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(54) **PROMOTEUR DU GENE DE PRESENILINE-1**

(54) **PRESENILIN-1 GENE PROMOTER**

(57) Cette invention concerne une molécule d'ADN isolée qui dirige la transcription à spécificité neuronale d'un segment d'ADN hétérologue situé en aval (un segment promoteur) dans une cellule de mamifère. La molécule d'ADN isolée contient un segment de promoteur provenant de l'ADN de préséniline-1 génomique de souris, ou une séquence d'ADN qui s'hybride à un tel ADN et dirige la transcription à spécificité neuronale d'un segment d'ADN hétérologue situé en aval dans une cellule de mamifère. Cette invention concerne également des structures d'ADN comprenant de tels segments de promoteur ainsi que les diverses utilisations de ces dernières.

(57) An isolated DNA molecule that directs neuron-specific transcription of a downstream heterologous DNA segment (a promoter segment) in a mammalian cell is disclosed. The isolated DNA molecule contains a promoter segment from the mouse genomic Presenilin-1 DNA, or a DNA sequence that hybridize to such DNA and directs neuron-specific transcription of a downstream heterologous DNA segment in a mammalian cell. DNA constructs comprising such promoter segments and various uses thereof are also disclosed.

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(54) Title: PRESENILIN–1 GENE PROMOTER

(57) Abstract

An isolated DNA molecule that directs neuron–specific transcription of a downstream heterologous DNA segment (a promoter segment) in a mammalian cell is disclosed. The isolated DNA molecule contains a promoter segment from the mouse genomic Presenilin–1 DNA, or a DNA sequence that hybridize to such DNA and directs neuron–specific transcription of a downstream heterologous DNA segment in a mammalian cell. DNA constructs comprising such promoter segments and various uses thereof are also disclosed.

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PRESENILIN-1 GENE PROMOTER

This invention was made with Government support under Grant RO1 AG-13839 from the National Institutes of health. The Government has certain rights to this invention.

Field of the Invention

This invention relates to genomic Presinilin-1 gene constructs, and the regulatory regions thereof, and recombinant DNA constructs employing the same.

Background of the Invention

Alzheimer's disease (AD) is a devastating neurological disorder and the most common cause of dementia. The genetics of this disorder suggest that multiple genes are involved. To date, mutations in four genes have been found to be associated with Alzheimer's disease phenotypes including the Amyloid Precursor Protein (APP) gene on chromosome 21 (Citron, M. et al., *Nature* **360**, 672-674 (1992); Suzuki, N. et al., *Science* **264**, 1336-1340 (1994)), the Apolipoprotein-E (APOE) gene on chromosome 19 (Corder, E. H. et al., *Science* **261**, 921-923 (1993); Corder, E. H. et al., *Nat. Genet.* 7, 180-184 (1994); Strittmatter, W. J. et al., *Proc. Natl. Acad. Sci. U. S. A.* **90**, 1977-81 (1993)), the Presenilin-1 (PS-1) gene on chromosome 14 (Sherrington, R. et al., *Nature* **375**, 754-760 (1995)) and the Presenilin-2 (PS-2) gene on chromosome 1 (Levy-Lahad, E.et al., *Science* **269**, 973-977 (1995)). An unknown gene on chromosome 12 appears to associate with a large percentage of late-onset AD patients (Stephanson, J. *J. Am. Med. Asoc.* **277**, 775 (1997)). The majority of familial Alzheimer's disease cases are associated with mutations in the PS-1. To date, over 30 independent mutations in the PS-1 gene have been described in unrelated Alzheimer's

families displaying an early-age-of-onset phenotype. Most of these mutations are missense mutations that result in single amino acid changes (Wasco, W. et al., *Nat. med.* **1**, 848 (1995); Alzheimer's Disease Collaborative Group, *Nat. Genet.* **11**, 219-222 (1995); Campion, D. et al., *Hum. Mol. Genet.* **4**, 2373-2377 (1995); Cruts, M.et al., *Hum. Mol. Genet.* **4**, 2363-2371 (1995); Boteva, K. et al., *Lancet* **347**, 130-131 (1996); Rossor, M. et al., *Lancet* **347**, 1560 (1996); Kamino, K. et al., *Neurosci. Let.* **208**, 195-198 (1996)).

Deletions found in Exons 4 and 9 cause additional mutations as do several truncations of the RNA transcripts arising by differential splicing (Perez-Tur, J. et al., *Neuroreport.* **7**, 297-301 (1995).). Although clustering of these mutations within the protein suggests the location of functionally important domains, the exact function of Presenilin proteins is a matter of active investigation.

One approach to find gene function is to study the regulation of PS-1 gene expression. Using *in situ* hybridization, we and others demonstrate that PS-1 mRNA is most highly expressed in neurons of the brain (Koracs, D. M. et al., *Nat. Med.* **2**, 224-229 (1996)). Immunohistochemistry revealed that the PS-1 protein was abundant in neurons, but was also associated with amyloid plaques and some glial cell types (Scheuner, D. et al., *Nat. Med.* **2**, 864-870 (1996); Lah, J. et al., *J. Neurosci.* **17**, 1971-1980 (1997)). In contrast, Sherrington et al. reported that PS-1 mRNA is widely expressed in a variety of organs throughout the body (*Nature* **375**, 754-760 (1995)). This raises the question as to why mutations in the PS-1 gene product appear to confer a disease state in familial Alzheimer's patients without apparent effect on their peripheral organs. The situation is further compounded because PS-1 mRNA and protein levels from FAD patients and age-matched healthy controls have not been reported, leaving open the possibility that aberrant regulation of PS-1 gene expression further contributes to the disease state.

Mutations in the PS-1 gene's promoter and non-protein encoding regions are not known and reports on the gene's wild-type sequence are lacking. Similarly, no functional analysis of the gene's ability to promote transcription have been reported. Combined with recent reports that PS-1 knockout mice are embryonic lethal (Shen, J. et al., *Cell* **89**, 629-639 (1997)), knowledge of the PS-1 gene sequence and its transcriptional regulation should be important clues that help to identify PS-1 function in both normal and diseased states.

Summary of the Invention

We herein described a complete sequence of the mouse Presenilin-1 gene. This sequence has shown that there are two independent transcription start sites. Functional testing of the DNA regions surrounding these start sites showed that they both were apparently controlled by a single, major promoter that includes the +1 position of Exon 1A. This promoter was also quite interesting because it is mostly active in neuron-like cells. Further characterization can now progress to a complete description of those positive and negative DNA elements and transcription factors which function to control Presenilin-1 gene expression.

A first aspect of the present invention is, accordingly, an isolated DNA molecule that directs neuron-specific transcription of a downstream heterologous DNA segment in a mammalian cell, said isolated DNA molecule having a sequence selected from the group consisting of:

(a) the sequence spanning position –327 to position –206 of the mouse genomic Presenilin-1 gene (with position +1 indicating the transcription start site of exon 1A);

(b) the sequence spanning position –449 to position +1171 of the mouse genomic Presenilin-1 gene;

(c) the sequence spanning position -9 to position +16, of the mouse genomic Presenilin-1 gene (the sequence AGGCCGGAAGTTGCGACACCGGTGA **(SEQ ID NO:1)**); and

(d) DNA sequences that hybridize to isolated DNA having a sequence of (a), (b), or (c) above, and which direct neuron specific transcription of a downstream heterologous DNA segment in a mammalian cell.

By "neuron specific" is meant any level of specificity, so long as the downstream heterologous DNA is preferentially transcribed or expressed in a neuron. By "neuron" is meant an excitable cell that sends a signal, as is well known in the art (*e.g.*, neurons of the brain cortex).

A second aspect of the present invention is a DNA construct comprising an expression cassette, which contains, in the 5' to 3' direction, a promoter segment consisting

of a DNA sequence as given above, and a heterologous DNA segment positioned downstream from said promoter segment and operatively associated therewith.

A third aspect of the present invention is a nerve cell containing a DNA construct as described above.

A fourth aspect of the present invention is a method of making a transgenic non-human animal. The method comprises transforming an animal cell with an expression cassette as described above, and then regenerating an animal from the transformed animal cell.

A fifth aspect of the present invention is a transgenic non-human animal, wherein some or all of the cells of the animal containing a heterologous expression cassette as described above.

The foregoing and other objects and aspects of the present invention are explained in detail in the drawings herein and the specification set forth below.

Brief Description of the Drawings

Figure 1. ***Structure of Three Different Presenilin-1 Transcripts from Mouse Brain.*** The cloned products of 5'-RACE of mouse brain cDNA and DNA sequencing revealed the presence of three independent transcripts (A, B and C) which appear to derive from two unique transcription start sites marked by the vertical arrows. The distance between two transcription start sites is 410 Bp. The sizes of Exon 1A, 1B and 1C are 141 Bp, 371 Bp and 139 Bp, respectively.

Figure 2. ***Cloning and Sequencing Strategy Elucidates the Mouse Presenilin-1 Gene's Exon-Intron Structure.***

Figure 2A. ***Screening Strategy:*** "Screening-A" utilized a fragment of the mouse PS-1 cDNA as Probe A (filled box) to identify lambda phage clones of the mouse PS-1 genomic DNA (represented as double lines). "Screening-B" utilized PCR primers to identify a P1 clone of the mouse PS-1 gene, P1-10809, as represented by the hatched horizontal box.

Figure 2B. ***Sequencing Strategy:*** Lambda phage clones and P1-10809 were restricted and subcloned into pBluescript II KS(+) vector. Thick lines correspond to

individual plasmid subclones from corresponding regions of PS-1 genomic DNA found in P1-10809. Double arrows represent PCR products from P1-10809 template which were sequenced directly. Restriction endonucleases abbreviated as: H, Hind III; E, EcoR I; N, Not I; X, Xho I.

Figure 2C. ***Exon-Intron Structure of the Mouse PS-1 Gene:*** Exons are boxed and double lines represent Introns. Filled boxes and open boxes correspond to the protein coding and untranslated regions, respectively. The translation start codon ATG begins at position +11,420, the translation termination codon TAG is at +45,627 and the putative polyadenylation signal (AATTAA) is at position +46,612.

Figure 3. ***Comparison of the Mouse and Human Presenilin-1 Promoters.*** Mouse PS-1 transcription begins with "G" at position +1 of Exon 1A and human PS-1 transcription begins with "A" (Data not shown.). By DNA sequence similarity searching with BLAST network service available from National Center Biotechnology Information, regions of mouse/human homology are found around the transcription iniation sites for both genes. Consensus binding sites for the transcription factors ETS1 and SP1 are underlined and are conserved in both mouse and human genes.

Figure 4. ***Nucleotide Sequence of the Mouse PS-1 Promoter Region.*** The sequence of mouse PS-1 gene flanking the two transcription initiation sites, marked with vertical arrows, is displayed. Some restriction endonuclease sites are underlined and various promoter elements are boxed and labeled. Exon 1A and Exon 1B are double-underlined.

Figure 5. ***Mouse Presenlin-1 Promoter-Reporter Constructs and their Relative Luciferase Activity (%RLA).***

Figure 5A. ***Structural Organization of PS-1 Promoter:*** Top line represents the region of the PS-1 gene which was analyzed for promoter activity where boxes for Exon 1A and Exon 1B are labeled. Open boxes represent genomic DNA fragments corresponding to the mouse PS-1 gene (top line) which were cloned upstream of the Firefly luciferase reporter gene in the plasmid pGL3-Basic (Promega). Open boxes are labeled on the left with the name of the promoter-reporter plasmid as LUC # and with a nucleotide number of the 5' end of the fragment based on +1 being the "G" at the beginning of Exon 1A. Letters above the open box refer to a restriction enzyme cleavage site. Numbers to the immediate right of the

open box denote the 3' end of the promoter fragment. The numbers on the left hand side are the percentage of Relative Luciferase Activity (%RLA) calculated as described in Materials and Methods followed by the number of times that construct has been transfected into cells and its activity measured which is in parentheses.

Figure 5B. ***Fine Structure Map of the PS-1 Promoter and Promoter-Reporter Construct Activity Strategy.*** Top line represents the region of the PS-1 gene with putative promoter elements, Exon 1A, Exon 1B and restriction enzyme site positions labeled. Open boxes of promoter-reporter constructs are as in (A). Letters above the open box refer to the end of the promoter fragment shown in Figure 3.

Figure 6. ***Cell-Type Specific PS-1 Promoter Activity.*** PS-1 promoter-reporter constructs LUC 29, LUC 27, LUC 4, LUC 3 and LUC 1 were transiently transfected into Neuro2a-neuroblastoma (N2a), undifferentiated P19 (P19), retinoic acid differentiated neuron-like P19 (P19N), dimethylsulfoxide differentiated muscle-like P19 (P19M) and NIH/3T3-fibroblast cells. An SV40-promoter driving Firefly luciferase in pGL3-Basic plasmid (Promega) and pRL-TK (Thymidine Kinase promoter driving Renilla luciferase gene) was also transfected into each cell line as external and internal controls, respectively. After measuring luciferase activity from all combinations of plasmids, the Index of Relative Luciferase Activity (IRLA) was calculated as RLA/ RLA_{SV40}, where RLA_{SV40} is the ratio of Firefly luciferase signal in the external control divided by the Renilla luciferase signal in the internal control, as to compare the activity of different promoter fragments in different cell lines. Plasmid LUC 29 transfected into N2a cells showed the greatest IRLA value which we defined as 100% activity.

Detailed Description of the Invention

Nucleotide sequences are presented herein by single strand only, in the 5' to 3' direction, from left to right. Nucleotides are represented herein in the manner recommended by the IUPAC-IUB Biochemical Nomenclature Commission.

Specific examples of neuron specific promoters of the present invention include, but are not limited to, DNA molecules that comprise mouse Presenilin-1 gene promoter segments from position:

-440 to +91;

-352 to +91;

-327 to +91;

-276 to +91;

-261 to +91;

-215 to +91;

-192 to +91;

-124 to +91;

-87 to +91; and

-32 to +91.

From position:

-276 to +519;

-276 to +206;

-276 to +148; and

-276 to +41.

And from position:

-87 to +41;

-9 to +16; and

-327 to +206.

The corresponding fragments from the Presenilin-1 gene of other mammalian species, such as rat, cat, dog, monkey, or human, may also be employed to carry out the present invention., as discussed in greater detail below.

Promoters of the present invention may be of any animal species of origin, but are preferably of mammalian origin (*e.g.,* mouse, rat, cat, dog, monkey, human). Promoters used to carry out the present invention are, in general, substantially homologous to the mouse segments described above. As used herein, such regions are "substantially homologous" when their DNA binding required for promoter function are homologous to eh various mouse promoter segments described herein. In general, such regions are at least 75%, and more preferably 80%, 85%, 90%, or even 95% homologous to the various mouse promoter segments described herein. It will be apparent that other sequence fragments of the regions

flanking the foregoing sequences, or minor additions, deletions or substitutions thereto, can be prepared which will also carry out the function of a Presenilin-1 gene promoter. They may also be identified by their binding to specific transcription factor proteins and/or their function to promote transcription.

Other DNA segments such as naturally occurring DNA segments or mammalian DNA segments, which encode neuron-specific gene promoters can be identified by their binding, or hybridization, to fragments as described above. Hybridization conditions which will permit such DNA sequence to hybridize to a DNA sequence as given herein are known in the art. For example, hybridization of such sequences to the DNA disclosed herein may be carried out in 25% formamide, 5X SSC, 5X Denhardt's solution, with 100 μg/ml of single stranded DNA and 5% dextran sulfate at 42° C, with wash conditions of 25% formamide, 5X SSC, 0.1% SDS at 42° C for 15 minutes, to allow hybridization of sequences of about 60% homology. More stringent conditions are represented by a wash stringency of 0.3M NaCl, 0.03M sodium citrate, 0.1% SDS at 60° C or even 70° C using a standard hybridization assay (*See* Sambrook et al., Molecular Cloning, A Laboratory Manual (2d Ed. 1989)(Cold Spring Harbor Laboratory)).

The term "operatively associated" as used herein refers to DNA sequences contained within a single DNA molecule that are associated so that the function of one is affected by the other. Thus, a promoter is operatively associated with a gene (or other DNA of interest) when it is capable of affecting the expression of that gene (i.e., the gene is under the transcriptional control of the promoter). The promoter is said to be "upstream" from the gene (or other DNA of interest), which is in turn said to be "downstream" from the promoter.

DNA constructs, or "expression cassettes," of the present invention include, 5' to 3' in the direction of transcription, a promoter of the present invention, a heterologous DNA segment operatively associated with the promoter, and, optionally, transcriptional and translational termination regions such as a termination signal and a polyadenylation signal. All of these regulatory regions should be capable of operating in the transformed cells. The 3' termination region may be derived from the same gene as the transcriptional initiation

region or from a different gene. The expression cassette may be provided in a DNA construct that also has at least one replication system.

As used herein, the term "heterologous gene" or "heterologous DNA segment" means a gene (or DNA segment) which is used to transform a cell by genetic engineering techniques, and which may not occur naturally in the cell. Structural genes are those portions of a genes which comprise a DNA segment coding for a protein, polypeptide, or portion thereof, possibly including a ribosome binding site and/or a translational start codon, but lacking a promoter. The term can also refer to copies of a structural gene naturally found within a cell but artificially introduced. Structural genes may encode a protein not normally found in the cell type into which the gene is introduced or in combination with the promoter to which it is operationally associated. As used herein, the term heterologous DNA segment also includes DNA segments coding for non-protein products, such as ribozymes or anti-sense RNAs (*see, e.g.,* U.S. Patent No. 4,801,540).

The various fragments comprising the various constructs, expression cassettes, markers and the like, may be introduced consecutively by restriction enzyme cleavage of an appropriate replication system and insertion of the particular construct or fragment into the available site. After ligation and cloning, the DNA construct may be isolated for further manipulation. All of these techniques are amply exemplified in the literature. *See, e.g.,* Maniatis et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 1982).

Promoters and the various constructs of the present invention have a variety of different uses. Nerve cells grown in culture may be transformed with constructs of the present invention and the heterologous DNA expressed therein to produce a protein or a peptide, and the protein or peptide then collected for subsequent use (for example, the protein or peptide could encode an antigen, which is used directly in diagnostic assays, or which is injected into an animal to produce antibodies thereto, which antibodies are used in diagnostic assays). Transgenic animals may be produced with the constructs of the invention, as discussed in greater detail below. The promoters may be used in vectors that comprise gene therapy vectors (*e.g.,* viral vectors such as herpesvirus vectors and RNA viruses such as retroviruse (wherein the promoter segment and the heterologous segment exist as an RNA transcript of

the DNA which is inserted into the host cell as a DNA transcript, or in the case of retroviruses as a provirus)) where the heterologous DNA encodes a therapeutic agent (*e.g.*, ApoE2 or ApoE3; nerve growth factor, ciliary neurotrophic factor, etc.); and where preferential expression of the heterologous DNA in nerve cells is desired . Numerous other uses for promoters of the instant invention will be readily apparent to those skilled in the art.

As noted above, a method of making a transgenic animal is also an aspect of the present invention. The method can be carried out on any suitable animal subject, but is preferably carried out with non-human mammals. Murine species or rodents (*e.g.*, mice, rats) are particularly preferred.

The method comprises transforming an animal cell with an expression cassette as described above, in an animal transformation vector, and then regenerating a transgenic animal from the transformed animal cell. The transformation step may be carried out by any suitable means, as discussed in detail below, and the regeneration step may also be carried out by any suitable means, as also discussed in detail below. Where chimeric animals are produced by the process, animals in which all cells (*e.g.*, including both somatic cells and germ cells) are transformed (and in which the expression cassette described above is stably integrated into the genome of the cells) may be regenerated from chimeric animals having transformed germ cells, as is known in the art.

The production of transgenic animals can be carried out by any suitable technique, such as pronuclear microinjection, infection of embryos with retroviruses, embryonic stem cell-mediated techniques, transfer of entire chromosomal segments and gamete transfection in conjunction with *in vitro* fertilization, etc. *See generally* Charles River Laboratories, *Transgenic Animal Science: Principles and Methods* (Summer 1991).

Transgenic animals that carry an expression cassette of the invention can be produced by the genetic transformation of zygotes, as described in T. Wagner et al., U.S. Patent No. 4,873,191 (applicant intends that the disclosure of all U.S. Patent References cited herein be incorporated herein by reference).

In another technique, a pluripotent embryonic stem cell from the species to be transformed may be derived, the expression cassette inserted into the stem cell, and one or more of the stem cells inserted into an early embryo such as a blastocyst of the animal to be

transformed, and the animal raised to birth in a suitable female host (*e.g.*, M. Evans, PCT Application WO90/03432). Methods of producing transgenic animals by subjecting a mixture of DNA and the embryo to an electric discharge are described in U.S. Patent No. 5,567,607 to X. Zhao et al. Mammalian expression vectors are described in U.S. Patent No. 5,627,033 to J. Smith et al.

Animals of the present invention are useful as laboratory models for studying the function of the Presenilin-1 gene, for studying the etiology of Alzheimer's disease, and for studying the activity of various drugs and drug candidates in treating Alzheimer's disease. In such animals the endogeneous Presenilin-1 gene may be active or inactive. The endogeneous Presenilin-1 gene may be inactivated by means of a "knock-out" of the Presenilin-1 gene in accordance with known techniques, such as homologous recombination. *See, e.g.*, O. Smithes, *Nature* **317**, 320 (1985).

In the Examples set forth below, the abbreviation used are: Bp, base pairs; PEA-3, polyoma virus enhancer activator-3; PS-1, presenilin-1; 5'-RACE, rapid amplification of 5'-cDNA ends; N2a, Neuro2a cells; P19N, neuron-like differentiated P19 cells; P19M, muscle-like differentiated P19 cells; RLA, relative luciferase activity; IRLA, Index of relative luciferase activity.

EXPERIMENTAL PROCEDURES

Isolation and characterization of genomic clones -- Labeled oligonucleotides and PCR products of the mouse PS-1 cDNA were used as probes to screen mouse libraries for genomic PS-1 clones. Based on the mouse PS-1 cDNA sequence (Genbank Accession #L42177), an upstream primer of sequence

5'- CGGAGAGAGAAGGAACCAAC-3' **(SEQ ID NO: 2)**

and a downstream primer of sequence

5'-TCAGCTCTTCGTCTTCCTCCTCATC-3' **(SEQ ID NO: 3)**

were used with Quick Clone Mouse Brain cDNA (Clontech) as template to amplify a portion of the mouse PS-1 cDNA by polymerase chain reaction (PCR). Amplification reactions were performed in 100 µl volume containing 1xPCR buffer II (Perkin Elmer), $MgCl_2$ (1.5mM), dATP, dGTP, dCTP and dTTP (0.2 mM each, Perkin Elmer), DNA primers at 0.5 µM, 1 µl cDNA template (0.1 ng) and Ampli-Taq DNA polymerase (5 Units, Perkin Elmer). The reaction cycle was 95°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes for a total of 30 cycles. This PCR product was gel purified and labelled with alpha-^{32}P-dCTP and a Random Primers DNA Labelling System (Gibco). Labelled probe was used to conventially screen a mouse strain 129/SVJ genomic library in Lambda Fix-II vector (Stratagene) as described (Sambrook, J. Fritsch, E. F., and Maniatis, T, Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).). Screening identified four independent phage clones designated Ph-1, Ph-2, Ph-3 and Ph-4 (**Figure 2**). Following digestion with Not I and/or EcoRI, restriction enzyme fragments were subcloned into pBluescript-II-KS(+) phagemid vector (Stratagene) using a DNA ligation kit (Stratagene). DNA sequence was determined using an Applied BioSystems model 373A automated DNA sequencer with dye terminator chemistry and protocols recommended by the manufacturer. Additional oligonucleotide probes from the 5'-untranslated region of the mouse PS-1 cDNA were labeled with ^{32}P-ATP and T4-polynucleotide kinase and used to

identify plasmid subclones by hybridization. Based on the partial sequence of phage clone Ph-2, the PCR primers 1C-US-for

GATCACAGTCTAGGTTGCTGGTGTG **(SEQ ID NO: 4)**

and 1C-US-rev

TGGGGCAAGGG ACACAAATAAG **(SEQ ID NO: 5)**

were used to further screen a mouse ES-129/SVJ genomic library in a P1 vector (Genome Systems Inc.) by PCR. Of the three P1 clones identified, P1-10809 was digested with EcoRI or Hind III and these restriction enzyme fragments subcloned and sequenced as described above.

5'-Rapid Amplification of cDNA Ends or RACE: -- The 5' end of PS-1 cDNA was identified using mouse-brain Marathon-Ready cDNA (male BALB/c, 9-11 weeks of age, Clontech). Briefly, a 50 µl PCR reaction containing 0.2 µM of a PS-1-specific reverse primer:

TGGCTCAGGGTTGTCAAGTC **(SEQ ID NO: 6)**

0.2 µM of the Clontech AP1 adaptor primer:

CCATCCTAATACGACTCACTATAGGGC **(SEQ ID NO: 7)**

2.5 ng Marathon-Ready cDNA, 1xPCR buffer (Gibco), $MgCl_2$ (1.5 mM), DMSO (5%), dATP, dGTP, dCTP and dTTP (0.2 mM each), and Taq DNA polymerase (5 Units, Gibco) was used with a reaction cycle of 95°C for 45 seconds, 55°C for 30 seconds and 72°C for 90 seconds for a total of 30 cycles in the first amplification step. The 100 µl second PCR amplification step contained 0.5 µM of a mouse PS-1-specific reverse primer, 151-130-reverse:

CAAACCTCTTGGGATTCTTTC **(SEQ ID NO: 8)**

and 0.5 μM of the nested Clontech adaptor primer AP2

ACTCACTATAGGGCTCGAGCGGC **(SEQ ID NO: 9)**

and 0.01 μl of the first PCR amplification, 1xPCR buffer (Gibco), $MgCl_2$ (1.5mM), DMSO (5%), dATP, dGTP, dCTP and dTTP (0.2 mM each) and Taq DNA polymerase (5 Units, Gibco) with the same cycling parameters as in the first amplification step. In some second PCR reactions, the PS-1 specific reverse primer 101-80-reverse:

AAGACCTCGAAGGGCTGCTGTC **(SEQ ID NO: 10)**

was used. RACE amplification products were electrophoresed on 2% agarose gels run in TAE, visualized with ethidium bromide and ultraviolet light, extracted from the gel matrix with a Wizard PCR Preps DNA Purification System (Promega), ligated into a pGEM-T vector (Promega) and transformed into competent DH5-alpha bacterial cells (Gibco). Ampicillin resistant colonies were characterized by restriction enzyme digestion, PCR amplification with a variety of primer combinations and DNA sequencing as above.

Computation of Sequence Similarities:---Comparison of the mouse PS-1 promoter with other eukaryotic promoter sequences were performed using the BLAST network service and the Eukaryotic Promoter database (EPD) Release 45 available from the National Center for Biotechnology Information.

Construction of PS-1 Promoter-Firefly Luciferase Reporters: -- Mouse genomic DNA fragments containing portions of the putative PS-1 promoter were subcloned into the promoterless pGL3-basic vector (Promega) upstream of the firefly luciferase gene. Based on the sequence of genomic DNA, PCR primers were designed to incorporate Xho I sites into the forward primers and Hind III sites into the reverse primers. These primers corresponding to different locations in the genomic DNA (see **Figure 2**) were used to PCR amplify

fragments as above which were purified with a Wizard Purification System (Promega), digested with the appropriate restriction enzymes and repurified with the Wizard kit. Cleaved PCR products were ligated into pGL3 plasmid cleaved with the same restriction enzymes, transformed into competent bacteria and clones containing plasmids with inserts verified by DNA sequencing.

Eucaryotic Cell Culture and Transfection:---Mouse Neuro2a-neuroblastoma cells, mouse P19-embryonal carcinoma and mouse NIH/3T3 fibroblast cells were obtained from the American Type Culture Collection (ATCC). Neuro2a cells were routinely propagated in Minimal Essential Medium with Earle's salt (Gibco) plus 10% fetal calf serum (Hyclone) plus 0.1 mM non-essential amono acids (Gibco). P19 cells were routinely propagated in alpha-MEM (Gibco) plus 2.5% fetal calf serum plus 7.5% bovine serum (Hyclone). Differentiation of P19 cells to neuron-like cells followed treatment with 0.5 μM trans-retinoic acid (Jones-Villeneuve, E. et al., *Mol. Cell. Biol.* 3, 2271-2279 (1983)). Differentiation of P19 cells to muscle-like cells followed treatment with 1% dimethylsulfoxide (Edwards, M. et al., *Mol. Cell. Biol.* 3, 2280-2286 (1983).). NIH/3T3 cells were routinely propagated in DMEM (Gibco) plus 10% fetal calf serum.

For transient transfection, Neuro2a, P19, retinoic acid treated-P19, DMSO-treated P19 and NIH/3T3 cells were plated in 6 well tissue culture dishes at 9×10^4 cells per well and allowed to recover for one day. Cells containing PS-1-promoter-reporter constructs were then co-transfected with 0.3 pmole of one of the Promoter-Firefly Luciferase plasmid constucts, pGL3 Basic vector or pGL3 Promoter plasmid (Promega), which contains an SV40 promoter upstream of the firefly luciferase gene, and 0.3 pmole of pRL-TK plasmid (Promega), which contains an herpes simplex virus thymidine kinase promoter upstream of the Renilla luciferase gene, using the Lipofectin procedure (Gibco) as described in the manufacture's protocol.

Relative Luciferase Activity Measures: -- Transfected cells were cultured for 24 hours, washed twice with 2 ml of Ca^{2+} and Mg^{2+} free PBS and lysed with Passive Lysis Buffer (Promega). Firefly luciferase and Renilla (sea pansy) luciferase activities were measured sequentially using a Dual-Luciferase Reporter Assay System (Promega) and a model TD-20E Luminometer (Turner Design). After measuring the Firefly luciferase signal

(LA_F) and the Renilla (sea pansy) luciferase signal (LA_R), the Relative Luciferase Activity (RLA) was calculated as: $RLA = LA_F / LA_R$, where relative RLA was caculated as a percentage, ie. $\%RLA = RLA / (RLA)_{max}$. To compare the Relative Luciferase Activity in one cell line with another, an Index of Relative Luciferase Activity was calculated as: IRLA = RLA/ RLA_{SV40} where RLA_{SV40} is the ratio of Firefly luciferase signal with an SV40 promoter in pGL3 divided by the Renilla luciferase signal in pRL-TK.

RESULTS

RACE Detects Multiple Transcipts: -- As a prelude to cloning the PS-1 promoter, the exact 5' end of mouse brain PS-1 mRNA was identified by the rapid amplification of cDNA ends (RACE) technique. 5'-RACE with the anti-sense oligonucleotide "101-80-reverse" found in exon 2 of mouse PS-1, gives a major broad band of 210 bp's and a minor band of 430 bp's from single-stranded cDNA templates complementary to mouse brain mRNA (Marathon Ready cDNA, Clontech, data not shown). Each of these bands was isolated from agarose gels, subcloned into the pGEM-T vector (Promega) and sequenced. Sequencing revealed the presence of three different PS-1 transcripts which appear to derive from 2 unique transcriptional start sites (**Figure 1**). This information suggests that the PS-1 gene may contain 2 promoters and that differential splicing generates multiple transcripts.

Isolation of the Mouse PS-1 Gene: -- A mouse genomic DNA library in Lambda-FIX II was screened with Probe A, a ^{32}P-labeled PCR probe corresponding to Exons 2, 3 and 4 of the murine PS-1 cDNA clone (**Figure 2A**). Of the positively hybridizing phage clones, four were selected for restriction mapping with EcoRI and Not I as shown **in Figure 2B**. Only one phage clone, Ph-2 **(Figure 2B)** hybridized to oligonucleotides from the 5'-untranslated region of the mouse PS-1 cDNA. Primers from the phage arms allowed sequencing into the genomic DNA insert. The insert's sequence allowed the PCR primer pair "1C-US-forward and 1C-US-reverse" to be chosen and used to identify a P1 clone of the mouse PS-1 genomic DNA as shown in **Figure 2A**. Clone P1-10809 was identified through a positive PCR reaction product with these primers and hybridization to the PS-1 cDNA fragment probe A (**Figure 2**). P1-10809 was then restricted, mapped and its entire sequence sublconed into multiple pBluescript-II-KS-(+) plasmid vectors as shown by the thick lines in Figure 2B.

Each subclone was sequenced on an Applied Biosystems 373A automated DNA sequencing machine using protocols supplied by the manufacturer.

Characterization of the PS-1 Gene's Exon-Intron Structure: -- The sequence of almost 50 kBp of the P1-10809 clone was compared to the mouse PS-1 cDNA sequence and regions of homology aligned with the MacVector DNA analysis program (IBI, New Haven, CT). The first nucleotide on the 5' end of the RNA transcript is usually designated as nucleotide +1 of exon 1 of a gene. In our case, PS-1 appears to have two different 5' end-sequences which are associated with three different length RNA transcripts which we named Transcript-A, Transcipt-B and Transcript-C. The alignment of Transcript-A with genomic sequence shows that a "G", designated conventionally as position +1 in Exon 1, corresponds to the transcription start site. The Presenilin gene's Exon 1A extends from position +1 to +141 which is spliced to Exon 2 whose 5' end begins at position +11,210 to give Transcript-A. The alignment of Transcript-B with genomic DNA shows that a "C" at position +411 corresponds to the alternative transcription start site. We define this second start site as beginning in Exon 1B which extends from position +411 to +781 and is spliced to Exon 2 (position +11,210) to give Transcript-B. The alignment of Transcript-C with genomic DNA shows the same "C" at position +411 as the alternative transcription start site. We define Exon 1C as extending from position +411 to +549 which is spliced to Exon 2 at position +11,210 to give Transcript-C. As shown in **Figure 2C** and summarized in **Table 1,** Exon 1A, Exon 1B, Exon 1C and Exon 2, together with a portion of Exon 3, comprise the 5' untranslated regions of PS-1 RNA transcripts.

Table 1. ***Numbering Scheme for the Mouse PS-1 Gene's Exon-Intron Structure.***[1]

	Size	Position
Exon 1A	141 bp	1 – 141
Exon 1B	371 bp	411 – 781
Exon 1C	139 bp	411 – 549
Exon 2	67 bp	11210 – 11276
Exon 3	140 bp	11367 – 11506
Exon 4	251 bp	23849 – 24099
Exon 5	142 bp	26311 – 26452
Exon 6	68 bp	34557 – 34624
Exon 7	221 bp	36060 – 36280
Exon 8	99 bp	39773 – 39871
Exon 9	87 bp	40245 – 40331
Exon 10	174 bp	42082 – 42255
Exon 11	119 bp	43217 – 43335
Exon 12		45459 --
A of ATG		11420
T of TAG		45627
AATTAA		46612

[1]The positions of the 5' end and the 3' end of each Exon were counted from the transcription start site of Exon 1A being defined as position +1. Note that this differs from the numbering in SEQ ID NO: 17.

The protein encoding portions of the gene begin with the ATG codon at position +11,420 where translation initiates in Exon 3 followed by exons encoding the remainder of the protein until stopping at a TAG codon (position +45,627) in Exon 12. 983 bp downstream from this TAG stop codon lies the putative polyadenylation signal AATTAA at position +46,612. Interestingly, the intron between Exon 1 and Exon 2 is about 10 kBp, between Exon 3 and Exon 4 is about 12 kBp and between Exon 5 and Exon 6 is about 8 kBp. The entire mouse PS-1 gene sequence is set forth as **SEQ ID NO: 17** herein, but with exon 1A begining at nucleotide 2234. Note in **SEQ ID NO: 17** that the A's of the start codons for all the transcripts, *i.e.*, A,B, and C are at the same position, 13653 in exon #, T's of the stop codons for all the transcripts are at the same position, 47860 in exon 12, and the polyadenylation signals for all the transcripts are at the same position, 48845, So the translations for all the transcripts are the same.

Characterization of the Mouse Presenilin-1 Promoter: -- 2300 bp of DNA sequence located upstream of the +1 initiation of transcription site for Transcript A was compared to its human PS-1 genomic DNA counterpart (**Figure 3**). The region of maximal similarity with human extends from postions -39 to +117 in the mouse sequence. This region is rich in Guanosine (G) and Cytosine (C) residues and contains the sequence motifs GCCGGAAGT (**SEQ ID NO: 11**) resembling an Ets 1-3 element (Fisher, R. et al., *Oncogene* **6**, 2249-2254 (1991)) and a GGGCGGG (**SEQ ID NO: 12**) motif resembling an Sp-1 hexanucleotide element commonly found in the promoters of other genes. The mouse sequences upstream of this region do not share similarity with the human sequence nor do they contain the most common eucaryotic promoter element, a TATA box (**Figure 4**). Instead, this unique mouse sequence contains two CAAATA motifs at postions -365 and -281 which resemble CAAT boxes found in other eucaryotic promoters. This unique region also contains an Ap-2 binding element at position -80 (CCCAGCCC) (**SEQ ID NO: 13**) and a sequence similar to a heat shock inducible element at position -220 (CTCGAATCGCAG) (**SEQ ID NO: 14**). Putative Sp-1 hexanucleotide binding sites with the sequence GGGCGG (**SEQ ID NO: 15**) or CCGCCC (**SEQ ID NO: 16**) are found downstream from the +1 site of transcription initiation with Exon 1A containing one Sp1 motif and Intron 1A containing five motifs. Also downstream of the Cap (transcription initiation) site are two additional Ap-2 sites and another Ets 1-3 motif.

To test whether these elements function to promote transcription, we employed a Dual-Luciferase Reporter Assay System (Promega). In general, we assayed the promoter activity of DNAs flanking the transcription initiation site of PS-1 by inserting these DNA fragments in front of a basic, promoterless firefly Luciferase reporter gene in plasmid pGL3. Constant amounts of pGL3 containing PS-1 promoter fragments and of pRL-TK plasmid containing a Herpes Simplex Virus Thymidine Kinase (HSV-TK) promoter driving expression of sea pansy Luciferase were co-transfected into a constant number of cells. After 24 hours, lysates of transfected cells were sequentially assayed for firefly luciferase (LA_F) and sea pansy luciferase activity (LA_R) so that a ratio of firefly to sea pansy activities could be calculated for each PS-1 promoter fragment as its Relative-Luciferase-Activity or RLA. Of all the fragments tested, plasmid LUC 29 with the fragment -327 to +206 showed the

greatest ratio of firefly to sea pansy activity (**Figure 5** and **Table 2**) which we defined as 100% activity. Larger fragments in LUC 1 (-2232 to +1436), LUC 3 (-499 to +1171) and LUC 16 (-276 to +519) display only a small percentage of the LUC 29 activity suggesting the presence of negative elements that apparently reduce their activities. Interestingly, the high activity of LUC 29 is not found in its flanking fragments such as LUC 2 (-2232 to -496) and LUC 23 (+188 to +519) which both lack significant promoter activity. The LUC 23 result is particularly interesting because the alternative transcription start site begins at position +411 of Exon 1B/Exon 1C and apparently lacks meaningful promoter activity.

Table 2. ***Neuron-Preferred Activity of Total and Core Promoter Regions of the Mouse PS-1 Gene.***[2]

Cell line	N2a	P19N	P19	P19M	NIH/3T3
Control	2.1	10.6	5.0	35.9	1.1
Luc29(total)	36.8	114.1	21.2	114.9	0.3
Luc27(core)	3.6	11.5	3.7	19.4	0.1
(IRLA)total	17.8	10.8	4.3	3.2	0.3
(IRLA)core	1.7	1.1	0.7	0.5	0.1
Total/core	10.2	9.9	5.8	5.9	3.4

[2]LUC29 and LUC27 were transiently transfected into the cell lines to measure the activities of Total- and Core-promoter, respectively. An SV40-promoter driving Firefly luciferase in pGL3-Basic plasmid (Promega) was also transfected as a control. An index of relative luciferase activity (IRLA) was calculated for the Total- and the Core-promoter as IRLA = RLA/ RLA_{SV40}.

To more accurately define the minimal or core regions conferring promoter activity, we studied the -327 to +206 region of the PS-1 gene in greater detail. Sequence comparision showed this region to contain a CAAT box (-281), a heat shock element (-220), an AP2 site (-80), a PEA-3 site (-53), an Ets 1-3 site (-7) and Sp1 sites (+25, +119 and +161). To find which of these elements and/or new elements were functionally active, we performed resection experiments to test smaller fragments within this region for promoter activity. Since LUC 24 and LUC 23 lacked significant activity, we initially focussed on the fragments from -440 to +91 as shown in **Figure 4**. The CAAT box at -281 plays an active role in the PS-1 promoter because LUC 8 (-261 to +91) has less activity than LUC 6 (-327 to +91) which contains this CAAT box. A negative element must reside upstream of this CAAT box

because the activity of LUC 4 (-440 to +91) is about half that of LUC 6 (-327 to +91). The Heat Shock element at -220 may not play a role in PS-1 promoter activity as fragments containing (LUC 8, -261 to +91) and lacking (LUC 10, -192 to +91) this element have similar activites. The AP2 site at -80 and/or the PEA-3 site at -53 appear to play positive roles in PS-1 promoter function as LUC 12 (-87 to +91) has about four fold more activity than LUC 13 (-32 to +91) which lacks these sites. Similarly, the Ets 1-3 site at position -7 plays a positive role as judged by the RLA activity of LUC 14 (-9 to +91) at 7.9% and LUC 15 (+42 to +91) at 0.7%. While the Sp1 site at position +161 does not appear to contribute when LUC 17 (-276 to +206) is compared to LUC 18 (-276 to +148), the Sp1 sites at +25 and +119 appear to be very active in the PS-1 promoter as negative and positive elements, respectively, when LUC 26 (-9 to +41) is compared to LUC 27 (-9 to +16) and LUC 7 (-276 to +91) is compared to LUC 18 (-276 to +148).

Based on these experiments, we tested whether the region from -87 to +41 could contain the core promoter activity in two ways. First, LUC 25 (-87 to +41) had an RLA promoter activity of 28%. Second, the deletion of this region to give LUC 30 (delete -87 to +41 from -327 to +206) decreased activity from 100% (LUC 29) to 0.2% (LUC 30). Taken together, these results strongly suggest that the Ap2, PEA-3, Ets 1-3 and Sp1 elements comprise the major functional elements of the PS-1 promoter in the region -87 to +41.

Cell-Specific Transcription: -- Using *in situ* hybridization to human brain slices, we found that PS-1 RNA was most abundant in neurons and below the limits of detection in other brain cells. This result suggested that the PS-1 promoter may preferentially function in neurons. To test this idea further, we compared the activity of the promoter-fragment/reporter plasmids LUC 1, LUC 3, LUC 4, LUC 27 and LUC 29 in different cell types. As reported above, the mouse Neuro-2A cell line of neuroectodermal lineage supports more RLA promoter activity from LUC 29 and LUC 4 than from LUC 27, LUC 3 and LUC 1 (**Figure 6** and **Table 2**). In contrast, the mouse NIH/3T3 fibroblast cell line supports only minimal promoter activity with each of these promoter/reporter constructs (LUC 29, LUC 27, LUC 4, LUC 3 or LUC 1). To further test the idea that the PS-1 promoter activity is great in neurons, we transfected the mouse embryonal carcinoma cell line P19 with these reporter constructs. P19 cells are uniquely differentiated by retinoic acid treatment into a neuron-like

phenotype (Jones-Villeneuve, E. et al., *Mol. Cell. Biol.* 3, 2271-2279 (1983)) or by dimethylsulfoxide treatment into a muscle-like phenotype (Edwards, M. et al., *Mol. Cell. Biol.* 3, 2280-2286 (1983).). Retinoic acid-treated P19 cells support as much as 2.5 fold more relative luciferase activity from plasmid LUC 29 compared to untreated P19 cells. Untreated P19 cells support as much as 1.3 fold more relative luciferase activity compared to dimethylsulfoxide-treated P19 cells

Discussion

From promoter to poly-adenylation signal, the full sequence of the mouse Presenilin-1 gene and its exon-intron structure set the stage to describe some of its unique functions. In contrast to the reported PS-1 cDNA sequence, 5' RACE (Rapid Amplification of cDNA Ends) surprised us by amplifying three different mRNA transcripts which share two unique transcription start sites. Sequence analysis showed that Transcript-A begins with Exon 1A while Transcript-B and Transcript-C begin with Exon 1B. Exon 1C is a fragment of Exon 1B sharing its 5' end at position +411, but only extending to position +549. This example of alternative splicing in Exon 1B versus Exon 1C to yield multiple RNA transcripts is well known in the field and has been described for Exon 9 in the human PS-1 gene (Perez-Tur, J. et al., *Neuroreport.* 7, 297-301 (1995)). Two distinct transcription start sites, however, have been reported for only a few genes including human catechol-O-methyl transferase (Tenhunen, J. et al., *Eur. J. Biochem.* **223**, 1049-1059 (1994)), mouse Neurotrophin-3 (Leingartner, A. et al., *Eur. J. Neurosci.* **223,** 1149-1159 (1994)) and rat aromatic L-amino acid decarboxylase (Albert, V. et al., *Proc. Natl. Acad. Sci. U. S. A.* **89**, 2053-12057 (1992)). In these cases, each transcriptional start site was associated with a distinct promoter so that a stoichiometry of one promoter per transcription start site was observed.

Our characterization of promoter activities for the PS-1 gene, however, revealed a much different picture. Using a promoter-fragment coupled to the Firefly luciferase reporter with sea pansy Renilla luciferase as an internal standard, we found that the -327 to +206 fragment (LUC 29) contains most of the PS-1 promoter activity. The known sequence motifs which apparently contain this activity are a CAAT box (-281), an Ap2 site (-80), a PEA-3 site (-53), an Ets 1-3 site (-7) and an Sp1 site (+25). While this region overlaps some of Exon

1A, deletion of the -87 to +41 region in LUC 30 reduces promoter activity by 50 fold. For the alternative transcription start site at position +410 in Exon 1B and Exon 1C, we tested LUC 23 (+118 to +519) containing Sp1, Ap2 and Ets 1-3 sites and found it to display about 1% of the activity surrounding the Exon 1A promoter. These results suggest to us that the region surrounding the +1 position of Exon 1A may promote the expression of Transcript-A, Transcript-B and Transcript-C. Alternatively, a weak promoter controlling transcription initiation at position +410 in Exon 1B/Exon 1C may amount to only 1% of the transcription initiation at position +1. By cloning all of the products of the 5' RACE into plasmid vectors and counting the number of clones carrying Exon 1C, we estimate the abundance of Transcript-C to approach 30% of all of the PS-1 transcripts (data not shown) further supporting the idea that the major promoter at +1 functions to control transcription initiation from both the +1 and the +410 sites. Quantitative measurement of Transcript-A, Transcript-B and Transcript-C levels will help to further resolve this issue. The high homology between human and mouse promoters, combined with our description of multiple start sites and alternative splicing for the mouse PS-1 gene, reasonably suggests how the human PS-1 promoter may function (**Figure 6**).

Recently, PS-1 was reported to be expressed predominantly in neurons of the central nervous system (Kovacs, D. et al., *Nat. Med.* **2**, 224-229 (1996)). This result matches our own data that PS-1 RNA, by *in situ* hybridization, is strongly expressed in neurons and at undetectable levels in other cell types. Similarly, several immunohistochemical studies report primarily neuronal localization of PS-1 protein with weak staining of amyloid plaques and some glia surrounding those plaques. On the other hand, Sherrington et al. showed that Northern blots of RNA from different organs all hybridized to a PS-1 cDNA probe suggesting that PS-1 RNA is ubiquitously expressed. At present, these results can not be easily reconciled.

Our data shows preferential promoter activity in neuron-like cells supporting a cell-type-specific pattern of PS-1 expression. We find the greatest amount of PS-1 promoter activity in the mouse Neuro2a-neuroblastoma cell line, followed by the P19 embryonal carcinoma cell line and almost no activity in the mouse NIH/3T3 fibroblast cell line (**Table 2**). To further confirm this finding, we employed the P19 mouse embryonal carcinoma cell

line because of its unique ability to be differentiated into a muscle-like phenotype (aka. P19-DMSO-muscle) following dimethylsulfoxide treatment or into a neuron-like phenotype (aka. P19-RA-neuron) following all trans-retinoic acid treatment (Jones-Villeneuve, E. et al., *Mol. Cell. Biol.* **3**, 2271-2279 (1983); Edwards, M. et al., *Mol. Cell. Biol.* **3**, 2280-2286 (1983)). If our hypothesis that PS-1 promoter activity is preferred in neuron-like cells, then we would predict that P19 cells differentiated with retinoic acid into neuron-like cells would display more PS-1 promoter activity than P19 cells differentiated with dimethylsulfoxide into muscle-like cells. As clearly shown in **Figure 5** and **Table 2**, P19-RA-neuron cells display the most PS-1 promoter activity followed by untreated P19 cells and the least activity in P19-DMSO-mucscle cells. These results combined with the Neuro2a and NIH/3T3 results indicate a clear pattern of PS-1 promoter activity which is preferred in neurons.

The mechanisms controlling neuron-specific promoter activity are poorly understood. The most direct mechanism would be for a positive regulator, that is only present in neuronal cells, to singularly activate the neuron-specific promoter. Alternatively, a negative regulator, that is only present in non-neuronal cells, could globally repress the neuron-specific promoter in all but neuronal cells. Depending upon the exact DNA elements within the promoter, some combination of positive and negative controls of transcriptional activity might also yield neuron-preferred promoter function. Going beyond our characterization of the regions conferring PS-1 promoter activity in Neuro2a cells, we may now look at the data to suggest which of the DNA elements might confer neuron-preferred promoter function. The region showing the highest activity in Neuro2a neuron-like cells extends from -329 to +206 (LUC 29) and contains a CAAT box at -281, a heat-shock inducible element at -218, an Ap2 site at -80, a PEA-3 site at -53, an Ets 1-3 site at -7 and an Sp1 site at +25. The CAAT box probably supports about a third of the positive control of neuron-specific activity as its deletion reduces promoter activity by about a third when comparing LUC 6 (-327 to +91) with LUC 8 (-261 to +91, **Figure 4**). The heat shock element probably does not contribute to neuron-specific activity under normal conditions (non-stressed) as its deletion does not affect promoter activity when comparing LUC 8 (-261 to +91) to LUC 10 (-192 to +91, **Figure 4**). Based on the four fold greater activity of LUC 12 (-87 to +91) compared to LUC 13 (-32 to +91), it appears that both the Ap2 site and the PEA-3 site are good candidates for the positive

control of neuron-specific promoter function. Ap2 sites are reported to be most frequently found in promoters active in cells of neural crest lineage and several examples exist of their involvement with neuron-specific activity (Sato, T. et al., *J. Biol. Chem.* **270**, 10314-10322 (1995); Petersohn, D. et al., *J. Biol. Chem.* **270**, 24361-24369 (1995); Chin, L. et al., *J. Biol. Chem.* **269**, 18507-18513 (1994)). In contrast, the five fold less activity of LUC 26 (-9 to +41) compared to LUC 27 (-9 to +16) implicates the Sp1 site at +25 as a negative regulator of neuron-specific promoter function. These same data could also be interpreted as the Ets 1-3 site having a positive function, possibly as part of a core promoter element from -9 to +16. Direct measurement of LUC 27 (-9 to +16) shows that Neuro2a and P19-RA-neuron cells have more activity than do P19-DMSO-muscle or NIH/3T3 non-neuronal cells supporting the idea that this 25 bp region contributes to neuron-preferred promoter activity. The major transcription start site at position +1 is located in this proposed core promoter element. The ETS-1 transcription factor prefers binding to the Ets 1-3 binding site found in this core by a ratio of five to one over the PEA-3 binding site (Fisher, R. et al., *Oncogene* **6**, 2249-2254 (1991)). This finding is particularly interesting as the ETS-1 transcription factor is thought to be specific for B cells and resting T cells of the immune system and not been previously described for neuronal cells. Sp1 binding sites appear to be ubiquitously distributed in all promoters of all cell types and their ability to function as negative elements appears to be novel.

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Roses, Allen D.

(ii) TITLE OF INVENTION: Presenilin-1 Gene Promoter

(iii) NUMBER OF SEQUENCES: 17

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

AGGCCGGAAG TTGCGACACC GGTGA 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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

CGGAGAGAGA AGGAACAAAC 20

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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

TCAGCTCTTC GTCTTCCTCC TCATC 25

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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

GATCACAGTC TAGGTTGCTG GTGTG 25

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TGGGGCAAGG GACACAAATA AG 22

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TGGCTCAGGG TTGTCAAGTC 20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCATCCTAAT ACGACTCACT ATAGGGC 27

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CAAACCTCTT GGGATTCTTT C 21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ACTCACTATA GGGCTCGAGC GGC 23

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AAGACCTCGA AGGGCTGCTG TC 22

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCCGGAAGT 9

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GGGCGGG 7

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CCCAGCCC 8

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTCGAATCGC AG 12

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGGCGG 6

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CCGCCC 6

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 48974 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 2234..2374
(D) OTHER INFORMATION: /product= "Exon 1A; initial exon of ps1 transcript A"

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 13443..13509
(D) OTHER INFORMATION: /product= "exon 2"

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 13600..13739
(D) OTHER INFORMATION: /product= "exon 3"

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 26082..26332
(D) OTHER INFORMATION: /product= "exon 4"

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 28544..28685
(D) OTHER INFORMATION: /product= "exon 5"

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 36788..36855
(D) OTHER INFORMATION: /product= "exon 6"

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 38293..38513
(D) OTHER INFORMATION: /product= "exon 7"

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 42006..42104
(D) OTHER INFORMATION: /product= "exon 8"

(ix) FEATURE:

(A) NAME/KEY: exon
(B) LOCATION: 42478..42564
(D) OTHER INFORMATION: /product= "exon 9"

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 44315..44488
(D) OTHER INFORMATION: /product= "exon 10"

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 45450..45568
(D) OTHER INFORMATION: /product= "exon 11"

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 47692..48974
(D) OTHER INFORMATION: /product= "exon 12"

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 13653..13739

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(ix) FEATURE:
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(B) LOCATION: 28544..28685

(ix) FEATURE:
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(B) LOCATION: 36788..36855

(ix) FEATURE:
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(B) LOCATION: 38293..38513

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(B) LOCATION: 42006..42104

(ix) FEATURE:
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(B) LOCATION: 42478..42564

(ix) FEATURE:
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(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 45450..45568

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 47692..48974

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 2644..2782
(D) OTHER INFORMATION: /product= "exon 1c; initial exon of PS1 transcript C"

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 2644..3014
(D) OTHER INFORMATION: /product= "exon 1b; initial exon of PS1 transcript B"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

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CCTAAGTGAC CTAGTCAATC AGGCCAAGTC ATTTCCTGCT TCACTCATTA GTTGCTACTG 180

GGTTTTCTCA TCTTCCTATT GCCAGGTTTT GAATAATGGG GAAAATAAAT GCTACACTGC 240

AGAAATTCTC ACTATTCTTT GGCACTGTTT GGTCATTCTT AGGTTTTAGT GTTTCTAGAC 300

CCTCAATGCT ATATTACATT GAAAAAATAG AAAATTATTT AGTATGTGCC TTTCAAATGT 360

CTCAGATCTT ATTCACATAG TTAGAAGTGT TGTGTGCCTT GATATTTCCG TGGGTGCCCG 420

AGTGGGATAC ATTAGCTTCT CTTGGCACTC TATAAACATT GTCTGTCTAT TTGATATTCA 480

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AGAAGCTTGA AGGTTACAGA GGTAAATTAT TTGCCTGGTC GCTTGCATGT GGCTTGACCT 40560

AAGAGTCTGA ATTCACTCCA TGAGTTACTC TCTCCACGAC CTTCAGCCTC TGCTCACAGC 40620

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GACATAGTGA CTCTCAACCT GTTCCTTACA AGGTCTCAAA ATGGTCTATC AGAAGTGTTC 40740

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TGGCAGCTTT AAAACAGCTG AAGATCTCAT TAGTTGCAAG CACAGGAAAG CTTGCTTTTC 40920

TCACTTGAAC ATGTAAATGG AAGTGTAGAG GAGTGTTACC ACTGTGCTGT TTTTACGTTC 40980

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AAAGAATTAA AGCGATGAGT GGCTGGTGAA GCGCTCTCTG TCCTGGCTGG TTTTTTGTCA 48900

CCGCAATCCA CTGCCAAAGG CAGAATTTAA ACTAGGGGCT GAAGGATAGC TCCGAGTAAT 48960

GTCCAATGCT GGGA 48974

That which is claimed is:

1. An isolated DNA molecule that directs neuron-specific transcription of a downstream heterologous DNA segment in a mammalian cell, said isolated DNA molecule having a sequence selected from the group consisting of:

(a) the sequence spanning position –327 to position –206 of the mouse genomic Presenilin-1 gene;

(b) the sequence spanning position –449 to position +1171 of the mouse genomic Presenilin-1 gene;

(c) the sequence spanning position -9 to position +16, of the mouse genomic Presenilin-1 gene; and

(d) DNA sequences that hybridize to isolated DNA having a sequence of (a), (b), or (c) above, and which direct neuron specific transcription of a downstream heterologous DNA segment in a mammalian cell.

2. An isolated DNA according to claim 1, said DNA having the sequence of a mammalian Presinilin-1 gene promoter from position:

-440 to +91;

-352 to +91;

-327 to +91;

-276 to +91;

-261 to +91;

-215 to +91;

-192 to +91;

-124 to +91;

-87 to +91; and

-32 to +91.

3. An isolated DNA according to claim 1, said DNA having the sequence of a mammalian Presenilin-1 gene promoter from position:

-276 to +519;

-276 to +206;

-276 to +148; and

-276 to +41.

4. An isolated DNA according to claim 1, said DNA having the sequence of a mammalian Presenilin-1 gene promoter from position:

-87 to +41;

-9 to +16; and

-327 to +206.

5. An isolated DNA according to claim 1, said DNA have the sequence of the mouse Presenilin-1 gene promoter from position -327 to +206.

6. A DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a promoter segment consisting of a DNA according to claim 1, and a heterologous DNA segment positioned downstream from said promoter segment and operatively associated therewith.

7. A DNA construct according to claim 6, wherein said construct comprises a plasmid.

8. A DNA construct according to claim 6, wherein said heterologous DNA segment encodes a protein.

9. A cell containing a DNA construct according to claim 6.

10. A gene transfer vector comprising a DNA construct according to claim 6.

11. A transgenic non-human mammal comprising cells that contain a DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a

neuron-specific promoter segment and a heterologous DNA segment positioned downstream from said promoter segment and operatively associated therewith;

said neuron-specific promoter having a sequence selected from the group consisting of:

(a) the sequence spanning position –327 to position –206 of the mouse genomic Presenilin-1 gene;

(b) the sequence spanning position –449 to position +1171 of the mouse genomic Presenilin-1 gene;

(c) the sequence spanning position -9 to position +16, of the mouse genomic Presenilin-1 gene; and

(d) DNA sequences that hybridize to isolated DNA having a sequence of (a), (b), or (c) above, and which direct neuron specific transcription of a downstream heterologous DNA segment in a mammalian cell.

12. A transgenic non-human mammal according to claim 11, wherein said mammal is a mouse.

13. A transgenic non human mammal according to claim 11, wherein said expression cassette is stably integrated into the genome of said cells containing the same.

14. A transgenic non-human mammal according to claim 11, wherein said expression cassette is carried by the somatic and germ cells thereof.

15. A transgenic non-human mammal according to claim 11, said heterologous DNA segment encoding a protein.

16. A transgenic-non-human mammal according to claim 11, wherein said heterologous DNA segment is transcribed in the cells thereof.

17. A transgenic-non-human mammal according to claim 11, wherein said heterologous DNA segment is preferentially transcribed in nerve cells thereof.

FIG. 1

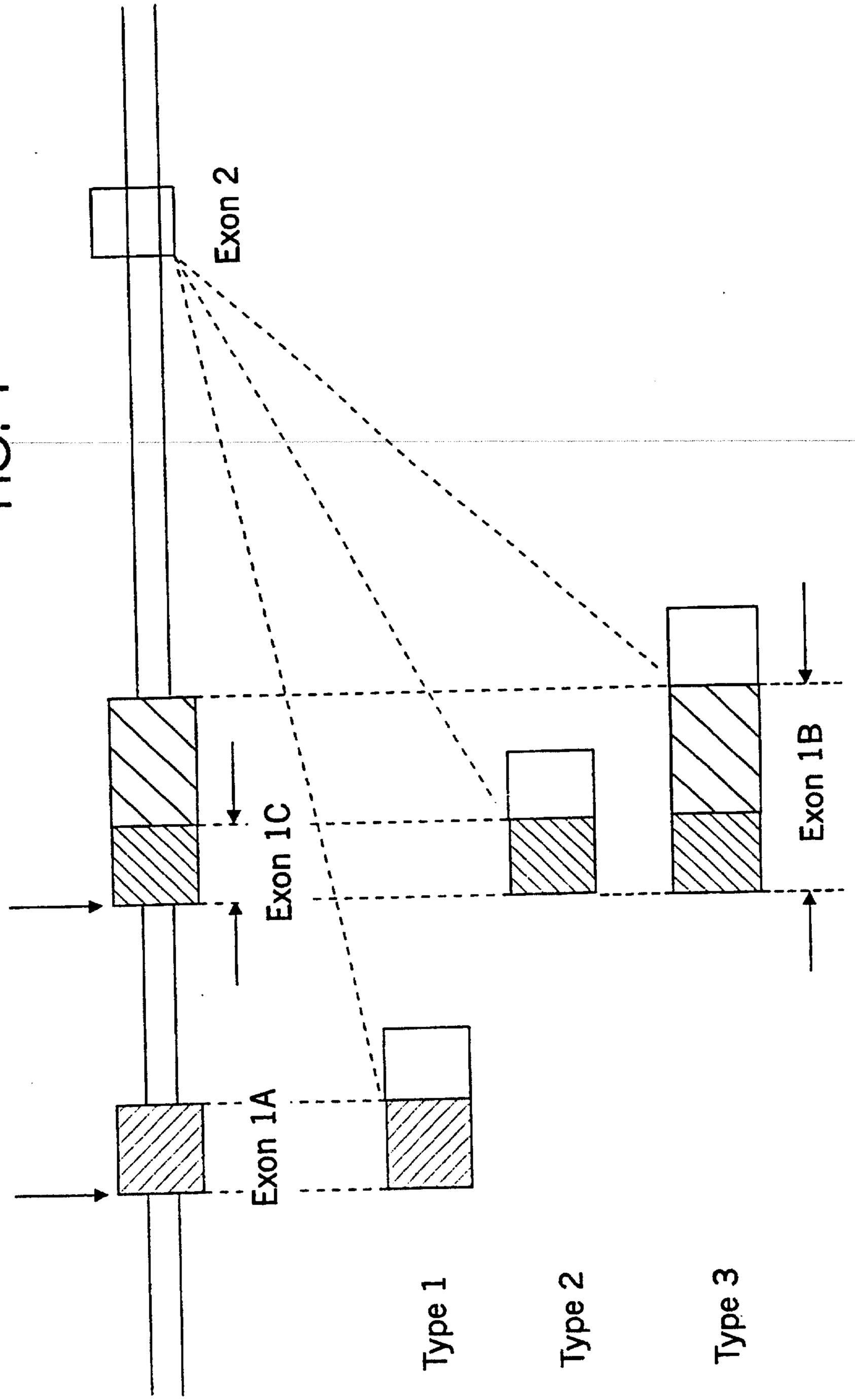
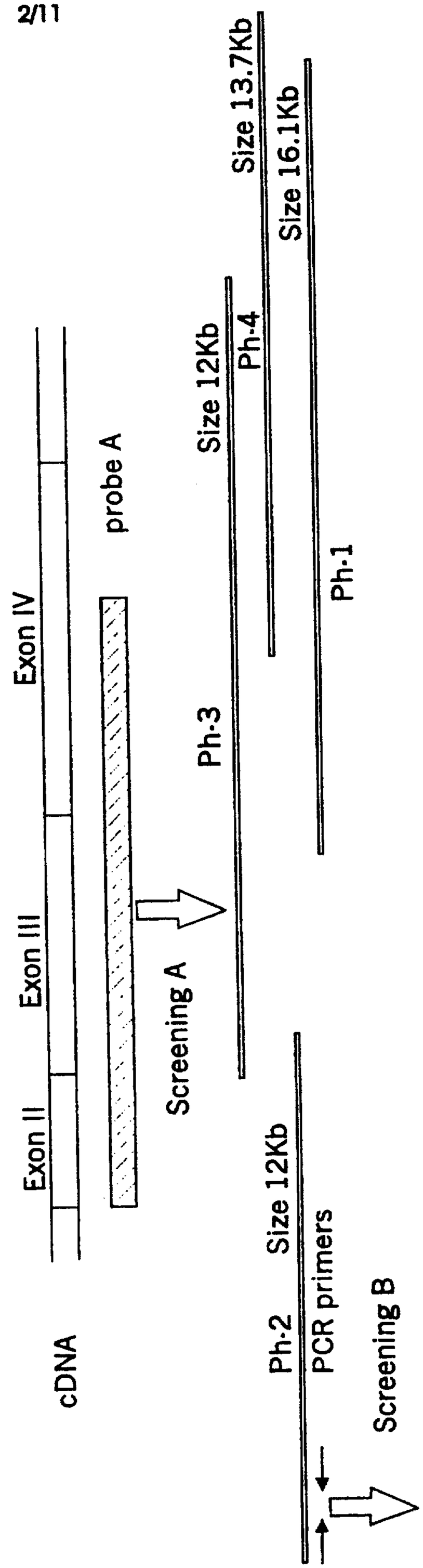


FIG. 2A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Size 16.1Kb
Exon IV
probe A
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Ph-4
Ph-1
Exon II
Exon III
Screening A
Ph-3
Size 12Kb
cDNA
Ph-2
PCR primers
Screening B

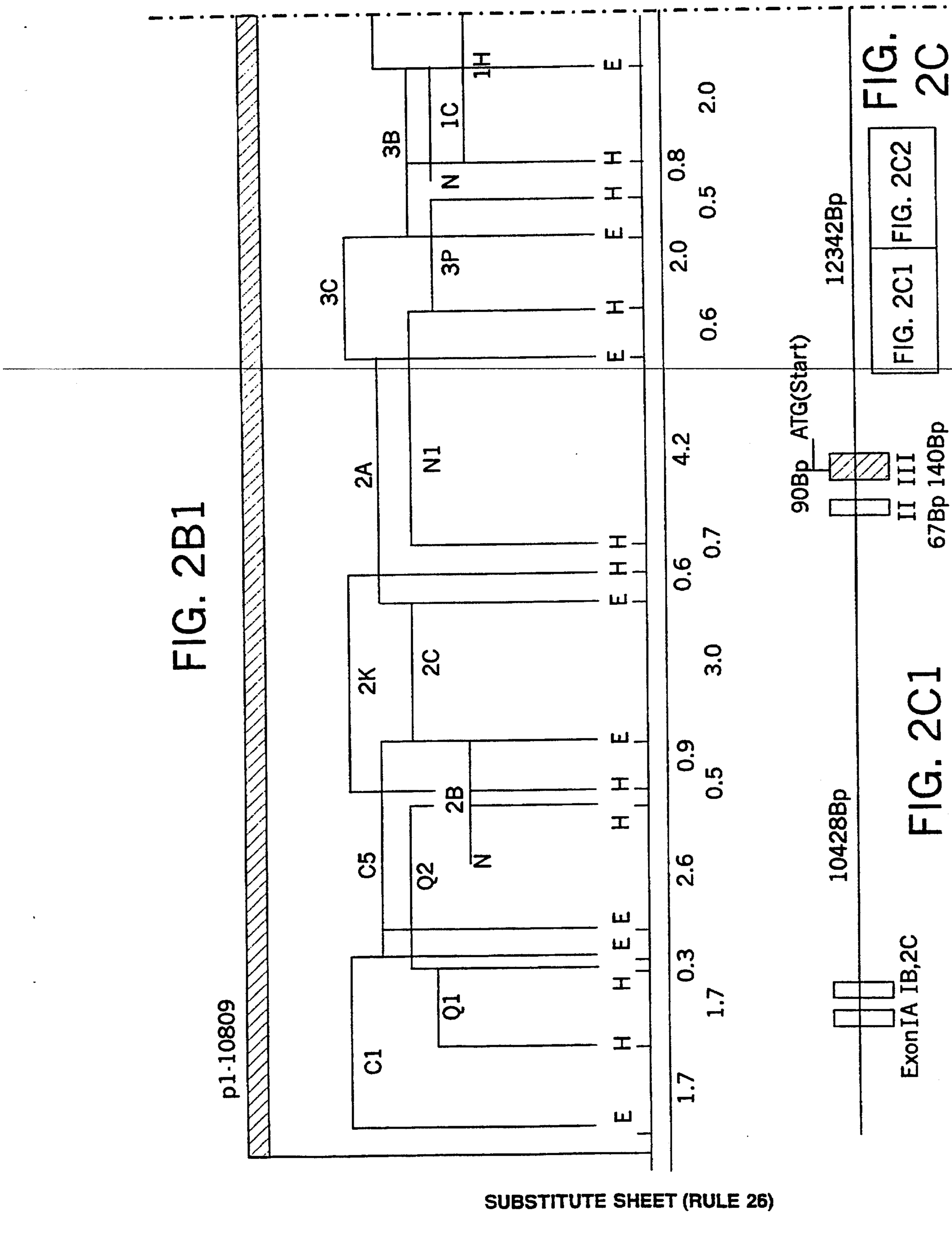


FIG. 2B1
FIG. 2C
FIG. 2C1
FIG. 2C2
p1-10809
C1
Q1
C5
Q2
2K
2C
2B
N
2A
N1
3C
3P
3B
1C
1H
E
H
N
2.0
0.8
0.5
0.6
4.2
0.6
0.7
3.0
0.9
0.5
2.6
0.3
1.7
1.7
12342Bp
90Bp ATG(Start)
II III
67Bp 140Bp
10428Bp
ExonIA IB,2C

FIG. 2B

FIG. 2B1	FIG. 2B2

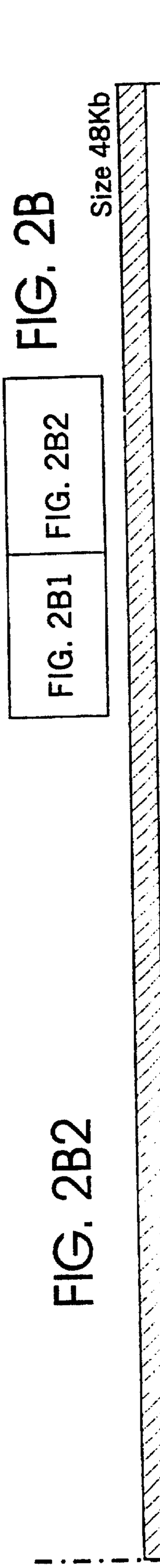


FIG. 2C2

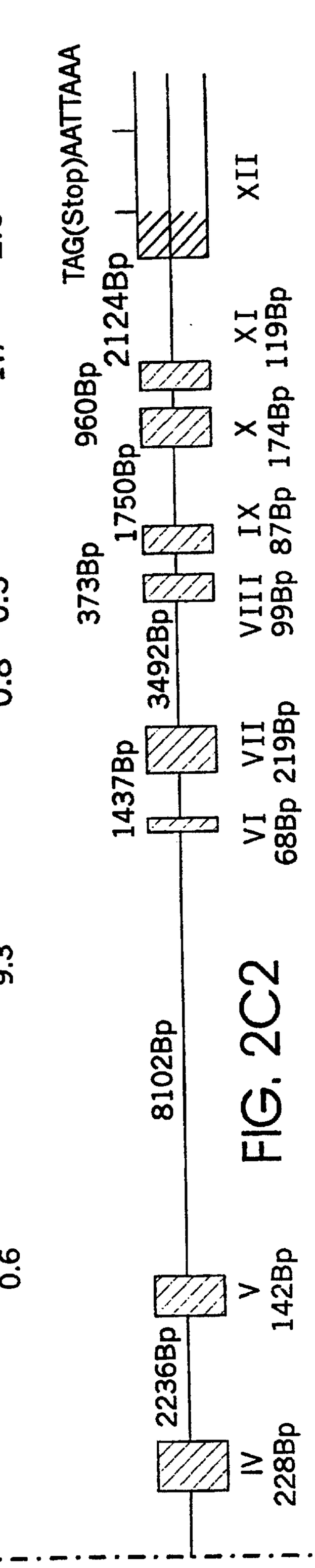


FIG. 3

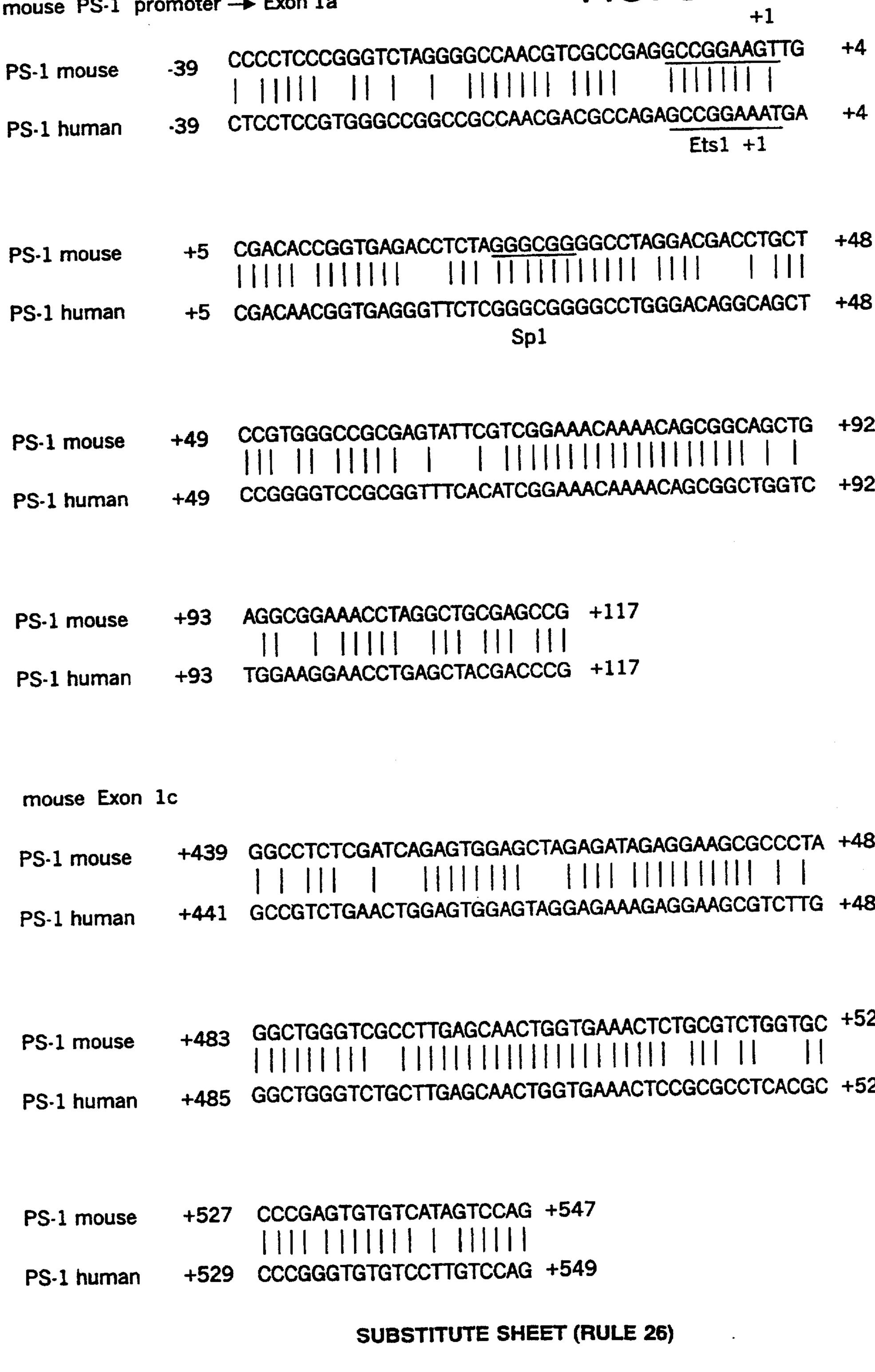
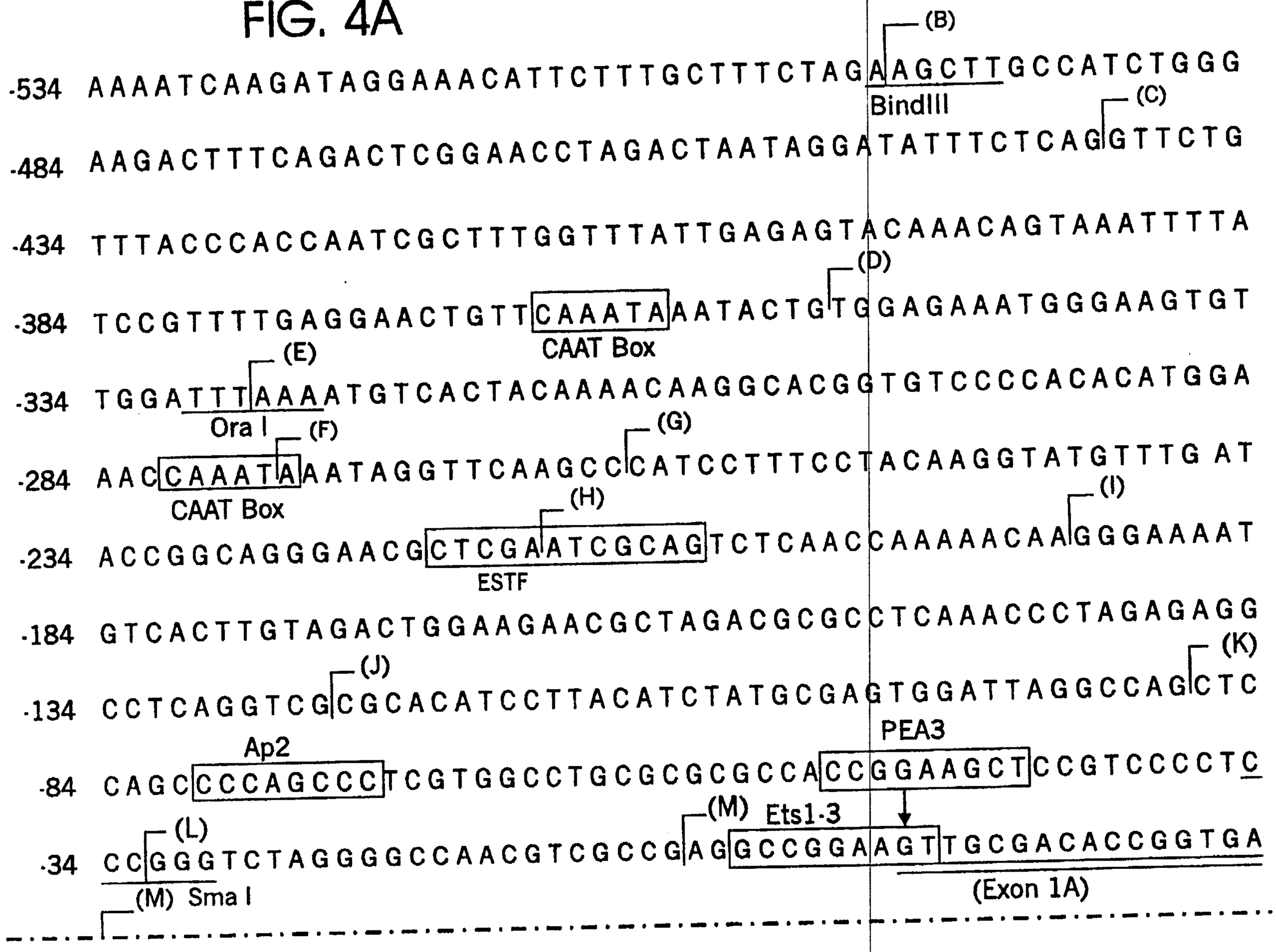

mouse PS-1 promoter → Exon 1a
+1
PS-1 mouse -39 CCCCTCCCGGGTCTAGGGGCCAACGTCGCCGAGGCCGGAAGTTG +4
PS-1 human -39 CTCCTCCGTGGGCCGGCCGCCAACGACGCCAGAGCCGGAAATGA +4
Ets1 +1
PS-1 mouse +5 CGACACCGGTGAGACCTCTAGGGCGGGGCCTAGGACGACCTGCT +48
PS-1 human +5 CGACAACGGTGAGGGTTCTCGGGCGGGGCCTGGGACAGGCAGCT +48
Sp1
PS-1 mouse +49 CCGTGGGCCGCGAGTATTCGTCGGAAACAAAACAGCGGCAGCTG +92
PS-1 human +49 CCGGGGTCCGCGGTTTCACATCGGAAACAAAACAGCGGCTGGTC +92
PS-1 mouse +93 AGGCGGAAACCTAGGCTGCGAGCCG +117
PS-1 human +93 TGGAAGGAACCTGAGCTACGACCCG +117
mouse Exon 1c
PS-1 mouse +439 GGCCTCTCGATCAGAGTGGAGCTAGAGATAGAGGAAGCGCCCTA +482
PS-1 human +441 GCCGTCTGAACTGGAGTGGAGTAGGAGAAAGAGGAAGCGTCTTG +484
PS-1 mouse +483 GGCTGGGTCGCCTTGAGCAACTGGTGAAACTCTGCGTCTGGTGC +526
PS-1 human +485 GGCTGGGTCTGCTTGAGCAACTGGTGAAACTCCGCGCCTCACGC +528
PS-1 mouse +527 CCCGAGTGTGTCATAGTCCAG +547
PS-1 human +529 CCCGGGTGTGTCCTTGTCCAG +549

FIG. 4A



SUBSTITUTE SHEET (RULE 26)

SUBSTITUTE SHEET (RULE 26)

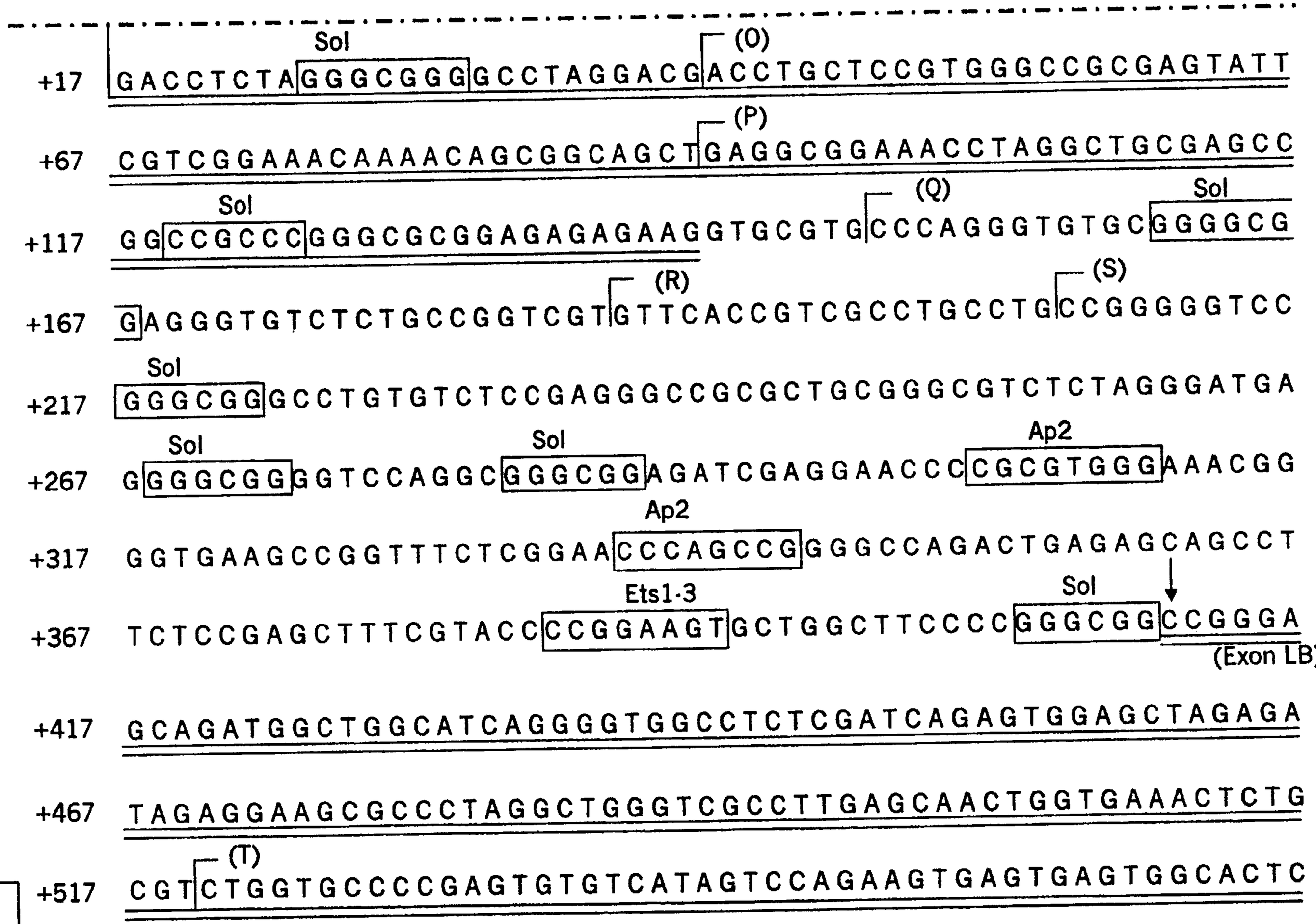


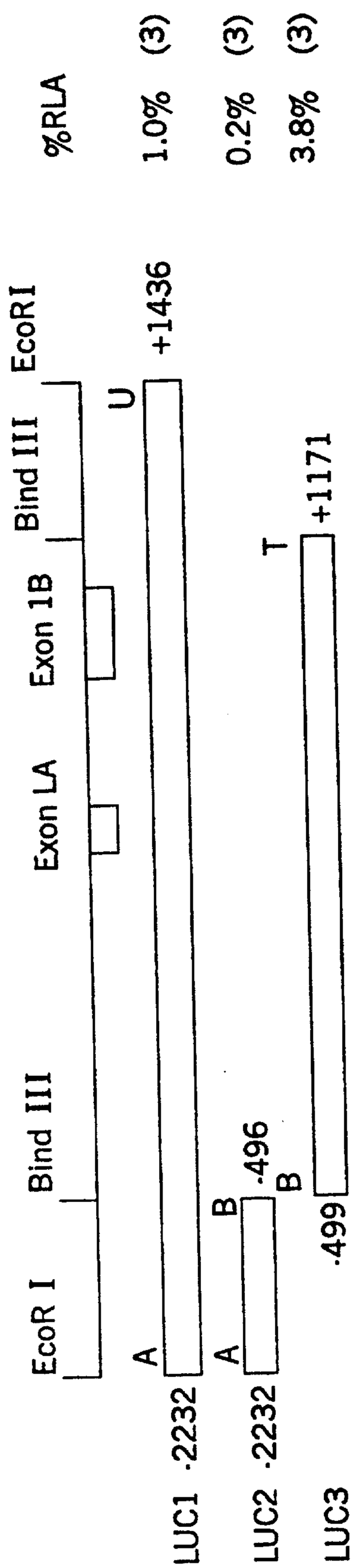
FIG. 4A

FIG. 4B

FIG. 4

FIG. 4B

FIG. 5A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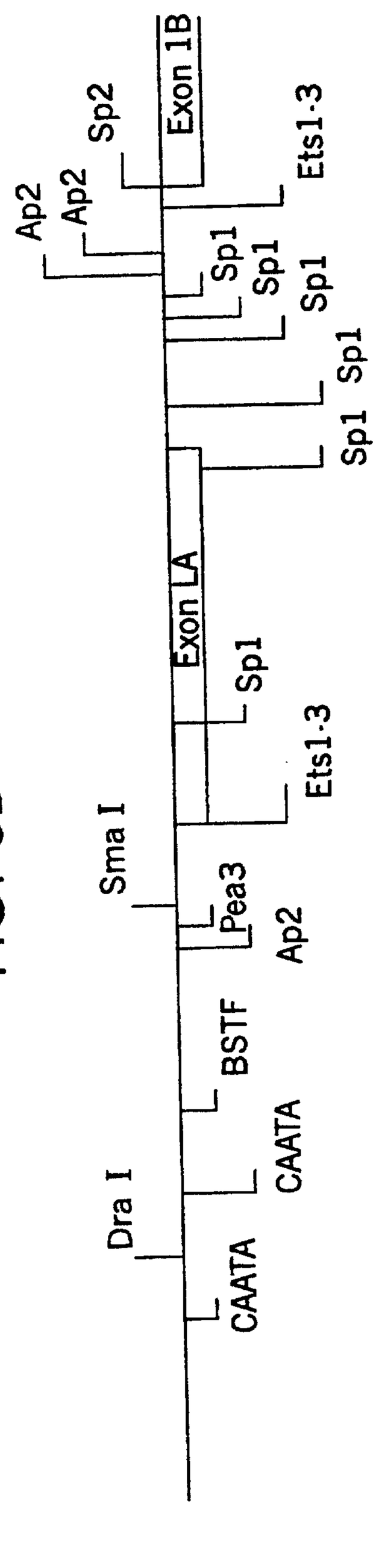
1.0%
0.2%
3.8%
EcoRI
Bind III
Exon 1B
U
+1436
T
+1171
Exon LA
Bind III
EcoR I
A
B
.496
.499
.2232
LUC1
LUC2
LUC3

FIG. 5B


Ap2
Ap2
Sp2
Exon 1B
Sp1
Sp1
Sp1
Ets1-3
Sp1 Sp1
Exon LA
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Sma I
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Ap2
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BSTF
Dra I
CAATA
CAATA

FIG. 5C

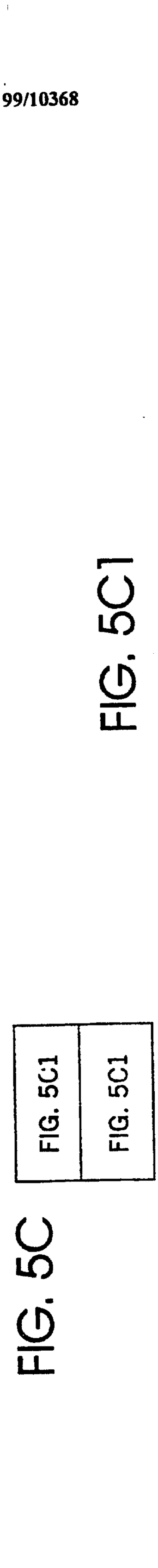


FIG. 5C1

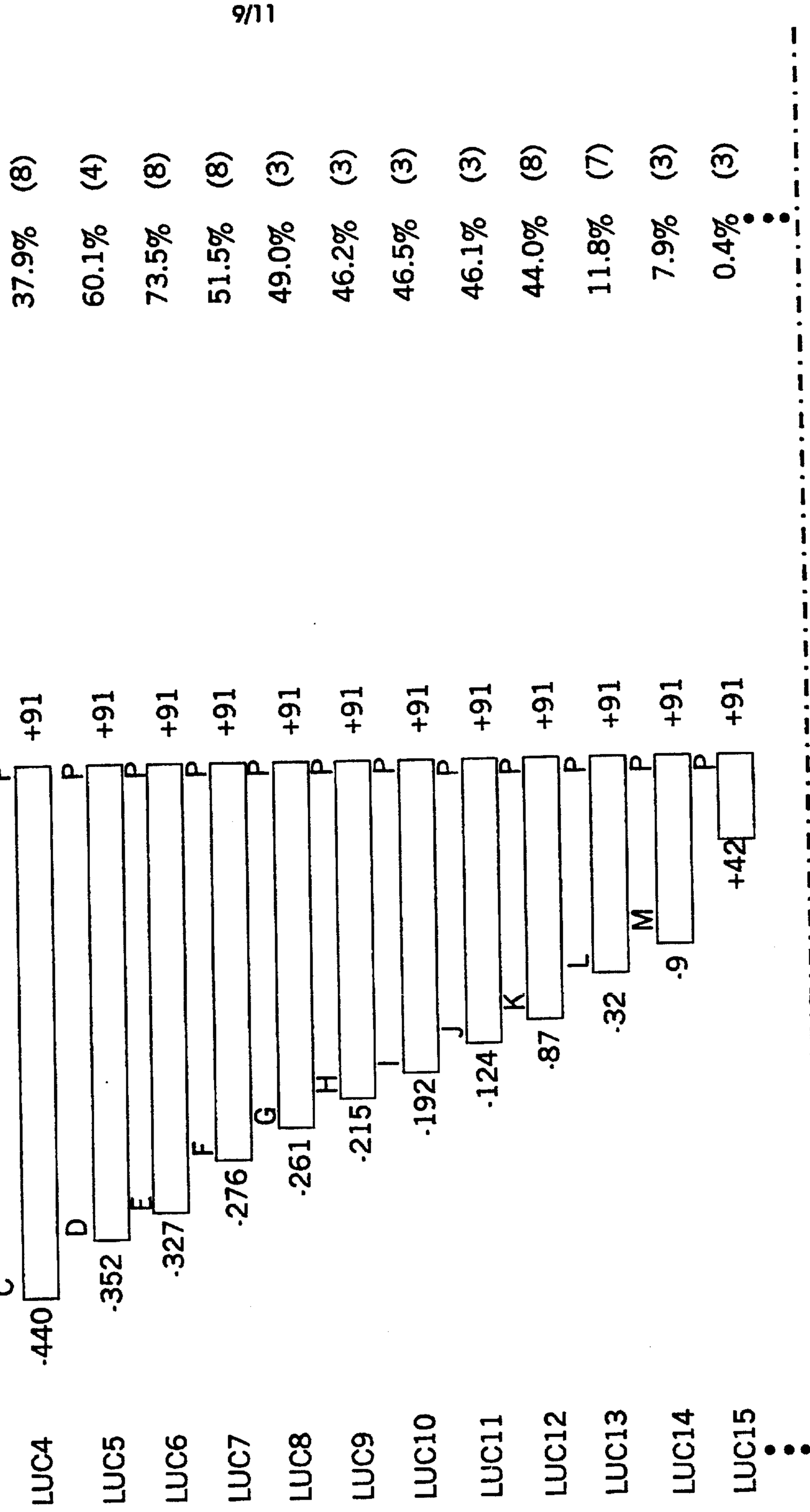
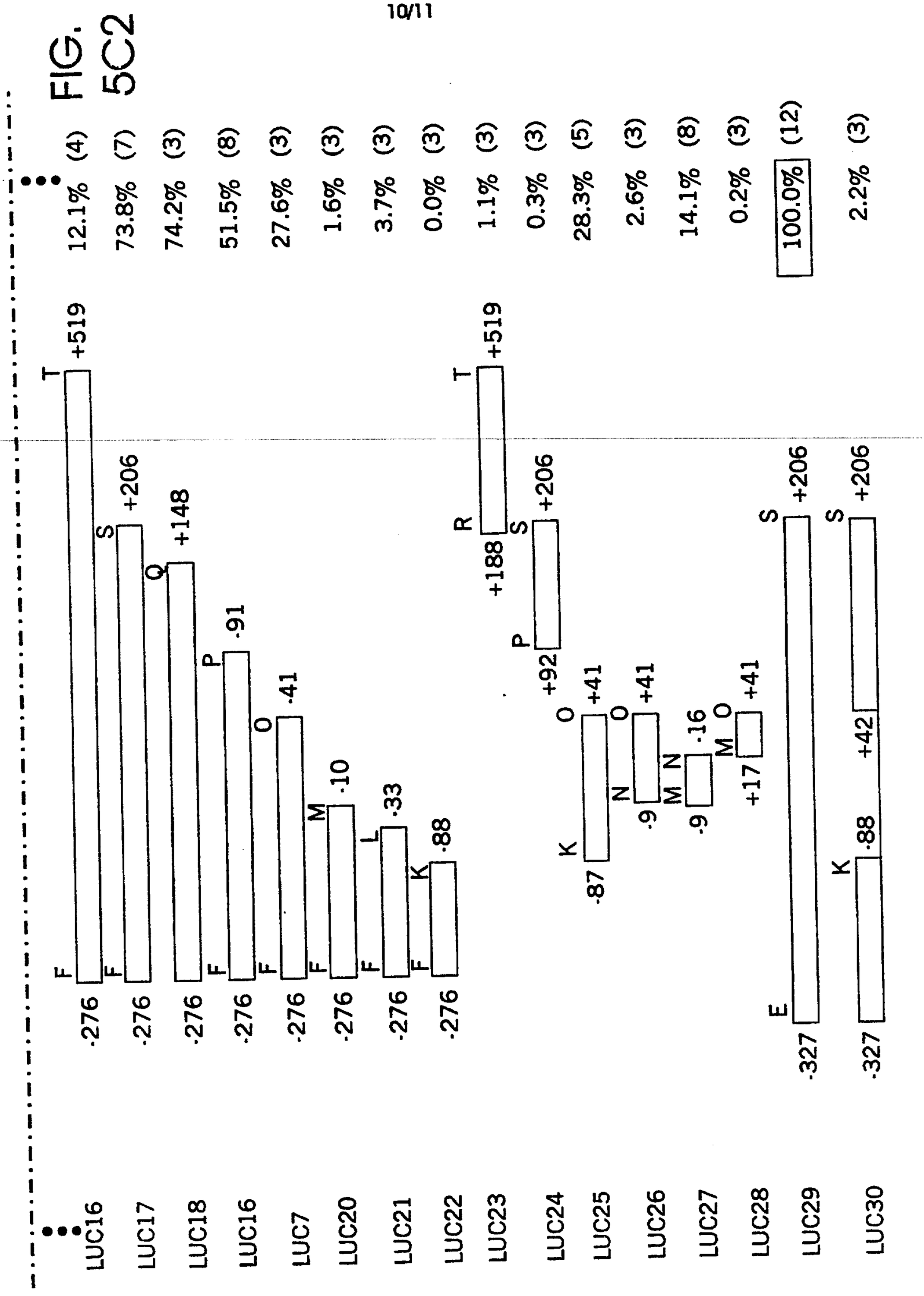


FIG. 5C2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LUC17
LUC18
LUC16
LUC7
LUC20
LUC21
LUC22
LUC23
LUC24
LUC25
LUC26
LUC27
LUC28
LUC29
LUC30
12.1% (4)
73.8% (7)
74.2% (3)
51.5% (8)
27.6% (3)
1.6% (3)
3.7% (3)
0.0% (3)
1.1% (3)
0.3% (3)
28.3% (5)
2.6% (3)
14.1% (8)
0.2% (3)
100.0% (12)
2.2% (3)
T +519
S +206
Q +148
P 91
F .276
O .41
M .10
L .33
K .88
R +188
P 92
O +41
K .87
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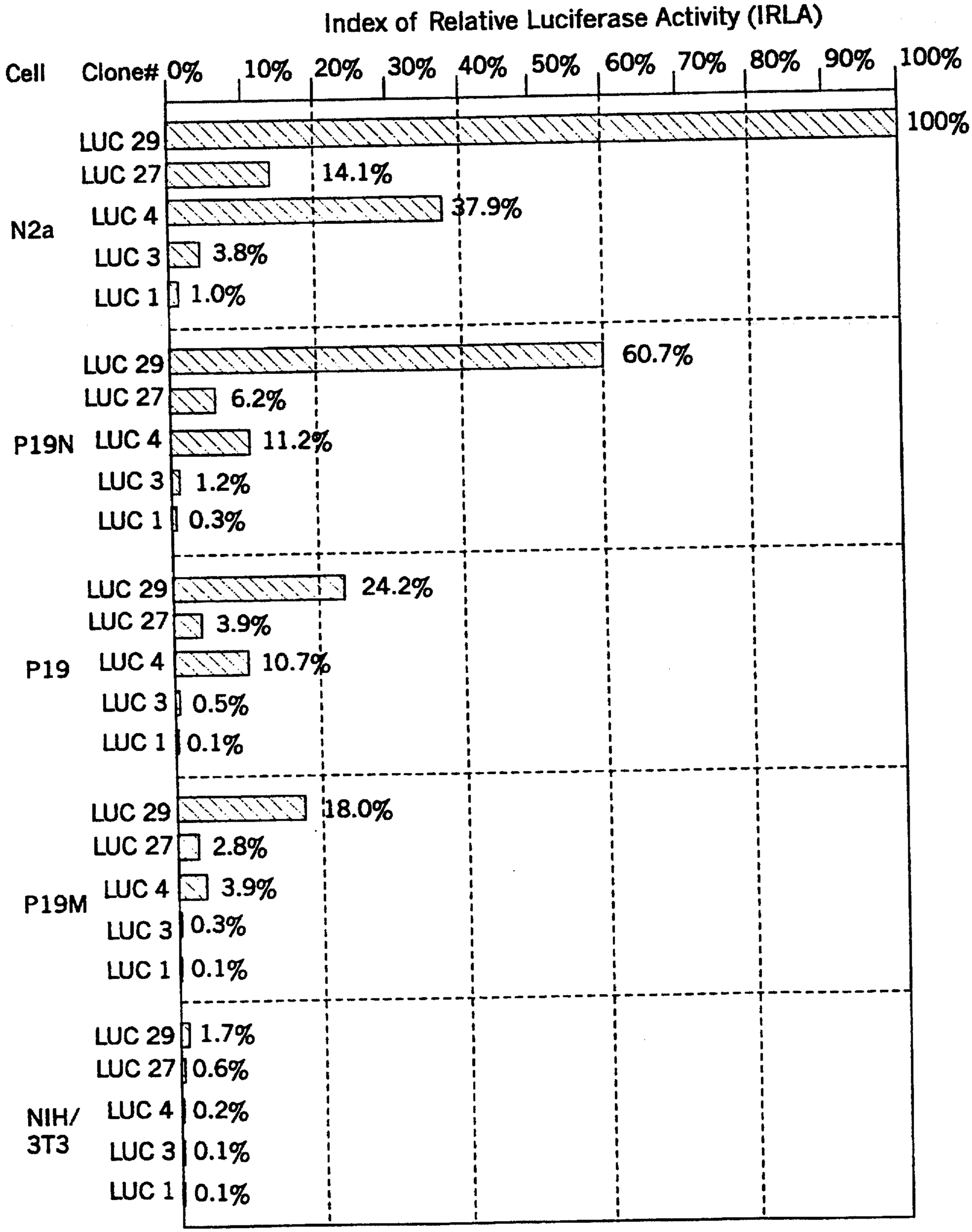
M N .16
M O +17
S +206
E .327
K .88
+42
.327

FIG. 6


Index of Relative Luciferase Activity (IRLA)
Cell
Clone#
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
N2a
LUC 29
100%
LUC 27
14.1%
LUC 4
37.9%
LUC 3
3.8%
LUC 1
1.0%
P19N
LUC 29
60.7%
LUC 27
6.2%
LUC 4
11.2%
LUC 3
1.2%
LUC 1
0.3%
P19
LUC 29
24.2%
LUC 27
3.9%
LUC 4
10.7%
LUC 3
0.5%
LUC 1
0.1%
P19M
LUC 29
18.0%
LUC 27
2.8%
LUC 4
3.9%
LUC 3
0.3%
LUC 1
0.1%
NIH/ 3T3
LUC 29
1.7%
LUC 27
0.6%
LUC 4
0.2%
LUC 3
0.1%
LUC 1
0.1%