sequence complementary to a leucine codon for residue 207.

5. DNA of claim 1 wherein the DNA is all or part of exon 6.

6. DNA of claim 5 wherein the DNA is the LPL coding strand having an asparagine codon for residue 250.

7. DNA of claim 5 wherein the DNA is the LPL non-coding strand having a sequence complementary to an asparagine codon for residue 250.

8. DNA according to any one of claims 1-7 wherein the DNA comprises at least 15 nucleotides.

9. A plasmid containing the DNA of any one of claims 1-8.

10. The pUC18 recombinant plasmid described in Figure 3, or one containing a complementary strand of exon 5 DNA.

11. The pUC18 recombinant plasmid described in Figure 4, or one containing a complementary strand of exon 6 DNA.

12. An oligonucleotide probe suitable for allele specific hybridization to the coding or the non-coding strand of the part of exon 5 of the human LPL gene which includes the codon for residue 207 of LPL (a Leu^{207} probe), wherein the specific allele is a T for C substitution in the codon.

13. An oligonucleotide probe suitable for allele specific hybridization to the coding or the non-coding strand of the part of exon 6 of the human LPL gene which includes the codon for residue 250 of LPL (a Asn^{250} probe), wherein the specific allele is a A for G substitution in the codon.

14. The probe of claim 12 or 13, wherein a nucleotide that is complementary to the substitution is at an intermediate position in the oligonucleotide.

15. The Leu^{207} probe of claim 12 comprising a sequence selected from the group consisting of:

(a) 5'-CATTTACCTGAATGGAG-3'; or

(b) 5'-CTCCATTCAGGTAAATG-3'.

16. The Asn^{250} probe of claim 13 comprising a sequence selected from the group consisting of:

(a) 5'-TCTTCATCAACTCTCTG-3'; or

(b) 5'-CAGAGAGTTGATGAAGA-3'.

17. The probe of any one of claims 12-16, having a length of 15-23 nucleotides.

18. The probe of any one of claims 12-17, that additionally comprises a radioactive label.

19. A method of screening for the presence of a Leu^{207} mutation in a patient that includes the steps of:

(a) amplification of exon 5 DNA of the patient using PCR;

(b) allele specific hybridization of the amplified DNA with a Leu^{207} probe of claim 12, 14, 15, 17, or 18;

(c) detecting whether the probe has hybridized to the DNA.

20. The method of claim 19 that additionally includes a method for screening for the Glu^{188} mutant that includes a step selected from the group comprising:

(a) allele specific hybridization of the amplified DNA with an oligonucleotide probe for Glu^{188} and detecting whether the probe has hybridized with the DNA, and

(b) digesting the amplified DNA with AvaII nuclease and determining the size of the resulting fragments.

21. A method of screening for the presence of an Asn^{250} mutation in a patient that includes the step of amplification of the exon 6 DNA of the patient using PCR; and additionally performing a step selected from:

(a) allele specific hybridization of the amplified DNA with an Asn^{250} probe of claim 13, 14, 16, 17 or 18, and detecting whether the probe has hybridized with the DNA, and

(b) digesting the amplified DNA with Taq1 nuclease and determining the size of the resulting fragments.

22. A kit for practising the method of claim 19 or 20, which includes PCR primers for exon 5 DNA; a Leu^{207} probe according to claim 12, 14, 15, 17 or 18, and, optionally, an additional component selected from the group comprising: an oligonucleotide probe for Glu^{188}; and AvaII nuclease.

23. A kit for practising the method of claim 21 which includes PCR primers for exon 6 DNA; and, an additional component selected from the group comprising: Asn^{250} probe according to claim 12, 14, 16, 17 or 18; and, Taq1 nuclease.

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FIGURE 1

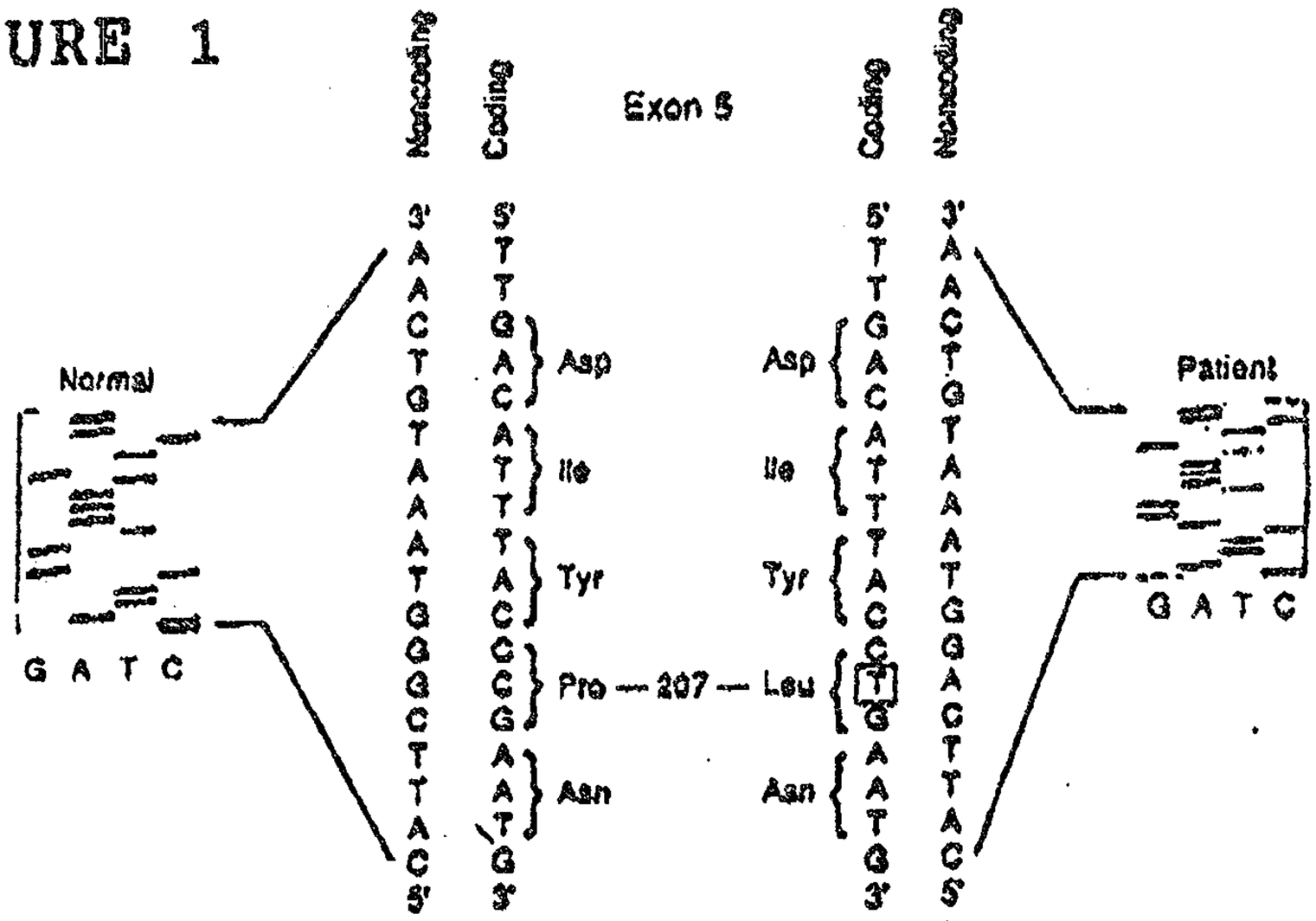
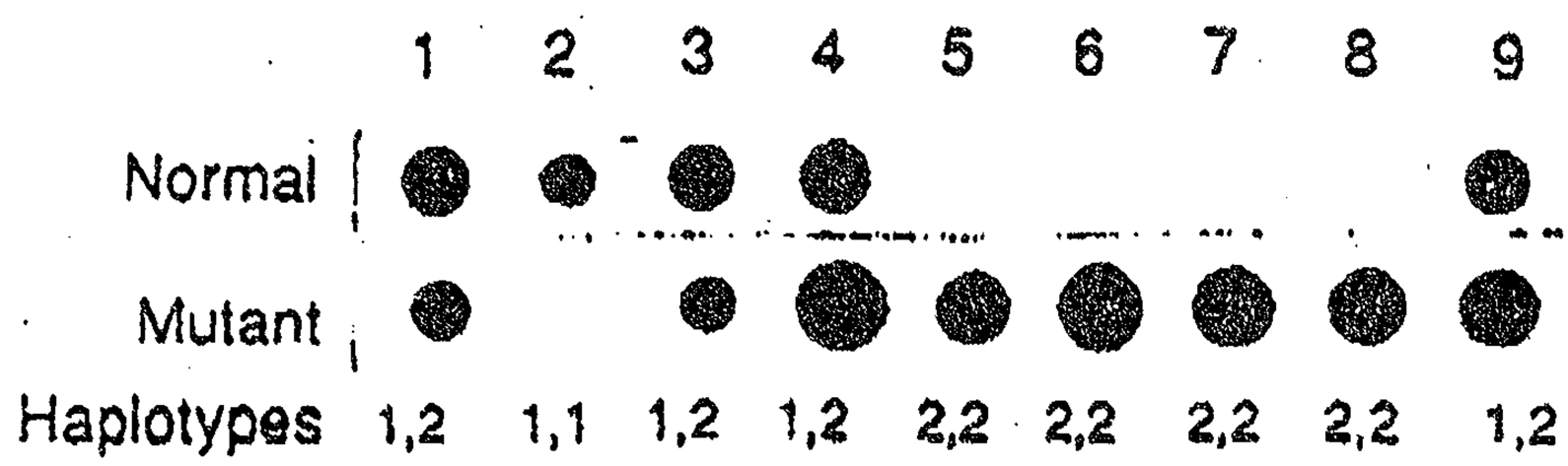


FIGURE 2



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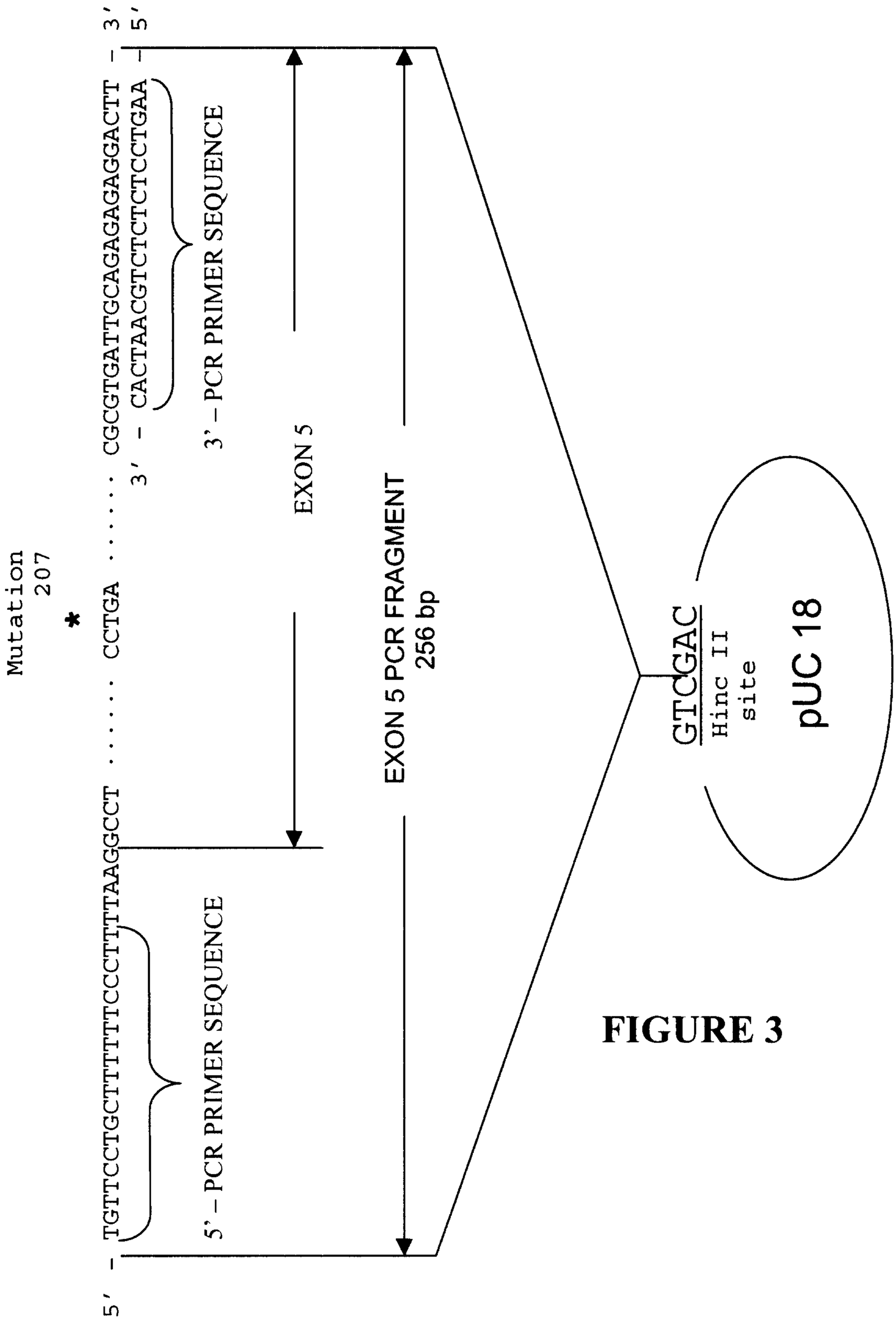
5' – TGTTCCTGCTTTTTTCCCTTTTAAGGCCT ..... CCTGA ..... CGCGTGATTGCAGAGAGGACTT – 3'
Mutation
207
*
5' – PCR PRIMER SEQUENCE
3' – PCR PRIMER SEQUENCE
EXON 5 PCR FRAGMENT
256 bp
EXON 5
GTCGAC
Hinc II
site
pUC 18

FIGURE 3

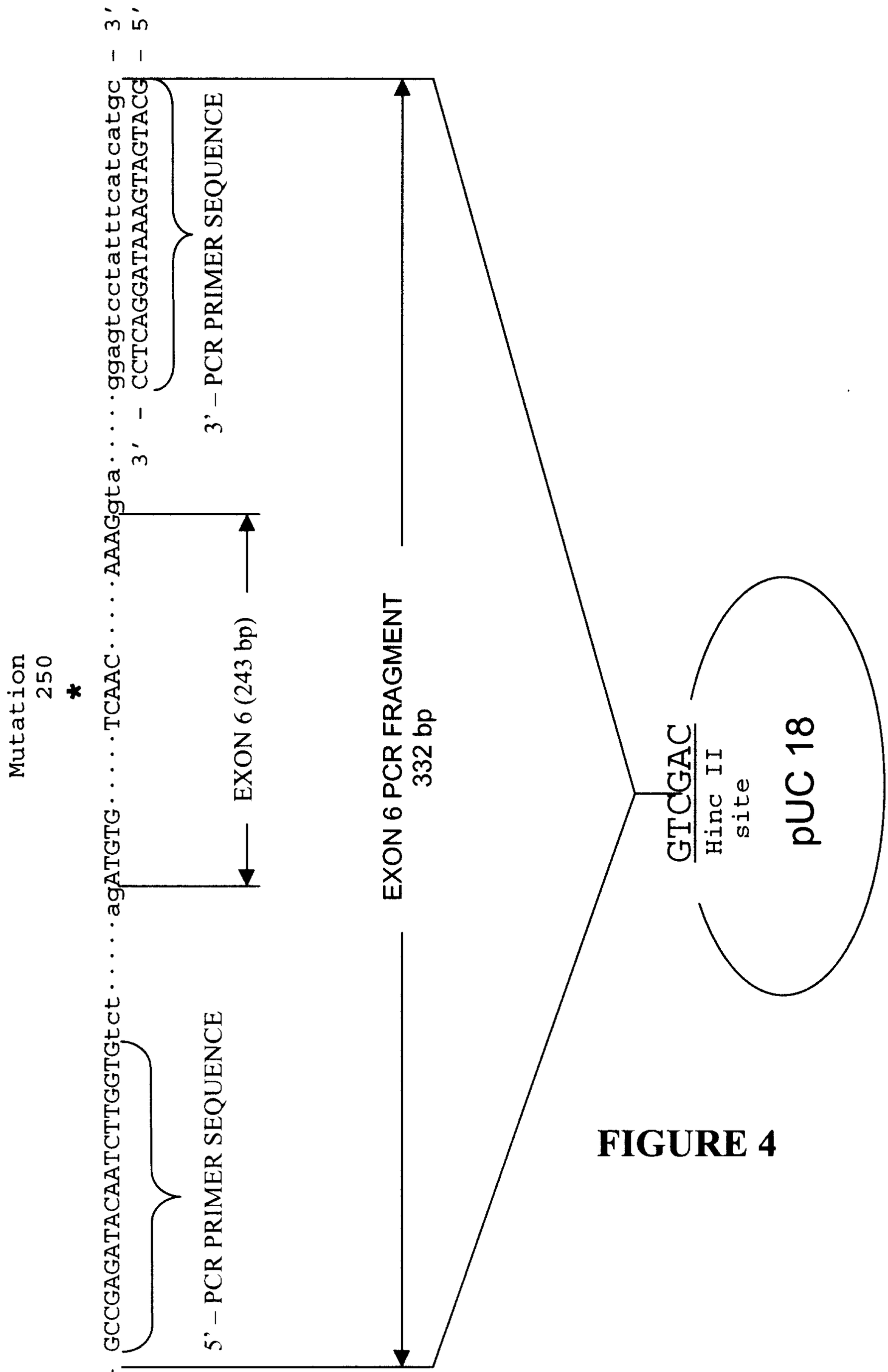
5' – GCCGAGATACAATCTTGGTGtct·····agATGTG·····TCAACAAC·····AAAGgta·····ggagtcctatcatgc – 3'
5' – PCR PRIMER SEQUENCE
3' – CCTCAGGATAGTACG – 5'
3' – PCR PRIMER SEQUENCE
Mutation
250
*
EXON 6 (243 bp)
EXON 6 PCR FRAGMENT
332 bp
GTCGAC
Hinc II
site
pUC 18

FIGURE 4